Epitopes specificity of antibodies to thyroid peroxidase in patients with Graves’ disease, Hashimoto’s thyroiditis and overlap-syndrome

Maira Espenbetova a, Nina Kuzmina b, Alexandr Zubkov c, Venera Akhmetova a, Zhanar Zamanbekova a, Ainur Krykpaeva d, Zhanar Zhumanbayeva e, Kuralay Amrenova f, Zhanargul Smailova g, Natalya Glushkova h,*,

a Department of Endocrinology, Semey Medical University, Semey, Kazakhstan
b Testing Laboratory for Evaluation of Medicinal Products’ Quality, I.I. Mechnikov Research Institute, Moscow, Russia
c Research Laboratory, Department of Immunology, I.I. Mechnikov Research Institute, Moscow, Russia
d Research Department, Semey Medical University, Semey, Kazakhstan
e Department of Nursing, Semey Medical University, Semey, Kazakhstan
f Department of Propaedeutics of Internal Diseases, Semey Medical University, Semey, Kazakhstan
g Department of Biochemistry and Chemical Disciplines Named After Doctor of Medical Sciences, Semey Medical University, Semey, Kazakhstan
h Department of Epidemiology, Biostatistics & Evidence Based Medicine, Al-Farabi Kazakh National University, Almaty, Kazakhstan

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ABSTRACT

Background: Antibodies against thyroid peroxidase (anti-TPO) serve as clinical markers of thyroid autoimmune diseases (TAIDs). By trying to elucidate the causes of heterogeneity in autoantibody levels among patients with different TAIDs it becomes possible to clarify the pathophysiology of GD and HT.

Objective: To investigate the heterogeneity of epitopes recognized by anti-TPO in patients with Hashimoto’s thyroiditis (HT), Graves’ disease (GD) and overlap-syndrome.

Methods: We carried out a cross-sectional study on 398 patients with GD, HT and overlap syndrome and analyzed the specificity of epitopes and binding constants of TPO with monoclonal antibodies (MAbs). Ten MAbs to TPO were used, of which five were reactive with native TPO and the rest were reactive with denatured TPO.

Results: The autoantibodies in blood serum of HT patients inhibited the binding of MAb63 more significantly than those in serum of GD patients: 59.62 % versus 54.02 %, respectively (p = 0.001). The anti-TPOs in serum of GD patients inhibited the binding of MAb77 more significantly than those in serum of HT patients: 54.36 % versus 51.13 %, respectively (p = 0.047). The binding of MAb45 was more inhibited in serum of patients with anti-TPO concentration over 1000 IU/ml (58.36 %). The blood serum of patients with overlap-syndrome showed less significant inhibition of MAb63 binding than that of patients with no overlap-syndrome: 52.47 % versus 58.81 %, respectively (p = 0.043).

Conclusion: Mapping the epitopes to TPO with the help of MAbs may improve the differential diagnosis between different thyroid autoimmunities.

Introduction

Perhaps, the most frequent organ-specific autoimmune disorders in humans are those affecting thyroid, i.e., Graves’ disease (GD) and Hashimoto’s thyroiditis (HT). These are observed more frequently than type 1 diabetes mellitus and multiple sclerosis: the prevalence of Graves’ disease in the general population is around 1 %, while autoimmune thyroiditis is presented in approximately 15 % of adult females, although predominantly in subclinical form [1–2]. Both HT and GD are accompanied by circulation of anti-thyroid auto-antibodies and infiltration of thyroid by auto-reactive lymphocytes. Females with a positive family history of autoimmune disease belong to the at-risk group [3].

Routinely, the development of HT was attributed to a cellular autoimmune response with inflammatory infiltration producing damage to thyroid that subsequently leads to inability to function properly. In contrast, humoral autoimmune response was attributed to GD because

* Corresponding author at: Department of Epidemiology, Biostatistics & Evidence Based Medicine, Al-Farabi Kazakh National University, Al-Farabi 71, Almaty 050040, Kazakhstan.
E-mail address: glushkovanatalyae@gmail.com (N. Glushkova).

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of thyrotropin receptor autoantibodies (TRAb) that suppress the functioning of thyroid follicular cells (TFC). In fact, both types of autoimmune responses are closely inter-related and stimulation of one type of autoimmune response leads to a cascade of molecular mechanisms causing inhibition of the other type [4].

Antibodies against thyroid peroxidase (anti-TPO), thyroid stimulating hormone receptor (anti-RTTG) and thyroglobulin (anti-Tg) serve as clinical markers of thyroid autoimmune diseases (TAIDs) [5–7]. There is a range of modern studies devoted to identification of anti-TG, anti-TPO and anti-RTTG epitopes, which may vary in different individuals [8]. Commonly, anti-TPOs recognize conformational epitopes located at the molecular surface but they are also capable of recognizing the linear determinants on TPO that appear at advanced stages of inflammation. These conformational epitopes are limited by two dominant determinants – immune-dominant domain regions A and B [9]. It remains unclear what is the exact contribution of anti-TPOs to the pathogenesis of TAIDs but it might be hypothesized that they directly destroy the TFC via cytotoxic mechanisms [6–7,10].

Currently, anti-TPOs are detected in blood serum of patients with HT in almost 100 % of cases and are slightly less prevalent in patients with GD – 50–90 %. However, anti-TPOs are also frequently detected in blood serum of the general population and they are 5 times more common in women as compared with men [2]. Although the clinical significance for individuals with unimpaired thyroid function remains unclear, anti-TPO levels in patients with GD correlate with the intensity of thyroid histological damage [11] Anti-TPOs are capable for complement fixation and thus it might be concluded that they take part in lysis of thyrocytes. Anti-microsomal antibodies (anti-MCAbs) are heterogeneous in terms of their cytotoxicity. Such, a threefold increase in the number of anti-TPO amino acids 513–633 was identified in blood serum of HT patients as compared with blood serum of GD patients [12]. In fact, by trying to elucidate the causes of such heterogeneity in autoantibody levels among patients with different TAIDs it becomes possible to clarify the pathophysiology of GD and HT and the use of monoclonal antibodies (MAb) could be helpful [13]. This study was aimed at investigating the heterogeneity of epitopes recognized by anti-TPO in patients with HT, GD and overlap-syndrome.

Materials and methods

Study design

This was a cross-sectional study that comprised data on 398 patients with GD and HT with mean age 47.18 ± 14.99 years. The patients were enrolled from January 1, 2016 to December 31, 2019.

Patients

The study participants were enrolled randomly, from the list of patients followed due to GD and HT by all endocrinologists at outpatient care settings. Before the decision on inclusion into the study was made, each patient was consulted by two experts in endocrinology. This consultation included verification of the diagnosis, objective clinical evaluation, thyroid function tests and thyroid ultrasonography. The same investigators examined each patient every six months to monitor his/her condition. According to their follow-up status all patients were classified as “primary patients” or patients with initial presentation of thyroid disease versus “patients under follow-up” or “patients with pre-existing thyroid disease”.

Thyroid function tests

Thyroid-stimulating hormone (TSH), free thyroxine (FT4), and ultrasound (US) were performed in all patients prior to study enrollment and on each subsequent visit (once in 6 months) within the study period.

Verification of overlap-syndrome in TAID

The diagnosis of overlap-syndrome was made when a patient had sufficient clinical and laboratory findings to diagnose certain TAID (for instance, HT) but also showed the signs of another disorder, like GD. Otherwise, overlap-syndrome was diagnosed when we observed a transformation of one disorder into another within the period of entire follow-up (for instance, GD transformed into HT) [14].

In the study group, 41 patients with an initial diagnosis of GD were diagnosed with the transition to the state of HT. Thus, in the general group of patients, a group with the development of overlap syndrome was identified.

Testing specificity of epitopes recognized by anti-TPO

Epitopes in TAID (GD and HT) and binding constants of TPO with monoclonal antibodies (MAbs) were studied in the laboratory of immunoenocrinology of Mechnikov’s Scientific Institute for Vaccines and Sera, Moscow, Russian Federation.

We used ten MAb to TPO, of which five were reactive with native TPO and the rest were reactive with denaturated TPO (Table 1). The epitope specificity was studied by the method of competitive ELISA. MAb in a dilution corresponding to 50 % binding to TPO was preliminarily introduced to the well plate sensitized with TPO at a concentration of 1.0 μg/ml. Wells containing MAb to horseradish peroxidase (HRP) were used as a control. The plate was incubated for 1 h at a temperature of 37°C. After the washing step, blood serum samples collected from TAID patients were added to the wells. The plate was kept for 1 h at 37°C washed, and mouse MAb conjugated with HRP to human IgG in a working dilution was added and incubated for 1 h at 37°C. The reaction was developed with a chromogenic substrate. The optical density of samples without antibodies was taken as 100%, inhibition of binding by 60% or more was considered as complete, 30–59 % inhibition was considered as partial, and less than 30 % inhibition was graded as unreliable. The degree of binding of MAb-HRP made it possible to determine the specificity of AT pair used [15–17]. The monoclonal antibodies used were synthesized by the laboratory of the Mechnikov’s Scientific Institute for Vaccines and Sera and are the part of its collection.

Anti-TPO in human serum was tested by ELISA. As a solid phase, we used the plates sensitized with human TPO at a concentration of 5.0–7.5 μg/ml. AT were detected with a conjugate of MAb to human IgG labeled with HRP in a working dilution. Calibration samples were prepared on the basis of human blood sera and evaluated according to the international standard (AT MTF No. 66/387). The method’s sensitivity was 10 IU/ml, and the determined concentrations ranged from 25 to > 1000 IU/ml. The level of anti-TPO in blood serum of 600 healthy donors did not exceed 50 IU/ml and thus, concentrations below 50 IU/ml were considered as normal [17].

Statistical analyses

The quantitative variables were presented as median with 25th – 75th percentiles in cases of asymmetrical distribution and as mean ± standard deviation when the data distribution was close to normal. The qualitative data were presented as absolute numbers and percentages.

| Antigen         | Number of MAb | MAb titers in supernatants | MAb titers in ascites |
|-----------------|---------------|----------------------------|-----------------------|
| Native TPO      | 3, 10, 45, 63, 77 | 10^6.10^3                  | 10^7.10^7             |
| Denaturated TPO | 1, 70, 82     | 10^6.10^2                  | 10^11.10^6           |
Mann-Whitney U test was used to compare the quantitative variables, while Pearson’s chi-squared test was used to compare the qualitative variables. The significance level of the differences observed was preset as p less than 0.05. All statistical tests were carried-out in SPSS 20 statistical software.

**Ethical statement**

The informed consent form was signed by each patient before the study beginning. The study protocol was considered by the Local Institutional Ethical Committee and the permission was granted (Protocol 2 dated October 25, 2018).

**Results**

Table 2 presents the demographic characteristic of study participants. The vast majority of patients were females both in the subgroup of HT (93.3 %) and in the subgroup of GD (70.7 %). The mean age of patients was very similar: 47.77 years for patients with HT and 45.75 years for patients with GD. Most patients in this study were of Kazakh ethnicity, which corresponds with the current demographic situation in the country of Kazakhstan (hereafter – Kazakhstan). The proportions of patients with urban/rural residence were very similar. Most patients were already followed-up at the moment of study enrollment: 69.9 % in the HT subgroup and 71.8 % in the GD subgroup.

The distribution of eight different MAb to TPO in patients with HT and GD is presented in Table 3. Such, the autoantibodies in blood serum of HT patients inhibited the binding of MAb63 more significantly than those in serum of GD patients: 59.62 % (48.99 %; 67.80 %) versus 54.02 % (46.32 %; 62.63 %), respectively. This difference allows supposing that MAb63 is a promising biomarker of HT. The anti-TPO in serum of GD patients inhibited the binding of MAb77 more significantly than those in serum of HT patients: 54.36 % (36.90 %; 68.03 %) versus 51.13 % (31.00 %; 62.25 %), respectively.

The only statistically significant difference in binding of MAb in dependence with the concentration of anti-TPO was observed in relation to MAb45, the binding of which was more inhibited in serum of patients having anti-TPO concentration over 1000 IU/ml (58.36 %) as compared with those presenting with anti-TPO concentration below 1000 IU/ml (52.37 %). Although the inhibition of MAb45 binding was more expressed in patients having anti-TPO concentration over 1000 IU/ml, no correlation between these indicators was identified and thus, it is rather unlikely that this biomarker is of clinical significance. However, more studies with larger sample sizes are needed to clarify this issue (Table 4).

Table 5 reflects the distribution of different MAbs to TPO in relation to overlap syndrome. The blood serum of patients with overlap-syndrome showed less significant inhibition of MAb63 binding (52.47 %) than that of patients with no overlap-syndrome (58.81 %). However, the anti-TPO in serum of patients with overlap syndrome inhibited the binding of MAb82 more significantly than those in serum of patients with no overlap syndrome: 67.68 % (50.02 %; 76.03 %) versus 56.51 % (28.37 %; 71.88 %), respectively.

**Discussion**

Since TPO is a thyroid specific autoantigen essential to its proper functioning, further research that may shed light on specificity of MAbs in various TAIIDs is strongly needed. For this reason, our study devoted to investigation of heterogeneity of epitopes recognized by anti-TPO in patients with HT, GD and overlap-syndrome may help to clarify this issue. By studying the MAbs to TPO, we identified that the significance of various MAbs in HT, GD and overlap-syndrome is different and this might be influenced by concentration of TPO in blood serum.

According to the clinical guidelines of the American Thyroid Association, a clinically expressed form of GD is treated with radioactive iodine, thyroid surgery, and/or methimazole [18]. The search for diagnostic markers useful for clinical decision-making about the choice of therapy is crucial.

The pronounced inhibition of MAb 82 binding in overlap syndrome can be considered as one of the potential marker of a favorable course of GD, in which aggressive therapy is not required with the development of significant consequences, such as secondary cancer, pulmonary fibrosis, permanent suppression of bone marrow function and genetic effects [19].

In fact, TPO presents a large membrane-bound glycoprotein, which contributes to the synthesis of thyroid hormones by inducing the iodination of tyrosyl residues. Also, TPO induces the synthesis of thyroglobulin mono-iodotyrosine and thyroglobulin di-iodotyrosine [20]. The molecule of TPO is composed of 933 amino acids and of these the first 735 are homologous with myeloperoxidase (MPO) [21]. Although the structure of TPO molecule is understood, there is lack of knowledge on its topography. Over the past decades a number of panels of MAbs were developed and most of them were isolated from patients with HT and GD. Anti-TPOs bind TPO on the surface of thyroid cells and predominantly interact with a specific part of TPO molecule called “immunodominant region”, which is mostly constituted by the MPO-like domain [22].

To a certain extent, our study complements the findings of several earlier studies. According to Zubkov and co-authors, eight MAbs to epitopes 1, 70, 82, 88, 2, 3, 77, and 79 are involved in the competition for TPO binding sites with autoantibodies in the blood serum of GD and HT patients. The maximum inhibition of binding was observed for autoantibodies directed against MAb3, which was 60.3 % for the patients with GD and 61.8 % for the patients with HT. Moreover, the degree of inhibition of binding did not depend on the concentration of anti-TPO in the blood serum of GD patients, in contrast with what has been observed in the serum samples of HT patients [23]. This was in agreement with our findings: autoantibodies in blood serum of HT patients inhibited the

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**Table 2** Demographic characteristics of the study participants in relation to their diagnosis (n = 398).

| Variable             | Diagnosis          | N  | %  | N  | %  |
|----------------------|--------------------|----|----|----|----|
|                      | Hashimoto’s thyroiditis |   |    |    |    |
|                      | Graves’ disease    |    |    |    |    |
| Sex                  | Male               | 19 | 6.7| 34 | 29.3|
|                      | Female             | 263| 82 | 70.7|
| Age (mean ± standard deviation) |                | 47.77 ± | 45.75 ± | 14.64 | 15.79|
| Ethnicity            | Kazakh             | 208| 73.8| 99 | 84.6|
|                      | Russian            | 74 | 26.2| 18 | 15.4|
| Country’s region     | East Kazakhstan region | 273| 96.8| 109| 93.2|
|                      | South Kazakhstan region | 5 | 1.8 | 6 | 5.1|
|                      | North Kazakhstan region | 4 | 1.4 | 2 | 1.7|
| Place of residence   | Urban              | 122| 43.3| 50 | 42.7|
| Follow-up status     | Rural              | 160| 56.7| 67 | 57.3|
|                      | Primary patient    | 85 | 30.1| 33 | 28.2|
|                      | Patient under follow-up | 197| 69.9| 84 | 71.8|
| Smoking              | No                 | 271| 96.1| 83 | 70.9|
|                      | Yes                | 11 | 3.9 | 34 | 29.1|
| Employment           | Unemployed         | 58 | 20.6| 35 | 29.9|
|                      | Employed           | 224| 79.4| 82 | 70.1|
binding of MAb3 more than those in serum of GD patients (62.93 % versus 60.90 %, respectively). Still, this difference did not reach the level of statistical significance. Also, Zubkov and co-authors noted that anti-TPO in blood serum of HT patients suppressed the binding of MAb77 more than those in the sera of GD patients: 36.3 % and 54.3 %, respectively [24]. This was not in line with our findings: there was more marked inhibition of MAb77 in serum of GD patients as compared with the serum of HT patients: 54.36 % versus 51.13 %, respectively. Ruf and co-authors [15] found out that both in blood sera of HT and GD patients there was only mild inhibition of binding for autoantibodies targeted against MAb1 and Bossowski with co-authors [16] came to a similar conclusion for blood serum of GD patients. Still, this was not confirmed by our findings as the degree of inhibition of MAb1 was stronger and constituted 57.05 % for HT and 62.65 % for GD patients.

According to the data of earlier studies, the degree of inhibition was higher in blood serum of patients containing high levels of antibodies to TPO (>1000 IU/ml) and constituted 70–90 % on average. Meanwhile, the samples containing medium (200–500 IU/ml) and low concentrations of autoantibodies (100–200 IU/ml) were characterized by the lower degree of inhibition: 40–60 % and 20–30 %, respectively [17]. This coincided with our findings but not completely: although blood sera containing high (>1000 IU/ml) levels of anti-TPO inhibited the binding of MAbs stronger, this inhibition did not exceed the level of 67.10 %. As for the coincidence of HT and GD, the so-called “overlap syndrome”, we failed to find a single study on heterogeneity of anti-TPO epitopes. For this reason, our study could be regarded as the first attempt to provide insights on epitopes specificity in this relatively uncommon thyroid autoimmunity.

Thyroid disorders, including TAIDs, are prevalent in Kazakhstan and their significance cannot be underestimated due to the activity of the former Semipalatinsk Nuclear Test Site – the largest Soviet nuclear weapons testing ground located in Eastern Kazakhstan, from where most of the study participants were enrolled [25]. Also, from 2010 to date Kazakhstan is the leading global producer of uranium ore and many mines are situated at the territory of Eastern Kazakhstan [26]. In general, the environmental status of Eastern Kazakhstan is rather

| Table 3 | Inhibition of MABs binding in relation to the type of thyroid autoimmune disorder (n = 398). |
|---------|------------------------------------------------------------------------------------------|
| Epitopes | Diagnosis | p-value | Hashimoto’s thyroiditis | | | | Graves’ disease | |
| MAB1 | Median(%) | 25th percentile(%) | 75th percentile(%) | Median(%) | 25th percentile(%) | 75th percentile(%) | |
| MAB3 | 62.93 | 44.50 | 72.65 | 60.90 | 42.85 | 68.79 | 0.146 |
| MAB10 | 66.92 | 45.83 | 75.44 | 65.18 | 42.30 | 73.57 | 0.272 |
| MAB45 | 54.99 | 38.80 | 63.33 | 50.49 | 37.45 | 60.11 | 0.113 |
| MAB63 | 59.62 | 48.99 | 67.80 | 54.02 | 46.32 | 62.63 | 0.001 |
| MAB70 | 63.05 | 55.20 | 68.81 | 65.76 | 54.52 | 72.70 | 0.057 |
| MAB77 | 51.13 | 31.00 | 62.25 | 54.36 | 36.90 | 68.03 | 0.047 |
| MAB82 | 57.27 | 30.08 | 73.31 | 56.63 | 32.33 | 70.33 | 0.442 |

| Table 4 | Inhibition of MABs binding in relation to the concentration of anti-TPO antibodies (n = 398). |
|---------|------------------------------------------------------------------------------------------|
| Epitopes | Concentration of anti-TPO antibodies | p-value | less than 1000 IU/ml | > 1000 IU/ml | |
| MAB1 | Median(%) | 25th percentile(%) | 75th percentile(%) | Median(%) | 25th percentile(%) | 75th percentile(%) | |
| MAB3 | 60.81 | 41.59 | 70.63 | 66.81 | 49.06 | 74.01 | 0.066 |
| MAB10 | 66.08 | 44.60 | 74.93 | 65.86 | 44.51 | 76.39 | 0.954 |
| MAB45 | 52.37 | 37.80 | 61.80 | 58.36 | 39.98 | 67.05 | 0.012 |
| MAB63 | 57.90 | 47.23 | 65.35 | 60.15 | 50.22 | 68.18 | 0.218 |
| MAB70 | 62.95 | 54.63 | 69.51 | 67.10 | 58.71 | 72.27 | 0.053 |
| MAB77 | 51.27 | 31.00 | 62.31 | 54.69 | 39.15 | 65.43 | 0.091 |
| MAB82 | 57.01 | 30.41 | 71.88 | 59.22 | 26.75 | 72.59 | 0.970 |

| Table 5 | Inhibition of MABs binding in dependence with the presence of overlap syndrome (n = 398). |
|---------|------------------------------------------------------------------------------------------|
| Epitopes | Overlap syndrome | p-value | Absent | Present | |
| MAB1 | Median(%) | 25th percentile(%) | 75th percentile(%) | Median(%) | 25th percentile(%) | 75th percentile(%) | |
| MAB3 | 62.48 | 44.20 | 71.95 | 57.71 | 41.21 | 69.22 | 0.375 |
| MAB10 | 66.74 | 45.83 | 75.44 | 56.71 | 33.45 | 73.57 | 0.070 |
| MAB45 | 54.95 | 39.00 | 63.11 | 47.29 | 27.24 | 57.79 | 0.078 |
| MAB63 | 58.81 | 47.61 | 66.16 | 52.47 | 45.47 | 62.00 | 0.043 |
| MAB70 | 63.54 | 55.06 | 69.70 | 63.83 | 55.32 | 70.23 | 0.945 |
| MAB77 | 52.21 | 32.20 | 63.61 | 50.98 | 36.47 | 61.95 | 0.925 |
| MAB82 | 56.51 | 28.37 | 71.88 | 67.68 | 50.02 | 76.03 | 0.008 |
compromised [27–28] and this might contribute to the incidence of TAIDs [29]. Besides, Kazakhstan is an iodine-deficient area [30] and this plays a role in the spread of thyroid disorders. Finally, Vitamin D deficiency is prevalent in Kazakhstan [31] and reduction of Vitamin D circulating levels has been recently reported to play a role in TAIDs [32]. This study has a number of drawbacks that deserve to be considered in detail. Firstly, the sample size was relatively small with respect to the prevalence of TAIDs. Secondly, most patients in our study were already under the follow-up at the moment of their enrollment. Thus, many of them were receiving treatment, which might have influenced the levels of anti-TPO. Thirdly, this study had a cross-sectional design and cohort studies are needed to analyze the associations between anti-TPO, MABs and thyroid function in patients with different TAIDs and at different treatment stages. Nevertheless, the studies targeted at improved diagnosis of different disorders are needed as they are associated with better patient satisfaction, among other benefits [33,34].

Conclusion

Thus, by mapping epitopes to TPO with the help of MABs it becomes possible to investigate the heterogeneity of human anti-TPO autoantibodies in patients with various thyroid autoimmune disorders. It should be assumed that evaluation of competitive interactions with other MABs that were not included in this study as well as the expansion of a panel of sera obtained from patients with various thyroid pathologies, can enable the understanding of immunodominant epitopes of autoantigens that determine the specificity of thyroid autoantibodies. In turn, this may improve the differential diagnosis between different thyroid autoimmunities.

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Clinical relevance

The pronounced inhibition of MAB 82 binding in overlap syndrome can be considered as one of the potential marker of a favorable course of GD, in which aggressive therapy is not required with the development of significant consequences, such as secondary cancer, pulmonary fibrosis, permanent suppression of bone marrow function and genetic effects.

CRedit authorship contribution statement

Maira Espenbetova: Conceptualization, Supervision, Nina Kuzmina: Methodology, Alexandr Zubkov: Methodology. Venera Akhmetova: Investigation, Data curation. Zhanar Zamanbekova: Investigation, Data curation. Ainaur Krykpaeva: Investigation, Data curation. Ainur Krykpaeva: Investigation, Data curation. Natalya Glushkova: Formal analysis, Software, Writing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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