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Original Paper

EXPRESSION LEVELS OF LOCAL PROTECTIVE FACTORS IN ORAL MUCOSA: A COMPARATIVE PILOT STUDY BETWEEN HEALTHY PATIENTS AND CHRONIC PERIODONTITIS PATIENTS

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This study examined the expression of local protective factors — Gal-10, hBD-3, hBD-2, LL-37, and CD163 — in the oral mucosa of chronic periodontitis patients compared to healthy patients. Seven chronic periodontitis patients and five control group patients of Latvian nationality were included, all meeting the selection criteria. Tissue samples underwent haematoxylin and eosin staining and immunohistochemical analysis. Results were analysed semi-quantitatively with Mann–Whitney U test and Spearman's correlation. Routine staining showed moderate to significant infiltration, without intergroup differences. Controls displayed low-moderate Gal-10, hBD-3, hBD-2 expression, while LL-37 and CD-163 were rare. Chronic periodontitis samples showed few Gal-10, hBD-3, hBD-2, LL-37 positive cells, with CD-163 only occasionally present. No significant differences were found via Mann–Whitney U test. Spearmen's rank correlation revealed significant correlations: in the control group — between hBD-2 and LL-37 (epithelium), hBD-3 (connective tissue) and CD-136 (macrophages), and LL-37 (epithelium) along with GAL-10 (connective tissue). In chronic periodontitis — one strong correlation between HBD-2 (connective tissue) and HBD-3 (epithelium). Similar local protective factors expression between chronic periodontitis patients and controls suggest comparable local immune status in the Latvians. These results highlight the complexity of oral immunity and the need for further studies on localised immune components in periodontitis.

Keywords: *chronic periodontitis, local protective factors, gingival tissue, inflammation.*

INTRODUCTION

Oral diseases are one of the world's most important problems. The state of the oral health status depends on a number of factors including hygiene practices, behaviours, and economic conditions (Desai and Nair, 2023). Typically, oral and periodontal diseases, as well as other inflammatory conditions affecting the tissues supporting teeth, result from an imbalance between the body's immune responses and environmental factors like smoking, inadequate nutrition, and elevated levels of periodontopathogenic bacteria (Isola, 2020).

Chronic periodontitis is most prevalent in adults but can occur in children and adolescents. It is an infectious disease resulting in inflammation within the supporting structures of teeth, progressive attachment, and bone loss. It is characterised by pocket formation and/or gingival recession. It is recognised as the most frequently occurring form of periodontitis. The prevalence and severity of the disease increases with age (Highfield, 2009).

The aetiology of periodontitis is multifactorial and is associated with the accumulation of dental plaque. Periodontitis affects select teeth or tooth surfaces, and rarely the entire dentition, and may approach the apex of one tooth while barely involving a neighbouring tooth sharing the same interdental space (Slots, 2017). Mostly the inflammatory process can cause progressive breakdown of the teeth-sup-

porting structures, including the periodontal ligament and alveolar bone. Disease pathogenesis involves complex dynamic interactions among specific periodontal pathogens, which produce harmful by-products and enzymes that break extracellular matrices, and destructive host immune response plus environmental factors like example smoking (Jain *et al*., 2008; Kwon *et al*., 2020).

The immune system is essential for survival and has evolved to defend against pathogens, as well as to maintain homeostasis within an organism, so there is production of over 40 antimicrobial peptides and proteins in the oral cavity (Bechinger and Gorr, 2016; Ebersole *et al*., 2016). Periodontal immunity includes overlapping innate and adaptive cellular and humoral responses, which, together with indigenous oral microbes, aim at controlling invading pathogens (Slots, 2017).

Current research indicates that alpha-defensins, beta-defensins, LL-37, and various other antimicrobial peptides and proteins play unique yet overlapping functions in maintaining oral health and inhibiting the adherence and infection of different microorganisms (Komatsuzawa *et al*., 2007).

This raises the question of how and what types of antimicrobial peptides are observed in patients with chronic periodontitis and how they differ from the healthy tissue, which would help to better understand the local immune response in cases of the disease.

LL-37 plays a protective role in maintaining oral health stability, which can be impaired by direct (e.g., enzymatic degradation by bacterial proteases) and indirect factors related to dysbiotic conditions (Tokajuk *et al*., 2022). LL-37 is an antimicrobial peptide with powerful skills to kill different microorganisms and to prevent the immunostimulatory effects of bacterial wall molecules like lipopolysaccharide (LPS), thereby LL-37 offers protection against lethal endotoxemia. In addition, LL-37 exhibits chemoattractant functions, which inhibit neutrophil apoptosis, and support angiogenesis, tissue regeneration, and cytokine release (Bucki *et al*., 2010). Also, LL-37 neutralises the activation of macrophages by blocking LPS from and other bacterial product-induced production of inflammatory cytokines (Scott *et al*., 2002). LL-37 is expressed in various type of cells like skin cells, epithelial cells of the intestine, respiratory system, genitals, ocular surface, differentiated surface and upper crypt epithelial cells in the colon, Brunner's glands in the duodenum and in eccrine glands and in the oral cavity (Bandurska *et al*., 2015). In relation to oral health, the LL-37 expression in the oral cavity is an important factor in oral homeostasis by supporting the physiological microbiota, while also involved in the development of oral dysbiosis, infectious diseases (including viral, bacterial, and fungal infections), autoimmune diseases, and oral carcinomas. Additionally, LL-37 has been suggested to be a marker of inflammation severity and treatment outcome (Tokajuk *et al*., 2022). However, nothing is known about LL-37 appearance in periodontitis.

In gingival cervical fluid of patients with periodontitis, Galectin-10 (GAL-10) is one of the most upregulated proteins (Kim *et al*., 2020). Galectin-10 belongs to a group of proteins known as lectins. Galectin-10 is found in humans mainly in eosinophils, basophils, and macrophages (Su, 2018). The present study suggests that GAL-10 could be a potential biomarker for periodontitis (Kim *et al*., 2020).

There are two different activated forms of macrophage: M1 that are classical activated macrophages and alternatively activated macrophages — M2. They play a role in late inflammatory response, where they remove debris, promote angiogenesis, tissue remodelling and repair, and act as Th2 cells of adaptive immune response (De Carli *et al*., 2015). CD163 is often used as a M2 marker and to find out if there is an inflammatory process in the tissue. CD163 is a haemoglobin scavenger receptor, whose high expression in macrophages indicates that the tissue is responding to inflammation, where CD163+ macrophages stimulate anti-inflammatory cytokines expression and produce anti-inflammatory haeme metabolites (Skytthe *et al*., 2020). There are also findings showing that the reduced tissue levels of CD163 are related to periodontitis, which might be due to its distinct characteristics (Yilmaz *et al*., 2022).

Human β-defensins (hBDs) belong to the group of antimicrobial peptides. Normal human oral epithelia have spinosum and granulosum layers that co-express hBD-1 and -2, while the basal layers of the epithelium create hBD-3 from mitotically active cells (Bissell *et al*., 2004; Li *et al*., 2016). In the oral cavity hBDs can be detected in soft tissues like gingiva, tongue, and oral mucosa and in oral fluids — saliva and gingival crevicular fluid. However, the undifferentiated junctional epithelium, where constant bacterial challenges and inflammatory responses occur, does not express hBDs (Atalay *et al*., 2024).

Human beta-defensin 2 is a vital protein that plays a key role in innate immune response that provides protection for the human organism against invading pathogens of bacterial, viral, fungal, and parasitical origin. hBD-2 are locally produced by the keratinocytes of various tissues, especially skin and mucosa and some have shown expression of these proteins in normal uninflamed tissues, which could be due to constant exposure of oral epithelium to microbial pathogens (Sidharthan *et al*., 2020). However other studies demonstrate that gingival biopsies in healthy individuals lack signs of inflammation, and that gingival tissue secretes hBD-2 at a very low level (Cieślik *et al*., 2021). However, the data about hBD-2 in periodontitis are unclear.

hBD-3 demonstrates the most potent antimicrobial activity among all hBDs (Sidharthan *et al*., 2020). Human beta-defensin 3 (hBD-3) is normally expressed in highly proliferating epithelial cells. It plays a role in normal wound healing, angiogenesis and induces activation of epidermal growth factor receptor (EGFR). Also, hBD-3 showed the strongest stimulatory effect on human fibroblasts — promotes its migration and proliferation (Takahashi *et al*., 2021; Ghosh *et al*., 2024). Also, decreased expression of

hBD-3 in the inflamed gingival tissues may result from the consumption of hBD-3 against chronic periodontitis in the immediate environment. The hBD-3 basal expression in periodontitis patient keratinocytes raised the possibility that hBD-3 is crucial for the immune response against periodontitis (Liu *et al*., 2013).

It is undeniable that the presence of gingival tissue protective factors such as Gal-10, hBD-3, hBD-2, LL-37 and (M2-anti-inflammatory) receptor CD163 is associated with the development of the inflammatory process. Therefore, the aim of the present study was to investigate the determination of local protective factors in periodontitis-affected oral mucosa and their comparison with healthy tissues.

MATERIALS AND METHODS

Characteristics of subjects. The patient gingival tissue samples used in this study were obtained from the archive of Rīga Stradiņš University Institute of Anatomy and Anthropology.

This study included seven chronic periodontitis patients and five control group patients, who met the selection criteria. The study included patients who were treated at the Periodontology Department of the RSU Institute of Dentistry and whose health status was determined to be healthy according to their medical history. The chronic periodontitis group consisted of patients after conservative periodontal treatment who had no reduction in periodontal pocket depth and required surgical treatment to reduce the pockets (Table 1). The control group consisted of patients who underwent clinical crown lengthening surgery to improve aesthetics for prosthodontic purposes. Tissue was obtained from the gingiva and dental sulcus (Table 2).

Inclusion and exclusion criteria of patients. The samples for the chronic periodontitis group were selected using the following inclusion criteria: (1) men and women requiring surgical periodontal treatment to reduce periodontal pockets, (2) good general health (patients free of chronic general diseases, *diabetes mellitus*, cardiovascular diseases, hepatitis, epilepsy, oncological diseases and do not smoke); (3) good oral hygiene; (4) gingival bleeding after periodontal probing is observed in some teeth; (5) periodontal pockets ≥ 5 mm are observed; (6) attachment loss is observed; (7) radiological bone loss is observed.

Inclusion and exclusion criteria of control subjects. The control samples were selected using the following inclusion criteria: (1) males and females for clinical crown lengthening, (2) good general health (patients free of chronic general diseases, diabetes mellitus, cardiovascular diseases, hepatitis, epilepsy, oncological diseases and non-smokers), (3) good oral hygiene, (4) no gingival bleeding after periodontal probing, (5) no periodontal pockets on probing, (6) no tooth mobility, (7) no bone loss (bone height radiographically 1.5–2 mm from the enamel cement border).

Table 1. Information about the patients

Nr.	Age (Years)	Sex	Manipulation
HP6	42	M	Surgery to reduce the periodontal pocket
HP7	44	F	Surgery to reduce the periodontal pocket
HP8	46	M	Surgery to reduce the periodontal pocket
HP9	48	M	Surgery to reduce the periodontal pocket
HP11	49	M	Surgery to reduce the periodontal pocket
HP12	51	F	Surgery to reduce the periodontal pocket
HP14	51	F	Surgery to reduce the periodontal pocket

M – male, F – female

Table 2. Information about the control group

Nr.	Age (Years)	Sex	Manipulation
K6	37	F	Crown lengthening surgery
K9	45	F	Crown lengthening surgery
K11	49	M	Crown lengthening surgery
K13	49	M	Crown lengthening surgery
K14	50	M	Crown lengthening surgery

M – male, F – female

Routine staining. The obtained tissue samples were fixed in a solution of 2% formaldehyde, 0.2% picric acid, and buffered to pH 7.2 with 0.1 M phosphate buffer. After that, the tissue samples were rinsed in Tyrode's solution. Finally, the tissue samples were encased in paraffin and sliced into sections measuring 3–5 μm in thickness. Haematoxylin and eosin staining were conducted to evaluate the standard morphology of the samples (Layton and Suvarna, 2019).

Immunohistochemical (IHC) analysis. Immunohistochemistry was performed to determine the presence of LL-37, GAL-10, CD-163, hBD-2, hBD-3 in the tissue samples in both patient and control groups (Vaivads and Pilmane, 2024). The materials used included antibody diluent, HiDef Detection™ reaction amplificator, HiDef Detection™ HRP Polymer Detector, DAB+ chromogenic liquid DAB Substrate Kit, haematoxylin, TRIS buffer, peroxide, ethanol, carboxylic acid, and xylol.

Firstly, the tissue samples were washed with TRIS buffer, then blocked using a 3% peroxide solution, and washed again with TRIS buffer. After that, primary antibodies were applied, then tissue was incubated for 1 hour and washed three times with TRIS buffer. The next step was application of HiDef Detection™ reaction amplificator for 10 minutes followed by another washing step with TRIS buffer. After that, HiDef Detection™ HRP Polymer Detector was used for 10 minutes, and the tissues were washed three more times with TRIS buffer. Next a DAB+ chromogenic liquid DAB Substrate Kit was applied to the samples for 10 minutes and then the samples were washed under running water and counterstained with haematoxylin. For the final step, the samples were dehydrated by using ethanol with increas-

ing concentrations (70% to 90%), clarified with carboxylic acid and xylol, and finally sealed with a coverslip.

Specific antibodies used for immunohistochemistry. Analysis of LL-37 (sc-166770, working dilution 1:100, mouse, Santa Cruz Biotechnology Inc., Dallas, TX, USA), GAL-10 (orb157023, working dilution 1:200, rabbit, Biorbyt LLC, St Louis, MO, USA), CD-163 (orb13303, working dilution 1:100, rabbit, Biorbyt LLC, St Louis, MO, USA), hBD-2 (sc-20798, working dilution 1:100, rabbit, Santa Cruz Biotechnology Inc., Dallas, TX, USA), and hBD-3 (orb183268, working dilution 1:100, rabbit, Biorbyt LLC, St Louis, MO, USA) was conducted by immunohistochemistry.

Assessment of local tissue defence factor quantity. The semi-quantitative counting method and light microscopy were used to assess the comparative amount of LL-37, GAL-10, CD-163, hBD-2, and hBD-3 positive cells in the epithelium and connective tissue of the tissue samples (Ozola and Pilmane, 2023). Semi-quantitative classifications were applied: 0 – no positive cells in the structure; 0/+ – occasional positive cells in the structure; + – few positive cells in the structure; +/++ – few to moderate positive cells in the structure; ++ – moderate positive cells in the structure; ++/+++ - moderate to numerous positive cells in the structure; and +++ – numerous positive cells in the structure. The tissue samples were observed by two independent morphologists to validate observational and accurate analysis. Images illustrating the stained tissue samples were captured using a Leica DC 300F digital camera (Leica Microsystems Digital Imaging, Cambridge, UK), then handled and evaluated using the Image Pro Plus software (Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis. The statistical assessment was conducted using Statistical Product and Service Solutions (SPSS) Statistics version 29.0.0.0 (IBM Company, Chicago, IL, USA). A p-value of < 0.05 was applied to evaluate the statistical significance of calculations, as required. Descriptive and analytical statistics methods were used for data analysis. The semiquantitative count of factor-containing cells within each visual field was evaluated with descriptive statistics methods, including median value calculation. The semiquantitative count of factor-containing cells in each visual field was analysed using descriptive statistical methods, including the calculation of the median. In order to quantify the results obtained by IHC positive staining of tissue samples, the observed data were converted into numerical values as follows: 0 = 0, 0/+ = 0.5, + = 1, +/++ = 1.5, ++ = 2, ++/+++ = 2.5, +++ = 3.

The Mann–Whitney U test was used to determine statistically significant differences between expression of factors in the patient group and control group tissue samples. Thus, its use enabled the assessment of whether the mean quantity of observed positive structures differed statistically significantly between the patient group and the control group. Spearman's Rank Correlation was used to determine and assess statistically significant correlations between factors in the patient group and in factors in the control group (Barton and Peat, 2014). Spearman's correlation coefficient (r_s) was interpreted with the following definition where $r_s = 0.0–0.2$ as a very weak correlation, $r_s = 0.2–0.4$ as a weak correlation, $r_s = 0.4–0.6$ as a moderate correlation, $r_s = 0.6–0.8$ as a strong correlation and $r_s = 0.8–1.0$ as a very strong correlation (Vaivads *et al*., 2023).

RESULTS

LL-37 immunohistochemistry. In the control group, the median quantity of LL-37 positive cells in the epithelium was from few to moderate (+/++) and with a variation from no positive cells to moderate number to numerous LL-37 positive cells (Fig. 1a). The median quantity of LL-37 positive connective tissue cells was few (+), with a variation from no positive cells to few to moderate number of LL-37 positive cells in the control group (Fig. 1b).

In the patient group, the median quantity of LL-37 positive epitheliocytes was few (+) and fluctuated from no positive cells to moderate number to numerous LL-37 positive epitheliocytes (Fig. 1c). The median quantity of LL-37 positive connective tissue cells was from few to moderate (+/++) positive cells and with fluctuation from few to moderate number of LL-37 positive cells in the patient group (Fig. 1d).

A comparison of the control and chronic periodontitis group using the Mann–Whitney U test demonstrated no statistically significant difference of positive cells ($U = 20.5$, $p = 0.639$) in the epithelium and no statistically significant difference ($U = 8.5$, $p = 0.149$) in the connective tissue (Table 3).

GAL-10 immunohistochemistry. In the control group, the median quantity of GAL-10 positive cells in epithelium was from few to moderate number (+/++) and without variation (Fig. 2a). The median quantity of GAL-10 positive connective tissue cells was few (+), with variation from few to moderate number of GAL-10 positive cells in the control group (Fig. 2b).

In the patient group, the median quantity of GAL-10 positive epitheliocytes was few (+) and fluctuated from no positive cells to few to moderate number of GAL-10 positive epitheliocytes (Fig. 2c). The median quantity of GAL-10 positive connective tissue cells was few (+) positive cells and with fluctuations from no positive cells to few to moderate number of GAL-10 positive cells in the patient group (Fig. 2d).

A comparison of the control and chronic periodontitis group using the Mann–Whitney U test demonstrated no statistically significant difference ($U = 27$, $p = 0.149$) between the cells in the epithelium and no statistically significant difference ($U = 27$, $p = 0.149$) in the connective tissue (Table 3).

CD-163 immunohistochemistry. In the control group, the median quantity of CD-163 positive macrophages was few (+) and with a variation from few (Fig. 3a) to moderate number of CD-163 positive macrophages (Fig. 3b).

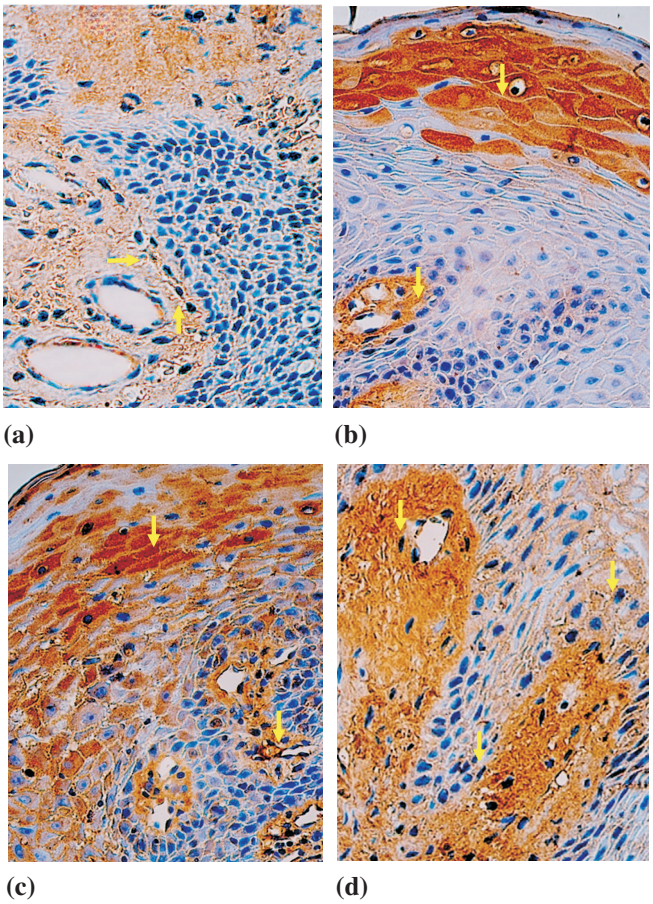

Fig. 1. Immunohistochemistry (IMH) of the LL-37 positive structures in the control and chronic periodontitis patient tissue samples. **(a)** Control sample with few LL-37 positive cells in the connective tissue and no LL-37 positive epitheliocytes (arrows). LL-37 IMH, 400×; **(b)** control sample with the few to moderate number of LL-37 positive in the connective tissue and moderate number of LL-37 positive epitheliocytes (arrows). LL-37 IMH, 400×; **(c)** chronic periodontitis patient sample with moderate number of LL-37 positive cells in the connective tissue and moderate number to numerous LL-37 positive epitheliocytes (arrows). LL-37 IMH, 400×; **(d)** chronic periodontitis patient sample with few to moderate number of LL-37 positive cells in the connective tissue and moderate number of LL-37 positive epitheliocytes (arrows). LL-37 IMH, 400×.

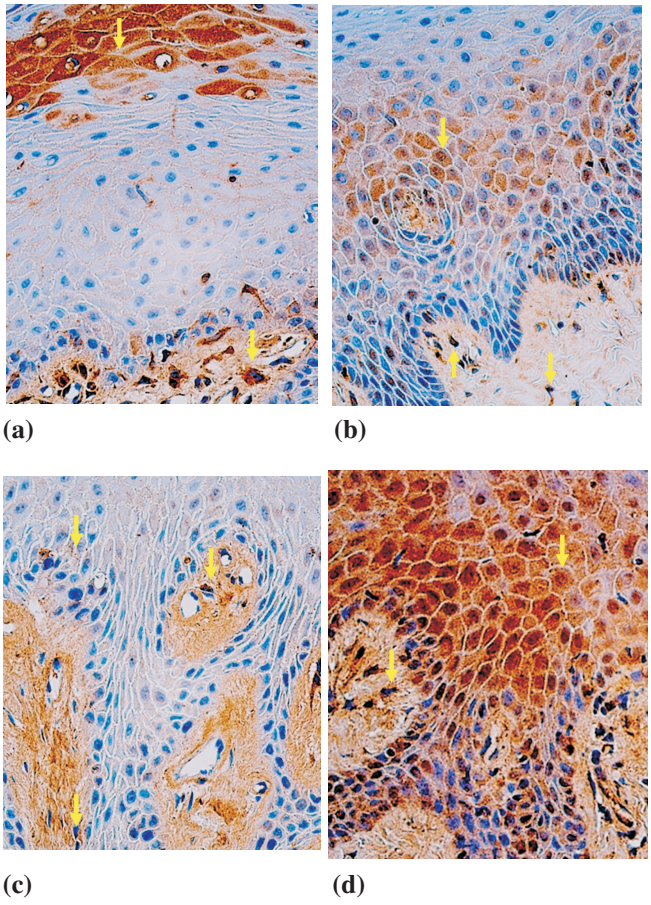

Fig. 2. Immunohistochemistry (IMH) of the GAL-10 positive structures in the control and chronic periodontitis patient tissue samples. **(a)** Control sample with moderate number of GAL-10 positive cells in the connective tissue and moderate number of GAL-10 positive epitheliocytes (arrows). GAL-10 IMH, 400×; **(b)** control sample with a few GAL-10 positive in the connective tissue and moderate number of GAL-10 positive epitheliocytes (arrows). GAL-10 IMH, 400×; **(c)** chronic periodontitis patient sample with a few GAL-10 positive cells in the connective tissue and a few GAL-10 positive epitheliocytes (arrows). GAL-10 IMH, 400×; **(d)** chronic periodontitis patient sample with moderate number of GAL-10 positive cells in the connective tissue and numerous GAL-10 positive epitheliocytes (arrows). GAL-10 IMH, 400×.

Table 3. Median values of semiquantitative evaluation for cathelicidin LL-37, galectin-10, human beta-defensin 2, human beta-defensin 3 and CD-163 within the chronic periodontitis and control tissue groups and macrophages

Structure	Factors								
	LL-37		GAL-10		hBD-2		hBD-3		CD-163
	ET	CT	ET	CT	ET	CT	ET	CT	M
CPP	+	+/++	+	+	+	+	+	+/++	+
CG	+/++	+	+/++	+	+	+	+/++	+	+
U test	20.5	8.5	27	27	22	16.5	26.5	27.5	25.5
p	0.639	0.149	0.149	0.149	0.530	0.876	0.149	0.106	0.202

LL-37 – cathelicidin LL-37, GAL-10 – galectin-10, hBD-2 – human beta-defensin-2, hBD-3 – human beta-defensin-3, CD-163 – haemoglobin scavenger receptor CD-163, ET – epithelium, CT – connective tissue, CPP – chronic periodontitis patients, CG – control group, U test – Mann–Whitney U test; *p* – *p*-value; 0 – no positive cells in the structure; 0/+ – occasional positive cells in the structure; + – few positive cells in the structure; +/++ – few to moderate positive cells in the structure; ++ – moderate number of positive cells in the structure; ++/+++ – moderate number to numerous positive cells in the structure; +++ – numerous positive cells in the structure

In the patient group, the median quantity of CD-163 positive macrophages was few (+) (Fig. 3b) and fluctuated from no positive cells to few to moderate number of CD-163 positive macrophages (Fig. 3d).

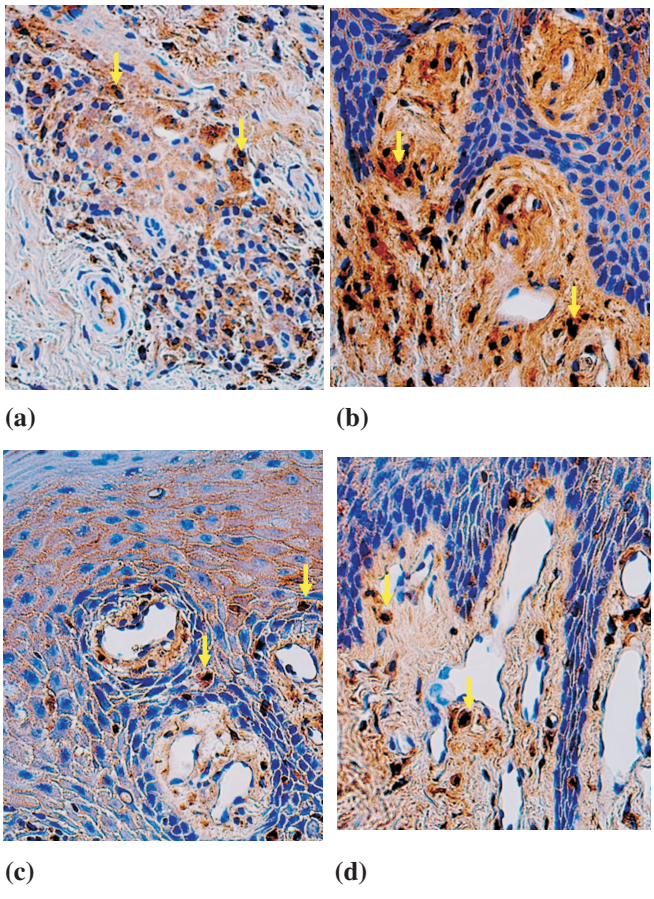

Fig. 3. Immunohistochemistry (IMH) of the CD-163 positive macrophages in the control and chronic periodontitis patient tissue samples. **(a)** Control sample with number of few to moderate number of CD-163 positive macrophages in the tissue sample (arrows). CD-163 IMH, 400×; **(b)** control sample with moderate number of CD-163 positive macrophages. CD-163 IMH, 400×; **(c)** chronic periodontitis patient sample with the few to moderate number of CD-163 positive macrophages (arrows). CD-163 IMH, 400×; **(d)** chronic periodontitis patient sample with moderate CD-163 positive macrophages in the connective tissue of chronic periodontitis patient (arrows). CD-163 IMH, 400×.

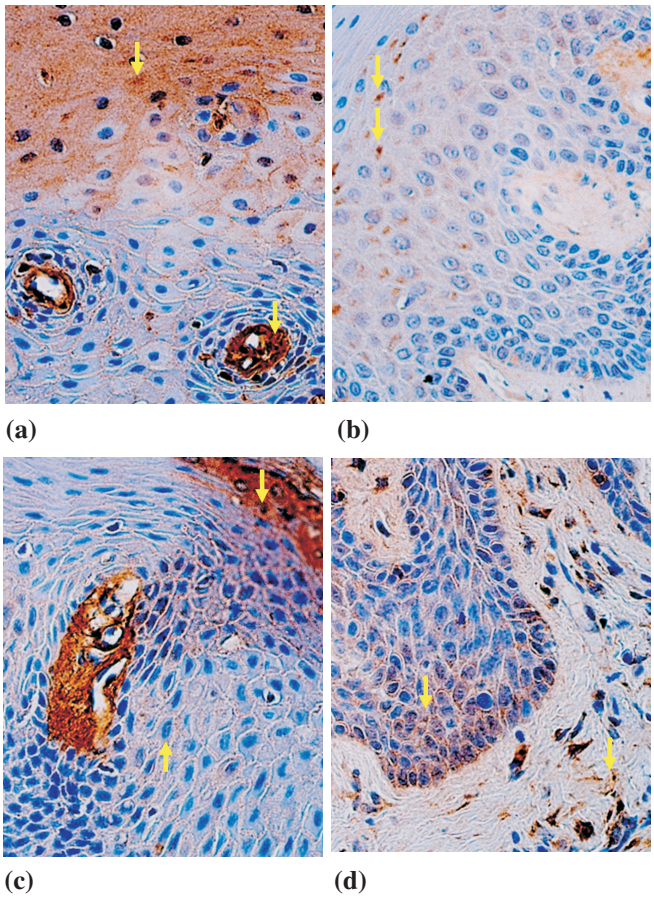

Fig. 4. Immunohistochemistry (IMH) of the hBD-2 positive structures in the control and chronic periodontitis patient tissue samples. **(a)** Control sample with moderate hBD-2 positive cells in the connective tissue and moderate number of hBD-2 positive epitheliocytes (arrows). hBD-2 IMH, 400×; **(b)** control sample with no positive cells to few hBD-2 positive cells in the connective tissue and no positive cells to few hBD-2 positive epitheliocytes (arrows). hBD-2 IMH, 400×; **(c)** chronic periodontitis patient sample with moderate number of hBD-2 positive cells in the connective tissue and moderate number of hBD-2 positive epitheliocytes (arrows). hBD-2 IMH, 400×; **(d)** chronic periodontitis patient sample with the few hBD-2 positive cells in the connective tissue and moderate number of hBD-2 positive epitheliocytes (arrows). hBD-2 IMH, 400×.

A comparison of the control and chronic periodontitis group positive cells using the Mann–Whitney U test demonstrated no statistically significant difference (U = 25.5, p = 0.202) in the macrophages (Table 3).

hBD-2 immunohistochemistry. In the control group, the median quantity of hBD-2 positive cells in epithelium was few (+) and with variation from no positive cells to few to moderate number of hBD-2 positive epitheliocytes (Fig. 4a). The median quantity of hBD-2 positive connective tissue cells was few (+), with variation from no positive cells to few to moderate number of hDB-2 positive cells in the control group (Fig. 4b).

In the patient group, the median quantity of hBD-2 positive epitheliocytes was few (+) and had fluctuated from no positive cells to few to moderate number of hBD-2 positive epitheliocytes (Fig. 4c). The median quantity of hBD-2 positive connective tissue cells was few (+) positive cells and with fluctuation from no positive cells to few to moderate number of hBD-2 positive cells in the patient group (Fig. 4d).

A comparison of the control and chronic periodontitis group positive cells using the Mann–Whitney U test demonstrated no statistically significant difference (U = 22, p = 0.530) in the epithelium and no statistically significant difference (U = 16.5, p = 0.876) in the connective tissue (Table 3).

hBD-3 immunohistochemistry. In the control group, the median quantity of hBD-3 positive cells in epithelium was few to moderate number (+/++) and with a variation from no positive cells to few to moderate number to numerous hBD-3 positive epitheliocytes (Fig. 5a). The median quantity of hBD-3 positive connective tissue cells was few to moderate number (+/++), with variation from no positive cells to few to moderate number of hDB-3 positive cells in the control group (Fig. 5b).

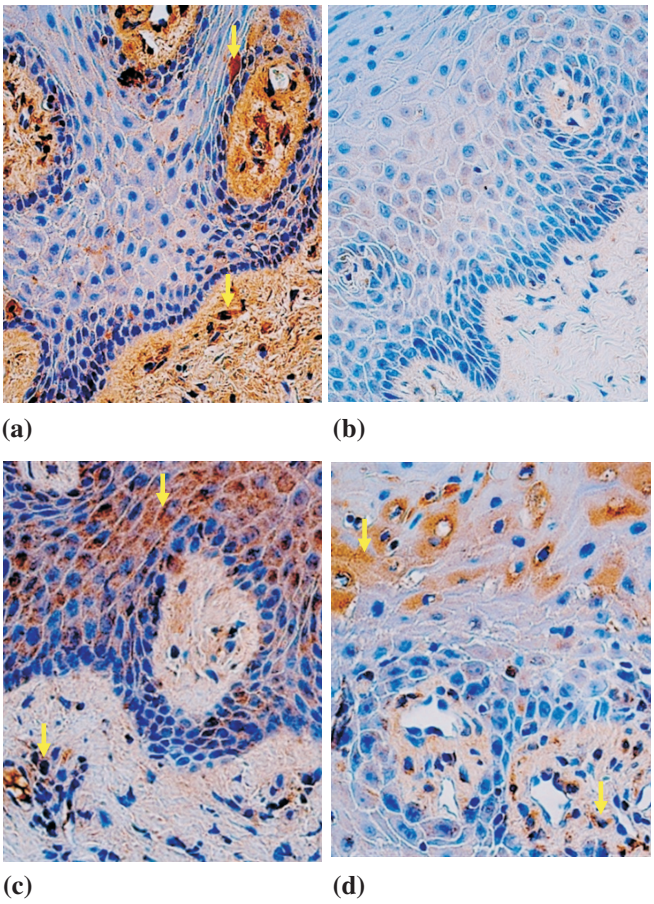

Fig. 5. Immunohistochemistry (IMH) of the hBD-3 positive structures in the control and chronic periodontitis patient tissue samples. **(a)** Control sample with the moderate number of hBD-3 positive cells in the connective tissue and the few to moderate number of hBD-3 positive epitheliocytes (arrows). hBD-3 IMH, 400×; **(b)** control sample with a lack of hBD-3 positive cells in the connective tissue and no positive cells to few hBD-3 positive epitheliocytes. hBD-3 IMH, 400×; **(c)** chronic periodontitis patient sample with the few hBD-3 positive cells in the connective tissue and few to moderate number of hBD-3 positive epitheliocytes (arrows). hBD-3 IMH, 400×; **(d)** chronic periodontitis patient sample with the few hBD-3 positive cells in the connective tissue and moderate number of hBD-3 positive epitheliocytes (arrows). hBD-3 IMH, 400×.

In the patient group, the median quantity of hBD-3 positive epitheliocytes was few (+) and had fluctuation from no positive cells to few to moderate number to numerous hBD-3 positive epitheliocytes (Fig. 5c). The median quantity of hBD-3 positive connective tissue cells was few (+) positive cells and with fluctuation from no positive cells to few to moderate number of hBD-3 positive cells in the patient group (Fig. 5d).

A comparison of the control and chronic periodontitis group positive cells using the Mann–Whitney U test demonstrated no statistically significant difference (U = 26.5, p = 0.149) in the epithelium and no statistically significant difference (U = 27.5, p = 0.106) in the connective tissue (Table 3).

Correlations in the epithelium and the connective tissue of control group. In the control group, a statistically significant negative correlation (r_s = ± 0.8 – ± 1.0>) was observed between the amount of hBD-2 in the epithelium and the expression of LL-37 in the epithelium (r_s = –0.921, p = 0.026), a statistically significant positive correlation (r_s = ± 0. 8 – ± 1.0>) between the amount of hBD-3 positive cells in connective tissue and the amount of CD-163 positive macrophages (r_s = 0.884, p = 0.047) and between LL-37 expression in epithelium and GAL-10 expression in connective tissue (r_s = 0.889, p = 0.044). Statistically significant correlations in the control group are summarised in Table 4.

Correlations in the epithelium and the connective tissue of chronic periodontitis group. Spearman's rank correlation coefficient was used to determine the correlations between antimicrobial peptides in the tissues of the patient and control group. A statistically significant negative correlation (r_s = ± 0.8 – ± 1.0>) was determined between hBD-3 expression in epithelium and hBD-2 expression in connective tissue (r_s = –0.933, p = 0.002) in the patient group. Statistically significant correlations in the patient group are summarised in Table 5.

DISCUSSION

In this study, we observed no statistically significant differences in the expression of HBD-3, HBD-2, LL-37, GAL-10, and CD163-positive cells between chronic periodontitis patients and the control group. This may suggest that chronic inflammation and immune responses to inflammation may be a common feature among these subjects, regardless of whether chronic periodontitis was the clinical diagnosis.

One possible explanation for the lack of significant differences between the control and chronic periodontitis groups could be that the general Latvian population may have a

Table 4. Statistically notable Spearman's rank correlation coefficient comparison analysis between tissue antimicrobial peptides in the control group

Strength of correlation	Correlations between tissue defense factors in control group	r_s	p-value
Very strong association (± 0.8 – ± 1.0>)	hBD-2 in epithelium and LL-37 in epithelium	–0.921	0.026
	hBD-3 in connective tissue and CD-163 in macrophages	0.884	0.047
	LL-37 in epithelium and GAL-10 in connective tissue	0.889	0.044

Table 5. Statistically significant Spearman's rank correlation coefficients between tissue antimicrobial peptides in the patient group

Strength of correlation	Correlations between tissue defense factors in patient group	r_s	p-value
Very strong association (± 0.8 – ± 1.0>)	hBD-3 in epithelium and hBD-2 in connective tissue	–0.933	0.002

low level of oral health, which therefore leads to a continuous stimulation of the immune system, even in subjects who were classified as "healthy" in this study and were included in the control group (Vidzis *et al*., 2011). Moreover, there are various underlying environmental and lifestyle factors, such as oral hygiene habits, diet and access to dental care, which could also influence the inflammatory process. Further studies are needed to clarify whether the expression of these antimicrobial markers in the oral mucosa influence the development of chronic periodontitis.

In addition, the absence of significant differences could suggest that the biomarkers selected, although important for the assessment of the immune response, may not be definitive markers to distinguish chronic periodontitis from a generally inflamed oral environment. Although previous studies have implicated these factors in the progression of periodontitis, their expression may be influenced by a variety of external and individual biological variables, including genetic predisposition, microbiome composition and systemic health conditions (Isola, 2020; Ravindran *et al*., 2024). It is possible that future studies should focus specifically on the level of genes that differentiate patients from controls in order to increase knowledge about the genetic predisposition to chronic periodontitis, as the currently known mutations are genetic polymorphisms in the interleukin-6 and matrix metalloproteinase enzymes — matrix metalloproteinase-1 and matrix metalloproteinase-2 (Munshi *et al*., 2024).

Interestingly, although no statistically significant changes were observed, trends in marker expression should nevertheless be taken into account. However, some statically significant correlations were found in both the patient group and the control group. Furthermore, the observed correlations between immune markers indicate the complexity of immune interactions in periodontal disease and warrant further investigation.

Another study, which also looked at hBD-3 and hBD-2 in patients with periodontitis and a control group, observed that their expression in keratinocytes was reduced, which was associated with a low response to inflammatory mediators (Liu *et al*., 2013). It is also important to mention that another study showed that hBD-3 and hBD-2 expression in periodontitis did not differ depending on the severity of inflammation (Özdemir *et al*., 2019). This could explain why the patient group did not differ from the control group, as inflammation was a common feature in both groups, regardless of the diagnosis of chronic periodontitis.

In the patient group, there was a negative correlation between hBD-3 expression in the epithelium and hBD-2 expression in the connective tissue. A negative correlation between these antimicrobial peptides was also observed in another study where hBD-2 may reduce hBD-3 expression (Winter *et al*., 2011). This suggests that there is a complex regulatory mechanism in the interaction between hBD-3 and hBD-2 in inflammation process.

In the control group, several significant correlations were observed. There was a statistically significant negative correlation between hBD-2 expression in the epithelium and LL-37 expression also in the epithelium, while there was a statistically significant positive correlation between GAL-10 in connective tissue and LL-37 expression in the epithelium. Another statistically significant positive correlation was between hBD-3 in connective tissue and CD-163 positive macrophages.

LL-37 plays an important protective role in oral health maintenance by arresting bacteria-induced immune-stimulating effects, stopping the production of inflammatory cytokines, and playing an important role in new blood vessel formation and regeneration (Scott *et al*., 2002; Bucki *et al*., 2010; Tokajuk *et al*., 2022). It is also known that LL-37 is found in neutrophils, so after neutrophil degranulation due to bacterial stimulation, LL-37 is released into tissues (Greer *et al*., 2013). It has been shown that the expression level of LL-37 increases in patients with aggressive or chronic periodontal disease, and its expression level also increases in inflammatory processes of different causes, because it influences both inflammatory cytokines and participates in the response to the inflammatory process (Madhwani and McBain, 2011; Leite *et al*., 2023). In contrast, hBD-2 is expressed more in the healthy oral cavity and its reduced expression could be related to an ongoing inflammatory process in the oral cavity (Greer *et al.,* 2013; Liu *et al.,* 2013). Therefore, a negative correlation between these two peptides may indicate an inflammatory process in the oral mucosa, even though this correlation was present in the control group, which showed a decrease in hBD-2 expression and an increase in LL-37 expression in response.

GAL-10 expression is associated with the development of inflammatory processes. GAL-10 was one of the most up-regulated proteins in periodontitis, as shown in a study analysing gingival crevicular fluid (GCF) (Kim *et al*., 2020). Also, GAL-10 is being applied as a new biomarker for eosinophilic diseases such as asthma, rhinitis, sinusitis, etc., as Tomizawa *et al.*, point out (Tomizawa *et al*., 2022). The results of the study by Altieri *et al.* suggest that LL-37 probably plays an important role in the pathogenesis of chronic inflammation-induced respiratory diseases such as asthma (Altieri *et al*., 2024). This may suggest that there may have been some unknown factors in the control group subjects that resulted in the inflammation that caused the positive correlation of GAL-10 and LL-37 in the control group tissues.

hBD-3 is an antimicrobial peptide involved in the maintenance of oral health, as it shows the most pronounced activity against pathogens and is also involved in tissue regeneration processes such as healing, angiogenesis and EGFR activation (Tokajuk *et al*., 2022; Atalay *et al*., 2024; Ghosh *et al*., 2024). CD-163 is a haemoglobin scavenger receptor used to identify M2 macrophages, which are also involved in tissue healing processes as they promote tissue remodelling and also angiogenesis (De Carli *et al*., 2015). CD-163 positive macrophages indicate that the tissue is mounting a response to inflammation and this is usually already characterised by late responses (Skytthe *et al*., 2020). A positive

correlation between hBD-3 expression and CD-163 positive macrophages could indicate that the regeneration process has started in the tissues of the control group, especially considering that the correlation was directly between hBD-3 expression in connective tissue and macrophages, which are also found in gingival connective tissue; this could be a direct description of the healing process after inflammation at a deeper tissue level.

LIMITATIONS OF THE STUDY

This study has several limitations that should be acknowledged. First, the use of a semi-quantitative method which may affect the precision of results. Although the tissue samples were observed by two independent morphologists to mitigate bias, there still could be the potential risk of interpretation variability. Also, the sample size was low. There were only five persons in the control group and seven in the patient group. That could restrict the statistical power of the findings. While the sample size is acceptable for morphological study, it may be insufficient for statistical analysis to yield statistically significant results in analyses. Another limitation could be that the study involved participants only from Latvia as there could be specific genetic or epidemiological traits within this population that may influence oral health, potentially including a naturally reduced defence against oral pathogens. A limitation could be ontogenetic that is presented by the age range of participants (37–51 years), since immune and regenerative capacities could vary across different life stages, potentially affecting local protective mechanisms in the oral mucosa. Furthermore, although the control group was considered healthy, there still could be undiagnosed or subclinical systemic conditions that may have influenced the local protective factors' expression. Vitamin D deficiency is another key element to consider because it impacts immunity and mucosal defence, and is common in Latvia. Given these limitations, further studies should investigate the role of genetic predisposition, the impact of regional epidemiological factors on oral pathology and the ontogenetic aspects that could affect the protective mechanisms in the oral mucosa.

CONCLUSIONS

No statistically significant differences in the expression of HBD-3, HBD-2, LL-37, GAL-10, and CD163-positive cells between chronic periodontitis patients and controls suggest the similar local immunity health status in the oral cavity of Latvian population. This also raises questions about the specificity of these biomarkers to differentiate chronic periodontitis from a generalised inflamed oral environment, highlighting the complexity of immune interactions in both periodontal disease-affected and seemingly healthy oral mucosa. Limited correlations between defensins in tissue of patients and controls indicate the practically balanced appearance in local defence factors in Latvian population oral cavities.

ETHICS

Permission to use patient gingival tissue samples in the study was received by the Ethics Committee of Rīga Stradiņš University (4 November 2003; 10 January 2008). Written informed consent for participation in the study was obtained from all patients.

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LOKĀLO AIZSARGFAKTORU EKSPRESIJAS LĪMENIS MUTES GĻOTĀDĀ: SALĪDZINOŠS PILOTPĒTĪJUMS STARP VESELIEM UN HRONISKA PERIODONTĪTA PACIENTIEM

Šajā pētījumā netika konstatētas statistiski nozīmīgas atšķirības HBD-3, HBD-2, LL-37, GAL-10 un CD163 pozitīvo šūnu ekspresijā starp hroniska periodontīta pacientiem un kontroles grupu, kas liecina par līdzīgu lokālās imunitātes stāvokli Latvijas iedzīvotāju mutes dobumā. Tas liek aizdomāties par šo biomarķieru specifiskumu hroniska periodontīta atšķiršanai no vispārēja mutes iekaisuma un uzsver imunitātes mijiedarbības sarežģītību gan periodontāli skartajā, gan šķietami veselīgajā mutes gļotādā. Neraugoties uz starpgrupu atšķirību trūkumu, katrā grupā tika novērotas nozīmīgas, tomēr atšķirīgas korelācijas starp lokālajiem aizsargfaktoriem hroniska periodontīta pacientu un kontroles grupas audos. Šie rezultāti apliecina komplicētu imūno mijiedarbību Latvijas iedzīvotāju mutes dobumā un uzsver nepieciešamību turpmākajos pētījumos izmantot lielāku paraugu izlasi.