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Persistent Oral Human Papillomavirus (HPV) Infection is Associated with Low Salivary Levels of Matrix Metalloproteinase 8 (MMP-8)

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ABSTRACT

Background: A persistent human papillomavirus (HPV) infection is a prerequisite for a HPV related cancer to develop. Asymptomatic, persistent HPV infections are not only found in genital tract, but also on oral mucosa. Oral HPV persistence may be associated with behavioural factors, but data on the role of innate immunity in oral HPV infections are still limited.

Objectives: Salivary concentrations of matrix metalloproteinases MMP-8 and MMP-9, tissue inhibitor of MMPs (TIMP-1), myeloperoxidase, and serum concentrations of MMP-8 were analysed in women with a persistent oral HPV infection and, as a control, in women who remained HPV DNA-negative during a 6-year follow-up. The effects of smoking, lactation and alcohol use on the salivary and serum parameters were assessed, too.

Study design: A nested case-control setting was used to select a subgroup of 57 women with a persistent oral HPV infection and 102 controls from the Finnish Family HPV Study.

Results: The salivary MMP-8/TIMP-1 molar ratio was lower in HPV DNA-positive women than in controls ($p = 0.036$). The difference was more pronounced in non-smoking women, in this group also the salivary MMP-8 levels differed ($p = 0.047$). There was a correlation between the salivary concentrations of myeloperoxidase and MMP-8 ($r = 0.567$, $p < 0.001$) or MMP-9 ($r = 0.234$, $p = 003$), but no correlation between salivary and serum MMP-8 levels. The MMP-9 concentration and the MMP-9/TIMP-1 molar ratio were significantly lower in smokers than in non-smokers ($p = 0.020$ and $p = 0.003$, respectively).

Conclusions: Persistent oral HPV infection was associated with a low salivary MMP-8 concentration indicating eventually a failure in oral anti-inflammatory defence.

1. Background

Approximately 24% of oral cavity squamous cell carcinomas (SCC) are human papillomavirus (HPV) DNA positive [1]. However, the detection of HPV-DNA only is not a sufficient indicator of causality, and it has been estimated that the HPV attributable fraction in oral cavity SCC could be as low as 3.0 – 6.8%, depending on the biomarkers used [1,2]. Furthermore, HPV-involvement is highly dependent on, e.g., cancer site or geographical region [1,2]. A persistent HPV infection is a prerequisite for a HPV-related cancer to develop. Importantly, persistent HPV infections are found also on healthy appearing oral mucosa [3–7,8]. Oral HPV persistence may be associated with behavioural factors such as smoking or sexual behaviour [9], but both innate and adaptive immunity are important in HPV clearance [10]. Although the role of innate immunity is difficult to predict from natural history studies [11], *e.g.* increased expression of toll like receptor RNAs seems to be associated with enhanced HPV clearance [12,13]. However, data on the role of innate immunity in oral HPV infections are still limited.

Matrix metalloproteinases (MMP) are a family of genetically distinct but structurally related zinc dependent endopeptidases important in tissue turn over and repair as well as in innate host defence [14]. The main part of salivary MMP-8 and MMP-9 originates from polymorphonuclear neutrophils (PMN) via gingival crevicular fluid, but they are secreted also by other non PMN-lineage cell types, including *e.g.* MMP-8 secretion by oral fibroblasts [15] and MMP-9 secretion by gingival keratinocytes and acinar cells [16]. In addition to its physiological role, up-regulation of MMP-9 is often associated with tumour invasion. Specifically, MMP-9 is overexpressed both in epithelial cells

Abbreviations: HPV, human papillomavirus; MMP, Matrix Metalloproteinase; TIMP, tissue inhibitor of MMPs; HNSCC, head and neck squamous cell carcinomas; SCC, squamous cell carcinoma; PMN, polymorphonuclear neutrophil; MPO, myeloperixidsase; WHIM, warts hypogammaglobulinemia infections and myelokathexis; ER, oestrogen (estrogen) receptors

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and in stromal tissue of human oral SCC [17]. The E2 proteins of HPV8 and HPV16 up-regulate MMP-9 expression possibly via MEK1-ERK1/2 pathway or p38 interaction [18,19]. *In vitro* overexpression of HPV16 E6 and E7 up-regulate MMP-9 expression in cervical cancer cells [20]. Furthermore, HPV16 E7 increases the activity of MMP-9 *in vitro* [21]. In contrast, MMP-8 seems to have a protective role in a variety of cancers [22–26] and inflammatory diseases [27–29]. In a population based study, MMP-8 levels were positively associated with better disease specific survival of the patients with a tongue SCC [22]. Furthermore, MMP-8 knock-out mice develop tongue SCC at a higher incidence rate than wild-type mice [22]. The activities of MMPs are inhibited by tissue inhibitors of MMPs (TIMPs) and the imbalance between MMPs and TIMPs is connected with *e.g.* periodontal disease, acute and chronic cardiovascular diseases, and cancer progression [30,31]. Salivary TIMP-1 may originate from pure glandular saliva secretions [32], gingival crevicular fluid [33] or even from carious dentine [34]. Tissue over-expression or elevated levels of TIMP-1 in serum have been associated with poor prognosis of many tumour types, including HNSCC [35].

In addition to MMPs, saliva contains also other proteins which originate from the PMNs. One of them is myeloperixidsase (MPO) [36]. Peroxidases in saliva oxidise thiocyanate or (pseudo)halides in the presence of hydrogen peroxide and thereby protect oral tissues from oxygen toxicity. Importantly, the oxidation products have a wide antimicrobial activity and a role in regulation of inflammatory proteolytic processes [36,37].

2. Objectives

Our aim was to compare salivary levels of MMP-8, MMP-9, TIMP-1, and MPO as well as the serum levels of MMP-8 in women with a persistent asymptomatic HPV infection in the oral mucosa and in women who had always tested HPV DNA negative during a 6-year follow-up.

3. Study design

3.1. Study population

The participants were selected among the study population of the Finnish Family HPV Study (University of Turku and Turku University Hospital, Turku, Finland); see [38,39]. The original study protocol and its amendments (#3/1998 and #2/2006) have been approved by the Research Ethics Committee of Turku University Hospital, Turku, Finland. A written informed consent was obtained from all participants on their first visit. The original study population consisted of 331 mothers, 131 fathers and 324 infants, and for this study a nested case-control setting was used with a subgroup of 57 women with a persistent oral HPV infection and of 102 women who were always HPV DNA-negative during the six-year follow-up. Oral HPV persistence was defined as at least two HPV DNA-positive samplings (any HPV type) from the oral mucosa during the 6-year follow-up. The mean persistent time was 35.7 ± 24.3 months, range 4–72 months (any type), or 33.6 ± 24.3 months, range 2–72 months (type specific). The women were examined clinically six years after entry to the study and no signs of premalignant changes of oral mucosa were found. The sub-group of women selected for this study is described in detailed in [40].

Lactation may affect the salivary MMP-9 and TIMP-1 concentrations [41], but the lactation status was not recorded in the original study questionnaire. However, breast milk samples were collected at the 2 month visit. For this study, we considered women who had given a breast milk sample as lactating.

There was no difference between the HPV-positive and HPV-negative groups with regards to age, lactation, as estimated by the given breast milk sample, or alcohol use. There were more smokers among HPV DNA-positive women than among HPV DNA-negative women (63% vs 42% respectively, p = 0.025, Chi square test).

3.2. Collection of salivary and serum samples

The salivary and serum samples used in this study were collected two months post-delivery. Paraffin stimulated whole saliva was collected on ice, centrifuged (1500 x g), refrigerated and stored at −70° C. Blood samples were centrifuged at 2400 r.p.m. for 10 min (Sorvall GLC-2; DuPont Instruments) divided into 1 ml aliquots and stored first at −20 ° C for one week maximum and then at −70 ° C until analysed.

3.3. Collection of oral scrapings and HPV DNA detection

The collection of oral scrapings from the buccal mucosa, as well as the extraction of HPV-DNA and HPV-testing and genotyping were performed as described earlier [3,5,42]. Multimetrix kit® (Multimetrix, Progen Biotechnik GmbH, Heidelberg, Germany) detecting altogether 24 low and high risk HPV-genotypes was used for HPV genotyping.

3.4. Detection of MMP-8, MMP-9, TIMP-1 and MPO in saliva and serum samples

Salivary and serum MMP-8 levels were measured by using time-resolved immunofluorometric assay (IFMA) and monoclonal MMP-8 specific antibodies obtained from Medix Biochemica, Espoo, Finland as described earlier [15,41].

Salivary MMP-9, MPO and TIMP-1 levels were measured by using commercial enzyme-linked immunosorbent assay (ELISA)-kits as described earlier: MMP-9 Quantikine ELISA (R & D Systems, Minneapolis, MN, USA) [43], MPO ELISA (Immundiagnostik AG, Bensheim, Germany) [41] and TIMP-1 Amersham ELISA (Human, Biotrak, ELISA system, GE Healthcare, Amersham, Buckinghamshire, UK) [41].

Vertebrate TIMPs interact with MMPs in a 1:1 stoichiometry [44] and the molar ratios of MMP-8/TIMP-1 and MMP-9/TIMP-1 were determined by dividing the concentrations with the corresponding molecular weights, 65 000 Da for MMP-8, 92 000 Da for MMP-9 and 28 000 Da for TIMP-1.

3.5. Statistics

The differences between salivary concentrations of MMP-8, MMP-9, TIMP-1 as well as their molar ratios, and between the salivary and serum concentrations of MPO in HPV DNA-positive and HPV DNA-negative groups or with regards to smoking, lactation or alcohol use were compared with Mann-Whitney U-test. A non-parametric test was chosen since a part of the data was not normally distributed (quantile-quantile plots and Shapiro-Wilk test), not even after a logarithmic transformation. Two of the saliva samples were reddish. In the first one, the concentrations of all measured markers were in line with other samples, but the other sample had a high MPO level, indicating possible contamination by blood. All analyses were done also by leaving this sample out, but this did not influence the interpretation of the results. Pearson correlation coefficients were calculated for the relationships between salivary and serum MPO, and salivary MPO and MMP-8 after logarithmic transformation of the data. The level of statistical significance was set at 0.05.

4. Results

The salivary concentrations of MMP-8, MMP-9, TIMP-1 and MPO, and the salivary MMP-8/TIMP-1 and MMP-9/TIMP-1 ratios and the serum concentration of MMP-8 in HPV DNA-positive and HPV DNA-negative women are presented in Table 1. The salivary MMP-8/TIMP-1 molar ratio was lower in HPV DNA-positive than in HPV DNA-negative women (p = 0.036). Similar trend was seen in the MMP-8 levels, although this lacked statistical significance (p = 0.057). The salivary MMP-9, TIMP-1 or MPO concentrations, the MMP-9/TIMP-1 molar ratios, or the serum MMP-8 concentrations did not differ between the

Table 1
The salivary concentrations of MMP-8, MMP-9, TIMP-1 and MPO as well as the salivary MMP-8/TIMP-1 and MMP-9/TIMP-1 molar ratios and serum MMP-8 concentrations in oral HPV DNA-positive and oral HPV DNA-negative women. Mean ± SD.

	MMP-8 (ng/ml)	MMP-9 (ng/ml)	TIMP-1 (ng/ml)	MMP-8/TIMP-1 molar ratio	MMP-9/TIMP-1 molar ratio	MPO (ng/ml)	Serum MMP-8 (ng/ml)
HPV DNA-positive	205 ± 286	289 ± 221	354 ± 161	0.34 ± 0.65	0.32 ± 0.32	3431 ± 2551	34.9 ± 23.5
HPV DNA-negative	273 ± 292	350 ± 280	305 ± 128	0.51 ± 0.72	0.46 ± 0.52	3538 ± 2841	33.7 ± 23.8
p-value (HPV+/−, Mann-Whitney U test)	0.057	0.273	0.110	0.036	0.091	0.790	0.628

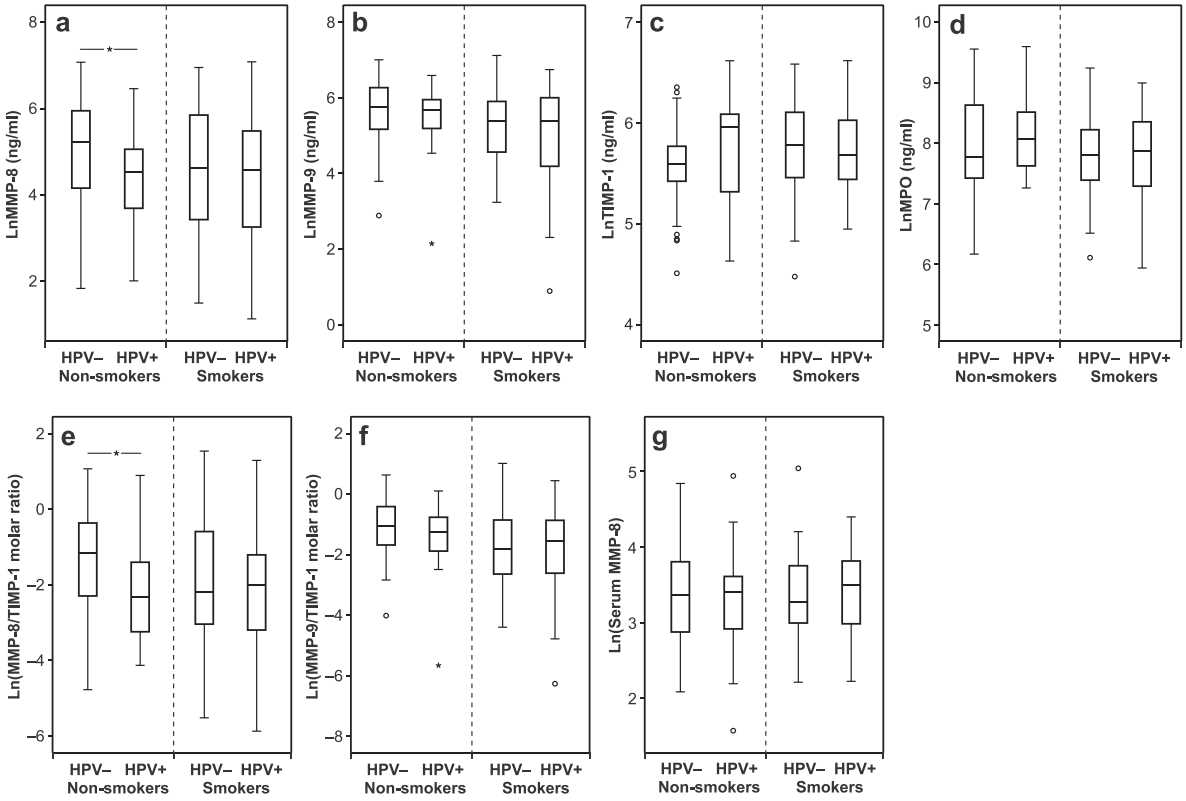

Fig. 1. The salivary concentrations of MMP-8 (a), MMP-9 (b), TIMP-1 (c) and MPO (d) as well as the molar ratios of MMP-8/TIMP-1 (e) and MMP-9/TIMP-1 (f) and the serum concentration of MPO (g) in HPV DNA-positive and HPV DNA-negative women with respect to their smoking status. *$p < 0.05$, Mann-Whitney U-test.

Table 2
The salivary concentrations of MMP-8, MMP-9, TIMP-1 and MPO as well as the salivary MMP-8/TIMP-1 and MMP-9/TIMP-1 molar ratios and serum MMP-8 concentrations with regards to lactation, alcohol use or smoking. Mean ± SD.

	Lactation		Alcohol use		Smoking	
	Yes (N = 101)	No (N = 58)	Yes (N = 135)	No (N = 14)	Yes (N = 74)	No (N = 75)
MMP-8 (ng/ml)	226 ± 264	287 ± 332	249 ± 304	232 ± 239	244 ± 326	251 ± 270
MMP-9 (ng/ml)	321 ± 254	340 ± 275	324 ± 271	326 ± 200	282 ± 259[a]	365 ± 264[a]
TIMP-1 (ng/ml)	313 ± 123	340 ± 171	324 ± 144	311 ± 125	343 ± 149	302 ± 131
MMP-8/TIMP-1 molar ratio	0.39 ± 0.54	0.57 ± 0.90	0.46 ± 0.73	0.42 ± 0.60	0.44 ± 0.80	0.46 ± 0.62
MMP-9/TIMP-1 molar ratio	0.37 ± 0.38	0.49 ± 0.57	0.40 ± 0.47	0.43 ± 0.48	0.34 ± 0.48[b]	0.47 ± 0.44[b]
MPO (ng/ml)	3466 ± 2743	3558 ± 2738	3548 ± 2780	3185 ± 2835	3229 ± 2397	3796 ± 3098
Serum MMP-8 (ng/ml)	34.4 ± 25.7	33.7 ± 19.5	34.4 ± 24.0	32.1 ± 17.9	34.6 ± 22.0	33.8 ± 24.9

[a] p = 0.020, Mann-Whitney U test.
[b] p = 0.003, Mann-Whitney U test.

oral HPV DNA-positive and HPV DNA-negative women.

The salivary MMP-8, MMP-9, TIMP-1 and MPO concentrations as well as the MMP-8/TIMP-1 and MMP-9/TIMP-1 molar ratios and the serum MMP-8 concentrations in HPV DNA-positive and HPV DNA-negative women with regards to the smoking status are shown in Fig. 1. Among non-smokers, there was a significant difference between the salivary concentrations of MMP-8 and the salivary MMP-8/TIMP-1 molar ratio in HPV DNA-positive and negative women (p = 0.047 and 0.026, respectively). In contrast, among smokers there was no difference. The salivary MMP-9 or MPO concentrations, salivary MMP-9/TIMP-1 molar ratio or serum MMP-8 concentrations did not differ between HPV DNA-positive and negative women even if the smoking status was taken into account.

There was a significant positive correlation between the salivary

concentrations of MPO and of MMP-8 ($r = 0.567$, $p < 0.001$), as well as a weak correlation between MPO and MMP-9 ($r = 0.234$, $p = 003$), but no correlation between salivary and serum MMP-8 levels ($r = 0.038$, $p = 0.636$). The positive correlation between salivary MPO and MMP-8 was found in the whole study group as well as among both HPV DNA-positive and HPV DNA-negative women separately ($r = 0.399$, $p = 0.002$ and $r = 0.652$, $p < 0.001$, respectively). The correlation between MPO and MMP-9 was statistically significant among HPV DNA-negative women only ($r = 0.225$, $p = 0.023$).

Lactation, as estimated by the given breast milk sample, or alcohol use did not affect the salivary concentrations of MMP-8, MMP-9, MPO or TIMP-1, nor did the MMP-8/TIMP-1 or MMP-9/TIMP-1 molar ratios or serum MMP-8 concentration differ between the groups (Table 2). There were no differences between smokers and non-smokers with regards to their salivary MMP-8, TIMP-1 or MPO concentrations, or the MMP-8/TIMP-1 molar ratio, but the MMP-9 concentration and the MMP-9/TIMP-1 molar ratio were significantly lower in smokers than in non-smokers ($p = 0.020$ and $p = 0.003$, respectively). The serum concentration of MMP-8 was not affected by smoking. (Table 2)

5. Discussion

To our knowledge, this is the first time when low salivary MMP-8 levels, indicating a lowered anti-inflammatory defence in the oral cavity, have been connected with an asymptomatic, persistent viral infection in the oral cavity.

The salivary MMP-8 levels were highest in non-smoking women without oral HPV infection. This result is in line with the earlier observations indicating the protective role of MMP-8 [22–29]. Based on this study, however, it cannot be concluded whether a low salivary MMP-8 concentration indicates a condition with an increased risk for chronic HPV infection or if the infection itself lowers MMP-8 expression. It is tempting to speculate that the lower MMP-8 concentration in saliva would indicate hampered entry or function of PMNs in the oral cavity. After all, PMN cells are the main source of salivary MMP-8. Secondly, warts, hypogammaglobulinemia, infections and myelokathexis (WHIM) syndrome is characterised by neutropenia together with extreme susceptibility for HPV infections [45] suggesting a role of PMN leukocytes in HPV clearance [10]. The mutation causing WHIM induces also impaired neutrophil motility and wound recruitment in zebrafish [46]. Finally, MMP-8 is needed for the early PMN recruitment in response to chemical carcinogens or after bacterial challenge [27,28]. On the other hand, our finding that there were no differences in salivary MPO concentrations in oral HPV DNA-positive and HPV DNA-negative women does not support this hypothesis.

The differences in the salivary MMP-8 concentrations in oral HPV DNA-positive and oral HPV DNA-negative women may also be due to inhibition of MMP-8 expression in infected epithelial cells, or effects of the infected cells on the fibroblasts in the near proximity. Part of salivary MMP-8 originates from other cell types than PMN, and a considerable proportion of salivary MMP-8 can be of fibroblast-type [15,43]. Furthermore, HPV positive cancer cell lines can transfer viral oncogenes to surrounding cells that lack receptors for HPV, and even express HPV oncogenes [47]. HPV positive cells may also affect their microenvironment by secreting soluble components. For example, HPV16 and HPV18 E6 up-regulate STAT3 pathway and consequently also secretion of interleukin (IL)-6 into the cell matrix [48]. IL-6 in turn down-regulates tumour necrosis factor TNF-α [49–51], which is needed for MMP-8 up-regulation in oral fibroblasts [15].

The protective action of MMP-8 may be in part oestrogen related. MMP-8 can cleave oestrogen receptors (ER), both ER-α and, perhaps in lesser amount, ER-β, and thereby MMP-8 regulates the amount of ERs [22]. Oestrogen is needed also for the malignant progression of HPV-related cervical cancer, this effect being ER-α dependent [52]. In this respect, there is no information on the role of ER-β, which is the dominating ER on healthy oral mucosa [53]. Epidemiological data suggest that oestrogen containing oral contraceptive use may contribute to the HPV persistence in the cervix [54,55], although the data is controversial. Our previous study suggests that oral contraceptives or second pregnancy reduced the risk for oral HPV persistence [42].

Salivary concentrations of MMPs and MPO are influenced also by numerous other factors than HPV infection in the oral mucosa. For example, salivary MMP-8 concentrations have been suggested as diagnostic biomarkers for periodontitis [56,57]. On the other hand, poor oral health is an independent risk factor of oral HPV infection [58], and recent studies suggest also an association between HPV related HNSCC and a history of oral infections or deepened periodontal pockets (i.e. periodontitis) [59,60]. In this study, however, persistent HPV infection was asymptomatic and the salivary concentrations of MMP-8 and MPO of participants of this study suggest that their gingival health was good. This implicates also, that oral HPV infection unlikely interferes with the use of MMP-8 as diagnostic marker for other purposes.

Lactation and smoking may affect the salivary MMP-levels [41,61]. With regards to smoking, our results are in line with earlier studies reporting lower MMP-9 levels in saliva of current smokers [61]. Interestingly, smoking, which is a risk factor for HPV persistence, masked the MMP-8 elevation, which was associated with HPV-absence in non-smoking women. Lactation, on the other hand, may lower the salivary TIMP-1 and [41] MMP-9 levels during pregnancy and lactation [41]. In this study, lactation did not have any effect on salivary concentrations of any of the measured markers. The discrepancy between our results and those reported in [41] may be explained by the differences between the studied women. This study included only women who had been pregnant, whereas the control group in the other study included non-pregnant, healthy age-match controls suggesting that the observed differences in [41] may be related to the pregnancy, not lactation *per se*.

To conclude, a non-symptomatic, persistent oral HPV infection was associated with lowered MMP-8 levels and lower MMP-8/TIMP-1 molar ratio in saliva. This may reflect lowered anti-inflammatory defence of the oral mucosa allowing an incident oral HPV infection to become chronic one.

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Competing Interest

TS is an inventor of US-patents 5652227, 5736341, 5866432 and 6143476. Other authors declare that they have no conflicts of interests.

Ethical Approval

The original study protocol and its amendments (#3/1998 and #2/2006) have been approved by the Research Ethics Committee of Turku University Hospital, Turku, Finland.

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