THE DIVERSITY OF IMMUNE REACTIONS TO BALNEOTHERAPY AND THEIR ACCOMPANIMENTS

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Abstract

Background. Earlier have been shown that the immune responses to course of drinking of Naftussya bioactive water from Truskavet’s spa are ambiguous and individual. However, at Truskavets’ spa water monotherapy is a rare exception for specific contingents, whereas the vast majority of patients use a balneotherapy complex: drinking of Naftussya, application of ozokerite and mineral baths. The immune responses to balneotherapeutic complex are also ambiguous which is a separate manifestation of the multivariate effects of balneological agents as well stressors on the body. Therefore, the purpose of this study is to analyze variants of immune responses to balneotherapeutic complex of Truskavets’ spa. Material and methods. The object of observation were 34 men and 10 women aged 24-70 years old, who came to the Truskavets’ spa for the treatment of chronic pyelonephritis combined with cholecystitis in remission. The survey was conducted twice, before and after balneotherapy (drinking Naftussya bioactive water three times a day, ozokerite applications, mineral baths every other day for 7-10 days). Immune status evaluated on a set of I and II levels recommended by the WHO. In portion of capillary blood we counted up Leukocytogram and calculated two variants of Adaptation Index as well as two variants of Strain Index by IL Popovych. We calculated also the Entropy of Immunocytogram and Leukocytogram. The condition of Microbiota is evaluated on the results of sowing of feces and urine. Results. Four variants of the immune responses to balneotherapeutic complex have been identified. In 40,9% of patients, initially normal immune status did not change significantly. In 31,8%, the lower boundary level of immunity is completely normalized. In 22,7% moderate immunosuppression is reduced, but not up to normal. However, in 4,5% of people, initially normal level of immunity are transformed into moderate immunosuppression. Discriminant analysis was conducted to identify exactly the parameters of the immunity and microbiota, in which the four immune response clusters differ significantly from each other. 24 parameters were characteristic, 12 of them related to the immune parameters of the blood, one of the saliva, 5 of the feces microbiota and 3 related to urinary syndrome as well as 4 parameters are information. The other 25 parameters were outside the discriminatory model. Conclusion.
The immunotrophic effect of balneotherapy on certain individuals is not effective enough, and in some cases even unfavorable.

**Key words:** Immunity, Microbiota, Urinary syndrome, Balneotherapy, Truskavets’ spa.

**INTRODUCTION**

Earlier have been shown that the immune responses to course of drinking of Naftussya bioactive water from Truskavet’s spa are ambiguous and individual [11,26-30,32]. However, at Truskavets’ spa water monotherapy is a rare exception for specific contingents, whereas the vast majority of patients use a balneotherapy complex: drinking of Naftussya, application of ozokerite and mineral baths [20]. The immune responses to ozokerite in partial [8,9,20,22] and balneotherapeutic complex in general [13,14,25] are also ambiguous which is a separate manifestation of the multivariate effects on the body of balneological agents as adaptogens as well as stressors [2,4,6,7,16,17,24]. Therefore, the purpose of this study is to analyze variants of immune responses to balneofactors of Truskavets’ spa.

**MATERIAL AND METHODS**

The object of observation were 34 men and 10 women aged 24-70 years old, who came to the Truskavets’ spa for the treatment of chronic pyelonephritis combined with cholecystitis in remission. The survey was conducted twice, before and after ten-day balneotherapy (drinking Naftussya bioactive water three times a day, ozokerite applications, mineral bathrooms every other day) [20].

In portion of capillary blood we counted up Leukocytogram (LCG) (Eosinophils, Stub and Segmentonuclear Neutrophils, Lymphocytes and Monocytes) and calculated two variants of Adaptation Index as well as two variants of Strain Index by IL Popovych [3,13,18].

\[
\text{Strain Index-1} = [(\text{Eo}/3,5-1)^2 + (\text{SN}/3,5-1)^2 + (\text{Mon}/5,5-1)^2 + (\text{Leu}/6-1)^2]/4
\]

\[
\text{Strain Index-2} = [(\text{Eo}/2,75-1)^2 + (\text{SN}/4,25-1)^2 + (\text{Mon}/6-1)^2 + (\text{Leu}/5-1)^2]/4
\]

Immune status evaluated on a set of I and II levels recommended by the WHO as described in the manuals [10,15]. For phenotyping subpopulations of lymphocytes used the methods of rosette formation with sheep erythrocytes on which adsorbed monoclonal antibodies against receptors CD3, CD4, CD8, CD22 and CD56 from company "Granum" (Kharkiv) with visualization under light microscope with immersion system. Subpopulation of T cells with receptors high affinity determined by test of “active” rosette formation. The state of humoral immunity judged by the concentration in serum circulating immune complexes (by polyethylene glycol precipitation method) and Immunoglobulins classes M, G, A (ELISA, analyser “ImmuNochem”, USA). In addition, the saliva level of secretory IgA, IgA and IgG was determined as well as lysozyme (by bacteriolyis of Micrococcus lysodeikticus).

We calculated also the Entropy (h) of Immunocytogram (ICG) and Leukocytogram (LCG) using formulas [19,21,34], adapted from classical CE Shannon’s formula [33]:

\[ h_{LCG} = - [\text{CD4}\log_2 \text{CD4} + \text{CD8}\log_2 \text{CD8} + \text{CD22}\log_2 \text{CD22} + \text{CD56}\log_2 \text{CD56}]\log_2 4 \]

\[ h_{ICG} = - [\text{L}\log_2 \text{L} + \text{M}\log_2 \text{M} + \text{E}\log_2 \text{E} + \text{SN}\log_2 \text{SN} + \text{Stub}\log_2 \text{Stub}]\log_2 5 \]

Parameters of phagocytic function of neutrophils estimated as described by SD Douglas and PG Quie [5] with moderately modification by MM Kovbasnyuk [23]. The objects of phagocytosis served daily cultures of Staphylococcus aureus (ATCC N 25423 F49) as typical specimen for Gram-positive Bacteria and Escherichia coli (O55 K59) as typical representative of Gram-negative Bacteria. Both cultures obtained from Laboratory of Hydro-Geological Regime-Operational Station JSC “Truskavets’kurort”. Take into account the following parameters of Phagocytosis: activity (percentage of neutrophils, in which found microbes -
Hamburger’s Phagocytic Index (PhI), intensity (number of microbes absorbed one phagocytes - Microbial Count MC or Right’s Index) and completeness (percentage of dead microbes - Killing Index KI). On the basis of the recorded partial parameters of Phagocytosis, taking into account the Neutrophils (N) content of 1 L blood, we calculated the integral parameter - Bactericidal Capacity of Neutrophils (BCCN) by the formula [13,23]:

\[
\text{BCCN (10}^9 \text{Bact/L)} = \text{N (10}^9/\text{L)} \times \text{PhI (\%)} \times \text{MC (Bact/Phag)} \times \text{KI (\%)} \times 10^{-4}
\]

In addition, the blood level of cytokines IL-1, IL-6 and TNF-α was determined (by the ELISA with the use of analyzer “RT-2100C” and corresponding sets of reagents from “Diaclone”, France).

The condition of Microbiota is evaluated on the results of sowing of feces and urine.

Norms are borrowed from the database of the Truskavets’ Scientific School of Balneology.

Results processed by methods of cluster [1] and discriminant [12] analyses, using the software package "Statistica 5.5".

**RESULTS AND DISCUSSION**

In order to evaluate the immune responses on a single scale according recommendation by IL. Popovych [7,13] immune variables (V) expressed as Z-scores calculated by formula:

\[
Z = (V/N – 1)/Cv,
\]

where

- N is Mean of Normal Variable,
- Cv is Coefficient its variation.

**Z-scores of eleven key immune parameters** were used to calculate the Immune Status Index (ISI) by the formula:

\[
\text{ISI} = (\text{BCCN vs St. aur.+BCCN vs E. coli}+\text{CIC}+\text{IgM}+\text{IgG}+\text{IgA}+\text{B}+\text{NK}+\text{Th}+\text{Tc}+\text{Ta})/11.
\]

Preliminary analysis [31] has shown that in different patients, individual ISI respond to balneotherapy not only in varying degrees, but even in the opposite way. The next phase was conducted Cluster analysis of ISI before and after balneotherapy. Clustering cohort of persons is realized by iterative k-means method. In this method, the object belongs to the class Euclidean distance to which is minimal. The main principle of the structural approach to the allocation of uniform groups consists in the fact that objects of same class are close but different classes are distant [1].

As a result, four groups of persons were created, significantly different from each other in terms of ISI (Table 1), while the differences between the members of each group were much smaller (Table 2).

**Table 1. Euclidean Distances between Clusters**

Distances below diagonal, Squared distances above diagonal

| Clusters | No. 1 | No. 2 | No. 3 | No. 4 |
|----------|-------|-------|-------|-------|
| No. 1    | 0,00  | 1,34  | .91   | 1,11  |
| No. 2    | 1,16  | 0,00  | .31   | .45   |
| No. 3    | 0,95  | 0,56  | 0,00  | 1,13  |
| No. 4    | 1,05  | 0,67  | 1,06  | 0,00  |
Table 2. Members of Clusters and Distances from Respective Cluster Center

Cluster Number 3 contains 18 cases

| Cas No. | Cas No. | Cas No. | Cas No. | Cas No. | Cas No. | Cas No. | Cas No. | Cas No. | Cas No. | Cas No. | Cas No. | Cas No. | Cas No. | Case No. | Case No. | Case No. | Case No. | Case No. | Case No. | Case No. | Case No. | Case No. |
|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| C18     | C19     | C20     | C21     | C22     | C23     | C24     | C25     | C26     | C27     | C28     | C29     | C30     | C31     | C32     | C33     | C34     | C35     | C36     | C37     | C38     | C39     | C40     |
| D       | .20     | .18     | .24     | .12     | .11     | .31     | .45     | .33     | .08     | .14     | .07     | .28     | .24     | .44     | .24     | .15     | .37     | .23     | .23     | .23     | .23     | .23     |

Cluster Number 2 contains 14 cases

| Case No. | Case No. | Case No. | Case No. | Case No. | Case No. | Case No. | Case No. | Case No. | Case No. | Case No. | Case No. | Case No. | Case No. | Case No. | Case No. | Case No. | Case No. | Case No. | Case No. | Case No. |
|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| C_6      | C_7      | C_8      | C_9      | C_10     | C_11     | C_12     | C_14     | C_15     | C_16     | C_17     | C_28     | C_30     | C_38     |          |          |          |          |          |          |          |          |          |          |
| Distance | .15      | .06      | .23      | .21      | .23      | .27      | .05      | .14      | .28      | .58      | .31      | .21      | .18      | .13      |          |          |          |          |          |          |          |          |          |          |

Cluster Number 4 contains 10 cases

| Case No. | Case No. | Case No. | Case No. | Case No. | Case No. | Case No. | Case No. | Case No. | Case No. | Case No. | Case No. | Case No. | Case No. | Case No. | Case No. | Case No. | Case No. | Case No. | Case No. | Case No. |
|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| C_2      | C_3      | C_4      | C_5      | C_13     | C_39     | C_41     | C_42     | C_43     | C_44     |          |          |          |          |          |          |          |          |          |          |          |          |
| Distance | .05      | .20      | .30      | .67      | .34      | .38      | .17      | .28      | .18      | .44      |          |          |          |          |          |          |          |          |          |          |          |          |

Cluster Number 1 contains 2 cases

| Case No. | Case No. | Case No. | Case No. | Case No. | Case No. | Case No. | Case No. | Case No. |
|----------|----------|----------|----------|----------|----------|----------|----------|----------|
|          |          | C_1      | C_27     |          |          |          |          |          |
| Distance | .37      | .37      |          |          |          |          |          |          |

Immune response options are visualized in Figs. 1 and 2. **Cluster №3** (40.9% of the sample), whose members are characterized by a stable normal (N) immune status, appeared to be the largest, which is quite expected given the remission phase of the chronic inflammatory process. In members of **cluster №2** (31.8%), the lower boundary level of immunity (N-) was completely normalized (N), indicating a **favorable** immunotropic effect of balneotherapy. In members of **cluster №4** (22.7%), moderate immunosuppression (S) was reduced but not sufficient. However, in two patients of **cluster №1** (4.5%), initially normal immune status (N) was transformed into moderate immunosuppression (S). Therefore, the immunotropic effect of balneotherapy on certain individuals is not effective enough, and in some cases even **unfavorable**.

Fig. 1. Individual immune status indexes (ISI) before (axis X) and after (axis Y) balneotherapy in members of different clusters of immune responses
Fig. 2. Average values (Mean±SE) of immune status indexes (ISI) before (axis X) and after (axis Y) balneotherapy in members of different clusters of immune responses

By constructing immune profiles, it was revealed (Fig. 3) that in patients of the N-/N cluster, moderately reduced bactericidality of neutrophils against both types of bacteria and lower-bound IgG levels increase to the upper zone of normal, whereas significantly expressed T-helper deficiency is only diminished, and the upper boundary level of NK cells and normal levels of the other major parameters of the immune status do not change significantly.
Fig. 3. Profiles of the parameters of immunity, by which the ISI is calculated

In the N/N cluster, all 11 immune parameters do not respond to balneotherapy factors, remaining in the range of ±1,5σ.

The stability of the immune status also occurs in the members of the S/S cluster, but negative, because along with the stable normal 7 parameters do not change significantly or significantly reduced levels of T-helper and bactericidity, nor moderately increased level of NK cells.

Changes in the key parameters of the immune status of the members of the last cluster are differently expressed and differently directed. In particular, significantly increased levels of B-lymphocytes are reduced to the normal range, moderate deficiency of IgA and T-helper cells deepens, and dramatically increased bactericidal activity is transformed into significantly reduced, while IgG level moves from the lower normal to the average.
Additional immune profiles (Fig. 4) make it clear that the members of the N-/N cluster increase the bactericidality of neutrophils due to the completion of phagocytosis and increase of their absolute content in the blood, especially rods, while the activity and intensity of phagocytosis remain normal. This is accompanied by a normalizing increase in the level of monocytes and a decrease in the level of eosinophils.

In the S/S cluster, neutrophil bactericidal activity against Staph. aureus increases slightly due to the normalizing increase in the killing index, despite a slight decrease within the norm absolute neutrophil content. Instead, a similar increase in the E. coli killing index is offset by a slight decrease within the norm of activity and intensity of phagocytosis, so that the decreased bactericidality capacity against this microbe remains unchanged.

The N/S cluster members have a dramatic drop in the bactericidal ability of neutrophils against E. coli due to a decrease in all three parameters of phagocytosis in combination with a decrease in the absolute content of neutrophils in the blood. Less pronounced drop in bactericidal activity against Staph. aureus is caused by the preservation at the initial levels of the phagocytic index and the microbial number of neutrophils.

Instead, only a slight decrease in monocyte blood content is expected in the N/N cluster.

The described changes in the parameters of systemic immunity are almost not reflected either at normal levels in lysozyme, IgG and sIgS saliva, or at reduced IgA level in members of all clusters (Fig. 5).

Instead, significant changes in the constellation of the feces microbiota, primarily in members of the N-/N cluster, have been identified. The reduced content of lactic acid microflora (Lactobacillus and Bifidobacterium) and normal E. coli reaches the lower zone of normal. However, the moderately elevated E. coli hemolytica content is completely normalized, the significantly increased E. coli content with impaired enzymatic activity is markedly reduced, and a similar level of Klebsiela&Proteus falls even to the lower normal range. Taken together, these data indicate a reduction in dysbiosis. Favorable changes in the composition of feces microbiota are accompanied by the reduction of moderate bacteriuria, leukocyturia and, to a lesser extent, erythrocyturia.

Less pronounced signs of dysbiosis and urinary syndrome in members of the N/N cluster exhibit less pronounced but favorable response to balneotherapy.

Instead, the S/S cluster members have no normalizing changes in fecal microbiota and leukocyturia.

In N/S cluster members, the development of marked bacteriuria and the growth of erythrocyturia, but not leukocyturia, is accompanied by a further increase in the content of Klebsiela&Proteus and E. coli with impaired enzymatic activity in combination with the lower boundary levels of other components of the microbiota.
Fig. 4. Profiles of other parameters of immunity of members of four clusters
Fig. 5. Profiles of immune parameters of saliva, microbiota of feces and urine as well as urinary syndrome of members of four clusters
We now turn to the analysis of the dynamics of the so-called information indicators, calculated on the parameters of Leukocytogram and Immunocytogram. Popovych's Strain Index-2 was the most sensitive. Severely elevated in N-/N cluster members, it is reduced under the influence of balneotherapy but not enough (Fig. 6 top). The less markedly higher index level of N/S cluster members is completely normalized (Fig. 6 lower), while the even lower initial index level of N/N cluster members decreases only to the upper normal range (Fig. 6 upper), and of cluster members S/S does not respond to balneofactors (Fig. 6 lower).

Popovych's Adaptation Index, by definition, exhibits a dynamic opposite to that of the Strain Index, but less clearly. Instead, entropy levels are almost unresponsive to balneofactors, remaining stably normal.

![Graphs showing the profiles of information parameters of Leukocytogram and Immunocytogram](image)

**Fig. 6. Profiles of information parameters of Leukocytogram and Immunocytogram of N-/N and N/N clusters (top) and S/S and N/S clusters (bottom)**

In order to give an overall impression of the reactions to the balneo factors of all registered indicators, individual profiles were combined into a panorama (Fig. 7).
Fig. 7. Integral parameter profiles of members of four clusters
Discriminant analysis was conducted to identify exactly the parameters of the immunity and microbiota, in which the four immune response clusters differ significantly from each other. 24 parameters were characteristic, 12 of them related to the immune parameters of the **blood**, one of the **saliva**, 5 related to the **feces microbiota** and 3 to **urinary syndrome** as well as 4 parameters are so-called **information**. The other 25 parameters were outside the discriminatory model (Tables 3 and 4).

**Table 3. Discriminant Function Analysis Summary for Changes in Variables of Immunity and Microbiota in Clusters**

Step 24, N of vars in model: 24; Grouping: 4 grps
Wilks' Λ: 0.00166; approx. F(72)=5.4; p<10^-6

| Variables currently in the model | Clusters of Immunity: Before/After (n) | Parameters of Wilks’ Statistics |
|---------------------------------|---------------------------------------|---------------------------------|
|                                 | N/S (2) | N/N (18) | S/S (10) | N-/N (14) | Wilks Λ | Partial Λ | F-remove (3.2) | p-level | Tole ran-cy | Norm Cv (30) |
| Bactericidity vs E. coli, 10^8 Bacteria/L | 154 | 99 | 71 | 80 | 0.0026 | 0.645 | 3.12 | 0.053 | 0.057 | 99 | 0.100 |
| Killing Index vs Staph. aureus, % | 64 | 48 | 47 | 44 | 0.0018 | 0.942 | 0.35 | 0.791 | 0.305 | 58.9 | 0.142 |
| CD4⁺CD3⁺ T-helper Lymphocytes, % | 31.5 | 35.5 | 26.2 | 26.2 | 0.0090 | 0.184 | 25.1 | 10^-5 | 0.040 | 39.5 | 0.082 |
| IgG Saliva, mg/L | 43.0 | 41.7 | 42.4 | 41.1 | 0.0029 | 0.568 | 4.30 | 0.020 | 0.109 | 36 | 0.222 |
| CD3⁺ T-active Lymphocytes, % | 33.0 | 30.0 | 27.0 | 28.1 | 0.0029 | 0.580 | 4.10 | 0.023 | 0.259 | 30 | 0.167 |
| Eosinophiles of Blood, % | 1.91 | 3.01 | 3.27 | 4.42 | 0.0033 | 0.498 | 5.72 | 0.007 | 0.139 | 2.75 | 0.318 |
| Lactobacillus faeces, Ig CFU/g | 7.04 | 6.04 | 6.21 | 5.05 | 0.0021 | 0.803 | 1.39 | 0.280 | 0.003 | 8.10 | 0.179 |
| Killing Index vs E. coli, % | 73 | 47 | 45 | 40 | 0.0072 | 0.229 | 19.0 | 10^-5 | 0.028 | 62.0 | 0.156 |
| Circulating Immune Complexes, units | 38 | 43 | 32 | 27 | 0.0075 | 0.220 | 20.0 | 10^-5 | 0.098 | 45 | 0.389 |
| Leukocyturia, Ig/L | 2.36 | 3.53 | 3.20 | 3.77 | 0.0036 | 0.462 | 6.59 | 0.004 | 0.076 | 3.00 | 0.167 |
| Popovych’s Leukocytary Adaptation Index-1, points | 0.62 | 1.32 | 0.84 | 0.95 | 0.0035 | 0.472 | 6.35 | 0.004 | 0.273 | 1.70 | 0.147 |
| Segmentonuclear Neutrophiles of Blood, % | 49.9 | 58.7 | 47.2 | 53.9 | 0.0040 | 0.417 | 7.94 | 0.002 | 0.080 | 55.0 | 0.100 |
| Stub Neutrophiles of Blood, % | 2.63 | 3.00 | 2.51 | 1.89 | 0.0032 | 0.516 | 5.31 | 0.009 | 0.160 | 4.25 | 0.147 |
| Popovych’s Leukocytary | 0.215 | 0.127 | 0.141 | 0.203 | 0.0032 | 0.517 | 5.30 | 0.009 | 0.333 | 0.067 |
| Strain Index-1, points | 0.081 | 0.082 | 0.168 | 0.166 | 0.0024 | 0.700 | 2.43 | 0.101 | 0.120 | 0.722 |
|------------------------|-------|-------|-------|-------|--------|-------|------|-------|-------|-------|
| E. coli faeces, Ig CFU/g | 8.83 | 8.32 | +0.11 | 8.04 | -0.037 |
|                        | 8.17 | 8.26 | +0.09 | 8.36 | +0.33  |
| IgG Serum, g/L         | 9.4  | 14.6 | +5.2  | 14.6 | +2.7   |
|                        | 16.5 | 16.5 | +0.0  | 12.2 | +2.7   |
| 0-Lymphocytes of Blood, % | -4.3 | 4.1  | +8.4  | 7.6  | +3.0   |
|                        | 0.1  | -0.8 | -0.9  | 3.9  | -1.8   |
| Entropy of Immunocytogram | 0.995 | 0.977 | -0.018 | 0.956 | +0.012 |
|                        | 0.957 | 0.952 | -0.004 | 0.970 | +0.002 |
| Erthrocytura, lg/L     | 3.00 | 3.42 | +0.42 | 3.07 | -0.14  |
|                        | 2.96 | 3.07 | +0.1  | 3.13 | -1.16  |
| Bacteriuria, Ig CFU/L   | 0.37 | 2.35 | +1.98 | 2.21 | 1.78   |
|                        | 1.06 | 0.56 | -0.50 | 2.11 | 0.61   |
| Bifidobacterium faeces, Ig CFU/g | 5.85 | 6.00 | +0.15 | 5.46 | +1.45  |
|                        | 5.32 | 5.73 | +0.41 | 4.59 | +0.14  |
| Phagocyte Index vs E. coli, % | 99.0 | 95.3 | -3.7 | 98.6 | 99.5 |
|                        | 99.4 | 99.9 | +0.1 | 97.7 | +0.4 |
| Hemolytic E. coli faeces, % | 2 | 0 | 2 | 15 | 45 |
|                        | 13 | 17 | +4 | 2 | 5 |
| Entropy of Leukocytogram | 0.665 | 0.663 | -0.001 | 0.677 | +0.016 |
|                        | 0.639 | 0.637 | -0.003 | 0.648 | +0.016 |
| Variables currently not in the model N/S (19) | N/N (18) | N/S (10) | N-N (14) | Wilks Λ | Partial Λ | F to enter | p-level | Tole-rance | Norm Cv (30) |
| Popovych's Leukocytary Strain Index-2, points | 0.261 | 0.105 | -0.156 | 0.202 | 0.014 |
|                        | 0.179 | 0.107 | -0.072 | 0.097 | 0.009 |
| Popovych's Leukocytary Adaptation Index-2, points | 0.74 | 0.62 | -1.10 | 0.62 | 0.016 |
|                        | 0.90 | 1.03 | -1.13 | 0.64 | 0.016 |
| Interleukin-1, ng/L | 5.41 | 4.31 | -1.10 | 4.79 | 0.016 |
|                        | 4.52 | 4.13 | -0.39 | 5.12 | 0.016 |
| Tumor Necrose Factor-α, ng/L | 6.84 | 6.00 | -0.84 | 5.07 | 0.016 |
|                        | 6.02 | 5.75 | -0.27 | 5.51 | 0.016 |
| Interleukin-6, ng/L | 5.91 | 5.36 | -0.55 | 5.16 | 0.016 |
|                        | 5.37 | 5.19 | -0.18 | 5.03 | 0.016 |
| Secretory IgA Saliva, mg/L | 390 | 335 | -55 | 499 | 0.015 |
|                        | 485 | 508 | -23 | 459 | 0.015 |
| Lysozyme Saliva, mg/L | 154 | 150 | -4 | 172 | 0.016 |
|                        | 169 | 172 | +3 | 167 | 0.016 |
| IgA Saliva, mg/L | 98 | 69 | -29 | 123 | 0.016 |
|                        | 149 | 156 | +7 | 125 | 0.016 |

*Table shows statistical data with values and p-values.*
| Bacteriuria, points | 0.05  | 0.50  | 0.24  | 0.15  | 0.53  | 0.48  | 0.41  | 0.13  | -0.28 | 0.0014 | 0.871 | 0.79  | 0.516 | 0.105 | 0  | 0.24 |
| Erthrocyturia, points | 0.10  | 0.19  | 0.09  | 0.12  | 0.09  | 0.09  | 0.13  | 0.09  | -0.04 | 0.0016 | 0.961 | 0.22  | 0.882 | 0.096 | 0  | 0.10 |
| Leukocyturia, points | 0.05  | 0.14  | 0.20  | 0.16  | 0.12  | 0.04  | 0.25  | 0.10  | -0.16 | 0.0014 | 0.847 | 0.96  | 0.434 | 0.085 | 0  | 0.15 |
| Attenuated E. coli faeces, % | 35.43 | 65.65 | 90.90 | 65.75 | 50.50 | -25.25 | 0.0015 | 0.924 | 0.44  | 0.726 | 0.038 | 17.4 | 0.500 |
| Klebsiela&Proteus faeces, % | 18.21 | 17.13 | 4.04  | 24.07 | 7.07  | -18.07 | 0.0015 | 0.932 | 0.39  | 0.763 | 0.035 | 10.0 | 0.500 |
| Phagocytose Index vs Staphylococcus aureus, % | 99.92 | 99.90 | +0.92 | 99.83 | 99.00 | +99.96 | 0.0015 | 0.920 | 0.47  | 0.710 | 0.357 | 98.3 | 0.018 |
| Microbial Count vs Staphylococcus aureus, Bact/Phagoc. | 66 63  | 60 64  | +4 4  | 61 58  | 65 64  | -1 -1  | 0.0016 | 0.982 | 0.10  | 0.959 | 0.589 | 61.6 | 0.160 |
| Microbial Count vs E. coli, Bacteria/Phagocyte | 70 61  | 62 66  | -3 -4  | 64 56  | 67 66  | -1 -1  | 0.0016 | 0.959 | 0.23  | 0.874 | 0.111 | 54.7 | 0.194 |
| Bactericidity vs Staphylococcus aureus, 10^9 Bacteria/L | 130 63 | 98 105 | +7 7 | 75 79 | 85 118 | +33.33 | 0.0016 | 0.983 | 0.09  | 0.964 | 0.280 | 106 | 0.100 |
| Leukocytes of Blood, 10^9/L | 7.25 5.89 | 5.81 5.56 | -0.25 -0.2 | 5.47 5.20 | 5.53 6.00 | +0.47 | 0.0016 | 0.956 | 0.25  | 0.863 | 0.206 | 5.00 | 0.100 |
| Monocytes of Blood, % | 8.2 7.1 | 5.6 4.9 | -0.7 -0.7 | 6.8 7.0 | 5.5 6.2 | +0.8 | 0.0014 | 0.873 | 0.78  | 0.524 | 0.053 | 6.0  | 0.083 |
| Pan-Lymphocytes of Blood, % | 37.3 42.4 | 29.3 30.4 | +5.1 +0.7 | 40.3 40.6 | 34.3 32.1 | -2.2 | 0.0014 | 0.873 | 0.78  | 0.524 | 0.007 | 32.0 | 0.174 |
| CD8⁺CD3⁺ T-cytolytic Lymphocytes, % | 25.5 25.0 | 23.8 25.7 | -0.5 +1.9 | 21.8 21.6 | 22.1 21.0 | -1.1 | 0.0016 | 0.991 | 0.05  | 0.986 | 0.242 | 23.5 | 0.138 |
| CD22⁺ B-Lymphocytes, % | 29.5 23.0 | 23.9 24.5 | -6.5 +0.6 | 20.4 23.8 | 23.4 23.8 | +0.4 | 0.0016 | 0.991 | 0.05  | 0.986 | 0.008 | 20.0 | 0.175 |
| IgA Serum, g/L | 1.40 1.20 | 1.93 1.93 | -0.20 -0.22 | 1.26 1.41 | 1.77 1.83 | +0.06 | 0.0015 | 0.928 | 0.42  | 0.743 | 0.101 | 1.875 | 0.167 |
| IgM Serum, g/L | 1.20 1.40 | 1.49 1.50 | 0.20 0.00 | 1.41 1.35 | 1.44 1.50 | +0.06 | 0.0015 | 0.900 | 0.59  | 0.631 | 0.361 | 1.15 | 0.239 |
| CD56⁺ Natural Killer Lymphocytes, % | 17.8 20.9 | 16.7 15.3 | +3.1 -1.5 | 24.0 23.7 | 23.7 22.1 | -1.6 | 0.0016 | 0.991 | 0.05  | 0.986 | 0.334 | 17.0 | 0.172 |
Table 4. Summary of Stepwise Analysis for Changes in Variables of Immunity and Microbiota in Clusters. The variables are ranked by criterion Lambda

| Variables currently in the model | F to enter | p-level | Λ  | F-value | p-level |
|--------------------------------|------------|---------|----|---------|---------|
| Bactericidity vs E. coli, 10⁹ Bacteria/L | 13.4 | 10⁻⁵ | .498 | 13.4 | 10⁻⁵ |
| Killing Index vs Staph. aureus, % | 4.8 | .006 | .363 | 8.6 | 10⁻⁶ |
| CD4⁺CD3⁺ T-helper Lymphocytes, % | 3.3 | .029 | .287 | 6.9 | 10⁻⁶ |
| IgG Saliva, mg/L | 3.1 | .040 | .230 | 6.1 | 10⁻⁶ |
| CD3⁺ T-active Lymphocytes, % | 2.2 | .106 | .195 | 5.4 | 10⁻⁶ |
| Eosinophiles of Blood, % | 2.1 | .116 | .165 | 4.9 | 10⁻⁶ |
| Lactobacillus faeces, lg CFU/g | 2.5 | .078 | .135 | 4.7 | 10⁻⁶ |
| Killing Index vsE. coli, % | 2.3 | .093 | .112 | 4.5 | 10⁻⁶ |
| Circulating Immune Complexes, units | 5.1 | .006 | .076 | 4.9 | 10⁻⁶ |
| Leukocyturia, lg/L | 3.6 | .025 | .056 | 5.1 | 10⁻⁶ |
| Popovych’s Adaptation Index-1, points | 2.8 | .055 | .044 | 5.1 | 10⁻⁶ |
| Segmentonuclear Neutrophiles of Blood, % | 2.8 | .058 | .034 | 5.1 | 10⁻⁶ |
| Stub Neutrophiles of Blood, % | 3.0 | .046 | .026 | 5.2 | 10⁻⁶ |
| Popovych’s Strain Index-1, points | 2.4 | .086 | .020 | 5.2 | 10⁻⁶ |
| E. coli faeces, lg CFU/g | 3.7 | .024 | .014 | 5.5 | 10⁻⁶ |
| IgG Serum, g/L | 2.3 | .106 | .011 | 5.5 | 10⁻⁶ |
| 0-Lymphocytes, % | 2.1 | .121 | .009 | 5.5 | 10⁻⁶ |
| Entropy of Immunocytogram | 3.4 | .036 | .006 | 5.8 | 10⁻⁶ |
| Erthrocyturia, lg/L | 3.2 | .045 | .004 | 6.1 | 10⁻⁶ |
| Bacteriuria, lg CFU/L | 1.7 | .202 | .003 | 6.0 | 10⁻⁶ |
| Bifidobacterium faeces, lg CFU/g | 1.5 | .256 | .003 | 5.9 | 10⁻⁶ |
| Phagocytose Index vs E. coli, % | 1.3 | .289 | .002 | 5.8 | 10⁻⁶ |
| Hemolytic E. coli faeces, % | 1.1 | .361 | .002 | 5.6 | 10⁻⁶ |
| Entropy of Leukocytogram | 1.0 | .405 | .002 | 5.4 | 10⁻⁶ |

Next, the 24-dimensional space of discriminant variables transforms into 3-dimensional space of canonical roots, which are a linear combination of discriminant variables. The canonical correlation coefficient is for Root 1 0.976 (Wilks' Λ=0.0017; χ²(72)=186; p<10⁻⁶), for Root 2 0.930 (Wilks' Λ=0.0345; χ²(46)=98; p<10⁻⁴) and for Root 3 0.863 (Wilks' Λ=0.254; χ²(22)=40; p=0.012). The major root contains 68% of discriminative properties, the second 22% and the minor 10%.

Table 5 presents standardized (normalized) and raw (actual) coefficients for discriminant variables. The calculation of the discriminant root values for each person as the sum of the products of raw coefficients to the individual values of discriminant variables together with the constant enables the visualization of each patient in the information space of the roots.
Table 5. Standardized and Raw Coefficients and Constants for changes in Variables of Immunity and Microbiota

| Variables                                      | Coefficients | Standardized | Raw |
|------------------------------------------------|--------------|--------------|-----|
| Bactericidity vs E. coli, $10^9$ Bacteria/L    |              | Root 1 | Root 2 | Root 3 | Root 1 | Root 2 | Root 3 |
| Killing Index vs Staph. aureus, %              | .188         | .085   | .448   | .022   | .010   | - .053 |
| CD4+CD3+ T-helper Lymphocytes, %               | 4.564        | - .929 | - .102 | .949   | - .193 | - .021 |
| IgG Saliva, mg/L                               | -1.666       | .623   | 1.138  | -5.76  | .216   | .393   |
| CD3+ T-active Lymphocytes, %                    | .474         | 1.044  | - .788 | .091   | 2.01   | - .152 |
| Eosinophiles of Blood, %                       | -1.196       | .434   | 1.678  | -5.96  | .216   | 836    |
| Lactobacillus faeces, lg CFU/g                 | - .763       | - .641 | 8.902  | -4.84  | .407   | 5.649  |
| Killing Index vs E. coli, %                     | -5.218       | - .378 | -1.519 | - .403 | -.029  | - .117 |
| Circulating Immune Complexes, units            | 2.739        | -.679  | .710   | 1.31   | -.032  | .034   |
| Leukocyturia, lg/L                             | -2.513       | -.823  | - .806 | -3.338 | -1.094 | -1.071 |
| Popovych’s Adaptation Index-1, points          | -.837        | 1.185  | - .274 | -1.303 | 1.845  | -.426  |
| Segmentonuclear Neutrophiles of Blood, %       | 2.461        | - .616 | 1.268  | .371   | -.093  | 191    |
| Stub Neutrophiles of Blood, %                  | -1.461       | .840   | .711   | -1.147 | .660   | .558   |
| Popovych’s Strain Index-1, points              | -1.009       | .524   | -.572  | -4.735 | 2462   | -2.684 |
| E. coli faeces, lg CFU/g                       | -.004        | 1.237  | 1.258  | - .011 | 3.384  | 34.40  |
| IgG Serum, g/L                                 | .431         | -.787  | -.256  | .101   | -.185  | -.060  |
| Ly-Lymphocytes, %                               | 1.707        | -1.889 | .554   | .268   | -.297  | .087   |
| Entropy of Immunocytogram                      | 1.341        | -.497  | .927   | 49.47  | 18.32  | 34.19  |
| Erhthrocyturia, lg/L                           | -.545        | 1.109  | .879   | -1.406 | 2.860  | 2.266  |
| Bacteriuria, lg CFU/L                          | .641         | -.133  | -1.056 | .749   | .155   | -1.234 |
| Bifidobacterium faeces, lg CFU/g               | 4.554        | -.420  | -7957  | 3427   | -.316  | -5.988 |
| Phagocytose Index vs E. coli, %                 | -.390        | .223   | .815   | -.268  | .153   | .560   |
| Hemolytic E. coli faeces, %                    | .466         | .369   | .604   | .010   | .008   | .013   |
| Entropy of Leukocytogram                       | .452         | -.941  | - .457 | 10.83  | -22.59 | -10.97 |
| Constants                                      | -2.514       | -.559  | -.421  | Cumulated Properties | .681 | .899 | 1.000 |

Table 5 shows the correlation coefficients of immunity and microbiota changes (discriminant variables) with canonical discriminant roots, the cluster centroids of both roots, and the normalized immunity and microbiota change values of the discriminant variables, as well as not included in the discriminant model because not getting a variable into the model does not always indicate a lack of recognition ability, but may be a consequence of redundancy of information.
Table 5. Correlations Variables-Canonical Roots, Means of Roots and Z-scores of changes in Variables of Immunity and Microbiota for Clusters

| Variables | Correlations Variables-Roots | N/S (2) | N/N (18) | S/S (10) | N/N (14) |
|-----------|-----------------------------|---------|----------|----------|----------|
| Root 1 (68%) |                             | R 1 | R 2 | R 3 | -6.50 | -3.47 | -0.73 | +5.91 |
| Killing Index vs Staph. aureus, % | .164 | .174 | -.251 | +0.64 | -1.32 | -1.39 | -1.83 |
| Bactericidity vs E. coli, 10^8 Bacteria/L | .153 | .290 | -.050 | +5.55 | -0.01 | -2.81 | -1.95 |
| CD4+CD3+ T-helper Lymphocytes, % | .104 | .049 | -.018 | -2.47 | -1.23 | -4.11 | -4.10 |
| Lactobacillus faeces, Ig CFU/g | .085 | -.009 | -.013 | -0.73 | -1.42 | -1.31 | -2.10 |
| Bifidobacterium faeces, Ig CFU/g | .081 | -.010 | -.026 | -0.96 | -1.42 | -1.30 | -2.07 |
| E. coli faeces, Ig CFU/g | .076 | .036 | .081 | -0.89 | -1.88 | -1.02 | -2.40 |
| Phagocytose Index vs E. coli, % | .072 | .230 | .055 | 0.59 | 0.93 | 0.25 | 0.65 |
| Stub Neutrophiles of Blood, % | .069 | .077 | .145 | -2.60 | -1.99 | -2.79 | -3.78 |
| Entropy of Leukocytogram | .049 | .005 | .095 | -0.59 | -0.47 | 0.04 | 0.03 |
| Circulating Immune Complexes, units | .045 | .019 | -.048 | -0.37 | -0.14 | -0.74 | -1.04 |
| Segmentonuclear Neutrophiles of Blood, % | .028 | .040 | -.044 | -0.92 | -1.14 | -1.34 | -3.03 |
| Bactericidity vs Staphylococcus aureus, 10^8 Bacteria/L | currently not in the model | +2.27 | -0.72 | -2.93 | -1.93 |
| Leukocytes of Blood, 10^7/L | currently not in the model | +4.50 | +1.62 | +0.94 | +1.06 |
| Popovych's Adaptation Index-2, points | currently not in the model | -3.84 | 1.11 | +0.40 | +0.31 |
| Monocytes of Blood, % | currently not in the model | +4.37 | +0.87 | +1.54 | +1.09 |
| IgA Saliva, mg/L | currently not in the model | -2.23 | 1.29 | +2.09 | +0.88 |
| Bacteriuria, Ig CFU/L | -1.33 | -.235 | -.054 | +0.38 | +1.07 | +2.25 | +1.81 |
| Leukocyturia, Ig/L | -0.96 | -.112 | .083 | -1.29 | +1.07 | +1.19 | +1.54 |
|                                |     |     |
|--------------------------------|-----|-----|
| **Hemolytic E. coli faeces, %**| -06| -012|
|                                | 006| 012 |
|                                | 000| 008 |
|                                | -010| -052|
| **Erthrocyturia, Ig/L**        | -076| -037|
|                                | 065| 017 |
|                                | 062| 065 |
|                                | 018| 023|
| **Eosinophiles of Blood, %**   | -031| -029|
|                                | 075| 097 |
|                                | 041| 059 |
|                                | 037| 061|
| **Bacteriuria, points**        | currently not in the model|
|                                | 021| 020 |
|                                | 088| 046 |
| **Leukocyturia, points**       | currently not in the model|
|                                | 033| 033 |
|                                | 095| 026 |
|                                | 062| 019|
| **Erthrocyturia, points**      | currently not in the model|
|                                | 000| 086 |
|                                | 193| 089 |
|                                | 093| 000 |
| **Pan-Lymphocytes of Blood, %**| currently not in the model|
|                                | 096| 041 |
|                                | 187| 029 |
|                                | 091| 012 |
| **CD56**                       | currently not in the model|
| **Natural Killer Lymphocytes, %**|     |     |
|                                | 027| 017 |
|                                | 134| 050 |
|                                | 007| 075|
| **Klebsiela&Proteus faeces, %**| currently not in the model|
|                                | 010| 132 |
|                                | 050| 070 |
|                                | 049| 053|
| **Attenuated E. coli faeces, %**| currently not in the model|
|                                | 016| 024 |
|                                | 050| 131 |
|                                | 049| 049|
| **Root 2 (22%)**               |     |     |
|                                | 031| 013 |
| **CD3**                       |     |     |
| **T-active Lymphocytes, %**    | currently not in the model|
|                                | 016| 039 |
|                                | 056| 039 |
|                                | 050| 039 |
| **IgG Saliva, mg/L**           | currently not in the model|
|                                | 007| 013|
|                                | 007| 013|
|                                | 007| 013|
| **Popovych’s Adaptation Index-1, points**|     |     |
|                                | 027| 030 |
|                                | 022| 022 |
|                                | 000| 030 |
| **Microbial Count vs E. coli, Bacteria/Phagocyte**| currently not in the model|
|                                | 042| 075 |
|                                | 056| 075 |
|                                | 111| 075 |
| **Microbial Count vs Staph. aureus, Bacteria/Phagocyte**| currently not in the model|
|                                | 041| 019 |
|                                | 012| 023 |
|                                | 029| 042 |
| **Secretory IgA Saliva, mg/L** | currently not in the model|
|                                | 008| 062 |
|                                | 067| 046 |
|                                | 058| 058 |
| **CD8’CD3’ T-cytolytic Lymphocytes, %**| currently not in the model|
|                                | 085| 085 |
|                                | 000| 000 |
| **Lysozime Saliva, mg/L**      | currently not in the model|
|                                | 044| 010 |
|                                | 069| 070 |
| **IgG Serum, g/L**             |     |     |
|                                | 044| 010 |
|                                | 069| 070 |
Extreme right localization along the axis of the first root of members of the N/N cluster (Figs. 8 and 10) reflects their maximally increase in parameters that correlate with the root positively, and maximally decrease in parameters that correlate with the root inversely. Instead, the leftmost localization of the N/S cluster members reflects the maximum decrease/increase in the same parameters. The members of other clusters do not differ in the totality of the parameters listed (mixed along the axis of the first root).

Instead, the members of the N/N and S/S cluster are clearly distinguished along the axis of the second root (Figs. 8 and 11). Higher N/N cluster localization reflects an increase in the parameters associated with this root, whereas in the lower members of the S/S cluster these parameters decrease under the influence of balneofactors (regarding IgG Serum changes on the contrary).

According to another constellation of parameters, these two clusters are delimited along the axis of the third root (Fig. 9 and 12).
Fig. 8. Scatterplot of individual values of the first and second roots in which condensed information about of the changes in Immunity and Microbiota of the members of the four clusters.

Fig. 9. Scatterplot of individual values of the first and third roots in which condensed information about of the changes in Immunity and Microbiota of the members of the four clusters.
Fig. 10. Patterns of changes in Immunity and Microbiota parameters, the information of which is condensed in the first root

Fig. 11. Patterns of changes in Immunity parameters, the information of which is condensed in the second root

Fig. 12. Patterns of changes in Immunity parameters, the information of which is condensed in the third root

In general, all four clusters on the planes of the discriminant roots are quite clearly delineated, which is documented by calculating the Mahalanobis distances (Table 6).
Table 6. Squared Mahalanobis Distances between Clusters, \textbf{F-values (df=24,2)} and \textbf{p-levels}

| Clusters | N/N | S/S | N/S | N-/N |
|----------|-----|-----|-----|------|
| N/N      | 0   | 33  | 155 | 101  |
| S/S      | 3.5 0.005 | 0   | 146 | 64   |
| N/S      | 2.6 0.024 | 2.3 0.038 | 0 | 268  |
| N-/N     | 13.2 10^{-6} | 6.0 0.0002 | 4.4 0.001 | 0 |

The same discriminant parameters can be used to identify the belonging of one or another person to one or another cluster. This purpose of discriminant analysis is realized with the help of classifying functions (Table 7).

Table 7. Coefficients and Constants for Classification Functions of Clusters

| Variables | Clusters | N/N | S/S | N/S | N-/N |
|-----------|----------|-----|-----|-----|------|
| Bactericidity vs E. coli, 10^9 Bacteria/L | \(p=0.409\) | \(p=0.227\) | \(p=0.045\) | \(p=0.318\) |
| Killing Index vs Staph. aureus, % | \(-0.020\) | \(0.250\) | \(-0.946\) | \(0.649\) |
| CD4^{+}/CD3^{+} T-helper Lymphocytes, % | \(0.003\) | \(0.218\) | \(-0.309\) | \(0.187\) |
| IgG Saliva, mg/L | \(1.693\) | \(-1.958\) | \(2.005\) | \(-4.114\) |
| CD3^{+} T-active Lymphocytes, % | \(2.070\) | \(-3.195\) | \(3.533\) | \(-3.887\) |
| Eosinophiles of Blood, % | \(2.072\) | \(-1.406\) | \(25.91\) | \(2.608\) |
| Lactobacillus faeces, Ig CFU/g | \(-1.520\) | \(1.768\) | \(-2.291\) | \(7.768\) |
| Bacteriuria, Ig/L | \(5.872\) | \(-1.958\) | \(2.005\) | \(-4.114\) |
| Popovych’s Adaptation Index-1, points | \(1.693\) | \(-1.958\) | \(2.005\) | \(-4.114\) |
| Circulating Immune Complexes, units | \(2.070\) | \(-3.195\) | \(3.533\) | \(-3.887\) |
| Leukocyturia, Ig/L | \(2.072\) | \(-1.406\) | \(25.91\) | \(2.608\) |
| Popovych’s Strain Index-1, points | \(5.872\) | \(-1.958\) | \(2.005\) | \(-4.114\) |
| Segmentonuclear Neutrophiles of Blood, % | \(-1.520\) | \(1.768\) | \(-2.291\) | \(7.768\) |
| Stib Neutrophiles of Blood, % | \(5.872\) | \(-1.958\) | \(2.005\) | \(-4.114\) |
| Entropy of Immunoecytogram | \(-89.93\) | \(-16.53\) | \(49.75\) | \(413.7\) |
| Erthrocyturia, Ig/L | \(10.81\) | \(-10.17\) | \(-11.29\) | \(-8.03\) |
| Bacteriuria, Ig CFU/L | \(-3.161\) | \(3.732\) | \(-6.757\) | \(4.080\) |
| Bifidobacterium faeces, Ig CFU/g | \(-10.83\) | \(20.65\) | \(-32.55\) | \(21.46\) |
| Phagocytose Index vs E. coli, % | \(0.717\) | \(-2.482\) | \(1.206\) | \(-2.068\) |
| Hemolytic E. coli faeces, % | \(0.033\) | \(-0.012\) | \(-0.056\) | \(0.115\) |
| Entropy of Leukocytogram | \(-92.43\) | \(48.14\) | \(100.1\) | \(54.35\) |
| Constants | \(-5.532\) | \(-6.945\) | \(-56.21\) | \(-37.96\) |

We can retrospectively recognize members of three clusters unmistakably, and only the cluster S/S is with one error (Table 8).
The next article will analyze the related changes in the parameters of the autonomic nervous and endocrine systems, as well as metabolism.

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ACCORDANCE TO ETHICS STANDARDS

Tests in patients are conducted in accordance with positions of Helsinki Declaration 1975, revised and complemented in 2002, and directive of National Committee on ethics of scientific researches. During realization of tests from all participants the informed consent is got and used all measures for providing of anonymity of participants. For all authors any conflict of interests is absent.

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