Original Article

OBSERVATIONAL STUDY OF FLAGELLIN, IL-1B, IL-6, AND TLR5 IN FLOOR-OF-THE-MOUTH SQUAMOUS CELL CARCINOMA

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ABSTRACT

This study aims to identify the relationships that patients' poor oral hygiene, flagellin, IL-1β, IL-6, and toll-like receptor 5 (TLR5) have with the progression of floor-of-the-mouth squamous cell carcinoma (FOM SCC). We collected venous blood samples, unstimulated saliva samples, and biopsies from the epithelial layer of the floor-of-the-mouth mucosa. Venous blood and unstimulated saliva samples were analysed using the ELISA kit protocol, and concentrations of flagellin, IL-1β, and IL-6 were calculated. In an immunofluorescence study, the biopsies were used to calculate TLR5. The average flagellin concentration value in saliva in the FOM SCC group and in the control, group was not significantly different. The average IL-1β, IL-6 concentrations in the saliva of the FOM SCC group were significantly higher than those of the control group. In the control group, the amount of TLR5 at a distance of 80 μm was lower compared with the experimental group on the healthy side and on the lesion side. After detecting changes in flagellin, IL-1β, IL-6, and TLR5, we determined that bacteria in the mouth containing flagellin may be involved in the pathogenesis of floor-of-mouth cancer.

Key words: Flagellin, Interleukins, TLR5, Epithelial squamous cells carcinoma, Oral cancer, Risk factors.

Introduction

Globally, oral cancer represents 2–4% of all malignant neoplasms; however, in certain regions, this incidence rises to as much as 10% [1]. Existing literature identifies several primary risk factors for the disease, including genetic predisposition, tobacco use, alcohol consumption, and poor oral hygiene [2-4].

No clear link exists between poor oral hygiene and the pathogenesis mechanism of the development of oral squamous cell carcinoma [2, 3]. In cases of poor oral hygiene, build-up of soft and hard plaque creates a favorable habitat for anaerobic bacteria that cause periodontitis. These bacterial species include Treponema denticola, whose metabolic products cause destruction of surrounding tissues and even bone loss [5]. Flagellated bacteria have flagella consisting of a flagellin, which is a solid, spatially structured protein. Patients with poor oral hygiene habits have a heavy plaque accumulation and a significantly higher anaerobic microflora of flagella compared to patients who maintain adequate oral health and hygiene [6-12].

When flagella bacteria die, gingival crevicular fluid and saliva wash away the flagellin as well as other bacterial fragments, endotoxins, and exotoxins. This mixture then settles on the floor of the mouth and starts to affect the local epithelium cells [13]. Smoking, alcohol consumption, poor oral hygiene, bacterial endotoxins (including lipopolysaccharides), and exotoxins irritate the mucosal epithelium, causing chronic inflammation to begin and persist [14]. In a healthy epithelium, the cells containing toll-like receptors 5 (TLR5) are only located at the basal layer. When an inflammation persists, these cells proliferate and migrate to the upper epithelial layer, where they make contact with oral fluids and can connect flagellin of the dead bacteria to the TLR5 receptors found in the membrane [2]. As stated in the literature, the TLR5–flagellin complex has various effects on different types of cells. For example, after they bind to flagellin, immune cells containing TLR5 become active and encourage the destruction of damaged, unhealthy, slowly proliferating, and undifferentiated cells. Meanwhile, after binding with flagellin, the epithelial cells containing TLR5 are encouraged to secrete cytokines and chemokines. These signalling proteins can lead to unregulated cell division and the development of carcinoma cells, as well as their invasive migration into the surrounding tissues. This balance can be disrupted by protracted or recurring inflammation [2, 15-17].

Furthermore, other authors have found a significant link between TLR5 and the flagellin protein in the carcinogenesis of the stomach, salivary glands, and tongue [2, 3, 18, 19]. The flagellin protein binds directly to TLR5 in the epithelial cells and initiates transcription in the nucleus, leading to the synthesis of interleukins 1β and 6 (IL-1β and IL-6), which have been found to significantly influence carcinogenesis [3, 6].

This study aims to identify the relationship between floor-of-mouth squamous cell carcinoma (FOM SCC) and patients' oral hygiene, particularly investigating their levels

of flagellin, TLR5, IL-1β, and IL-6.

Materials and Methods

Patients and study design

Experimental groups

The Lithuanian University of Health Science's (LUHS) Bioethics Center's permission (No. BEC–OF–04) was obtained for this study. The study involved 45 patients who were hospitalised in the Facial and Maxillofacial Surgery Department of the Hospital of LUHS.

The inclusion criteria for the experimental group were as follows:

diagnosis of primary tumors in the floor of the mouth or sublingual mucosa, no previous history of malignancy before diagnosis of FOM SCC, completed histologic examination for squamous cell carcinoma, and no radiation or chemotherapy before surgery [20-25].

The inclusion criteria for the control group were as follows:

older than 40 years, no history of malignancy, hospitalised for cysts, neuralgia, benign structures, or fractures of a facial bone (lower jaw, cheekbone, or zygomaticomaxillary complex).

Patients with facial and maxillary abscesses or osteomyelitis were excluded from the control group.

Patients voluntarily agreed to participate in the experimental study, providing written confirmation of informed consent. All 45 patients were interviewed and then completed questionnaires.

Questionnaire

A 15-item survey was developed to gather demographic data and assess potential risk factors contributing to the development of floor-of-mouth squamous cell carcinoma (FOM SCC). Evaluated variables encompassed lifestyle habits, such as daily cigarette usage, alcohol intake—quantified against World Health Organization benchmarks—and oral hygiene standards measured via the Silness-Loe Plaque Index. Additionally, the questionnaire captured pertinent social and demographic details, including the participants' age, gender, educational background, and residential location.

The dental formula of each patient was recorded in writing, and the number of teeth remaining in their mouths was counted.

Saliva and blood sample collection

Unstimulated saliva was collected from patients between 7:00 and 7:30 a.m. Immediately after waking up, while fasting, and without having cleaned their teeth, patients had to spit saliva into a sterile cup in a sitting position. The saliva-sample collection process took 15–20 minutes. The volume of saliva samples was 4–7 ml. According to the literature, flagellin is a soluble protein [26]. On the same day of the collection, the saliva was centrifuged at 6,800 rpm in a Thermo Scientific IEC FL40R centrifuge (Thermo Fisher Scientific, USA) and cooled to 4°C for 10 minutes. Using this method, saliva fluid was separated from the sediment that remained on the bottom of the tube after centrifugation. The biological fluid was separated from the sediment and placed in 1.5 ml tubes. 1.5 µl of proteinase inhibitor (Sigma Aldrich, USA) was added, and a test number was placed on the tube. The prepared samples were immediately frozen at −80°C and stored until the day of testing.

Venous blood was collected from all patients into vacutainers with a red cap. Fifteen to 20 minutes after the collection, the samples were centrifuged at 1,500 rpm, cooled to 4°C for 10 minutes. The obtained serum was separated from erythrocytes and transferred into 1.5 ml sterile tubes, frozen at −80° C, and stored until the day of testing.

Determination of flagellin, IL-1β, and IL-6 in blood and saliva samples

The 96-well ELISA kit (sensitivity range 0.1–10 ng/ml) for detecting bacterial flagellin in human samples was used (Catalogue No. ABX051553) to determine the concentration of flagellin in human saliva. In addition, the 96-well IL-1β ELISA kit (Catalogue No. KAC1211) and the 96-well IL-6 ELISA kit (Catalogue No. KHC0061) were used.

All collected saliva and blood samples were thawed using the ELISA kit protocol, and both groups' samples and standard solutions were dispensed into the wells. After the wells were filled and the reaction occurred, light absorption was measured at 450 nm. The concentrations of flagellin, IL-1β, and IL-6 (pg/ml) in each patient's sample were calculated based on the calibration curves.

Immunofluorescence

Biopsies of the oral mucosa of the floor of the mouth were taken from both groups. Two biopsy samples were taken from the experimental group (i.e., one sample of clinically healthy oral mucosa and one sample of lesions of the oral mucosa), and one sample was taken from the floor-of-the-mouth mucosa of the control group. Samples were fixed in 10% formalin solution, and after preparation, they were fixed in wax. To ensure that samples had all epithelial layers clearly visible, they were incised, stained with haematoxylin, and examined under a microscope. The following antibodies were used: 100 µg each of primary rabbit anti-TLR5 pAb (C-terminal region) from Thermo Fisher Scientific (catalogue number PA1-41139, identified as the most plausible match based on vendor listings) and secondary donkey anti-rabbit IgG (H+L), Alexa Fluor 488 conjugate (Thermo Fisher Scientific, USA), and 0.5 ml coating fluid (4′,6-diamidino-2-phenylindole) (Thermo

Fisher Scientific, USA). Nuclei were counterstained with DAPI. Samples were deparaffinised, and immunofluorescence was performed. Photographs of the preparations were taken using an ApoTome 2 fluorescence microscope (ZEISS, Deutschland) and the Axiovision program. ImageJ 1.52a software was used for calculating concentrations of positive fluorescent cells, indicating TLR5. Because it is known that the average thickness of the oral epithelium is 124.09 μm (SD 13.53) [27], we decided to form squares and calculate the quantity of TLR5 in the floor-of-the-mouth epithelium within these squares, at average distances of 40 μm and 80 μm from the base membrane, including the sides of the square. Calculations of 10 different, randomly assigned sites were made for the oral mucosa of each healthy subject and the squamous cell carcinoma groups of both healthy and affected sides. The investigator was unaware of which sample belonged to which group during calculations. Interference from measurement was eliminated manually with the help of threshold adjustments in ImageJ.

Statistics

Statistical analysis of the survey responses and biomarker data (flagellin, IL-1β, IL-6, and TLR5) was conducted using SPSS. Relationships between categorical variables were assessed via the chi-square test, while differences between groups were determined using the student's t-test. Furthermore, TLR5 concentrations were analysed utilising both Kruskal-Wallis and Mann-Whitney tests.

Results and Discussion

Study groups analysis

The study involved 45 patients who were hospitalised in the Facial and Maxillofacial Surgery Department of the Hospital of LUHS.

In the experimental group, 81.3% were current smokers compared to 55.2% of the control group. Moreover, 81.3% of the experimental group used alcohol, whereas 58.6% of the control group used it. In addition, we found that 56.3% of experimental group patients brushed their teeth less than once a day compared to 17.2% of the control group. In the experimental group, the patients had an average of 10.88 ± 2.05 teeth, whereas in the control group, the average was 18.66 ± 3.25 teeth. All study group analyses are presented in **Table 1**.

Table 1. Questionnaire data from the experimental and control groups

Patient characteristics	Experimental group	Control group
Patients (*n*)	16	29
Age (years)	57.81±11.94	57.29±12.05
Mean	57.81 (SD 11.94)	57.29 (SD 12.05)
Min	44	40
Max	82	78
Gender		
Female	5	15
Male	11	14
Nicotine use (%)		
Nonsmokers	18.7	44.8
Smokers	81.3	55.2
Alcohol use (%)		
Do not use alcohol	18.7	41.4
Use alcohol	81.3	58.6
Education degree (%)		
School education	87.5	72.4
Higher education	12.5	27.6
Lives in (%)		
City	62.5	27.6
Village	37.5	72.4
Teeth brushing (%)		
Once a day	43.7	82.8
Less often	56.3	17.2
Uses oral hygiene products (%)		
Mouthwash	18.7	24.1
Floss/interdental brush	12.5	17.2

Do you go to the dentist regularly? (%)			
Yes	6.2	68.7	
No	93.8	31.3	
Silness-Loe plaque index	2.31	1.53	$P < 0.05$
Teeth quantity	10.88 (SD 2.05)	18.66 (SD 3.25)	$P < 0.05$

No stage I tumors were found in the FOM SCC patients in the experimental group, but stage II was diagnosed for 17.65%, stage III for 50.75%, and stage IV for 31.60% of patients.

Flagellin concentration in blood and saliva

In the experimental group, the average flagellin concentration value in saliva was 0.311 ng/ml (SD 0.128), or 2 pg/mg of saliva proteins. In the control group, the average value was 0.3565 ng/ml (SD 0.162), or 3.3 pg/mg of saliva proteins. The results were not significantly different between the two groups.

IL-1β and IL-6 concentration in blood and saliva

Using the ELISA method, no flagellin was found in the blood serum in either of the groups. In the experimental group, the average IL-1β concentration in the saliva was significantly higher than in the control.

group. Also, the concentration of IL-6 in the saliva and blood serum of the experimental group was significantly higher than it was in the control group. Analyses of flagellin, IL-1β, and IL-6 concentrations in blood and saliva from the experimental and control groups are presented in **Table 2**.

Table 2. Analyses of flagellin, IL-1β, and IL-6 concentration in blood and saliva from the experimental and control groups

	Flagellin		**IL-1β**		**IL-6**	
	Blood serum	Saliva	Blood serum	Saliva	Blood serum	Saliva
Experimental group	0	0.311 ng/ml (SD 0.128), or 2 pg/mg	4.275 pg/ml (SD 0.893)	1297.9 pg/ml (SD 216.5) *	7.289 pg/ml (SD 0.976) *	353.27 pg/ml (SD 45.275) *
Control group	0	0.3565 ng/ml (SD 0.162), or 3.3 pg/mg	3.449 pg/ml (SD 0.847)	580.42 pg/ml (SD 96.1) *	4.914 pg/ml (SD 1.254) *	210.518 pg/ml (SD 56.384) *

*P < 0.05

An analysis of interleukin levels within the experimental cohort revealed that saliva contained markedly higher concentrations of both IL-1β and IL-6 compared to blood serum, a difference that reached statistical significance (P < 0.05, **Figure 1**).

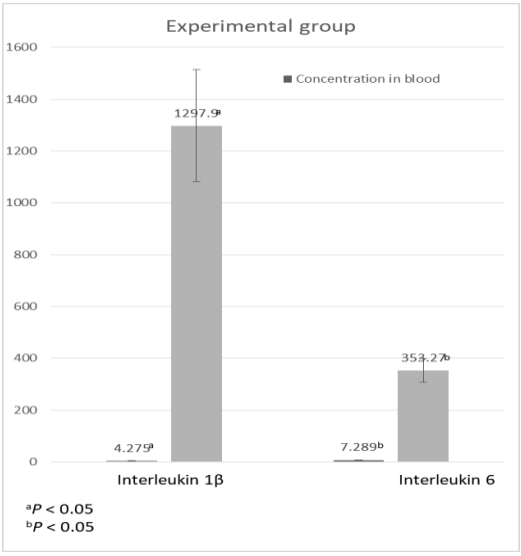


Figure 1. The experimental group's IL-1β and IL-6 concentration comparison in the blood serum and saliva

TLR5

In an immunofluorescence study, the epithelial layer of the floor-of-the-mouth mucosa was examined to assess changes in the quantity and distribution of TLR5 **(Figure 2)**. For the immunofluorescence study of the experimental group, there were 32 biopsies (16 biopsies of the healthy area and 16 biopsies of the altered mucosa), and for the control group, there were 16 biopsies.

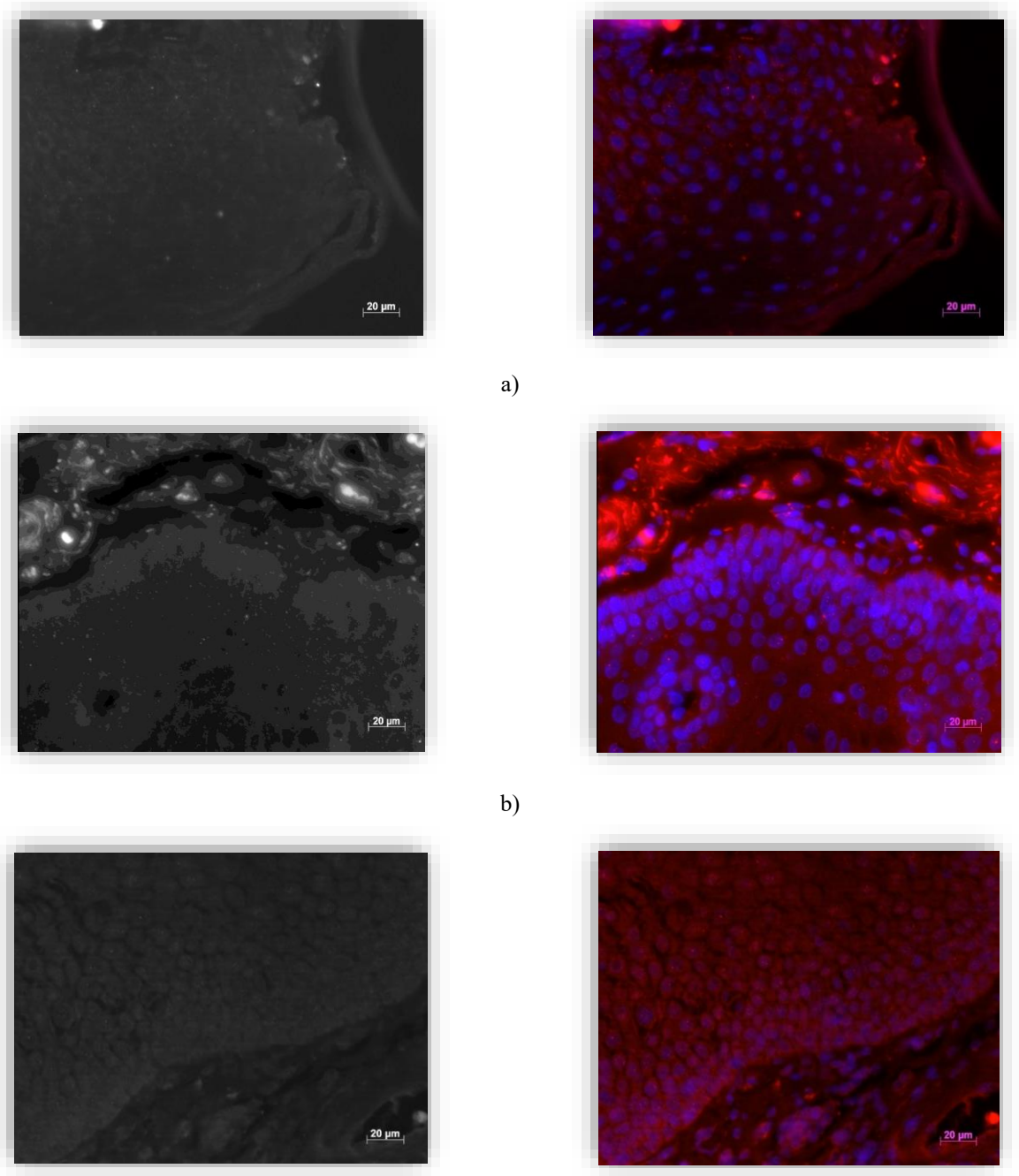
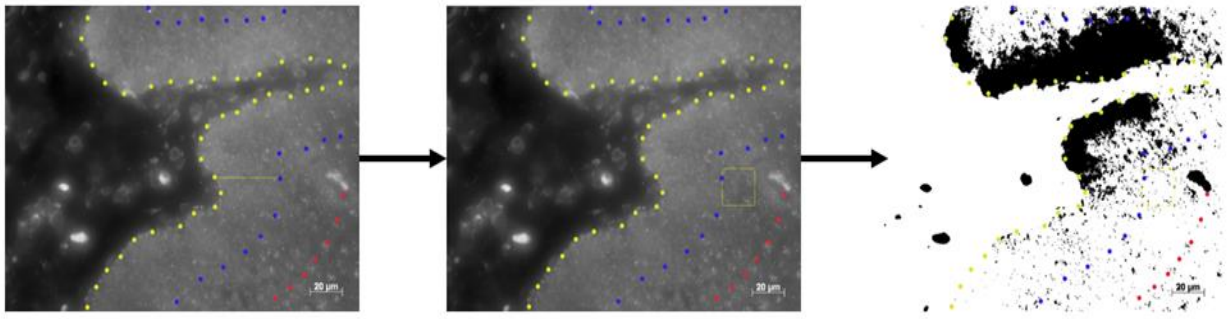


Figure 2. Immunofluorescence of the epithelial layer of the floor of the mouth mucosa near the basement membrane. The images in A show a sample of the control group biopsy. The images in B show a sample of the healthy area of the experimental group biopsy. The images in C show a sample of the lesion area of the experimental group biopsy. Nuclei were counterstained with DAPI (blue). The Alexa Fluor 488 channel was pseudocolored in red for visualization clarity (despite its native green emission).

Measurements were performed at 40 µm and 80 µm from the basement membrane boundary towards the surface of the epithelium **(Figure 3)**.



a) b) c)

Figure 3. Quantification of TLR5 in epithelial layers. Raw fluorescence image with the basement membrane (yellow dashed line) indicated. Semi-transparent shading highlights the regions measured at 40 µm above the basement membrane (blue) and at 80 µm (red) above the basement membrane. (a) yellow line measuring 40 µm in length, drawn from above the basement membrane. (b) Representative 20 µm × 20 µm regions of interest (ROIs) used for quantification are shown as yellow squares. (c) Binary mask of TLR5-positive signal after background subtraction and thresholding (isotype + 3 SD). Quantification was performed as $TLR5^+$ area fraction and $TLR5^+$ cells per 100 $DAPI^+$ nuclei within each layer.

Measurements were made on 10 randomly selected ROIs per sample under blinded conditions.

In the final analysis, we evaluated 10 biopsies from the healthy areas and 10 biopsies from the altered mucosa of the experimental group, as well as 11 biopsies from the control group. A total of 480 photos were taken during the study.

Other preparations used for immunofluorescence examination could not be evaluated due to a failed reaction, the absence of an epithelial layer in the preparation, or excessive disintegration, mostly in the preparations of the experimental group.

In the control group, the amount of TLR5 at a distance of 40 µm from the basement membrane boundary was statistically lower at 32.4 (SD 9.09) compared to both biopsies of the experimental group. In the healthy mucosa, the average was 42.84 (SD 3.89), and in the lesion, the average was 44.1 (SD 4.58) ($P < 0.05$ and $P < 0.01$, comparing the control to the experimental healthy and the experimental lesion, respectively).

Within the control group, TLR5 expression levels measured 80 µm from the basement membrane boundary were significantly reduced at 13.66 (SD 2.6) compared to both biopsy types in the experimental set. Specifically, the healthy mucosa averaged 27.63 (SD 4.7), while the lesion site averaged 46.88 (SD 8.13), with statistical significance noted at $P < 0.05$ and $P < 0.01$, respectively. Furthermore, the density of modified mucosal receptors was markedly higher than that observed in the healthy mucosal samples of the experimental group. Although some sections, particularly those in the lesion group (Panel C), exhibited prominent nuclear-localized fluorescence, these findings are viewed with skepticism; given that TLR5 is typically a membrane-bound protein, such signals may be indicative of fixation artifacts or nonspecific binding. Consequently, all formal quantification was restricted exclusively to patterns of cytoplasmic and membrane-bound fluorescence.

In both the control group and the clinically healthy mucosal tissues of the experimental group, TLR5 expression showed a statistically significant decline ($P < 0.05$) as the distance from the basal membrane toward the epithelial surface increased. Conversely, the lesion mucosa of the experimental group exhibited an opposing trend, with receptor density rising as measurements moved closer to the epithelial surface **(Figure 4)**.

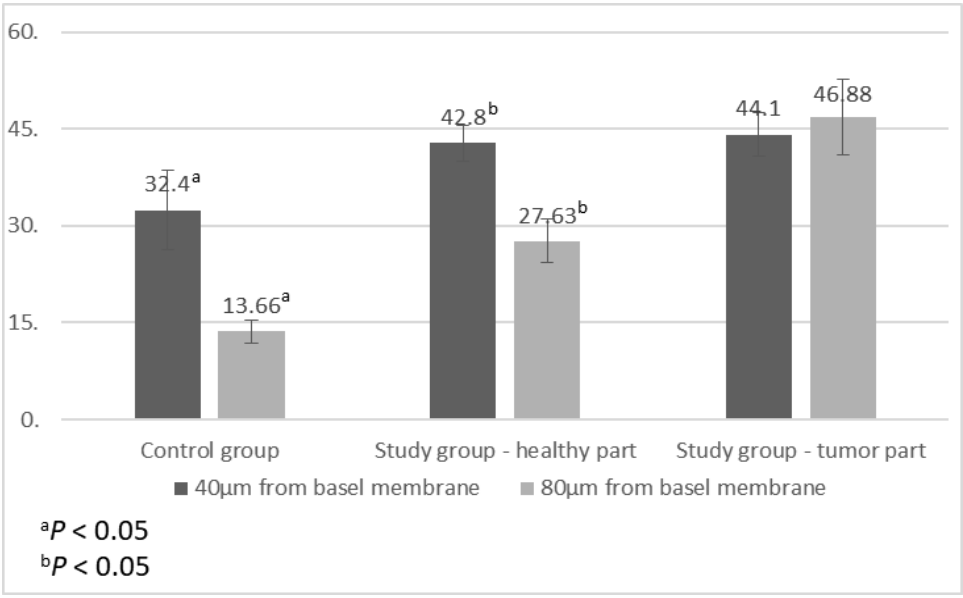


Figure 4. Comparison of TLR5 quantities measured at 40 µm and 80 µm from the basement membrane boundary

The aim of this study was to identify the relationship between FOM SCC and patients' oral hygiene, particularly investigating their levels of flagellin, TLR5, IL-1β, and IL-6.

As mentioned before, a significant link between TLR5 and the flagellin protein in the carcinogenesis of the stomach, salivary glands, and tongue is already known [2, 3, 6]. Our study showed that patients with FOM SCC have significantly more IL-1β in saliva and IL-6 in saliva and blood serum compared with the control group, but it doesn't show a connection with carcinogenesis. After performing an immunofluorescence study, it was statistically significantly determined that the amount of TLR5 at 80 µm from the basement membrane boundary towards the surface of the epithelium is higher in the experimental group.

During our research, it was found that patients with FOM SCC consume alcohol (81.3%) and smoke (81.3%). Found in alcoholic beverages, ethanol is classified as carcinogenic to humans and can promote the development of this pathology [28]. The risk of head and neck cancers increases with the use of tobacco or alcohol [29]. Cordeiro-Silva *et al*. [30] assessed that a high proportion of patients with SCC consume alcohol (63%), smoke (75%), or do both (57%). 1. Additionally, elevated catecholamine levels in oral cancer patients have been associated with impaired quality-of-life domains, suggesting that stress-related neuroendocrine factors may also contribute to disease progression and patient outcomes [31].

In this study, we found that patients with FOM SCC were less likely to have regularly scheduled dental appointments before FOM SCC was diagnosed ($P < 0.05$). Moreover, 56.3% of patients do not brush their teeth or brush them less than once per day [32, 33]. This is in line with the findings of Konduru *et al*. [34], who showed that 43.5% of patients with oral cancer brushed their teeth less than once per day ($P = 0.522$), whereas 47% of patients did not have annual dental check-ups ($P = 0.568$).

The experimental group's poor oral hygiene indicated a high Silness-Loe Plaque Index score ($P < 0.05$) with a mean of 2.31 and fewer remaining teeth ($M = 10.88$, SD 7.92; $P < 0.05$). Guha *et al*. [35] determined that patients with oral, laryngeal, or oesophageal cancer and poor oral hygiene have fewer teeth in their mouths compared to healthy patients. One limitation of our study is that the periodontal status of patients was not assessed; only oral hygiene status. Therefore, the elevated IL-6 and IL-1β levels observed in the FOM SCC group may be influenced by underlying periodontal disease rather than carcinoma alone. Future studies should control for or stratify by periodontal status to better clarify this relationship

The advanced stage at which floor of the mouth squamous cell carcinoma (FOM SCC) is typically identified serves as further evidence of compromised oral hygiene among affected patients. Research indicates that approximately 82.35% of these tumors are detected in their late stages (III or IV). This trend is consistent with findings from Mahmood *et al*. [36] in Pakistan, where 69.6% of oral cancer diagnoses were documented at the advanced third stage [37-44].

It has been established that TLR5 is important in regulating immune reactions [45]. Chantratita *et al*. [46] have shown that the relationship between TLR5 and flagellin affects the levels of interleukins in the blood. According to Song *et al*. [47], toll-like receptors have a significant effect on the development process of tumors. Moreover, recent evidence shows that TLR5 is a ubiquitously expressed extracellular receptor for bacterial flagellin, present in many tissues and

immune cells such as macrophages and T cells, and it triggers inflammatory responses primarily through the MyD88-dependent pathway, leading to NF-κB activation and the release of cytokines including IL-1β and IL-6. Alecu *et al.*'s [48] patients with scleroderma had increased quantities of interleukins in the blood compared to the control group. However, these blood interleukin levels remained within the normal range. During this research, we observed a significant increase of IL-1β and IL-6 quantities in the saliva of patients diagnosed with FOM SCC, which likely contributed to the development of their lesions [49]. Interleukin quantities increased locally and not systemically in the saliva, with the concentration 10 times lower in the blood serum compared to the saliva.

Unexpectedly, the concentration of flagellin in saliva, which depends on the presence of bacteria with flagella, was lower in the experimental group compared to the control group. We believe that the decreased flagellin concentration in the experimental group was caused by its usage as an active component of TLR5 [50, 51].

When using the immunofluorescence method, it was observed that TLR5 migrated towards outer epithelial cells in the experimental group more than it did in the control group. Moreover, a significantly higher quantity of TLR5 was found 80 μm from the basement membrane in the experimental group compared to the control group. This may have led to the observed lower flagellin concentration in the saliva, because flagellin binds to TLR5. Previous studies have also identified the increased quantity of TLR5 and its migration towards the outer layer in gastric cancer [6, 52, 53]. Interestingly, TLR5 changes are noticeable in patients with gastritis, with an increased concentration of TLR5 at the basement membrane (basolateral pole) [54]. However, the relatively small sample size (45 patients) and the reduction in effective statistical power due to subgroup analyses (for example, stage distribution and biopsy samples lost to processing) should be acknowledged as limitations of this study. After flagellin has bound to TLR5 in epithelial cells, changes in transcription and translation occur, leading to increased quantities of IL-1β and IL-6. This contributes to carcinogenesis and invasiveness of the cells **(Figure 5)** [55]. Moreover, studies have shown that molecular changes such as altered expression of p53, p21^CIP1/WAF1^, and eIF4E occur in tissues adjacent to oral SCC, suggesting a progressive molecular transformation at the tumor margins that may drive carcinogenesis [56]. This manuscript is an extended version of our previously presented conference paper, incorporating additional experimental data, more detailed analyses, and an expanded discussion of the results [57].

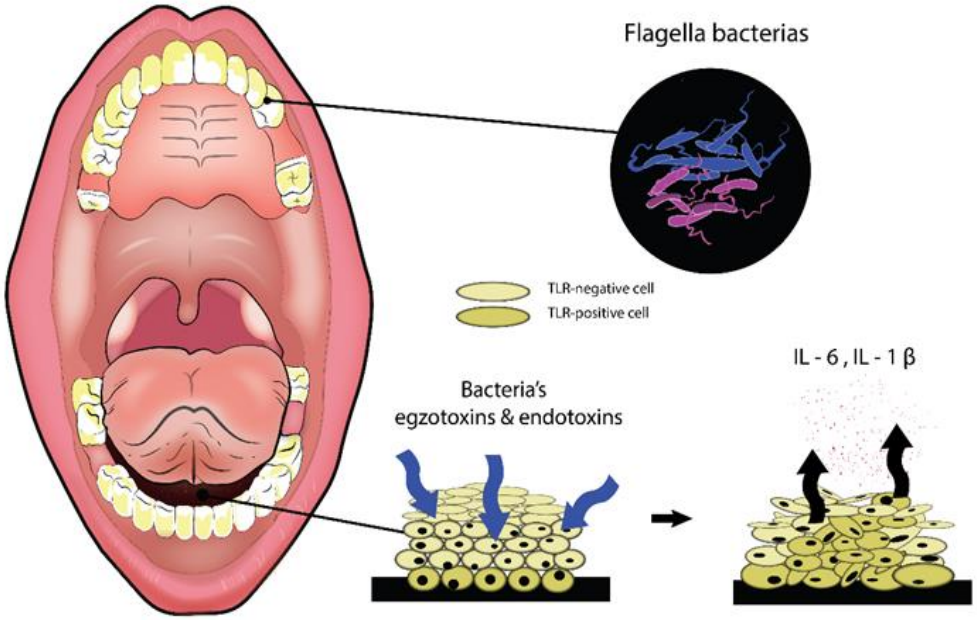


Figure 5. Millions of bacteria with the flagellin protein exist on the floor of the mouth. TLR5 is only located at the basal layer, but due to high concentrations of the flagellin of the dead bacteria, TLR5 expression increases and redistributes from the basal to the upper epithelial layers in response to bacterial flagellin. TLR5 initiates transcription in the nucleus, followed by translation in the cytoplasm via the proteins found there. As a result, IL-1β and IL-6 are synthesized.

Conclusion

This study identified alterations in flagellin, TLR5, IL-1β, and IL-6 in patients with floor-of-the-mouth squamous cell carcinoma. However, given the cross-sectional nature of this study, no causal relationships can be inferred. Changes in

the localization of flagellin, IL-1β, IL-6, and TLR5 highlight the need for larger-scale studies to further explore these associations and to develop potential methodologies for the early diagnosis of floor-of-the-mouth cancer.

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Conflict of interest: None

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Ethics statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Lithuanian University of Health Sciences' (LUHS) Bioethics Center (No. BEC–OF–04). Informed consent was obtained from all subjects involved in the study.

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