

# The effect of obesity and periodontitis on the expression of antimicrobial peptides in gingival tissues

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## ABSTRACT

**الأهداف:** مقارنة ظهور بروتينات البيتا ديفنسين في أنسجة اللثة لدى الأشخاص المصابين بالسمنة والأشخاص ذو الوزن الطبيعي

**الطريقة:** أجريت هذه الدراسة في كلية طب الاسنان، جامعه الملك عبد العزيز، جدة، المملكة العربية السعودية وذلك خلال الفترة من مارس 2010م إلى نوفمبر 2011م. شملت الدراسة 20 شخصا من أصحاب الوزن الزائد و 20 شخصا من ذوي الوزن الطبيعي. وتم أخذ عينات لثوية لتحديد مقدار ظهور بروتينات البيتا ديفنسين.

**النتائج:** دلت نتائج هذه الدراسة على وجود بروتينات البيتا ديفنسين في أنسجة اللثة لدى الأشخاص المصابين بالسمنة والأشخاص ذو الوزن الطبيعي. كما لم تظهر الدراسة على وجود اختلاف في مقدار ظهور بروتينات البيتا ديفنسين بين الأشخاص المصابين بالسمنة والأشخاص ذو الوزن الطبيعي.

**خاتمة:** أظهرت الدراسة عدم وجود علاقة بين السمنة وأمراض اللثة وظهور بروتينات البيتا ديفنسين.

**Objectives:** To compare the expression of human  $\beta$ -defensins (HBD) in the gingival tissues of obese and normal weight systemically healthy subjects.

**Methods:** This cross-sectional study was conducted at the Faculty of Dentistry, King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia from March 2010 to November 2011. Twenty obese (10 affected with periodontitis and 10 periodontally healthy), and 20 normal weight subjects (10 affected with periodontitis and 10 periodontally healthy) were selected. Gingival tissue samples were collected and subjected to immunohistochemistry assay to evaluate the expression of HBD-1 and -2.

**Results:** In the epithelium, the staining was almost limited to the basal and supra-basal cells, while the superficial layers and keratin were negative. In the connective tissue, some of the chronic inflammatory cells and fibroblasts were also stained. Endothelial

cells lining blood or lymphatic vessels were mostly positive with HBD-2 and were almost negative with HBD-1. The percentage of positively stained areas did not show any differences in the expression of HBD-1 and -2 in obese compared to normal weight subjects whether they were affected or not affected with periodontitis.

**Conclusion:** The results of this study did not demonstrate an association between the expression of HBD-1 and -2 and obesity and periodontitis.

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The reported prevalence of obesity in the Middle East and especially in Saudi Arabia is high.<sup>1</sup> Obesity is considered a major contributor to the development of a number of health conditions, including atherosclerosis, hypertension, cardiovascular disease, dyslipidemia, and diabetes.<sup>2-4</sup> Furthermore, obesity has also been suggested as a risk factor for periodontal disease.<sup>5-7</sup> Periodontal disease results from the interaction between pathogenic bacteria and the host's immune response.<sup>7</sup> The association between obesity and periodontal disease has long been suspected but gained considerable attention in recent years.<sup>8</sup> Saito et al<sup>9</sup> showed higher periodontitis prevalence among those with abdominal adiposity as compared to those without this condition. This report was followed by a study in the US population using

data from the third National Health and Nutrition Examination survey on 13,665 individuals.<sup>10</sup> The study reported an association between overall and abdominal obesity. Since then several cross-sectional studies have confirmed the association between obesity and periodontitis.<sup>11-17</sup> Several mechanisms have been suggested to explain the association between obesity and periodontitis. Obesity has been shown to affect host immunity and increase susceptibility to diseases.<sup>18</sup> Furthermore, obesity has been shown to affect the individuals' immune response to several pathogens (for example, *Porphyromonas gingivalis* [*P. gingivalis*]).<sup>19,20</sup> Another possible mechanism is by the increase secretion of several proinflammatory cytokines that are known to be associated with periodontal disease.<sup>8,21</sup> The response of mammalian hosts to microbial pathogens involves the activation of both innate and adaptive components of the immune system. An integral part of the innate immunity is antimicrobial peptides.<sup>22</sup> Antimicrobial peptides such as human  $\beta$ -defensins-1 and -2 (HBD-1 and HBD-2) are expressed in human gingival tissues, and are suggested to play a role in the host defense mechanism against periodontal infection.<sup>23,24</sup> Obesity disturbs the host' immunity and impairs the innate immune system, which could affect the function and/or expression of HBD.<sup>18,25</sup> Nevertheless, no published data is available on the potential role and expression of HBD in the gingival tissues of obese subjects. The objective of this study therefore was to examine the association between obesity and expression of HBD-1 and -2 in the gingival tissues.

**Methods. Sample selection.** Subjects for this cross-sectional study were selected from patients who were seeking treatment in the Periodontology Clinics at the Faculty of Dentistry, King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia from March 2010 to November 2011. Inclusion criteria included subjects who were: older than 30 years of age; presented with healthy periodontal status, or diagnosed with generalized moderate chronic periodontitis; and needed tooth extraction or pre-prosthetic periodontal surgery.

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Exclusion criteria were: known systemic diseases; history and/or presence of other infections, systemic antibiotic or anti-fungal treatment in the preceding 6 months; any concomitant medical therapy; pregnancy or lactation in females; and those who require antibiotic prophylaxis before periodontal treatment. The study was reviewed and approved by the Research Ethics Committee at the Faculty of Dentistry, King Abdulaziz University and was in agreement with the Helsinki Declaration. After obtaining informed consent, weight and height were recorded for each participant, and body mass index (BMI) was calculated. Subjects were considered obese when their BMI was  $>30 \text{ kg/m}^2$ . A total of 40 patients were selected and assigned to one of the following 4 groups. First group were normal weight subjects not affected with periodontitis ( $n=10$ ). Second group were obese subjects not affected with periodontitis ( $n=10$ ). Third group were normal weight subjects affected with periodontitis ( $n=10$ ). Finally, the fourth group were obese subjects affected with periodontitis ( $n=10$ ).

**Gingival tissue sample collection.** Gingival tissue samples from each subject were collected during the extraction of non-restorable teeth in periodontitis patients, and during the pre-prosthetic surgery in periodontally healthy subjects. The tissue samples were fixed immediately in freshly prepared 1% paraformaldehyde in phosphate buffered saline (PBS; pH: 7.4) for later use. The samples were coded so that the technicians performing the laboratory work were blinded to the subjects' identities and group assignments.

**Immunohistochemistry.** Tissue samples were embedded in paraffin wax, sectioned by microtome, placed on poly-L-lysine coated slides and stained by Hematoxylin and Eosin (H&E) then coverslipped by Leica Multistainer and Coverslipper (Leica ST5020 and CV5030, Leica Microsystems, Germany) for observation and validating the orientation of the gingival tissue. Immunohistochemistry for the validated samples to determine HBD-1 and -2 expressions was performed according to the manufacturer instruction as follows: paraffin-embedded sections of tissue were deparaffinized by washing them with xylene 3 times for 5 minutes each and hydrated by washing them 2 times in 100% ethanol for 10 minutes each, 2 times in 95% ethanol for 10 minutes each, 2 times in de-ionized water ( $\text{dH}_2\text{O}$ ) for 5 minutes each, then 2 times in PBS for 5 minutes each. For antigen unmasking, sections were heated in 10 mM sodium citrate buffer (pH: 6.0) for 1 minute at 600 kilowatts in a conventional microwave followed by 3 minutes at 800 kilowatts, then 10 minutes at 400 kilowatts. Slides were cooled for 20 minutes to room

temperature, washed in dH<sub>2</sub>O 3 times for 5 minutes each then incubated in 1% hydrogen peroxide for 10 minutes. Sections were then washed in dH<sub>2</sub>O 3 times for 5 minutes each, and washed in PBS for 5 minutes each. Sections were blocked with horse serum for one hour at room temperature, followed by incubation with primary antibodies (in blocking solution) overnight at 4°C. The primary antibodies used were anti HBD-1 and HBD-2 (Abcam, Cambridge, UK). Antibody solutions were removed and sections were washed in PBS 3 times for 5 minutes each. Secondary biotinylated IgG antibody (Vector Laboratories, UK) were pre-diluted in blocking solution for each section. Sections were then incubated for 30 minutes at room temperature. Secondary antibody solutions were removed and sections were washed 3 times with PBS for 5 minutes each. The Avidin/Biotinylated enzyme Complex (ABC reagent) (Vector Laboratories, Peterborough, UK) was then added to the sections, and they were incubated for 30 minutes at room temperature, then washed 3 times in PBS for 5 minutes each, and 3,3'-diaminobenzidine (DAB) reagent (Vector Laboratories, Peterborough, UK) was added and staining was monitored closely. To stop the reaction, slides were immersed in dH<sub>2</sub>O. Sections were then washed in dH<sub>2</sub>O 2 times for 5 minutes each, and dehydrated in 95% ethanol 2 times for 10 seconds each in 100% ethanol 2 times for 10 seconds each, and xylene 2 times for 10 seconds each. Slides were then coverslipped.

**Histomorphometric quantification.** Image analysis was performed using the Olympus DP25 Microscope Digital Camera with color rendition fixed on Olympus BX61 fully motorized research microscope (Olympus, Southend on Sea, UK). Four regions of interest were selected from each slide. These were selected to represent the highest chromogen density of the immunostained area. Images were then analyzed using an image analysis software<sup>26</sup> with the aid of the ImmunoRatio plugin.<sup>27,28</sup> No attempts were carried out to discriminate between epithelial and connective tissue immunostaining. Likewise, no attempts were made to discriminate between nuclear and cytoplasmic immunostaining. Both epithelial and connective tissue, nuclear and cytoplasmic staining were calculated.

**Statistical analysis.** Data were collected and tabulated using Microsoft® Excel 2003, and then were statistically analyzed using the Statistical Package for Social Science version 16 (SPSS Inc, Chicago, IL, USA). Independent sample t-test and one-way ANOVA were used to compare the percentage of positively stained areas (representing the expression of HBD) in

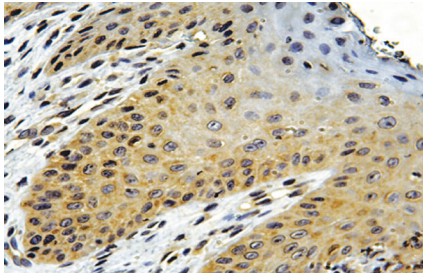
obese compared to normal weight subjects. Data are presented as mean  $\pm$  standard deviation (SD). Statistical significance was considered at the 0.05 alpha level.

**Results.** The mean age of the study sample was  $40 \pm 8.2$  years. Sixty percent of the participants were females. The expression of HBD-1 and HBD-2 revealed a positive nuclear and cytoplasmic staining of some epithelial and connective tissue cells. In the epithelium, the staining was almost limited to the basal and supra-basal cells, while the superficial layers and keratin were negative (Figure 1). In the connective tissue, some of the chronic inflammatory cells and fibroblasts were also stained (Figure 2). Endothelial cells lining blood or lymphatic vessels were mostly positive for HBD-2 (Figure 3) and were almost negative with HBD-1 (Figure 4). The cytoplasmic expression was brownish and granular (Figure 2 and Figure 4).

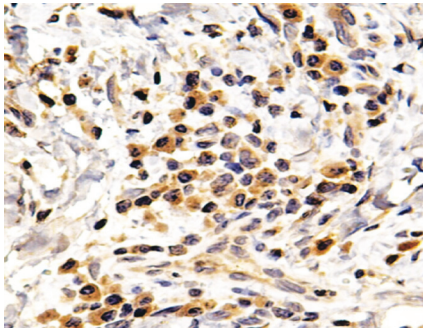
**Histomorphometric results.** The expression of HBD-1 and HBD-2 by estimating the percentage of positively stained area is shown in Tables 1-3. The mean ratio of expression of HBD-1 was  $25.5 \pm 2.05$ , and HBD-2 was  $25.5 \pm 1.9$  in the total sample. The expression of HBD-1 and HBD-2 between males and females was not statistically significant ( $p=0.23$ ). Table 1 shows the mean  $\pm$  SD of the percentage of immunopositive area for HBD in subjects with and without periodontitis. A slightly lower expression of HBD (as measured by the percentage of positively stained area) was noted in periodontitis compared to periodontally healthy patients, however, this was not statistically significant. Table 2 shows the mean  $\pm$  SD of the percentage of immunopositive area for HBD's in obese and non-obese subjects regardless of their periodontal status. A slightly higher expression of HBD (as measured by the percentage of positively stained area) was noted in normal weight subjects compared to obese ones, but this was not statistically significant. Table 3 shows the mean  $\pm$  SD of the percentage of immunopositive area for HBD in the 4 study groups. Normal weight subjects with no periodontitis had the highest expression whereas obese with periodontitis showed the lowest expression for HBD-2 but it was not statistically significant.

**Discussion.** This study was conducted to characterize the mechanism of the reported association between obesity and periodontal disease. Specifically, the expression of antimicrobial peptides (HBD-1 and -2) was compared in the gingival tissues of obese and normal weight subjects. The results of this study

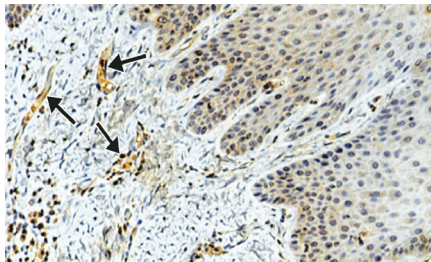




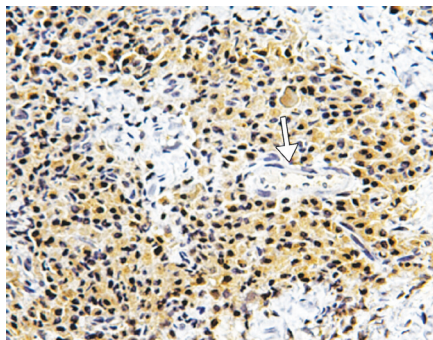
**Figure 1** - The human  $\beta$ -defensins-1 in a patient showing positive nuclear and brownish granular cytoplasmic staining of basal and supra-basal epithelial cells. Note the absence of the stain in the superficial layers of the epithelium (x400).



**Figure 2** - The human  $\beta$ -defensins-2 in a patient showing positive nuclear and brownish granular cytoplasmic staining of some of the connective tissue and inflammatory cells (x400).



**Figure 3** - The human  $\beta$ -defensins-2 in a patient showing positive reaction in the endothelial cells lining the blood vessel (arrows) (x200).



**Figure 4** - The human  $\beta$ -defensins-1 in a patient showing negative reaction in the endothelial cells lining the blood vessel (arrows) (x200).

**Table 1** - Percentage of immunopositive area for human  $\beta$ -defensins (HBD) in subjects with and without periodontitis included in a study conducted at the Periodontology Clinics, of the Faculty of Dentistry, King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia.

HBD	Periodontitis Mean $\pm$ standard deviation		P-value
	Yes	No	
1	25.5 $\pm$ 2.1	25.6 $\pm$ 2.1	0.9
2	25.2 $\pm$ 2.0	26.0 $\pm$ 1.8	0.2

**Table 2** - Percentage of immunopositive area for human  $\beta$ -defensins (HBD) in obese and normal weight subjects included in a study conducted at the Periodontology Clinics of the Faculty of Dentistry, King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia..

HBD	Obesity Mean $\pm$ standard deviation		P-value
	Yes	No	
1	25.1 $\pm$ 2.3	26.0 $\pm$ 1.8	0.2
2	25.3 $\pm$ 1.9	25.7 $\pm$ 1.9	0.5

**Table 3** - Percentage of immunopositive area for human  $\beta$ -defensins (HBD) in the 4 study groups included in a study conducted at the Periodontology Clinics of the Faculty of Dentistry, King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia.

Variables	HBD-1 Mean $\pm$ standard deviation	HBD-2 Mean $\pm$ standard deviation
Non-obese without periodontitis	26.6 $\pm$ 1.5	26.2 $\pm$ 2.0
Non-obese with periodontitis	26.0 $\pm$ 1.9	25.5 $\pm$ 1.9
Obese without periodontitis	24.4 $\pm$ 2.5	25.6 $\pm$ 1.5
Obese with periodontitis	25.4 $\pm$ 2.3	25.2 $\pm$ 2.1
P-value	0.2	0.7

revealed no differences in the expression of HBD-1 and -2 between obese and normal weight participants. In both groups, the expression of HBD in the epithelium was almost limited to the basal and supra-basal cells, while the superficial layers and keratin were negative. Endothelial cells lining blood or lymphatic vessels were mostly positive with HBD-2, and were almost negative with HBD-1.

In the present study, the expression of HBD-2 was slightly lower among the periodontitis patients than periodontally healthy subjects, however, this difference was not statistically significant. Earlier studies have demonstrated that the expression of HBD was changed and altered based on the microorganism, host condition, and different HBD molecule. In one study, an increase of HBD-2 mRNA expression was found in tissue biopsies from subjects with gingivitis and periodontitis.<sup>23</sup> Also, it has been shown that *P. gingivalis* induces the expression

of HBD-1,<sup>29</sup> and HBD-2<sup>30</sup> in cultured human gingival epithelial cells (HGEC), while *Fusobacterium nucleatum* and *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*) stimulate the production of HBD-2.<sup>23,31</sup> However, HBD response to *A. actinomycetemcomitans* was found to be strain-specific and variable between individuals.<sup>23,31</sup> Furthermore, HBD response was shown to be reduced in cells from a person with localized aggressive periodontitis.<sup>32</sup> The induction of these responses was not mediated by lipopolysaccharides, which propose that regulation of the induction of these mediators of innate immunity in the oral mucosa is controlled by more complex mechanisms of the innate immune system.

In the current study, the immune reaction was calculated as a surface area percentage rather than to quantify the immunostaining intensity. This was preferred for the following reasons: 1) Antigen-antibody reactions are not stoichiometric, hence, "darkness of stain" does not mean "amount of reaction products";<sup>26</sup> 2) the DAB does not follow the Beer-Lambert law,<sup>33</sup> where the brown DAB reaction product is not a true absorber of light, but a scatterer of light and has a broad, featureless spectrum; 3) differences in the thickness of the cut sections resulting from inadequate precision and accuracy of microtomes can result in deviations in stain intensity.

Obesity is the hallmark of the metabolic syndrome that leads to the increasing infiltration of proinflammatory immune cells into adipose tissue causing chronic, low-grade inflammation. Furthermore, cytokines and chemokines produced by immune cells influence localized and systemic inflammation.<sup>25</sup>

Obesity is a multisystem condition and considered a major contributor to several systemic diseases, as well as being suggested as a risk factor for periodontal disease.<sup>7,8</sup> Periodontal disease, which is an inflammatory disease, induce the production of proinflammatory cytokines, such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 and interleukin-6.<sup>8</sup> It has been proposed that the secretion of TNF- $\alpha$  by adipose tissue, triggered by lipopolysaccharides from periodontal gram-negative bacteria, promotes hepatic dyslipidemia and decreases insulin. Type 2 diabetes and decreased insulin sensitivity are associated with the production of advanced glycation end products (AGE), which trigger inflammatory cytokine production, thus predisposing to inflammatory diseases such as periodontitis. Consequently, in addition to being a risk factor for Type 2 diabetes and coronary heart disease, obesity-related inflammation may also promote periodontitis.

Conversely, periodontitis, once it exists, may promote systemic inflammation and thereby increase the risk of coronary heart disease.<sup>4</sup>

Currently, the mechanism clarifying the relationship between obesity and periodontal disease is poorly understood. This study looked at the expression of HBD as a possible mechanism, whereby the innate immune system affects the individuals' susceptibility to periodontitis in obese subjects. However, no differences were found between the expression of both HBD-1 and HBD-2 in the studied groups. One limitation to this study was that the sample size was small. Another is that only a semi-quantitative method was used to measure the expression of HBD in the studied samples.

In conclusion, the results of this study did not demonstrate an association between the expression of antimicrobial peptides (HBD-1 and HBD-2), and obesity and periodontitis. Further studies are warranted that include a larger sample size and use quantitative methods, such as real time PCR and flow cytometric analyses to compare the expression of HBD in obese and non-obese participants.

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