Results of the study of factors predicting the risk of the development of grade III radiation-induced mucositis during radiation or chemoradiation therapy in patients with oral cavity and oropharynx cancer

Výsledky studie faktorů predikujících riziko vzniku poradiační mukozitidy stupně III během radioterapie nebo chemoradioterapie u pacientů s karcinomem ústní dutiny a orofaryngu

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Summary

Background: Today, a number of methods and ways of prevention and treatment of radiation-induced mucositis of the oral cavity and oropharynx have been developed, but the represented approaches are still not effective enough. Therefore, to increase the effectiveness of the prevention and treatment of radiation-induced mucositis, it is necessary to approach this problem comprehensively and individually, and to evaluate the factors affecting the development of mucositis. ***Materials and methods:*** In this single-center prospective controlled non-randomized clinical trial, the results of clinical observation of the development of complications of radiation and chemoradiation therapy in 105 patients with a newly diagnosed squamous cell cancer of the oral cavity and oropharynx were analyzed. Factors affecting the risk of the development of grade III radiation-induced mucositis including the age, gender of the patients, their general condition before the treatment according to World Health Organisation scales, type of the treatment and its doses, additional use of immunotherapy with alpha/beta defensins, characteristic signs of the tumor process and all indices of the immune status of the patients before the treatment have been analyzed. ***Results:*** The method of construction and analysis of one-factor logistic regression models, where 24 indices were analyzed as factorial features, showed that the reduction of the risk of the development of grade III radiation-induced mucositis is predicted by several factors: immunotherapy, gender, serum concentrations of IgG and IgA. A decrease ($P < 0.001$) in the risk of the development of grade III radiation-induced mucositis was revealed if immunotherapy with alpha/beta defensins (with a total dose of 40 mg) was included into the treatment scheme (relative odds (RO) 0.05; 95% reference interval (RI) 0.02–0.18), in comparison with patients of the groups where it was not present or this immune agent was used in a total dose of 60 mg ($P = 0.001$, RO 0.06; 95% RI 0.01–0.30). The next factorial sign was gender, namely the risk of the development of grade III radiation-induced mucositis was lower for men ($P = 0.003$; RO 0.15; 95% RI 0.04–0.53) compared to women. An increase ($P = 0.024$) in the risk of the development of grade III radiation-induced mucositis with an increase in the initial level of IgG serum concentration was revealed, (RO 1.08; 95% RI 1.01–1.16) for each 1 mg/mL, as well as an increase ($P = 0.044$) in the possibility of the appearance of grade III radiation-induced mucositis with an increase in the serum concentration of IgA (RO 1.23; 95% RI 1.01–1.50) for every 1 mg/mL also before the beginning of the treatment. Multifactorial analysis has also confirmed that the risk of the development of grade III radiation-induced mucositis increases ($P = 0.008$) with a high serum IgG concentration before the treatment or with an increase in this index during therapy (RO 1.13; 95% RI 1.03–1.09) for

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every 1 mg/mL (when standardized by other risk factors). It was determined that when standardizing according to other factors (gender, IgG level), the risk of the development of grade III radiation-induced mucositis in the use of the immune agent alpha/beta defensins in a total dose of 40 mg per course decreases (P < 0.001; RO 0.08; 95% RI 0.02–0.27) compared to patients with oral cavity and oropharynx cancer who were not treated with immunotherapy. The risk of the development of grade III radiation-induced mucositis also decreases (P = 0.001) in the use of immunotherapy in a higher dose, i.e. 60 mg per course (RO 0.03; 95% RI 0.004–0.24 compared to patients whose treatment did not include immunotherapy (when standardized by other factors). ***Conclusion:*** As a result of this controlled clinical study, some factors were determined in addition to the radiation as those affecting the risk of the development of grade III radiation-induced mucositis in patients with oral cavity and oropharynx cancer during special treatment. These factors comprise the inclusion of immunotherapy with alpha/beta defensins into the specific treatment; gender, and baseline levels of serum IgG and IgA concentrations suggest a pattern in which the higher the serum IgG and IgA concentrations are before the start of the treatment, the greater is the likelihood of severe radiation-induced mucositis degree during special therapy. The results of the study of humoral state of the immune system in patients with oral cavity and oropharynx cancer before the beginning of chemoradiation therapy can be used as prognostic risk factors for the development of severe gamma-irradiation-induced mucositis of the oropharyngeal area, as well as an indication for the use of immunotherapeutic agents (in particular, alpha/beta defensins) that are able to polarize the immune response towards type 1 T-helpers through their immunomodulatory action.

Key words
cancer – oral cavity – oropharynx – chemoradiation – immunotherapy – alpha/beta defensins – immunity – mucositis

Souhrn
Východiska: K dnešnímu dni byla vyvinuta řada metod a technik pro prevenci a léčbu poradiační mukozitidy ústní dutiny a orofaryngu, ale uvedené přístupy stále nejsou dostatečně účinné. Pro zvýšení účinnosti prevence a léčby poradiační mukozitidy je proto nutné přistupovat k tomuto problému komplexně a individuálně a posoudit faktory ovlivňující vznik tohoto onemocnění. ***Materiál a metody:*** Tato prospektivní nerandomizovaná kontrolovaná klinická studie z jednoho centra analyzovala výsledky klinického sledování rozvoje komplikací po radioterapii a chemoradioterapii u 105 pacientů s nově diagnostikovaným dlaždicobuněčným karcinomem ústní dutiny a orofaryngu. Byly analyzovány faktory ovlivňující riziko vzniku poradiační mukozitidy stupně III, vč. věku, pohlaví pacientů, jejich celkového stavu před léčbou podle stupnice Světové zdravotnické organizace, typu léčby a její dávky, dodatečného podání imunoterapie alfa/beta defenziny, charakteristických příznaků nádorového procesu a všech ukazatelů imunitního stavu pacienta před léčbou. ***Výsledky:*** Metoda konstrukce a analýzy jednofaktorových logistických modelů regrese, ve kterých bylo analyzováno 24 ukazatelů jako faktoriální prvky, ukázala, že snížení rizika vzniku poradiační mukozitidy stupně III je predikováno několika faktory: imunoterapií, pohlavím a sérovými koncentracemi IgG a IgA. Bylo zjištěno snížení (p < 0,001) rizika vzniku poradiační mukozitidy stupně III, pokud byla do léčby pacientů zařazena imunoterapie alfa/beta defenziny v celkové dávce 40 mg (relativní poměr šancí (relative odds – RO) 0,05; 95% referenční interval (RI) 0,02–0,18) oproti pacientům ve skupině bez imunoterapie a ve skupině s touto imunoterapií v celkové dávce 60 mg (p = 0,001; RO 0,06; 95% RI 0,01–0,30). Dalším faktorem bylo pohlaví, konkrétně u mužů bylo ve srovnání s ženami riziko vzniku poradiační mukozitidy stupně III nižší (p = 0,003; RO 0,15; 95% RI 0,04–0,53). Bylo zaznamenáno zvýšení rizika (p = 0,024) mukozitidy stupně III při zvýšení výchozí hladiny sérové koncentrace IgG (RO 1,08; 95% RI 1,01–1,16) na každý 1 mg/ml a zvýšení (p = 0,044) pravděpodobnosti vzniku mukozitidy stupně III se zvýšením hladiny sérové koncentrace IgA (RO 1,23; 95% RI 1,01–1,50) na každý 1 mg/ml, také před léčbou. Multivariační analýza rovněž potvrdila, že riziko vzniku poradiační mukozitidy stupně III se zvyšuje (p = 0,008) s vysokou hladinou sérové koncentrace IgG před léčbou nebo se zvýšením tohoto ukazatele během léčby (RO 1,13; 95% RI 1,03–1,09) na každý 1 mg/ml (při standardizaci na ostatní rizikové faktory). Bylo zjištěno, že při standardizaci na další faktory (pohlaví, koncentrace IgG) je riziko vzniku mukozitidy stupně III při použití imunosupresiva alfa/beta defenzinů v celkové dávce 40 mg na kúru sníženo (p < 0,001; RO 0,08; 95% RI 0,02–0,27) ve srovnání s pacienty s karcinomem ústní dutiny a orofaryngu, kteří imunoterapii nedostávali. Podobně je riziko vzniku poradiační mukozitidy stupně III sníženo (p = 0,001) při použití imunoterapie ve vyšší dávce, tj. 60 mg na jeden cyklus (RO 0,03; 95% RI 0,004–0,24) ve srovnání s pacienty, kteří imunoterapii nedostali (při standardizaci na ostatní faktory). ***Závěr:*** Tato kontrolovaná klinická studie identifikovala další faktory, které kromě samotného ozařování ovlivňují riziko vzniku poradiační mukozitidy stupně III u pacientů s karcinomem ústní dutiny a orofaryngu během speciální léčby. Mezi tyto faktory patří zařazení imunoterapie alfa/beta defenziny do speciální léčby, pohlaví a výchozí sérové koncentrace IgG a IgA, přičemž platí, že čím vyšší jsou sérové koncentrace IgG a IgA v době zahájení léčby, tím větší je pravděpodobnost vzniku závažnější poradiační mukozitidy během speciální léčby. Výsledky studie humorálního stavu imunitního systému pacientů s karcinomem ústní dutiny a orofaryngu před zahájením chemoradioterapie lze využít jako prognostické rizikové faktory pro vznik závažné poradiační mukozitidy orofaryngu a také jako indikaci pro použití imunoterapeutických látek, zejména alfa/beta-defenzinů, které jsou díky imunomodulačnímu účinku schopny polarizovat imunitní odpověď směrem k pomocným T-lymfocytům typu 1.

Klíčová slova
karcinom – ústní dutina – orofarynx – chemoradioterapie – imunoterapie – alfa/beta defenziny – imunita – epitel

Introduction

During radiation or chemoradiation therapy of patients with oral cavity or oropharynx cancer, the development of radiation-induced mucositis of any grade of severity leads to a decrease in the quality of life of the patients and is the most exhausting of the complications of study treatment [1,2]. Therefore, the occurrence of grade III radiation-induced mucositis forces the premature completion of chemoradiation therapy at a dose that is insufficient to achieve an optimal antitumor result, which increases the risk of recurrence of neoplasia, or to take a break only during the radiation stage of therapy, which can cause secondary radio-resistance of the tumor, which will be manifested later upon resumption of the irradiation courses after recovery from mucositis [3,4].

Due to the concentration of many efforts in performance the clinical research, a number of methods and ways of prevention and treatment of radiation-induced mucositis of the oral cavity and oropharynx have been developed, since this approach can be the key one in the results of treatment improvement and prolonging the endurance of patients with cancer of the oral cavity and oropharynx [5,6]. However, the represented approaches are still not effective enough; thus the results of their application in clinical practice leave unsatisfactory results in many oncological patients having mucositis as a complication of chemotherapy. The evidence obtained in recent studies indicates that in order to increase the effectiveness of the prevention and treatment of radiation-induced mucositis, it is necessary to approach this problem comprehensively and individually and to evaluate other known factors influencing the development of the inflammatory reaction of oral cavity mucous membrane of a severe grade, in addition to the source of ionizing radiation. As it was shown by Sougiannis et al., the effect of radiation therapy is associated with the induction of immune inflammation reactions on the mucous membranes, the nature and intensity of which is determined not only by the properties of the radiation, but also by the immune status of the human body. Therefore, it is advisable to search for potential immune factors that can contribute to the development of grade III mucositis during the exposure to radiation therapy in cancer patients, which would allow to find methods of influencing the immune status to prevent complications of study treatment. It is also known that some immunotherapeutic agents, such as interferons of various classes [7,8], thymosin-alpha1 [9], interleukin 2 [10], which have demonstrated effectiveness in oncology as antitumor agents, also have adjuvant and tolerogenic effects on chemoradiation therapy, which improves the tolerability of study therapy and can potentially be associated with a reduction in the risk of radiation-induced mucositis development. In such cases, their positive effect is found in the immunomodulatory effect, which rebuilds the nature of the immune system response to radiation damage of the mucous membrane in the oral cavity during chemoradiation therapy. Nevertheless, the side effects of tested immunotherapeutic agents [7] as well as frequent cases of resistance to their immunomodulatory action [8] force us to look for new, more effective and safer ways of immunotropic influence in oncology. As it is indicated by Adyns et al., natural and recombinant alpha/beta defensins are currently among the most promising immunomodulatory agents in oncology [11]. Therefore, in the course of specially planned controlled clinical studies, it seems promising to determine the potential protective effects of these agents regarding the development of radiation-induced mucositis in patients with oral cavity and oropharynx cancer during courses of study treatment.

Aim of the study

This study aimed to determine the prognostic factors of the risk of the development of grade III radiation-induced mucositis in patients with oral cavity and oropharynx cancer during the radiation or chemoradiation stage of treatment in order to improve the endurance of patients by improving the methods of prevention and treatment of radiation damage to the mucous membrane in the oropharyngeal area.

Materials and methods

In this single-center prospective controlled non-randomized clinical trial, there were analyzed the results of laboratory examination, treatment, and clinical observation of 105 patients with a newly diagnosed squamous cell carcinoma of the oral cavity and oropharynx. The study was performed on the basis of the surgical department of head and neck pathology and the radiological department of the communal nonprofit enterprise Precarpathian Clinical Oncology Center of the Ivano-Frankivsk Regional Council from 2017 to 2022. All patients were acquainted with the treatment plan, which was certified by signing a written informed consent, following the principles of the Declaration of Helsinki. The Ethics Committee of Ivano-Frankivsk National Medical University approved the research protocol (protocol No. 94/17 dated 16.11.2017)

The age of the patients ranged from 33 to 85 years, and the average index was 60.3 ± 9.8 years (± SD). A total of 87.6% were men (92 patients) and 12,4 % were women (13 patients). During the additional examination, more than 90% had an unsanitated oral cavity, which, accordingly, was rectified before the beginning of the treatment, namely by professional oral hygiene, treatment of chronic foci of infection or tooth root extraction. In case of the presence of metal dental crowns, protective plastic mouth guards were made for the patients during radiation therapy to prevent secondary irradiation of the oral cavity mucous membrane.

All patients were divided into the groups depending on the therapy scheme at the first stage of the study treatment. Group I (RT+IT) included 25 patients receiving radiation therapy and immunotherapy with alpha/beta defensins (40 mg); group II (CH/RT+IT) included 20 patients receiving radiation therapy with intra-arterial chemotherapeutic potentiation and immunotherapy (40 mg); group III (RT+2IT) included 20 patients receiving gamma therapy against the background of immunotherapy in an increased dose (60 mg); group IV (RT) included 20 patients receiving radiation therapy only; group V (CH/RT) included 20 patients receiving radiation therapy with regional intra-arterial chemotherapy without immunotherapy. Groups IV and V were comparators.

The choice of the treatment method was regulated by the "Standards of diagnosis and treatment of cancer patients"; in particular, by the clinical protocol for providing medical care to patients with oral cavity and oropharynx cancer – Order of the Ministry of Health of Ukraine No. 554, dated September 17, 2007, with changes and additions made by Order of the Ministry of Health of Ukraine No. 247, dated March 28, 2016.

The method for performing the radiation course consisted in the application of remote gamma irradiation using Cobalt-60 device to the tumor area and

Tab. 1. Allocating patients to treatment groups depending on the histological differentiation of the tumor.

Groups	Highly differentiated G1		Moderately differentiated G2		Low-differentiated G3		P
	abs.	%	abs.	%	abs.	%	0.705
I – RT+IT	8	32	10	40	7	28	
II – CH/RT+IT	5	25	11	55	4	20	
III – RT+2IT	6	30	11	55	3	15	
IV – RT	5	25	13	65	2	10	
V – CH/RT	8	40	10	50	2	10	
total	**32**	**31**	**55**	**52**	**18**	**17**	

% – frequency, abs. – absolute number of patients, CH/RT – chemoradiotherapy, IT – immunotherapy, RT – radiation therapy

Tab. 2. Allocating patients to treatment groups depending on the form of tumor growth.

Groups	Exophytic tumor growth		Endophytic tumor growth		Mesophytic tumor growth		P
	abs.	%	abs.	%	abs.	%	0.917
I – RT+IT	1	4	16	64	8	32	
II – CH/RT+IT	1	5	14	70	5	25	
III – RT+2IT	3	15	12	60	5	25	
IV – RT	3	15	11	55	6	30	
V – CH/RT	2	10	13	65	5	25	
total	**10**	**9**	**66**	**63**	**29**	**28**	

% – frequency, abs. – absolute number of patients, CH – chemoradiotherapy, IT – immunotherapy, RT – radiation therapy

the area of regional lymph drainage up to a total dose of 36–40 Gy (single focal dose 2–2.5 Gy, 5 sessions per week).

Depending on the location of the tumor in the oral cavity or oropharynx, patients of the groups underwent catheterization of the superficial temporal artery or the external carotid artery. Regional intra-arterial chemotherapy with cisplatin at a dose of 20 mg/m^2 was performed since the first day of radiation therapy for 5 days with the duration of cisplatin administration for 2–3 hours using a DSh-08 syringe pump. At the same time, patients received remote gamma therapy (RGT). After completion of chemotherapeutic potentiation, radiation therapy was continued up to a total dose of 40 Gy with control of general as well as local changes and blood parameters once a week.

Patients of groups I, II and III were additionally given immunotherapy with an alpha/beta defensins medicine, according to two schemes of its administration to prevent and reduce the complications of radiation and chemoradiation therapy. The first is 2.0 mL intramuscularly twice a day for 5 days, starting 2 days before the study treatment, and 2.0 mL once a day during the next 10 days (total dose (TD) 40 mg). The second scheme of the administration of alpha/beta defensins consisted in increasing the dose to 2.0 mL intramuscularly twice a day for 10 days, starting 2 days before the beginning of study treatment, and 2.0 mL once a day during the next 10 days (TD 60 mg).

All patients had squamous cell carcinoma according to histological structure, and the grade of differentiation among the groups was various (Tab. 1). In general, there were most patients with histological differentiation G2 (52%) the observation, and the least with G3 (17%). According to the analysis performed (comparison by the chi-square test), the study groups were compared according to the histological differentiation of the tumor before the treatment ($P > 0.05$).

The analysis of the distribution of patients depending on the form of tumor growth shows that the majority (66) of patients (63%) had endophytic tumors and least of them (10) had exophytic tumors (9%) (Tab. 2). There were 3 (15%) patients with an exophytic tumor in each of groups III and IV, 1 patient in each of groups I and II, and 2 (10%) patients in group V. Endophytic tumors were most common in group I – 16 patients (64%), and least common in group IV – 11 patients (55%). The mixed form of the tumor was distributed between the groups as follows: 8 (32%) patients in the group I and 5 (25%) patients in each of the groups II, III, V and 6 (30%) patients in group IV.

The comparison between the groups depending on the nature of tumor growth was performed using the chi-square test and no differences were found (P > 0.05).

In our study, there were 38 and 67 patients (36 and 64%) with oropharyngeal and oral cavity cancer, respectively (Tab. 3). According to tumor localization, the groups were comparable by the chi-square test (P > 0.05).

Staging of oral cavity and oropharynx cancer was carried out according to the international classification of TNM-7 (Tab. 4) [12]. In general, the study included 94 (89.5%) patients in an extensive-stage of the disease (III, IV) and only 11 (10.5%) patients had stages I–II. Only 18 (17.1%) patients did not have the spread of the tumor process to adjacent anatomical sites, but 87 (82.9%) patients had. In 68 (64.8%) patients, the tumor spread beyond more than one anatomical site. In 18 (17.1%) patients the tumor spread into the full-thickness of the bone tissue together with cancer of the mucous cellular part of the lower or upper jaws, the floor of the oral cavity or the hard palate.

Tab. 3. Allocating patients to treatment groups depending on localization.

Groups	Oral cavity		Oropharynx		P
	abs.	%	abs.	%	0.308
I – RT+IT	13	52	12	48	
II – CH/RT+IT	14	70	6	30	
III – RT+2IT	11	55	9	45	
IV – RT	13	65	7	35	
V – CH/RT	16	80	4	20	
total	67	64	38	36	

% – frequency, abs. – absolute number of patients, CH – chemoradiotherapy, IT – immunotherapy, RT – radiation therapy

Tumor spreading to regional lymph nodes was noted in 82 (78.1%) patients, which was confirmed by diagnostic methods such as lymph node puncture biopsy, ultrasound, multi-slice CT or MRI examination. After performance of the analysis according to the chi-square test, the groups were compared according to the rate of metastasis into the regional lymph nodes (P > 0.442).

As it can be seen from Tab. 4, there were 7 patients (28%) with $T_3N_1M_0$ tumor disease in group I and 8 patients (32%) in the same group who were not diagnosed with regional metastasis of oral cavity and oropharynx squamous cell cancer. Also, there was 1 (4%) patient with IVb (i.e. $T_4aN_3M_0$) tumor stage in this same group. In groups II, III and IV, there was also a majority of patients with tumors in $T_3N_1M_0$ stage, with 60, 30 and 25%, respectively. There were 4 (20%) patients in group V and the tumor did not form regional metastases into the lymphatic

Tab. 4. Allocating patients to treatment groups according to the international TNM-7 classification [12].

Staging		Group I RT-IT N = 25		Group II CH/RT-IT N = 20		Group III RT-2IT N = 20		Group IV RT N = 20		Group V CH/RT N = 20		Total
		abs.	%	abs.	%	abs.	%	abs.	%	abs.	%	abs.
$T_1N_0M_0$	I	1	4									1
$T_1N_1M_0$	III							1	5			1
$T_2N_0M_0$	II	5	20	1	5	2	10			1	5	9
$T_2N_1M_0$	III					1	5	2	10	3	15	6
$T_2N_2M_0$	IVa	1	4			1	5			1	5	3
$T_3N_0M_0$	III	2	8	1	5	2	10	2	10	4	20	11
$T_3N_1M_0$	III	7	28	12	60	6	30	5	25	3	15	33
$T_3N_2M_0$	IVa	2	8	2	10	4	20	5	25	3	15	16
$T_3N_3M_0$	IVb					1	5	1	5			2
$T_4aN_0M_0$	IVa							1	5	1	5	2
$T_4aN_1M_0$	IVa	3	12	2	10	3	15			3	15	11
$T_4aN_2M_0$	IVa	3	12	2	10			3	15	1	5	9
$T_4aN_3M_0$	IVb	1	4								1	

% – frequency, abs. – absolute number of patients, CH – chemoradiotherapy, IT – immunotherapy, RT – radiation therapy

Tab. 5. Allocating patients to treatment groups and indices according to ECOG, Karnofsky index.

Indices of general condition		Group I RT+IT N = 25		Group II CH/RT+IT N = 20		Group III RT+2IT N = 20		Group IV RT N = 20		Group V CH/RT N = 20		Total
		abs.	%	abs.	%	abs.	%	abs.	%	abs.	%	
ECOG before treatment	1	21	84	19	95%	18	90%	12	60	15	75	0.073
	2	3	12	1	5%	1	5%	8	40	4	20	
	3	1	4	–	–	1	5%	–	–	1	5	
Karnofsky index before the treatment, %		90 (80–90)		90 (80–90)		90 (90–90)		80 (70–90)		80 (70–90)		0.005

% – frequency, abs. – absolute number of patients, CH – chemotherapy, ECOG – Eastern Cooperative Oncology Group, IT – immunotherapy, RT – radiation therapy

Tab. 6. Characteristics of patients according to groups and study treatment received. For indices (distribution law other than normal), the medians and the interquartile ranges (QI–QIII) are given. The comparison was performed according to the Kruskal-Wallis test.

1st stage of study treatment	Group I RT+IT N = 25	Group II CH/RT+IT N = 20	Group III RT+2IT N = 20	Group IV RT N = 20	Group V CH/RT N = 20	Level of significance for difference, P
RGT dose, Gy	40 (39.5–40)	40 (38–40)	40 (36–40)	40 (37–40)	38 (36–40)	0.267
dose of cisplatin, mg		100 (100–175)			152.5 (100–172.5)	0.616

% – frequency, abs. – absolute number of patients, CH – chemotherapy, IT – immunotherapy, RGT – remote gamma therapy, RT – radiation therapy

nodes – $T_3N_0M_0$, and in a single quantity, 3 (15%) patients with each of the tumor diagnosis according to the international classification: $T_2N_1M_0$, $T_3N_1M_0$, $T_3N_2M_0$, $T_4aN_1M_0$. Only 1 patient in the study was diagnosed with oral cavity or oropharynx cancer of $T_1N_0M_0$ and $T_1N_1M_0$ stage.

Before the beginning of the treatment, all patients were assessed for their general condition and quality of life, using the World Health Organisation scale according to Eastern Cooperative Oncology Group – ECOG) for assessing the general condition of the patient (performance status); the results are shown in Tab. 5. The majority of patients in all groups had ECOG1, but in groups IV and V, their percentages were 60 and 70%, respectively. In groups I–III, these percentages were 84–95%. Accordingly, there were more patients in the comparison groups with ECOG2 – 8 (40%) and 4 (20%) patients, respectively. And there was 1 patient in each of groups I, III and V with ECOG3. After the statistical analysis, no differences were found between the groups according to the ECOG index before the treatment, P > 0.05 (Tab. 5).

In group I, 18 (72%) patients were admitted for the treatment; they had Karnofsky index 90%. In groups II and III, the majority of patients had also 90% – 14 (70%) and 16 (80%) patients, respectively, in contrast to patients of groups IV and V, where there were 7 (35%) such patients in each of them. Only in groups I, III and V, there was 1 patient in each of them who had low indices at the time of admission – the Karnofsky index was 50%. Comparison of the Karnofsky index rates before the treatment between groups was performed according to ANOVA. The groups actually differ according to this index (Tab. 5). The median (Me) and interquartile range (Q_I–Q_{III}) are given for the Karnofsky index (the distribution law is different from normal). The comparison was performed according to the Kruskal-Wallis test.

Thus, during the first stage of study treatment, 18 (72%) patients of group I (RT+IT) received a radiation therapy dose of 40 Gy, 1 (4%) patient received 44 Gy, 4 (16%) patients received 38 Gy, 1 (4%) patient received 34 Gy and 1 (4%) patient 26 Gy (Tab. 6). Also, all patients of this group were administered 40 mg of an immune agent with alpha/beta defensins per course of the treatment.

Patients of group II (CH/RT+IT) had chemoradiation therapy against the background of immunotherapy with an alpha/beta defensins agent in a total

dose of 40 mg: during the first stage, 14 (70%) of them received 40 Gy of remote gamma therapy, 4 (20%) patients received 38 Gy, 1 (5%) patient received 36 Gy and 1 (5%) patient received 20 Gy. The average dose of administered cisplatin in the group was 125 mg, the minimum and maximum doses were 100 and 200 mg, respectively (Tab. 6).

In group III (RT+2IT), most (11) of the patients (55%) also received the planned treatment – 40 Gy of radiation therapy – and 1 patient (5%) received 44 Gy. In 4 (20%) patients, the treatment ended at the first stage with a total dose of RGT of 38 Gy, and 2 (10%) patients each had a total focal dose of 36 and 34 Gy.

In group III, all patients were performed telegamma therapy against the background of a higher dose of immunotherapy with an alpha/beta defensins agent (60 mg).

Group IV was a comparison group whose patients received only the radiation component of treatment; 12 (60%) of them received 40 Gy, 3 (15%) patients received 38 Gy, 3 (15%) patients received 36 Gy, 1 (5%) patient received 22 Gy and 1 (5%) patient received 20 Gy RGT (Tab. 6).

In group V, 9 (45%) patients received 40 Gy of remote gamma therapy, 4 (20%) patients received 38 Gy, 6 (30%) patients received 36 Gy, and 1 (5%) patient received 34 Gy. The average total dose of cytostatic in the group was 140 mg, the smallest total dose administered was 80 mg per course and the largest was 210 mg.

The patients who participated in this study before the beginning of the treatment were evaluated for systemic cellular (T-cell, NK-cell, T-NK-cell) and humoral (IgG, IgM, IgA) immunity. T-, NK-, and T-NK-cells were determined in blood serum with an Epics XL (USA) flow cytofluorimeter using IO Test CD3--FITC/CD (16^{+}CD56) monoclonal antibodies, and with the use of Beckman Coulter (USA) reagents. The indices of humoral immunity – IgG, IgM, IgA – were determined. Venous blood sampling was performed in the morning, on an empty stomach, in two separate special test tubes. After that, one of them was centrifuged, blood serum was collected and stored in a refrigeration system (freezer) at a temperature of about –20 °C until the performance of analytical procedures. The venous blood from the second test tube was examined for the quantitative content of T-, NK-, and T-NK-cells within 24 hours, according to the implementation protocol.

Evaluation of local cellular and humoral immunity (INF-α, INF-γ, IL-6, sIgA) before the beginning of the treatment was performed by studying these indices in oral fluid using an Epics XL (USA) flow cytofluorimeter and a human ELISA Kit. The saliva collection procedure was performed in the morning, on an empty stomach, before oral hygiene and without the use of stimuli for salivation. The amount of collected saliva was approx. 5–10 mL. Saliva samples were also stored in a refrigeration system in accordance with the requirements for direct analysis.

All immunological studies were performed on the basis of Research Institute of Experimental and Clinical Medicine, O. O. Bohomolets National Medical University.

The study of NK-cell indices before the treatment among the groups shows that their concentrations were lowest in groups III and IV with median values 232 cells/μL and 235 cells/μL, respectively, and highest in group V with a value of 390.5 cells/μL. Also, the T-NK-cell concentrations in all groups were within the range 61.5–98 cells/μL (Tab. 7). According to these two indices, no differences between the groups were found after the statistical analysis performed ($P > 0.05$). However, the difference was found between groups II and III, as well as between groups II and IV according to the median T-cell index before the treatment (where $P < 0.02$) because the median values of the absolute numbers of T-cells in groups II, III and IV were 1,382; 928; and 827.5 cells/μL, respectively.

After statistical processing of the median indices of humoral and cellular immunity, no differences between the groups were found; for details, see Tab. 7. However, there is a difference between the groups in the value of IgG before the treatment – 10.507 and 5.35 mg/mL in groups III and I, respectively, ($P = 0.04$). In other groups, it ranged from 7.09 to 9.945 mg/mL.

Statistical method of data processing

Statistical analysis of the results was performed using the Statistical software package EZR v. 1.54 (graphical user interface for R statistical software version 4.0.3, R Foundation for Statistical Computing, Vienna, Austria). Statistical analysis of indices of systemic cellular natural antitumor immunity was performed using the IBM SPSS Statistics 23 package.

The distribution of quantitative indices was tested for normality using the Shapiro-Wilk test. In a normal distribution, quantitative indices are measured by the mean ± standard deviation (± SD), and in the non-normal distribution – through the median (Me) and interquartile range (Q_I–Q_{III}). Most of the parameters were not subject to the normal distribution law, so non-parametric criteria were used: the Kruskal-Wallis criterion and the Dunn or Mann–Whitney test were used for pairwise comparisons, taking into account the Bonferroni correction. The chi-square test was used to compare qualitative characteristics, and the posterior comparison was performed according to Fisher's exact test, taking into account the Bonferroni correction. The differences in the groups were indicated as P with an indication of the level of significance. The data were considered to be different at $P < 0.05$. The diagrams were provided in the form of bars with an indication (CI 95%).

Results

After the completion of the first stage of radiation or chemoradiation therapy, we determined the grade of mucositis in each of the groups. Mucositis phenomena were not observed in 12% of patients of group I, in 25% of group II and in 5% of group III until the end of the treatment; in these groups, 10–44% of patients had grade I radiation-induced mucositis in comparison with the absence of such patients in group IV and the presence of only 10% of patients in group V. It should be noted that in groups IV and V, there were 75 and 45% of patients with grade III mucositis (Fig. 1, 2), and in groups I, II and III,

Tab. 7. Median values of the indices of systemic cellular (T-cell, NK-cell, T-NK-cell), systemic humoral (IgG, IgM, IgA), local cellular (INF-α, INF-γ, IL-6) and humoral (sIgA) immunity in blood serum and oral fluid to oral cavity and oropharynx cancer before the study treatment in groups. The Kruskal-Wallis test was used for comparison. Posterior comparisons were performed according to the Dunn test.

	Group I RT+IT N = 25	Group II CH/ RT+IT N = 20	Group III RT+2IT N = 20	Group IV RT N = 20	Group V CH/RT N = 20	P
T-cell ($CD3^+$), cells/μL	1,103	1,382[3]	928[2]	827.5[2]	1,093.5	**0.002**
NK-cell ($CD3^-$/ $CD16^+$/$CD56^+$), cells/μL	279	306	232	235	390.5	0.272
T-NK-cell ($CD3^+$/ $CD16^+$/$CD56^+$), cells/μL	84	98	78	79,5	61.5	0.241
IgG, mg/mL	5.35[3]	7.09	10.507[1]	9.945	8.889	**0.004**
IgM, mg/mL	0.861	1.035	1.302	1.024	1.004	0.300
IgA, mg/mL	2.019	3.756	4.704	3.771	4.534	0.089
INF-α, pg/mL	0.01	0.563	3.561	0.01	0.564	0.081
INF-γ, pg/mL	1.824	2.251	3.217	2.91	2.196	0.168
IL-6, pg/mL	3.78	5.39	4.47	5.16	4.605	0.576
sIgA, mg/L	274.16	245.02	321.85	263	354.9	0.393

[1–3] The difference between the groups is statistically significant, $P < 0.05$.
CH – chemotherapy, ECOG – Eastern Cooperative Oncology Group, IL – interleukin, INF – interferon, IT – immunotherapy, NK – natural killer, RT – radiation therapy

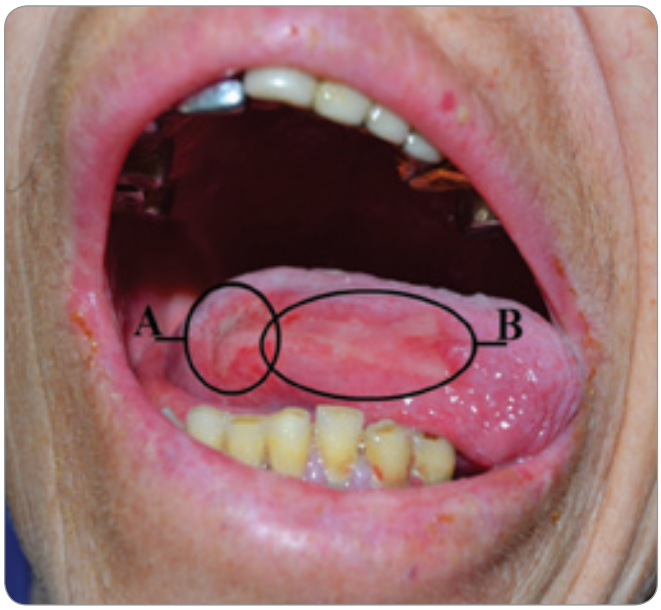


Fig. 1. Endophytic tumour of the lateral surface of the tongue on the right (A); erosion of the lateral surface of the tongue without ulceration and bleeding – grade III radiation mucositis (B).

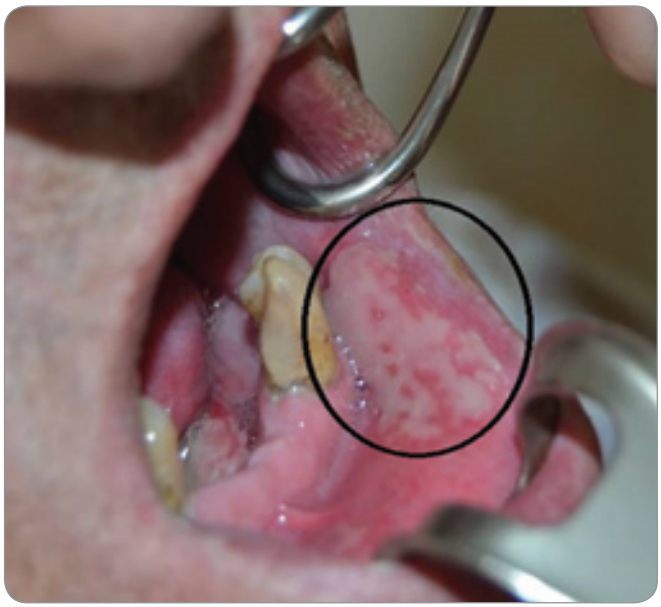
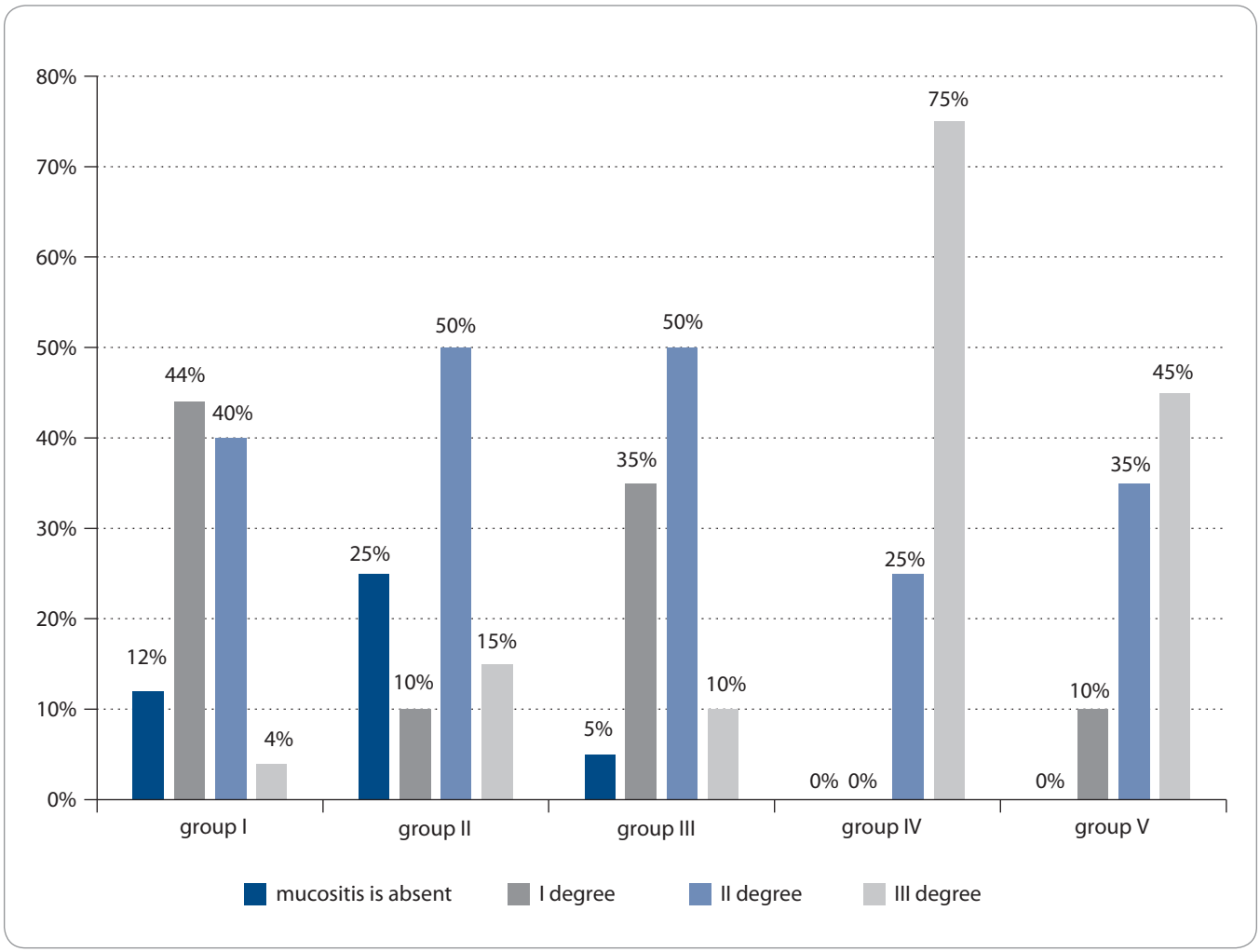


Fig. 2. Diffuse erosion, more than 1.5 cm covered with fibrinous plaque – grade III radiation mucositis.

the numbers of such patients were significantly lower – 4, 15 and 10%, respectively. It means that in each of the groups where the combination of the treatment included immunotherapy with alpha/beta defensins, there was a greater proportion of patients with grade II mucositis, but less with grade III radiation-induced mucositis (Graph 1). All data in groups are statistically reliable ($P < 0.001$).

A detailed search for factors influencing the risk of the development of grade III radiation-induced mucositis was performed by us. For this, the method of building and analyzing univariate logistic regression models was used. There were analyzed 24 indices as factor characteristics, namely: use of immunother-



Graph 1. Indices of radiation mucositis according to its grade in groups of patients with oral cavity and oropharynx cancer after the first stage of treatment.

apy as a method of accompanying study treatment, type of treatment – radiation or chemoradiation therapy, age, gender, ECOG and Karnofsky index before the beginning of the treatment, localization, stage, tumor size (T), presence of regional metastasis (N), nature of tumor growth, histological differentiation of the tumor, dose of RGT at the first stage of treatment, dose of regional intra-arterial chemotherapeutic potentiation, blood parameters before the treatment, namely T-cells ($CD3^+$), NK-cells ($CD3^-CD16^+/CD56^+$), T-NK-cells ($CD3^+CD16^+/CD56^+$), IgG, IgM, IgA and saliva indices before the treatment with INF-α, INF-γ, IL-6, and sIgA. The results of univariate analysis are shown in Tab. 8.

As a result of the univariate analysis (Tab. 8), it can be concluded that the reduction of the risk of developing grade III radiation-induced mucositis is predicted by several factors: immunotherapy, gender, and blood serum concentrations of IgG and IgA. A decrease ($P < 0.001$) in the risk of the development of grade III radiation-induced mucositis was revealed if immunotherapy with alpha/beta defensins (in a total dose of 40 mg) was included into the treatment of patients (RO 0.05; 95% RI 0.02–0.18) compared with the patients of the groups where it was not administrated. Also, this tendency is observed when using this immune agent in a total dose of 60 mg ($P = 0.001$; RO 0.06; 95% RI 0.01–0.30; patients of group III) compared with patients of the groups without the use of immunotherapy. The next factor sign was gender: the risk of the development of grade III mucositis was lower in men ($P = 0.003$; RO 0.15; 95% RI 0.04–0.53) compared to women. An increase (P 0.024) in the risk of the development of grade III mucositis with an increase in the initial level of IgG serum concentration was revealed (RO 1.08; 95% RI 1.01–1.16) for each 1 mg/mL, as well as an increase ($P = 0.044$) in the possibility of the appearance of grade III mucositis with an increase in the serum concentration of IgA (RO 1.23; 95% RI 1.01–1.50) for every 1 mg/mL also before the beginning of the treatment. In this study, we have not found a connection ($P > 0.05$) between the increased risk of the development of grade III radiation-induced mucositis and the type of treatment, the dose of chemotherapeutic potentiation or radiation therapy, localization, size and dif-

Tab. 8. Analysis of univariate logistic regression models for predicting the risk of developing grade III radiation-induced mucositis in patients with cancer of the oral cavity and oropharynx.

Factor sign		Coefficient of the model, b ± m	Level of the significance of RO difference from 1, P	Model of odds ratio index, RO (95% RI)
immunotherapy	IT, none	referential		
	IT, 40 mg	–2.94 ± 0.62	**< 0.001**	0.05 (0.02–0.18)
	IT, 60 mg	–2.82 ± 0.82	**0.001**	0.06 (0.01–0.30)
type of treatment	RGT	referential		
	RGT+IACT	0.40 ± 0.43	0.355	–
gender	female	referential		
	male	–1.91 ± 0.65	0.003	0.15 (0.04–0.53)
age		0.011 ± 0.022	0.605	–
Karnofsky index before the treatment		–0.031 ± 0.021	0.150	–
ECOG before the treatment		0.19 ± 0.43	0.661	–
localization	oral cavity	referential		
	oropharynx	–0.11 ± 0.44	0.798	–
stage of the disease		0.43 ± 0.34	0.203	–
T (tumor)		–0.10 ± 0.33	0.762	–
N (nodulis)		0.44 ± 0.27	0.109	–
nature of tumor growth	exophyte	referential		
	endophyte	–0.29 ± 0.70	0.680	–
	mixed	–0.94 ± 0.79	0.236	–
histological differentiation of the tumor	G1	referential		
	G2	0.13 ± 0.49	0.784	–
	G3	0.24 ± 0.65	0.700	–
RGT, dose		0.025 ± 0.052	0.636	–
chemotherapeutic potentiation, dose		0.004 ± 0.003	0.211	–
Indices of blood and saliva analysis before the treatment				
T-cell ($CD3^+$), cells/μL		–0.0010 ± 0.0006	0.064	–
NK-cell ($CD3^-CD16^+/CD56^+$), cells/μL		–0.0008 ± 0.0013	0.531	–
T-NK-cell ($CD3^+CD16^+/CD56^+$), cells/μL		–0.0046 ± 0.0036	0.207	–
INF-α in saliva, pg/mL		–0.031 ± 0.064	0.634	–
INF-γ in saliva, pg/mL		–0.028 ± 0.081	0.731	–
IL-6 in saliva, pg/mL		–0.0032 ± 0.0082	0.700	
sIgA, mg/L		0.0022 ± 0.014	0.114	–
IgG in blood serum, mg/mL		0.079 ± 0.031	0.024	1.08 (1.01–1.16)
IgM in blood serum, mg/mL		0.11 ± 0.20	0.565	–
IgA in blood serum, mg/mL		0.20 ± 0.10	0.044	1.23 (1.01–1.50)

b – regression coefficient, ECOG – Eastern Cooperative Oncology Group, IACT – intra-arterial chemotherapeutic potentiation, IL – interleukin, INF – interferon, IT – immunotherapy, m – median, NK – natural killer, RGT – remote gamma therapy, RI – reference interval, RO – relative odds

Tab. 9. Analysis of a three-factor logistic regression model for predicting the risk of the development of grade III radiation-induced mucositis in patients with oral cavity and oropharynx cancer.

Factor sign		Coefficient of the model, b ± m	Level of the significance of RO difference from 1, P	Model of odds ratio index, RO (95% RI)
immunotherapy	IT, none	referential		
	IT, 40 mg	−2.58 ± 0.65	**< 0.001**	0.08 (0.02–0.27)
	IT, 60 mg	−3.47 ± 1.04	**0.001**	0.03 (0.004–0.24)
gender	female	referential		
	male	−1.51 ± 0.82	0.064	0.22 (0.04–1.09)
IgG index in the blood serum before treatment, mg/mL		0.12 ± 0.05	0.008	1.13 (1.03–1.24)

b – regression coefficient, IT – immunotherapy, m – median, RI – reference interval, RO – relative odds

ferentiation of the tumor and other factors. Having performed such analysis, it can be assumed that the use of an immune agent based on alpha/beta defensins during radiation and chemoradiation therapy reduces the risk of the development of grade III mucositis.

During the second stage of the analysis, a selection of indices significantly related to the resulting sign was performed by multivariate logistic regression models in order to identify a set of signs associated with the risk of the development of grade III mucositis. The method of stepwise inclusion/exclusion of factor features was used for the selection (the inclusion and exclusion thresholds $P < 0.1$ and $P > 0.2$, respectively). When performing the analysis of 24 factors that can theoretically influence the development of grade III radiation-induced mucositis, three independent characteristics were identified: the use of immunotherapy, gender, and the level of serum IgG concentration before the beginning of the therapy.

The results of the multivariate analysis (Tab. 9) indicate an increase ($P = 0.008$) in the risk of the development of grade III radiation-induced mucositis with a high level of serum concentration of IgG before the treatment or an increase in this index during the therapy (RO 1.13; 95% RI 1.03–1.09) for every 1 mg/mL (when standardized by other risk factors). It was determined that when standardized according to other factors (gender, IgG level), the risk of the development of grade III mucositis decreases with the use of the immune agent with alpha/beta defensins in a total dose of 40 mg per course ($P < 0.001$; RO 0.08; 95% RI 0.02–0.27) compared to patients with oral cavity and oropharynx cancer who were not treated with immunotherapy. The risk of the development of grade III radiation-induced mucositis also decreases ($P = 0.001$) in the use of the immunotherapy in a higher dose – 60 mg per course (RO 0.03; 95% RI 0.004–0.24) compared to patients treated without the inclusion of immunotherapy (standardized according to other factors).

The three-factor model built on the selected features is adequate, the area under the curve of operating characteristics (Graph 2) AUC = 0.89 (95% RI 0.82–0.95), which indicates the presence of a very strong connection with the risk of the development of grade III radiation-induced mucositis by the above defined three factors.

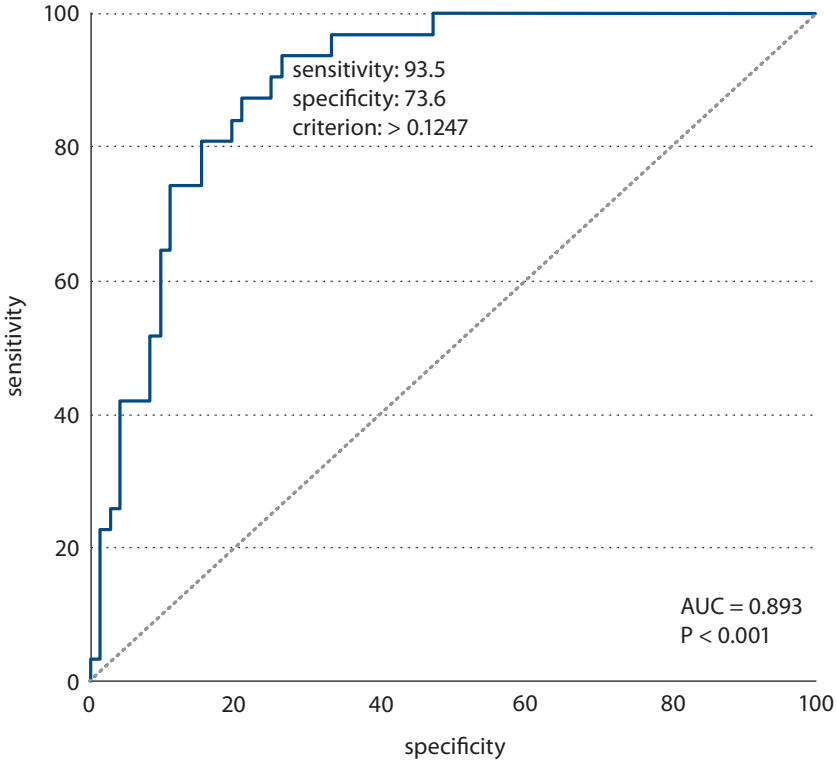
The obtained data indicate that immunotherapy in both lower and higher doses leads to a decrease in the risk of the development of grade III mucositis, and this effect is associated precisely with the immunomodulatory effect of the agent with alpha/beta defensins, which consists, in particular, in the effect on the level of serum concentration of IgG.

To select the optimal threshold of the test for predicting the risk of the development of grade III radiation-induced mucositis in the three-factor model, the optimization of the Youden index test was used with the critical threshold $Y_{crit} = 0.1247$ (Graph 2).

When choosing the optimal test threshold, its sensitivity is 93.5% (95% RI 78.6–98%), the specificity is 73.6% (95% RI 61.9–83.3%), the predictive significance of a positive result is 60.4% (95% RI 50.7–69.4%), and the prognostic significance of a negative test result is 96.4% (95% RI 87.3–99.0%).

Discussion

The development of radiation-induced mucositis during chemoradiation therapy of patients with cancer of the oral cavity and oropharynx is associated with radiation induction of a reactive immune inflammatory reaction in the mucous membrane of the oropharyngeal area [13]. The use of modern highly conformal irradiation methods is more protective for the surrounding healthy mucous membrane of the oral cavity and oropharynx, therefore it is not always possible to fully investigate and evaluate changes in such irradiated tissues. And the use of an older technique of radiation therapy with the use of appropriate devices such as in our study allows for more revealing monitoring of changes in the immune response to radiation. So far, data on various mechanisms of the formation of immune inflammation in response to damaging microbial and non-microbial factors have been confirmed, depending on the type of regula-



Graph 2. Curve of the operational characteristics of the three-factor model for the prediction of the risk of grade III radiation mucositis development in patients with oral cavity and oropharynx cancer.

tory immunocompetent cells, which are mainly involved in the coordination of the pathological process development. Type 1 T-helpers are isolated, which due to the production of a set of cytokines, in which the leading role is given to interleukin-2, gamma-interferon and lymphotoxin, contribute to the maturation of cytotoxic $CD8^+$ T-lymphocytes, polarizing the immune response towards cellular immunity [14]. Instead, type 2 T-helpers produce mostly interleukins 4, 5 and 6, activate B-lymphocytes, promote the synthesis of immunoglobulins of different classes, thus polarizing the immune response towards humoral immunity [15]. At the same time, one of the leading mechanisms of the known immunomodulatory effect of immunotherapeutic agents used in clinical practice, including oncology, is precisely the influence on the process of immune dichotomy during the implementation of the immune response, mediated by the oppositional functioning of types 1 and 2 T-helpers [10]. In previous controlled clinical trials, medicines with alpha/beta defensins demonstrated the ability to strengthen the cellular link of immunity, which implies the polarization of the immune response when they are used in the direction of type 1 T-helpers [16,17].

In particular, in *in vitro* and *in vivo* studies, Kim et al. showed the ability of beta-defensin in response to MERS-CoV infection to enhance the production of a number of antiviral (IFN-β, IFN-γ, MxA, PKR, and RNaseL) and primary immune-inducing (NOD2, TNF-α, IL-1β, and IL-6) molecules, which leads to the activation and proliferation of monocytes/macrophages, natural killer cells, granulocytes, T-cells, and dendritic cells [18]. This indicates beta-defensin-mediated modulation of the innate and adaptive antiviral immune response. Accordingly, Owusu et al. showed in a controlled clinical study that in patients with viral hepatitis C, the production of type 1 T-helper cytokines, especially interleukin 2, is associated with increased synthesis of both alpha- and beta-defensins [19]. Judge et al. showed that beta-defensin-3 activates NK-cells and thereby stimulates the production of gamma-interferon, a key cytokine in the polarization of the immune response to type 1 T-helper cells, through a direct effect on NK-cells through TLR1, TLR2 and CCR2 receptors and through the indirect mechanism including the mDC-dependent activation pathway [20].

On the other hand, the risk of the development of radiation-induced mucositis during study treatment of oral cavity and oropharynx cancer, as shown by the data of this trial, is highest with increased production of immunoglobulins, namely IgG and IgA molecules, which indicates the predominant activation of type 2 T-helpers. Therefore, it seems obvious that the preventive clinical effect of the medicine of alpha/beta-defensins regarding the development of grade III radiation-induced mucositis during study treatment in patients with oral cavity and oropharynx cancer demonstrated in the results of this study that it is related to the switching-over to immune response from the initial unfavourable prognostic direction, mediated by type 2 T-helpers, to inflammation regulated by type 1 T-helpers and, accordingly, associated with a lower frequency and severity of radiation-induced inflammatory reactions of the mucous membrane of the oropharyngeal area.

Currently, the specified immune switch from type 2 T-helpers to type 1 T-helpers is described as one of the main pleiotropic immunomodulatory effects of a number of immunotherapeutic agents used in clinical practice both in the treatment of neoplasia and other immune-dependent human pathologies.

The disadvantages of the used immunotherapy agents are the pronounced pro-inflammatory side effects, as, for example, in interferon-alpha medicines [7], frequent cases of rapid induction of secondary resistance to the immunotherapeutic medicine during the course of

the treatment, e.g. due to the phenomenon of N-glycosylation, as it happens when using interferon-gamma [8], or the induction of autoimmune reactions to autoantigens of the connective tissue, internal organs and/or endocrine glands, typical for human recombinant interleukin-2 medicines [10].

Potential advantages of alpha/beta defensins, with similar clinical efficacy to the above-mentioned immunotropic medicines, include safety of their use and rare cases of induction of the resistance [11], which, however, still requires significant clarification due to the lack of a sufficient number of large randomized clinical trials in this area.

Conclusions

As a result of this controlled clinical study, the additional factors, aside from radiation, were determined as those affecting the risk of the development of grade III radiation-induced mucositis in patients with oral cavity and oropharynx cancer during study treatment. These factors comprise the inclusion of immunotherapy with alpha/beta defensins in the specific treatment, gender, and baseline levels of serum IgG and IgA concentrations with a pattern in which the higher the serum IgG and IgA concentrations are before the beginning of treatment, the greater is the likelihood of severe radiation-induced mucositis grade during study therapy.

The results of the study of the humoral state of the immune system in patients with oral cavity and oropharynx cancer before the beginning of chemoradiation therapy can be used as prognostic risk factors for the development of severe gamma-irradiation-induced mucositis of the oropharyngeal area, as well as an indication for the use of immunotherapeutic agents, i.e. alpha/beta defensins, in particular, that are able to polarize the immune response towards type 1 T-helpers through their immunomodulatory action.

Further clinical studies in this direction with a more sophisticated design are needed to clarify the results of this work.

Dedication

This work is a fragment of the scientific-research work "Individualization of treatment for cancer of the organs of the reproductive system and the gastrointestinal tract by studying prognostic factors and improving diagnostic methods", state registration number 0121U109033.

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