# Clinicolaboratory criteria of prediction of disease progression in course of interferontherapy in patients with cutaneous regional metastatic melanoma

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Summary.

Authors made the immunologic monitoring of 40 patients with regional metastatic melanoma (RMM) in dynamics of the combined treatment and it was found that the decreased levels circulating NK cells, Treg, T-helper cells with the receptor for IL-2 and the increased mitogen-induced proliferation of T cells *in vitro* during the first 3 months of adjuvant IFN-α2b therapy were the immunological criteria for unfavorable prognosis for a disease. Probably, the using these immunological criteria for investigation of RMM patients will allow to determine individual peculiarity of disease course, to predict the response of organism on biotherapy and so to optimize the treatment tactics.

**Key words**: cutaneous melanoma, interferontherapy, immunological criteria of prediction of efficiency of treatment

## INTRODUCTION

Cutaneous melanoma remains a major cause of death among patients with malignant tumors of the skin [1]. In Ukraine, only 41,8 % of patients refers to oncologist at early stages of the cutaneous localized melanoma and melanoma diagnoses on II-IV stage in 58 % of patients, when the further development of the disease is almost inevitable [2]. In this situation, therapeutic options are limited and directed on increase of antitumor resistance of an organism and improvement of quality of patient’s life [3].

At the same time, the achievement of immunotherapy in treatment of this patient category remains quite modest, and the views of different authors at this method of

antitumor treatment do not coincide. In particular, the results of adjuvant interferon(IFN)-α therapy are ambiguous and cause discussions concerning expediency of its appointment by all patient [4]. Different views at this problem, first of all, are connected to empirical appointment IFN without a sufficient substantiation. Probably, for this reason application of modifiers of biological reactions does not give desirable results, and in some cases can worsen efficiency of the basic treatment. Experts of the International Society Immunotherapy of Cancer (SITC) agree with it [5]. In opinion of authors of this investigation, wide introduction of immunotherapy that has a direct effect on the antitumor resistance of the organism prevents the lack of a unified methodology to assess the immune status of patients and a consensus concerning potential biomarkers which research will allow to predict current of disease and the answer to an immunotherapy. In turn, the low predictive accuracy of traditional assessment systems that are based on a study of tissue samples obtained during surgical excision of the primary tumor is also associated, as determined solely histopathological features of the tumor without morphological features of the formation of the anti-tumor immune response of the organism in different activity of tumor process [6].

Given the above, the aim of this study is the definition of immunological criteria for prediction of disease progression in the background of IFN therapy in patients with regional metastatic melanoma (RMM) to optimize the combined treatment.

## PATIENTS AND METHODS

Patients. The present study was based on 40 patients with histologically confirmed diagnosis RMM IIIB-IIIC stage. Written informed consent was obtained from all patients. The patients included 23 men and 17 women. The middle age of patients was 54,9 ± 2,7 years (raging in age from 30 to 71 years). In addition, 60 healthy normal subjects (HNS) in the same age-old group were included this study.

By randomization patients were divided into control (20 patients) and basic (20 patients) groups. The control group patients after 8-10 days surgical treatment received the induction course of recombinant IFN-α2b (“Laferobion” subcutaneously with 9 million IU daily for 23 days), after which they received supporting course of IFN therapy (“Laferobion” subcutaneously 3 million IU three times a week for 12 months). The basic group patients started treatment with induction course IFN (“Laferobion”

subcutaneously with 9 million IU daily for 23 days), and then received surgical treatment, in 8-10 days after which started supporting course of IFN therapy ("Laferobion" subcutaneously 3 million IU three times a week for 12 months).

Blood samples obtained from patients 3 times: 8-10 days after surgery, at 3 and 12 months from the beginning of supporting course IFN-α2b therapy.

Conducting this study was approved by the Local Ethics Committee of the National Cancer Institute.

Research methods. The absolute number of leukocytes in peripheral blood and leukocyte formula were determined standard method [7]. The following anti-human monoclonal antibodies were used for immunophenotyping of peripheral blood lymphocytes (PBL) [8, 9] by flow cytometry: fluoresceinisothiocyanate-labeled anti-CD3, anti-CD19, anti-CD4, anti-CD8, anti-CD16, anti-HLA-DR, anti-CD95 ("Сорбент", Russia), phycoerythrocyanin-5-labeled anti-CD25, phycoerythrin-labeled anti-CD127 ("Beckman Coulter", USA). Staining PBL was done as described [10]. Data acquisition and analysis were performed using FACScan flow cytometer ("Becton Dickinson", USA) and CellQuest software. Forward and side scatter were used to gate lymphocytes [11]. To detect main subset of lymphocytes $2\times10^3$ events were acquired for each sample. To detect regulatory T cells (Treg, $CD4^{+}25^{high}127^{low\text{-}neg}$) $10\times10^3$ events were analyzed by flow cytometry. Immunoregulatory index (IRI) was determined as the ratio of CD4/CD8.

The functional status of lymphocytes was tested in cytotoxic assay (results are represented as cytotoxic index in percentage) [8], proliferation assay with PHA (10 μg/ml, results are represented percentage of blasttransfomed lymphocyte) [12] and reaction of inhibition of leukocyte migration with PHA (10 μgml, results are represented as migration index in percentage) [13].

Statistical analysis. Analysis was performed using Excel software (MS Office 2003, XP) and STATISTICA software version 6,0 (StatSoft Inc., USA). Based on distribution level, differences in means and correlation analyses were evaluated with Mann-Whitney, Wilcoxon and Spearman's tests. For all statistical analysis the level of significance was set at $p < 0,05$.

RESULTS AND DISCUSSION

In the previous research [10] we reported on features of immunoreactivity at patients RMM in conditions of above schemes IFN therapy for 3 months. In summary: it was found that the use of neoadjuvant induction IFN-α2b course promoted recovery after surgical treatment of T cells, but did not eliminate the increased content Treg. Conversely, a similar IFN-α2b course, conducted in adjuvant, did not restore the reduced T lymphocytes in the circulation, however reduced the level of Treg.

Further monitoring of these patients for 12 months showed that regardless of the application of the scheme of IFN in 50 % of patients developed distant metastases. In this connection, to determine the immunological criteria predicting of the combined treatment patients of control and basic groups at stage after carrying out of surgical treatment were combined into one group (40 patients) with the subsequent distribution to 2 subgroups depending on presence (20 patients) or absence (20 patients) distant metastases which developed within 12 months from the beginning of treatment.

As a result of analysis of researched immune system parameters at distribution of patients the following in such a way was revealed. After surgical treatment the number of leukocytes and the absolute counts of lymphocytes in peripheral blood of patients were within the limits of physiological level (fig. 1A). While there was a relative lymphopenia with preservation of the main lymphocyte subsets (T and B cells and natural killer (NK) cells) on level of HNS. However, T cell compartment of the immune system underwent a series of certain changes: the frequency and the absolute number of cytotoxic T lymphocytes (CTL) with increase in IRI were reduced, and the frequency of Treg was increased ($p < 0,05$). The increased frequency of Treg is the characteristic sign of metastatic melanoma which indicates a deepening of immune dysfunction in this category of patients as we have shown earlier [16]. For data Cesana G.C. and et. al. [17], the increased number of peripheral blood Treg (identified by marker FOXP3) correlates with poor prognosis of diseases.

As show in Fig. 1B at 3 months after treatment with IFN the leukocyte counts and total number of lymphocytes were significantly reduced in patients with favorable course of disease in comparison with pre-treatment and normal levels ($p < 0,05$). The absolute lymphopenia was caused by reduction of number of T lymphocytes (in both T helper and CTL compartments) and NK cells ($p < 0,05$). However, the frequency of

lymphocytes and their main subsets did not show difference between patients and HNS but the frequency B cells was increased. The percentage of Treg was significantly lower than pre-treatment level ($p < 0,05$) but remained higher than normal level.

At this stage of treatment in patients with generalized process also it was observed leukopenia and lymphopenia by reduced the absolute number of main lymphocyte subsets ($p < 0,05$; Fig. 1B). However, leukopenia and the decreased number of NK cells in this group of patients were more pronounced than in group of patients with a favorable course of disease ($p < 0,05$). Profound changes in lymphocyte subset composition were revealed: the frequency of NK cells was significantly decreased but the frequency of T-lymphocytes was significantly increased namely T helper cells. While there was a disproportion content of $CD4^+$ and $CD8^+$ cells which represented increase of IRI ($p < 0,05$). It is important to note that generalization of tumor process in patients this group leaded to significant decrease in frequency of Treg in comparison with pre-treatment level ($p < 0,05$) but it was not showed in patients with favorable course of disease. This was contrary to the above facts, which was connected with the different methods of determination of Treg and status of immune system of patients in conditions of different course IFN therapy in our study.

So, irrespective of efficiency of treatment use of adjuvant IFN therapy within 3 months leaded to development of leukopenia with lymphopenia by reduced number of T and NK cells. These effects were more pronounced in patients with generalized process than in patients with a favorable course of disease. In addition, for patients with disease progression characteristic was more profound changes in T-cell component of immune system which might indicate the exhaustion of compensatory mechanisms of immune system.

That was shown by results of the correlation analysis of data which derived after 3 months of adjuvant IFN. It were established, that between total number of leucocytes, the frequency of NK cells and Treg in peripheral blood and the disease progression there was a return correlation dependence (accordingly $r = - 0,548$, $r = - 0,550$, $r = - 0,557$; $p < 0,05$). In contrast, between the frequency of circulating T cells and the efficiency of treatment there was a direct correlation dependence ($r = 0,504$; $p < 0,05$).

After end of IFN therapy in patients with a favorable course of the disease the number of leukocytes and lymphocytes in the peripheral blood was restored up to the

physiological level but there were preconditions to development of lymphopenia (Fig. 1B). In particular, there was reduced the absolute number of NK cells and CTL ($p < 0,05$). Thus, the frequency of NK cells in patients was significantly lower than in HNS ($p < 0,05$). In patients with progressive disease also the leucopenia eliminated but in contrast to patients with a favorable course of disease it was remained absolute lymphopenia by reduced number of T and B cells ($p < 0,05$). In addition, the frequency of circulating lymphocytes was significantly decreased in patients in comparison with HNS ($p < 0,05$). In lymphocyte subset composition the percentage of T cells was decreased to the physiological level with increased frequency of T helper cells and reduced frequency of CTL. That was displayed on IRI which was significantly increased in patients than in HNS ($p < 0,05$). At the same time the percentage and absolute number of NK cells restored up to physiological level. It is important to note, that at this stage of monitoring the level of peripheral blood Treg was not depended on efficiency of treatment and was significantly increased than in patients after 3 months of IFN therapy and in HNS ($p < 0,05$).

Analysis of the content of activated lymphocytes ($CD25^+$, $CD4^+25^+$, $HLA\text{-}DR^+$, $CD95^+$) in peripheral blood of patients RMM depending on the efficiency of treatment showed that after carrying out of surgical treatment there was a significant increase in the frequency of all researched activated lymphocytes ($p < 0,05$, Fig. 2A). After 3 months of IFN therapy (Fig. 2B) in patients with a favorable course of disease the frequency of lymphocytes with late activation markers ($HLA\text{-}DR^+$, $CD95^+$) was reduced to the physiological norm and the lymphopenia was associated with a decrease of absolute number of these cells ($p < 0,05$). At the same time, the content of $CD25^+$ and $CD4^+25^+$ lymphocytes did not experience significant changes. The patients with progressive disease had similar changes in the frequency of $HLA\text{-}DR^+$ and $CD95^+$ lymphocytes. The frequency of $CD25^+$ lymphocytes was decreased to the physiological level; however, the percentage of $CD4^+25^+$ lymphocytes remained elevated. In patients of this subgroup lymphopenia accompanied by a reduction of the absolute number of all researched activated lymphocytes ($p < 0,05$).

So, lymphopenia which was formed within 3 months of adjuvant IFN therapy regardless of the efficiency of treatment was accompanied by a decrease in the percentage of activated $HLA\text{-}DR^+$ and $CD95^+$ lymphocytes with preservation of

increased level of $CD4^+25^+$ lymphocytes. In addition, in patients with generalized process in contrast to patients with a favorable course of the disease the frequency of lymphocytes with receptors for IL-2 ($CD25^+$) was decreased, that on background of severe lymphopenia might also indicate the depletion of compensatory mechanisms of immune system. Results of the correlation analysis showed that between the absolute number of $CD4^+25^+$ lymphocytes and progressive disease was an return correlation dependence ($r = - 0,567$; $p < 0,05$).

After the completion of IFN therapy (Fig. 2B) in patients with a favorable clinical course content of activated lymphocytes essentially did not change, except increasing the frequency of HLA-$DR^+$ lymphocytes ($p < 0,05$). In patients with generalized process an increase in the frequency of $CD25^+$ , $CD4^+25^+$ and HLA-$DR^+$ lymphocytes was observed ($p < 0,05$) but in contrast to patients with a favorable course of the disease the content of lymphocytes predisposed to apoptosis ($CD95^+$) was significantly increased.

The research of lymphocyte functional activity showed (Fig. 2A) that after surgical treatment cytotoxicity and mitogen-induced proliferation of cells were significantly suppressed ($p < 0,05$). In the dynamics of the treatment the cytotoxic activity of lymphocytes did not experience significant changes (Fig. 2 B, C) in both subgroups in patients. After 3 months of IFN therapy in patients with a favorable course of the disease mitogen-induced proliferation of lymphocytes was reduced, after the completion of treatment it was restored (Fig. 2 B, C). In contrast, in patients with disease progression after 3 months of IFN therapy the lymphocyte response to PHA *in vitro* was increased to physiological level but after the completion of treatment it was decreased ($p < 0,05$).

A similar dependence is observed in the study of lymphocyte function in relation to production of factor which inhibits the leukocyte migration. No difference in the migration index was found between patients with a favorable course of the disease after surgery and 3 months of IFN therapy and HNS but post-treatment migration index was significantly higher in these patients relative to HNS ($p < 0,05$). Conversely, in patients with generalized process after 3 months of IFN therapy this T cell function was suppressed ($p < 0,05$) and remained so until the end of the monitoring.

So, the researched parameters of the functional activity of lymphocytes (except for cytotoxic activity) in patients after 3 months of adjuvant IFN therapy differently

changed depending on efficiency of treatment. However connection with disease progression was established only for response of lymphocytes in vitro to PHA ($r = 0,730$; $p < 0,05$).

Thus, the reduced content of circulating NK cells, Treg, T helper cells with the receptor for IL-2 and the increased frequency of T cells with the raised ability to mitogen-induced proliferation in vitro on a background leukopenia during the first 3 months of adjuvant IFN-α2b therapy was the immunological criteria for poor prognosis of the disease in patients RMM who receive combined treatment with IFN-α2b.

## CONCLUSIONS

1. The research of parameters of immune system in patients with cutaneous melanoma is the important component of laboratory-diagnostic complex to monitor the efficiency of treatment.

2. The monitoring current of disease on the basis of definition the change of immunological parameters in patients RMM during the combined treatment allows to predict its progression before the onset of clinical symptoms.

3. The decreased levels circulating NK cells, Treg, T-helper cells with the receptor for IL-2 and the increased mitogen-induced proliferation of T cells *in vitro* on a background leukopenia during the first 3 months of adjuvant IFN-α2b therapy are the most informative criteria for poor prognosis of the disease in RMM patients

## REFERENCES

1. Федоренко З.П., Гайсенко А.В., Гулак Л.О. та ін. (2012) Рак в Україні, 2010–2011. Захворюваність, смертність, показники діяльності онкологічної служби. Бюл. Нац. канцер-реєстру України. К., 13: 124 с.

2. Коровин С.И., Гулак Л.О., Федоренко З.П. и др. (2010) Проблема меланомы кожи в Украине. Онкология (спец. выпуск), 12 (1): 46-52.

3. Mimeault M., Batra S.K. (2012) Novel biomarkers and therapeutic targets for optimizing the therapeutic management of melanomas. World J. Clin. Oncol., 10(3): 32–42.

4. Коровин С.И., Кукушкина М.Н., Паливец А.Ю. и др. (2012) Интерферон в лечении меланомы кожи. Клиническая онкология, 5(1): 38–42.

5. Fox B.A., Schendel D.J., Butterfield L.H. et al. (2011) Defining the critical hurdles in cancer immunotherapy. J. Translat. Med., 9: 214–253. Doi: 10.1186/1479-5876-9-214.

6. Wolchok J.D., Hoos A., O'day S. et al. (2009) Guidelines for the evaluation of immune therapy activity in solid tumors: Immune-related response criteria. Clin. Cancer Res., 15(23): 7412–7420.

7. Руководство по клинической лабораторной диагностике: в 2 ч. (1982) Под ред. М.А. Базарновой. К. : Вища школа, 2-я ч.: 175 с.

8. Пинегин Б. В., Ярилин А. А., Симонова А. В. и др. (2001) Применение проточной цитометрии для оценки функциональной активности иммунной системы человека. Пособие для врачей-лаборантов. М., 55 с.

9. Вопросы современной проточной цитометрии. Клиническое применение (2008) Под ред. С.В. Хайдукова, А.В. Зурочки. Челябинск, 195 с.

10. Фільчаков Ф.В., Шуміліна К.С., Кукушкіна С.М. та ін. (2011) Особливості імунореактивності організму хворих на меланому шкіри з метастазами в регіональні лімфовузли в умовах дії різних схем інтерферонотерапії. Клиническая онкология, 4 (4): 102–106.

11. Хайдуков С.В. (2007) Подходы к стандартизации метода проточной цитометрии для иммунофенотипирования. Настройка цитометров и подготовка протоколов для анализа. Мед. иммунол., 9(6): 569–574.

12. Фільчаков Ф.В., Шуміліна К.С., Льон Г.Д. та ін. (2011) Вплив різних схем інтерферонотерапії на функціональну активність лімфоцитів у хворих на меланому шкіри. Імунологія та алергологія, 2: 120–125.

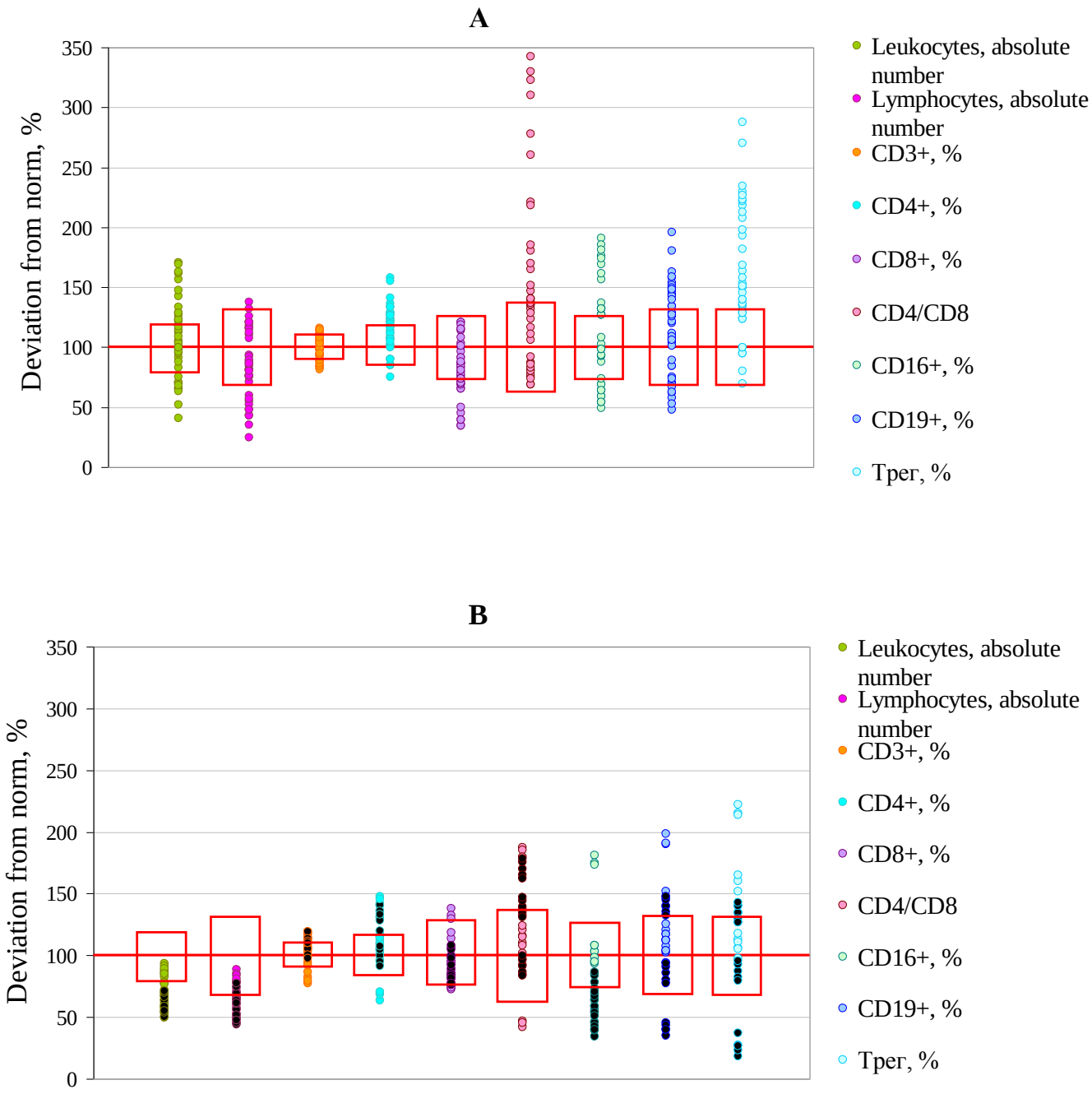
13. Лаповець Л. Є., Луцик Б. Д., Лебедь Г. Б. та ін. (2008) Посібник з лабораторної імунології. К.: 268 с.

14. Лапач С.Н., Чубенко А.В., Бабич П.Н. (2001) Статистические методы в медико-биологических исследованиях с использованием Excel.– К.: Морион, 408 с.

15. Мінцер О.П., Вороненко Ю.В., Власов В.В. (2003) Оброблення клінічних і експериментальних даних у медицині. Кн. 5: Навч. посіб. Інформаційні технології в охороні здоров'я і практичній медицині: У 10 кн. За ред. О.П. Мінцера. К.: Вища школа, 350 с.

16. Фильчаков Ф.В., Кукушкина С.Н., Шумилина Е.С. и др. (2012) Клинико-лабораторные критерии диагностики иммунной дисфункции у больных меланомой кожи. Онкология, 14 (2): 139–144.

17. Cesana G.C., DeRaffele G., Cohen S. et. al. (2006) Characterization of CD4+CD25+ regulatory T cells in patients treated with high-dose interieukin-2 for metastatic melanoma or renal cell carcinoma. J. Clin. Oncol., 24: 1169–1177.


A
Deviation from norm, %
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200
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100
50
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Leukocytes, absolute number
Lymphocytes, absolute number
CD3+, %
CD4+, %
CD8+, %
CD4/CD8
CD16+, %
CD19+, %
Tper, %
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Deviation from norm, %
350
300
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200
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100
50
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Leukocytes, absolute number
Lymphocytes, absolute number
CD3+, %
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CD8+, %
CD4/CD8
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CD19+, %
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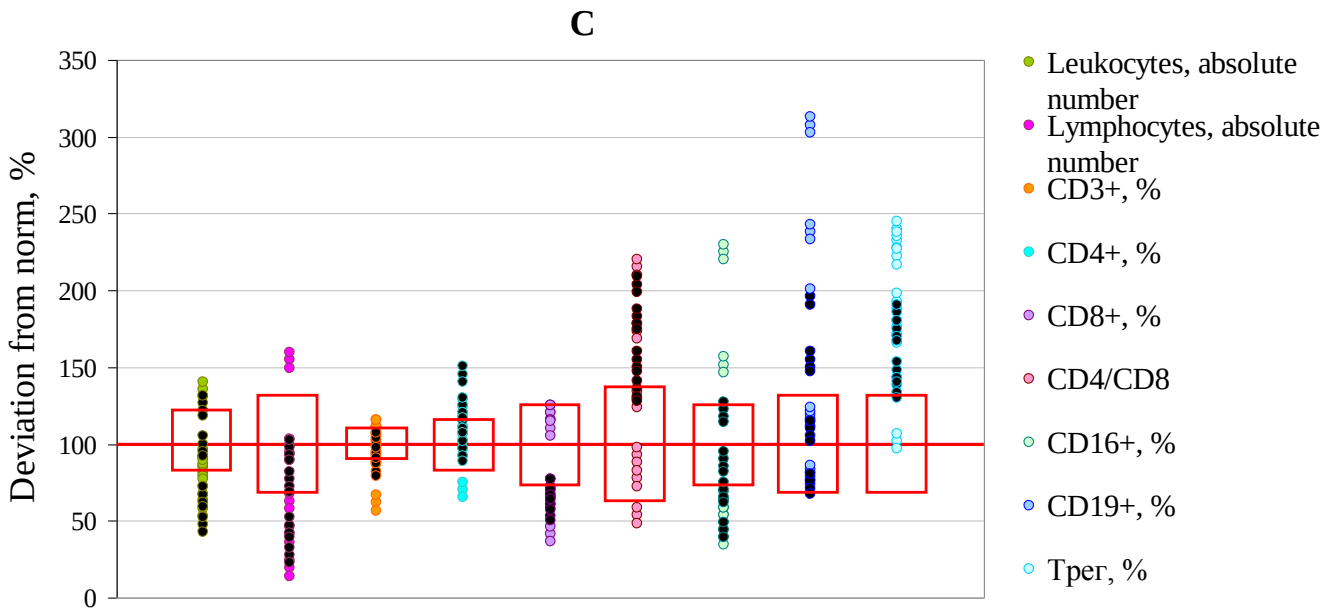


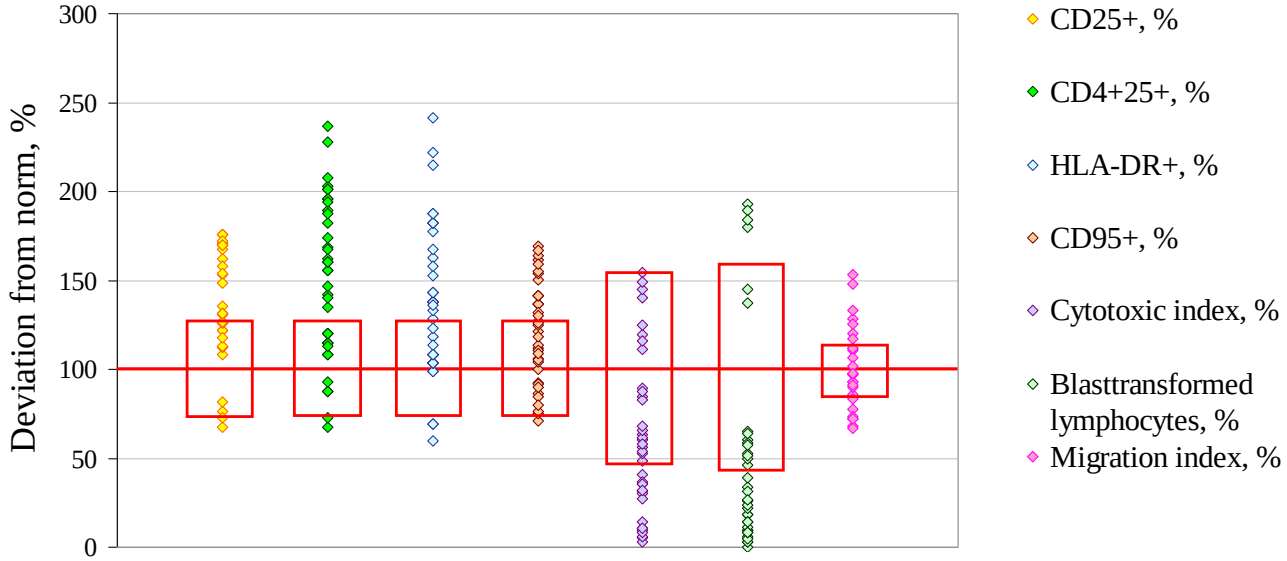

Fig. 1. Changes (% deviation from norm) of subset composition of LPB in patients RMM during the combined treatment (A – after surgical treatment; B – after 3 months IFN therapy; C – after 12 months IFN therapy).

Note to Fig. 1 and Fig 2:

1. Red line (100 %) on *y* axis shows average of parameters in HNS, box indicates its standard deviation;

2. Meanings of parameters in patients with disease progression are painted over black color.

**A**


Deviation from norm, %
300
250
200
150
100
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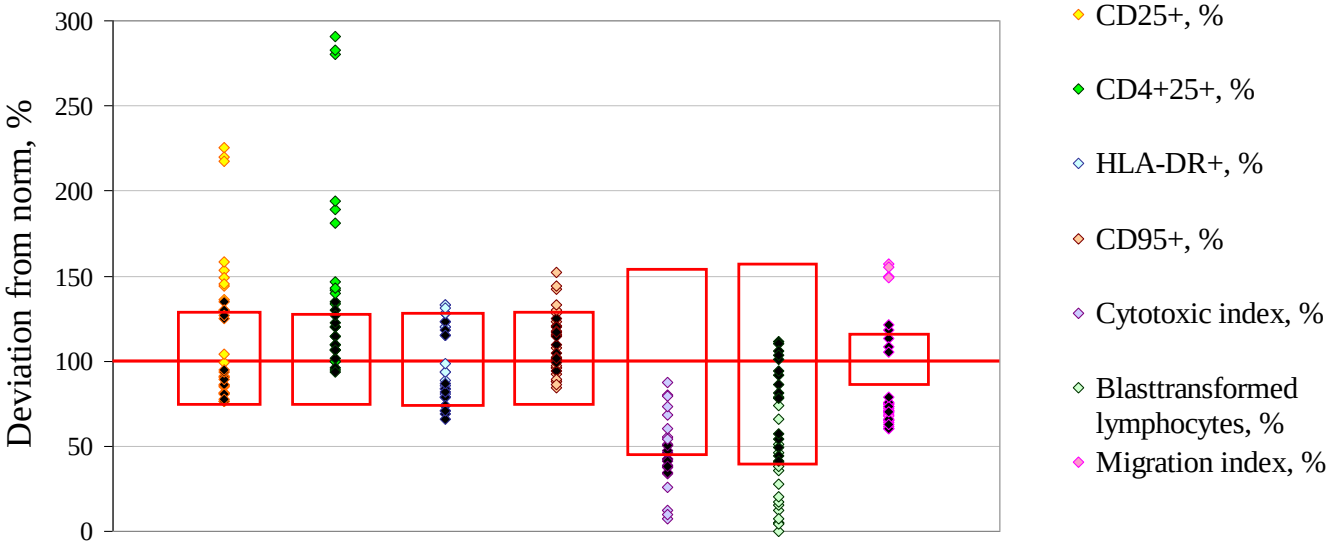
CD25+, %
CD4+25+, %
HLA-DR+, %
CD95+, %
Cytotoxic index, %
Blasttransformed lymphocytes, %
Migration index, %

**B**


Deviation from norm, %
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CD25+, %
CD4+25+, %
HLA-DR+, %
CD95+, %
Cytotoxic index, %
Blasttransformed lymphocytes, %
Migration index, %

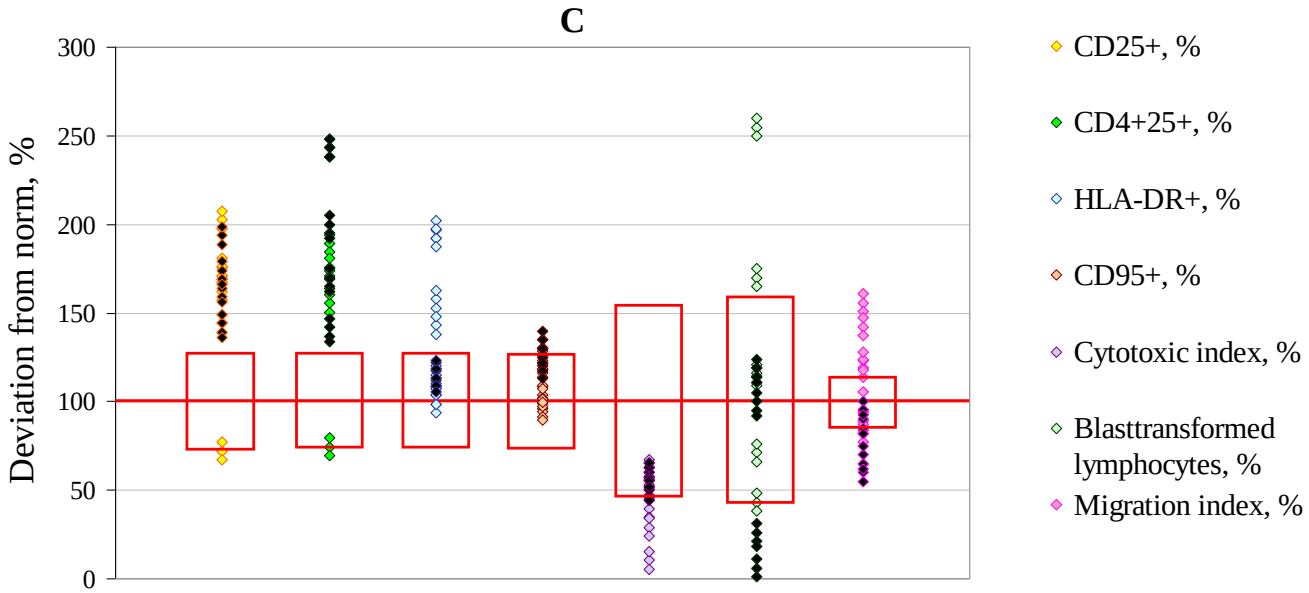


Fig. 2. Changes (% deviation from norm) of activated phenotype and functional activity of LPB in patients RMM during the combined treatment (A – after surgical treatment; B – after 3 months IFN therapy; C – after 12 months IFN therapy).