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LYMPHOCYTE SUBSET DISTRIBUTION AFTER COMBINED CHEMO- AND RADIOTHERAPY IN PATIENTS WITH CANCER OF THE ORAL CAVITY, OROPHARYNX, AND LARYNGOPHARYNX

Aim. To study the prognostic value of the lymphocyte subset distribution to predict the overall survival and its association with the clinicopathologic characteristics and treatment in patients with cancer of the oral cavity, oropharynx, and laryngopharynx. **Materials and Methods.** 44 patients were examined. Immunophenotyping of lymphocyte subsets was performed in peripheral blood samples using flow cytometry. The lymphocyte subset distribution was analyzed depending on the clinicopathological characteristics and treatment outcome, as well as the overall survival. **Results.** The changes in $CD3^+$ T-cells and $CD3^+57^+$ NKT counts were associated with the sex of the patients, $TCR\alpha\beta^+$ T-cells — with the stage of the disease, $CD4^+8^+$ T-cells and $CD3^-16^+57^+$ NK — with the tumor size and differentiation grade, and $CD3^+HLA\text{-}DR^+$, $CD8^+$ T-cells, and $CD4^+/CD8^+$ ratio — with lymph node involvement. The content of $CD3^+HLA\text{-}DR^+$ and $TCR\alpha\beta^+$ T-cells, $CD3^-16^+57^+$ NK, and $CD3^+57^+$ NKT differed in patients depending on the tumor location. There were changes in $CD19^+$ and $HLA\text{-}DR^+$ B-cells, $CD3^+$, $CD4^+$, $CD4^+25^+$ and $TCR\alpha\beta^+$ T-cells, $CD3^-CD16^+57^+$ NK, and $CD3^+57^+$ NKT during treatment, with the most pronounced changes after the first stage of RT. The relative number of $CD3^+HLA\text{-}DR^+$ and tumor size T4 influenced the overall survival of patients ((HR = 0.798, 95% CI, 0.658—0.967, $p = 0.021$) and (HR = 3.015, 95% CI, 1.303—6.975, $p = 0.009$), respectively). **Conclusion.** Parameters of lymphocyte subsets can be promising prognostic markers.

Keywords: lymphocytes, oral cancer, prognosis, immune system, biomarkers.

According to the International Agency for Research on Cancer, malignant neoplasms of the lip and oral cavity rank 16th in terms of morbidity and mortality [1]. In 2020, there were 747,316 cases of lip, oral cavity, and pharynx cancers (global ASR rate of 8.1 new cases per 100,000 population) and 367,285 deaths [2]. Every year, more than 2,200 new cases of malignant neoplasms of the oral cavity are registered in Ukraine. The incidence of this pathology in men is 5.0—5.8 times higher than in

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women [3]. Most often, at the advanced stage, malignant neoplasms of the tonsil (76.1%) and oropharynx (82.0%) are detected. In 2022, 63.1% of cases of malignant neoplasms of the oral cavity were registered at the advanced stage [4]. Despite improvements in 5-year survival in recent decades, treatment results remain disappointing, especially in patients with advanced disease [5].

The functional state of the immune system influences the clinical course of cancer, which allows its components to be considered biomarkers associated with tumor progression and survival [6]. The immune response to head and neck carcinomas is complex [7], and the T-cell component of immunity plays an important role in the antitumor immune response [8]. However, the determination of molecular biomarkers of the immune response in tissue is a highly invasive method, and testing in peripheral blood takes many hours [9].

Determination of changes in the phenotype of peripheral blood lymphocytes by flow cytometry is a minimally invasive and rapid method that makes it possible to sequentially monitor immunological changes before and after each stage of treatment [10].

Materials and Methods

The study included 44 patients with histologically confirmed keratinized and non-keratinized variants of squamous cell carcinoma of the oral cavity, oral and laryngeal part of the pharynx (ICD-10: C01–02, C04–06, C09–10, C12–14), at the stages III, IVA, and IVB (without distant metastases), who were treated at the Zaporizhzhia Regional Cancer Center from 2020 to 2021. The study was approved by the Institutional Review Board of the State Institution «National Research Center for Radiation Medicine of the National Academy of Medical Sciences of Ukraine» (protocol No. 4 from 12 February 2020). Each patient signed an informed consent to participate in the study.

36 patients out of 44 underwent complex treatment, which consisted of two induction systemic courses of polychemotherapy (CT) and three stages of external beam radiation therapy (RT). The course of CT consisted of the intravenous administration of cisplatin 100 mg/m^2 on day 1 and 5-fluorouracil 600 mg/m^2 (continuous infusion) on days 1—5. At the first stage of RT, the course was divided into the tumor and lymphatic drainage pathways up to a total focal dose of 30 Gy. The interval between CT courses and the start of RT was 19—21 days.

Immunophenotyping of lymphocytes was performed in peripheral blood samples collected in 6 mL K_2EDTA tubes using a set of monoclonal antibodies BD Simultest IMK Plus (Becton Dickinson (BD), USA) and additional antibodies CD4 FITC, CD25 PE, and TCRαβ FITC/TCRγδ PE/CD3 PerCP-Cy 5.5 (BD, USA) by flow cytometry. Counting of 5,000 events and analysis were carried out in the dot plot mode using BD CellQuest Pro software (BD, USA) on an FACSCalibur laser flow cytometer (BD, USA).

Statistical analysis was carried out using Statistica 8.0 software (StatSoft, Inc., USA), IBM SPSS Statistics Software version 27.0.1.0 (IBM GmbH, Germany), and MedCalc® Statistical Software version 22.026 (MedCalc Software Ltd, Belgium). The normality of the distribution of quantitative variables was determined using the Kolmogorov — Smirnov test with the Lilliefors correction. To compare indicators characterized by a normal distribution, Student's *t*-test or the main effect ANOVA with Tukey's HSD post hoc test for unequal samples was used. For parameters whose distribution differed from normal, the Mann — Whitney test or non-parametric Kruskal —Wallis rank analysis of variations and the median test with the pairwise comparison of average ranks were used. Dependent samples were compared using the repeated measures ANOVA with Tukey's post hoc test or Friedman's ANOVA with Dunn's post hoc test. The relationship between continuous and ordinal parameters was calculated using point-biserial correlation. The level of statistical significance was set at $p < 0.05$, and $0.06 \leq p \leq 0.10$ was accepted as a sign of a trend.

The sensitivity and specificity of lymphocyte subsets in predicting the prognosis were assessed by receiver operating characteristic (ROC) curves, and the area under the ROC curve (AUC) was calculated as well. Kaplan — Meier survival curves and log-rank analysis were performed to estimate the survival curves and compare the differences between them. The Cox proportional hazard regression model was used to assess the effect of each variable on the overall survival (OS).

Results

The clinical and histopathological characteristics of the patients are presented in the Table.

Based on the results of the study of the distribution of the main subsets of lymphocytes ($CD3^+$ T-cells, $CD19^+$ B-cells, $CD4^+$ T-cells, $CD8^+$ T-cells, $CD4^+$/ $CD8^+$, $CD3^-16^+57^+$ NK, $CD3^+57^+$ NKT) depending on the clinicopathological characteristics, a decrease in the relative number of $CD3^+57^+$ NKT (0.85 ± 0.81 vs 3.92 ± 3.16, $p < 0.05$) and a trend toward a decrease in $CD3^+$ T-cells in women compared to men were revealed (58.72 ± 17.02 vs 64.48 ± 9.92, $0.06 \le p \le 0.10$). No differences were found in the parameters of the main subsets of lymphocytes of patients depending on the stage of the disease and nodal status. The content of $CD3^-16^+57^+$ NK was the lowest in patients with T4 tumors compared to patients with T2 tumors (2.47 ± 1.20 vs 7.68 ± 4.09, $p < 0.05$) and T3 tumors (5.68 ± 3.71). The $CD4^+/CD8^+$ ratio tended to decrease in patients with N2—3 tumors compared to patients with Nx—N0 (0.96 ± 0.42 vs 1.46 ± 0.54, $0.06 \le p \le 0.10$) or N1 tumors (1.06 ± 0.50). A decrease in $CD3^-16^+57^+$ NK was observed in patients with low-grade tumors compared to patients with intermediate-grade tumors (4.70 ± 3.60 vs 7.79 ± 3.84, $0.06 \le p \le 0.10$). The relative numbers of $CD3^-16^+57^+$ NK and $CD3^+57^+$ NKT (2.45 ± 1.98 and 0.59 ± 0.38, respectively, $p < 0.05$) were reduced in patients with palatine tonsil tumors compared to patients whose tumor was located in the tongue (2.45 ± 1.98 and 0.59 ± 0.38 vs 6.65 ± ± 5.04 and 3.52 ± 2.62, respectively), oral cavity (6.38 ± 5.47 and 4.61 ± 3.30, $p < 0.05$), and larynx (6.15 ± 2.40 and 3.37 ± 2.19).

Clinical and histopathological characteristics of patients with cancer of the oral cavity, oropharynx, and laryngopharynx

Characteristics	Number of patients (%)
	44 (100 %)
Sex	
male	38 (86.4)
female	6 (13.6)
Age	
<60	23 (52.3)
≥60	21 (47.7)
Stage	
III	17 (38.6)
IV	27 (61.4)
Tumor size	
T2	9 (20.5)
T3	22 (50)
T4	13 (29.5)
Nodal status	
Nx-N0	10 (22.7)
N1	17 (38.6)
N2	15 (34.1)
N3	2 (4.6)
Differentiation grade	
Low grade	32 (76.2)
Intermediate grade	10 (23.8)
Localization	
Oral cavity	15 (34.1)
Tongue	10 (22.7)
Oropharynx and laryngopharynx	11 (25)
Palatine tonsils	8 (18.2)

Next, we studied the changes in minor lymphocyte subsets ($CD3^+$HLA-DR^+ T-cells, $CD3^-$HLA-DR^+ B-cells, $CD4^+25^+$ T-cells, $CD4^+8^+$ T-cells, $TCR\alpha\beta^+$ T-cells, $TCR\gamma\delta^+$ T-cells) depending on the clinicopathologic characteristics of patients with cancer of the oral cavity, oropharynx, and laryngopharynx. There were no differences between HLA-DR^+ T- and B-cells, $CD4^+25^+$, $CD4^+8^+$, $TCR\alpha\beta^+$, and $TCR\gamma\delta^+$ T-cells depending on sex. When comparing parameters depending on the stage and grade of differentiation, only $TCR\alpha\beta^+$ T-cells tended to decrease in patients with stage IV compared to stage III (49.53 ± 13.32 vs 57.10 ± 11.75, $0.06 \le \le p \le 0.10$) and in patients with low-grade tumors compared to patients with intermediate-grade tumors (43.74 ± 13.11 vs 54.60 ± 12.89; $0.06 \le p \le \le 0.10$). In patients with T4 tumors, the content of $CD4^+8^+$ T-cells decreased compared to that in patients with T3 tumors (1.14 ± 0.81 vs 2.50 ± 1.93, $p < 0.05$). The relative number of $CD3^+$HLA-DR^+ T-cells decreased in patients with N1 tumors compared to patients with Nx—N0 (2.54 ± 1.65 vs 5.22 ± 3.47, $p < 0.05$) and N2—N3 tumors (4.03 ± ± 2.59). The lowest rate of $CD3^+$HLA-DR^+ T-cells was found in patients with tongue tumors compared to patients with oral cavity and larynx tumors (1.49 ± 1.01 vs 4.33 ± 2.69 and 4.35 ± 2.95, $p < 0.05$). Patients with oral cavity tumors tended to have a decreased relative number of $CD3^-$HLA-DR^+ B-cells compared to patients with oropharynx, laryngopharynx, and palatine tonsil tumors (4.54 ± ± 2.01 vs 8.19 ± 5.43 and 9.70 ± 6.61; $0.06 \le$

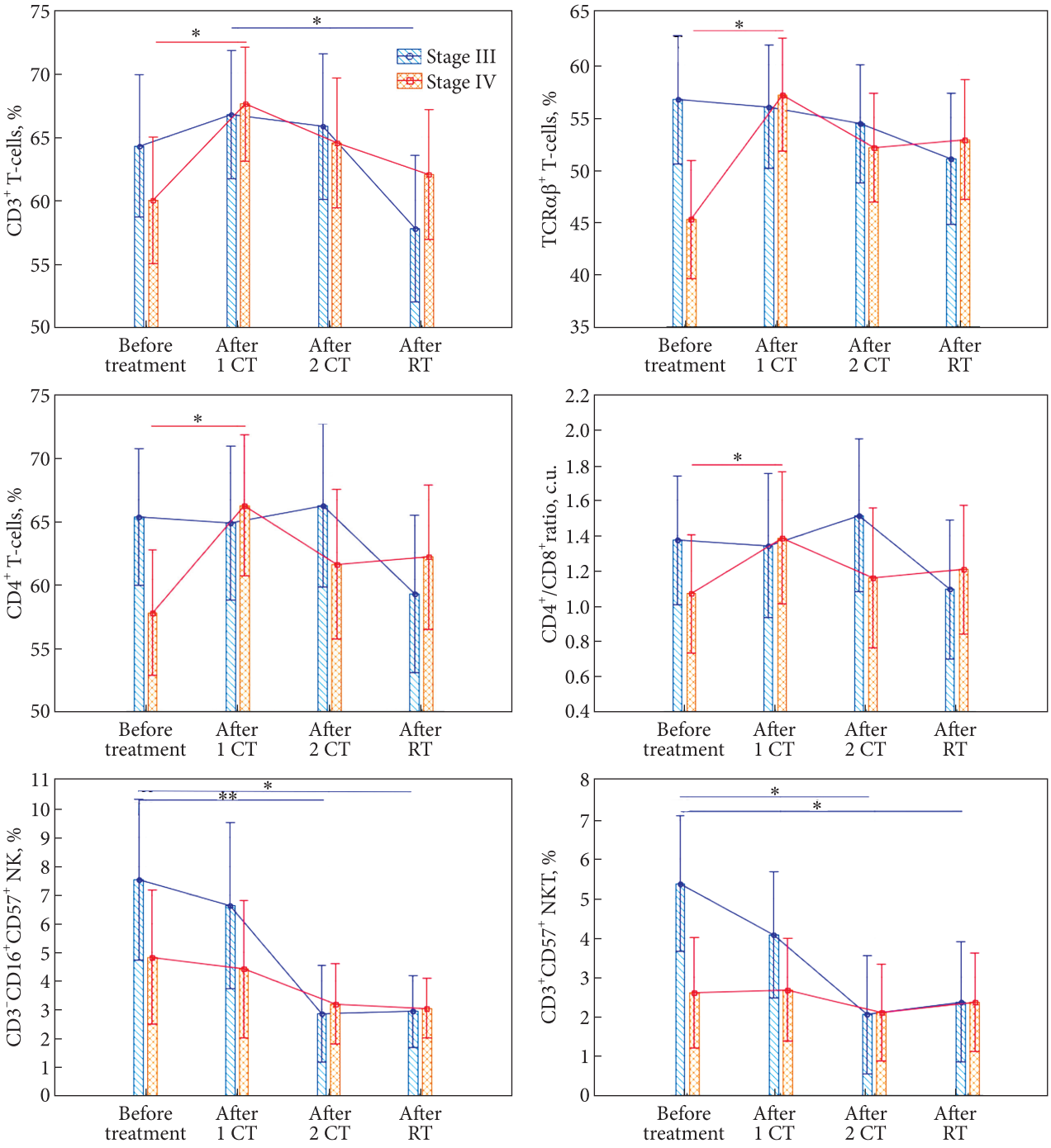

Fig. 1. Percentage of lymphocyte subsets, dependent on treatment and stage of tumor in patients with cancer of the oral cavity, oropharynx, and laryngopharynx. * $p < 0.05$, ** $p < 0.01$ — the difference is significant

$\leq p \leq 0.10$). The relative number of $CD3^+HLA\text{-}DR^+$ T-cells in patients with tongue tumors was lower than in patients with oral cavity tumors or oropharynx and laryngopharynx tumors (1.49 ± 1.01 vs 4.33 ± ± 2.69 and 4.35 ± 2.95, $p < 0.05$). No statistically significant correlation was found between clinicopathological characteristics and lymphocyte subsets.

An increase in the relative number of $CD3^+$ T-cells after the first course of CT compared to the parameters before the treatment was detected (67.23 ± 9.47 vs 61.89 ± 10.71; $p < 0.05$). Compared to the levels of $CD3^+$ T-cells after the first and second courses of CT, the parameters after the first stage of RT were the lowest (60.18 ± 10.99 vs 67.23 ± ± 9.47, $p < 0.001$ and 65.10 ± 10.78, $p < 0.05$, respectively). The lowest parameters of the relative number of $CD19^+$ B-cells were observed after the first stage of RT compared to the parameters before

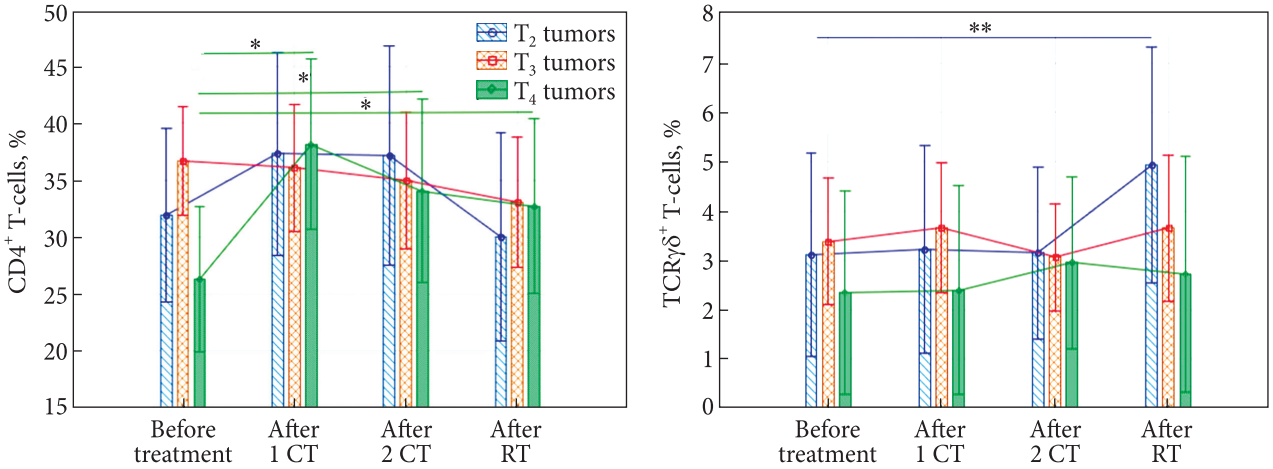

Fig. 2. Percentage of lymphocyte subsets dependent on treatment and tumor size in patients with cancer of the oral cavity, oropharynx, and laryngopharynx. * $p < 0.05$, ** $p < 0.01$ — the difference is significant

treatment (2.67 ± 1.71 vs 5.21 ± 3.96, $p < 0.01$) or after the first (5.91 ± 3.82, $p < 0.001$) and second (5.53 ± 3.54, $p < 0.01$) courses of CT. A trend toward an increase in $CD4^+$ T-cells was detected after the first course of CT compared to the parameter before treatment and after the first stage of RT. The relative number of $CD3^-CD16^+57^+$ NK decreased after the second course of CT and the first stage of RT compared to the parameters before treatment (2.21 ± 1.72 and 3.49 ± 1.83 vs 5.54 ± 3.81, $p < 0.01$ and $p < 0.05$, respectively) and after the first course of CT (5.45 ± 4.28, $p < 0.05$). A decrease in the $CD3^+57^+$ NKT score was observed after the second course of CT compared to the values before treatment (1.31 ± 1.20 vs 3.71 ± 2.91, $p < 0.05$). The higher levels of $HLA\text{-}DR^+$ B-cells were observed after the second course of CT compared to the values before treatment (8.41 ± 5.24 vs 6.45 ± 4.11) and after the first stage of RT (5.74 ± 3.55), without statistical significance. There was a tendency toward a decrease in $CD4^+25^+$ T-cells after the first stage of RT compared with values before treatment (1.48 ± 1.07 vs 2.47 ± 1.75), first (2.11 ± 1.70) and second (1.94 ± 1.67) course of CT. After the first course of CT, the proportion of $TCR\alpha\beta^+$ T-cells tends to be higher than before treatment (56.61 ± ± 11.42 vs 50.47 ± 13.21).

In patients with stage III, the relative number of $CD3^+$ T-cells decreased after the first stage of RT compared to the parameters after the first ($p < 0.05$) and second courses of CT (Fig. 1). Parameters of $CD4^+$T-cells and $CD4^+/CD8^+$ ratio tended to decrease after the first stage of RT. The relative number of $CD3^-16^+57^+$ NK decreased after the second course of CT ($p < 0.01$) and the first stage of RT ($p < 0.05$) compared to the values before treatment. Changes in $CD3^+57^+$ NKT scores were similar ($p < 0.05$). In patients with stage IV, the relative number of $CD3^+$, $TCR\alpha\beta^+$ and $CD4^+$ T-cells, and $CD4^+/CD8^+$ ratio increased after the first course of CT compared to the parameters before treatment ($p < 0.05$).

The content of $CD4^+$ T-cells was higher during treatment than before treatment ($p < 0.05$) in patients with T4 tumors. The relative number of $TCR\gamma\delta^+$ T-cells increased after the first stage of RT ($p < 0.01$) compared to the value before treatment in patients with T2 tumors (Fig. 2).

In patients with intermediate grade tumors, the relative number of $CD3^+$ T-cells was lower after the first stage of RT than after the first ($p < 0.01$) and second course of CT (Fig. 3). The relative number of $TCR\gamma\delta^+$ T-cells ($p < 0.05$) was higher after the first stage of RT compared to the parameters before treatment and after two courses of CT. A decrease in the content of $CD3^-16^+57^+$ NK ($p < 0.05$) was also revealed after the second course of CT and the first stage of RT compared to the value before treatment. The content of $CD3^+57^+$NKT decreased after the first stage of RT compared to the values before treatment ($p < 0.01$) and after the first course of CT ($p < 0.05$).

Using ROC curve analysis of lymphocyte subsets to predict overall survival, optimal thresholds were determined for $CD3^+HLA\text{-}DR^+$ and $CD4^+8^+$ T-cells only (Fig. 4). The area under the ROC curves for $CD3^+HLA\text{-}DR^+$ T-cells was 0.692 (95% CI, 0.532—0.823), for $CD4^+8^+$ T-cells — 0.690

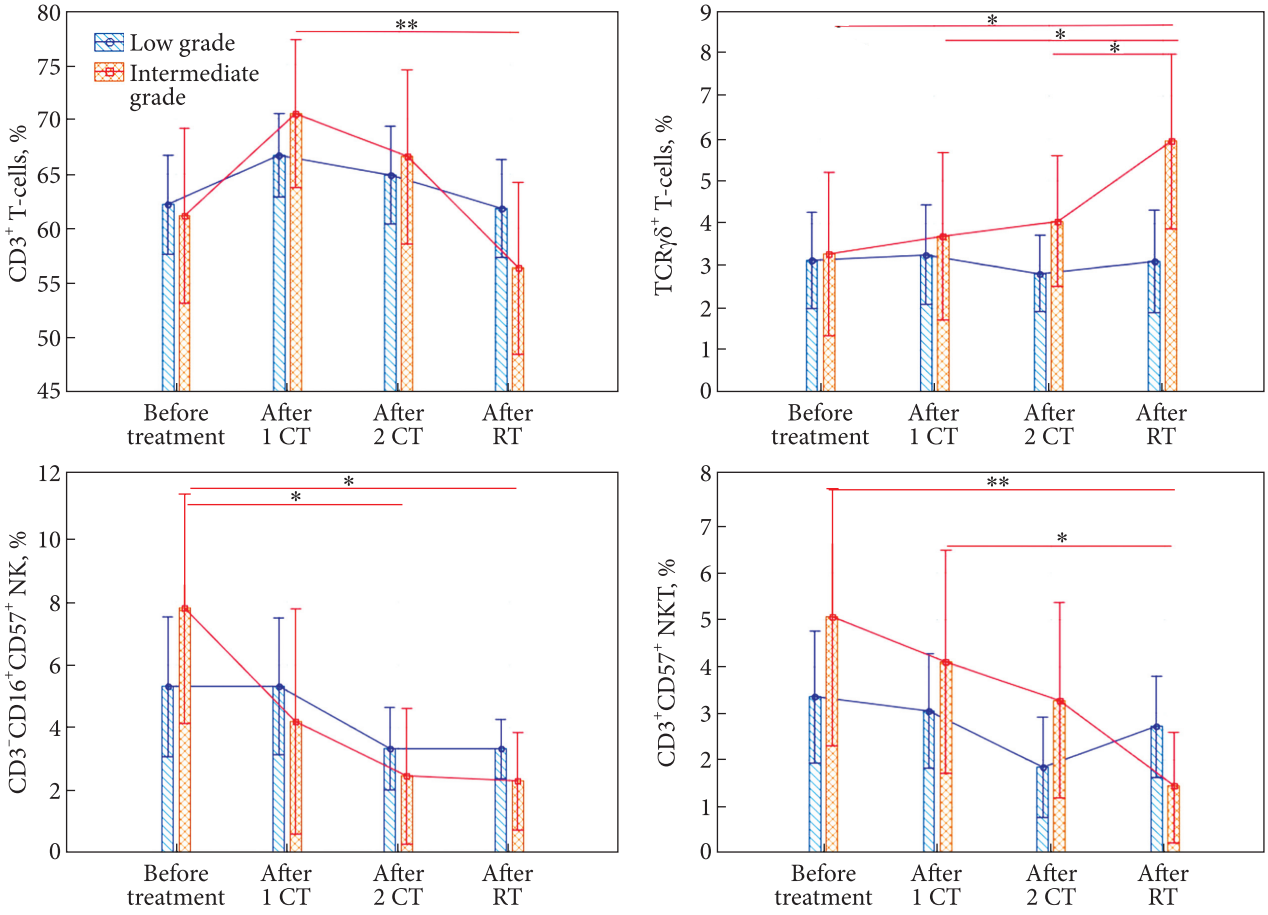

Fig. 3. Percentage of lymphocyte subsets dependent on treatment and pathological grading in patients with cancer of the oral cavity, oropharynx, and laryngopharynx. * $p < 0.05$, ** $p < 0.01$ — the difference is significant

(95% CI, 0.529—0.824). The threshold value for $CD3^+HLA\text{-}DR^+$ T-cells was ≤ 2.61 with a sensitivity of 68.2% and specificity of 71.4%, for $CD4^+8^+$ T-cells it was ≤ 1.06 (sensitivity of 61.9% and specificity of 81.0%).

Patients were divided into two subgroups based on the cutoff values of $CD3^+HLA\text{-}DR^+$ and $CD4^+8^+$ T-cells. The Kaplan — Meier analysis showed that patients with lower $CD3^+HLA\text{-}DR^+$ (HR = 3.447, 95% CI, 1.435—8.280) and $CD4^+8^+$ T-cells (HR = = 4.318, 95% CI, 1.679—11.102) had worse 2-year overall survival (Fig. 5).

Univariate Cox-proportional hazard regression analysis was performed to determine whether sex, age, stage, tumor size, nodal status, differentiation grade, localization, and lymphocyte subsets were factors influencing patient overall survival. Only $CD3^+HLA\text{-}DR^+$ T-cells (HR = 0.798, 95% CI, 0.658—0.967, $p = 0.021$) and tumor size T4 (HR = = 3.015, 95% CI, 1.303—6.975, $p = 0.009$) influenced the overall survival of patients. Because only two factors were found to be significant in the univariate analysis, the multivariate Cox proportional hazard regression analysis was not applied.

To date, the use of the TNM classification to predict the clinical outcomes of patients with malignant neoplasms, in particular oral carcinoma, is insufficient [11, 12]. Malignant cell transformation and tumor progression are closely related to the functioning of the immune system, which influences prognosis, so the patient's immune status is considered a prognostic marker [13].

Thus, changes in the proportion of T-cells, in particular $CD4^+25^+$ Tregs, $CD16^+56^+$ and $CD57^+$ NK, among the total number of peripheral blood lymphocytes depending on the clinical stage in patients with oral carcinoma have been shown [6, 14—17]. The parameters of $CD4^+$, $CD8^+$, $CD4^+CD25^+$ T-cells, $CD16^+CD56^+$, and $CD57^+$ NK changed depending on the tumor size [6, 14, 18]. In patients with different nodal status, changes were also observed in the parameters of $CD19^+$ B-cells [15], $CD4^+$ and $CD8^+$, $CD4^+CD25^+$ T-cells, and $CD16^+CD56^+$ NK [14, 15, 18]. No correlation

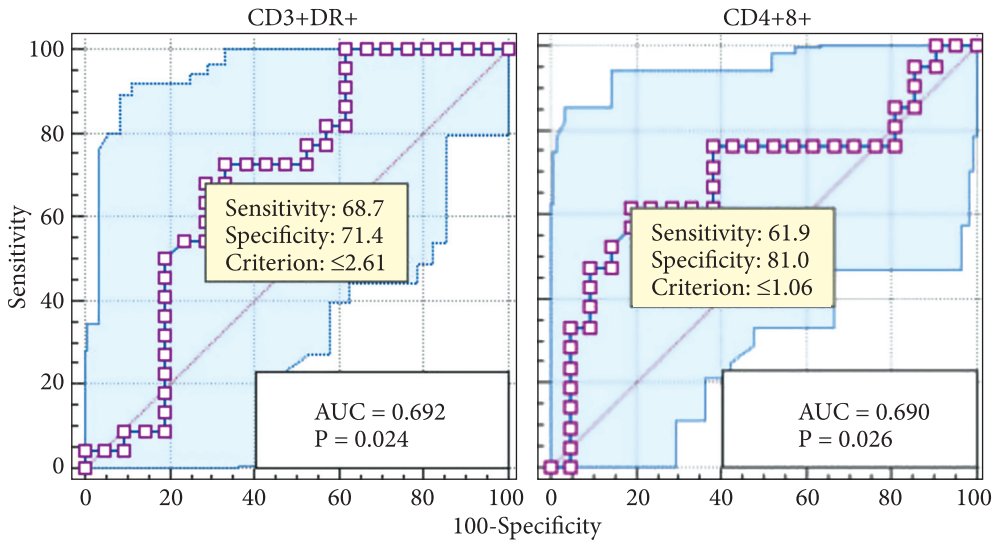

Fig. 4. The ROC curve of lymphocyte subsets to predict the overall survival of patients with cancer of the oral cavity, oropharynx, and laryngopharynx. The abscissa represents the false positive rate and the ordinate represents the true positive rate

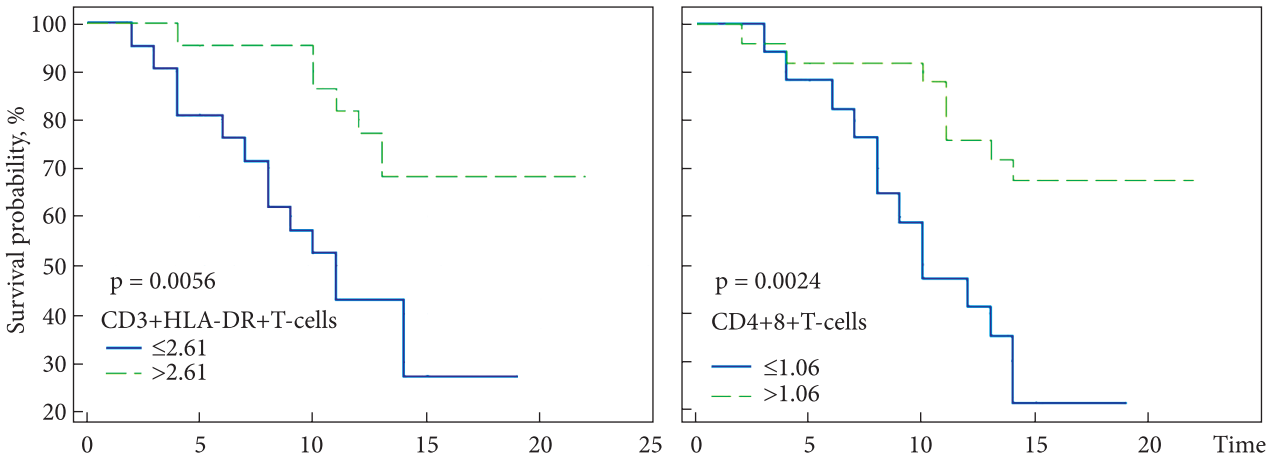

Fig. 5. Two-year overall survival by of patients with cancer of the oral cavity, oropharynx, and laryngopharynx dependent on $CD3^+HLA\text{-}DR^+$ and $CD4^+8^+$ T-cells status

between the $CD57^+$ NK parameter and the nodal status has been determined [6]. There was a difference between the histological classification and the parameters of $CD8^+$ T-cells and the $CD4^+/CD8^+$ ratio [19]. Other studies have not revealed a relationship between the content of $CD3^+$ T-cells, their subsets $CD4^+$ and $CD8^+$, $CD4^+CD25^+$ and $TCR\gamma\delta^+$ T-cells, NK, and $CD4^+/CD8^+$ ratio and the clinicopathological characteristics of patients [7, 18—23].

A decrease in the relative numbers of $CD19^+$ B-cells, $CD3^+$, $CD4^+$, and $CD8^+$ T-cells after RT [24, 25], which persisted three months after treatment for B-cells [26] and after a year for T-cells [27], was shown. Studies of the effects of combination RT and CT with cisplatin or cetuximab combined with RT on $CD19^+$ B-cells and $CD4^+$ and $CD8^+$ T-cells indicate that RT may cause changes in the proportions of these lymphocyte subsets [28]. Increased $CD4^+$ T-cell counts are associated with better survival of patients with laryngeal cancer treated with induction CT [29]. Other studies have shown changes in the distribution of T- and B-cells and NK after CT and surgery [18], as well as their association with the development of side effects after CT [30]. The proportion of $TCR\gamma\delta^+$ T-cells was higher after treatment [23]. NK content increased after RT one year later to levels higher than before treatment [27], although in another study, the relative number of NK was independent of RT [24], but the increased NK content persisted throughout the treatment period [26].

Our study has several limitations that may reduce the power of statistical analysis: a small sample of

patients and the fact that not all patients received comprehensive treatment. The discrepancy between the results of our studies and those of other investigators may be explained by the variability of the patient groups evaluated and the methodology used, which emphasizes the importance of larger study groups and multicenter studies. Based on the results obtained in our and other studies, it is possible to trace the presence of changes in the subset structure of lymphocytes both with different clinicopathological characteristics and at different stages of treatment. Therefore, it is important to continue research and validation of immune status markers for early diagnosis, disease assessment, and prognosis.

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СУБПОПУЛЯЦІЙНИЙ РОЗПОДІЛ ЛІМФОЦИТІВ ПІСЛЯ КОМБІНОВАНОЇ ХІМІО- ТА ПРОМЕНЕВОЇ ТЕРАПІЇ У ХВОРИХ ЗІ ЗЛОЯКІСНИМИ НОВОУТВОРЕННЯМИ ПОРОЖНИНИ РОТА, РОТОВОЇ ТА ГОРТАННОЇ ЧАСТИН ГЛОТКИ

Мета. Визначити значення субпопуляційного розподілу лімфоцитів для прогнозування загальної виживаності та його зв'язку з клініко-патологічними характеристиками та проведеним лікуванням у хворих на злоякісні новоутворення порожнини рота, ротової та гортанної частин глотки. **Матеріали та методи.** Імунофенотипування субпопуляцій лімфоцитів проводили на зразках периферичної крові за допомогою проточної цитометрії. Аналіз включав визначення залежності від клініко-патологічних характеристик та курсів лікування, а також залежність загальної виживаності від досліджуваних параметрів. **Результати.** Показано зміни відносної кількості $CD3^+$ Т-лімфоцитів і $CD3^+57^+$ НКТ залежно від статі, $TCR\alpha\beta^+$ Т-лімфоцитів — від стадії захворювання, $CD4^+8^+$ Т-лімфоцитів, $CD3^-16^+57^+$ НК — від розміру пухлини і ступеню диференціювання, $CD3^+HLA\text{-}DR^+$, $CD8^+$ Т-лімфоцитів і співвідношення $CD4^+/CD8^+$ — від наявності метастатичного ураження в регіонарні лімфатичні вузли. Вміст $CD3^+HLA\text{-}DR^+$, $TCR\alpha\beta^+$ Т-лімфоцитів, $CD3^-16^+57^+$ НК та $CD3^+57^+$ НКТ відрізнявся в пацієнтів залежно від локалізації пухлини. Спостерігалися зміни в розподілі $CD19^+$ і $HLA\text{-}DR^+$ В-лімфоцитів, $CD3^+$, $CD4^+$, $CD4^+25^+$ і $TCR\alpha\beta^+$ Т-лімфоцитів, $CD3^-CD16^+57^+$ НК і $CD3^+57^+$ НКТ під час лікування з найбільш вираженими змінами після першого етапу променевої терапії. Відносна кількість $CD3^+HLA\text{-}DR^+$ і розмір пухлини Т4 впливали на загальну виживаність пацієнтів ((HR = 0,798, 95% ДІ, 0,658–0,967, p = 0,021) і (HR = 3,015, 95% ДІ, 1,303–6,975), p = 0,009), відповідно). **Висновок.** Показники субпопуляцій лімфоцитів можуть бути перспективними прогностичними маркерами, які в майбутньому, після валідації, можна буде використовувати додатково для персоналізації лікування.

Ключові слова: лімфоцити, новоутворення порожнини рота, прогнозування, імунна система, біомаркери.