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# Characteristics of Tumor Infiltrating Lymphocytes in Patients with Benign and Malignant Sinonasal Neoplasms

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**Abstract**

***Introduction***. Tumor infiltrating lymphocytes are a major component of sinonasal neoplasms, actively populating tumor tissue, yet their role remains poorly understood. The aim is to characterize subsets and functional profiles of tumor infiltrating lymphocytes in patients with benign and malignant sinonasal tumors. ***Materials and methods***. Tumor infiltrating lymphocytes were isolated from the biopsy material of 58 patients: 18 with sinonasal malignancies, 23 with inverted papilloma and 17 with polypous rhinosinusitis (control group). Flow cytometry was used for tumor infiltrating lymphocytes immunophenotype estimation. The production of γ-interferon in tissue homogenates was measured by enzyme-linked immunosorbent assay kit. Further, statistical analysis was done using GraphPad Prism 8. ***Results and discussion***. The microenvironment of sinonasal malignancies is characterized by increased $CD3^+$T cells ($p = 0.049$) among tumor infiltrating lymphocytes as well as high levels of extracellular γ-interferon in tissue ($p = 0.044$). In this group, the decreased γ-interferon-positive $CD3^+$T cells ($p = 0.010$) and $CD56^+CD16^+$NK cells ($p = 0.027$) were detected as compared to control group. In patients with benign and malignant sinonasal tumors, a significant elevation of regulatory T cells and increased cytotoxic T lymphocyte associated protein 4-positive $CD3^+$T cells have been observed. ***Conclusions***. The application of tissue resident regulatory T cells, cytotoxic T lymphocyte associated protein 4-, and CD16-positive $CD3^+$TILs, as well as intracellular γ-interferon production may be considered as potential prognostic biomarkers for malignancy in sinonasal tumors and requires further investigation.

**Keywords:**
tumor infiltrating lymphocytes; sinonasal malignancies; immunophenotype; regulatory T cells; γ-interferon; cytotoxic T lymphocyte associated protein 4; mucosal tumor immunity

## 1. Introduction

Tumors of the nasal cavity and paranasal sinuses are rare (account for 1–3% of malignant tumors of all localizations), and are heterogeneous neoplasms that are characterized by multifactorial etiology. Their development is influenced by genetic predisposition (such as associations with HLA-B17 and HLA-Bw26), environmental factors, and chronic viral infections, including human papillomavirus (HPV) and Epstein-Barr virus (EBV) [1–3]. Although tumors of the nasal cavity and paranasal sinuses are presented with different histological features and clinical behavior, squamous cell carcinoma accounts for 90% of head and neck cancer [4]. Inverted papilloma (IP) represents the predominant type of benign tumors in the sinonasal tract [5]. Due to the poverty of prospective and large clinical trials, the main problem is the lack of biomarkers for early detection of the malignant process.

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Thereby, late diagnosis results in poor effectiveness of treatment and low survival of patients. From a diagnostic point of view, it is mandatory not only to perform an immunohistochemical tumor assessment but also to understand the immunological peculiarities of malignant and benign sinonasal neoplasms. Thereby, the local microenvironment and tumor-infiltrating lymphocytes (TILs) are of high scientific and practical interest.

TILs are immune cells that migrate to the area of the primary neoplasm and actively infiltrate it as well. They can also be identified in metastases and regional lymph nodes, making them the objects of increased researcher's attention worldwide. However, the understanding of TILs role in the pathogenesis of cancer is still poorly investigated [6–8]. Initial studies suggested TILs to be cytotoxic to cancer cells, having almost no effect on healthy tissues. Later, the dual role of TILs in the tumor microenvironment was confirmed: on the one hand, TILs can suppress tumor growth by destroying transformed cells or inhibiting their growth, and, on the other hand, TILs promote tumor progression via selecting immune resistant clones or creating conditions in the tumor microenvironment that promote its growth [9–11]. Such a wide functional spectrum is primarily due to the heterogeneity of TILs, which mainly include subpopulations of T lymphocytes, and to a lesser extent, B lymphocytes, NK cells and innate lymphoid cells. The same populations of immune cells can exhibit both anti- and pro-tumorigenic effects. Their composition and functional state can vary significantly depending on the tumor type, disease stage, therapy, and the method of cell isolation and cultivation [6,12].

TILs prognostic value is still under debate due to contradictory data. Many authors have demonstrated TILs to correlate with clinical outcome in different malignant neoplasms, and have shown that the degree of tumor infiltration by lymphocytes determines the disease prognosis [13–15]. According to some data, TILs are recognized as a more significant predictor of patient's survival than the TNM classification of malignant tumors [10]. Nonetheless, most of these studies were conducted using immunohistochemical analysis of fixed blocks that allows comparing laboratory and clinical results. However, this methodological approach provides information only about the presence of infiltration, its exact localization and severity, but does not characterize the subpopulation composition and TILs functional profile. Disadvantages of the current methodology for TILs determination as a prognostic biomarker include the lack of quantitative immuno(cyto)histochemistry approaches, insufficient reproducibility of results, and labor-intensive procedures for TILs measuring that can be improved by using the flow cytometry. Thus, the determination of the immunological characteristics of benign and malignant sinonasal neoplasms is a highly relevant area that will allow clarifying the specific contribution of immune parameters to systematization and timely diagnostics of sinonasal malignant and benign tumors.

In this article, the characteristics of TILs subsets and function profile in patients with sinonasal neoplasms is presented for the first time, aimed at assessing the TILs differences in malignant and benign tumor-associated pathological process in the nasal cavity and paranasal sinuses.

## 2. Materials and Methods

***Patients.*** The biopsy material was obtained from 58 patients (37 men and 21 women, average age of 56.5 (43.5; 64.3) y.o.) hospitalized at N.N.Alexandrov National Cancer Centre of Belarus or the Republican Scientific and Practical Center of Otorhinolaryngology from January 2022 to December 2024. All subjects were divided into three groups: group 1–18 patients with sinonasal malignant neoplasms; group 2–23 patients with IP; group 3–17 patients with polypous rhinosinusitis (PRS) as a control group. The group of malignant tumors of the nasal cavity and paranasal sinuses included patients with the following diagnoses: squamous cell carcinoma (n = 10), melanoma (n = 5), sarcoma (n = 2), neuroendocrine cancer (n = 1). Clinical and demographic characteristics of patients are presented in Table 1.

***TILs isolation protocol.*** Biopsy materials were routinely processed within 1–2 h of surgical excision using standard biosafety procedures for human tissues. Sinonasal tumors were weighed, minced into small fragments of 2–4 mm, transferred into the gentleMACS C tube («Miltenyi Biotec», Bergisch Gladbach, Germany) containing 4.7 mL RPMI-1640 («Bio-Whittaker», Walkersville, MD, USA) along with an enzyme mix of 200 μL enzyme H, 100 μL enzyme R, and 25 μL enzyme A (Human Tumor Dissociation Kit, «Miltenyi Biotec», Bergisch Gladbach, Germany) per 0.2–1.0 g of tumor sample. The samples then underwent enzymatic tissue digestion using the gentleMACS Dissociator («Miltenyi Biotec», Bergisch Gladbach, Germany). The dissociation was carried out by running the gentleMACS program «h_tumor_01» followed by samples incubation for 30 min at 37 °C under continuous rotation. Cell suspension was applied onto a 70 μm cell strainer («Millipore», Burlington, MA, USA) and washed with 20 mL of RPMI-1640 at 300 g for 7 min for further immunophenotyping or cell culturing.

***Flow cytometry method.*** The immunophenotype of TILs basic subsets was determined using the next CYTOSTAT tetraCHROME monoclonal antibody panels: CD45-FITC/CD4-RD1/CD8-ECD/CD3-PC5 and CD45-FITC/

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**Table 1:** Clinical and demographic characteristics of patients.

| Parameters | | Group 1 (n = 18) | Group 2 (n = 23) | Group 3 (n = 17) |
|---|---|---|---|---|
| Age, years, Me (Q25; Q75) | | 59 (49; 66) | 53 (38; 67) | 56 (45; 63) |
| Gender, n (%) | male<br>female | 11 (61.1)<br>7 (38.9) | 16 (69.6)<br>7 (30.4) | 10 (58.8)<br>7 (41.2) |
| Smoking, n (%) | yes<br>no | 4 (22.2)<br>14 (77.8) | 7 (30.4)<br>16 (69.6) | 4 (23.5)<br>13 (76.5) |
| Bleeding, n (%) | yes<br>no | 5 (27.8)<br>13 (72.2) | 3 (13.0)<br>20 (87.0) | 1 (5.9)<br>16 (94.1) |
| Anosmia, n (%) | yes<br>no | 4 (22.2)<br>14 (77.8) | 8 (34.8)<br>15 (65.2) | 10 (58.8)<br>7 (41.2) |
| Ophthalmological symptoms, n (%) | yes<br>no | 6 (33.3)<br>12 (66.7) | 1 (4.3)<br>22 (95.7) | 2 (11.8)<br>15 (88.2) |
| Negative localization, n (%) | yes<br>no | 7 (38.9)<br>11 (61.1) | 2 (8.7)<br>21 (91.3) | 6 (35.3)<br>11 (64.7) |
| Fibrinogen, g/L, Me (Q25; Q75) | | 3.62 (3.05; 4.25) | 3.15 (2.97; 4.60) | 3.17 (2.75; 3.61) |
| T classification, n (%) | T0<br>T1<br>T2<br>T3<br>T4, n (%) | 2 (11.1)<br>3 (16.7)<br>1 (5.5)<br>2 (11.1)<br>10 (55.6) | n.d. | n.d. |

Notes: group 1—patients with sinonasal malignant tumors; group 2—patients with inverted papilloma; group 3—patients with polypous rhinosinusitis; Me (Q25; Q75)—median (lower quartile; upper quartile); n—number; negative localization—location of pathological focus in sphenoid sinus, orbital or cranial cavity; T—refers to the tumor size and extent in TNM system for classifying a malignancy; n.d.—not determined.

CD56-RD1/CD19-ECD/CD3-PC5 («Beckman Coulter», Indiana, IN, USA). γδT lymphocytes subsets were identified using a DuraCloneIMTCRs monoclonal antibody panel: γδTCR-FITC/αβTCR-PE/Vδ1TCR-PC7/CD4-APC/CD8-AF700/CD3-AF750/Vδ2TCR-PB/CD45-KrO («Beckman Coulter», Indiana, IN, USA). $CD19^+CD5^+B_1$ lymphocytes were estimated using CD45-FITC/CD5-PE/CD19-ECD panel («Beckman Coulter», Indiana, IN, USA). Regulatory T cells (Treg) were detected as $CD3^+CD4^+CD127^{low}CD25^{hi}$ cells using CD127-FITC/CD25-PE/CD4-PC7/CD3-APC/CD45-PB panel («Beckman Coulter», Indiana, IN, USA). Programmed cell death 1 (PD-1)-positive or CD16-positive cells were measured with CD4-FITC/CD279-PE/CD8-ECD/γδTCR-PC7/CD3-APC/CD45-PB or CD3-FITC/CD56-PE/CD16-ECD/γδTCR-PC7/CD45-PB panels, respectively («Beckman Coulter», Indiana, IN, USA; «BioLegend», San Diego, CA, USA). Monoclonal antibody reagents were added according to the manufacturer's instructions to 100 μL of TILs suspension, and reaction mixtures were incubated at 20–25 °C for 15 min in the dark. Results were measured using a 10-channel Cytoflex flow cytometer («Beckman Coulter», Brea, CA, USA) and analyzed on 10,000 $CD3^+$T lymphocytes or 1,000 γδT lymphocytes using CytExpert software (version 2.3.0.84, «Beckman Coulter», Brea, CA, USA).

The intracellular γ-interferon (γIFN) and cytotoxic T lymphocyte-associated protein 4 (CTLA-4) production was evaluated in 24 h TILs cultures in RPMI-1640 medium («Bio-Whittaker», USA) completed with 10% fetal calf serum («Gibco», Waltham, MA, USA), 2 mM L-glutamine («Bio-Whittaker», Walkersville, MD, USA), 1% antibiotic-antimycotic («Gibco», Waltham, MA, USA) in $CO_2$-incubator («Thermo Fischer Scientific», Waltham, MA, USA) with 5% $CO_2$ and 37 °C. For quantitative intracellular γIFN and CTLA-4 determination 4 ng/mL phorbol 12-myristate 13-acetate («Sigma», Darmstadt, Germany), 1 μg/mL of ionomycin calcium salt and 10 μg/mL brefeldin A («Cayman Chemicals», Ann Arbor, MI, USA) were added in the last 4 h of cell culture activation. Then, TILs were stained with monoclonal antibodies panel CD3-FITC/CD8-PC5/ γδTCR-PC7/CD45-PB («Beckman Coulter», Indiana, IN, USA) at 20–25 °C for 15 min in the dark, fixed with 4% paraformaldehyde («Sigma», Darmstadt, Germany), permeabilized with 2% Triton X («Sigma», Darmstadt, Germany). Intracellular staining was performed using monoclonal antibodies γIFN-PE or CTLA-4-PE

(«Beckman Coulter», Indiana, IN, USA). The results were analyzed on 10,000 $CD3^+$T lymphocytes or 1,000 γδT lymphocytes using flow cytometer.

***ELISA.*** γIFN concentration was measured in supernatants of tissue homogenates using «gamma-Interferon-ELISA» kit (A-8752, «Vector-Best», Novosibirsk, Russian Federation, diagnostic sensitivity—2.0 pg/mL) according to the manufacturer's manual. For tissue homogenates preparation tissue pieces were weighed and then homogenized in phosphate buffered saline (PBS) as tissue weight (g): PBS (mL) volume = 1:9 using homogenization program in gentleMACS Dissociator («Miltenyi Biotec», Bergisch Gladbach, Germany). To further break down the cells, the suspensions were subjected to freeze-thaw cycles. Then, homogenates were centrifuged for 5–10 min at 5000× *g* at 2–8 °C to get supernatants. The optical density values were measured with a microplate reader Sunrise («Tecan», Grödig, Austria) set to 450 nm.

***Statistical method.*** Statistical data processing was performed using GraphPad Prism 8 («GraphPad Software Inc.», Boston, MA, USA). Data were tested for normality with a Shapiro-Wilk test. The median (Me), lower (Q25) and upper (Q75) quartiles were used as descriptive statistics of the studied groups. Significant differences between investigated groups were determined by nonparametric Kruskal-Wallis test with Dunn post hoc test. Significance levels were set at $p < 0.05$. The correlation was estimated using Spearman's rank coefficient (rho). RStudio version 2024.09.1 («Posit Software», Boston, MA, USA) with corrplot package version 0.95 was used to produce the correlation matrix. Positive correlations were displayed in blue, while negative correlations were shown in red. The color intensity and size of the circles were proportional to the correlation coefficients. Correlation results were considered significant at $p < 0.05$.

## 3. Results and Discussion

***TILs number and subsets characteristics in patients with sinonasal tumors.*** A comparative analysis of TILs concentration and phenotype was performed in investigated groups. A significant increase in TILs number per 1 g of tissue was detected in both patient groups with malignant sinonasal neoplasms, and IP as compared to patients with PRS: 23.6 (11.7; 28.5) × $10^6$ (group 1, $p = 0.044$) and 26.8 (16.0; 34.4) × $10^6$ (group 2, $p = 0.039$) versus 17.4 (12.8; 25.4) × $10^6$ (group 3), respectively, indicating the immune system activity in the tumor tissue.

The obtained results were consistent with the data of other authors, stating an increased lymphoid infiltration of sinonasal neoplasm tissue, along with the number of migrating cells into the lesion. This reflected the active involvement of the cellular immune compartment in the anti-tumor response [6,10,16]. Many studies have described not only TILs infiltration in the head and neck squamous cell carcinoma but also their correlation with outcome, since Wolf first had documented it in 1986 [11,14,15,17]. However, conflicting data have been reported about TILs association with improved prognosis that can be explained by differences in subsets, tumor compartment localization (intra-tumoral versus stromal) and HPV-positive/negative patients' status [7]. Thus, further progression of sinonasal neoplasms is largely determined by the TILs subpopulation composition, the functional profile of which can be characterized by both pro- and anti-tumorigenic effects.

To characterize TILs subsets in patients with sinonasal tumors, the relative numbers of the major and minor populations of T, B and NK cells among TILs were investigated. The results are presented in Figure 1.

Increased $CD3^+$TILs (Figure 1a, $p = 0.003$) in combination with decreased $CD19^+$TILs (Figure 1e, $p = 0.002$) and $CD56^+$TILs (Figure 1g, $p = 0.035$) were detected in patients with malignant neoplasms, as compared to IP patients. Similar differences in T cells (Figure 1a, $p = 0.049$) and a tendency toward a decreased number of B cells (Figure 1e, $p = 0.073$) were observed in TILs of Groups 1 and 3, indicating a redistribution of lymphoid populations with predominant infiltration of T lymphocytes into the tumor tissue. In IP patients, a feature of TILs subsets composition was an increase in $CD56^+$NK cells (Figure 1g, $p = 0.035$), including $CD3^+CD56^+$TNK cells (Figure 1h, $p = 0.047$) as compared to patients with malignant tumors. Meanwhile, the significant change in $CD3^+CD4^+$T helpers/$CD3^+CD8^+$T killer ratio (Figure 1b,c) was observed, with a predominance of T helper cells in Group 2 (Figure 1b, $p = 0.043$) compared to the control group. It reflected the predominant activation of innate but not adaptive killer effector mechanisms of anti-tumor immunity in patients with IP.

The investigation of minor lymphoid cells subsets revealed increased Treg numbers in patients with malignant and benign tumors, as compared to the control group (Figure 1i, respectively, $p = 0.044$ and $p = 0.041$). Currently, Treg are of a particular interest among lymphoid cell populations, as they play a decisive role in the selection of highly avid cytotoxic $CD8^+$T lymphocytes, reducing the functional activity of tumor-specific cytotoxic cells.

Meanwhile, there were no significant differences in the percent of $B_1$ cells (Figure 1f) and γδT cells (Figure 1d) among TILs of the investigated groups. However, the assessment of γδT cells subsets based on Vδ repertoire of T cell receptors (tissue-resident $V\delta1^+$ or $V\delta1^-V\delta2^-$T cells and circulating $V\delta2^+$T cells) showed changes: in group 1,

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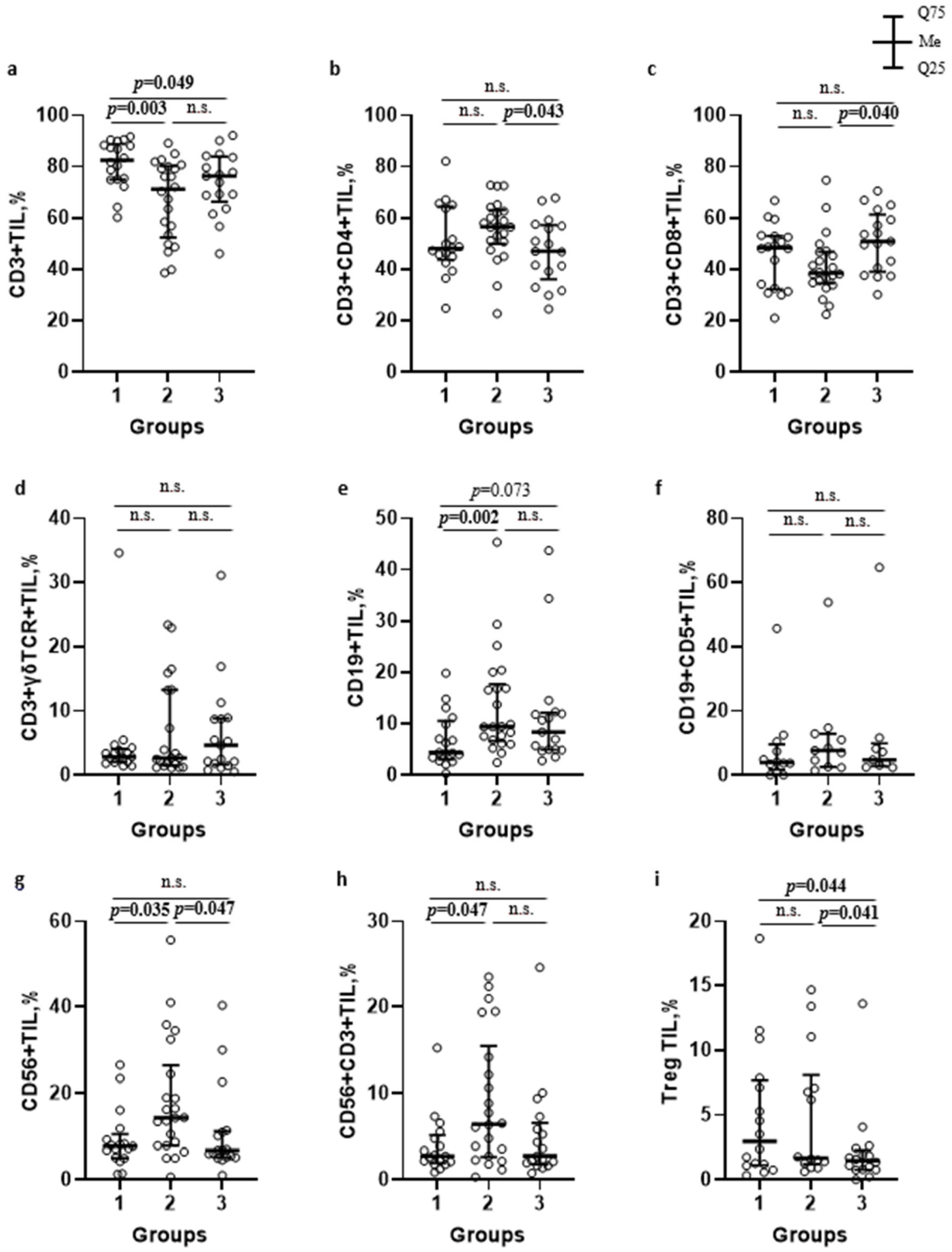


**Figure 1:** TILs subsets numbers in biopsy materials of patients with malignant and benign tumors and PRS: (**a**) $CD3^+$TILs, (**b**) $CD3^+CD4^+$TILs, (**c**) $CD3^+CD8^+$TILs, (**d**) $CD3^+\gamma\delta TCR^+$TILs, (**e**) $CD19^+$TILs, (**f**) $CD19^+CD5^+$TILs, (**g**) $CD56^+$TILs, (**h**) $CD56^+CD3^+$TILs, (**i**) $CD3^+CD4^+CD127^{low}CD25^{hi}$Treg TILs. Notes: group 1—patients with sinonasal malignant tumors; group 2—patients with inverted papilloma; group 3—patients with polypous rhinosinusitis; *p*—*p*-value; n.s.—not significant. Data are represented as individual points and whisker plots showing the median (Me), lower (Q25) and upper (Q75) quartiles.

$V\delta2^+$T cells subset prevailed among others (44.3 (27.3; 53.4) %, $p = 0.001$), while the dominated subsets in group 2 and 3 were $V\delta1^+$T cells (54.3 (27.1; 70.4) % and 40.1 (33.8; 49.0) %, respectively, $p < 0.001$ and $p = 0.002$).

Based on the prevalence of immune cells inside and outside the tumor, Kather et al. assessed a domination of different immune cells in various cancer types and classified the tumors into three groups i.e., «cold» (presence of fewer immune cells both outside and inside the tumor), «immune excluded» (presence of fewer immune cells inside and more immune cells outside of the tumor), and «hot» (presence of more immune cells inside regardless immune cell density outside) [18]. According to the data, the identification of $CD3^+$T lymphocytes absolute majority among TILs, in combination with up-regulated tissue-resident Treg in patients with malignancies, corresponded to Kather et al., who had established a high incidence of CD3-hot, CD8-hot, FOXP3-hot and PD-1-hot tumors in head and neck squamous carcinoma. In contrast to a positive outcome in patients with active TILs infiltration, meta-analysis data indicate that $FOXP3^+$Treg in tumor tissue predicted worse survival rates in the majority of solid tumors, including head and neck cancers [19]. Moreover, according to Lou et al., high levels of $CD8^+$TILs infiltration were related to poor differentiation and lymph node metastases in patients with sinonasal squamous cell carcinoma. The paradox of $CD8^+$TILs and tumor progression coexistence may be explained by a complex crosstalk between tumor and infiltrating immune cells [20]. In contrast, Yin et al. demonstrated that $CD8^+$T cells and TNK cells were highly expressed in patients with no sinonasal mucosal melanoma progression, while Th2 T cells, macrophages and M2 macrophages were highly expressed in patients with disease progression [21]. Liu et al. reported that HPV-positive patients with head and neck squamous cell carcinoma exhibit higher expression of $CD8^+$T cells, Treg, dendritic cells, and $CD56^{dim}$NK cells [22].

The human immune system uses a wide range of cellular factors and molecules to implement mechanisms of anti-tumor immunity, resulting in effective destruction of tumors. The main mechanisms are contact killing mediated $CD8^+$T lymphocytes or NK/TNK cells, as well as antibody-dependent cellular cytotoxicity. Upon activation and recognition of tumor cells, cytotoxic lymphocytes can induce tumor cells death via various pathways, including perforin/granzyme B, FasL-Fas, TRAIL, or inflammatory cytokines. Another effector mechanism is mediated by antibodies, binding to which results in tumor cells destruction via activating the complement cascade, or the transmission signals through Fc receptors on immune cells. It results in antibody-dependent cellular phagocytosis or cellular cytotoxicity involving neutrophils and NK cells [1,6,23]. Due to the fact that the tumor microenvironment forms an unfavorable surrounding, and cells are immature or tolerant, it is crucial to investigate not only the composition of cell subsets but also the functional changes in anti-tumor effector mechanisms. In this regard, TILs functional profile, including the estimation of the cytotoxic innate and adaptive TILs potential, as well as mechanisms of TILs silencing were further investigated in patient groups.

***The characteristics of effector cytotoxicity mechanisms of immune killer TILs in patients with sinonasal tumors.*** To assess the effector mechanisms of specific immunity in patients with sinonasal tumors, the intra- and extracellular γIFN production (Figure 2) as well as CD16 (Fc receptor to IgG fragment) expression (Table 2) as key regulators of anti-tumor immunity were characterized.

A down-regulation of intracellular γIFN production in $CD3^+$TILs of patients with malignant neoplasms was determined as compared to group 3 ($p = 0.010$). A similar decrease in the percentage of $CD3^+\gamma IFN^+$TILs in the local microenvironment was detected in patients with benign tumors as compared to group 3 ($p = 0.043$). Moreover, decreased number of γIFN-positive T cells in the tumor tissue of group 1 was observed in 61.5% of cases, while in patients of group 2 in 35.7%. A moderate positive correlation was established between tissue resident $CD3^+\gamma IFN^+$TILs and circulating peripheral blood $CD3^+\gamma IFN^+$T cells in both groups: in patients with malignant (rho = 0.43, $p = 0.048$) as well as benign (rho = 0.51, $p = 0.048$) neoplasms. At the same time, the number of the major γIFN-producer cells subsets among T lymphocytes —γδ$TCR^+$T cells—were not significantly different in all groups: 43.0 (26.9; 57.5) % in group 1; 34.7 (14.8; 45.0) % in group 2; 39.0 (33.4; 45.3) % in group 3 ($p = 0.616$). In addition, an increase in extracellular γIFN synthesis in tumor tissue was found in patients with malignancies as compared to patients with IP ($p = 0.011$) and PRS ($p = 0.044$), while the level of tissue γIFN production did not differ significantly in patients with IP as compared to the control group (Figure 2, lower panel).

Thus, the microenvironment of sinonasal malignant tumors is characterized by a high TILs level as well as extracellular γIFN synthesis in tissue, along with a markedly decreased population of γIFN-producing T cells. This may reflect the activation of the tumor-specific immune response and suggest that T cells fully exert their effector γIFN-mediated potential at the site of the pathological process. This can lead to failure due to immunological depletion of cytotoxic T lymphocytes and decreased reserve capacity of cellular immunity. Kondoh et al. have also obtained similar results, reporting a high γIFN-producing capability among patients with oral squamous cell carcinoma at stage I, but it was decreased among patients whose tumor progressions were at stages II and III [24].

Norouzian et al. have demonstrated that larger tumor size, higher stage of the disease and/or lymph nodes

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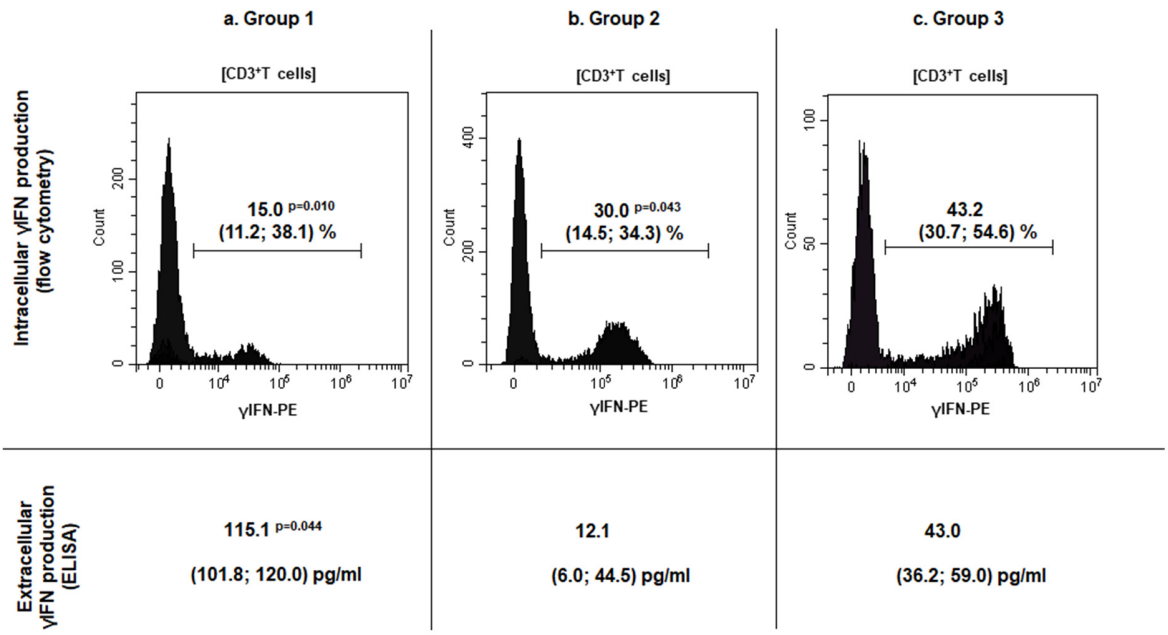


**Figure 2:** $CD3^{+}\gamma IFN^{+}T$ cells (%) in TILs (upper panel) and tissue γIFN concentration (pg/mL) (lower panel) in patient groups, Me (Q25; Q75). Notes: (**a**) group 1 (patients with sinonasal malignant tumors); (**b**) group 2 (patients with inverted papilloma); (**c**) group 3 (patients with polypous rhinosinusitis); *p*—*p*-value as compared to group 3; Me (Q25; Q75)—median (lower quartile; upper quartile). Upper panel represents individual histograms with descriptive statistics as Me (Q25; Q75) of intracellular γIFN production in tumor infiltrated $CD3^{+}T$ lymphocytes measured with flow cytometry, lower panel—extracellular γIFN production in tissue homogenates measured with enzyme-linked immunosorbent assay (ELISA).

**Table 2:** CD16- and γIFN-positive TILs subsets and tissue γIFN production in patients with sinonasal tumors, Me (Q25; Q75).

| TILs Subsets | | Patients' Groups | | | *p*-Value | |
|---|---|---|---|---|---|---|
| | | Group 1 | Group 2 | Group 3 | *Kruskal-Wallis Test* | *Dunn Post-Hoc Test* |
| $CD16^{+}$ cells | $CD3^{+}CD16^{+}$ TILs, % | 1.4 (0.5; 2.7) | 1.6 (0.7; 7.8) | 3.0 (1.1; 5.3) | $p = 0.098$ | $p_{1-2} = 0.832$<br>$p_{1-3} = 0.094$<br>$p_{2-3} > 0.999$ |
| | $\gamma\delta TCR^{+}CD16^{+}$ TILs, % | 15.4 (4.5; 23.4) | 25.3 (9.6; 34.0) | 12.1 (9.5; 16.4) | $p = 0.383$ | $p_{1-2} = 0.803$<br>$p_{1-3} > 0.999$<br>$p_{2-3} = 0.587$ |
| | $CD56^{+}CD16^{+}$ TILs, % | 20.7 (17.5; 40.4) | 27.0 (21.8; 45.1) | 39.2 (32.0; 58.2) | **$p = 0.031$** | $p_{1-2} > 0.999$<br>**$p_{1-3} = 0.027$**<br>$p_{2-3} = 0.386$ |

Notes: TILs—tumor-infiltrating lymphocytes; group 1—patients with sinonasal malignant tumors; group 2—patients with inverted papilloma; group 3—patients with polypous rhinosinusitis; Me (Q25; Q75)—median (lower quartile; upper quartile). Significant *p*-values are shown in bold.

involvement were associated with lower frequencies of $CD8^{+}\gamma IFN^{+}T$ cells [25].

In patients with IP, a decrease in intracellular γIFN production, in association with normal $CD3^{+}$TILs and extracellular γIFN level at the site of the tumor may indicate insufficient stimulation of effector reactions, mediated by type II interferon from antigen-presenting cells. This mechanism requires further detailed study at the level of induction and regulation mechanisms of interferon synthesis.

In addition to its autocrine effect on primary γIFN producing cells, this cytokine can also influence stromal cells in the inflammatory or tumor environment, such as macrophages, myeloid-derived suppressor cells, dendritic cells, and B cells. γIFN acts as a cytotoxic cytokine together with granzyme B and perforin initiating apoptosis in tumor cells, but, on the other hand, it can also promote the synthesis of PD-L1 and indoleamine 2,3-dioxygenase, thereby stimulating other immunosuppressive mechanisms [24–26].

One of the actively studied areas in cancer immunological research is assessment of expression of CD16 receptors on subpopulations of lymphoid cells. Low-affinity receptors type III of immunoglobulin G Fc-fragments (CD16, FCγR3) are predominantly expressed as CD16A protein (FCγR3A) on NK cells, and may be found on the membrane of monocytes, tissue-specific macrophages, γδT lymphocytes and dendritic cells. The presence of CD16A on TNK and γδT lymphocytes allows these cells to recognize tumor cells without MHC I, unlike T killers, and to carry out antibody-dependent cellular cytotoxicity due to the presence of the Fc receptor on their membrane. Evaluation of membrane CD16 expression is used to determine the population composition and their functional status in various diseases, including oncology [27,28]. In this regard, the expression of the CD16 receptor on subpopulations of lymphoid cells in the tumor tissue was assessed in patients with sinonasal tumors (Table 2).

Decreased $CD56^+CD16^+$NK cells among TILs in tumor tissue of patients with sinonasal malignancies were shown, as compared to patients with PRS ($p$ = 0.027). While there were no significant differences in CD16 expression on $CD3^+$TILs and $\gamma\delta TCR^+$TILs in group 1 (Table 2). The results indicate the failure of antibody-dependent cytotoxicity at the level of innate immune cells, contributing to the protumorigenic pathogenesis of neoplasms. Decreased antibody-dependent cellular cytotoxicity is likely to occur under the influence of inhibitory receptors in the tumor microenvironment.

To our knowledge, no studies have yet been published on the specific function of $CD16^+$T cells in sinonasal malignancies. However, Zöphel et al. have found that the percentage of $CD16^+$T cells (>1.6% $CD16^+$ T cells) is a protective predictor for the progression-free survival of patients with non-Hodgkin B cell lymphoma over a 24-month period [29]. Meanwhile, Lalos et al. reported that a high density of CD16-expressing TILs in recurrent ovarian cancer is associated with improved recurrence-free survival and overall survival [30].

***The expression of CTLA-4 and PD-1 on TILs in patients with sinonasal tumors.*** Immune checkpoint inhibitory molecules are a system of regulatory mechanisms acting through the activation of immunosuppressive signaling, resulting in the formation of peripheral tolerance and promoting the development of neoplasms [22,31]. The expression of most studied checkpoint inhibitory molecules—cytotoxic T lymphocyte associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1)—was investigated on TILs membrane in patients with sinonasal tumors. The results are presented in Table 3.

Increased CTLA-4 expression on $CD3^+$TILs was established in both groups of patients with sinonasal tumors, malignant ($p$ = 0.036) as well as benign ($p$ = 0.047), as compared to patients with PRS. Herewith, in group 1 changes occurred mainly due to CTLA-4 expression elevation on T helpers ($p$ = 0.049) and cytotoxic T cells ($p$ = 0.033) subpopulations, while in group 2—on T helpers ($p$ = 0.018) and γδT cells ($p$ = 0.021) subsets of TILs (Table 3). Moreover, the higher the percentage of CTLA-4-positive $CD3^+$TILs and $CD3^+CD4^+$TILs was detected in the tissue of patients with malignancies, the largest amount established in circulating peripheral blood T lymphocytes (rho = 0.48, $p$ = 0.047 and rho = 0.55, $p$ = 0.032, respectively). Increased CTLA-4 expression on $CD3^+CD4^+$TILs can presumably occur due to the pool of Treg, which regulate the anti-tumor immune response. The number of these cells was significantly higher among TILs in patients from Groups 1 and 2 compared to the control group (Figure 1g). According to literature data, CTLA-4 is a transmembrane glycoprotein that plays a key role in the development of peripheral tolerance to self-proteins by neutralizing the function of the co-stimulatory molecule CD28, while being a homologue of the latter one. Unlike CD28, CTLA-4 has a higher binding affinity for CD80/CD86 on antigen-presenting cells, so there is a competition for binding. The mechanisms of CTLA-4 inhibitory effect on T cells are still a subject of debate in the investigation of tumor pathogenesis. The extracellular mechanism of inhibition includes the secretion of a soluble form of CTLA-4, the production of indoleamine-2,3-dioxygenase, and the involvement of Treg in the immune response. The intracellular type of this effect involves competition with CD28 for binding to the ligand, blocking the expression of lipid rafts and the formation of an immunological synapse, as well as association with phosphatases. Moreover, CTLA-4-mediated trans-endocytosis can occur on the T cell surface, removing the ligand from the surface of the antigen-presenting cell [11,22,32]. Saini et al. have reviewed the majority of head and neck squamous cell carcinoma overexpressed actionable immunity genes, including CTLA-4, which are associated with poor prognosis [33]. Moreover, the exhausted T cells are characterized by CTLA-4 expression that negatively regulates their response, as along with reduced secretion of cytokines and cytolytic molecules [34].

There were no significant differences in the expression of the checkpoint inhibitory molecule PD-1 on $CD3^+$ TILs and their subsets across all patient groups (Table 3), which contradicts the findings of some authors [11,18,28]. PD-1 is a membrane protein of the immunoglobulin superfamily that negatively regulates T cell activation by inducing dephosphorylation and inactivation of the T cell kinase ZAP70, thereby reducing the production of inflammatory cytokines and cell survival proteins [35]. Along

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**Table 3:** PD-1- and CTLA-4-positive TILs subsets in patients with sinonasal tumors, Me (Q25; Q75).

| | TILs Subsets | Patients Groups | | | *p*-Value | |
|---|---|---|---|---|---|---|
| | | Group 1 | Group 2 | Group 3 | *Kruskal-Wallis Test* | *Dunn Post-Hoc Test* |
| CTLA-4$^+$ cells | $CD3^+CTLA\text{-}4^+$ TILs, % | 43.0 (29.2; 56.8) | 36.7 (34.2; 45.1) | 23.6 (17.2; 43.1) | **$p = 0.019$** | $p_{1\text{-}2} > 0.999$<br>**$p_{1\text{-}3} = 0.036$**<br>**$p_{2\text{-}3} = 0.047$** |
| | $CD4^+CTLA\text{-}4^+$ TILs, % | 55.0 (39.8; 68.1) | 55.4 (49.4; 62.2) | 34.2 (29.3; 51.2) | **$p = 0.013$** | $p_{1\text{-}2} > 0.999$<br>**$p_{1\text{-}3} = 0.049$**<br>**$p_{2\text{-}3} = 0.018$** |
| | $CD8^+CTLA\text{-}4^+$ TILs, % | 26.9 (15.1; 33.4) | 13.6 (7.8; 35.1) | 13.1 (7.9; 17.5) | **$p = 0.039$** | $p_{1\text{-}2} = 0.450$<br>**$p_{1\text{-}3} = 0.033$**<br>$p_{2\text{-}3} = 0.616$ |
| | $\gamma\delta TCR^+CTLA\text{-}4^+$ TILs, % | 44.3 (25.6; 59.2) | 53.7 (36.4; 70.3) | 31.2 (20.8; 44.9) | **$p = 0.023$** | $p_{1\text{-}2} = 0.332$<br>$p_{1\text{-}3} = 0.865$<br>**$p_{2\text{-}3} = 0.021$** |
| PD-1$^+$ cells | $CD3^+PD\text{-}1^+$ TILs, % | 80.4 (71.4; 85.8) | 70.3 (57.2; 87.8) | 77.2 (72.8; 82.7) | $p = 0.646$ | $p_{1\text{-}2} > 0.999$<br>$p_{1\text{-}3} > 0.999$<br>$p_{2\text{-}3} > 0.999$ |
| | $CD4^+PD\text{-}1^+$ TILs, % | 80.6 (60.6; 84.8) | 68.2 (58.5; 81.8) | 76.4 (66.1; 81.6) | $p = 0.701$ | $p_{1\text{-}2} > 0.999$<br>$p_{1\text{-}3} > 0.999$<br>$p_{2\text{-}3} > 0.999$ |
| | $CD8^+PD\text{-}1^+$ TILs, % | 75.9 (69.2; 85.8) | 77.8 (60.6; 85.1) | 80.9 (72.6; 86.3) | $p = 0.617$ | $p_{1\text{-}2} > 0.999$<br>$p_{1\text{-}3} > 0.999$<br>$p_{2\text{-}3} = 0.980$ |
| | $\gamma\delta TCR^+PD\text{-}1^+$ TILs, % | 71.4 (57.6; 87.6) | 66.4 (48.9; 87.2) | 80.2 (60.3; 83.9) | $p = 0.862$ | $p_{1\text{-}2} > 0.999$<br>$p_{1\text{-}3} > 0.999$<br>$p_{2\text{-}3} > 0.999$ |

Notes: TILs—tumor-infiltrating lymphocytes; PD-1—programmed cell death 1; CTLA-4—cytotoxic T-lymphocyte-associated protein 4; group 1—patients with sinonasal malignant tumors; group 2—patients with inverted papilloma; group 3—patients with polypous rhinosinusitis; Me (Q25; Q75)—median (lower quartile; upper quartile). Significant *p*-values are shown in bold.

with CTLA-4, PD-1 represents a promising target for the development of new therapeutic strategies for anticancer therapy, but its role in sinonasal malignancies is still controversial.

***The correlation of clinical and immunological parameters in patients with sinonasal tumors.*** To identify potential predictors for possible malignancy of the tumor, a correlation analysis of TILs number and their functional profile with patients' clinical parameters including smoking, bleeding, anosmia, ophthalmological symptoms, tumor size and extent (T) was performed. The results are presented in Figure 3.

In patients of group 1, the number of $CD3^+$TILs correlated with negative localization (R = 0.53; $p = 0.021$) and serum fibrinogen level (R = 0.56, $p = 0.035$), while the percentage of $CD19^+$TILs was inversely dependent on bleeding (R = −0.53; $p = 0.029$), anosmia (R = −0.56; $p = 0.011$) and negative localization (R = −0.45; $p = 0.047$). The established correlation of $CD3^+$TILs number with serum fibrinogen levels, as well as negative tumor localization in patients with malignant neoplasms, suggests a possible role of T cells functional profile in tumor progression. Fibrinogen may serve as a scaffold that facilitates the binding of tumor cells and platelets, thereby contributing to migration and promoting tumor angiogenesis.

## 4. Limitations of the Study

The present study is associated with some limitations. Firstly, this was a single-center retrospective study with a limited number of patients and a heterogeneous group of malignancies. On the other hand, this study was preliminary and paves the way towards further investigation, including prospective studies with a larger number of more homogeneous groups, and broader clinical sample validation that is necessary to confirm prognostic significance of immunological biomarkers. Secondly, the study did not assess the clinical importance of the association between TILs subsets and their localization in tumor tissue using hematoxylin-eosin stained sections. However,

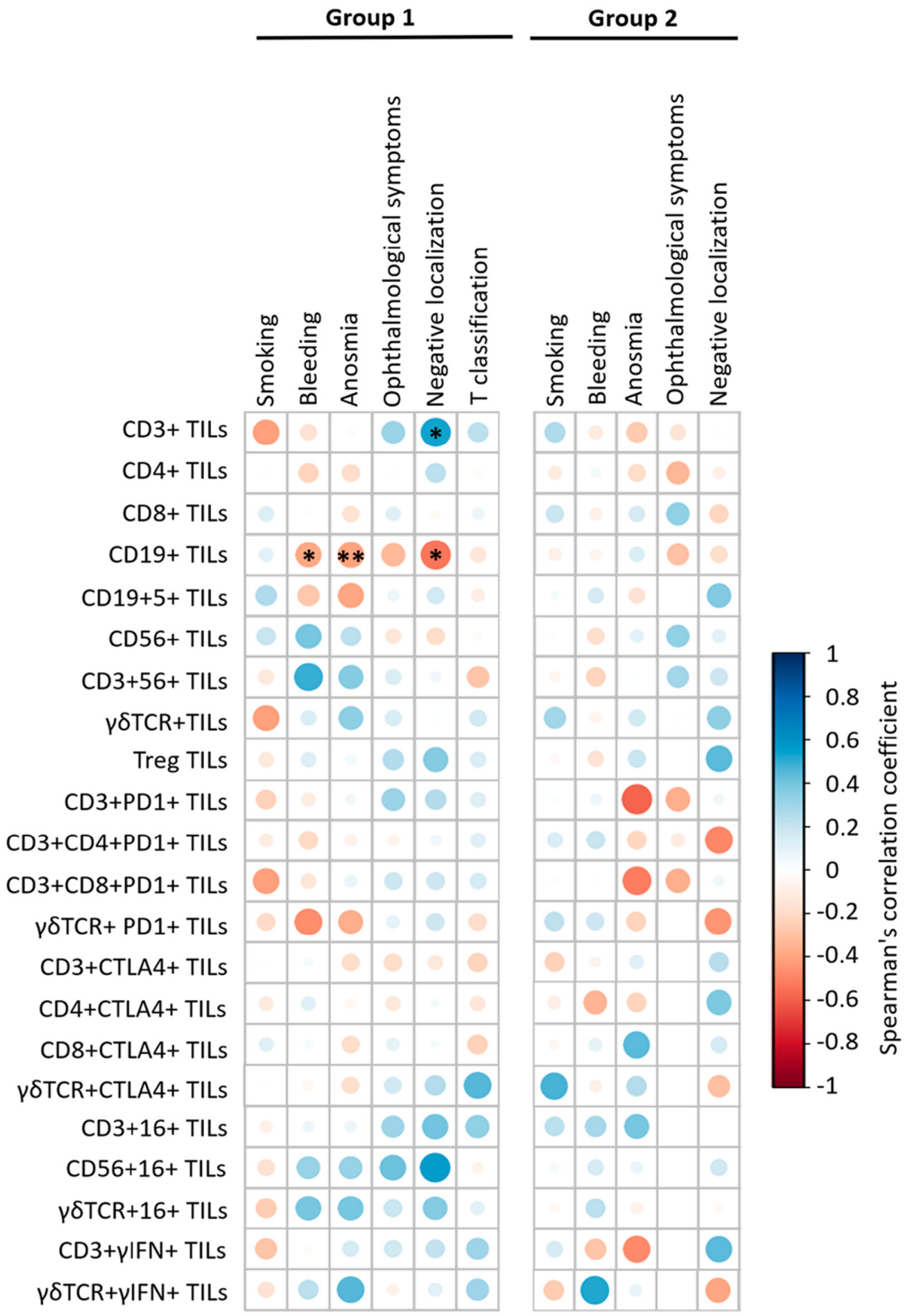


**Figure 3:** The correlation of clinical and morphological parameters in patients with malignant and benign tumors. Notes: positive correlations are displayed in blue and negative correlations in red color. Color intensity and the size of the circle are proportional to the correlation coefficients. Correlation results were considered significant at $p$-value: * $p$-value $< 0.05$, ** $p$-value $< 0.01$.

the International TILs Working Group does not currently recommend that immunohistochemistry be used to detect specific subpopulations. In the near future, this association will be clarified. Thirdly, many mechanisms for immunosuppressive tumor microenvironment are already known, but the study investigated only γIFN- and CD16-

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mediated TILs effector anti-tumor mechanisms, as well as role of checkpoint inhibitory molecules CTLA-4 and PD-1 in immunological tolerance formation. Therefore, further comprehensive research is needed to better understand cytotoxic mechanisms in sinonasal tumors, including TILs functions exhaustion, not only at the level of immune cells, but also tumor cells.

## 5. Conclusions

TILs play a central role in the surveillance of malignant and benign sinonasal neoplasms. However, currently the generally accepted approach to the immune status assessment focuses on the characterization of peripheral blood lymphoid cells, and the phenotypic and functional TILs characteristics are still not fully investigated. In this study, the involvement of various mechanisms of cellular cytotoxicity and tolerance formation to tumor antigens has been demonstrated in patients with benign and malignant sinonasal neoplasms.

The microenvironment tissue in patients with malignant sinonasal tumors is accompanied by decreased TILs cytotoxicity mechanisms, due to FcRγ (CD16) down regulation on NK cells and T lymphocytes, along with increased Treg and CTLA-4-positive $CD3^+$TILs. The cytokine profile of sinonasal malignancies is characterized by a significantly high level of local extracellular γIFN production, in combination with a pronounced decrease in the number of γIFN-producing T cells. This indicates the implementation of the effector γIFN-mediated potential by T cells in the focus of the pathological process, potentially leading to immune response failure as a result of cytotoxic T lymphocytes immunological depletion and a decreased reserve capacity of cellular immunity.

Decreased CD16 expression on NK cells and increased CTLA-4 expression only on T helper TILs subset have been established in IP patients. The detected significant decrease in both intracellular and extracellular γIFN production in the tumor tissue may indicate insufficient stimulation of γIFN-mediated effector reactions in patients with a benign neoplasm.

The application of tissue resident Treg, CTLA-4- and CD16-positive $CD3^+$TILs, as well as intracellular γIFN production may be considered as potential prognostic biomarkers for malignancy in sinonasal tumors and requires further investigation.

## Abbreviations

| | |
|---|---|
| CD | Cluster of Differentiation |
| CTLA-4 | Cytotoxic T Lymphocyte Associated Protein 4 |
| FasL | Fas Ligand |
| Fas | Fas Receptor |
| FOXP3 | Forkhead Box P3 |
| Fc | Crystallisable Fragment of Immunoglobulin |
| HLA | Human Leukocyte Antigen |
| HPV | Human Papillomavirus |
| γIFN | γ-Interferon |
| IgG | Immunoglobulin G |
| IP | Inverted Papilloma |
| MHC | Major Histocompatibility Complex |
| NK | Natural Killer |
| PD-1 | Programmed Cell Death 1 |
| PD-L1 | Programmed Cell Death Ligand 1 |
| PRS | Polypous Rhinosinusitis |
| TCR | T-Cell Receptor |
| TILs | Tumor-Infiltrating Lymphocytes |
| TNK | Natural Killer T Cells |
| TRAIL | Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand |
| Treg | Regulatory T Cells |
| ZAP-70 | Zeta-Chain-Associated Protein Kinase 70 |

## Author Contributions

M.Z., D.N. and Z.K. designed the study; D.N. and M.Z. drafted the manuscript; N.M., V.K. and Z.K. performed surgical procedures; D.N., E.N., G.I. and M.Z. carried out tissue samples proceeding and immunological experiments; D.N., E.N. and M.Z. analyzed and discussed the data and generated the figures and tables. All authors have read and agreed to the published version of the manuscript.

## Availability of Data and Materials

Data supporting the results of this study are available upon request from the corresponding author.

## Consent for Publication

Not applicable.

## Conflict of Interest

The authors declare that they have no conflicts of interest.

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## Ethics Committee Approval and Consent to Participate

The study was approved by the Ethics Committee of N. N. Alexandrov National Cancer Center of Belarus (protocol No. 17 dated 07.06.2022) and all patients provided written informed consent to participate in the study.

## Human Rights Statement

The study was performed following the Declaration of Helsinki (1975, revised in 2013).

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