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MEDICINE FIELD

Immune microenvironment and PD-L1 expression study in laryngeal carcinomas

DOCTORAL THESIS - SUMMARY

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INTRODUCTION

Laryngeal cancer is one of the most common head and neck cancers and is one of the few oncological conditions in which 5-year survival rates have fallen over the past 40 years from about 66% to 63%, although the incidence has also decreased. . The results of the treatment of laryngeal carcinomas remain modest globally, the overall incidence rates at 5 years being in 2018 of 154,977 cases in men and 22,455 cases in women. Also in 2018, there were 81,806 deaths in males and 12,965 deaths in females, according to data published by GLOBOCAN [1-5].

Carcinogenesis is a multifactorial process that has not been fully elucidated, and the role of the immune system in the etiopathogenesis and progression of malignant tumors is currently a topic of primary interest. This aspect has already been studied in many types of solid tumors, which has resulted in the introduction into therapy of drugs with immunomodulatory and / or immunostimulatory action.

The theory of immunological control / immunological surveillance assigns specific roles for different types of inflammatory cell populations (neutrophils, macrophages and lymphocytes) to induce an environment with cytokines that favors the occurrence of chronic inflammatory response typical of tumor diseases, while other cellular populations NK killer, CD8 + lymphocytes and major cytotoxic response factors are suppressors of tumor cell populations. Among inhibitory immune mediators, the PD-1 receptor pathway and its ligands - PD-L1 / PD-L2 play an important role in inducing and maintaining peripheral immunological tolerance and in maintaining the stability and integrity of the T lymphocyte. mediates potentially inhibitory signals to prevent the proliferation and functioning of effector T cells, having adverse effects on antiviral and antitumor immunity.

Studies performed so far on the tumor immune microclimate on different types of tumors have highlighted the prognostic role of the various components that make up this microenvironment (CD3, CD4, CD8 lymphocytes), but also the prognostic role of PD-L1 expression, there are studies on cancers laryngeal, but with contradictory results. The initial idea of this study started from the role of Human Papilloma Virus (HPV) infection in laryngeal carcinogenesis and its correlation with p16 protein expression, but studies in large cohorts of patients showed that the percentage of laryngeal malignancies associated with viral infection is one reduced. Laryngeal carcinomas are part of head and neck cancers, and numerous studies have been performed on the latter related to the immune microenvironment and PD-L1

expression, which caught my attention. In the current study we analyzed the immune microenvironment in laryngeal carcinomas through immunohistochemical examination using CD3, CD4, CD8, p16 markers, but also PD-L1 expression correlated with CD8 lymphocytes. The case belongs to the County Emergency Clinical Hospital "St. Apostle Andrei ”Constanța. The paper is structured in two parts, with 9 chapters, the first 5 representing the current state of knowledge, and the last 4 personal contributions.

The actual state of knowledge

In the first chapter of the paper we reviewed data on embryology, anatomy and physiology of the larynx, histological aspects, and in the second chapter we brought data on the epidemiology of laryngeal cancer and addressed the etiological factors. In Chapter 3 we addressed the WHO updated classification of laryngeal carcinomas and the pTNM classification, but also the histopathological aspects of laryngeal carcinomas, with emphasis on conventional squamous cell carcinomas and their morphological variants. We described the usual staining aspects, immunohistochemical aspects and differential diagnosis. Chapter 4 contains data on the tumor immune microclimate, focusing on the cellular component, especially stromal lymphocytes and the current importance of PD-L1, the link between laryngeal cancer-HPV viral etiology and the relationship between p16 expression and HPV infection in laryngeal carcinomas. The last part (chapter 5) of the current state of knowledge highlights the current role of digital pathology in the current diagnosis of laryngeal tumors.

Personal contributios

The *aim* of the current study is to correlate the clinical-morphopathological aspects with the immune microclimate, through the stromal lymphocytes evaluated using CD3, CD4 and CD8 markers and with the expression of PD-L1 in laryngeal carcinomas.

The objectives of the doctoral study:

- Clinical-morphopathological analysis of the studied cases;
- Tumoral immune microenvironment analysis in laryngeal carcinomas with the help of the expression of the markers CD3, CD4, CD8, p16 and PD-L1, but the correlation of the TIME classes with the morpho-pathological factors.
- statistical analysis of the obtained data

MATERIAL AND METHOD

The research for the doctoral study was based on the retrospective analysis of the laryngeal tumors biopsied / operated in the ENT clinic of the Emergency Clinical Hospital “St. Apostol Andrei ”Constanța, for a period of 9 years (01.01.2011-31.12.2019), with

histopathological diagnosis in the Clinical Service of Pathological Anatomy Constanța. The unique registry of the pathological anatomy service and the computerized database of the hospital were the sources from which were extracted the clinical data of patients (age, sex, location of tumors, date of diagnosis, subsequent treatment), anatomopathological information (type of surgical specimen - biopsy, laryngectomy with or without regional lymph node dissection), anatomopathological diagnosis, tumor grade, staging of pTNM and subsequent evolution of the cases that could be followed.

During the study period, 404 cases of laryngeal lesions and malignancies were diagnosed and treated, of which 370 biopsies and 34 laryngectomies. From these, we selected a batch of 76 biopsies, for which we performed the immunohistochemical examination for the biomarkers studied and also a batch of 23 laryngectomy cases for which PD-L1 and CD8 were performed and also the classification in the TIME classes. . For this group, follow-up could be performed for a period of at least 12 months, in order to determine whether the short-term prognosis of these tumors is influenced by the composition of the immune microclimate and PD-L1 expression.

After processing the examined pieces, the microscopic evaluation followed, finalized with the elaboration of the histopathological report which included the obligatory and additional elements, necessary for the most detailed evaluation of the tumor lesion [6-7].

Immunohistochemical examination Immunostaining was performed on a fully automatic immunohistochemistry device Ventana BenchMark GX Automated system, with previously validated antibodies CD3, CD4, CD8, p16 and PD-L1.

The quantification of the expression of CD3, CD4 and CD8 immunomarkers was performed after scanning the slides using the scanner was performed after scanning the HURON Tissuescope 4000x scanner (CEDMOG Constanța-Development Research Center for Morphological and Genetic Study in Malignant Pathology). The images were then interpreted using the QuPath digital quantization program, a program developed by Peter BankHead in 2017, which allowed me to count positive lymphocytes per mm2 [8].

Antibodies	**Cut-off value*10^2/mm^2**	**Low level**	**High level**
CD3	**5,7**	**0< 5,7**	**≥5,7**
CD4	**4,3**	**0 < 4,3**	**≥ 4,3**
CD8	**3,6**	**0< 3,6**	**≥ 3,6**

Table nr. 11. Cutt-off values of the antibodies

Tabe nr. 12. TIME clasiffication depending on PD-L1 expression and TILs level[14].

I	II	III	IV
PD-L1 negativ TIL negativ/nivel scăzut **Immune ignorance**	PD-L1 pozitiv TILpozitiv/nivel crescut **Adaptative resistance**	PD-L1 negativ TILpozitiv/nivel crescut **Tolerance**	PD-L1pozitiv TILnegativ/nivel scăzut **Intrinsic induction**

Quantification of PD-L1 in head and neck cancer, including larynx

PD-L1 expression is quantified using the combined positive score (CPS), which represents the number of PD-L1 positive cells (tumor cells, lymphocytes and macrophages) divided by the total number of viable cells, multiplied by 100. Although the result may exceed 100, the maximum score is defined as CPS 100. The formula for calculating CPS:

CPS = # positive cells x 100

total # of viable tumor cells

Positive cells = tumor cells, lymphocytes, macrofaphes

p16: Interpretation of staining: CINtec p16 histology is a nuclear and cytoplasmic staining. p16 was considered positive if more than 70% of tumor cells had a moderate or severe positive nuclear reaction [15].

Statistical analisys of clinical, morphopathological and immunohistochemical data were introduced and analyzed in the electronic database of the SPPSS platform (Statistical Package for the Social Sciences).

RESULTS AND DISCUSSIONS BASED ON CLINICALMORPHOLOGICAL STUDY and IMMUNOHISTOCHEMISTRY

Results and discussions based on clinical-morphological analisys

During 01.01.2011-31.12.2019, within the Clinical Pathological Anatomy Service of the County Emergency Clinical Hospital „St. Apostol Andrei „Constanța was diagnosed 404 patients, of both sexes, with preneoplastic and malignant laryngeal lesions, all benefiting in advance of hospitalization and minimal surgical treatment or resections in the ENT Clinic of our hospital, then a complete pathological examination. These predominated in males, registering a number of 367 cases, in females in number of 37. Of these, 58 were premalignant lesions and 346 invasive carcinomas, the latter including the diagnosed variants of CCS: 7 cases

of basal cell SCC, 2 cases of warty SCC, 3 of papillary SCC, 3 of SCC, 3 of spindle cell SCC and 1 case of adenoscuamos carcinoma. In men, 367 lesions were diagnosed, of which 50 (13.62%) premalignant lesions and 317 (86.37%) invasive carcinomas, and in women 37 lesions, of which 7 (18.91%) premalignant and 30 invasive carcinomas (81.08%). Based on the eligibility criteria, in my study I included 99 cases, of which 76 biopsies and 23 total laryngectomies.

I mention that, out of the 404 cases in total, 370 were biopsy fragments and 34 total laryngectomies. In the case of total laryngectomies, not all were able to benefit from follow-up. As a percentage, 9.2% were registered in women (37 cases), and 90.8% in men (367 cases). What is highlighted in the graph is an oscillation in the number of cases in men over the study period, but also an increase in the number of cases in women, with a peak in 2019, probably due to changing behaviors in women, in the sense of increasing consumption of alcohol and tobacco, as other studies have shown [17]. The youngest patient was 33 years old (0.2%) and the oldest 85 years old (3 patients, 0.7%). Conventional CCS predominated, as evidenced by other studies in our country [18]. The other types of SCC were much rarer, of which the most common was basaloid SCC (9 cases) and the rarest adenoscuamos SCC with a single case.

From a histopathological point of view, out of a total of 346 diagnosed carcinomas, SCC was predominant, of which 91.77% in males, basaloid SCC, also predominant in men (77.8%, 7 cases out of 9 diagnosed). The other variants - verrucos, papillary, fusiform, adenoscuamos were much rarer and all diagnosed in males, ranging from one to 3 cases each. The mean age of the patients was 60.59 ± 8.38, in women it was 59.37 years, and in men it was slightly higher, at 60.70 years. The Chi-Square test showed a direct link between symptoms and location, consistent with the results of other studies [19], its value being 0.01 (<0.05).

Results and discussions based on immunohistochemical analisys

The immune microenvironment evaluation based on CD3, CD4, CD8 and p16 markers

In the current study, the level of CD3 + lymphocytes is defined as low at less than 5.7 * 102 / mm2 and high at values exceeding 5.7 * 102 / mm2, the CD4 + level is low at values below 4.3 * 102 / mm2 and increased to values above 4.3 * 102 / mm2, and the cut-off value of CD8 + is 3.6 * 102 / mm2.

Classification of cases according to the level of antibodies (dichotomized score according to the average value of lymphocytes / mm2).

Markeri TIL	**CD3**	**CD4**	**CD8**
Scor IHC dihotomizat	< 5,7 ≥	< 4,3 ≥	< 3,6 ≥
Scor scăzut	59	57	31
Scor crescut	17	19	45
Total	76	76	76

In the current study, of the 76 cases studied, 59 cases (77.6%) had low CD3 levels, and 17 cases (22.36%) had high levels, results similar to those of Balermpas et al [26]. Regarding the distribution by degrees of differentiation, the crosstabulation statistical analysis showed that out of the 59 cases (77.6%) with low CD3 level, the highest percentage belonged to SCC G2, and out of the 17 cases with high (22.4%), the highest percentage belonged to SCC G3, without a statistically significant association (chi-square test: $\chi 2$ (df2) = 2; p = 0.316).

The age distribution of CD3 was high in 17 cases, of which 8 cases under 60 years and 9 cases over 60 years, and low in 59 cases, of which 24 under 60 years, results that correlate with those of Balermpas' study [26]. The age of 60 has been considered a cut-off point, as there are studies that have shown that it can be an independent prognostic factor for survival without events in both neck and laryngeal cancer [27]. Also, the platelet-lymphocyte ratio is lower in patients over 60 years, according to recent studies investigating the inflammatory microclimate and based on peripheral blood elements [28].

There was also a high level of CD3 in 13 cases in men, compared to only 4 cases in women, while a low level of CD3 was noted in 55 men and only 4 women. The statistical analysis revealed a statistically significant association between the 2 parameters, the test $\chi 2$ (df2) = 0.047, between the 2 parameters there was a negative correlation (Spearman Rs correlation = -0.222, p <0.05). This result does not overlap with the data of other studies in the literature [26,29]. The relationship between CD3 and tumor location highlights the predominantly glottic origin for increased CD3 cases (15 cases, 88.23%), followed by supraglottic (2 cases, 11.77%), and the behavior regarding risk factors shows that of those 76 patients, 59 were smokers (heavy and moderate smoking) and only 8 were non-smokers. Of those who smoked, 12 showed increased CD3 expression (85.3%), and of non-smokers only 2 (25%). No statistically significant association between these parameters was identified. The initial clinical stage was T1-T2 for 18 cases and advanced T3-T4 for 36 cases. Of the T1-T2, 12 cases (66.66%) were low CD3 and 6 cases (33.33%) were high CD3. And among the advanced stage cases, 29 (80.55%) were low CD3 and only 7 (19.44%) high CD3.

Nodal metastases were in the N0-N1 stage in 15 cases, N2a-N2b in 8 cases and N2c-N3 in only 5 cases. CD3 expression was increased in 5 cases (62.5%) NO-N1, in 2 cases (25%) N2a-N2b and only 1 case (12.5%) N2c-N3. There were no statistically significant correlations between CD3 expression and clinical factors, results that overlap with Karpathiou's 2017 study [30]. This study showed that elevated CD3 + lymphocyte levels are associated with better overall survival, and the prognostic impact is strong for lymphocytes in the stromal compartment compared to those in the tumor front compartment. CD3 + lymphocyte infiltration has been studied as a prognostic factor in small series of cases of squamous cell carcinoma of the neck and neck, and the results have been extremely variable as some studies have shown them as a positive prognostic factor [31] and others they showed no correlation with survival. In Balermpas' 2016 study, conducted on a cohort of 161 patients, elevated CD3 levels were a positive prognostic factor [32].

CD4 expression was decreased in 57 cases (75%) and increased in 19 cases (25%), and the distribution by degrees of differentiation showed the most low CD4 cases in the SCC G2 category, and the most high CD4 in the SCC G3, results inconsistent with Balermpas' study [26], which shows a statistically significant association between the two variables, with a positive correlation between the two parameters, test value $\chi 2$ (df2) = 6,350; p = 0.012), and the Sperman Rs correlation having the value 0.288.

In terms of age, the highest numbers of increased CD4 + were in patients over 60 years of age, so that of the 19 cases, 12 (63.15%) belonged to patients in the 7th decade and only 7 cases (36 , 85%) patients under 60 years of age. CD4 recorded a high level in our study in 19 cases, of which 18 in men and only one in women, and of the low-level cases most belonged to males, the correlation statistic showing no statistically significant correlation, test $\chi 2$ (df2) = 0.338. The localization correlated with the level of CD4 lymphocytes did not show any statistically significant association, aspect superimposable with other studies [26], most cases with increased CD4 + level being localized at glottic level (17 cases, 94.44%). Risk factors, smoking and alcohol consumption were not statistically significantly correlated with CD4 + levels, similar to other studies [30]. Thus, all patients were smokers (14 cases, 100%) and 17 (100%) of them denied alcohol consumption or declared moderate consumption, none with high consumption.

In the current study, most tumors were in advanced stages of T3-T4, and of the 11 cases with elevated CD4 + lymphocytes, 5 cases (45.45%) were included in the advanced stage. And N2c-N3 stage lymph node metastases showed only 2 cases out of 5 with high CD4 + level (40%). There are studies showing that elevated CD4 + levels do not affect the patient's

prognosis, but correlate with the absence of distant metastases [33]. CD4 + T lymphocytes together with dendritic cells support the response of cytotoxic T cells and help other cytotoxic cells of the immune system, such as NK cells and macrophages [34].

CD8 expression was decreased in 31 (41%) of cases and increased in 45 (59%) of cases, and statistical analysis showed that 76.08% of cases expressed increased CD8 and degree of G1 differentiation -G2 (35 cases), and 16 cases (21.05%) are poorly differentiated G3 carcinomas and high CD8 levels. There is no correlation between the two variables, test $\chi 2$ (df2) = 0.485. Increased CD8 expression predominated in patients over 60 years (32 cases, 71.11%) and it is interesting that most cases had a high CD8 level (45 compared to only 31 cases with low CD8). Statistical crosstabulation analysis did not reveal any statistically significant correlation. Differences related to decades of age have indicated that with age, the map of patients' immune microclimate changes, which influences their antitumor response and response to treatment [35].

CD8 showed a high level in 51 cases, of which 48 in men (94.11%) and only 3 in women (5.89%), the number of cases with low CD8 being much lower. It is noted that the number of increased CD8 cases far exceeds the number of increased CD3 and CD4 cases, statistically there is no significant association between the parameters. The data do not overlap with those in the literature [26]. Tumors infiltrated with a high level of CD8 + T lymphocytes were located predominantly at the glottic level (45 cases, 88.23%) and only 6 at the supraglottic level (11.77%).

The increased level of CD8 + lymphocytes (Fig. 84) predominates in smoking patients - 39 cases (88.63%), non-smokers being only 5 cases (11.36%). Also, 4 patients (9.09%) are consumers of alcohol in large quantities, the remaining 45 cases (91.83%) being consumers of moderate amounts. Regarding the clinical stage of pT, 24 cases (68.57%) showed increased CD8 + lymphocyte level and advanced stage T3-T4, and 11 cases (31.42%) early stages T1-T2. N1 or N0 lymph node metastases predominated in tumors with high CD8 + levels (13 cases, 72.22%).

In the current study, regarding the tumor localization and the studied markers, it is observed that all 3 CD3, CD4 and CD8 markers registered an increased number of cases with increased level for glottic localization, and the fewest for subglottic localization. Statistical analysis did not show a statistically significant association between the 2 parameters.

Correlating the initial clinical stage with the level of CD markers, it was found that their increased level is recorded for the advanced tumor stage, for each marker. Thus, out of 18 T1-

T2 cases, 6 of them were CD3 and CD4 elevated and 11 increased CD8. The chi-square test did not indicate any statistically significant association.

The initial dominant clinical stage N was N0-N1 (15 cases), followed by N2a-2b (8 cases) and N2c-N3 with only 5 cases. For N0-N1 cases, increased CD8 predominated (13 cases out of 15), while the other 2 CD3 and CD4 markers recorded an increased level in 5 and 2 cases, respectively. For stages N2a-2b and N2c-N3, CD3 and CD4 had a dominant low level. These data partially overlap with those in the literature [26]. There are studies that show that tumors infiltrated with numerous T lymphocytes have a better prognosis compared to patients whose tumors are devoid of T lymphocytes in large numbers [30]. As in our study, nor in that of Karpathiou (2017), CD3 and CD8 did not correlate with many clinical or histological factors (location, T, N, histological degrees of differentiation, risk factors, age, sex) . This suggests that the CD3 and CD8 immune response is rather a constitutive feature, independent of known tumor clinicohistological features. However, in the current study CD4 was statistically significantly correlated with the degree of differentiation ($p = 0.012$), and CD3 with the patient's sex ($p = 0.047$). Karpathiou also pointed out that the increased density of CD4 + lymphocytes was associated with the absence of distant metastases [30].

Affara et al studied CD3 in association with CD20 and CD163, highlighting a link between the T-lymphocyte reaction and B lymphocytes and macrophage. Experimental data on squamous cell carcinomas suggest that B lymphocytes promote tumor growth especially in dysplasia and that anti-CD20 monoclonal antibodies increase responsiveness to chemotherapy. The latter effect requires the action of CD8 macrophages and lymphocytes [36].

p16 was positive in 17 (22.3%) cases and negative in 59 (77.63%) cases, 12 cases (70.58%) p16 positive registering in the age group under 60 years and only 5 cases (29.42%) at over 60 years of age. The distribution by sex includes 15 cases in males (88.23%) and 2 cases in females (18.75%). The localization was predominantly glottic (13 cases, 81.25%), at supraglottic level being located only 3 cases (17.64%), and subglottic only 1 case (5.88%). These results differ from the recent 2019 study [37] by Dogantemur et al., In which most p16-positive cases were localized at the supraglottic level.

Of the 17 p16 positive cases, 10 were poorly differentiated G3 (58.8%), and 7 (41.17%) were well and moderately differentiated G1-G2. 3 positive (81.25%) p16 cases were detected in smoking patients, and 15 cases (88.23%) in non-alcoholic or moderate-use patients. The results are partially correlated with those of other studies of p16 expression [38]. The statistical analysis showed a statistically significant association only in the case of the degree of

differentiation, the test $\chi 2$ (df2) = 5.89, p = 0.015 <0.05, between the 2 parameters there is a negative correlation, the Spearman index Rs = - 278.

Immune microenvironment evaluation baseb on CD8 and PD-L1 markers

The group for the TIL CD8 + study correlated with PD-L1 expression included 23 cases of laryngeal squamous cell carcinomas (total laryngectomies) that presented the eligibility criteria. The clinical-pathological characteristics of the analyzed cases are presented below.

Of these, the highest frequency was carcinomas in patients over 60 years (56%), 96% male. The mean age was 61.48 ± 4.42. The predominant location was glottic (61%), followed by transglottic (17%) and infraglotic (13%). The supraglottic location was the rarest (9%). Dysphonia was the most common symptom (78%), and smokers with more than 40 cigarettes / day recorded 43% of cases, and non-smokers 57%. From the point of view of the degree of differentiation, moderately differentiated G2 carcinomas predominated in 61% of cases, followed by the poorly differentiated G3 carcinomas by 30%, the rest being well differentiated G1 (9%). 57% of the carcinomas studied were diagnosed in early T1-T2 stages, the remaining 43% in advanced T3-T4 stages.

Correlations of CD8 + density with clinical and pathological characteristics

All cases included in the 2nd study group benefited from the quantification of TIL CD8 + lymphocytes using the QuPath digital quantification program, which allowed the counting of lymphocytes per mm2, on 3 areas for each case, peritumor and stromal. The average number was the cut-off point, against which we established the low and high level of CD8 + lymphocytes [39].

In the current study, most cases had increased CD8 + levels (15 cases, 65.21%), predominantly in patients over 60 years, all male. In patients under 60 years of age (where the only female patient falls), the number of cases with high CD8 + was equal to that of low CD8 + cases (5 cases, 21.73%). Of the 15 cases of increased CD8 +, the highest share is represented by SCC G1-G2 (11 cases, 47.82%), compared to only 4 cases (17.39%) of SCC G3. Although the statistical analysis did not show a statistically significant association, it should be noted that the increased CD8 + level predominated in non-smokers (8 cases, 34.78%), exceeding the number of smoking patients with high CD8 + levels. Also, the increased number of CD8 + cases in patients with CCS in the T1-T2 stage (8 cases, 34.78%) slightly exceeded that of patients with advanced stages of T3-T4 disease (7 cases, 30.43%) . Low CD8 + levels predominated in patients with SCC in the early stages of T1-T2 disease (5 cases, 21.73%). The number of metastatic lymph nodes was lower in patients with increased CD8 + levels (9 cases,

39.13%) compared to patients with high metastatic lymph nodes (N2c-N3 stage) - only 2 cases (8 , 69%).

Statistical analysis between CD8 + level and PD-L1 expression demonstrated a statistically significant association (chi-square test; $\chi 2$ (df2) = 2; p = 0.019), with a positive correlation between the two parameters (Spearman correlation rs = 0.516) . The statistical analysis between the CD8 + level and the other clinico-pathological parameters did not reveal statistically significant associations, which overlaps with the data obtained by Vassilakopoulou [40]. As in this study and in the current study, there was a statistically significant association between CD8 + level and PD-L1 expression. The biological causes / determinants of this association are not fully known. For example, in melanomas infiltrated with stroma-positive CD8 T lymphocytes, there was a high expression of immunosuppressive elements such as Treg lymphocytes, PD-L1 protein and mRNA. The induction of these immune inhibitory pathways in the tumor microclimate depends on the presence of CD8 + and IFN T lymphocytes [41].

All cases underwent immunohistochemical examination to determine PD-L1 expression. This was quantified using the combined positive score (CPS), represented by the number of PD-L1 positive cells (tumor cells, lymphocytes and macrophages), divided by the total number of viable tumor cells, multiplied by 100, this being the score used after using clone 22C3. Tumors were classified according to the system: CPS <1 (PD-L1 negative), CPS = 1-19 (PD-L1 + positive) and CPS≥20 (PD-L1 + high level).

Of the 23 cases studied, the majority were PD-L1 negative (14 cases, 60.86%), the positive ones having a lower share (9 cases, 39.13%). Of the positive ones, 5 cases (21.73%) had the value PD-L1> 1, and 4 cases (17.39%) had the value over 20, the latter being distributed equally between the 2 age categories (under and over 60 years). In men, most cases were negative (13 cases, 56.52%), the only case in females was also negative. The degree of tumor differentiation registered differences in PD-L1 expression, thus, both well and moderately differentiated tumors (G1 and G2) and poorly differentiated (G3) tumors were predominantly PD-L1 negative (9 cases, 39.13% , respectively 5 cases, 21.73%). Among the differentiated salb tumors, the percentage of positive tumors as a whole is small, with only 1 case for each category (2 positive cases, 8.69%). Depending on the tumor stage (pT), of the 10 cases (43.47%) of PD-L1 positive tumors, most were tumors in the early stages (T1-T2) (6 cases, 26.08%), those in advanced stages T3-T4 registering only 4 cases (17.39%).

The statistical analysis did not show statistically significant associations between PD-L1 expression and smoking-related behavior, the number of positive and negative cases being equal in non-smokers (5 cases, 21.73%), and in heavy smokers, most cases are negative (9

cases, 39.13%), only 4 being positive (17.39%). The most PD-L1 positive cases were those with N0-N1 lymph node status (6 cases, 26.08%), compared with only 3 positive cases (13.04%) for N2a-N2b lymph node status and 0 cases for N2c N 3. Regarding perineural invasion, it is observed that the number of negative cases IPN0 and IPN1 are equal (7 cases, 30.43%), and most positive PD-L1 cases did not associate perineural invasion (7 cases, 30.42%) , compared to positive PD-L1 cases, with associated perineural invasion, with low number (2 cases, 8.69%). Also, the most cases that associated angiolymphatic invasion were PD-L1 negative (9 cases, 39.13%) and 8 positive (34.78%) (4 cases PD-L1 1-19, 4 cases PD -L1> 20). The crosstabulation statistical analysis showed a statistically significant association between the PD-L1 expression and the CD8 + level, the value of the chi-square tets being 0.019, with a negative association, the value of the Pearson Rs test = - 0.537.

To date, very few studies of laryngeal carcinomas have been performed on PD-L1 and TIL expression. In 2015 and 2019, respectively, Vassilakopoulou et al [40] and Ismail et al [42] studied the correlation between PD-L1 expression and TIL stromal lymphocytes in laryngeal carcinomas. In the first study, a cohort of patients with laryngeal SCC was selected who underwent total laryngectomy (86.2%) or conservative procedures (13.8%), while postoperative radiotherapy was administered in 32.7%. of these, and the dissection of the lymph nodes was performed in 28.5% [40] The patients selected in our cohort underwent total laryngectomy and lymph node dissection, these being also eligibility criteria. The radiotherapy treatment was not followed, being partly applied to the patients, therefore it was not introduced in the correlation with other parameters. The mean age of the patients was 61.48 ± 4.42 years, a mean age lower than the 63 years of the study. The preferential location of SCC in the current study is glottic and supraglottic (70%), which overlaps with the data highlighted by the 2015 study, where it also predominated in a proportion of 90%. Tumors in advanced stages accounted for 43%, a lower percentage compared to the mentioned study (79.6%). 14 cases (60.86%) did not show lymph node metastases, a lower percentage compared to 84.6% in Vassilokopoulou's study.

Regarding the level of CD8 + lymphocytes, in our study 65.21% of cases had a high level, while in the reference study 36.1%, a much lower percentage, and the number of cases with low CD8 + level was 63 , 8%, while in the current study of only 34.78%. No statistically significant correlation was observed between CD8 + level and tumor stage (T), differentiation, nodal status and smoking behavior, the current results overlapping with those of 2015. The same result was obtained by Karpathiou et al., in 2016, the level of CD8 lymphocytes did not correlate with the mentioned parameters [30]. In Ismail's 2019 study, PD-L1 was positive in a

proportion of 92.5%, and while in the current study the percentage of PD-L1 positive was only 39.13%. In both reference studies, the level of TIL (2015) and TIL CD8 + (2019) were correlated with the expression PD-L1, a result obtained in the current study. Also, Vassilokopoulou's study analyzed the correlation between TIL and PD-L1 protein level, the correlation being a positive one, patients with high TIL levels having high levels of PD-L1 protein [40]. Statistically significant correlations between PD-L1 expression and lymph node status and smoking behavior were obtained in the reference study, without being found in the current cohort of the pacients.

In the 2015 study, TIL level and PD-L1 expression were independent predictors of overall survival and event-free survival in patients with laryngeal SCC, with stromal TIL levels being closely associated with better survival. Kaplan-Meier curves showed a longer survival interval in patients with high TIL levels. Also, a better prognosis was observed in patients with increased PD-L expression, with favorable intervals without the appearance of a new event and significantly lower risk for recurrence were highlighted by Kaplan-Meier curves and univariate cox-regression analysis. In terms of overall survival, Ismail's study showed that overall survival correlated positively with high levels of CD8 + and low levels of PD-L1 [42].

In 2019, a meta-analysis conducted by Jia et al [43], studied survival rates, including overall and no signs of disease survival or tumor progression without a history of events, but also survival without distant metastases of patients with different levels. of immunological verification molecules. The study was based on 52 prospective papers in the literature and included oral, head and neck pharyngeal carcinomas (7127 patients), all articles published between 2010 and 2018. Most studies also included PD-L1, In laryngeal carcinomas, increased PD-L1 expression is associated with better disease-free survival (DFS).

Kaplan-Meier curves and the log-rank test were performed to analyze disease-free survival and overall survival. The level of CD8 + lymphocytes was correlated with disease free survival, without a statistically significant association ($p = 0.98 > 0.05$), which shows that the distribution of survival is the same in the analyzed groups.

Thus, the mean survival without signs of disease was 9 months in patients with low CD8 + level and 16 months in patients with high CD8 level, and the maximum survival without signs of disease was 11 and 21, respectively. Analysis of survival without signs of disease disease correlated with PD-L1 expression showed a favorable prognosis in patients with elevated PD-L1 levels (PD-L1> 1 and PD-L1> 20), compared to patients with PD-L1 negative tumors. The value $p = 0.006 < 0.05$ shows a statistically significant association between the two parameters, so it can be concluded that the distribution of survival is different in population groups. The

results correlate with those obtained by Vassilakopoulou in 2015 in terms of PD-L1 expression [40]. Also, the results are contradictory to those obtained for other types of malignancies (renal, colorectal, lung) [44], which show that an increased PD-L1 expression is associated with an unfavorable prognosis. There are also studies in which clone 5H1 was used for PD-L1 immunohistochemical examination, in metastatic malignant melanoma, non-small lung cancers, Merkel cell carcinomas, and these showed a positive association between high PD-L1 expression, elevated blood TIL and patient prognosis [45,46].

We also found a positive association between PD-L1 expression and CD8 + lymphocyte levels. To date, the biological mechanism of the association between PD-L1 expression, TIL level and favorable prognosis has not been fully understood. For example, in melanomas in which the stromal inflammatory infiltrate is dominated by CD8 + T cells, an association with a high level of Treg cells, PD-L1 protein and mRNA has been found. Moreover, the induction of immune inhibitory pathways in the tumor microclimate was dependent on the presence of CD8 + and IFN T lymphocytes [47]. Thus, it is possible that rather than an indication of total immune deprivation, PD-L1 expression of tumor cells reflects the presence of antigen-induced antitumor immune pressure mediated by TIL lymphocytes. Although only partially efficient, recruiting TIL from the tumor microclimate into the tumor response to preserve chemotactic signals could induce a partially antitumor effect and explain the improvement in survival rates [48]. Thus, the levels of TIL / CD8 + lymphocytes and PD-L1 expression may be selection criteria for the treatment of patients with anti-PD-L1 monoclonal antibodies [40].

Many of the correlative predictive studies performed on multi-institutional trials focused on PD-L1 expression, being known to be associated with TIL, but also with the composition of the stromal infiltrate, a result obtained by Kluger in 2015 [49], in malignant melanoma, suggesting that TIL may be a predictive biomarker in studies that include patients treated with anti-PD-L1 inhibitors. It also assessed the prognostic value of T cell subsets in malignant melanoma, demonstrating that the percentage of CD8 + T cells is an independent factor for better survival. In Taube's 2014 study, PD-L1a expression was the strongest predictor of patients' response to nivolumab treatment, but he showed at the time of the study that a combined model (PD-L1 and TIL expression) might be the most effective. good model for predicting response to treatment [50]. In the current study, the positive, statistically significant correlation between PD-L1 expression and CD8 + lymphocyte levels falls within this prediction model.

Balermpas et al. studied the immune microclimate in head and neck cancers and showed that tumors with stromal inflammatory infiltrate rich in cytotoxic lymphocytes CD3 and CD8

are associated with a better prognosis after chemoradiotherapy treatment [51], and the prognostic importance of tumor infiltration with CD3 T lymphocytes and CD8 has also been found in breast, esophageal, lung, ovarian, colon and anal cancers [52]. Not only the composition of the tumor microclimate has prognostic importance, but also the location of inflammatory cells within the tumor (near the tumor islands, in the stroma or periphery), which may be the subject of future study on laryngeal tumors [51].

Laryngeal tumors TIME classification (Tumor Immunity in the MicroEnvironment). Corelations between TIME classes and the main clinical-morphological factors

Starting from the studies of Taube [48] and Zhang [53], we classified laryngeal carcinomas according to PD-L1 and TIL CD8 + (TIME) levels, as follows: TIME I class PD-L1 negative / low CD8 + level (tolerance class immunological); TIME class II PD-L positive / CD8 level + high level (adaptive resistance class); negative TIME class III PD-L1 / increased CD8 + level (immunological tolerance class); TIME class IV PD-L1 positive / low CD8 + level (intrinsic induction class) [12]. There was an increased frequency of TIME IV class cases (39%), of which the share of female cases was very low (1 case), included in TIME IV class, the rest of male cases, most being TIME class IV.

By age, TIME IV carcinomas were the most common in patients under 60 years of age (5 cases, 21.73%), and TIME II class carcinomas predominated in patients over 60 years of age (6 cases, 26). , 08%). The degree of tumor differentiation was statistically significantly associated with the TIME class, as shown by the crosstabulation correlation, the value of the chi-square test being $\chi 2 = 0.019 < p = 0.05$, between the 2 variables there is a low level of correlation and negative, (Spearman r = - 0.053). The most common carcinomas G1-G2 belonged to class TIME IV (8 cases, 34.78%), and the poorly differentiated G3 had the same number in classes II and III (3 cases, 13.04%). Also, the classification in the TIME class was statistically significantly associated with the tumor stage pT, the value of the test $\chi 2 = 0.029 < p = 0.05$, between the two variables there is a low, negative correlation level highlighted by the value of the Spearman index r = - 314. Most carcinomas in early stages T1-T2 belonged to class IV (8 cases, 34.78%), and most in advanced stages T3-T4 fell into class II (5 cases, 21.73%) .

Table nr. 34. TIME classes and clinico-morphological characteristics correlations

	TIME I	TIME II	TIME III	TIME IV	P value (Chi-square test)
Age					0,320
≤ 60	2	2	0	5	
>60	1	6	3	4	
Differentiation grade					0,019
G1-G2	3	5	0	8	
G3	0	3	3	1	
pT					0,029
T1-T2	2	3	0	8	
T3-T4	1	5	3	1	
pN					0,149
N0	2	7	2	3	
N1-N3	1	1	1	6	
IPN					0,269
IPN0	0	3	2	6	
IPN1	1	5	1	3	
ILV					0,708
ILV0	1	2	0	3	
ILV1	2	6	3	6	
Level					0,287
Supraglottic	1	1	0	0	
Glotic	1	5	1	7	
Infraglotic	1	1	0	1	
Transglotic	0	1	2	1	

Perineural invasion was not statistically significantly associated with the TIME class, most carcinomas with perineural invasion belonging to class II (5 cases, 21.73%), and most without perineural invasion (6 cases, 26.08%) were classified in class TIME IV. Vascular invasion was frequent (15 ILV1 cases, 65.21%), of which most belonged to classes II and IV (6 cases each, 26.08%). Most cases that did not associate perineural invasion belonged to class

IV (3 cases, 13.04%). The preferred glottic location of laryngeal carcinomas was divided between second and fourth class carcinomas, with 7 cases each (30.43%). The other locations recorded between 0 and 2 cases (maximum 8.69%).

For the analysis of general short-term survival correlated with TIME classes, all 23 cases studied, diagnosed with SCC and followed for a period of 24 months were analyzed. Within this group, 30.43% of patients died due to SCC, the distribution by TIME classes being: class I - 33.33%, class II - 33.33%, class III - 66, 66%, class IV - 55.55%. Kaplan-Meier curves and the log-rank test were performed for overall survival. TIME immunological classes were statistically significantly correlated with overall survival ($p = 0.05$), showing that the distribution of overall survival is different in different population groups. Analyzing the general survival curve, it is observed that class IIa tumors (PD-L1 positive, high CD8 + level) had the best maximum survival (18 months), and those in class I (PD-L1 negative, CD8 + low) the shortest survival period (8 months). The overall survival rate per group of patients was 56.52%, and the classification by TIME classes was as follows: class I - 14.28%, class II 42.85%, class III 14.28 % and 4th grade 28.57%. Thus, class TIME II had the best survival rate, and the lowest survival rate had SCC in classes I and III, these differences being statistically significant (Log Rank test, $p = 0.011$).

The results of our study are consistent with those published by Vassilokopoulou et al. [40], who point out that in laryngeal carcinomas, elevated TIL levels and increased PD-L1 expression correlate with a better prognosis. In addition to this, there was a positive correlation between CD8 + TIL levels and PD-L1 expression [42], results obtained in the current study.

In the last 2 years, starting from 2018 until now, several studies have been conducted that analyzed the expression of PD-L1 correlated with TIL, how these variables influence survival and the possibility of being used as biomarkers to stratify patients with laryngeal SCC , head-neck carcinomas but also nasopharyngeal carcinomas, for unique immunotherapy treatment or combined with chemoradiotherapy. Thus, Ma et al reported that a 1% PD-L1 expression on tumor cells and stromal lymphocytes did not influence overall or disease-free survival, and PD-L1 positivity rates ranged from 20 to 40 percent. % [54-56]. Other retrospective studies have reported a 34-46% positivity of PD-L1, with increased expression in 70-100% of positive cases [57]. In 2019, the study by Minichsdorfer et al, on nasopharyngeal carcinomas showed a positivity rate of 83%, but also the fact that PD-L1 expression correlates in the Caucasian population with overall survival and no signs of disease [58], and a meta-analysis of the prognostic significance of PD-1 / PD-L1 in Asian patients did not show any correlation between PD-L1 expression and prognosis [59]. In the meta-analytical study

conducted by Jia et al in 2019, the general conclusion regarding head and neck carcinomas and laryngeal carcinomas, that increased PD-L1 expression correlates positively with a better prognosis [43].

Even in early 2020, Hu et al published the results on hypopharyngeal carcinomas and the correlation between PD-L1 expression and TIL lymphocyte levels, without introducing them into immunological classes. It showed that PD-L1 was not associated with the prognosis, and PD-L1 positivity in combination with CD8 + TIL levels may have predictive potential [60]. Although the number of cases analyzed in the current study is not high, we developed a predictive model based on variables that allowed correlations of this type (Annex 1 and 2), through statistical analysis of logistic binary regression. The predictability of the model as a whole is 60.9%, which is a good percentage of prediction - in about 60% of cases, based on CD8 + values and PD-L1 expression, so TIME classes, it will be possible to make a prediction of short-term survival of patients.

CONCLUSIONS

The current clinical-statistical study was performed on a group of 404 patients diagnosed at the histopathological examination in usual hematoxylin-eosin staining with invasive squamous cell carcinomas of the larynx during 2011-2019. Following the study we drew the following conclusions:

1. The distribution of cases in the period 2011-2019 recorded a maximum number of cases diagnosed in 2013 (66 cases; 16.33%). Out of the total of 346 cases of laryngeal carcinomas, 317 cases were diagnosed in males (86.37%), in women the number being much smaller (30 cases, 81.08% of lesions diagnosed in women and only 8.67 % of carcinomas). Although there is a decrease in the number of invasive lesions in females in the first part of the period, from 6 cases in 2011 to 1 case in 2015, there is then a fluctuation in the number of cases, so that in 2019 the number will be increased again - 8 cases, the highest number recorded in women in one year).
2. The histopathological analysis highlighted the preponderance of cases of conventional squamous cell carcinomas (328 out of 346 cases, 94.79%). Moderately differentiated G2 carcinomas predominated (153 cases, 46.64%), followed by G3 carcinomas (128 cases, 39.02%). The least common were well-differentiated G1 carcinomas (48 cases, 14.63%).

3. Carcinoma variants were much less common, in descending order of basaloid SCC 7 cases, spindle cell SCC 3 cases, papillary SCC 3 cases, warty SCC 2 cases and adenoscuamos SCC 1 case.
4. Most tumors were included in T1-T2 tumor stages (13 cases, 56.52%) and only 10 cases (43.47%) in advanced T3-T4 tumor stages.
5. Components of the immune microclimate, CD3 lymphocytes showed an increased level in 22.36% of cases, a low level in 77.6% of cases. The level of CD3 + lymphocytes was correlated with the degree of tumor differentiation without a statistically significant association. Thus, of the cases with high CD3 level, most were poorly differentiated G3 carcinomas, and of those with low level the most common were well and moderately differentiated. Statistical analysis showed a statistically significant association between the level of CD3 lymphocytes and the sex of the patient test $\chi 2$ (df2) = 0.047, between the 2 parameters there is a negative correlation (Spearman Rs correlation = -0.222, $p < 0.05$). Most commonly in men, the level of CD3 lymphocytes was low (55 cases), compared to only 13 cases with high CD3 levels. of women the number of those with low and high CD3 levels was the same (4 cases).
6. The level of CD4 + lymphocytes, another component of the immune microclimate was decreased in 75% of the studied cases and increased in 25%, this being statistically significantly associated with the degree of tumor differentiation, between the two variables there is a positive correlation - test value $\chi 2$ (df2) = 6.350; p = 0.012), and the value of the Sperman Rs index having the value 0.288. The most common cases with CD4 + level belonged to decades of age over 60 years (63.15%), in the decade under 60 years only 35, 85% of cases being CD4 + increased. For the other clinical factors, there were no statistically significant associations with CD4 + lymphocyte levels.
7. CD8 + lymphocytes were elevated in 59% of cases and predominated in the age group over 60 years (71.11%) and in tumors with glottic location (88.23%), in patients consuming moderate amounts of alcohol (91.83%) and smoking patients (88.63%) and decreased by 41%. Increased CD8 + levels showed tumors in advanced stages T3-T4 (68.57%) and those without lymph node metastases or with lymph node metastases stage N1 (72.22%).

8. The expression of p16 protein was low in most cases (77.63%) and positive in only 22.37%, the positivity being higher in patients under 60 years (70.58%), in patients over 60 years the positivity decreasing to 29.42% of cases. The preferred location of p16-positive carcinomas was glottic (81.25%). Statistical analysis showed a statistically significant association between p16 expression and the degree of tumor differentiation, test $\chi 2$ (df2) = 5.89, p = 0.015 <0.05, between the 2 parameters there is a negative correlation, Spearman index Rs = - 278. Slightly differentiated G3 tumors predominated (58.8%), with a slightly higher percentage compared to the bime and moderately differentiated G1-G2 tumors (41.17%). Risk factors (alcohol, smoking) were not statistically significantly associated with the expression of p16 protein, but it should be noted the positivity in a higher percentage of tumors in smoking patients (81.25%) and in patients with non-alcohol consumption or with moderate alcohol consumption (88.23).
9. In the group of patients for whom we analyzed the correlation of CD8 + lymphocyte levels with PD-L1 expression, elevated CD8 + levels predominated in patients over 60 years (65.21%), in well and moderately differentiated squamous cell carcinomas G1-G2 (47 , 82%), in the T1-T2 stage (34.78%) and in tumors not associated with lymph node metastases (39.13%).
10. The expression PD-L1 was negative in 60.86% of cases and positive in 39.13% of cases, between the latter 21.73% with a value of less than 20 and 17.39% over 20. Depending on of the degree of differentiation, the results did not show statistically significant differences, so that in the case of bime and moderately differentiated tumors and in the case of poorly differentiated, PD-L1 was predominantly negative by 39.13% and 21.73% of cases, respectively . Most PD-L1 positive tumors were diagnosed in the T1-T2 stage (26.08%). The most PD-L1 positive cases were those with N0-N1 lymph node status (26.08%), compared to only 3 positive cases (13.04%) for N2a-N2b lymph node status and 0 cases for N2c-N3. The number of negative PD-L1 cases IPN0 and IPN1 are equal (30.43%), and most positive PD-L1 cases did not associate perineural invasion (30.42%), compared to positive PD-L1 cases with associated perineural invasion , with a small number (8.69%). Also, the most cases that associated angiolymphatic invasion were PD-L1 negative (39.13%) and 8 positive (34.78%) (4 cases PD-L1 1-19, 4 cases PD-L1> 20).
11. The statistical analysis between the CD8 + level and the PD-L1 expression showed a statistically significant association (chi square test; $\chi 2$ (df2) = 2; p = 0.019),

between the two parameters showing a positive correlation (Spearman correlation rs = 0.516).

12. The originality of the thesis is the classification of laryngeal squamous cell carcinomas according to TIME class (CD8 + level and PD-L1 expression), which highlighted the following: the most common were carcinomas of class TIME IV (intrinsic induction class) (39 %), followed by Class II (Adaptive Resistance Class) (35%) and Class I (Immune Tolerance Class) and Class III (Adaptive Resistance Class) (13% each). The statistical analysis showed a statistically significant association between the TIME classes and the degree of tumor differentiation, the value of the chi-square test being $\chi 2 = 0.019 < p = 0.05$, between the 2 variables there was a low level of negative correlation, (Spearman r = - 0.053). The most common carcinomas G1-G2 belonged to class TIME IV (34.78%), and the poorly differentiated G3 had the same number in classes II and III (13.04%). Also, the TIME class was statistically significantly associated with the tumor stage pT, the value of the test $\chi 2 = 0.029 < p = 0.05$, between the two variables there is a low level, negative correlation highlighted by the value of the Spearman index r = - 314. Other parameters histopathological (pN, ILV, IPN) and tumor location or age of patients did not show statistically significant associations with TIME classes.
13. Analysis of Kaplan Meier survival courses showed that the level of CD8 + lymphocytes was correlated with survival without signs of disease (this being higher in patients with elevated CD8 + lymphocytes), without a statistically significant association ($p = 0.98 > 0.05$), which shows that the distribution of survival is the same in the analyzed groups. Analysis of survival without signs of disease correlated with PD-L1 expression revealed a favorable prognosis in patients with elevated PD-L1 levels (PD-L1> 1 and PD-L1> 20), compared to patients with PD-L1 negative tumors. The value $p = 0.007 < 0.05$ shows a statistically significant association between the two parameters, so it can be concluded that the distribution of survival is different in population groups.
14. Analysis of Kaplan-Meier survival curves by TIME classes showed that they were statistically significantly correlated with overall survival ($p = 0.05$), with class II TIME having the best survival rate and the lowest houses I and III, the differences being statistically significant (Log Rank test, $p = 0.011$).
15. The predictive model based on the analyzed parameters has an overall predictability of 60.9%, a good percentage of prediction, which shows that based on the level of

CD8 lymphocytes and correlated PD-L1 expression (based on the TIME classification) can be make a prediction of short-term survival, so the TIME class can become a predictive factor of the patient's prognosis.

16. For the setting of PD-L1 and / or TIME classes as predictive biomarkers to help stratify patients for immunotherapeutic treatment, new studies are needed, conducted in large cohorts of patients to help standardize results, current data from the literature being contradictory, both on head and neck carcinomas as a whole and on those of the larynx.

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ANNEXES

ICONOGRAPHY. The paper includes 121 original macro- and microscopic images, belonging to the studied cases, 38 tables and 136 figures.