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ORIGINAL ARTICLE

Association between Volatile Sulfur Compounds *Prevotella intermedia* and Matrix Metalloproteinase-8 Expression

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ABSTRACT

Objective: To determine the correlation between levels of methyl mercaptan (CH_3SH) hydrogen sulfide (H_2S), the proportion of *Prevotella intermedia* (*Pi*), and matrix metalloproteinase-8 (MMP-8) gene expression levels in periodontitis patients accompanied by halitosis. **Material and Methods:** Samples were obtained from gingival crevicular fluid (GCF) in the deepest pocket and by swabbing in the tongue coating area in patients with periodontitis presenting with halitosis (n = 23) and healthy subjects as controls (n = 7). The values of CH_3SH and H_2S were obtained using Oral Chroma. The proportion of *Pi* and MMP-8 expression levels were evaluated using PCR-RT. All the result was statistically analyzed using SPSS software. **Results:** The levels of CH_3SH and H_2S in participants with PD ≥ 6 mm showed a robust negative correlation with the proportion of *P. intermedia* in GCF and tongue coating. No statistically significant association was detected between CH_3SH and H_2S levels and MMP-8 expression levels ($p>0.05$). **Conclusion:** There is no association between CH_3SH and H_2S levels, the proportion of *P. intermedia*, and MMP-8 expression in patients with periodontitis accompanied by halitosis.

Keywords: Halitosis; Periodontitis; *Prevotella intermedia*; Matrix Metalloproteinase-8.

Introduction

Periodontitis is a condition where inflammation affects the supporting structure of the tooth, which could be caused due to a specific group of microorganisms, leading to progressive damage in both the periodontal ligament and the alveolar bone. This damage is also accompanied by an increase in pocket depth (PD) during probing, recession, and both. Periodontitis could affect a person's quality of life, especially from the psychological aspect associated with halitosis. Halitosis is a condition manifested by an unpleasant odor arising from the oral cavity that can make a person less confident, thereby affecting his/her psychological condition. Halitosis is also recognized as bad oral breath or oral malodor [1,2].

The onset of periodontitis begins with microbial shifting from healthy oral microbes to being dominated by Gram-negative bacteria and obligate anaerobes. These two bacterial complexes that have an essential role in the development of periodontitis are red complex and orange complex bacteria, respectively, and are closely associated with periodontal PD [3,4].

Gram-negative anaerobic bacteria are the major producers of gas products that can cause halitosis, such as volatile sulfur compounds (VSCs) that can be toxic to the periodontal tissues and aggravate periodontal abnormalities [5,6].

Degradation of proteins from substrates containing sulfur, such as those in food debris, epithelial cells, or blood, could trigger the formation of VSCs, primarily in the form of methyl mercaptan (CH_3SH), hydrogen sulfide (H_2S), and dimethyl sulfide, which are gases that cause halitosis. The presence of periodontal abnormalities and the formation of periodontal pockets is one of the ideal environments for the development of sulfur-producing bacteria, which can produce VSCs that cause halitosis [5,7].

VSCs also influence the formation of the extracellular matrix from the fibroblast tissue. It has been demonstrated that both H_2S and CH_3SH can reduce protein production, reduce protein synthesis and increase tissue protein degradation, thereby playing a possible role in influencing periodontal tissue damage in patients with periodontitis. In addition, CH_3SH, together with LPS and IL-1ß, can increase the secretion of prostaglandin E2 and collagenase, which are essential mediators in the development of inflammation and tissue damage [5,6].

The metalloproteinase matrix is a part of a protease that functions in physiological development and tissue reformation and when the tissue is at risk of damage due to pathological inflammation. MMP-8 is the most predominant protease in the periodontal tissue that can reflect the severity, progressiveness, and response to the treatment of periodontitis. MMP-8 is a part of the collagenase family and is secreted through the infiltration of leukocyte PMNs, macrophages, plasma cells, and resident cells such as fibroblasts, keratinocytes, endothelial cells, and bone cells [8,9].

In Indonesia, to our knowledge, there has yet been no study investigating halitosis associated with periodontitis by directly analyzing the effects of CH_3SH and H_2S levels on the quantity of *Prevotella intermedia* bacteria and MMP-8 expression levels associated with the status of periodontal tissue damage in patients in periodontitis and halitosis.

Therefore, this study was conducted limitedly to determine the association between the levels of CH_3SH and H_2S, the proportion of *P. intermedia*, and MMP-8 gene expression levels in patients with periodontitis accompanied by halitosis.

Material and Methods

Study Design

In this cross-sectional study, the subjects were recruited from patients who visited the specialist Periodontics Education Clinic at the Universitas Indonesia Dental and Oral Hospital for treatment. Patients who complained of bad breath were tested for halitosis conditions and then assessed for the presence of periodontitis.

This study was conducted from May to June 2019 at the specialist Periodontics Education Clinic at the University of Indonesia Dental and Oral Hospital. This study began with an interview process on the patients' halitosis condition, followed by an in-depth history-taking, intraoral examination, and halitosis examination using Oral Chroma™, after which the patients' gingival crevicular fluid (GCF) and tongue coating samples were collected.

Sampling

The sample size for this study was determined using the formula {(Z∝+Zβ)/(0.5 ln((1+r)/(1-r))}^2 with r as a correlation coefficient defined as 0.88 [10], which resulted in a minimum sample number of 24 for three groups. The GCF and tongue coating samples were collected from 30 participants aged 17–55 years, comprising 8 (27%) males and 22 (73%) females. The proportion of GCF taken from a healthy sulcus (<4 mm) was as much as 7 samples (23.3%) as the healthy control subject, medium pocket (4–5 mm) as much as 16 samples (53.3%), and deep pocket (>5 mm) as much as 7 samples (23.3%).

Participants aged >55 years who had a history of systemic diseases, smoking habits, tonsillitis, gastrointestinal disorders, antibiotic usage in the past 3 months, and received periodontal treatment during the past 3 months were excluded from the study.

Data Collection

First, all the study participants were interviewed for an in-depth history-taking associated with the condition of halitosis and their oral health. Than, they were asked not to speak or open their mouth for approximately 3 min. Then, an air sample was collected from the patient's mouth to measure the levels of CH_3SH and H_2S using Oral Chroma™ (Abimedical, Abilit Corporation, Osaka City, Japan). Next, clinical measurements of the periodontal condition of the participants were conducted using pocket depth (PD) and the scope of the coating area on the tongue. Finally, pocket depth measurements were performed using the UNC 15 Periodontal Probe (Osung Mnd Co. Ltd, Gimpo-si, Korea).

After conducting a clinical examination, GCF samples were collected from the deepest pocket of each respondent. The tooth's surface was first dried and carried out with a cotton roll. Then, 3 (numb 30) paper points were inserted into the part of the gingival sulcus that had the deepest pocket for approximately 15–30 s. Next, tongue coating samples were carried out using a sterilized cotton swab. All these samples were inserted into Eppendorf tubes, each containing 1000 µl of Ringer's solution, which were immediately stored at −20 °C for further analysis in the Oral Biology Laboratory (Lab-OB) Faculty of Dentistry, Universitas Indonesia.

Total DNA and RNA were extracted using GENEzol™ Reagent (Geneaid Biotech Ltd., New Taipei City, Taiwan). The extracted RNA was then reverse-transcribed using TaqMan Reverse Transcription Kit (Applied Biosystems, USA), which resulted in cDNA. The concentration of these cDNA samples was then estimated using a Qubit® 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). The cDNA was then amplified by RT-PCR using MMP-8 primers shown in Table 1 and SYBR Green Master Mix (Applied Biosystems, Waltham, MA, USA) according to the protocol set by the manufacturer with the PCR conditions as follows: predenaturation stage at 95 °C for 10 min, followed by 40 cycles of amplification at 95 °C for 30 s

with an annealing cycle at 61 °C–62 °C for 1 min and 72 °C for 30 s. For the melt curve profile-making phase, the PCR conditions were set at 95°C for 15 s, 60 °C for 60 s, and 95 °C for 15 s.

Table 1. Sequences of *Prevotella intermedia*, 16s RNA, MMP-8, and GAPDH primers.

Primer Name	Sequences	Reference
Prevotella intermedia	Forward: 5′-CAG CAC CCA CAA CGA TAT GA-3′ Reverse: 5′-TTC CAC CTT CTC TGC CTG TC-3′	[11]
16s RNA	Forward: 5′-TTA AAC TCA AAG GAA TTG ACG G-3′ Reverse: 5′-CTC ACG ACA CGA GCT GAC GAC-3′	[12]
MMP-8	Forward: 5′-GCT GCT TAT GAA GAT TTT GAC AGA G- 3′ Reverse: 5′-ACA GCC ACA TTT GAT TTT GCT TCA G-3′	[13]
GAPDH	Forward: 5′-CCC AAA ACA TCA TCC CAT CTT C-3′ Reverse: 5′-GGA ACA CGG AAC GCC ATA-3′	[14]

On the other hand, after the process of total DNA extraction, the concentration was estimated using a Qubit® 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and then analyzed in the ABI StepOnePlus Real-Time PCR System using SYBR Green PCR Master Mix (Applied Biosystems, Waltham, MA, USA) and *P. intermedia* primers as shown Table 1, according to the protocol set by the manufacturer. The PCR conditions were as follows: predenaturation at 95 °C for 2 min, followed by 40 amplification cycles at 95 °C for 5 s with an annealing cycle at 57 °C for 10 s and 72 °C for 15 s. For the melt curve profile-making phase, the PCR conditions were 95 °C for 15 s, 60 °C for 60 s, and 95 °C for 15 s. The $2^{-\Delta\Delta CT}$ method was used for analyzing the proportion of *P. intermedia* and the relative gene expression level of MMP-8 [15].

Data Analysis

The data were analyzed using the SPSS software program (IBM SPPS Corp., Chicago, USA). The data between participants with PD 4–5 mm and those with PD >5 mm were analyzed using the Mann-Whitney test to determine any significant difference in CH_3SH and H_2S levels, the proportion of *P. intermedia*, and the gene expression levels of MMP-8. In addition, Spearman's correlation test was used to determine the correlation between CH_3SH and H_2S levels, the proportion of *P. intermedia*, and the gene expression levels of MMP-8 for each group. The results were considered to be significant when $p < 0.05$.

Ethical Clearance

This study was approved by the Ethical Committee of Dental Research (KEPKG), Faculty of Dentistry, Universitas Indonesia (Protocol number 090460419). Furthermore, all research participants agreed and signed an informed consent before the study was conducted.

Results

The mean levels of CH_3SH (2.44 ng/ml) and H_2S (5.66 ng/ml) in participants with PD >5 mm were higher than those in participants with PD 4–5 mm (0.76 and 1.35 ng/ml, respectively).

The level of CH_3SH and H_2S score among participants is presented in Table 2. Results of the Mann–Whitney test revealed a significant difference ($p < 0.005$) in H_2S score among participants with PD 4–5 mm and those with PD >5 mm, shown in Table 2, where the levels were higher in those with PD >5 mm than in the other group (median 0.4700 (0.01–8.88) ng/ml). On the other hand, there were no statistically significant differences in CH_3SH levels between participants with PD 4–5 mm and those with PD >5 mm ($p>0.05$).

Table 2. The level of methyl mercaptan and hydrogen sulfide score between participants with pocket depth 4–5 and those with pocket depth >5 mm.

Variables	Median [Min–Max]	p-value
Methyl Mercaptan Score		
Pocket depth 4–5 mm	0.27 [0.01-2.33]	
Pocket depth >5 mm	0.47 [0.01-8.88]	0.55
Hydrogen sulfide Score		
Pocket depth 4–5 mm	0.80 [0.07-6.40]	0.03
Pocket depth >5mm5 mm	2.30 [0.28-23.15]	

The proportion of *P. intermedia* in GCF between participants is shown in Table 3. The proportion of *P. intermedia* in participants with PD 4–5 mm was 12.5 times relatively higher than that in control subjects. Participants with a deeper pocket (>5 mm) had 2.3 times relatively higher *P. intermedia* proportion than control subjects. The median value was used for descriptive statistics as the data were nonparametric. The maximum score for *P. intermedia* proportion in participants with PD >5 mm was 6710 times higher than in control subjects. The Mann-Whitney test results revealed no statistically significant difference in *P. intermedia* proportion in GCF between participants with PD 4–5 mm and those with PD >5 mm (p>0.05).

Table 3. The proportion of *Prevotella intermedia* in GCF between participants with pocket depth 4–5 mm and those with pocket depth > 5mm.

Variables	Median [Min–Max]	Normality Test	p-value
Pocket depth 4–5 mm	12.49 [0.06-1004.06]	<0.05	0.79
Pocket depth >5 mm	2.27 [0.01-6709.71]		

The proportion of *P. intermedia* in the tongue coating of participants with PD 4–5 mm was five times higher than in control subjects. In those with PD >5 mm, the proportion of *P. intermedia* in the tongue coating was 29 times higher than that in control subjects. There was no statistically significant difference (p>0.05) in *P. intermedia* proportion in GCF between participants with PD 4–5 mm and those with PD >5mm according to the Mann-Whitney test results, as shown in Table 4.

Table 4. The Proportion of *Prevotella intermedia* in tongue coating between participants with pocket depth 4–5 mm and those with pocket depth >5 mm.

Variables	Median [Min–Max]	Normality Test	p-value
Pocket depth 4–5 mm	5.04 [0.02-550.16]	<0.05	0.39
Pocket depth >5 mm	28.64 [1.73-566.05]		

The expression level of MMP-8 in GCF between participants is shown in Table 5. The relative MMP-8 gene expression level in GCF in participants with PD 4–5 mm was decreased by 0.02-fold compared to that in the control group. However, the relative MMP-8 gene expression level in those with PD >5 mm was increased by 1.33-fold compared to that in the control group. The Mann-Whitney test results showed no significant differences in MMP-8 gene expression levels between those with PD 4–5 mm and participants with PD >5 mm, but a higher gene expression level was detected in the latter group (median 1.33).

Table 5. The expression level of MMP-8 in GCF between participants with pocket depth 4–5 mm and those with pocket depth >5 mm.

Variables	Median [Min–Max]	Normality Test	p-value
Pocket depth 4–5 mm	0.02 [0.00-0.86]	<0.05	0.29
Pocket depth >5 mm	1.33 [0.00-5.38]		

The correlation between CH_3SH, H_2S levels, the proportion of *P. intermedia* and the relative expression gene level of MMP-8 is presented in Table 6. The correlation between CH_3SH and H_2S with the proportion of *P. intermedia* in GCF was extremely weak and negative, with no statistical significance ($p>0.05$) in participants with PD 4–5 mm. However, this correlation in the tongue coating was very strong and positive, although still lacking statistical significance between CH_3SH and proportion of *P. intermedia*. Furthermore, the correlation between H_2S and the proportion of *P. intermedia* was moderate and positive. The negative correlation implies that an increase in methyl mercaptan or hydrogen sulfide levels can decrease the proportion of *P. intermedia* and vice versa. A negative and moderate correlation was also found between CH_3SH and H_2S levels with the relative gene expression of MMP-8, but it was not statistically significant.

Table 6. Correlation between methyl mercaptan and hydrogen sulfate levels with the proportion of *Prevotella intermedia* and relative expression gene level of MMP-8 in participants with pocket depth 4–5 mm.

Variables	Correlation (*r*)*	p-value**
Methyl Mercaptan Level		
Proportion of *Prevotella intermedia* in GCF	-0.21	0.44
Proportion of *Prevotella intermedia* in Tongue Coating	0.94	0.73
MMP-8 Relative Gene Expression	-0.34	0.20
Hydrogen Sulfate Level		
Proportion of *Prevotella intermedia* in GCF	0.04	0.88
Proportion of *Prevotella intermedia* in Tongue Coating	0.27	0.32
MMP-8 Relative Gene Expression	-0.37	0.16

*With Spearman's Correlation Test; **Significance level $p<0.05$

Table 7 shows the correlation between variables in participants with PD > 5mm. The correlation between CH_3SH level and the proportion of *P. intermedia* in GCF and tongue coating was very strong, negative, and statistically significant ($p<0.05$) in participants with PD >5 mm. A similar association was found between the H_2S level and the proportion of *P. intermedia* in GCF. On the other hand, the correlation between H_2S level and the proportion of *P. intermedia* in the tongue coating of participants was found to be strong, negative, but not statistically significant. The correlation between CH_3SH and H_2S levels with the relative gene expression of MMP-8 was also very weak and not statistically significant.

Table 7. Correlation between methyl mercaptan and hydrogen sulphate level with proportion of *Prevotella intermedia* and relative gene expression of MMP-8 in pocket depth > 5mm.

Variables	Correlation (r)	p-value
Methyl Mercaptan Level		
Proportion of Prevotella intermedia in GCF	-0.79*	0.03**
Proportion of Prevotella intermedia in Tongue Coating	-0.87*	0.01**
MMP-8 Relative Gene Expression	-0.09	0.85
Hydrogen Sulfide Level		
Proportion of Prevotella intermedia in GCF	-0.93*	0.003**
Proportion of Prevotella intermedia in Tongue Coating	-0.64*	0.12
MMP-8 Relative Gene Expression	-0.14*	0.76

*With Spearman's Correlation Test; **Significance level $p<0.05$.

Discussion

In this study, three groups were investigated: control participants with no pocket or only healthy sulcus (1–3 mm), those with a medium PD of 4–5 mm, and finally, those with a deep PD of >5 mm. CH_3SH and H_2S levels were significantly different in participants with PD 4–5 mm compared to those with PD >5 mm. However, regarding the proportion of *P. intermedia* in the GCF and tongue coating and the relative gene

expression level of MMP-8, there was no statistically significant difference between participants with PD 4–5 mm and those with PD >5 mm.

There was also no significant correlation between methyl mercaptan and hydrogen sulfide levels with the proportion of *P. intermedia* in the GCF and tongue coating and also with the relative gene expression level of MMP-8 in those with PD 4–5 mm. However, in participants with PD >5 mm, there was a statistically significant and very strong negative correlation between methyl mercaptan level and the proportion of *P. intermedia* in the GCF and tongue coating and also between hydrogen sulfide level and the proportion of *P. intermedia*.

Makino et al. [16] reported a significant correlation between the concentration of volatile sulfur compound gases and clinical attachment loss, especially in participants with pocket depth ≥4 mm, which indicated the presence of a relationship between periodontal disease condition and levels of volatile sulfur compounds.

Takeshita et al. [17] evaluated 240 subjects with a mean PD of 3.4 mm and found that *Prevotella* species had a low capacity to produce CH_3SH or H_2S. Even the primary producer, such as *Porphyromonas* or *Fusobacterium*, is not the only primary producer that significantly impacts on CH_3SH and H_2S production. This is because CH_3SH and H_2S are primarily produced by degrading food debris, serum, and other substances with the breakdown of acids containing sulfur. The primary producer was also found to be minorities compared with the microbiota that produced CH_3SH and H_2S [17].

This finding explains why in participants with PD 4–5 mm, there was no significant and robust correlation between CH_3SH and H_2S levels and the proportion of *P. intermedia* in the GCF or even in tongue coating. This could also explain the strong negative correlation between CH_3SH and H_2S levels and the proportion of *P. intermedia*. Therefore, it needs a synergistic interaction between the bacterial population that could affect the production of CH_3SH and H_2S that might cause *P. intermedia* to be detected but has a negative correlation with the level of CH_3SH and H_2S.

A very strong correlation was also detected between CH3SH level and the proportion of *P. intermedia* in the tongue coating. Ren et al. found that when there is a deep fissure in the tongue, it could provide an anaerobic environment with low oxygen level so that it becomes an ideal place for anaerobic bacteria to grow, such as *P. intermedia* that was found to have a correlation with the level of methyl mercaptan [18].

PD has an effect on the correlation between CH_3SH and H_2S levels with the proportion of *P. intermedia*, as it was observed that in participants with PD >5 mm, there was a strong and negative relationship but statistically not significant. Therefore, the clinical attachment loss parameter could be used in further study to determine the strong association with MMP-8 relative gene expression.

In their meta-analysis study, Silva et al. [19] found that the criteria set for diagnosing periodontitis could affect the association between halitosis and periodontitis. Using clinical attachment loss in the study could provide more magnitude on the association than using only the PD [19]. This study used only PD as a basic measurement for assessing periodontal abnormalities and not clinical attachment loss. This could have also been the reason for the absence of a statistically significant correlation between the relative gene expression level of MMP-8 and the levels of methyl mercaptan and hydrogen sulfide in this study.

Conclusion

There was no statistically significant association between hydrogen sulfide and methyl mercaptan levels, the proportion of *P. intermedia*, and matrix metalloproteinase-8 (MMP-8) gene expression levels in

patients with periodontitis (PD 4–5 mm or >5 mm) presenting with halitosis. This finding also indicates that hydrogen sulfide and methyl mercaptan levels could not be used to describe the proportion of *P. intermedia* and MMP-8 expression levels in patients with periodontitis accompanied by halitosis. Therefore, it might be better for future research to use clinical attachment loss as guidance for evaluating the periodontal status rather than PD.

Authors' Contributions

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All authors declare that they contributed to critical review of intellectual content and approval of the final version to be published.

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Conflict of Interest

The authors declare no conflicts of interest.

Data Availability

The data used to support the findings of this study can be made available upon request to the corresponding author.

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