<https://helda.helsinki.fi>

In HPV-negative oropharyngeal squamous cell carcinoma,
elevated toll-like receptor 2 immunoexpression may increase
the risk of disease-specific mortality

Kylmä, Anna Kaisa

2020-08

Kylmä , A K , Jouhi , L , Mohamed , H , Randen-Brady , R , Mäkitie , A , Atula , T , Haglund ,
C , Sorsa , T & Hagström , J 2020 , ' In HPV-negative oropharyngeal squamous cell
carcinoma, elevated toll-like receptor 2 immunoexpression may increase the risk of
disease-specific mortality ' , Oral Oncology , vol. 107 , 104778 . <https://doi.org/10.1016/j.oraloncology.2020.104778>

<http://hdl.handle.net/10138/329757>

<https://doi.org/10.1016/j.oraloncology.2020.104778>

cc_by_nc_nd

acceptedVersion

Downloaded from Helda, University of Helsinki institutional repository.

This is an electronic reprint of the original article.

This reprint may differ from the original in pagination and typographic detail.

Please cite the original version.

**In HPV-negative oropharyngeal squamous cell carcinoma, elevated toll-like receptor
immunoexpression may increase the risk of disease-specific mortality**

Anna Kaisa Kylvä,^a Lauri Jouhi,^b Hesham Mohamed,^a Reija Randén-Brady,^a Antti Mäkitie,^{b,c,d} Timo
Atula,^b Caj Haglund,^{e,f} Timo Sorsa,^{g,h} Jaana Hagström^{a,f,i}

Affiliations

^aDepartment of Pathology, University of Helsinki, HUSLAB and Helsinki University Hospital, P. O.
Box 21, 00014 University of Helsinki Helsinki, Finland

^bDepartment of Otorhinolaryngology - Head and Neck Surgery, University of Helsinki and Helsinki
University Hospital, P. O. Box 263, 00029 HUS Helsinki, Finland

^cDivision of Ear, Nose and Throat Diseases, Department of Clinical Sciences, Intervention and
Technology, Karolinska Institutet and Karolinska Hospital, Stockholm, Sweden

^dResearch Program in Systems Oncology, Faculty of Medicine, University of Helsinki, Helsinki, Finland

^eDepartment of Surgery, University of Helsinki and Helsinki University Hospital, P. O. Box 20, 00014
University of Helsinki, Helsinki, Finland

^fResearch Programs Unit, Translational Cancer Medicine, University of Helsinki, P. O. Box 63, 00014
University of Helsinki, Helsinki, Finland

^gDepartment of Oral and Maxillofacial Diseases, University of Helsinki and Helsinki University Central
Hospital, P. O. Box 41, 00014 University of Helsinki, Helsinki, Finland

^hDivision of Periodontology, Department of Dental Medicine, Karolinska Institutet, Huddinge, Sweden

21 ⁱDepartment of Oral Pathology and Radiology, Institute of Dentistry, Faculty of Medicine, University
22 of Turku, Turku, Finland

23

24

25 **Corresponding author**

26 Anna Kaisa Kylmä, MS, BDS

27 Department of Pathology, University of Helsinki, HUSLAB and Helsinki University Hospital,

28 P.O. Box 21, FI-00014 University of Helsinki, Helsinki, Finland, Tel +358-50 4015651

29 Fax +358-9-47175010, E-mail address: anna.kylma@helsinki.fi

30

31

32

33 **Word count**

34 3082 words

35

36 **Conflict of interest**

37 None declared.

38

39 **Abstract**

40 **Objectives:** In oropharyngeal squamous cell carcinoma (OPSCC), toll-like receptors (TLR) 5 and 7
41 associate with the tumor's human papilloma virus (HPV) status [1]. TLR 2, on the other hand, has been
42 linked to head and neck squamous cell carcinoma (HNSCC), and to oral carcinogenesis [2,3]. Here we
43 investigated the presence of TLR 2 and 4 in HPV-positive and HPV-negative OPSCC, and their
44 relationship to opportunistic oral pathogen *Treponema denticola* chymotrypsin-like protease (Td-CTLP)
45 immunoexpression, clinical parameters, and patient outcome.

46 **Materials and methods:** Clinicopathological data of 198 unselected consecutive OPSCC patients came
47 from hospital registries. Immunoexpression of TLRs 2 and 4 we evaluated by immunohistochemistry,
48 and earlier in this patient series we studied immunoexpression of Td-CTLP and HPV DNA, HPV mRNA,
49 and p16 status.

50 **Results:** Immunoexpression of both TLRs 2 and 4 showed a significant association with HPV-status.
51 Strong expression was associated with HPV-positivity and mild expression with HPV-negativity.
52 Patients with strong TLR 2 immunoexpression in the HPV negative subgroup had significantly poorer
53 5-year DSS (58%) than did patients with mild TLR 2 expression (77%), and strong TLR 2
54 immunoexpression remained as an independent factor linked to increased disease mortality in the
55 multivariable setting ($P=0.019$). No association existed between TLR 2 or 4 and Td-CTLP expression.

56 **Conclusion:** Our results support the role of TLR 2 receptor as a possible target for development of
57 therapeutics as earlier proposed [2]. The involvement of Td and other oral pathogens in carcinogenesis
58 of OPSCC, remains open and calls for further study.

59

60

61 **Keywords**

62 Oropharyngeal squamous cell carcinoma, human papillomavirus, toll-like receptor, Treponema
63 denticola, chymotrypsin-like protease, dentilisin

64

65

66

67

68

69

70

71

72

73

74

75

76

77

78

79

80 **Abbreviations**

81	CRT	Chemoradiotherapy
82	DSS	Disease-specific survival
83	DAMP	Danger-associated molecular pattern
84	HNSCC	Head and neck squamous cell carcinoma
85	HPV	Human papillomavirus
86	HR	Hazard ratio
87	OPSCC	Oropharyngeal squamous cell carcinoma
88	PAMP	Pathogen-associated molecular pattern
89	SCC	Squamous cell carcinoma
90	Sx	Surgery
91	RT	Radiotherapy
92	TLR	Toll-like receptor
93	TMA	Tissue microarray
94	TNM	Tumor lymph node metastasis

95

96

97

98 **Introduction**

99 According to the latest WHO classification of head and neck tumors, the human papilloma virus (HPV)-
100 associated oropharyngeal squamous cell carcinoma (OPSCC) and the HPV-unrelated OPSCC are distinct
101 entities [4]. In developed countries, incidence of HPV-positive OPSCC is increasing [5-12], whereas
102 incidence of HPV-negative OPSCC has remained stable or even declined [7,8,12]. Typically, patients
103 carrying an HPV-positive tumor have better prognosis than patients with HPV-negative disease [5,6,11].
104 The differences now evident in the etiology and course of HPV-positive and -negative OPSCC will call
105 for more detailed understanding of their cellular mechanisms and underlying causalities.

106 Toll-like receptors (TLRs) 1-10 participate in the initiation of innate immune cascades by recognizing
107 pathogen-associated molecular patterns (PAMPs) of bacteria, viruses, fungi, and parasites, and
108 recognizing endogenous danger-associated molecular patterns (DAMPs) in damaged or dying cells [13-
109 15]. TLRs expressed in the immune cells contribute to chronic inflammation and an to attacking of tumor
110 cells, whereas TLRs expressed during carcinogenesis by tumor cells may promote cancer-cell survival
111 and chemoresistance [16,17]. The role of TLRs is, however, controversial, since TLR-activated immune
112 cells may also show pro-tumorigenic effects [18,19]. The expression of TLRs may be prognostic in
113 several cancers including colorectal-, salivary gland-, and non-small cell lung cancer, and squamous cell
114 carcinoma of the tongue [20-24]. Recently, TLR5 and 7 in HPV-positive OPSCC have both shown the
115 likelihood for providing prognostic value [1].

116 In oral and gastrointestinal tract cancers, the presence of several oral pathogens has been evident.
117 Epidemiological studies have shown an association between the periodontal pathogen Porphyromonas
118 gingivalis and increased risk of mortality from orodigestive cancer [25], and increased incidence of
119 pancreatic cancer [26]. According to Narikiyo et al., [27] the oral pathogens Streptococcus mitis,

120 Streptococcus anginosus, and Treponema denticola (Td) are frequent in esophageal cancer tissue.
121 Recently, Td has appeared also in oral- and gastrointestinal-tumor samples [28,29]. Furthermore,
122 Fusobacterium nucleatum has been associated with colorectal cancer and shown to promote colorectal
123 carcinogenesis [30]. Oral carcinogenesis, on the other hand, was in in vitro and in vivo studies promoted
124 by F. nucleatum and P. gingivalis via their interaction with oral epithelial cells through TLR 2 [3]. In an
125 in vitro and in vivo model of head and neck squamous cell carcinoma (HNSCC), the activation of TLR
126 2 promoted organoid growth, supporting its pro-tumorigenic role [2].

127 Earlier, we showed an association between Td key virulence factor, a chymotrypsin-like protease (Td-
128 CTLP), and HPV-negative OPSCC [29]. We additionally observed that strong Td-CTLP expression
129 associated with poor disease-specific survival. Here we aimed to investigate the presence of TLRs 2 and
130 4 in HPV-positive and HPV-negative OPSCC, and the relation of these to Td-CTLP expression, clinical
131 parameters, and patient outcome in vivo in a series of 198 unselected consecutive OPSCC patients.
132 Furthermore, we earlier proposed the use of HPV E6/E7 mRNA ISH detection method as a verifying test
133 for p16-positive tumors [31], the concept which we here evaluate as a HPV classification method in
134 parallel with the classification method proposed by Smeets et al. [32].

135

136

137 **Materials and methods**

138 **Patients and clinicopathological data**

139 This patient cohort, as previously described, [1] comprised 331 consecutive patients with oropharyngeal
140 cancer diagnosed and treated at the Helsinki University Hospital (Finland) between 2000 and 2009.
141 Included in the study were patients with ICD-10 codes C01, C02.4, C05.1, C05.2, C05.8, C05.9, C09.0,

142 C09.1, C09.8, C09.9, C10.0, C10.2, C10.3, C10.8, and C10.9 comprising squamous cell carcinomas
143 (SCC) and subtypes of SCC. Patients treated with palliative treatment intent (44), with concurrent head
144 and neck squamous cell carcinoma (HNSCC) (5), earlier treated HNSCC (11), histology other than SCC
145 (18), or tumor-tissue unavailability (55) were considered ineligible for our investigation. The study series
146 thus comprised 198 patients with treatment-naïve OPSCC treated with curative intention.

147 Clinicopathological data from patient records included patient age, sex; smoking and alcohol
148 consumption; tumor histology, grade, UICC 7th edition TNM stage [33]; primary treatment, i.e. surgery
149 (Sx), radiotherapy (RT), and chemoradiotherapy (CRT); tumor recurrence, treatment of recurrent disease,
150 and status at last follow-up [1]. Follow-up of all patients was at minimum three years or until death. Data
151 on dates and causes of death came from Statistics Finland. This study received the approval of the
152 Research Ethics Board of the Hospital District of Helsinki and Uusimaa and was granted institutional
153 permission.

154 Among the 198 patients studied, 129 underwent primary surgery, and among these, 115 also were
155 receiving adjuvant oncological treatment, either RT or CRT. Five patients received no postoperative
156 oncological therapy because of their stage I-II disease and nine for patient-related reasons. Definitive
157 CRT or RT was the treatment for 69 patients, and among these, 11 underwent salvage surgery in the
158 primary treatment phase (primary site, one; neck only, 7; primary site and neck, 3). Tissue samples
159 collected before RT/CRT involved all except the two patients who had only post-treatment samples
160 available for immunohistochemistry.

161 Available from our earlier analysis were HPV DNA, HPV mRNA, p16^{INK4a}(p16) and Treponema
162 denticola chymotrypsin-like protease (Td-CTLP) status [1,29,31]. Briefly, HPV DNA status was defined
163 by Ventana Inform HPV in situ hybridization (ISH) assay using a high-risk HPV probe and an iVIEW

164 Blue detection kit in Benchmark XT series stainer machine (Ventana Medical Systems, Inc., Tuscon,
 165 AZ, USA). The assay has affinity to the high-risk HPV subtypes: 16, 18, 31, 33, 35, 39, 45, 51, 56, 58,
 166 and 66. HPV DNA status was regarded as positive if any spot in the ISH assay was positive. HPV mRNA
 167 status was defined manually by the RNAscope®2.5 HD Reagent kit (Advanced Cell Diagnostics, Inc.,
 168 Hayward, CA, USA) having affinity for E6/E7 HPV mRNA. HPV p16 and Td-CTLTP
 169 immunohistochemical staining was performed on a tissue microarray (TMA) block slides prepared and
 170 stained as described previously [28,34]. We used monoclonal mouse anti-human p16^{INK4a} (9517 CINtec
 171 Histology Kit, Roche Diagnostics, Basel, Switzerland) and polyclonal anti-CTLTP IgG prepared
 172 according to the method of Grenier et al. [35] for immunostaining.
 173 For evaluation purposes, three HPV-status classification methods were used. Data classification for
 174 Method A was as proposed by Smeets et al. [32] by dividing the samples into an HPV-positive group of
 175 99 that included only p16-positive and HPV DNA-positive samples, and an HPV-negative group that
 176 included 99 either p16-positive but HPV DNA-negative samples, p16-negative and HPV DNA-negative
 177 samples, or p16-negative but HPV DNA-positive samples (Table 1). Similarly, a second classification
 178 (Method B) was as proposed by Randén-Brady et al., in which HPV DNA status was replaced by HPV
 179 mRNA status. The HPV-positive group included 106 p16-positive and HPV mRNA-positive samples;
 180 the HPV-negative group included 92 either p16-positive but HPV mRNA-negative samples, p16-
 181 negative and HPV mRNA-negative samples, or p16-negative but HPV mRNA -positive samples (Table
 182 1). In third classification (Method C), the HPV-positive group included 109 HPV mRNA positive
 183 samples and HPV-negative group 89 HPV mRNA negative samples (Table 1).

184

185 **Immunohistochemistry**

186 We prepared TMA blocks, and stained slides by immunohistochemistry as described previously [34].
187 For expression of TLR 2 in the tumor TMA samples, the immunohistochemical staining was performed
188 with polyclonal rabbit anti-human TLR 2 IgG (1:200, sc-10739, Santa Cruz Biotechnology, Inc., Dallas,
189 TX, USA), and for TLR 4 with monoclonal mouse anti-human TLR 4 IgG (1:2000, sc-293072, Santa
190 Cruz Biotechnology).

191

192 **Immunoscore**

193 The decoded TMA slides immunostained with TLR 2 and 4 antibody separately underwent scoring by
194 two researchers (J.H. and A.K.K.). In case of discordance, the slides were reassessed to achieve
195 consensus. TLR scoring was based on intensity of positivity in tumor tissue: none (0), mild (1), moderate
196 (2), or strong (3). The highest immunoscore value for each TLR was chosen for analysis when several
197 tumor spots were available for selection. Positivity in TLR 2 and 4 staining was localized in the cytoplasm
198 of the carcinoma cells. In addition to carcinoma cells, TLR2 was detected in tumor infiltrating
199 lymphocytes and TLR4 especially in plasma cells, but these were not scored, and hence are not included
200 in analysis. (Figure 1)

201

202 **Statistical analysis**

203 We used SPSS version 20.0 (IBM SPSS Statistics, IBM Corporation, New York, NY, USA) for statistical
204 data analysis. Differences between categorical variables we evaluated using the Chi-square test with
205 asymptotic or exact P-value, whichever appropriate. The Kaplan-Meier (KM) estimator served in
206 calculation of 5-year disease-specific survival (DSS) rates. In evaluation of statistically significant

207 between-group differences in DSS, we used the log-rank test. We defined follow-up time in the DSS as
208 the period between the last treatment day and the last day of follow-up or date of death from the disease.
209 To minimize bias in the follow-up, the maximum follow-up period for the analysis was five years. The
210 Cox proportional hazard model we utilized for multivariable survival analysis. This analysis included
211 clinically relevant variables with a P-value less than 0.1. The proportional hazard assumptions were
212 tested with KM curves. A two-sided P-value less than 0.05 was considered statistically significant.

213

214

215 **Results**

216

217 **TLR 2 and 4 association with patient and tumor characteristics in OPSCC**

218 TLR 2 and TLR 4 expression appeared in the cytoplasm of OPSCC cells. Among the 198 samples, TLR
219 2 was immunoexpressed as follows: 7 (4%) had none, 63 (32%) were mild, 98 (49%) moderate, and 30
220 (15%) strong. As for TLR 4 immunoexpression: 22 (11%) had none, 71 (36%) were mild, 69 (35%)
221 moderate, and 36 (18%) strong.

222 TLR 2 showed a significant association with patient alcohol consumption (Table 2). Mild and moderate
223 TLR 2 immunoexpression prevailed among all alcohol-consumption groups (none, former, and current
224 excess alcohol consumption habit). Strong immunoexpression of TLR 2 was observable only among
225 patients with none or former excess alcohol consumption. Among tumors with strong TLR 2
226 immunoexpression, 89% were those of patients with no excess alcohol consumption.

TLR 4 was associated with patient's alcohol consumption and grade of tumor differentiation (Table 3). As with TLR 2, mild and moderate TLR 4 immunoexpression prevailed among all alcohol consumption groups, but strong TLR 4 immunoexpression appeared among all alcohol consumption groups. Grade 1 tumors had none to moderate TLR 4 immunoexpression (score 0-2), with mild expression dominating. Strong TLR 4 immunoexpression existed only in tumors of grade 2 (23%) and 3 (77%) (Table 3).

232

TLR 2 and 4 association with HPV status

Immunoexpression of both TLR 2 and TLR 4 showed significant association with HPV status when method A and B were used for classification (Table 4). A significant association was also detectable when method C served as a classification method (TLR2, P=0.009; TLR4, P=0.005). Strong expression was associated with HPV positivity and mild expression with HPV negativity (Table 4).

238

TLR 2 and 4 association with Td-CTL

No association existed between TLR 2 or 4 and Td-CTL immunoexpression (Table 4).

241

TLR 2 and 4 association with survival

In survival analysis of all OPSCC patients, and in comparison of sample subgroups with TLR 2 staining of none to mild (0-1) and moderate to strong (2-3), no statistically significant difference emerged in the 5-year DSS (P=0.074). Among patients with HPV-negative OPSCC according to method A, the 5-year DSS was 77% among patients with TLR 2 score 0-1 and 58% among those of score moderate to strong

TLR 2 ($P=0.032$). When method B was used for HPV status classification, the association of TLR 2 with DSS in the HPV-negative group was non-significant ($P=0.057$) (Figure 2). When HPV status was classified according to method C, the association between TLR 2 score and DSS among HPV-negative OPSCC was again significant ($P=0.046$). Among patients with HPV-positive OPSCC, TLR 2 had no association with DSS ($P=0.092$ (A); $P=0.054$ (B); $P=0.052$ (C)). TLR 4 had no associations with DSS among all OPSCC or in any of the subgroups based on classification methods A, B or C.

In multivariable analysis of all 198 patients, sex, TLR 2, and HPV (method A) were independent prognostic factors having respective hazard ratios of 2.5, 2.6 and 3.0 ($P=0.028$; $P=0.010$; $P=0.007$) (Table 5).

256

257

258 Discussion

We show here that mild TLR 2 immunoexpression associated with negative HPV status in OPSCC, and further, that patients with strong TLR 2 immunoexpression in the HPV-negative subgroup had significantly poorer 5-year DSS (58%) than did patients with mild TLR 2 expression (77%). Strong TLR 2 immunoexpression remained as an independent factor and was related to increased disease mortality in the multivariable setting. Patients with strong TLR 2 expression had a trend towards a poorer DSS also in the HPV-positive OPSCC subgroup, and among all OPSCC patients, but these differences were not statistically significant. Our findings are in line with findings of Farnebo et al. indicating that TLR 2-receptor activation mediates pro-tumorigenic effects in vivo in HNSCC xenograft tumors [2]. In their study, inhibition of TLR 2-receptor also inhibited tumor growth in tumor xenografts in vivo and in HNSCC cell lines in vitro [2]. Similarly, according to findings of Binder Gallimidi et al., TLR 2 inhibition

269 resulted in an anti-tumorigenic effect on cell proliferation and inhibited the oral pathogen-induced Il-6
270 expression in oral SCC cell lines [3]. Here we may hypothesize that strong TLR2 expression among
271 HPV-negative OPSCC is related mainly to carcinogenesis, whereas among HPV-positive OPSCC there
272 may be a component of TLR2 expression related to virus infection.

273 The significance of the association of TLR 2 immunoexpression with DSS in HPV-negative OPSCC
274 patients was dependent on the method used for HPV status classification. According to classification
275 method A proposed by Smeets et al. the number of HPV-positive OPSCC samples was smaller than when
276 method B proposed by Randén-Brady et al. was used for classification [31,32]. This is explained by the
277 higher sensitivity of the HPV mRNA method compared with that of the HPV DNA method [31]. The
278 number of HPV-positive OPSCC samples was even greater when HPV mRNA alone in method C was
279 used for classification. However, the differences in significance were relatively small ($P=0.032$ (A);
280 $P=0.054$ (B); $P=0.046$ (C)) and reflect our limited number of OPSCC samples in this study, only 198.
281 We may speculate that with a higher number of OPSCC samples, the association of TLR 2 expression
282 with DSS would be statistically significant in HPV-negative OPSCC independent of classification
283 method. Hence, our results comprise no contraindication to our earlier proposal to use HPV mRNA
284 detection method to verify p16-positive tumors [31].

285 Of the OPSCC samples TLR 2 was expressed in 96% and TLR 4 in 89%, but their expression showed
286 no association with Td-CTLP immunoexpression, although both TLR 2 and 4 recognize bacterial
287 components [36]. Furthermore, Td is able to trigger TLR 2 and 4 in murine macrophages [37].
288 Interestingly, strong expression of TLR 2 and 4 was associated with HPV positivity. Since HPV infection
289 has an association with periodontitis [38,39], a dysbiosis contributed to several opportunistic oral
290 pathogens, the strong TLR 2 and 4 may result from activation by bacteria other than those studied here.
291 The role of oral pathogens in triggering TLR 2 receptor gained earlier support when oral SCC cell lines

292 derived from tongue cancer tissue were exposed to the oral pathogens *Porphyromonas gingivalis* or
293 *Fusobacterium nucleatum*, or both of them [3]. TLR 2 and 4 positivity is usually detectable on cell
294 membranes [40] whereas here the immunopositivity was cytoplasmic. This may reflect the more diverse
295 role of TLRs in carcinogenesis when compared with their role in microbial activation [41].

296 TLR 2 immunoexpression was associated with alcohol consumption, and 89% of OPSCC samples with
297 strong TLR 2 expression occurred among those patients with no excess alcohol consumption. This is
298 consistent with earlier results, in which HPV-positive OPSCC is more likely to occur among patients
299 using less alcohol [5], and strong TLR 2 expression was in our series more common among HPV-positive
300 OPSCC.

301 We also showed that TLR 4 was associated with alcohol consumption and with tumor grade of
302 differentiation. Over 50% of the tumors expressing strong TLR 4 appeared among non-alcohol users and
303 were either grade 2 or 3. As strong TLR 4 expression was also associated with HPV positivity, these
304 findings are in line with the current clinicopathological characteristics reported for HPV-positive
305 OPSCC, indicating that patients with HPV-positive tumors are less often heavy alcohol users [42,43].
306 The association between TLR 2 and 4 and alcohol consumption itself may be related to alcohol mediated
307 inflammatory responses, and thus will be the subject of another study.

308 In our recent study with the same patient series, we observed Td-CTLTP in 81% of the OPSCC samples
309 [29], and in the HPV-negative subgroup of OPSCC samples showed an association of strong Td-CTLTP
310 immunoexpression with strong TLR 5 expression and with mild TLR 7 expression [1,29]. Further, strong
311 Td-CTLTP immunoexpression among patients with HPV-negative OPSCC was associated with a poor
312 DSS [29]. Our current results show an association between strong TLR 2 immunoexpression and poor

313 DSS among the same HPV-negative subgroup of OPSCC and support the role of TLR 2 receptor as a
314 possible target for development of therapeutics as proposed earlier [2].

315 Based on the present results it is impossible to conclude that involvement of Td would trigger the TLR
316 2 or TLR 4 receptors. However, earlier findings have demonstrated the triggering of TLR 2 receptor by
317 the oral pathogens *P. gingivalis* and *F. nucleatum* [3]; this may suggest that Td and other oral pathogens
318 also play a role in oral carcinogenesis. In periodontitis, the periodontopathogens act in combinations of
319 several bacteria, leading the dysbiosis in the gingival microbiota [26]. Interestingly, here we observed
320 that strong Td-CTL expression was more common in OPSCC samples which showed no expression to
321 mild TLR 4 expression, although that association was statistically non-significant ($P=0.080$). This is in
322 line with our earlier finding of strong Td-CTL expression as being associated with HPV negativity [29].
323 Nonetheless, the involvement of Td and other oral pathogens in carcinogenesis of OPSCC, and also in
324 host immunological responses, remains an open possibility.

325

326

327 **Conclusions**

328 The present results show an association between strong TLR 2 immunoexpression and poor DSS among
329 the HPV-negative subgroup of OPSCC patients and thus support the role of TLR 2 receptor as a possible
330 target for the development of therapeutics for this disease as proposed earlier [2]. This view is further
331 strengthened here, because strong TLR 2 immunoexpression remained in multivariable analysis an
332 independent factor that elevated disease mortality.

333 The involvement of Td and other oral pathogens in carcinogenesis of OPSCC, and also in host
334 immunological responses, calls for further study.

335

336 **Ethics approval**

337 The study received institutional permission and the approval of The Research Ethics Board of the
338 Hospital District of Helsinki and Uusimaa, Finland.

339

340 **Funding**

341 This work was supported by the Minerva Foundation Institute for Medical Research, grant number
342 701337.

343

344 **Role of the Funding Source**

345 Minerva Foundation provided partial funding for the research work based on grant application and a
346 research plan.

347

348 **Acknowledgements**

349 We thank Päivi Peltokangas and Pia Saarinen for technical assistance.

350 **References**

- 351 [1] Jouhi L, Mohamed H, Makitie A, Remes SM, Haglund C, Atula T, et al. Toll-like receptor 5 and 7
352 expression may impact prognosis of HPV-positive oropharyngeal squamous cell carcinoma patients.
353 *Cancer Immunol.Immunother.* 2017.
- 354 [2] Farnebo L, Shahangian A, Lee Y, Shin JH, Scheeren FA, Sunwoo JB. Targeting Toll-like receptor 2
355 inhibits growth of head and neck squamous cell carcinoma. *Oncotarget* 2015;6:9897-907.
- 356 [3] Binder Gallimidi A, Fischman S, Revach B, Bulvik R, Maliutina A, Rubinstein AM, et al. Periodontal
357 pathogens *Porphyromonas gingivalis* and *Fusobacterium nucleatum* promote tumor progression in an
358 oral-specific chemical carcinogenesis model. *Oncotarget* 2015;6:22613-23.
- 359 [4] El-Naggar AK, Chan J, Takata T, Grandis J, Slootweg P. The 4th Edition of the Head and Neck WHO
360 Blue Book: Editor's Perspectives. *Hum.Pathol.* 2017.
- 361 [5] Gillison ML, Koch WM, Capone RB, Spafford M, Westra WH, Wu L, et al. Evidence for a causal
362 association between human papillomavirus and a subset of head and neck cancers. *J.Natl.Cancer Inst.*
363 2000;92:709-20.
- 364 [6] Ang KK, Harris J, Wheeler R, Weber R, Rosenthal DI, Nguyen-Tan PF, et al. Human papillomavirus
365 and survival of patients with oropharyngeal cancer. *N.Engl.J.Med.* 2010;363:24-35.
- 366 [7] Chaturvedi AK, Engels EA, Pfeiffer RM, Hernandez BY, Xiao W, Kim E, et al. Human
367 papillomavirus and rising oropharyngeal cancer incidence in the United States. *J.Clin.Oncol.*
368 2011;29:4294-301.
- 369 [8] Nasman A, Attner P, Hammarstedt L, Du J, Eriksson M, Giraud G, et al. Incidence of human
370 papillomavirus (HPV) positive tonsillar carcinoma in Stockholm, Sweden: an epidemic of viral-induced
371 carcinoma? *Int.J.Cancer* 2009;125:362-6.
- 372 [9] Nichols AC, Palma DA, Dhaliwal SS, Tan S, Theuer J, Chow W, et al. The epidemic of human
373 papillomavirus and oropharyngeal cancer in a Canadian population. *Curr.Oncol.* 2013;20:212-9.
- 374 [10] Rietbergen MM, Leemans CR, Bloemena E, Heideman DA, Braakhuis BJ, Hesselink AT, et al.
375 Increasing prevalence rates of HPV attributable oropharyngeal squamous cell carcinomas in the
376 Netherlands as assessed by a validated test algorithm. *Int.J.Cancer* 2013;132:1565-71.
- 377 [11] Reuschenbach M, Tinhofer I, Wittekindt C, Wagner S, Klussmann JP. A systematic review of the
378 HPV-attributable fraction of oropharyngeal squamous cell carcinomas in Germany. *Cancer.Med.*
379 2019;8:1908-18.
- 380 [12] Wittekindt C, Wagner S, Bushnak A, Prigge ES, von Knebel Doeberitz M, Wurdemann N, et al.
381 Increasing Incidence rates of Oropharyngeal Squamous Cell Carcinoma in Germany and Significance of
382 Disease Burden Attributed to Human Papillomavirus. *Cancer.Prev.Res.(Phila)* 2019;12:375-82.

383 [13] Janeway CA, Jr. Approaching the asymptote? Evolution and revolution in immunology. Cold Spring
384 Harb. Symp. Quant. Biol. 1989;54 Pt 1:1-13.

385 [14] Matzinger P. The danger model: a renewed sense of self. Science 2002;296:301-5.

386 [15] Kawai T, Akira S. Pathogen recognition with Toll-like receptors. Curr. Opin. Immunol. 2005;17:338-
387 44.

388 [16] Basith S, Manavalan B, Yoo TH, Kim SG, Choi S. Roles of toll-like receptors in cancer: a double-
389 edged sword for defense and offense. Arch. Pharm. Res. 2012;35:1297-316.

390 [17] Ioannou S, Voulgarelis M. Toll-like receptors, tissue injury, and tumourigenesis. Mediators
391 Inflamm. 2010;2010:10.1155/2010/581837. Epub 2010 Sep 14.

392 [18] Suttmüller RP, den Brok MH, Kramer M, Bennink EJ, Toonen LW, Kullberg BJ, et al. Toll-like
393 receptor 2 controls expansion and function of regulatory T cells. J. Clin. Invest. 2006;116:485-94.

394 [19] Hussaini HM, Parachuru VPB, Seymour GJ, Rich AM. Forkhead box-P3(+) regulatory T cells and
395 toll-like receptor 2 co-expression in oral squamous cell carcinoma. Acta Histochem. 2017;119:205-10.

396 [20] Grimm M, Kim M, Rosenwald A, Heemann U, Germer CT, Waaga-Gasser AM, et al. Toll-like
397 receptor (TLR) 7 and TLR8 expression on CD133+ cells in colorectal cancer points to a specific role for
398 inflammation-induced TLRs in tumourigenesis and tumour progression. Eur. J. Cancer 2010;46:2849-57.

399 [21] Korvala J, Harjula T, Siirila K, Almangush A, Aro K, Makitie AA, et al. Toll-like receptor 9
400 expression in mucoepidermoid salivary gland carcinoma may associate with good prognosis. J. Oral
401 Pathol. Med. 2014;43:530-7.

402 [22] Kauppila JH, Mattila AE, Karttunen TJ, Salo T. Toll-like receptor 5 (TLR5) expression is a novel
403 predictive marker for recurrence and survival in squamous cell carcinoma of the tongue. Br. J. Cancer
404 2013;108:638-43.

405 [23] Mäkinen LK, Atula T, Hayry V, Jouhi L, Datta N, Lehtonen S, et al. Predictive role of Toll-like
406 receptors 2, 4, and 9 in oral tongue squamous cell carcinoma. Oral Oncol. 2015;51:96-102.

407 [24] Zhou H, Chen JH, Hu J, Luo YZ, Li F, Xiao L, et al. High expression of Toll-like receptor 5
408 correlates with better prognosis in non-small-cell lung cancer: an anti-tumor effect of TLR5 signaling in
409 non-small cell lung cancer. J. Cancer Res. Clin. Oncol. 2014;140:633-43.

410 [25] Ahn J, Segers S, Hayes RB. Periodontal disease, Porphyromonas gingivalis serum antibody levels
411 and orodigestive cancer mortality. Carcinogenesis 2012;33:1055-8.

412 [26] Michaud DS, Izard J, Wilhelm-Benartzi CS, You DH, Grote VA, Tjønneland A, et al. Plasma
413 antibodies to oral bacteria and risk of pancreatic cancer in a large European prospective cohort study.
414 Gut 2013;62:1764-70.

415 [27] Narikiyo M, Tanabe C, Yamada Y, Igaki H, Tachimori Y, Kato H, et al. Frequent and preferential
416 infection of *Treponema denticola*, *Streptococcus mitis*, and *Streptococcus anginosus* in esophageal
417 cancers. *Cancer.Sci.* 2004;95:569-74.

418 [28] Nieminen MT, Listyarifah D, Hagstrom J, Haglund C, Grenier D, Nordstrom D, et al. *Treponema*
419 *denticola* chymotrypsin-like proteinase may contribute to orodigestive carcinogenesis through
420 immunomodulation. *Br.J.Cancer* 2018;118:428-34.

421 [29] Kylma AK, Jouhi L, Listyarifah D, Mohamed H, Makitie A, Remes SM, et al. *Treponema denticola*
422 chymotrypsin-like protease as associated with HPV-negative oropharyngeal squamous cell carcinoma.
423 *Br.J.Cancer* 2018;119:89-95.

424 [30] Rubinstein MR, Wang X, Liu W, Hao Y, Cai G, Han YW. *Fusobacterium nucleatum* promotes
425 colorectal carcinogenesis by modulating E-cadherin/ β -catenin signaling via its FadA adhesin. *Cell host*
426 *& microbe* 2013;14:195-206.

427 [31] Randen-Brady R, Carpen T, Jouhi L, Syrjanen S, Haglund C, Tarkkanen J, et al. In situ hybridization
428 for high-risk HPV E6/E7 mRNA is a superior method for detecting transcriptionally active HPV in
429 oropharyngeal cancer. *Hum.Pathol.* 2019;90:97-105.

430 [32] Smeets SJ, Hesselink AT, Speel EJ, Haesevoets A, Snijders PJ, Pawlita M, et al. A novel algorithm
431 for reliable detection of human papillomavirus in paraffin embedded head and neck cancer specimen.
432 *Int.J.Cancer* 2007;121:2465-72.

433 [33] Anonymous In:Sobin LH, Gospodarowicz MK, Wittekind C, editors. TNM classification of
434 malignant tumours, Hoboken, New Jersey, USA:Wiley-Blackwell;2010,.

435 [34] Makinen LK, Hayry V, Atula T, Haglund C, Keski-Santti H, Leivo I, et al. Prognostic significance
436 of matrix metalloproteinase-2, -8, -9, and -13 in oral tongue cancer. *J.Oral Pathol.Med.* 2012;41:394-9.

437 [35] Grenier D, Uitto VJ, McBride BC. Cellular location of a *Treponema denticola* chymotrypsinlike
438 protease and importance of the protease in migration through the basement membrane. *Infect.Immun.*
439 1990;58:347-51.

440 [36] Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, et al. Differential roles of TLR2
441 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity*
442 1999;11:443-51.

443 [37] Nussbaum G, Ben-Adi S, Genzler T, Sela M, Rosen G. Involvement of Toll-like receptors 2 and 4
444 in the innate immune response to *Treponema denticola* and its outer sheath components. *Infect.Immun.*
445 2009;77:3939-47.

446 [38] Ortiz AP, Gonzalez D, Vivaldi-Oliver J, Castaneda M, Rivera V, Diaz E, et al. Periodontitis and
447 oral human papillomavirus infection among Hispanic adults. *Papillomavirus Res.* 2018;5:128-33.

448 [39] Tezal M, Scannapieco FA, Wactawski-Wende J, Hyland A, Marshall JR, Rigual NR, et al. Local
449 inflammation and human papillomavirus status of head and neck cancers.
450 Arch.Otolaryngol.Head.Neck.Surg. 2012;138:669-75.

451 [40] Takeda K, Kaisho T, Akira S. Toll-like receptors. Annu.Rev.Immunol. 2003;21:335-76.

452 [41] Jouhi L, Renkonen S, Atula T, Makitie A, Haglund C, Hagstrom J. Different Toll-Like Receptor
453 Expression Patterns in Progression toward Cancer. Front.Immunol. 2014;5:638.

454 [42] Nauta IH, Rietbergen MM, van Bokhoven AAJD, Bloemena E, Lissenberg-Witte BI, Heideman
455 DAM, et al. Evaluation of the eighth TNM classification on p16-positive oropharyngeal squamous cell
456 carcinomas in the Netherlands and the importance of additional HPV DNA testing. Ann.Oncol.
457 2018;29:1273-9.

458 [43] Gillison ML. Human Papillomavirus and Oropharyngeal Cancer Stage. J.Clin.Oncol. 2016;34:1833-
459 5.

460

461 **Table 1** Classification of oropharyngeal squamous cell carcinoma (OPSCC) according to methods A, B
 462 and C based on results of detection methods p16^{INK4a}, HPV DNA and HPV mRNA.

463

HPV-status	Detection method			All patients (N =198)
	p16INK4a	HPV DNA	HPV mRNA	n
Method A (Smeets <i>et al.</i> , 2007)				
HPV-positive	positive	positive	-	99
HPV-negative	positive	negative	-	99
	negative	negative	-	
	negative	positive	-	
Method B (Randén-Brady <i>et al.</i> , 2019)				
HPV-positive	positive	-	positive	106
HPV-negative	positive	-	negative	92
	negative	-	negative	
	negative	-	negative	
Method C				
HPV-positive	-	-	positive	109
HPV-negative	-	-	negative	89

464

Table 2 Toll-like receptor 2 (TLR 2) immunoexpression in relation to patient and tumor characteristics; patient sex, smoking and alcohol consumption, tumor site of origin, grade of differentiation, T class (primary tumor size), N class (presence of regional lymph node metastasis), and tumor stage.

	Total	TLR 2 score								P value
		None (0)		Mild (1)		Moderate (2)		Strong (3)		
		n	%	n	%	n	%	n	%	
Sex										0.470
Male	146	5	3.4	48	32.9	73	50.0	20	13.7	
Female	52	2	3.8	15	28.8	25	48.1	10	19.2	
Smoking										0.575
None	26	1	3.8	6	23.1	15	57.7	4	15.4	
Finished	48	1	2.1	15	31.3	23	47.9	9	18.8	
Current	94	3	3.2	30	31.9	47	50.0	14	14.9	
Excess alcohol consumption										0.001
None	60	1	1.7	16	26.7	26	43.3	17	28.3	
Finished	24	0	0.0	8	33.3	14	58.3	2	8.3	
Current	37	5	13.5	11	29.7	21	56.8	0	0.0	
Tumor site										0.579
Anterior wall	59	3	5.1	16	27.1	31	52.5	9	15.3	
Lateral wall	115	3	2.6	37	32.2	57	49.6	18	15.7	
Posterior wall	3	1	33.3	1	33.3	1	33.3	0	0.0	
Superior wall	21	0	0.0	9	42.9	9	42.9	3	14.3	
Tumor grade										0.080
Grade 1	18	1	5.6	4	22.2	11	61.1	2	11.1	
Grade 2	77	4	5.2	28	36.4	40	51.9	5	6.5	
Grade 3	103	2	1.9	31	30.1	47	45.6	23	22.3	
T class										1.000
T1-2	112	5	4.5	35	31.3	54	48.2	18	16.1	
T1-3	86	2	2.3	28	32.6	44	51.2	12	14.0	
N class										0.471
N0	38	1	2.6	15	39.5	17	44.7	5	13.2	
N+	160	6	3.8	48	30.0	81	50.6	25	15.6	
Tumor stage										0.284
I-II	29	1	3.4	12	41.4	13	44.8	3	10.3	
III-IV	169	6	3.6	51	30.2	85	50.3	27	16.0	

Table 3 Toll-like receptor 4 (TLR 4) immunoexpression in relation to patient and tumor characteristics; patient sex, smoking and alcohol consumption, tumor site of origin, grade of differentiation, T class (primary tumor size), N class (presence of regional lymph node metastasis), and tumor stage.

	Total	TLR 4 score								P value
		None (0)		Mild (1)		Moderate (2)		Strong (3)		
		n	%	n	%	n	%	n	%	
Sex										0.717
Male	146	15	10.3	58	39.7	49	33.6	24	16.4	
Female	52	7	13.5	13	25.0	20	38.5	12	23.1	
Smoking										0.119
None	26	1	3.8	8	30.8	14	53.8	3	11.5	
Finished	48	4	8.3	17	35.4	16	33.3	11	22.9	
Current	94	15	16.0	34	36.2	30	31.9	15	16.0	
Excess alcohol consumption										0.022
None	60	8	13.3	17	28.3	25	41.7	10	16.7	
Finished	24	2	8.3	10	41.7	9	37.5	3	12.5	
Current	37	9	24.3	17	45.9	7	18.9	4	10.8	
Tumor site										0.928
Anterior wall	59	9	15.3	22	37.3	26	44.1	2	3.4	
Lateral wall	115	11	9.6	39	33.9	33	28.7	32	27.8	
Posterior wall	3	1	33.3	1	33.3	1	33.3	0	0.0	
Superior wall	21	3	14.3	9	42.9	8	38.1	1	4.8	
Tumor grade										0.001
Grade 1	18	4	22.2	10	55.6	4	22.2	0	0.0	
Grade 2	77	6	7.8	37	48.1	26	33.8	8	10.4	
Grade 3	103	14	13.6	24	23.3	38	36.9	27	26.2	
T class										0.695
T1-2	112	15	13.4	38	33.9	36	32.1	23	20.5	
T1-3	86	9	10.5	33	38.4	32	37.2	12	14.0	
N class										0.338
N0	38	5	13.2	16	42.1	12	31.6	5	13.2	
N+	160	19	11.9	55	34.4	56	35.0	30	18.8	
Tumor stage										0.092
I-II	29	4	13.8	15	51.7	7	24.1	3	10.3	
III-IV	169	20	11.8	56	33.1	61	36.1	32	18.9	

475 **Table 4** Toll-like receptor 2 (TLR 2) and TLR 4 immunoexpression in relation to oropharyngeal squamous cell carcinoma (OPSCC) human
 476 papillomavirus (HPV) status classified using p16, HPV DNA, and HPV mRNA results, and Treponema denticola chymotrypsin-like protease (Td-
 477 CTLP) immunoexpression.

478

	Total	TLR2 score								P value	TLR4 score								P value
		None (0)		Mild (1)		Moderate (2)		Strong (3)			None (0)		Mild (1)		Moderate (2)		Strong (3)		
		n	%	n	%	n	%	n	%		n	%	n	%	n	%	n	%	
HPV status ^A										0.001									P < 0.001
HPV-negative	99	5	5.1	42	42.4	41	41.4	11	11.1		20	20.2	46	46.5	24	24.2	9	9.1	
HPV-positive	99	2	2.0	21	21.2	57	57.6	19	19.2		4	4.0	25	25.3	44	44.4	26	26.3	
HPV status ^B										0.007									P < 0.001
HPV-negative	92	3	3.3	39	42.4	41	44.6	9	9.8		19	20.7	41	44.6	23	25.0	9	9.8	
HPV-positive	106	4	3.8	24	22.6	57	53.8	21	19.8		5	4.7	30	28.3	45	42.5	26	24.5	
Td-CTL expression										0.634									0.080
0 - 1	98	5	5.1	28	28.6	47	48.0	18	18.4		9	9.2	32	32.7	37	37.8	20	20.4	
2 - 3	99	2	2.0	34	34.3	51	51.5	12	12.1		15	15.2	38	38.4	31	31.3	15	15.2	

^A = HPV classification method A

^B = HPV classification method B

479

480 **Table 5** Multivariable Cox regression analysis for Disease specific survival (DSS) in a series of all 198
481 oropharyngeal squamous cell carcinoma (OPSCC) patients.

482

Multivariable analysis			
All patients (N =198)			
	HR	95% CI	P
Sex			
Female vs male	2.5	1.1 - 5.6	0.028
Smoking			
Currently vs earlier/never	1.4	0.7 - 3.1	0.362
T class			
T3-4 vs T1-2	1.3	0.7 - 2.4	0.35
N class			
N+ vs N0	1.8	0.7 - 4.5	0.203
TLR 2			
2-3 vs 0-1	2.6	1.3 - 5.3	0.010
HPV status^A			
HPV-negative vs HPV-positive	3.0	1.4 - 6.5	0.007

^A = HPV classificatio method A

483

484

485 **Figure 1.** Immunohistochemical staining of oropharyngeal squamous cell carcinoma (OPSCC) samples
486 with Toll-like receptor (TLR) 2 and TLR4 antibody. OPSCC with none **A**, mild **B**, moderate **C**, and
487 strong **D** expression of TLR2, and OPSCC with none **E**, mild **F**, moderate **G**, and strong **H** expression
488 of TLR4. Arrowheads point to the tumor tissue, and arrows to the infiltrating lymphocytes and plasma
489 cells. Magnification $\times 200$.

490

491 **Figure 2.** The 5-year disease-specific survival (DSS) rates calculated by Kaplan-Meier (KM) estimation
492 for the entire sample (A) and for the human papillomavirus (HPV) -positive (B) and the HPV-negative
493 (C) subgroups classified by p16- and HPV DNA results. Samples grouped according to toll-like receptor
494 2 (TLR 2) immunoexpression into the categories none to mild (score = 0 – 1) and moderate to strong
495 (score 2 - 3).