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A Point-Of-Care Test of Active Matrix Metalloproteinase-8 (Ammp-8) Predicts Triggering Receptor Expressed on Myeloid Cells-1 (TREM-1) Levels in Saliva

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2,891 **Words**, 3 **Figures**, 1 **Table**, and 50 **References**

Short Running Title: POC test of aMMP-8 predicts TREM-1 levels in saliva

One-Sentence Summary: The present study validated usability of aMMP-8 point-of-care test for predicting pro-inflammatory profile in saliva of adolescents.

ABSTRACT

Background: This cross-sectional study aims to investigate if a point-of-care (PoC) test of active matrix metalloproteinase-8 (aMMP-8) predicts levels of inflammation amplifier TREM-1 and its putative ligand the neutrophil peptidoglycan recognition protein 1 (PGLYRP1) in saliva.

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Methods: Forty-seven adolescents, aged 15-17, were tested with aMMP-8 PoC test, which was followed by a full-mouth clinical examination of the assessment of periodontal, mucosal and oral health. TREM-1 and PGLYRP1 levels were analyzed by ELISA. The IFMA assay specific for aMMP-8 was used as the reference method.

Results: 14 saliva samples out of total of 47 samples showed positivity for aMMP-8 PoC test. Both the TREM-1 and the aMMP-8 (IFMA) levels were significantly elevated among the aMMP-8 PoC test positives compared to the PoC test negatives (P<0.05). Moreover, aMMP-8 levels assessed by IFMA showed a strong positive correlation with TREM-1 levels in saliva (r=0.777, P < 0.001). The number of periodontal pockets with ≥4mm was significantly lower among the adolescents with a negative aMMP-8 PoC test result and TREM-1 levels below 75 pg/mL (P<0.05). In contrast, adolescents with a positive aMMP-8 PoC test result (i.e. elevated aMMP-8 levels) together with elevated TREM-1 levels had significantly higher number of periodontal pockets with ≥4mm (P<0.001).

Conclusions: The present study validated usability of aMMP-8 PoC test for predicting "pro-inflammatory" salivary profile and periodontal health status in adolescents.

KEYWORDS: Biomarkers; Periodontitis; Triggering Receptor Expressed on Myeloid Cells-1; Matrix Metalloproteinases; Point-of-Care Testing

1 INTRODUCTION

Periodontitis is a common oral inflammatory disorder induced by impaired host response to periodontal pathogens leading to a loss of connective tissue and bone support.[1] Severe periodontitis, which eventually results in tooth loss, has prevalence of 5-20% in most adult populations worldwide.[1] Children and adolescents can also suffer from periodontal attachment loss.[2] According to Heikkinen et al. (2008)[3] prevalence of initial or early periodontitis (called subclinical periodontitis), where the clear clinical and x-ray manifestations of deterioration of periodontal health are yet lacking, was 10-15% among 15-16-year-olds of Kotka, Finland.

The triggering receptor expressed on myeloid cells-1 (TREM-1) is a cell surface receptor of the immunoglobulin superfamily expressed on monocytes, macrophages and neutrophils during inflammatory and infectious processes.[4] Yet, TREM-1 expression is not limited to myeloid cells as TREM family members have been detected also in epithelial cells e.g. in gingiva.[5] TREM-1 plays an important signaling role as it regulates the magnitude of the host's inflammatory response to bacteria, viruses and fungi.[4-8] Activation of TREM-1 leads to a cascade of intracellular signaling events resulting in inflammatory effects, such as enhanced cytokine production, activation and degranulation of neutrophils and phagocytosis.[9] In addition to the membrane-bound form of TREM-1, there also exists a soluble form of TREM-1.[10] Its concentration in saliva, gingival crevicular fluid

(GCF) and serum has been shown to be risen at the presence of periodontitis, proposing it to be a potential biomarker for periodontitis in adults.[11-14]

More recently, the neutrophil peptidoglycan recognition protein 1 (PGLYRP1) has been identified as a functional ligand for TREM-1 and capable of inducing TREM-1 activation.[15] PGLYRP1 is known as an antibacterial protein against Gram-positive and Gram-negative bacteria.[16] Other ligands for TREM-1 include high-mobility group box 1 protein (HMGB1), heat shock protein 70 (hsp 70), a ligand expressed on platelets, extracellular actin, as well as bacterial peptidoglycans and lipopolysaccharides.[17-21]

There are studies that have identified proteolytic matrix metalloproteinases (MMPs) to be responsible for processing membrane bound TREM-1 to its soluble form.[22,23] Activated MMP-8 (aMMP-8) is already known as one of the most validated periodontitis biomarkers.[24,25] Several studies have reported that the progression of periodontitis is reflected as an excessive elevation and activation of total MMP-8 and aMMP-8 in oral fluids.[24-35] This stage can be measured using a point-of-care (PoC) lateral flow collagenase-2 (aMMP-8) immunotest.[24,29,30,36-46] Moreover, previously there have been found a moderate positive association / correlation between MMP-8/PGLYRP1 axis and TREM-1 levels among adults,[14] but to the best of author's knowledge, this association hasn't been studied among adolescent subjects before. A non-invasive, quick, easy to use and inexpensive aMMP-8 PoC test, which is currently available also as a quantitative point-of-care aMMP-8 test, may be also a potential tool for point of care detection of salivary TREM-1 levels and as such for detection of periodontal and systemic inflammation. Thus, our aim was to study whether the concentrations of TREM-1 and its natural ligand PGLYRP1 could be linked to the aMMP-8 PoC/ chairside test result. We used the aMMP-8 IFMA levels as a reference method. In this regard, our hypothesis was that the salivary concentrations of TREM-1 and PGLYRP1 has a positive association with the aMMP-8 PoC test result and the aMMP-8 (IFMA) levels. Another hypothesis was that the aMMP-8 PoC test has a positive association with the number of ≥4 mm deep periodontal pockets.

MATERIALS AND METHODS

1.1 Study cohort

Forty-seven patients of a birth cohort of 15- to 17-year-olds participated in this cross-sectional study, which was carried out in Kotka Health Center, Kotka, Eastern Finland in 2014 and 2015. One participant had been excluded due to gender check.[29,30] The study was approved by the Ethical Committee of the Helsinki and Uusimaa Hospital District (Diary number 260/13/03/00/13) and was conducted according to the principles of the Declaration of Helsinki of 1975, as revised in 2000.[47] All participants (their parents/guardians) gave their written informed consent and were examined for a full-mouth clinical examination of periodontal, mucosal and oral health as well as health habits as described earlier by Heikkinen et al.[29,30] In detail, periodontal probing depth (≥4 mm), bleeding on probing (BOP), root calculus (RC) and visible plaque index (VPI) were recorded at four sites per tooth. RC and VPI were recorded from index teeth.[29,30] Additionally, patient's smoking history and oral hygiene habits were included in the dental health care system electronic records.

1.2 aMMP-8 PoC testing

Saliva samples were collected and the aMMP-8 PoC test was performed as described earlier. [29,30,45] The same person (AMH) performed all the clinical measurements and the collection of saliva samples. Briefly, aMMP-8 PoC test result was read in 5-7 minutes as a color change resulting from immunoreactions after placing maximum of 4 drops of mouthrinse in the lateral flow immunoassay system by a trained assistant. One single blue line on the test device indicated a negative test result, and no risk for active periodontal tissue destruction and periodontitis; while two blue lines indicated a positive test result, and an increased risk for active periodontal tissue destruction, and periodontitis.[29,30,46] An independent consulting company has defined and validated the cut-off point (20 ng/mL) of the aMMP-8 lateral flow point-of-care test before it was made available commercially.[29,39,40,44,46] AMH was unaware of the aMMP-8 PoC test result when performing each clinical examination. Saliva samples were further analyzed for the presence and the levels of aMMP-8 (IFMA), TREM-1 and PGLYRP1 as described earlier by Nylund et al. (2018).[14] Moreover, MMP-8 concentration was determined in accordance with the manufacturer's instructions by time-resolved immunofluorometric assay (IFMA, Medix Biochemica, Espoo, Finland) and lateral flow chairside/point-of-care immunoassay utilizing the same antibody.[33,44-46,48] The inter-assay coefficient of variation (CV)% was 7.3% (n=28) and the detection limit was 0.08 µg/L for the assays in IFMA.[33,36,48] Information regarding aMMP-8 (IFMA) was available for 47 patients, and TREM-1 and PGLYRP1 for 43 patients.

1.3 Statistical analysis

The relationship among the concentrations of TREM-1, PGLYRP1 and aMMP-8 was assessed by Spearman rank correlations (r_s). Normality assumption was assessed graphically with a histogram and a Q–Q plot, and numerically with the Shapiro-Wilk test. As a result, Mann-Whitney U test was used for testing the association between two groups of aMMP-8 Point-of-Care (PoC) test results ("positive", "negative") on aMMP-8, TREM-1 and PGLYRP1 levels. Kruskal Wallis test was used for testing differences in the number of ≥4 mm deep periodontal pockets, BOP, VPI, RC, and toothbrushing frequency per week between the groups classified by the aMMP-8 PoC test result, and TREM-1 levels together. Pairwise testing was adjusted with Bonferroni correction. Data analysis was performed, and figures plotted with statistical software. [‡,§] A two-tailed *P* value less than 0.05 was considered as statistically significant.

2 RESULTS

2.1 Association between the aMMP-8 PoC test and aMMP-8 (IFMA), TREM-1, and PGLYRP1

Figure 1 illustrates the concentrations of aMMP-8 (IFMA), TREM-1 and PGLYRP1 according to aMMP-8 PoC test result. The median concentrations of aMMP-8 (IFMA) were 103.4 ng/mL (interquartile range [IQR], 56.7 to 211.8), TREM-1 145.2 pg/mL (IQR, 75.0 to 400.7) and PGLYRP1 6.1 ng/mL (IQR, 1.5 to 7.5) for the aMMP-8 PoC test negatives, and aMMP-8 (IFMA) 207.9 ng/mL (IQR, 131.0 to

[‡] SPSS Statistics, Version 25.0. Armonk, NY: IBM Corp

[§] R version 3.3.3: A language and environment for statistical computing. Vienna, Austria; 2017. Available at: https://www.R-project.org/.

235.4), TREM-1 407.6 pg/mL (IQR, 254.2 to 610.5) and PGLYRP1 6.9 ng/mL (IQR, 4.1 to 25.2) for the aMMP-8 PoC test positives (Figure 1). The concentrations of TREM-1 and aMMP-8 (IFMA) were statistically significantly elevated among the aMMP-8 PoC test positives compared to the test negatives (P = 0.016 and P = 0.018, respectively), whereas the difference in PGLYRP1 concentrations according to the aMMP-8 PoC test was not significant (P = 0.242).

2.2 Correlation analysis among aMMP-8 (IFMA), TREM-1 and PGLYRP1

There seemed to be a possible nonlinear relationship between TREM-1, PGLYRP1 and aMMP-8 (IFMA) concentrations, which was confirmed with Spearman's rank correlations (Figure 2). There was a strong positive correlation between TREM-1 and aMMP-8 (IFMA) concentrations (r_s = 0.777, P < 0.001), a moderate positive correlation between PGLYRP1 and aMMP-8 (IFMA) (r_s = 0.526, P < 0.001), and also a strong positive correlation between TREM-1 and PGLYRP1 (r_s = 0.663, P < 0.001) (Figure 2). Thus, the association between TREM-1 and the aMMP-8 PoC test result was verified with aMMP-8 (IFMA).

2.3 Association of TREM-1 and the aMMP-8 PoC test result with the number of ≥4mm periodontal pockets

All of the adolescents with TREM-1 levels below 75 pg/mL (n = 7) were in safe zone having less than two ≥4mm periodontal pockets (six adolescents with zero pockets and one adolescent with one pocket). In other words, a cut-off point of 75 pg/mL for TREM-1 resulted in sensitivity of 100%. However, the specificity of this cut-off point was only 31.8%. Figure 3 illustrates the number of at least two ≥4mm periodontal pockets against aMMP-8 PoC test result clustered according to TREM-1. Median of the number of ≥4mm periodontal pockets for the aMMP-8 PoC test negative adolescents was zero pockets (IQR, 0 to 0) for TREM-1 levels below 75 pg/mL (n = 7) and a one pocket (IQR, 0.0 to 2.0) for TREM-1 levels ≥75 pg/mL (n = 22), and for the aMMP-8 PoC test positive adolescents the median was eleven pockets (IQR, 5.8 to 15.3) for TREM-1 levels ≥75 pg/mL (n = 14). There were zero aMMP-8 PoC test positive adolescents with TREM-1 levels below 75 pg/mL. The number of ≥4mm periodontal pockets was significantly lower among adolescents with an aMMP-8 PoC negative test result and TREM-1 levels below 75 pg/mL (n = 7) compared to adolescents with TREM-1 levels ≥75 pg/mL and a positive test (n = 14) result (P < 0.001). Further, adolescents with TREM-1 levels ≥75 pg/mL had significantly less ≥4mm periodontal pockets when the aMMP-8 PoC test was negative compared to adolescents with a positive test (P < 0.001). But the difference in the number of ≥4mm periodontal pockets was not significant (after Bonferroni correction) between adolescents classified by the TREM-1 cut-off of 75 pg/mL with a negative aMMP-8 PoC test result. There was not a statistically significant difference in the medians of BOP%, VPI%, RC% when classified by the aMMP-8 PoC test result, and TREM-1, and the cut-off point of 75 pg/mL (p>0.05). However, the medians were markedly different for adolescents with a positive aMMP-8 PoC test result compared to adolescents with a negative test result (with either TREM-1 levels over or under the cut-off of 75 pg/mL) (Table 1).

2.4 Association of TREM-1 and the aMMP-8 PoC test result with oral hygiene habits

Finally, there was not a statistically significant difference in the medians of toothbrushing frequency per week when classified by the aMMP-8 PoC test result, and TREM-1, and the cut-off point of 75 pg/mL. (Table 1).

3 DISCUSSION

The present study is the first to investigate association between salivary concentrations of TREM-1, its natural ligand PGLYRP1 and the aMMP-8 PoC/ chairside test result among adolescents. Firstly, we found a significant association between the aMMP-8 PoC test result and the concentrations of TREM-1 and aMMP-8 (IFMA), but not between the aMMP-8 PoC test result and the concentration of PGLYRP1. Both the TREM-1 and the aMMP-8 (IFMA) levels were significantly higher among the aMMP-8 PoC test positives compared to the test negatives. Previous studies have found TREM-1 to be a potential risk indicator, in addition to periodontitis,[12] in different systemic inflammatory diseases such as in human sepsis and pneumonia.[10,19,49,50] Further, Bostanci et al.[12] found a significant, moderate positive correlation between the salivary and serum concentrations of TREM-1 among systemically healthy periodontitis patients and their healthy controls. Thus, a non-invasive, quick, easy to use and inexpensive aMMP-8 PoC test that is currently available also as a quantitative point-of-care aMMP-8 test seems to be a potential tool for point of care detection of systemic TREM-1 levels and as such detection of systemic inflammatory diseases.

Secondly, there was also a strong positive correlation not only between the concentrations of TREM-1 and PGLYRP1, which probably results from PGLYRP1 being a functional ligand for TREM-1,[15] but also between the concentrations of TREM-1 and aMMP-8 (IFMA). As the aMMP-8 (IFMA) levels increased this seemed to result in an exponential increase in the TREM-1 levels verifying the association between the aMMP-8 PoC test and the TREM-1 levels. Further, this suggests that there may be an aMMP-8 cleavage site in TREM-1 that allows aMMP-8 to process or shed membrane-bound TREM-1 to its soluble form. Although the approach in our study cannot directly reveal the cause–effect relationship between aMMP-8, TREM-1 and its soluble form, the result is in agreement with the findings of Gómez-Piña et al. (2007)[22] that one or several of the proteolytic matrix metalloproteinases (MMP-1, MMP-2, MMP-3, MMP-8 and MMP-9) are responsible for this cleavage or shedding process of TREM-1. Also, a recent study identified a MMP-9 cleavage site in TREM-1 and that the increase of MMP-9 in bronchoalveolar lavage fluid reflects MMP-dependent release of TREM-1.[23] Moreover, we found also a moderate positive correlation between the aMMP-8 (IFMA) and PGLYRP1 levels, but the functional form of this relationship was not as clear and strong as between aMMP-8 (IFMA) and TREM-1 levels in our cohort of adolescents.

Finally, in the current literature, the role of soluble TREM-1 is still unclear, but previous studies have proposed it acting as a negative regulator of the TREM-1 pathway by competing from the TREM-1 signaling receptor with ligand(s).[9] Thus, soluble TREM-1 may very well be regulating negatively the effect of aMMP-8 (and other MMPs) through preventing excessive activation of TREM-1 pathway. In the present study, we found that adolescents with low salivary TREM-1 levels had significantly fewer ≥4mm periodontal pockets compared to adolescent with elevated TREM-1 levels (over a safe zone cut-off point of 75 pg/mL). However, this difference in TREM-1 levels was not significant anymore after adjusting for multigroup comparisons. Further, the number of ≥4mm periodontal pockets were significantly higher among adolescents with a positive aMMP-8 PoC test result compared to adolescents with a negative test result regardless of TREM-1 levels (with cut-off point of 75 pg/mL). Even though low TREM-1 levels seemed to be associated with low amount of ≥4mm periodontal (six adolescents with zero pockets and one adolescent with one pocket), there were also some adolescents who had no pockets while having elevated TREM-1 levels, and also a negative aMMP-8 PoC test result. This suggests that low levels of TREM-1 may be a good thing for periodontal health,

and therefore the true value of TREM-1 for periodontal diagnostics is mainly in assessing the absence of disease. The positive aMMP-8 PoC test result (indicating elevated aMMP-8 levels) seemed to have a greater association to periodontal health situation among adolescents than elevated TREM-1 levels. The subclinical periodontal destruction and the progression towards periodontitis seem to be more strongly associated with the positive aMMP-8 PoC test result, in other words, with elevated concentrations of aMMP-8.

This study has some limitations mainly related to the sample size. Our original aim was to get a whole birth cohort to participate in this study, but the inclusion of subjects for the aMMP-8 PoC testing (chairside) were based on approval of parents/guardians.[29,30,45] Total of 47 adolescents´s parents gave consent for the test, which may lead to limit the power of statistical tests to avoid the type two error. For example, the difference between PGLYRP1 levels according to the aMMP-8 PoC test result was not significant, even though there was a significant correlation between aMMP-8 (IFMA), TREM-1 and PGLYRP1 levels. However, the correlation between PGLYRP1 and aMMP-8 (IFMA) was not as clear and strong as between TREM-1 and aMMP-8 (IFMA). Still, we cannot exclude the possibility that the association between PGLYRP1 and the aMMP-8 PoC test exists. Also, there were some extreme TREM-1 and PGLYRP1 values, yet they were rare (7-10%), and their influence on the significance tests was minimized by using the non-parametric tests. Further, there were a few unexpectedly large values for aMMP-8 (IFMA) among adolescents with a negative aMMP-8 PoC test, when considering the plaque status, the number of deep periodontal pockets and the oral hygiene habits. Moreover, we found that the aMMP-8 PoC test had a significant association with BOP and VPI, while aMMP-8 (IFMA) did not (calculations not shown here). This suggests that there may have been some measurement errors related to aMMP-8 (IFMA) instead of false negative aMMP-8 PoC test results. Yet, it should be noted that although immunofluorometric assay and lateral immunoassay utilized the same antibody, [33,44-46,48] they still are based on different kinds of technologies, which may also explain some of this phenomenon.

4 CONCLUSIONS

In summary, our study demonstrates the usability of aMMP-8 PoC/ chairside test in predicting pro-inflammatory profile in saliva of adolescents within only 5-7 minutes non-invasively and causing no discomfort to patient. There was also a significant strong positive correlation between the aMMP-8 (IFMA) and the TREM-1 levels. As the aMMP-8 (IFMA) levels increased the TREM-1 levels seemed to increase in exponential manner, suggesting an aMMP-8 cleavage site in TREM-1 that allows aMMP-8 to process or shed membrane-bound TREM-1 to soluble TREM-1. Moreover, the effect of increasing aMMP-8 levels assessed by the aMMP-8 PoC test seemed to outweigh the effect of increasing TREM-1 levels to the deterioration of periodontal health. Nevertheless, due to the cross-sectional nature of this study, the results are not definitive, and more research with other populations and larger sample sizes is needed to confirm our results.

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CONFLICT OF INTEREST

The other authors report no conflicts of interest related to this study.

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FIGURE LEGEND

Figure 1: A) aMMP-8, **B)** TREM-1 and C) PGLYRP1 levels according to aMMP-8 Point-of-Care (PoC) test result. The box-and-whiskers plots denote the median, quartiles and extreme values. P values were obtained from Mann Whitney U test.

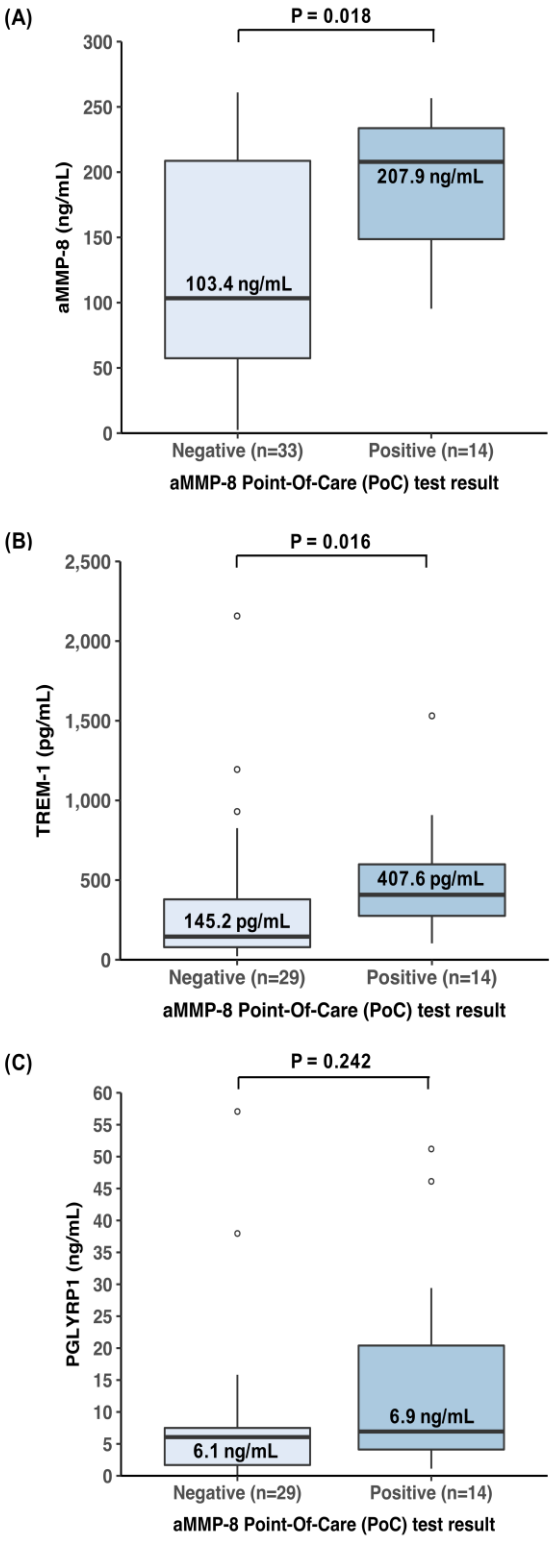


Figure 2: The relationship between **A)** TREM-1 and aMMP-8, **B)** PGLYRP1 and aMMP-8 and **C)** TREM-1 and PGLYRP1. Spearman's correlation coefficient (r_s) calculated for all three combinations. An exponential (first-order) fit for panels A and B and a linear fit for panel C with 90% confidence interval superimposed on the scatter plot was estimated by Generalized Linear Model.

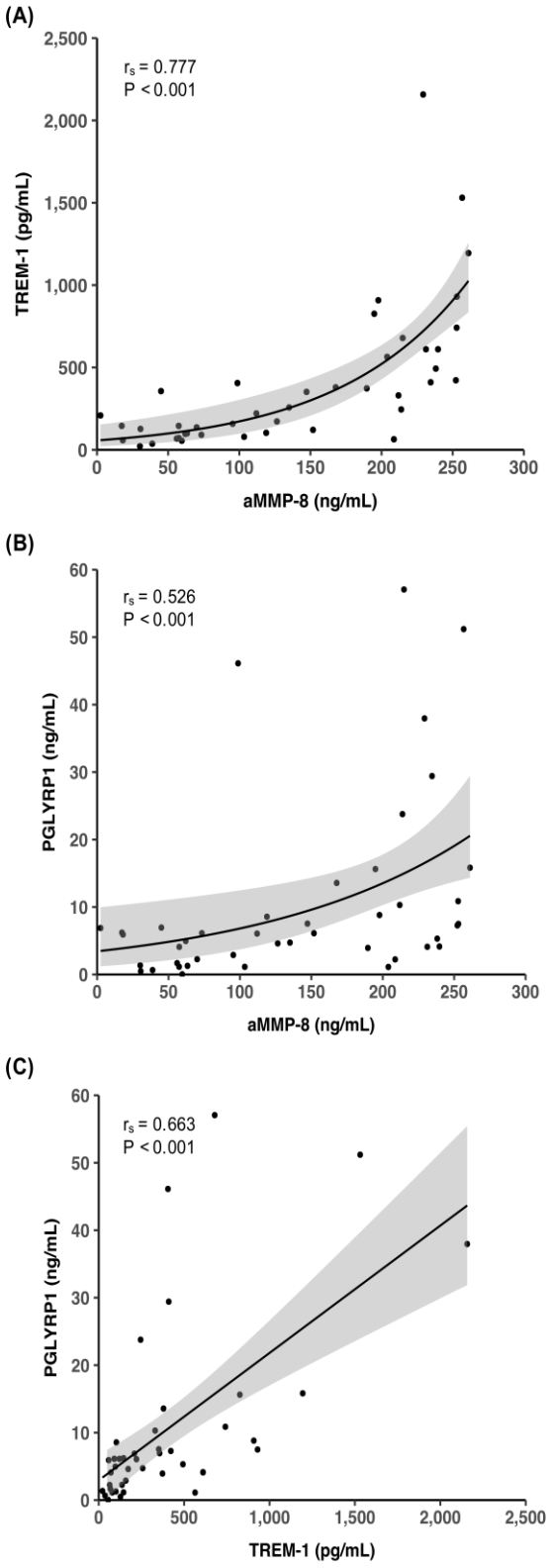
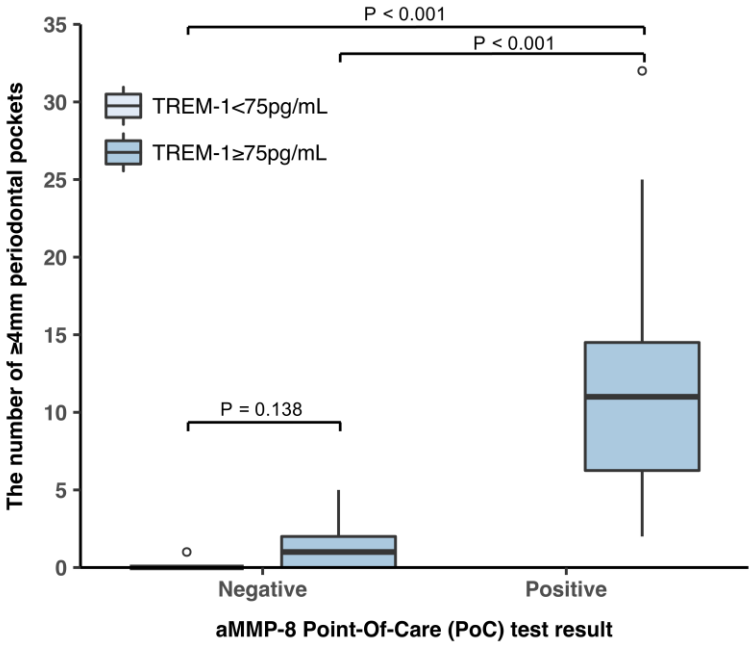


Figure 3: aMMP-8 Point-of-Care (PoC) test result ("positive", "negative") against the number of ≥4mm periodontal pockets (PD) clustered according to TREM-1's safe zone cut-off point of 75 pg/mL. The box-and-whiskers plots denote the median, quartiles and extreme values.



TABLES

Table 1: Periodontal inflammatory status (bleeding on probing, visual plaque index and root calculus) and oral health habits (toothbrushing) of the adolescents classified by the aMMP-8 PoC test result (positive negative) and TREM-1 (cut-off point 75 pg/mL).

	aMMP-8 PoC - (N = 29)		aMMP-8 PoC + (N = 14)		
	TREM-1 < 75 pg/mL (N = 7)	TREM-1 > 75 pg/mL (N = 22)	TREM-1 < 75 pg/mL (N = 0)	TREM-1 > 75 pg/mL (N = 14)	Kruskal-Wallis Test
	Median (IQR)	Median (IQR)	Median (IQR)	Median (IQR)	*P* value
BOP (%)	7.10 (1.80-11.10)	7.60 (3.00-13.83)	-	12.50 (8.63-33.05)	0.050
VPI (%)	10.70 (8.30-14.80)	8.95 (5.68-16.30)	-	19.05 (9.08-48.00)	0.117
RC (%)	3.60 (0.00-5.60)	0.00 (0.00-4.80)	-	3.65 (0.00-7.40)	0.296
Toothbrushing	14.00 (7.00-14.00)	14.00 (7.00-14.00)	-	10.50 (7.00-14.00)	0.891

BOP: bleeding on probing; VPI: visual plaque index; RC: root calculus; Toothbrushing: toothbrushing frequency per week.

aMMP-8 PoC - = aMMP-8 point-of-care test negative; aMMP-8 PoC + = aMMP-8 point-of-care test positive.