

Inflammatory activity and markers of extracellular matrix destruction in pulmonary tuberculoma

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ABSTRACT

Aim. To correlate the concentration of markers of extracellular matrix (ECM) destruction in peripheral blood with morphological characteristics of inflammatory activity and to evaluate their applicability in determining treatment strategy for patients with pulmonary tuberculoma (TUB).

Materials and methods. Peripheral blood samples were taken from 87 patients diagnosed with TUB. The concentrations of matrix metalloproteinases (MMPs), such as collagenases (MMP-1 and MMP-8), stromelysin (MMP-3), gelatinase (MMP-9), and tissue inhibitors of metalloproteinases (TIMP-1), were measured using the ELISA method (R&D Systems, Minneapolis, MN, USA). The activity of α_2 -macroglobulin (MG), neutrophil elastase (NE) and proteinase inhibitor (PI) were measured using enzyme assays; acute phase reactants (APR) – haptoglobin (GP) and α_1 -acid glycoprotein (AGP) – were measured using immunoturbidimetric assays (Thermo Fisher Scientific, USA). Statistica 7 software package and the predictive classification method (PCM) were employed for data analysis.

Results. It has been established that TUB as a clinical form of pulmonary tuberculosis (TB) is characterised by enzyme imbalance between MMP, NE and their inhibitors, namely, by an increase in the levels of MMP-1, MMP-8, MMP-9, and NE and a decrease in MG without changes in MMP-3, TIMP-1 and PI. There is a clear correlation between markers of ECM destruction in blood and morphological characteristics of inflammatory activity. The combinations of MMP-1 and MG can serve as a diagnostic criterion for caseous necrosis in the TUB centre (the alterative component of inflammation), while the levels of MMP-8 and MG can be indicative of granulomatous changes in the capsule (the productive component of inflammation). Various combinations of markers of ECM destruction (with or without APR) enable to predict a particular morphological pattern with accuracy from 80% up to 92%.

Conclusions. When determining a treatment strategy for patients with TUB, biochemical data which allow to assess the tempo and intensity of the inflammation process should be taken into account along with a dataset of clinical and radiological features.

Key words: extracellular matrix, matrix metalloproteinases, proteinase inhibitors, pulmonary tuberculoma.

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

Source of financing. The authors state that they received no funding for the study.

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Conformity with the principles of ethics. All patients signed an informed consent to participate in the study. The study was approved by the Ethics Committee at St. Petersburg Scientific Research Institute of Phthisiopulmonology.

For citation: Esmedlyeva D.S., Alekseeva N.P., Novitskaya T.A., Dyakova M.Ye., Ariel I.V., Grigoriev B.M., Sokolovich E.G. Inflammatory activity and markers of extracellular matrix destruction in pulmonary tuberculoma. *Bulletin of Siberian Medicine*. 2020; 19 (2): 112-119. <https://doi.org/10.20538/1682-0363-2020-2-112-119>.

Активность воспалительного процесса и маркеры деструкции внеклеточного матрикса при туберкулезе легких

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РЕЗЮМЕ

Цель. Сопоставить уровень маркеров деструкции внеклеточного матрикса (ВКМ) в периферической крови с морфологическими характеристиками активности воспалительного процесса и определить возможность их использования при выборе тактики лечения больных с туберкулезом легких (ТУБ).

Материалы и методы. В периферической крови 87 больных (55 мужчин и 32 женщины) с верифицированным диагнозом ТУБ иммуноферментным методом определяли концентрацию коллагеназ (матриксные металлопротеиназы (ММП) 1, 8), стромелизина (ММП-3), желатиназы (ММП-9), тканевого ингибитора ММП-1 (ТИМП-1) с использованием наборов R&D Systems (США); энзиматически – активность нейтрофильной эластазы (НЭ), протеиназного ингибитора (ПИ) и $\alpha 2$ -макроглобулина (МГ); иммунотурбидиметрически – концентрацию реактанты острой фазы воспаления (РОФ): гаптоглобина (ГП), $\alpha 1$ -кислого гликопротеина (АГП) с использованием наборов Thermo Fisher Scientific (США). Применяли пакет программ Statistica 7 и метод проективной классификации.

Результаты. Установлено, что ТУБ как клиническая форма туберкулеза легких характеризуется нарушением баланса ММП и НЭ с ингибиторами: повышением уровня ММП-1, -8, -9, НЭ и снижением МГ при отсутствии изменений ММП-3, ТИМП-1 и ПИ. Показано соответствие маркеров деструкции ВКМ в крови морфологическим характеристикам активности процесса. Информативными показателями для оценки альтернативного компонента воспаления (наличия казеоза в центре ТУБ) и его продуктивного компонента (гранулематозных изменений в капсуле) является как сочетание ММП-1 с МГ, так и ММП-8 с МГ. Различные комбинации показателей маркеров деструкции ВКМ (в сочетании с РОФ или без) дают возможность прогнозировать ту или иную морфологическую картину с точностью 80–92%. **Заключение.** При выборе тактики лечения больных с ТУБ следует принимать во внимание биохимические данные с их оценкой активности воспалительного процесса наряду с комплексом клинико-рентгенологических характеристик.

Ключевые слова: внеклеточный матрикс, матриксные металлопротеиназы, ингибиторы протеиназ, туберкулеза легких.

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

Источник финансирования. Авторы заявляют об отсутствии финансирования при проведении исследования.

Соответствие принципам этики. Исследования выполнены с информированного согласия пациентов. Исследование одобрено локальным этическим комитетом СПб НИИФ.

Для цитирования: Эсмедляева Д.С., Алексеева Н.П., Новицкая Т.А., Дьякова М.Е., Ариэль Б.М., Григорьев И.В., Соколов Е.Г. Активность воспалительного процесса и маркеры деструкции внеклеточного матрикса при туберкулезе легких. *Бюллетень сибирской медицины*. 2020; 19 (2): 112-119. <https://doi.org/10.20538/1682-0363-2020-2-112-119>.

INTRODUCTION

The search for various markers that would enable early diagnosis and prediction of treatment effectiveness for various pathologies has not lost relevance. The complexity of the problem is explained by the fact that most of the markers are highly sensitive, yet not specific enough. This determines the potential of their aggregate isolation and as a clinical prognostic tool [1]. Currently, one of the methods for assessing the intensity of the inflammatory and destructive process is evaluating the activity of various classes of blood proteinases: serine, cysteine, aspartic, and matrix metalloproteinases (MMPs). The latter are divided into several subgroups based on their substrate specificity: collagenases, gelatinases, stromelysins, etc. [2]. Proteins of the MMPs perform a double role in the pathogenesis of inflammation, causing the destruction of all components of the ECM and regulating the immune response in the inflammatory processes. The ultimate effect of the proteolytic systems depends on the proteinase and proteinase inhibitor correlation. Neutrophils, monocytes, macrophages, fibroblasts, and epithelial cells are the sources of MMPs. At the post-translational level, serine proteinases are involved in the pro-enzyme activation, while specific tissue inhibitors (TIMPs) and α_2 -macroglobulin (MG) regulate their activity [3]. *Mycobacterium tuberculosis* (MTB) regulates matrix metalloproteinase gene expression along with tumour necrosis factor- α (TNF α) and interleukin-1 (IL-1) [4].

A correlation between the morphological characteristics of the inflammatory activity and the functional and metabolic properties of phagocytes in different forms of pulmonary tuberculosis (PT) has been reported in a few works [5], while data on their association with the markers of ECM destruction cannot be found in the literature.

Pulmonary tuberculoma (TUB) is a clinical manifestation of secondary PT. The term TUB is used to describe a caseous necrotic mass located in the lungs,

over 12 mm in diameter, encapsulated by multiple layers of fibrous connective tissue. In the capsule, single Langhans cells, surrounded by epithelioid cell tubercles (in the case of rapidly progressing inflammation process), are sometimes found. However, the progression of TUB may be slow and torpid [6]. According to the National Clinical Guidelines for Surgical Treatment of PT, surgery is recommended for TUB after 4–6 months of chemotherapy with no effect [7]. The absence of clinical and radiological signs of disease activity does not exclude the presence of its morphological manifestations. In recent years, the morphological features of TUB have become especially well known. It is mainly determined by the fact that the evidence of mass lesions serves as an indication for surgery, while the resected sections of lung tissue undergo a thorough pathological examination [8].

The aim of the study was to correlate the changes in the markers of ECM destruction (the activity of MMPs and serine proteinase) in peripheral blood with the morphological characteristics of the inflammation process and to evaluate their applicability in determining a treatment strategy for patients with TUB.

MATERIALS AND METHODS

The study included 87 patients (55 men and 32 women) diagnosed with TUB (based on clinical assessment and morphological examination), treated at St. Petersburg Scientific Research Institute of Phthisiopulmonology, Department of Thoracic Surgery. The average age of the patients was 35.3 ± 1.2 years. All the study participants were eligible for surgical treatment (2011–2017). The control group consisted of 20 healthy donors whose demographic characteristics were consistent with those in the patient cohort. In most cases, TUB formed due to the involution of infiltrative PT (95%) following chemotherapy for up to 1.5 years. CT scanning of the chest cavity revealed that TUBs located in the upper lobe, lower lobe, and bilaterally account for 70.2%, 17.2%, and 12.5% of cases, respec-

tively. TUBs with a size of 1–2 cm, 2–4 cm and more than 4 cm were found in 57.14%, 28.51% and 14.35% of cases, respectively. Before treatment, bacteriological examination of the sputum was performed, and MTB, mainly multidrug-resistant strains (MDR), was detected in 34.9% of cases, which is typical of present-day tuberculosis, regardless of its clinical and anatomical forms [9].

Biochemical tests were performed no earlier than 7 days prior to the surgery. The enzyme-linked immunosorbent assay (ELISA) was used to measure the MMP concentrations in blood serum with R&D Systems reagents (Minneapolis, MN, USA). Representatives of three MMP subfamilies – collagenases MMP-1 and MMP-8; gelatinase MMP-9; and stromelysin MMP-3 – were measured, as well as their inhibitor TIMP-1. The concentrations of acute-phase reactants (APR) – haptoglobin (GP) and α_1 -acid glycoprotein (AGP) – were determined using immunoturbidimetric assays (Thermo Fisher Scientific, USA), according to the manufacturer's protocols. The enzyme methods were used to assess the activity of serine proteinase – neutrophilic elastase (NE) [10], proteinase inhibitor (PI), and MG [11].

All TUBs showed morphological features of caseoma (Table 1)*. The inflammatory activity was assessed according to the B.M. Ariel classification (1998) [6], based on the correlation between the parameters of caseous necrosis, capsules and surrounding lung tissue.

Table 1

Morphological characteristics of TUB inflammatory activity		
Morphological characteristics		Characteristic frequency (absolute (<i>n</i>), relative %)
Number of tuberculomas	Single	35 (40.2)
	Multiple	41 (47.0)
	Conglomerate	11 (12.8)
Parameters of caseous necrosis	Without melting	23 (26.8)
	With melting	64 (73.2)
Capsule changes	One layer	34 (39)
	Two layers	53 (61)
Inflammatory activity level	2	23 (26)
	3	52 (45)
	4	24 (27.5)
	5	1 (1.5)

For statistical data analysis, the Statistica 7.0 software package was used. Qualitative attributes were presented in the form of absolute (*n*) and relative values (%). The indicators were presented as medians (*Me*) and the interquartile range (25%; 75%) (Q_1 ; Q_3). For a number of indicators, logarithmic data

transformation – $\log_2 (x + 1)$ (*MeL*) – was used to reduce the skewness of distributions. The significance of the relationship between qualitative variables was tested using the Fisher's exact test. The hypothesis of homogeneity was estimated for two and several samples according to the criteria of the Mann–Whitney U test and the Kruskal – Wallis test, respectively. Correlation analysis was performed using the Spearman's rank correlation coefficient. Differences in indicators were considered significant at a level of statistical significance $p < 0.05$.

The objective assessment of morphological data was carried out through analysis of a set of ECM destruction markers using the predictive classification method (PCM) with a linear discriminant analysis algorithm. The advantage of the method is the possibility of analysis regardless of the completeness of the data presented [12]. Typically, the discriminant function (DF) is calculated simultaneously for all variables. PCM computes the set of most significant correlating DFs, constructed from different subsets of markers. Due to the small number of markers included in the DFs, they are easier to interpret. Moreover, this allows to examine the diversity of biochemical manifestations of the studied process from different points of view. Discriminant weights (standardised coefficients) enable identification of the variables that contribute the most to the discrimination between groups. Positive DF values in patients provide evidence to assign them to a group of patients with less severe manifestations of the disease.

RESULTS

TUB, as a clinical form of PT, was characterised by a moderate increase in blood concentrations of collagenases (MMP-1 and MMP-8) and a significant increase in gelatinase (MMP-9), while concentrations of stromelysin (MMP-3) and TIMP-1 remained at the control level. Additionally, there was a decrease in the activity of another MMP inhibitor – MG. A statistically significant increase in the activity of serine proteinase (NE) was established with no changes in its inhibitor (PI) activity (Table 2)*.

Table 2

Concentrations of the analysed biochemical parameters in patients with TUB, <i>Me</i> and <i>MeL</i> [Q_1 ; Q_3]		
Marker	Patients with TUB <i>n</i> = 87	Healthy donors <i>n</i> = 20
MMP-1L (ng/mL)	1.74 [1.31; 2.30] <i>p</i> = 0.002	1.17 [0.89; 1.72]

Table 2 (continued)

Marker	Patients with TUB <i>n</i> = 87	Healthy donors <i>n</i> = 20
MMP-8L (ng/mL)	3.27 [2.64; 3.94] <i>p</i> = 0.003	2.58 [2.22; 2.70]
MMP-9 (ng/mL)	1638.00 [950.80; 2557.69] <i>p</i> = 0.00004	71.99 [51.33; 73.94]
MMP-3L (ng/mL)	1.55 [1.07; 2.16]	1.87 [1.57; 2.07]
TIMP-1L (ng/mL)	6.72 [6.58; 6.89]	6.66 [6.55; 6.80]
MG (ME)	1.70 [1.40; 2.16] <i>p</i> = 0.00003	3.00 [2.46; 3.28]
NE (ME)	195.60 [173.90; 217.30] <i>p</i> = 0.0002	163.00 [152.10; 173.90]
PI (ME)	1.82 [1.27; 2.17]	1.20 [0.91; 1.31]
GP (g/L)	1.56 [1.08; 2.14] <i>p</i> = 0.01	1.04 [0.90; 1.10]
AGP (g/L)	1.10 [0.86; 1.69]	0.96 [0.88; 1.08]

Note: TUB – pulmonary tuberculoma; MMP – matrix metalloproteinases; TIMP-1 – tissue inhibitor of metalloproteinases; MG – α_2 -macroglobulin; NE – neutrophil elastase; PI – proteinase inhibitor; GP – haptoglobin; AGP – orosomucoid; *p* – a statistical significance threshold compared to healthy donors (Mann – Whitney U test).

We found a direct correlation between the concentrations of MMP-9 and MMP-8 ($r = 0.44$, $p \leq 0.014$) and the NE activity ($r = 0.23$, $p \leq 0.05$), as well as between the levels of TIMP-1 and MMP-9 ($r = 0.31$,

$p \leq 0.009$). Given the ability of NEs to act as a pro-MMP activator in the blood, it can be assumed that the absence of changes in the concentrations of its main inhibitor PI indirectly contributed to the growth of MMP-1. A negative correlation ($r = -0.46$, $p \leq 0.004$) between PI and MMP-1 [13] was found. According to the significant differences between the increase in MMP-1 and MMP-8 concentrations and a decrease in TIMP-1 in the blood, the proteolytic processes intensified as the TUB size grew. Consequently, the degradation of ECM during the formation of TUB was associated with an imbalance between proteinases and inhibitors, namely, the prevalence of the two classes of proteinase (MMP and NE) concentrations. This information is consistent with the reports stating that mycobacterial infection is able to enhance the expression and secretion of MMP, but not TIMP-1 [14].

The statistical analysis showed that morphologically, neither gender (Fisher's test), nor age or duration of chemotherapy (Kruskal – Wallis test) correlate with the intensity of the TUB inflammation process: all results had statistical significance over 0.70. At the same time, analysis of the interrelation between the biochemical parameters of destruction and the morphological signs of the inflammatory activity revealed associations with changes in the MMPs levels (Table 3)*.

Table 3

Concentrations of the analysed biochemical parameters with regard to the morphological signs of the inflammatory activity in TUB, Me and MeL [Q_1 ; Q_3]				
Marker	Parameters of caseous necrosis (Signs of melting)		Changes in the capsule	
	No <i>n</i> = 23	Yes <i>n</i> = 64	Single-layered <i>n</i> = 34	Double-layered <i>n</i> = 53
MMP-1L (ng/mL)	1.59 [1.10; 1.83]	1.96 [1.48; 2.46] <i>p</i> = 0.04	1.82 [1.57; 2.40]	1.78 [1.26; 2.69]
MMP-8L (ng/mL)	3.28 [2.87; 3.87]	2.96 [2.46; 3.52]	2.84 [2.62; 3.26]	3.37 [2.92; 3.73] <i>p</i> = 0.02
MMP-9 (ng/mL)	1575.63 [916.30; 2195.86]	2,060.67 [913.68; 2,988.27]	1,647.07 [936.91; 2,228.03]	1,623.43 [916.29; 2,253.56]
MMP-3L (ng/mL)	1.56 [1.10; 1.84]	1.50 [0.86; 2.13]	1.41 [0.87; 1.77]	1.64 [1.07; 2.14]
TIMP-1L (ng/mL)	6.70 [6.60; 6.86]	6.66 [6.57; 6.86]	6.79 [6.65; 6.82]	6.64 [6.65; 6.93]
MG (ME)	1.84 [1.40; 2.12]	2.20 [1.36; 3.09] <i>p</i> = 0.04	1.82 [1.6; 2.22]	2.11 [1.44; 2.72] <i>p</i> = 0.04
NE (ME)	197.33 [173.90; 217.30]	205.68 [162.90; 241.83]	196.61 [179.30; 217.30]	201.02 [168.43; 225.47]
PI (ME)	1.69 [1.27; 2.15]	1.80 [1.55; 2.09]	1.63 [1.45; 1.84]	1.66 [1.22; 2.15]

Note. TUB – pulmonary tuberculoma; MMP – matrix metalloproteinases; TIMP-1 – tissue inhibitor of metalloproteinases; MG – α_2 -macroglobulin; NE – neutrophil elastase; PI – proteinase inhibitor; GP – haptoglobin; AGP – orosomucoid; * *p* – a statistical significance threshold between the groups (Mann – Whitney U test).

With perceptible signs of melting, the values of MMP-1 and MG were higher (although the latter was lower than the control level in both cases) than with no signs of melting. The obtained results appear logically conclusive, as the centre of the granuloma, represented by caseous masses, is formed from destroyed macrophages that die upon contact with the MTB and release of proteinases, which violates the protease/antiprotease balance. This is consistent with the opinion of A. Kubler (2015) on the leading role of MMP-1 in the formation of the caseous centre [15].

A double-layered capsule composed of collagenous connective tissue (in the outer layer) and granulation tissue with macrophages, epithelioid cells and Langhans cells (in the inner layer) is formed during the transition from stabilisation to progression phase. Compared to the single-layered fibrous capsule, the double-layered one is associated with a more pronounced increase in MMP-8 / MG values. This is consistent with the published data on the increase in the number of granulocytes and their phagocytic activity with active TUBs [5].

Similarly, in contrast to the inflammation process of moderate intensity (level 3 of disease activity) and low intensity (level 2 of disease activity), acute inflammation process of high intensity (levels 4 and 5 of disease activity) revealed an increase in the concentrations of MMP-1 ($p = 0.03$) and MMP-9 ($p = 0.04$) and a decrease in the MG activity ($p = 0.003$). Besides, the values of MMP-3, TIMP-1 and PI were within the specific reference ranges, while the concentrations of NE were above the upper end of the reference range, regardless of the intensity of the inflammatory activity in terms of morphology and its constituent characteristics (Kruskal–Wallis test).

Consequently, the progression of the inflammation process is reflected in a more pronounced imbalance in the proteinase/inhibitor system, which is consistent with the literature data [16]. Moreover, this is true for all patients with at least one TUB lesion. In other words, the number of TUBs is irrelevant to the case.

PCM was used to classify the patients according to the degree of the inflammatory activity level. 8 markers of ECM destruction were analysed. For a more complete assessment, the analysis included data on the status of such multifunctional APRs as GP (one of its functions is the activation of pro-MMP-1) and AGP (it activates fibrogenesis) (Table 2). 32 DFs, separating patients with the lowest inflammatory activity level 2 (DF1) and the highest levels 4 and 5 (DF2) from the rest of the patients with the accuracy 80–92%, were

obtained as outcome indicators of PCM associated with different levels of disease activity (Table 4)*.

Table 4

The most informative combinations of the analysed biochemical parameters, which determine the accuracy of inflammatory activity assessment in TUB			
Parameters	Most informative combinations of concentrations of the analysed biochemical parameters and their weight	Level of inflammatory activity	Prediction accuracy (%)
DF 1	MMP-8 (1.13). NE (0.02). PI (–1.03).	2/3–5	86
	MMP-8 (1.34). MG (1.14). AGP (–1.29).	2/3–5	83
DF 2	MMP-1(0.55). MMP-3 (0.089). MG (–1.53)	2–3/4–5	92

Note. TUB – pulmonary tuberculoma; MMP – matrix metalloproteinases; MG – α_2 -macroglobulin; NE – neutrophil elastase; PI – proteinase inhibitor; AGP – orosomucoid.

Taking into consideration high interdependence among the DFs, determined by the method of their construction, we will limit ourselves to interpreting only a few of them. Separation of the patients with low-level inflammatory activity (level 2 of disease activity) from the rest of the patient population (levels 3 to 5) reflects neutrophil characteristics (DF1). This is characterised by lower values of neutrophil collagenase (MMP-8) and neutrophil degranulation marker (NE) in combination with a high concentration of its inhibitor (PI) according to the values of the correlation coefficient between proteinases and the inhibitor. When we consider the progression from inflammation of low and moderate intensity (levels 2-3 of disease activity) to severe inflammation (levels 4-5 of disease activity) (DF2), the combination of the concentrations of MMP-1 and stromelysin is of utmost significance. This is consistent with the published reports on the decisive role of the MMP-3 / TIMP-1 correlation in the destruction of ECM [17].

The use of PCM for assessing the morphological signs of the inflammation process enabled to identify the areas typical of patients with level 2 and levels 4–5 of the disease activity. Classification of patients with level 3 of the disease activity posed the greatest challenge. The disease in this group of patients may develop in either direction. This indicates not so much the limitations of the method as the unavoidable simplification of the mathematical model of the polymorphic picture of the inflammation process. Only two

morphological parameters – ‘the nature of the caseous masses’ and ‘the state of the capsule’ – were taken into account. Apparently, for a clearer classification, it is required to analyse more data with the inclusion of additional variables, such as screenings in the surrounding lung tissue, bronchial lesions, and regional lymph nodes.

With the example of TUB, the applicability of the PCM for assessing the inflammatory activity with the use of combinations of three ECM destruction markers (with or without APR) with prediction accuracy of 80-92% was shown. The proposed method enables a deeper understanding of a variety of biochemical manifestations of tissue and cellular inflammatory mechanisms. The absence of significant differences in most indicators of ECM destruction in their isolated assessment based on the morphological characteristics does not exclude their significant contribution to the formation of different morphological features.

In conclusion, it should be noted that when choosing the treatment strategy for patients with TUB, biochemical data assessing the intensity of the inflammation process should be taken into consideration along with a set of clinical and radiological features.

CONCLUSION

TUB, as a clinical form of secondary PT, is characterised by an increase in the levels of different classes of proteinases in peripheral blood. This contributes to a shift in the proteinase/inhibitor system balance towards proteinases. An increase in the concentrations of collagenases (MMP-1, MMP-8) and gelatinase (MMP-9) is observed, while stromelysin (MMP-3) and TIMP-1 remain at the control level along with low MG activity and uncompensated increase in the NE level.

There is a correlation between the markers of ECM destruction in peripheral blood and the morphological characteristics of the disease activity. Thus, the combination of MMP-1 and MG is an indicator for assessing the alternative component of inflammation (presence of caseous necrosis in the TUB centre), while the MMP-8 / MG system serves as a diagnostic criterion for its productive component (granulomatous changes in the capsule).

Using TUB as an example, the applicability of PCM for assessing the morphological features of the inflammation process using a number of combinations of three markers of ECM destruction (with or without APR) in peripheral blood with prediction accuracy of

80–92% was shown. They can be used as additional criteria in determining the treatment strategy for patients with TUB.

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Esmedlyeva D. S. – conception and design, preparation of samples, collection of materials, carrying out of biochemical research, analysis of literature, analysis and interpretation of data, drafting of the manuscript. Alekseeva N. P. – statistical analysis of the findings, drafting of the manuscript. Novitskaya T. A. – morphological research. Dyakova M. E. – biochemical research. Ariel B. M. – interpretation of data, drafting of the manuscript. Grigoriev I.V. – conception, editing of the manuscript. Sokolovich Ye. G. – conception, final approval of the manuscript for publication.

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Received 19.03.2019

Accepted 25.12.2019