

Tonsillar swabs and sputum predict SLPI- and AnxA2 expression in tonsils: A prospective study on smoking dependent SLPI- and AnxA2-expression, and tonsillar HPV infection

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Abstract. Previous retrospective studies have elucidated a correlation between secretory leucocyte protease inhibitor (SLPI) and Annexin A2 (AnxA2), patient smoking status and tonsillar human papilloma virus (HPV) status. The current study assessed these parameters prospectively and to the best of our knowledge, analyzed SLPI-/AnxA2-expression for the first time in tonsillar swabs and sputum. Samples were obtained from 52 patients with tonsillar squamous cell carcinoma and 163 patients with tonsillar hyperplasia (H; n=56) and chronic or recurrent tonsillitis (CRT; n=107). HPV-DNA, SLPI and AnxA2 gene expression was analyzed in sputum, tonsillar swabs and tissue by performing reverse transcription-quantitative PCR. Results were compared with smoking status, revealing that smoking resulted in significantly increased SLPI gene expression in all biomaterials of all cases. SLPI-gene expression was significantly decreased in all HPV-DNA-positive samples (tissue/swab/sputum), while AnxA2 was significantly increased in all HPV-DNA-positive samples. Results from swabs and sputum were able to predict SLPI- and AnxA2 gene expression of the corresponding tonsil. The current prospective study confirmed previous retrospective results underlining this hypothesis: Smoking enhances SLPI-expression, preventing HPV-binding to AnxA2. HPV-binding to AnxA2 appears essential for successful

cell-entry. SLPI/AnxA2-gene expression in swabs and sputum reflect their expression in tonsillar tissue. Accordingly, a positive AnxA2/SLPI-ratio in sputum/swabs could possibly be used to reduce HPV-associated carcinogenesis, by performing tonsillectomy or HPV-vaccination in patients with positive AnxA2/SLPI-ratios.

Introduction

The majority of head and neck squamous cell carcinomas (HNSCC) is causally linked to enhanced tobacco and alcohol consumption of individuals which especially holds true for cancers of the larynx and hypopharynx and HPV-negative oropharyngeal carcinomas (1). Pronounced in Asia and East Africa, oral cancer might also be caused by betel nut chewing (2). Carcinogenesis of the lingual and specifically palatine tonsils, however, is to a substantial proportion linked to infections with human papillomaviruses (HPV) (3-6). 30-90% of tonsillar SCC (TSCC) can be attributed to HPV16 infections with the infection rate diversity depending on the geographical region the tested patients live in (7,8). It is well established that, among others, patients with HPV-driven TSCC show significantly better survival rates and are predominantly non-smokers (9-11). Analysis of the association between HPV infection and smoking of the here described study population has confirmed that HPV-negative and HPV-positive patients are smokers vs. non-smokers, respectively, reaching strong statistical significance (for details on the latter and on further epidemiologic data see Ref. 12). The reason for this reciprocal correlation between HPV-status and smoking is up to date only poorly understood.

Previously analyzing altogether 928 tissue samples from 892 patients in retrospective studies (13 and references therein), we have consistently shown a significant link between a) smoking habit, b) expression levels of the human secretory leucocyte protease inhibitor (SLPI) and Annexin A2 (AnxA2), and c) HPV infections in various SCCs and also benign tonsillar lesions (12). Basically, for these two proteins it is described,

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that SLPI as a serine protease inhibitor potently inhibits (among others) neutrophil elastase and thereby protects skin and mucosa from proteolysis. Its antiviral activity is mediated by affecting the host cells rather than the virus itself. AnxA2 is involved in various cell functions, among others endocytosis and exocytosis [for both see References listed in (13)]. Data from meanwhile 892 patients with various diseases of either the head and neck or anogenital region analyzed for smoking habit, expression levels of SLPI and AnxA2, and HPV infection status of the investigated tissues prompted us to formulate the following hypothesis: Smoking leads to increased SLPI and AnxA2 expression in mucosal tissues with significant SLPI excess. SLPI binds to AnxA2, which consecutively inhibits the binding of HPV, if present, to AnxA2. HPV binding to AnxA2 is crucial for successful HPV infection of mucosal cells. Conversely, in non-smokers with significantly higher levels of AnxA2 compared to SLPI, HPV can bind more readily to unoccupied-non-SLPI-bound-AnxA2, and successful infection of cells is likely. This hypothesis is supported by experimental evidence provided by U.S. American groups regarding the binding capacity of SLPI and HPV to AnxA2 (14-16) and by other groups describing elevated SLPI expression in smokers (17,18). Moreover, similar observations as described above have been made by Ma and coworkers (19) already in 2004 and later on by others (20,21) in terms of infections with HIV. Only recently, we published a summary of six preceding retrospective studies highlighting the aforementioned hypothesis (13).

Here, for the first time in a prospective study design the association of smoking habit, SLPI- and AnxA2-expression as well as HPV infection of 215 patients with benign and malignant diseases of the palatine tonsils have been investigated. Moreover, it was tested whether or not SLPI- and AnxA2-gene expression levels measured in sputum or tonsillar swabs are suitable as surrogate marker for SLPI- and AnxA2-gene expression in the respective tonsillar tissue of the same individual. The latter was prompted by studies reporting on 73% oral gargle/tumor biopsy HPV agreement in oropharyngeal cancer patients (22). This gave rise to hope for superior agreement ratios for SLPI and AnxA2 in different biomaterial e.g. tonsillar tissue, swab and/or sputum since SLPI and AnxA2 are intrinsic proteins, i.e. produced by mucosal cells, instead of extrinsic factors such as viral infections.

Materials and methods

Study design. In a prospective, consecutive setting, patients with tonsillar squamous cell carcinoma (TSCC) or non-neoplastic tonsillar lesions, namely tonsillar hyperplasia (H) or chronic or recurrent tonsillitis (CRT), were enrolled between February 2016 and April 2018 when treated at the ENT departments of the university hospitals of Kiel, Rostock or Oldenburg or in the ENT department of the Asklepios-Clinic, Hamburg-Harburg, Germany. In the study presented the main focus was to study benign vs. malignant tonsillar lesions. Since it is described that p16^{INK4A} and SLPI might be elevated by inflammation as might be the case in patients with chronic or recurrent tonsillitis (23,24) we additionally enrolled patients with tonsillar hyperplasia, a tonsillar lesion not showing high-grade signs of inflammation. All patients were

asked about their smoking habit and their age was recorded. Each patient was asked to deliver a sputum sample directly pre-operatively, which was immediately taken to the laboratories of the respective clinics. For later isolation of DNA and RNA from the sputum, the sputum sample was supplemented with 2 ml nucleic acid stabilizer (AmpTec). All samples were stored at room temperature until nucleic acid extraction, which was performed in the Kiel laboratories. Intraoperatively, patient material was extracted in different ways depending on the diagnosis: In patients with benign tonsillar lesions tonsils of both sides were swabbed and resected; in TSCC-patients only the affected tonsil was swabbed and resected. From each tonsil that was eligible for analysis, 2 swabs were taken with the Buccalyse swabs (Isohelix). One swab was used to extract DNA, the other swab was transferred in 700 µl of the above-mentioned stabilizer (AmpTec) to extract RNA. All swabs were stored at room temperature until nucleic acid extraction in Kiel; samples were once every fortnight send to Kiel. Tissue specimens (~1 cm³) of each tonsil was shock frozen in liquid nitrogen and stored at -80°C until nucleic acid extraction in Kiel. A second piece of tissue was transferred to formalin for immunohistochemistry. All samples were obtained following informed written consent and approval by the local Ethics Committee (D 429/14).

Nucleic acid extraction. DNA and RNA from all sputum samples and RNA from the tonsillar swabs was extracted using the ExpressArt Mag RNA+DNAready kit (AmpTec), according to the manufactures protocol. DNA of the tonsillar swabs was isolated using the Buccalyse DNA Release kit (Isohelix), according to the manufactures protocol. To isolate DNA and RNA from the tissue samples, 20-30 mg tissues were transferred into a Precellys[®] ceramic-Kit 1,4 mm tube (VWR International), containing 600 µl RLT-buffer part of the AllPrep DNA/RNA Mini Kit (Qiagen). The tubes were transferred into the Precellys[®] tissue homogenizer and were homogenized using the program 1 x 5,000 for 30 sec. Afterwards the lysates were centrifuged in an Eppendorf centrifuge 5417R at maximum speed for 3 min at 4°C. DNA and RNA was isolated from the supernatants using the AllPrep DNA/RNA mini kit (Qiagen) according to the manufacture's protocol. All RNA samples were stored at -80°C and all DNA sample at -20°C until further analysis. Nucleic acid-quantity and -quality was assessed using the NanoDrop 1000 (PqLab) and the Tapestation 2200 (Agilent), respectively.

cDNA synthesis and SLPI and AnxA2 gene expression. RNA (200 ng) was transcribed into cDNA (TR-cDNA synthesis kit; AmpTec) under the following reaction conditions: 30 min at 16°C, 30 min at 42°C, 5 min at 85°C, using a Biometra T1 cyclor (Analytik Jena; Jena Germany) followed by 5 min storage on ice. RT-qPCR was performed as described, previously (25) using the following primers: SLPI Forward: 3'-AAT GCCTGGATCCTGTTGAC-5'; SLPI Reverse: 3'-AAAGGA CCTGGACCACACAG-5'; AnxA2 Forward: 3'-AACCGA CGAGGACTCTCTCA-5'; AnxA2 Reverse: 3'-CGCTGATCC ACTTGGGAACAT-5' (26). RT-qPCR using a Rotorgene 3000 (Corbett, LTF) was performed amplifying 2.5 µl each of the abovementioned cDNA (=10 ng RNA) under the following PCR conditions: 10 min initial denaturation at 95°C, followed

by 40 cycles: 20 sec denaturation at 95°C, annealing 20 sec at 60°C and elongation: 20 sec at 72°C followed by melt curve analysis from 60°C to 95°C ramping: 5°/sec. Primers for the housekeeping genes 18S rRNA (P-030126), β -actin (P030124) and b-2-microglobulin (B2M; P-030127) were purchased from Promolgene (with catalogue numbers given in parenthesis) and used according to the manufacturer's protocol. RT-qPCR data were analyzed according to the $\Delta\Delta C_t$ method (26) using the mean C_t value of the housekeeping genes. Fold changes of expression levels were calculated using the following equations: $2^{-(\Delta C_t \text{ HPV-positive} - \Delta C_t \text{ HPV-negative})}$ for decreases in HPV-related gene expression; $1/2^{-(\Delta C_t \text{ HPV-positive} - \Delta C_t \text{ HPV-negative})}$ for increases in HPV-related gene expression; $1/2^{-(\Delta C_t \text{ active/former smoker} - \Delta C_t \text{ never smoker})}$ for increases in smoking-related gene expression; $2^{-(\Delta C_t \text{ AnxA2} - \Delta C_t \text{ SLPI})}$ for negative AnxA2/SLPI ratios and $1/2^{-(\Delta C_t \text{ AnxA2} - \Delta C_t \text{ SLPI})}$ for positive AnxA2/SLPI ratios (26).

SLPI immunohistochemistry. Paraffin-embedded tissue specimens were cut into 2 μm sections and stained for SLPI. Immunostaining was performed and evaluated according to Cordes *et al* (27). In brief: Paraffin-embedded tissue specimens were deparaffinized, and rehydrated, followed by heat-induced epitope retrieval. Methanol containing 1% hydrogen peroxide was used to block the endogenous peroxidase. Sections were blocked with the corresponding pre-immune serum for 15 min and incubated for 1 h with monoclonal primary antibody directed against SLPI (LifeSpan BioSciences) followed by incubation with a biotin-conjugated rabbit anti-mouse IgG secondary antibody (Dako) at room temperature for 30 min. After washing with tris-buffered saline, a labelled peroxidase complex system (ABC-Vectorstain; Dako) was used to visualize all immune reactions. To assess SLPI protein levels, 300 cells in at least five areas was analyzed (x200 magnification). A mean percentage of positive cells were determined and cases were assigned to one of the following categories: negative (-) <5%, weak (+) 5-30%, moderate (++) 31-75% and strong (+++) >75%.

HPV detection. HPV DNA detection was performed by PCR amplifying 50 ng DNA per sample using the primers GP5+/GP6+, as described previously (28). DNA integrity was analyzed using genomic B2M primers (Promolgene; P-030127) according to the manufacturer's protocol. Additionally, a positive control (a synthetic oligonucleotide of the HPV L1 gene, covered by the GP5+/GP6+ primers; Eurofins; Ebersberg Germany) was amplified in the GP5+/GP6+ PCRs. Amplification products were sequenced by Sanger sequencing and alignments were obtained from the GenBank online BLAST server (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). A representative Sanger sequencing trace of a HPV16-positive cancer tissue sample is shown in Fig. S1.

Statistical analyses. Firstly, demographic differences between the patient cohorts suffering from either tonsillar hyperplasia (H) or chronic or recurrent tonsillitis (CRT), were analyzed using Fisher's exact tests. Next the demographic characteristics of the cancer patients and of all patients with non-neoplastic lesions (H and CRT) were compared using Fisher's exact tests. Student's t-test was performed to assess

age-related differences first between the H and CRT patients and later between all patients with benign lesions and the TSCC patients. Fisher's exact test was performed relating SLPI protein expression to HPV-positivity and smoking habit. For analysis of the RT-qPCR data ΔC_t values [C_t value of the target genes (SLPI and AnxA2) corrected for mean C_t values of the housekeeping genes (18S rRNA, β -actin B2M)] were used. Since data were, as confirmed by Kolmogorov-Smirnov test, non-parametric Mann-Whitney- and Kruskal-Wallis test was performed to analyze differences between 2 and more than 2 groups, respectively. All tests were performed using SPSS 20.0; P-values ≤ 0.05 were considered statistically significant, confirmed by Bonferroni post-testing for multiple comparisons where appropriate.

Results

Patient demographics. A total of 215 patients was enrolled in the study. 52 patients (age: 63.03 ± 8.99 years) were treated for cancer of the palatine tonsil [(TSCC) 9/52 (17.2%) never smokers, 19/52 (36.5%) active smokers, and 22/52 (42.3%) had given up smoking at least two years before diagnosis; in two (3.8%) cases, data regarding smoking habit were missing]. Further 163 patients (age 25.69 ± 15.10 years) with benign tonsillar lesions were enrolled. [97/163 (59.5%) never smokers; 63/163 (38.7%) active smokers; two patients (1.2%) had given up smoking at least 2 years prior to diagnosis; for one case (0.6%) no data regarding the smoking habit was available]. Patients with benign tonsillar lesions were comprised of 56 patients (age: 29.30 ± 21.47 years) suffering from tonsillar hyperplasia [(H) 31/56 (55.4%) never smokers; 23/56 (31.1%) active smokers; one patient (1.8%) had given up smoking at least 2 years prior to diagnosis and for one case (1.8%) no data regarding the smoking habit was available]. 107 patients (age: 24.05 ± 10.78 years) were treated for non-neoplastic chronic or recurrent tonsillitis [(CRT) 66/107 (61.7%) never smokers; 40/107 (37.4%) active smokers; one patient (0.9%) had given up smoking at least 2 years prior to diagnosis; age is in all cases given as mean \pm standard deviation]. No significant differences between patient demographics were observed between CRT and H patients (Table I). As expected TSCC patients are older and this group is comprised of significantly fewer never smokers but also more former smokers when compared to the CRT and H patients. Given the different disease entities studied, the number of HPV-positive cases is significantly higher in the TSCC patients (Table I). The two cases with benign tonsillar lesions who had given up smoking were, due to too small numbers, excluded from further analysis regarding the effect of smoking on SLPI- and AnxA2-expression.

HPV DNA-status in tonsillar tissue. The tonsils of 23/163 (14.1%) patients treated for benign tonsillar lesions were HPV DNA-positive [12/107 (11.2%) CRT- and 11/56 (19.6%) H-patients]. Of the 52 TSCC-patients, 21/52 (40.4%) were HPV DNA-positive. Given the underlying hypothesis that SLPI expression levels influence the initial HPV cell-entry in human mucosa, we focused on the presence of exclusively HPV DNA in the present study. Since in this context it is not important whether or not HPV cell-entry finally results in an inactive or active HPV infection of the tonsils, direct or indirect markers

Table I. Patient demographics (n=215).

Variable	Cohort				Significance	
	CRT (n=107)	H (n=56)	All benign (n=163) ^c	TSCC (n=52)	CRT vs. H	All benign vs. TSCC
Age (years)	24.05±10.78	29.30±21.47	25.68±15.10	63.03±8.99	n.s.	P<0.0001
Smoking status at time of diagnosis ^a					n.s.	P<0.0001
Active	40 (37.4)	23 (31.1)	63 (38.7)	19 (36.5)		
Never	66 (61.7)	31 (55.4)	97 (59.5)	9 (17.2)		
Former ^b	1 (0.9)	1 (1.8)	2 (1.2)	22 (42.3)		
HPV DNA					n.s.	P<0.0001
Negative	95 (88.8)	45 (80.4)	140 (85.9)	31 (59.6)		
Positive	12 (11.2)	11 (19.6)	23 (14.1)	21 (40.4)		
SLPI IHC					n.s.	n.s.
Negative/weak	62 (57.9)	36 (64.3)	98 (60.1)	31 (59.6)		
Moderate/strong	45 (42.1)	20 (35.7)	65 (39.9)	21 (40.4)		

^aThe smoking status data of three patients could not be retrieved, including two patients with TSCC and one with H. ^bPatients were considered former smokers if that had stopped smoking at least 2 years prior to diagnosis. ^cAll benign represents the sum of patients with CRT and H. SLPI IHC results are scored as follows according to Cordes *et al* (27): Negative, <5%; weak, 5-30%; moderate, 31-75%; and strong, >75%. To increase numbers per groups, sections with negative and weak, and sections with moderate and strong staining, were analyzed together. Numbers in parenthesis are percent per disease entity. CRT, chronic or recurrent tonsillitis; H, tonsillar hyperplasia; HPV, human papilloma virus; IHC, immunohistochemistry; n.s., not significant; SLPI, secretory leucocyte protease inhibitor; TSCC, tonsillar SCC.

of HPV activity were neglected here. These HPV-related results along with further details on the study population are described elsewhere (12, and are briefly summarized in Table I).

SLPI and AnxA2 gene expression. As shown in Fig. 1 smoking resulted in tonsillar tissue, swabs and sputum of TSCC-patients (Fig. 1A-C) and patients with benign tonsillar lesions (Fig. 1D-F) in significantly increased SLPI gene expression levels. In TSCC-patients who quit smoking at least 2 years prior to diagnosis (former smoker) SLPI gene expression was still significantly higher than in never smokers albeit not as high as in still active smokers; approx. 2.5-fold increase in former and 3.5 to 5.2-fold increase in active smokers (Fig. 1A-C). Analyzing patients with begin lesions separated by disease type, it can be seen that in CRT-patients (Fig. 1G-I) smoking resulted in similarly increased SLPI gene expression levels as seen in TSCC-patients. In H-patients (Fig. 1J-L), the smoking related increase in SLPI gene expression was less pronounced and was only significantly increased in tonsillar tissue, while gene expression in swabs and sputum of these patients was not significantly altered. Smoking resulted in only insignificantly increased AnxA2 levels, in all patient groups and biomaterial analyzed (Fig. 1A-L).

Next, the effect of HPV on SLPI and AnxA2 gene expression was tested. SLPI gene expression was significantly decreased in all HPV-positive samples (tissue, swab and sputum) in TSCC-patients and in patients with benign lesion, when analyzing the latter as one group. AnxA2 gene expression, on the other hand, was significantly increased in HPV-positive when compared to HPV-negative samples of these patient groups (Fig. 2A-F). Analyzing H- and CRT-patients separately, it can be seen that in CRT-patients

(Fig. 2G-I) HPV-positivity resulted in similarly decreased SLPI gene expression levels as already seen in TSCC-patients. AnxA2 gene expression in CRT-patients was significantly increased in HPV-positive samples when compared to HPV-negative ones. In H-patients, on the other hand, neither SLPI nor AnxA2 gene expression was significantly different between HPV-positive and HPV-negative samples, irrespective of the biomaterial analyzed (Fig. 2J-L). It should, however, be mentioned that SLPI gene expression in all HPV-positive biomaterials of H-patients was decreased, albeit not significantly. Likewise, AnxA2 levels of HPV-positive biomaterials of H-patients showed small but insignificant increases in comparison to HPV-negative materials (Fig. 2J-L).

To determine the relation between SLPI and AnxA2 gene expression, the fold change of AnxA2 gene expression in relation to SLPI gene expression was calculated (for mathematical details see Material & Methods and Legend to Fig. 3). The biomaterial of all never smoking patients had significantly more AnxA2 than SLPI (Fig. 3A-L). In active and former smokers, with the exception of the tonsillar swabs of TSCC-patients who reported to have stopped smoking at least 2 years prior to diagnosis, AnxA2 and SLPI gene expression were not significantly different. HPV-negativity resulted in all biomaterials, with the exception of the swabs obtained from H-patients, in significantly reduced AnxA2 gene expression in comparison to SLPI gene expression (Fig. 3A-L). It should, however, be noted that AnxA2 gene expression in comparison to SLPI gene expression in tonsillar swabs of H-patients was also reduced, albeit not significantly. In all HPV-positive biomaterials with the exception of the sputum samples obtained from H-patients (Fig. 3L), AnxA2 gene expression was significantly increased in comparison to SLPI gene expression (Fig. 3A-K).

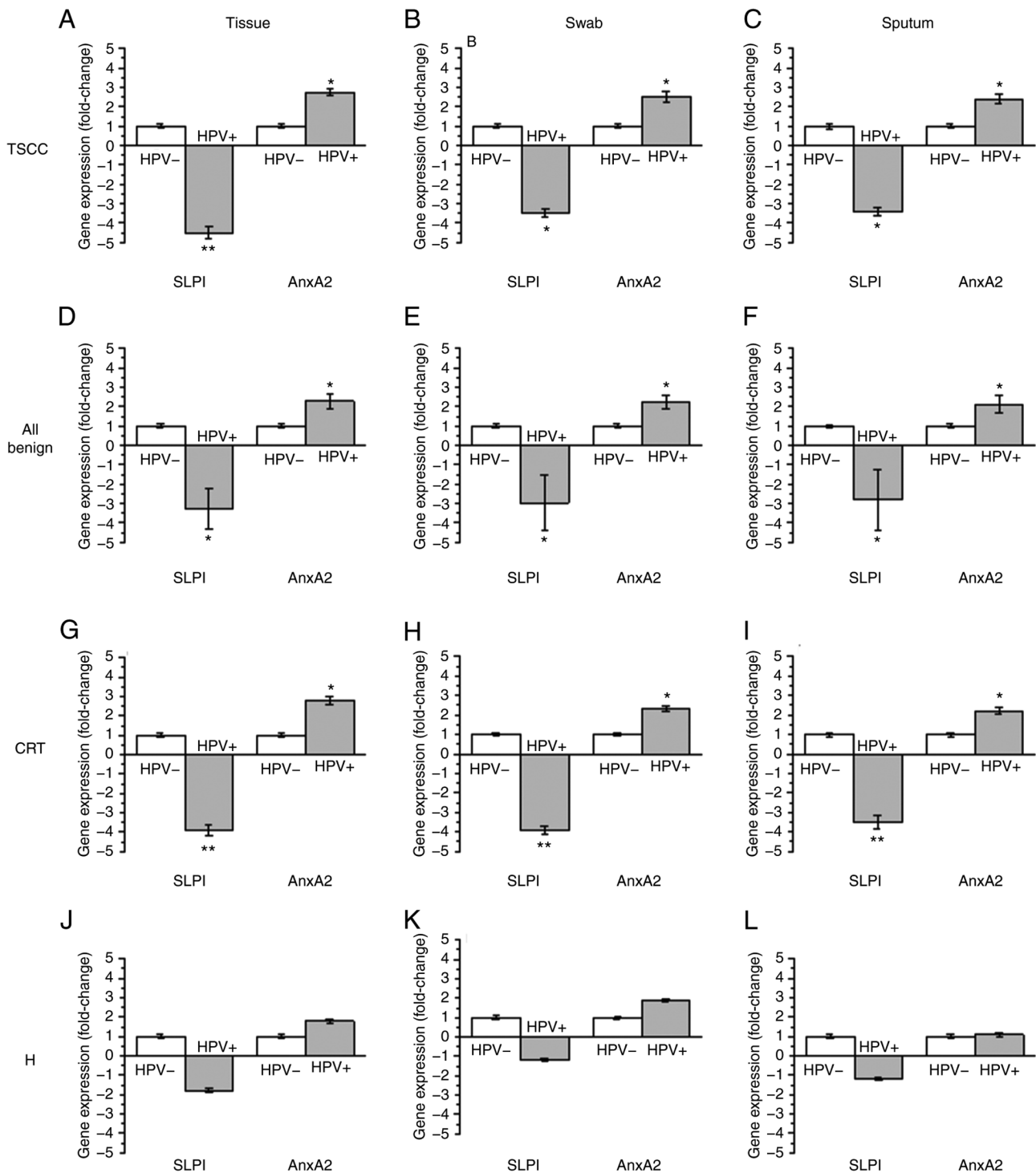


Figure 1. Effect of smoking status on SLPI- and AnxA2-gene expression in tonsillar tissue, tonsillar swabs and the sputum of patients with malignant and benign tonsillar lesions. Fold changes in SLPI and AnxA2 gene expression in (A) tissue (B) swab and (C) sputum samples dependent on the smoking habits of patients with TSCC. Fold changes in SLPI and AnxA2 gene expression in (D) tissue (E) swab and (F) sputum samples dependent on the smoking habits of all patients with benign tonsillar lesions (CRT and H). Fold changes in SLPI and AnxA2 gene expression in (G) tissue (H) swab and (I) sputum samples dependent on the smoking habits of patients with CRT. Fold changes in SLPI and AnxA2 gene expression in (J) tissue (K) swab and (L) sputum samples dependent on the smoking habits of patients with H. Fold changes in gene expression levels in active smokers and former smokers (TSCC only) were calculated using the following equation: $1/2^{(\Delta\text{Ct active/former smoker} - \Delta\text{Ct never smoker})}$. For better comparisons, ΔCt values obtained in non-smoking patients were set as 1 (26). Patients in the former smoker group had stopped smoking at least 2 years prior to diagnosis. * $P < 0.05$ and ** $P < 0.001$ vs. never smokers. AnxA2, Annexin A2; CRT, chronic or recurrent tonsillitis; H, tonsillar hyperplasia; SLPI, secretory leucocyte protease inhibitor; TSCC, tonsillar SCC.

SLPI protein expression. To corroborate tonsillar SLPI gene expression levels by protein levels, the SLPI protein expression of the tonsils was measured by means of immunohistochemistry. Representative cases for

negative (<5% cells were stained) and strong (>75% cells were stained) SLPI staining in tonsillar tissue of patients with TSCC, patients with chronic or recurrent tonsillitis, and patients with tonsillar hyperplasia are shown in Fig. 4.

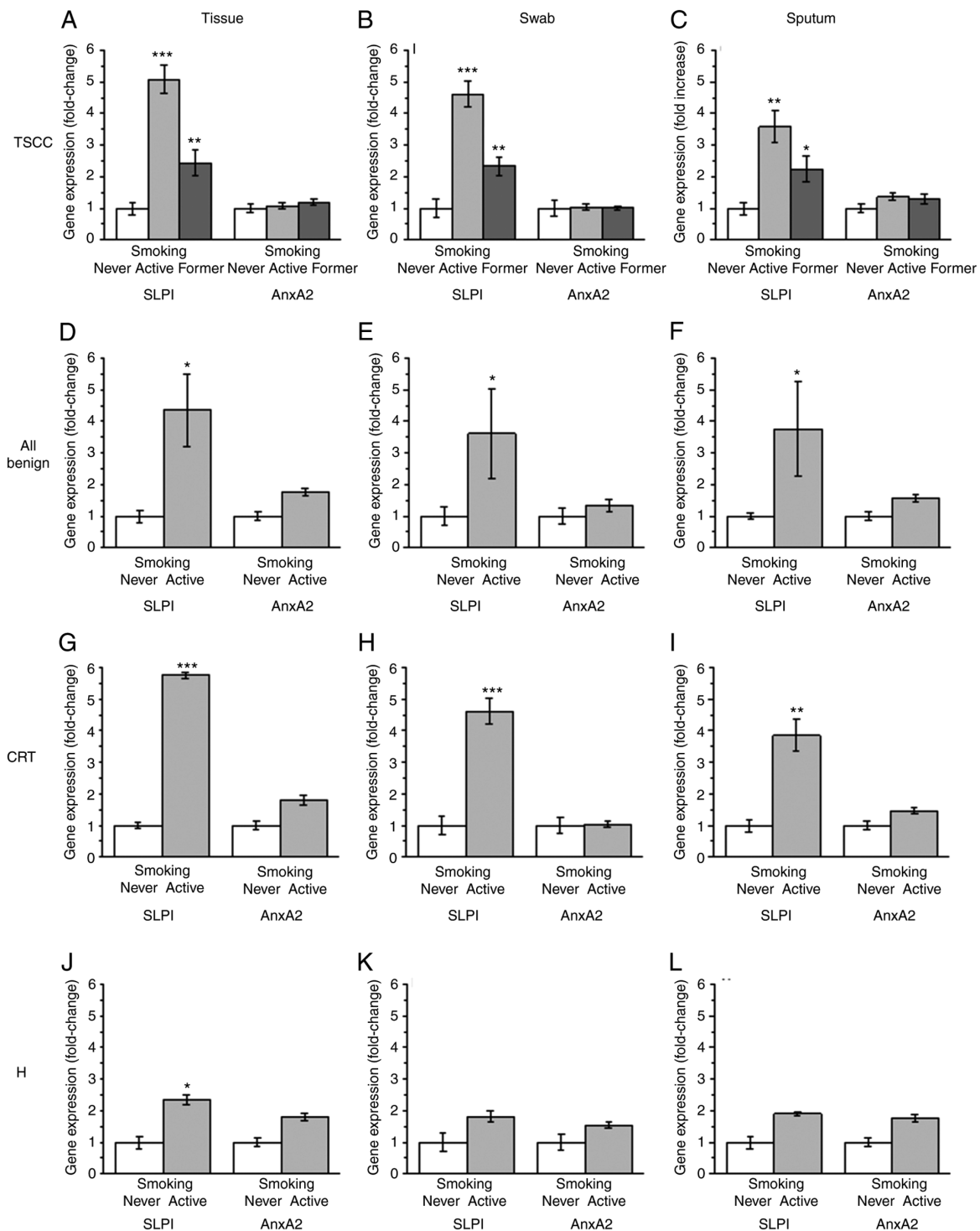


Figure 2. Effect of HPV status on SLPI- and AnxA2-gene expression in tonsillar tissue, tonsillar swabs and the sputum of patients with malignant and benign tonsillar lesions. Fold changes in SLPI and AnxA2 gene expression in (A) tissue (B) swab and (C) sputum samples dependent on the HPV status of patients with TSCC. Fold changes in SLPI and AnxA2 gene expression in (D) tissue (E) swab and (F) sputum samples dependent on the HPV status of all patients with benign tonsillar lesions (CRT and H). Fold changes in SLPI and AnxA2 gene expression in (G) tissue (H) swab and (I) sputum samples dependent on the HPV status of patients with CRT. Fold changes in SLPI and AnxA2 gene expression in (J) tissue (K) swab and (L) sputum samples dependent on the HPV status of patients with H. Fold-changes of gene expression in HPV-positive samples were calculated using the following equations: $2^{\Delta(\Delta Ct \text{ HPV-positive} - \Delta Ct \text{ HPV-negative})}$ for decreases in HPV-related gene expression; $1/2^{\Delta(\Delta Ct \text{ HPV-positive} - \Delta Ct \text{ HPV-negative})}$ for increases in HPV-related in gene expression. For better comparisons, ΔCt values obtained in HPV-negative patients were set as 1 (26). * $P < 0.05$, ** $P < 0.001$ and *** $P < 0.0001$ vs. HPV-negative samples. AnxA2, Annexin A2; CRT, chronic or recurrent tonsillitis; H, tonsillar hyperplasia; HPV, human papilloma virus; SLPI, secretory leucocyte protease inhibitor; TSCC, tonsillar SCC.

Immunohistochemical analysis could be performed on all 215 samples; the results relating SLPI protein expression to smoking habit are presented in Table II. In the tonsillar tissue of TSCC-patients, a significant correlation between

SLPI-IHC and smoking habit could be found; $P = 0.0022$. Similarly, in the benign samples, when analyzed as one group, a significant correlation between SLPI protein expression and smoking habit could be found ($P = 0.0009$).

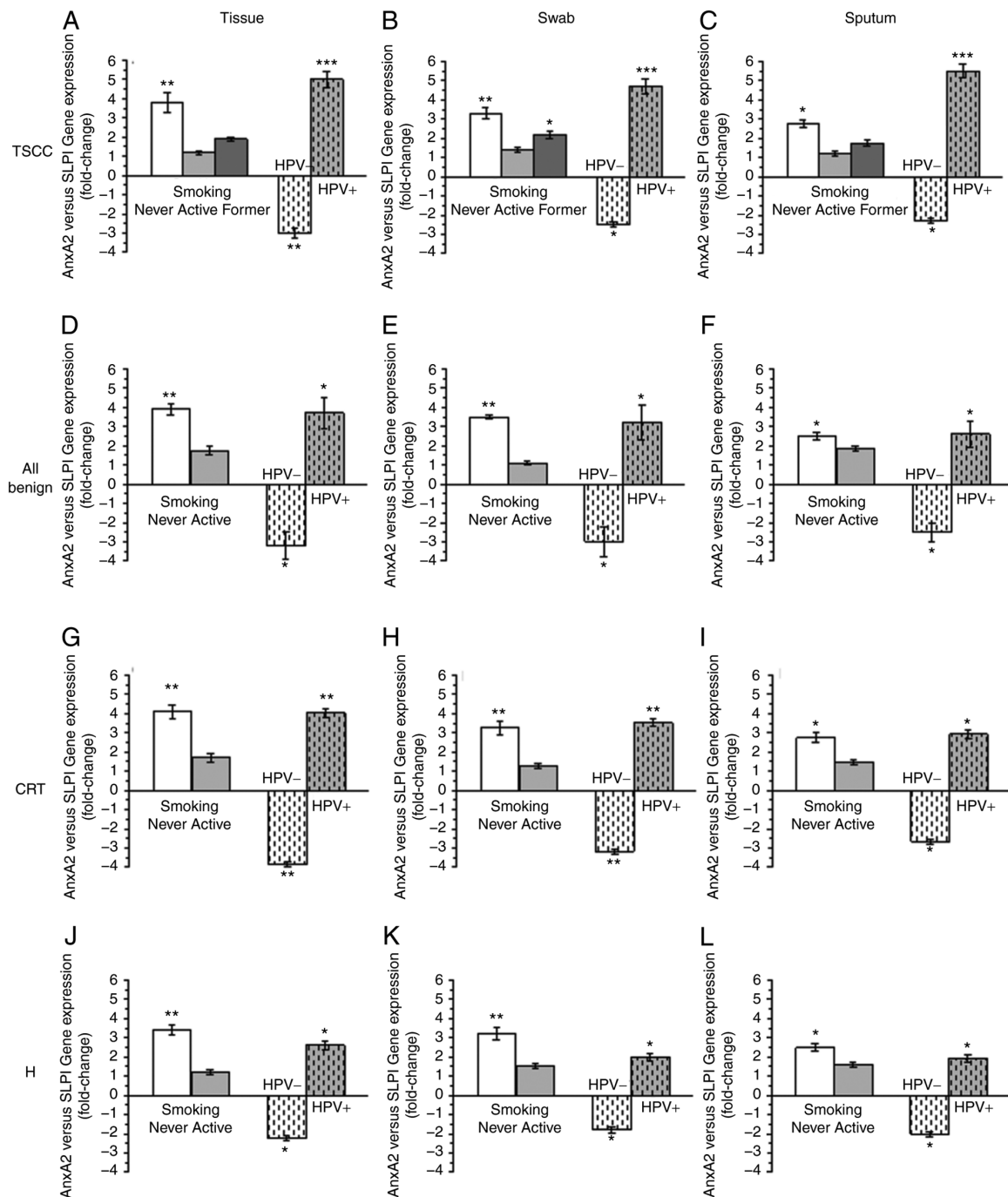


Figure 3. Comparison of AnxA2- and SLPI-gene expression in tonsillar tissue, tonsillar swabs and the sputum of patients with malignant and benign tonsillar lesions. Relationship between AnxA2 and SLPI gene expression in (A) tissue (B) swab and (C) sputum samples dependent on the smoking habit and HPV status of patients with TSCC patients. Relationship between AnxA2 and SLPI gene expression in (D) tissue (E) swab and (F) sputum samples dependent on the smoking habit and HPV status of all patients with benign tonsillar lesions (CRT and H). Relationship between AnxA2 and SLPI gene expression in (G) tissue (H) swab and (I) sputum samples dependent on the smoking habit and HPV status of patients with CRT patients. Relationship between AnxA2 and SLPI gene expression in (J) tissue (K) swab and (L) sputum samples dependent on the smoking habit and HPV status of patients with H. Fold-change expression of AnxA2 in relation to SLPI gene expression was calculated using the following equations: $2^{-(\Delta\text{Ct AnxA2} - \Delta\text{Ct SLPI})}$ for negative AnxA2/SLPI ratios and $1/2^{(\Delta\text{Ct AnxA2} - \Delta\text{Ct SLPI})}$ for positive AnxA2/SLPI ratios (26). To compare the AnxA2 and SLPI gene expression levels, SLPI gene expression for each group [never smoker, active smoker and former smoker (TSCC patients only) or never and active smoker; HPV- and HPV+] was set as '1'. The SLPI data are not displayed. Former smokers had stopped smoking at least 2 years prior to diagnosis (only TSCC). * $P < 0.05$, ** $P < 0.001$ and *** $P < 0.0001$ AnxA2 vs. SLPI gene expression. AnxA2, Annexin A2; CRT, chronic or recurrent tonsillitis; H, tonsillar hyperplasia; HPV, human papilloma virus; SLPI, secretory leucocyte protease inhibitor; TSCC, tonsillar SCC.

Analyzing the two subgroups of benign lesion, separately, revealed a significant correlation between SLPI-IHC and smoking habit for CRT-but not for H-patients ($P = 0.0022$ and $P > 0.05$ respectively; Table II).

The correlation between HPV DNA-status and SLPI protein expression was also calculated and the results are presented in Table III. In the majority of patients with HPV-positive tonsillar tissue the tonsillar tissue was characterized by

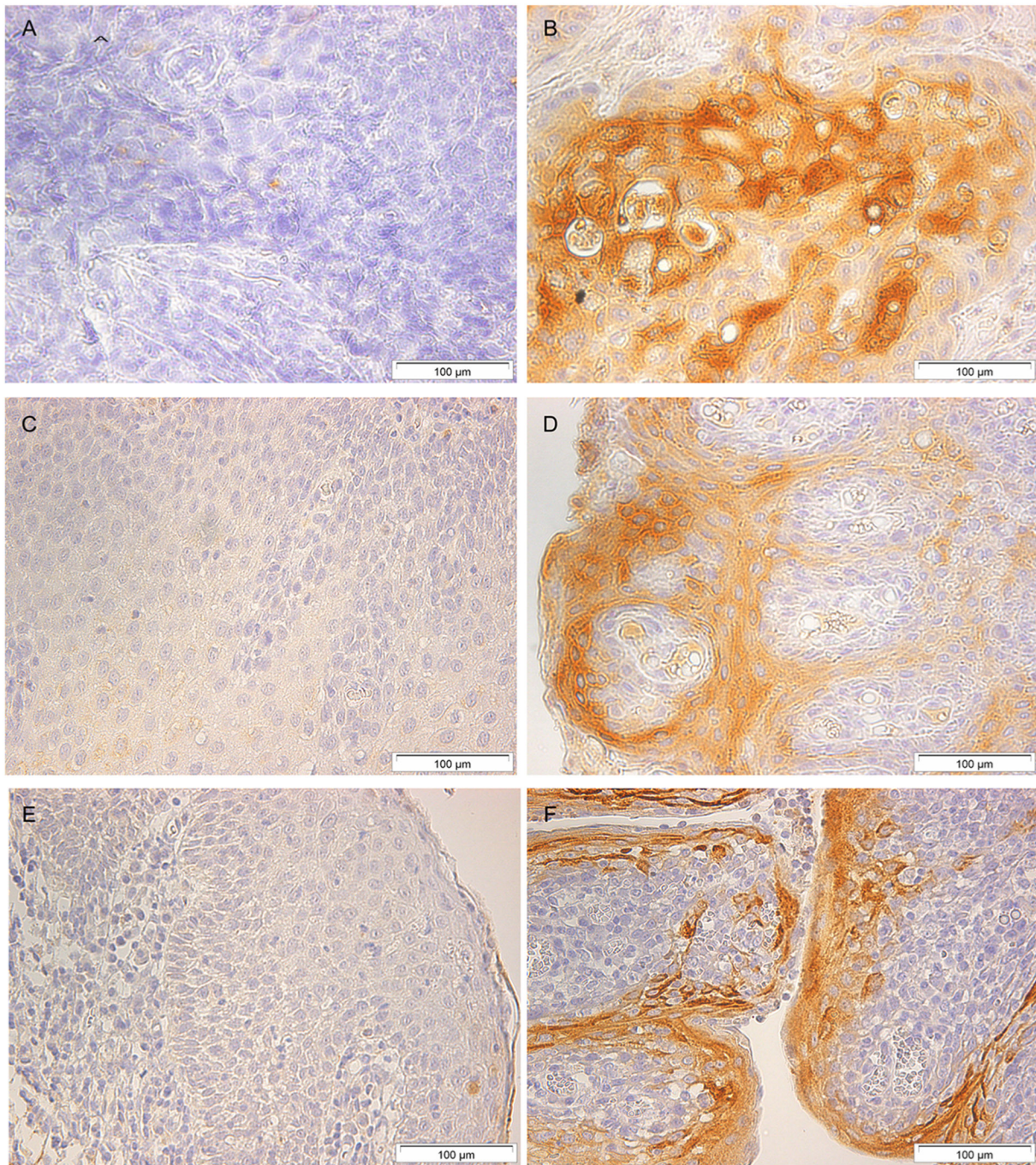


Figure 4. Immunohistochemical staining for SLPI (A, C and E) Representative cases for negative (<5% cells were stained) and (B, D and F) strong (>75% cells were stained) SLPI staining is shown. Tonsillar SCC tissue is displayed in A and B, chronic or recurrent tonsillitis tissue in C and D and tonsillar hyperplasia tissue in E and F (magnification, x200; scale bars, 100 μ m). SLPI, secretory leucocyte protease inhibitor.

negative/weak SLPI-IHC staining, while HPV-negative tissue showed a relative even distribution between negative/weak and moderate/strong SLPI-IHC staining, resulting in significant correlations in TSCC-patients and patients with benign tonsillar lesions ($P < 0.0001$ and $P = 0.0209$, respectively; Table III). Analyzing the different subgroups of benign lesion separately revealed a significant correlation between SLPI-IHC and HPV DNA-status for CRT- but not for H-patients ($P = 0.001$ and $P > 0.05$ respectively; Table III).

Correlating SLPI and AnxA2 expression in the different biomaterials analyzed. To assess the validity of SLPI and

AnxA2 gene expression in tonsillar swabs and/or sputum samples as surrogate marker for SLPI and AnxA2 gene expression in tonsillar tissue we focused on those samples where we previously found incongruent HPV DNA-results in the analyzed biomaterials (12). Among the 52 patients with TSCC 40 patients showed concordant results in all biomaterials analyzed. The remaining 12 cases are depicted in Table IV. In 97/107 patients with CRT and in 48/56 patients with H concordant results in all biomaterials analysed were obtained. The remaining 10/107 CRT and 8/56 H, where differences between the tissue HPV-status, the sputum and swab results were detected are presented in Table V. Of note: cases 9-12 in

Table II. Relationship between SLPI immunohistochemistry and the smoking status of patients.

Pathology	Smoking status			P-value
	Active	Never	Former	
TSCC SLPI-IHC				0.0022
Negative/weak	6 (12.0)	9 (18.0)	14 (28.0)	
Moderate/strong	13 (26.0)	0 (0.0)	8 (16.0)	
CRT + H SLPI-IHC				0.0009
Negative/weak	27 (16.9)	68 (42.5)	N/A	
Moderate/strong	36 (22.5)	29 (18.1)	N/A	
CRT SLPI-IHC				0.0022
Negative/weak	15 (14.1)	46 (43.4)	N/A	
Moderate/strong	25 (23.6)	20 (18.9)	N/A	
H SLPI-IHC				n.s.
Negative/weak	12 (22.1)	22 (40.8)	N/A	
Moderate/strong	11 (20.4)	9 (16.7)	N/A	

One patient with CRT and one patient with H that stopped smoking at least 2 years prior to diagnosis were excluded from analysis, as the dataset was too small to be analyzed. For two patients with TSCC and one patient with H, no information regarding their smoking status was available. SLPI-IHC results are scored as negative/weak and moderate/strong according to Cordes *et al* (27). Numbers in parenthesis are percent per disease entity. CRT, chronic or recurrent tonsillitis; H, tonsillar hyperplasia; IHC, immunohistochemistry; N/A, not applicable; n.s., not significant; SLPI, secretory leucocyte protease inhibitor; TSCC, tonsillar SCC.

Table IV as well as cases 5-10 and 14-18 in Table V were characterized by HPV-negative tissue but HPV-positive sputum/swab samples. These samples were reanalyzed by re-extracting DNA and RNA from the tissues. DNA was analyzed again by performing GP5+/GP6+ PCRs and RNA was transcribed into cDNA. RT-qPCR was performed using HPV16 and HPV18 specific primers, in case of the TSCC cases and HPV 6, 11 and 18 specific primers in case of the CRT and H cases, depending on the HPV types found in the corresponding HPV-positive samples, assuming that in these cases, as in all other cases, all HPV positive materials would have been affected with the same HPV type. However, no HPV-DNA or HPV-RNA could be detected in these tissues, despite positive signals for the positive controls. Furthermore, in Tables IV and V AnxA2 gene expression in relation to SLPI gene expression is shown. All cases are characterized by the same AnxA2/SLPI ratio in tonsillar swabs, sputum and the respective tissue samples.

Discussion

This prospective study investigating 215 patients confirms the significant association between (a) smoking, (b) SLPI- and AnxA2-expression and (c) HPV infection in benign and malignant tonsillar lesions as we have previously shown in various, yet, retrospective studies (13). For TSCC- and CRT-cases both, i) the correlation between a positive smoking habit and high SLPI-expression levels and vice versa as well as ii) the

Table III. Relationship between SLPI immunohistochemistry and tonsillar HPV DNA status.

Pathology	HPV-DNA		P-value
	Positive	Negative	
TSCC SLPI-IHC			<0.0001
Negative/weak	20 (38.5)	11 (21.1)	
Moderate/strong	1 (1.9)	20 (38.5)	
CRT+H SLPI-IHC			0.0209
Negative/weak	19 (11.6)	79 (48.5)	
Moderate/strong	4 (2.5)	61 (37.4)	
CRT SLPI-IHC			0.001
Negative/weak	12 (11.2)	50 (46.7)	
Moderate/strong	0 (0.0)	45 (42.1)	
H SLPI-IHC			n.s.
Negative/weak	7 (12.5)	29 (51.8)	
Moderate/strong	4 (7.1)	16 (28.6)	

SLPI IHC results are scored as negative/weak and moderate/strong according to Cordes *et al* (27). Numbers in parenthesis are percent per disease entity. CRT, chronic or recurrent tonsillitis; H, tonsillar hyperplasia; HPV, human papilloma virus; IHC, immunohistochemistry; n.s., not significant; SLPI, secretory leucocyte protease inhibitor; TSCC, tonsillar SCC.

correlation between high SLPI-expression and fewer HPV infections and vice versa, show strong statistical significance. However, this is not the case in H-cases, although of the 11 HPV-positive cases fittingly 8 are non-smokers and 7 of these show negative/weak SLPI staining (Ref. 13 and Tab 3). Possibly with n=54, the number of investigated H-cases is not sufficient for statistical analysis to reach significance. Moreover, chronic inflammation which is present in TSCC and CRT, yet, to a lesser extent in H, might be additionally involved in the interaction of the here investigated parameters (17,18). Among the benign cases 29/163 show strong staining for SLPI in immunohistochemistry even among never smokers. The latter has not been detected in a single case among never smokers of the TSCC-group. Thus, inflammation cannot be ruled out to trigger tissue expression of SLPI and AnxA2 in non-malignant tonsils. Perhaps, the comparably small number of H-cases accompanied by inflammation-induced SLPI expression is responsible for the lack of significance correlating smoking, SLPI/AnxA2 expression and HPV infection in H-cases. However, analyzing all cases with benign tonsillar lesions as one group showed similar results as seen in the TSCC-patients.

As specifically depicted by the tables and figures in the results section, the here performed analysis affirms the positive correlation between a positive smoking history with elevated SLPI- and AnxA2-expression (and vice versa) with yet a significant surplus of SLPI (13). Furthermore, HPV DNA-presence in the investigated tissue specimens is associated with low SLPI-expression, a positive AnxA2/SLPI ratio and vice versa. These results were, as already mentioned, expected due to comparable results obtained from 6 retrospective studies on altogether 892 patients performed,

Table IV. Ratio of AnxA2/SLPI gene expression levels in patients with tonsillar SCC that exhibit differing HPV results in different biomaterials.

Case	Tonsillar tissue			Tonsillar swab		Sputum	
	HPV	SLPI-IHC	AnxA2/SLPI	HPV	AnxA2/SLPI	HPV	AnxA2/SLPI
1	16	-/+	+5.23	16	+4.58	-	+5.32
2	16	-/+	+5.64	16	+5.03	16	+5.63
3	16	-/+	+4.86	16	+4.34	16	+5.10
4	16	-/+	+5.98	16	+5.14	16	+5.71
5	18	-/+	+4.86	18	+4.56	-	+5.13
6	18	-/+	+5.32	-	+4.94	-	+5.41
7	16	-/+	+5.74	-	+5.08	-	+5.68
8	18	-/+	+5.50	18	+4.98	18	+5.58
9	-	++/+++	-2.90	18	-2.51	18	-2.33
10	-	++/+++	-2.93	16	-2.53	16	-2.40
11	-	++/+++	-2.87	16	-2.48	16	-2.11
12	-	++/+++	-3.13	16	-2.58	-	-2.41

In cases of HPV-positivity, the HPV-type is given. HPV-negativity is indicated by '-'. SLPI-IHC results are scored as negative/weak: -/+ and moderate/strong: ++/+++ according to Cordes *et al* (27). Moreover, the ratio of AnxA2/SLPI expression levels are provided and indicate that the ratio between the two genes is the same as that found in tonsillar tissues, irrespective of the HPV-status of the tonsillar swabs and/or sputum. AnxA2, Annexin A2; HPV, human papilloma virus; IHC, immunohistochemistry; SLPI, secretory leucocyte protease inhibitor.

previously (13). With the prospective validation of the results of these 6 previous studies, in total now adding up to 1.107 investigated patients, epidemiologic aspects of the here tested parameters and correlations seem to be described sufficiently, at least for respective disease entities of the head and neck. While own studies on mechanistic aspects in terms of HPV transfection assays in the presence and absence of SLPI are ongoing, we encourage others to test the findings described by us in different geographical regions of the world and for other neoplastic and non-neoplastic HPV-associated disease entities.

Data derived from the present study population on HPV-epidemiology and on the validity of the detection methods used (12) showed i) a 14% dis-agreement of HPV- and p16^{INK4A}-status among the various tested biomaterials and ii) with 26% an even higher mis-match ratio between p16^{INK4A}-staining and the true HPV-status (HPV-DNA and HPV-RNA positive) of the TSCC-cases analyzed (12). However, analyzing SLPI- and AnxA2-gene expression, sputum and tonsillar swabs precisely match the results of SLPI- and AnxA2-gene expression levels measured in the respective tonsillar tissue specimens. The latter instance represents the most intriguing result of the present study. The fact that analysis of the AnxA2/SLPI ratio in sputum or oral swabs precisely predict the expression ratio in the respective tonsillar tissue appears promising. As shown in Tables III and IV hardly any differences can be found between the AnxA2/SLPI ratio in the different biomaterials, hence one can argue that sputum might be the better biomaterial to analyze since sputum samples are often easier obtainable than tonsillar swabs.

According to the presented results, AnxA2/SLPI ratios determined in sputum and tonsillar swabs resemble the ratio in the respective tonsillar tissue to an extent that both sputum and swab analysis qualify as a surrogate marker for the situation in

the tonsillar tissue. There seem to be two measures that could possibly derive from the knowledge of the AnxA2/SLPI ratio in tonsils of a person determined by the surrogate sputum or tonsillar swab: i) It could be discussed whether or not persons with a positive AnxA2/SLPI ratio should possibly be tonsillectomized, precautionary. This notion is supported by the finding that within this study population out of in total 163 patients with benign tonsillar lesions 23 (14.1%) were HPV-positive, five (21.7%) of these also being positive for HPV mRNA, classifying these HPV infections as active ones (12). Moreover, three of these five HPV RNA-positive cases showed HPV16-infections classified as a high-risk virus type with a high potential that infection will progress to carcinogenesis. The latter two facts proof that active high-risk HPV infections in non-malignant tonsils exist and it might be assumed that in these cases progression to carcinogenesis would have been likely if the patients were not tonsillectomized prior to that possible progression. The idea that tonsillectomy might prevent from TSCC is supported by a nation-wide cohort study in Sweden comparing cancer incidence in a tonsillectomy cohort (n=225.718) with Sweden's general population. The authors detected a reduced risk of tonsillar cancer in the tonsillectomy cohort but unrelated to other oropharyngeal or other head and neck cancers (29). The cases with active HPV16 infection in the non-malignant tonsils, indeed, showed a positive AnxA2/SLPI ratio in tonsillar swab and sputum samples in the present study which identifies them at risk for an HPV infection. However, it must be critically noted that tonsillectomies are not without danger due to possible complications, in particular postoperative secondary bleeding with sometimes even fatal outcome (30). Focusing on the here investigated patients with benign tonsillar lesions by tonsillectomy on the basis of a positive AnxA2/SLPI ratio 87% (20/23) would have been

Table V. Ratio of AnxA2/SLPI gene expression in patients with CRT and H with different HPV results in different biomaterials.

Case	Pathology	Tonsillar tissue						Tonsillar swab					
		Right			Left			Right			Left		
		HPV	SLPI-IHC	AnxA2/SLPI	HPV	SLPI-HPV	AnxA2/IHC	HPV	AnxA2/SLPI	HPV	AnxA2/SLPI	HPV	AnxA2/SLPI
1	CRT	6	-/+	+4.44	6	-/+	+4.29	-	+3.77	-	+3.92	6	+3.48
2	CRT	6	-/+	+4.32	6	-/+	+4.23	6	+3.71	6	+3.76	-	+3.39
3	CRT	11	-/+	+3.56	11	-/+	+3.03	-	+3.71	11	+3.39	11	+2.73
4	CRT	11	-/+	+3.58	11	-/+	+3.88	11	+3.86	11	+3.63	-	+3.18
5	CRT	-	++/+++	-4.03	-	++/+++	-4.10	6	-3.45	6	-3.58	6	-2.83
6	CRT	-	++/+++	-3.87	-	++/+++	-3.95	6	-3.14	6	-2.99	6	-2.50
7	CRT	-	++/+++	-3.71	-	++/+++	-3.81	6	-2.97	6	-2.91	6	-2.38
8	CRT	-	++/+++	-3.93	-	++/+++	-3.97	6	-3.18	6	-3.05	6	-2.57
9	CRT	-	++/+++	-3.95	-	++/+++	-4.01	11	-3.25	11	-3.28	11	-2.57
10	CRT	-	++/+++	-4.03	-	++/+++	-4.05	11	-3.25	11	-3.39	11	-2.64
11	H	11	-/+	+2.39	11	-/+	+2.16	11	+2.09	-	+1.91	11	+2.06
12	H	18	-/+	+2.25	18	-/+	+2.14	18	+1.95	18	+1.89	-	+2.03
13	H	6	-/+	+2.10	6	-/+	+2.06	6	+1.93	6	+1.78	-	+2.02
14	H	-	++/+++	-2.03	-	++/+++	-2.08	11	-2.02	11	-2.07	11	-2.19
15	H	-	++/+++	-1.99	-	++/+++	-1.97	6	-1.85	6	-1.96	-	-1.95
16	H	-	++/+++	-1.96	-	++/+++	-1.92	11	-1.72	11	-1.67	-	-1.82
17	H	-	++/+++	-2.03	-	++/+++	-2.04	11	-1.97	11	-2.03	-	-2.04
18	H	-	++/+++	-2.00	-	++/+++	-1.99	18	-1.96	18	-1.99	-	-2.00

In case of HPV-positivity the HPV-type is provided. HPV-negativity is indicated by '-'. SLPI-IHC results are scored as negative/weak: -/+ and moderate/strong: ++/+++ according to Cordes *et al* (27). The ratio of the AnxA2/SLPI gene expression is provided, showing that the expression ratio between the two genes is the same as found in the tonsillar tissues, irrespective of the HPV-status of the tonsillar swab and/or sputum samples. AnxA2, Annexin A2; CRT, chronic or recurrent tonsillitis; H, tonsillar hyperplasia; HPV, human papilloma virus; IHC, immunohistochemistry; SLPI, secretory leucocyte protease inhibitor.

overtreated, however, it would possibly have saved 13% (3/23) of patients from developing tonsillar cancer. Since the latter seems intriguing, more epidemiologic data are required to further provide a risk-benefit assessment before tonsillectomy can be recommended solely based on AnxA2/SLPI ratios in a cancer prevention scenario. ii) A second measure that could possibly derive from the AnxA2/SLPI levels of the tonsillar swab and sputum samples is HPV vaccination of individuals with positive AnxA2/SLPI ratios, since these are at higher risk to be successfully infected by HPV. The latter would be particularly interesting for economically disadvantaged developing countries (31-33), which could use the AnxA2/SLPI analysis to identify a subgroup of the population as such a group most likely to benefit from HPV vaccination. Thereby, the relatively cost-intensive vaccines could be better targeted, which should positively influence the cost-effectiveness ratio in the countries concerned. Following our results, one could argue to include only non-smokers in vaccination strategies, as they are associated with lower SLPI levels. However, there are also smokers with low and non-smokers with high SLPI levels. Fittingly, Carpen and coworkers (34) found that smoking and alcohol consumption is also common in patients with HPV-positive, p16^{INK4A}-positive oropharyngeal carcinomas, as we also have observed in comparable study populations investigated, previously (7,10). Therefore, stratifying by smoking habit of individuals would not be precise enough for a vaccination campaign. Indeed, we believe that this second aspect is worth further evaluation. Since at present HPV vaccination is predominantly targeted to prevent cervical cancer, it might be of interest that we previously found similar associations between SLPI, AnxA2 and tissue HPV-status when analyzing 99 vulvar carcinomas (35). Based on these findings, analysis of AnxA2/SLPI ratio of cervical swabs might also be suitable as a surrogate marker for the situation in the cervical tissue, hence identifying women who might be at risk developing cervical cancers and thereby contribute to better targeted HPV vaccination strategies.

A limitation of the study is the rather small number of patients with tonsillar hyperplasia which is due to the fact that the vast majority of tonsils histopathologically show to some degree inflammation. We aimed to enroll H-patients as 'counterparts' to CRT-patients (lower versus higher degree of inflammation) but were unable to enroll more than the here analyzed 56 patients with tonsillar hyperplasia. The above discussed measures in case of a positive AnxA2/SLPI ratio is significantly supported by all data except for analysis of H-cases, separately. Focusing on H-cases, data show at least a to the other entities' results comparable trend (decreased and increased changes in gene expression levels as seen in CRT- and TSCC-patients), yet, without significance. Significance also in H-cases possibly would have been reached if a larger number of H-patients could have been enrolled.

In conclusion, the data prospectively collected here confirm the previous retrospective results and thus underline the plausibility of the hypothesis put forward. According to this hypothesis, smoking leads to enhanced SLPI expression, which in turn prevents HPV from binding to cellular AnxA2. However, binding of HPV to AnxA2 appears to be essential for successful HPV cell-entry and, thus, active infection prompting carcinogenesis. Most interestingly, SLPI- and AnxA2-gene

expression levels measured in tonsillar swabs and sputum accurately reflect the expression status of both genes in tonsillar tissue. Accordingly, the AnxA2/SLPI ratio in sputum and/or tonsillar swab might be used to possibly identify patients which could be subjected to either tonsillectomy or HPV vaccination, thereby making HPV-associated carcinogenesis less likely.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MH and ESQ designed, supervised and/or performed the experiments. ESQ analyzed the data, generated the figures and wrote the first draft of the manuscript. ML and PA made substantial contributions to data acquisition. MH and ML performed plausibility checks and reviewed and edited the first draft, which was further edited and later approved for publication by all authors. AH, AK, FH and RM provided patient data and material, and performed or supervised the initial experimental sampling procedures before samples were sent to Kiel. The laboratories in Kiel are part of the Department of Otorhinolaryngology, Head and Neck Surgery, University Clinic Schleswig-Holstein, directed by PA. MH was the principal investigator and acquired the project funding. MH and ESQ confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The current study was approved by the Ethics Committee of the Medical Faculty of the Christian-Albrechts-University of Kiel, Germany (approval no. D 429/14). Informed consent was obtained from all subjects involved in the present study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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