Is periodontitis a prognostic factor in order to indicate antibodies against citrullinated peptides in patients with rheumatoid arthritis?

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Abstract

Objective

In this cross-sectional study we investigated antibody titres against cyclic citrullinated peptides derived from filaggrin (anti-CCP) and citrullinated α-enolase (anti-CEP-1) among patients with RA as a function of periodontal findings.

Methods

107 patients with RA (median age 56 years, 75% females) were included. For periodontal diagnoses missing teeth, periodontal epithelial surface area, periodontal inflamed surface area and periodontal diagnosis according to the working group's guidelines of the Center for Disease Control and Prevention were determined. Subgingival bacterial DNA of five periodontopathic bacteria was assessed by PCR with sequence-specific oligonucleotides. Anti-CCP and anti-CEP-1 antibodies in plasma samples were investigated using enzyme-linked immunosorbent assays. Low resolution human leukocyte antigen (HLA) typing was carried out using PCR with sequence-specific primers.

Results

*PESA was found associated with a low adjusted odds ratio for anti-CCP positivity (OR=1.002, p=0.040). All patients who were infected with Aggregatibacter actinomycetemcomitans were simultaneously anti-CCP positive (p=0.043). HLA-DRB1*13 lowered the adjusted odds ratio for anti-CCP (OR=0.073, p=0.002) and anti-CEP-1 (OR=0.068, p=0.018) positivity whereas HLA-DRB1*07 indicated a lower risk only for demonstrable anti-CCP antibodies (OR=0.079, p=0.004). HLA-DRB1*04 was associated with increased adjusted odds ratio for anti-CEP-1 positivity (OR=4.154, p=0.005) and the simultaneous proof of both investigated autoantibodies (OR=3.725, p=0.011).*

Conclusion

Among patients with RA periodontitis may be a minor risk factor for anti-CCP positivity. Our data first provide evidence that an infection with A. actinomycetemcomitans is associated with an increased formation of anti-CCP. HLA phenotype proved to be a significant risk indicator for both investigated antibodies.

Key words

periodontitis, rheumatoid arthritis, cyclic citrullinated peptides, citrullinated α-enolase, *P. gingivalis, A. actinomycetemcomitans,* HLA

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Introduction

Rheumatoid arthritis (RA) and periodontitis are characterised by similar pathogenic mechanisms. In both diseases chronic inflammation is maintained by pro-inflammatory cytokines leading to connective tissue breakdown and bone erosion (1). A lot of epidemiologic studies reported an increased prevalence/severity of periodontitis among patients with RA (2-9) and vice versa (10-12). There are a number of hypotheses in order to explain the relationship between these two diseases. For instance, they share risk factors such as age, smoking (13, 14), certain human leukocyte antigen (HLA) alleles (15) or cytokine polymorphisms (16). Moreover, a large number of studies indicate a potential influence of periodontal bacteria on the aetiology of RA. Antibodies against periodontological pathogens were detected at a significantly increased frequency in both, serum (17-21) and synovial fluid (22) of patients suffering from RA in comparison to controls. Furthermore, DNA from periodontopathogens was found at an elevated level in synovial fluid from patients with rheumatic diseases in comparison to controls (23, 24). Cell culture studies strongly suggest that *Porphyromonas gingivalis*, a major pathogen of periodontitis, may infect human chondrocytes. This infection led to changes in the cell cycle as well as an increased apoptosis rate of the cells (25). The latter result indicates that articular tissues may be directly damaged by living periodontal bacteria. So far only one study has been published to show the cultivation of the periodontopathic bacteria *Peptostreptococcus micros* (now *Parvimonas micra*) out of the synovial fluid from a patient with a total knee joint prosthesis (26).

Further studies addressed the question whether periodontal pathogens could promote the formation of citrullinated antigenic peptides and thereby trigger autoimmune processes that are important in RA aetiology. Most of these studies were done with regard to *P. gingivalis*.

P. gingivalis expresses its own unique peptidylarginine deiminase (PPAD) (27)}. PPAD citrullinates endogenous bacterial as well as human host peptides such as fibrinogen and α-enolase (28) and may thus have an impact on the development of anti-citrullinated peptide/protein antibodies (ACPA) (29-33). In addition, it is assumed that *P. gingivalis* induces neutrophil extracellular trap (NET) formation which could be another source of citrullinated autoantigens (34).

Aggregatibacter actinomycetemcomitans produces leukotoxin A (LTxA) which forms pores in the cell membrane of neutrophils at the crevicular fluid of patients with periodontitis. This leads to neutrophil PAD activation and citrullination of many peptides released from neutrophils (35).

Antibodies against the periodontal pathogen *Prevotella intermedia* were found to be associated with autoantibodies against the two citrullinated peptides cytokeratin (cCK13-1) and tenascin-C (cTNC5). Autoantibodies against both citrullinated peptides correlated with each other. CCK13 was found in gingival crevicular fluid in patients who suffered from RA and periodontitis. As an extracellular matrix protein, cTNC5 is expressed at high levels in the joints of patients suffering from RA and, furthermore, was identified in RA patients as target for autoantibodies (36).

The formation of citrullinated peptides and autoantibodies against them is known to be relevant in the aetiology of RA (37). The identification of ACPA attains sensitivity for RA of more than 70% and specificity that approaches 96–98% (38). ACPAs both precede the onset of RA (39, 40) and are associated with the severity of this disease (41). Additionally, they promote the production of pro-inflammatory cytokines which contribute to joint destruction (42).

According to the cited literature periodontitis or periodontal bacteria may represent a risk factor for ACPA seropositivity. A previous work of our group (43) aimed to investigate levels of autoantibodies against citrullinated α-enolase (anti-CEP-1) and cyclic citrullinated peptides derived from filaggrin (anti-CCP) within patients suffered from generalised aggressive periodontitis (GAgP) and generalised chronic periodontitis (GChP) in comparison to a non-periodontitis control group. All individuals had no RA or

other systemic diseases for which an association with RA is known. As a result, we found no association between periodontitis and ACPA levels. Thus, periodontitis in subjects without RA does not appear to be a risk factor for increased ACPA levels. In comparison to our previous work the current study aimed to investigate a putative relationship between the periodontal status and/or the detection of five periodontal pathogenic bacteria in subgingival plaque with autoantibody levels against CEP-1 and/or CCP in patients with RA. Known risk factors for ACPA formation such as age, smoking and HLA-DRB1* alleles should be additionally taken into consideration. The main question to answer was whether periodontitis is beside other factors an additional risk factor for ACPA positivity in patients with RA.

Methods

Study population and clinical investigations

The study was approved by the local ethics committee and was carried out in accordance with the ethical guidelines of the Declaration of Helsinki 1975 and its amendment in "Tokyo and Venice".

From May 2012 to April 2016, 116 patients with RA were tested for fulfilling the criteria of the study. Both patients with newly diagnosed RA and those with longer existing RA were included. They had to meet the following inclusion criteria: Caucasian descent, not related to each other, at least eighteen years old, at least four own teeth. The following criteria led to the exclusion from the study: diabetes type 1 or 2, coronary heart disease, pregnancy, antibiotic regimen within 3 months before beginning of the study, subgingival scaling 6 months prior the examination, consumption of drugs that could lead to gingival overgrowth such as nifedipine, cyclosporine A and hydantoin. 107 patients met the inclusion criteria and were included in the study. Rheumatologically diagnosis and treatment was performed at the Clinic of Internal Medicine II, Department of Rheumatology, Martin-Luther University Halle-Wittenberg (Prof. G. Keyßer, Dr C. Schäfer), at a Department of Rheumatology (professional association Dr M. Bohl-Bühler and Dr S. Reckert, Rheumahaus, Potsdam) and at three internal-rheumatologic practices in Halle/Saale and Magdeburg according to current criteria for classifying RA and spondyloarthropathies (44, 45). If, from the rheumatologist's point of view, a patient was suitable for the study and the patient consented to the study enrolment the dental examiners were informed. All periodontal examinations took place where the patients were also treated rheumatologically. They were performed by two dentists (Dr. M. Haffner and E. Jurianz) and comprised assessment of smoking anamnesis (current, past, never smoker), approximal plaque index (API) (46), percentage of sites with bleeding upon probing (BOP), pocket depth (PD = distance between gingiva margin and apical stop of the probe), clinical attachment loss (CAL= distance between cement-enamel junction and apical stop of the probe), and number of missing teeth.

BOP, PD and CAL were determined after six-point measurement with a pressure-sensitive periodontal probe (TPS-probe Vivicare, Vivadent, Schaan, Liechtenstein or DB764R Aesculap AG & Co. KG, Tuttlingen, Germany). The maximum values for PD and CAL per tooth were included for statistically comparisons. Before the start of the study the examiner were instructed in the use of the both periodontal probes. Their application was trained on a phantom model A-PB (frasaco GmbH, Tettnang, Germany) and under clinical conditions. In previous studies the overall reproducibility of the UNC 15 probe and TPS-probe was already demonstrated (47, 48).

The clinical periodontitis case definition was held according to the guidelines of the working group of the Centers for Disease Control and Prevention (CDC) (49). A severe periodontitis case was diagnosed if at least ≥2 interproximal sites with CAL ≥6mm (not on same tooth) and ≥1 interproximal site with PD ≥5mm were present. A moderate periodontitis case was diagnosed if at least ≥2 interproximal sites with CAL ≥4mm (not on same tooth) or ≥2 interproximal sites with PD ≥5mm (not on one tooth) were present. If no severe or moderate periodontitis was present, periodontitis was diagnosed as mild or absent. For a more accurate quantification of the root surface affected by attachment loss and quantification of the inflamed epithelial surface, both the periodontal epithelial surface area (PESA) and the periodontal inflamed surface area (PISA) were calculated (50). For that purpose, a freely downloadable (www.parsprototo.info<http://www.parsprototo.info) Excel spreadsheet was used. In order to calculate PESA, data of CAL and recession have to be entered. For the calculation of PISA, sites with BOP have to be recorded additionally.

Detection of five periodontal marker bacteria from subgingival plaque specimens, measurements of anti-CCP and anti-CEP-1 antibody serum levels, and determination of HLA class 2 alleles has previously been described by us (43). Therefore, these steps are only briefly explained.

Molecular assessment of periodontopathic bacteria

In 104 of 107 patients with RA, the analysis of the subgingival plaque could be performed. After removal of supragingival plaque and relative drying, microbial samples were taken from the deepest pocket of each quadrant by insertion of a sterile paper point for 20 seconds. All bacterial plaque samples of each individual were pooled in one tube. The preparation of bacterial DNA was carried out using a commercial DNA kit (QIAamp DNA Mini Kit; Qiagen, Hilden, Germany) according to the manufacturer's instructions. A molecular biological test (Micro-Ident test, Hain Lifescience, Nehren, Germany) was used for the identification of the five periodontogenic bacterial species (*A. actinomycetemcomitans, P. intermedia, P. gingivalis, Treponema denticola, Tannerella forsythia*) according to the manufacturer's protocol.

Determination of anti-CCP and anti-CEP-1

Antibody concentrations were determined using enzyme-linked immunsorbent assays (ELISA) (EUROIMMUN, Lübeck, Germany). In both

ELISAs only the immunoglobulin class G (IgG) was detected, *i.e.* anti-CCP ELISA (IgG) and anti-CEP-1 ELISA (IgG). The cut-off values were 5 RE/ml for anti-CCP and 20 RE/ml for anti-CEP-1. If a study participant had an antibody concentration in range of the cut-off value, this person was considered as positive for the respective antibodies.

Genomic HLA typing

Preparation of genomic DNA from fresh human venous EDTA-blood was carried out by using a salting-out procedure to extract DNA from human nucleated cells (51). 99 out of 107 included patients were HLA-DRB1*/DR) typed by PCR with sequence-specific primers (SSP) using commercial kits (Histotype-DR,-DQ, BAG, Lich) at the level of low resolution according to the manufacturer's protocol.

Statistical analysis

Statistical analyses were carried out using commercially available software (SPSS v. 25.0 package, IBM, Chicago, IL). Values of $p<0.05$ were considered as significant.

Metric demographic and clinical data were checked for normal distribution using both, the Kolmogorov-Smirnov test and the Shapiro-Wilk test. As all metric values were not normally distributed, they were plotted as median and 25th/75th percentiles. Mann-Whitney U-test was performed for bivariate comparisons.

Categorical variables were plotted as number and percentage in parentheses. Differences between cases and controls were evaluated using Chi-squared analysis. Only in the case of 2x2 tables the *p*-values could be corrected with Yates continuity correction. If the expected number in one group was less than 5, Fisher's exact test was performed.

Multivariate analyses were conducted with binary logistic regression analyses with respect to putative confounders for a positive anti-CCP or anti-CEP-1 serum level such as age, gender, smoking, HLA-DRB1* alleles, administration of disease-modifying anti-rheumatic drugs (DMARDs), and PESA/PISA.

Table I. Demographic and clinical conditions of the entire study cohort.

Demographic and clinical parameters	Entire study cohort n=107
Age (years) median (25th/75th percentile)	56 (48/63)
Females, n (%)	75 (70.1)
Disease duration (month)	
median (25th/75th percentile)	1 (0/14)
minimal/ maximal (month)	0 / 576
Smoking history, n (%)	
- current	26 (24.3)
- past	36 (33.6)
- no	45 (42.1)
Periodontal conditions median (25th/75th percentile)	
Approximale plaque index (%)	34.8 (11.5/63.2)
Bleeding upon probing (% sites)	8.9 (3.5/19.1)
Pocket depth (mm)	3.0 (2.7/3.7)
Attachment loss (mm)	3.4 (2.9/4.3)
Missing teeth exception of 3rd molars	5 (2/10)
Periodontal epithelial surface area (mm^2)	1066.9 (745.4/1336.7)
Periodontal inflamed surface area (mm^2)	80.6 (30.1/205.3)
CDC classification, n (%)	
No or mild periodontitis	38 (35.5)
Moderate periodontitis	40 (37.4)
Severe periodontitis	29 (27.1)
Positive antibody level against	
CEP-1, n (%)	50 (46.7)
CCP, n (%)	67 (62.6)
Periodontal bacteria, n (%)	n=104 (%), missing 3
A. actinomycetemcomitans	7 (6.5)
P. gingivalis	46 (43.0)
P. intermedia	25 (23.4)
T. forsythia	85 (79.4)
T. denticola	70 (65.4)
Medication, n (%)	n=104, missing 3 (exception: steroids, no missing patient)
NSAIDs	50 (46.7)
DMARD	72 (67.3)
Biologicals	46 (43.0)
Steroids	62 (57.9)

CDC: Centres for disease control; CCP: cyclic citrullinated peptides; CEP: citrullinated α-enolase; NSAIDs: non-steroidal anti-inflammatory drugs; DMARDs: disease-modifying anti-rheumatic drugs.

Results

Univariate analyses

The demographic and clinical conditions of the entire study cohort are presented in Table I. Because the metric variables were not normally distributed they are displayed as median (25th/75th) percentiles. More women than men were investigated. 57.9% of study participants were current or past smokers. According CDC classification 64.5% of RA patients suffered from a severe or moderate periodontitis. 46.7% of the entire study cohort was positive for anti-CEP-1 antibodies whereas 62.6% exhibited anti-CCP antibodies. 42.1 % were positive for both autoantibodies. The detection of anti-CEP-1 antibodies was closely associated with those of anti-CCP ($p<0.0001$). *T. forsythia* was most prevalent in subgingival plaque followed by *T. denticola, P. gingivalis, P. intermedia* and *A. actinomycetemcomitans*.

At first, we compared all recorded demographic and clinical parameters based on detection of anti-CEP-1 and anti-CCP autoantibodies. Regarding presence of anti-CEP-1 and the simultaneous occurrence of both autoantibodies (anti-CCP+anti-CEP-1) we did not find any significant differences between seropositive and seronegative patients (Tables II and IV). In contrast anti-CCP-positive-patients harbored always *A. actinomycetemcomitans* in the subgingival plaque and took more frequently DMARDs than those in-

Table II. Demographic, periodontal and rheumatological conditions in anti-CEP-1 positive in comparison to anti-CEP-1 negative patients with rheumatoid arthritis.

Demographic and clinical parameters	Anti-CEP-1 positive n=50 (46.7%)	Anti-CEP-1 negative n=57 (53.3%)	*p*-value
Age (years) median (25^{th}/75^{th} percentile)	56.5 (50/63.3)	56 (47.5/65)	0.881*
Disease duration (month) median (25^{th}/75^{th} percentile)	0 (1/24)	0 (1/10.3)	0.900*
Females, n (%)	33 (66)	42 (73.7)	0.513**
Smoking history, n (%)			
- current	10 (20)	16 (28.1)	
- past	20 (40)	16 (28.1)	
- no	20 (40)	25 (43.9)	0.380***
Periodontal conditions median (25^{th}/75^{th} percentile)			
Approximale plaque index (%)	44 (14.4/70.8)	27.8 (10.3/59)	0.261*
Bleeding upon probing (% sites)	9 (3.7/21.3)	8.3 (2.95/17.9)	0.413*
Pocket depth (mm)	3.1 (2.7/3.8)	2.9 (2.6/3.65)	0.361*
Attachment loss (mm)	3.32 (2.9/4.33)	3.5 (2.9/4.3)	0.712*
Missing teeth exception of 3rd molars	5 (2/10)	5 (2/10)	0.923*
Periodontal epithelial surface area (mm^2)	1091.6 (761.9/1395.7)	1044.9 (735/1188.4)	0.541*
Periodontal inflamed surface area (mm^2)	89.2 (30.1/209.7)	63 (29.6/206)	0.451*
CDC classification, n (%)			
No or mild periodontitis	21 (42)	17 (29.8)	
Moderate periodontitis	17 (34)	23 (40.4)	
Severe periodontitis	12 (24)	17 (29.8)	0.421***
Periodontal bacteria, n (%)	n=48, missing 2	n=56, missing 1	
A. actinomycetemcomitans	5 (10.4)	2 (3.6)	0.244**
P. gingivalis	20 (41.7)	26 (46.4)	0.772**
P. intermedia	13 (27.1)	12 (21.4)	0.658**
T. forsythia	38 (79.2)	47 (83.9)	0.710**
T. denticola	30 (62.5)	40 (71.4)	0.448**
Medication, n (%)	n=49, missing 1	n=55, missing 2	
NSAIDs	24 (49)	26 (47.3)	1.000**
DMARD	38 (77.6)	34 (61.8)	0.128**
Biologicals	22 (44.9)	24 (43.6)	1.000
Steroids	28 (56.0)	34 (59.6)	0.703**

*Mann-Whitney U test; **Chi-Square test with Yates correction or Fisher's exact test; ***Chi-Square test (uncorrected).
CEP: citrullinated α-enolase; NSAIDs: non-steroidal anti-inflammatory drugs; DMARD: disease-modifying anti-rheumatic drugs.

dividuals negative for anti-CCP antibodies. There was a trend for a higher PESA in RA patients who were anti-CCP positive (Table III)

Secondly we investigated putative associations between HLA-DRB1* alleles and anti-CCP, anti-CEP-1 and anti-CCP+anti-CEP-1 positivity. RA patients who were HLA-DRB1*04 positive exhibited anti-CCP and anti-CEP-1 antibodies at a significantly higher frequency. On the contrary the HLA-DRB1*13 genotype was negatively associated with both investigated antibodies (Fig. 1-2-3). The HLA-DRB1*07 genotype was negatively associated only with anti-CCP antibodies (Fig. 1).

In order to evaluate a putative genedose effect of HLA on ACPA positivity we compared RA patients who were carrier of no, one or two copies (homozygosity) of susceptibility (HLA-DRB1*04) or protective (HLA-DRB1*13) HLA alleles. We obtained that the percentages of ACPA positive individuals depends on the number of predisposing (Table V) or protective (Table VI) HLA alleles.

Multivariate analyses

The multivariate analysis was designed to assess whether the periodontal parameters PESA and PISA were associated to the ACPA levels stratified for age, gender, smoking, DMARDs and HLA-alleles DRB1*04, DRB1 * 13, and -DRB1 * 07 (Table VII). The detection of *A. actimomycetemcomitans* in subgingival plaque was not included as cofactor because only seven patients were infected with this bacterium and all of them were positive for anti-CCP antibodies.

For anti-CCP positivity, intake of DMARDs and PESA were associated with an increased adjusted odds ratio (OR) whereas expression of HLA-DRB1*13 and -DRB1*07 were associated with a lower OR. For HLA-DRB1*04 genotype we evaluated a trend for association with anti-CCP serum level (Tables VII, model 1). For anti-CEP-1 antibodies a positive association with HLA-DRB1*04 genotype and a negative association with HLA-DRB1*13 genotype was demonstrable (Table VII, model 2). Only HLA-DRB1*04 was positive associated with the simultaneous presence of both autoantibodies (Table VII, model 3).

Discussion

According to epidemiologic studies, periodontitis is more prevalent in patients with RA and vice versa. There is evidence to suggest that periodontitis and/or certain periodontal marker bacteria induce citrullination of various peptides thereby promoting the forma-

Table III. Demographic, periodontal and rheumatological conditions in anti-CCP positive in comparison to anti-CCP negative patients with rheumatoid arthritis. Significant *p*-values are indicated in bold.

Demographic and clinical parameters	Anti-CCP positive n=67 (62.6%)	Anti-CCP negative n=40 (37.4%)	*p*-value
Age (years) median (25^{th}/75^{th} percentile)	0 (1/45)	58 (42/67)	0.854*
Disease duration (month) median (25^{th}/75^{th} percentile)	56 (52/62)	0 (1/10.3)	0.782*
Females, n (%)	48 (71.6)	27 (67.5)	0.815**
Smoking history, n (%)			
- current	16 (23.9)	10 (25)	
- past	24 (35.8)	12 (30)	
- no	27 (40.3)	18 (45)	0.820***
Periodontal conditions median (25^{th}/75^{th} percentile)			
Approximale plaque index (%)	28.6 (14.3/58)	33.3 (5.3/70.4)	0.982*
Bleeding upon probing (% sites)	8.9 (3/16.7)	7.1 (3.6/19.1)	0.887*
Pocket depth (mm)	3.1 (2.7/3.8)	2.8 (2.5/3.5)	0.103*
Attachment loss (mm)	3.5 (2.9/4.3)	3.3 (2.8/4.2)	0.335*
Missing teeth exception of 3rd molars	5 (2/10)	4.5 (1.3/10)	0.639*
Periodontal epithelial surface area (mm^2)	1111.3. (792.6/1403.3)	914.5 (686.3/1157.3)	0.074*
Periodontal inflamed surface area (mm^2)	86.1 (26.1/204)	59.5 (30.2/178.6)	0.598*
CDC classification, n (%)			
No or mild periodontitis	22 (32.8)	16 (40)	
Moderate periodontitis	29 (43.3)	11 (27.5)	
Severe periodontitis	16 (23.9)	13 (32.85)	0.257***
Periodontal bacteria, n (%)	n=65, missing 2	n=39, missing 1	
A. actinomycetemcomitans	7 (10.8)	0 (0)	**0.043****
P. gingivalis	30 (46.2)	16 (34.8)	0.760**
P. intermedia	17 (26.2)	8 (20.5)	0.678**
T. forsythia	51 (78.5)	34 (87.2)	0.394**
T. denticola	42 (64.6)	28 (7 1 . 8)	
0.589**			
Medication, n (%)	n=66, missing 1	n=38, missing 2	
NSAIDs	28 (42.4)	22 (57.9)	0.188**
DMARD	51 (77.3)	21 (55.3)	**0.034****
Biologicals	32 (48.5)	14 (36.8)	0.344**
Steroids	39 (58.2)	23 (57.5)	1.000**

*Mann-Whitney U test; **Chi-Square test with Yates correction or Fisher's exact test; ***Chi-Square test (uncorrected).
CCP: cyclic citrullinated peptides; NSAIDs: non-steroidal anti-inflammatory drugs; DMARD: disease-modifying anti-rheumatic drugs.

tion of autoantibodies against these citrullinated peptides (37). Therefore we investigated, whether periodontal status or periodontal bacteria are associated to ACPA-positivity among patients with RA. In this context two different ACPAs, anti-CCP and anti-CEP-1 antibodies, were chosen. Anti-CCP antibodies are highly sensitive (65–70%) and specific for RA (85%) and thus have a high predictive relevance for early diagnosis (52). Regarding anti-CEP-1 antibodies a specificity of 97.1% (53) was obtained for a cohort of early RA patients. In the context of periodontitis alpha-enolase is of particular relevance because antibodies to the immunodominant epitopes of human CEP-1 cross-react with a sequence of the citrullinated enolase of *P. gingivalis* (42).

Both RA patients with short and very long disease duration (0 to 576 month) were included. Since this could affect ACPA levels, ACPA positive and ACPA negative patients were compared in terms of disease duration. However, there were no significant differences (Tables II-IV).

Periodontal diagnosis was performed using the CDC-classification and, additionally, the determination of values for PESA and PISA. The CDC-classification allows the definition of three different disease categories based on certain threshold values for CAL and PD (49). This classification has been developed for studies investigating the prevalence of periodontitis in different populations and therefore is internationally comparable. The disadvantage of this classification (and others) is in particular that the area of the periodontal inflamed tissue which may associate with the systemic inflammatory burden could not be detected. For that reason PESA and PISA were additionally determined as continuous values. PESA allows determining the amount of periodontal altered pocket epithelium whereas PISA allows determining the amount of inflamed pocket epithelium (50).

As main result we obtained a weak but significant association between periodontitis assessed as PESA and the prevalence of anti-CCP positivity (Table VII, model 1). Moreover, all patients who were infected with *A. actinomycetemcomitans* in the subgingival plaque were as well positive for anti-CCP antibodies (Table III). All the other periodontal conditions were not significantly associated to anti-CCP or anti-CEP-1 levels. Regarding the genetic background we revealed a positive association between HLA-DRB1*04 and

Table IV. Demographic, periodontal and rheumatological conditions in patients who were anti-CCP and anti-CEP-1 positive in comparison to patients who were negative for both autoantibodies.

Demographic and clinical parameters	Anti-CCP and anti-CEP-1 positive n=45 (42.1%)	Anti-CCP and anti CEP-1 negative n=62 (57.9%)	*p*-value
Age (years) median (25^{th}/75^{th} percentile)	56 (51.5/63)	57 (47/64.5)	0.947*
Disease duration (month) median (25^{th}/75^{th} percentile)	0.5 (0/22.5)	1.0 (0/11.5)	0.909*
Females, n (%)	29 (64.4)	46 (74.2)	0.382**
Smoking history, n (%)			
- current	9 (20)	17 (27.4)	
- past	18 (40)	18 (29.0)	
- no	18 (40)	27 (43.5)	0.449***
Periodontal conditions median (25^{th}/75^{th} percentile)			
Approximale plaque index (%)	41.5 (15.9/65.8)	29.9 (8.7/60.4)	0.254*
Bleeding upon probing (% sites)	9 (3.4/20)	7.8 (3.4/17.3)	0.551*
Pocket depth (mm)	3.1 (2.7/3.8)	2.9 (2.6/3.6)	0.214*
Attachment loss (mm)	3.4 (2.9/4.3)	3.4 (2.9/4.3)	0.810*
Missing teeth exception of 3rd molars	5 (1.8/10)	5 (2/10)	0.807*
Periodontal epithelial surface area (mm^2)	1126.4 (797.1/1400)	1038.8 (731.1/1183.7)	0.350*
Periodontal inflamed surface area (mm^2)	99.6 (29.6/212.5)	61.2 (30.1/205.2)	0.432*
CDC classification, n (%)			
No or mild periodontitis	17 (37.8)	21 (33.9)	
Moderate periodontitis	17 (37.8)	23 (37.1)	
Severe periodontitis	11 (24.4)	18 (29)	0.853***
Periodontal bacteria, n (%)	n=43, missing 2	n=61, missing 1	
A. actinomycetemcomitans	5 (11.6)	2 (3.3)	0.122**
P. gingivalis	20 (46.59	26 (42.6)	0.847**
P. intermedia	12 (27.9)	13 (21.3)	0.588**
T. forsythia	33 (76.7)	52 (85.2)	0.397**
T. denticola	26 (60.5)	44 (72.1)	0.300**
Medication, n (%)	n=44, missing 1	n=60, missing 2	
NSAIDs	19 (43.2)	31 (51.7)	0.511**
DMARD	35 (79.5)	37 (61.7)	0.082**
Biologicals	20 (45.5)	26 (43.3)	0.988**
Steroids	26 (57.8)	36 (58.1)	1.000**

*Mann-Whitney U-test; **Chi-Square test with Yates correction or Fisher's exact test; ***Chi-Square test (uncorrected).
CCP: cyclic citrullinated peptides; NSAIDs: non-steroidal anti-inflammatory drugs; DMARD: disease-modifying anti-rheumatic drugs.

anti-CEP-1 or anti-CEP-1 + anti-CCP seropositivity. HLA-DRB1*13 was negatively associated to both investigated autoantibodies. No RA patient who harboured simultaneous both autoantibodies was HLA-DRB1*13 positive. HLA-DRB1*07 was negatively associated with anti-CCP antibodies (Table VII, models 1 to 3). Regarding the use of anti-rheumatic drugs it could be shown that RA patients who took DMARDs were positive for anti-CCP antibodies at a significantly higher frequency (Table VII, model 1).

In comparison to our previous study (43) where no association between periodontitis patients without RA and ACPA was shown we obtained a slight positive association (OR=1.002, *p*=0.040) between periodontitis assessed as PESA and anti-CCP positivity. An association between periodontitis and anti-CCP concentration was found also in another previous study among patients with RA (54) as well as among individuals who had no RA (55, 56). However, the strength of the identified associations varies between the studies. Gonzales *et al*. (54) obtained a relatively closely relationship between degree of alveolar bone loss according periodontitis and anti-CCP concentration (*p*=0.004). Janssen *et al*. (55) revealed only a relationship with borderline significance between periodontitis and anti-CCP seropositivity (OR=5.2, 95% CI 0.99-27, *p*=0.05). Terao *et al*. (56) showed in a large-scale study among systemically healthy Japanese significant associations between three periodontal conditions (missing teeth, community periodontal index, attachment loss) and anti-CCP positivity. However, the obtained odd ratios were very low (1.03 to 1.23) like our results. In a study among Malaysian RA-patients (57) no association between moderate and severe periodontitis regarding anti-CCP levels was obtained. The reasons for the partially inconsistent results may be differences in the definition of periodontitis, the definition of study groups (patients with or without RA) or the number of patients and controls who were included. Furthermore, there were differences between further risk factors for ACPA, which were taken into account such as smoking, administration of anti-rheumatic drugs, disease duration and HLA-DRB1 alleles.

The results of our study suggest that compared to other factors (genetic background) periodontitis is if any only a minor risk factor for anti-CCP antibody positivity among patients

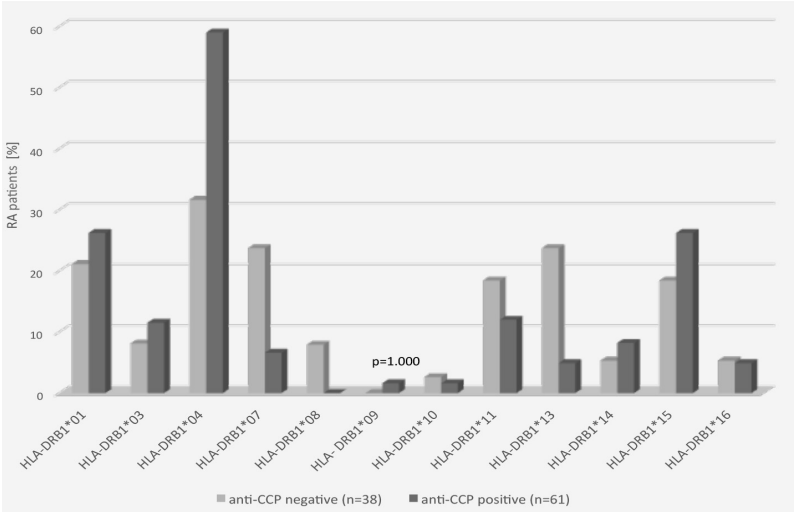

Fig. 1. Distribution of anti-CCP positive and negative RA patients in dependence of expression of HLA-DRB1* alleles. Significant *p*-values are indicated in bold.

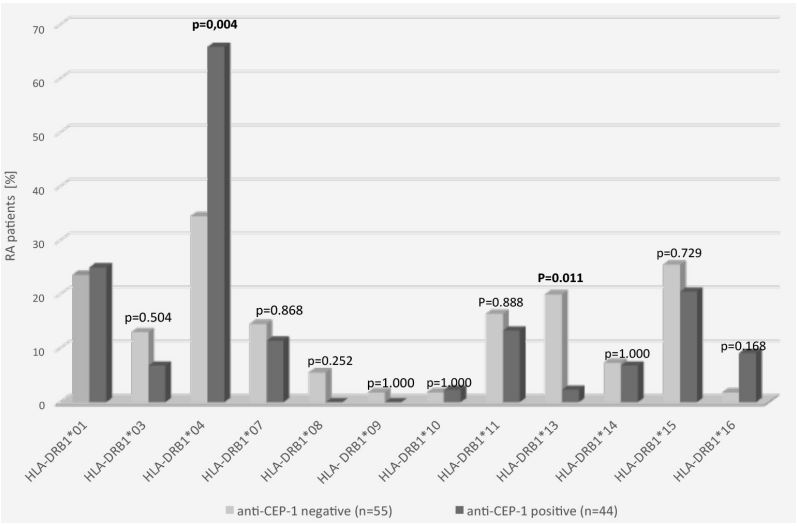

Fig. 2. Distribution of anti-CEP-1 positive and negative RA patients in dependence of expression of HLA-DRB1* alleles. Significant *p*-values are indicated in bold.

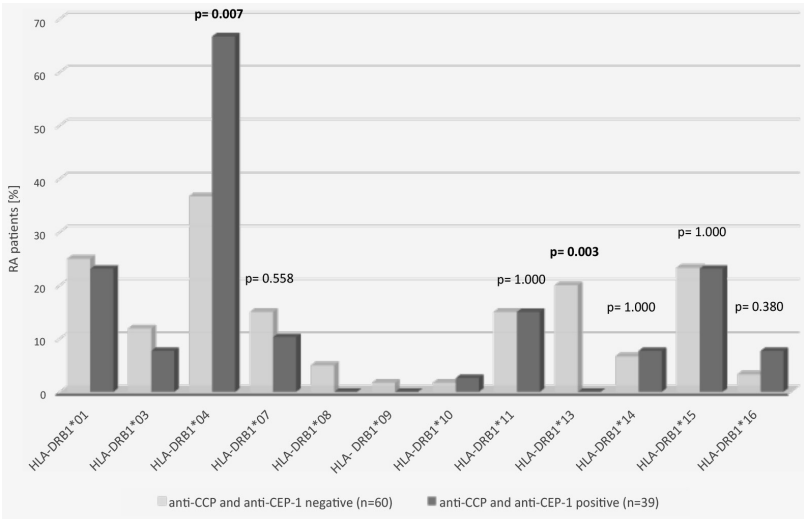

Fig. 3. Distribution of RA patients who were positive or negative for both investigated antibodies in dependence of expression of HLA-DRB1* alleles. Significant *p*-values are indicated in bold.

with RA. Interestingly, an infection with *A. actinomycetemcomitans* has always been associated with seropositivity for anti-CCP. Due to the previously described ability of A. *actinomycetemcomitans* to promote the citrullination of peptids in the oral cavity (35), a replication of this association in a large-scale study would be useful.

We did not show, as expected, significant associations between *P. gingivalis* and *P. intermedia* with ACPA-positive RA. The postulated role of both bacteria in the aetiology of RA could not be confirmed by our study. It has to be considered that only a small part of the oral microbiome was analysed with our applied microbiological test. For some times Next Generation Sequencing (NGS) has been able to capture the oral microbiome in its entirety. This provides the opportunity to identify previously unknown periodontal pathogens, which may possibly be important in aetiology of RA. A cohort study of 78 patients with chronic RA elucidated that the disease status was associated with a specific composition of the subgingival microbiome (58). Furthermore a pilot study in periodontally healthy patients with RA (n=22) and periodontal healthy subjects without RA (n=19) revealed that the composition of the subgingival microbiome differs depending on the RA diagnosis (59). Beside bacteria, also viral antigens are likely to influence the formation of ACPAs. A recent study demonstrated associations between low anti- Epstein-Barr virus (EBV)/anti- parvovirus B19 antibody levels and ACPA-positive RA, in particular when HLA-DRB1 SE was present. The authors concluded that high anti-viral antibody levels would be protective against ACPA-positive RA (60). Beside bacterial and viral infections many environmental factors such as cigarette smoking and occupational and atmospheric agents have been proposed as trigger stimuli for the development of RA in genetically predisposed individuals (61). Cigarette smoking is a strong risk factor for periodontitis at all (62) and the interrelationship between periodontitis and RA may also be due to the existence of common risk factors. Our findings show that individual ex-

Table V. Percentage of ACPA positive and negative individuals depending on the number of expressed HLA-DRB1*04 alleles.

Antibodies	HLA-DRB1*04 negative, n=36	HLA-DRB1*04 one allele, n=36	HLA-DRB1*04 two alleles, n=12	*p*-value*
Anti-CCP (% individuals)				
positive	49.0	72.2	83.3	
negative	51.0	27.8	16.7	0.023
Anti-CEP-1 (% individuals)				
positive	29.4	58.3	66.7	
negative	70.6	41.7	33.3	0.007
Anti-CCP + anti-CEP-1 (% individuals)				
positive	25.5	50.0	66.7	
negative	74.5	50.0	33.3	0.008

ACPA: anti-citrullinated peptide/protein antibodies; CCP: cyclic citrullinated peptides; CEP-1: citrullinated α-enolase; *Chi-Square test.

Table VI. Percent ACPA positive and negative individuals in depending on the number of expressed HLA-DRB1*13 alleles.

antibodies	HLA-DRB1*13 negative, n=86	HLA-DRB1*13 one allele, n=11	HLA-DRB1*13 two alleles, n=3	*p*-value*
Anti-CCP (% individuals)				
positive	67.4	30.0	0.0	
negative	32.6	70.0	100.0	1.000
Anti-CEP-1 (% individuals)				
positive	50.0	10.0	0.0	
negative	50.0	90.0	100.0	0.016
Anti-CCP + anti-CEP-1 (% individuals)				
positive	45.3	0.0	0.0	
negative	54.7	100.0	100.0	0.008

ACPA: anti-citrullinated peptide/protein antibodies; CCP: cyclic citrullinated peptides; CEP-1: citrullinated α-enolase; *Chi-Square test.

pression of certain HLA-alleles is closely associated with higher or lower risk for ACPA positivity. Furthermore, a gene dose effect could be shown obvious the differences between the subjects who were carriers of one or two predisposing or protective HLA alleles were not very high (Tables V and VI). The positive association of HLA-DRB1*04 alleles to anti-CCP positivity is supported by numerous previous studies (63-69) whereas another study confirmed the positive association between HLA-DRB1*04 and anti-CEP-1 positivity (70). A meta-analysis (71) including four European populations revealed, in best accordance with our results, a negative association of HLA-DRB1*13 and -DRB1*07 to anti-CCP positivity. In a case-control study the protective role of HLA-DRB1*13 and -DRB1*07 for RA was confirmed (72). The association between certain HLA alleles and ACPA could be explained by the "shared epitope" hypothesis, *i.e.* certain HLA alleles encode a conserved sequence of amino acids in the third hypervaribale region (amino acids 67-74) (73). These HLA-alleles influence binding and presentation of arthritogenic antigens. It is noteworthy in this context that citrullination of peptides such as vimentin significantly increases peptide-binding and presenting affinity to RA-associated HLA alleles such as HLA-DRB1*0101, -DRB1*0401 and -DRB1*0404 in comparison to non-citrullinated vimentin. In HLA alleles which were not associated with RA (*e.g.* HLA-DRB1*0802, -DRB1*1101 and -DRB1*1302), no differences in binding affinity between citrullinated and not citrullinated peptides were in contrast demonstrable. Like the HLA-alleles rather representing RA-susceptibility, so-called protective alleles showed a high degree of homology of certain amino acids in the third hypervariable region (74).

The expression of HLA shared epitopes

Table VII. Logistic regression analysis for anti-CCP (model 1), anti-CEP-1 (model 2) and anti-CCP+anti-CEP-1 (model 3) serum level stratified for periodontal epithelial surface area (PESA), periodontal inflamed surface area (PISA) and other putative confounders. Significant *p*-values are indicated in bold.

	Model 1 $R^2 = 0.42$	anti-CCP	positive		Model 2 $R^2 = 0.29$	anti-CEP-1	positive		Model 3 $R^2 = 0.34$	anti-CEP-1 anti-CCP	and positive	
Variable	OR	Lower CI	Upper CI	*p*	OR	Lower CI	Upper CI	*p*	OR	Lower CI	Upper CI	*p*
Age	0.981	0.928	1.037	0.499	0.997	0.950	1.048	1.046	1.020	0.967	1.076	0.471
Gender	1.244	0.359	4.318	0.730	0.880	0.310	2.497	0.880	0.860	0.297	2.493	0.781
Smoking	1.234	0.394	3.871	0.781	2.199	0.760	6.357	0.146	1.984	0.673	5.851	0.214
DMARD	4.869	1.567	15.123	**0.006**	2.322	0.836	6.466	0.106	2.777	0.940	8.204	0.065
DRB1*04	2.240	0.782	6.414	0.133	4.154	1.540	11.205	**0.005**	3.725	1.354	10.244	**0.011**
DRB1*13	0.073	0.014	0.369	**0.002**	0.068	0.007	0.625	**0.018**	0.000	0.000	n.d.	0.998
DRB1*07	0.079	0.014	0.452	**0.004**	0.761	0.166	3.491	0.725	0.724	0.153	3.435	0.685
PESA	1.002	1.000	1.005	**0.040**	0.999	0.997	1.001	0.476	1.000	0.998	1.002	0.972
PISA	0.998	0.994	1.001	0.173	1.001	0.998	1.004	0.695	1.000	0.997	1.003	0.999
Missing teeth	1.133	0.985	1.304	0.080	0.986	0.873	1.114	0.824	0.986	0.870	1.118	0.825

CEP: citrullinated α-enolase; CCP: cyclic citrullinated peptides; CI: confidence interval; DMARD: disease-modifying anti-rheumatic drugs; ND: not determined; OR: odds ratio. Nagelkerkes R^2 to assess goodness of fit.

predisposing ACPA positive RA may not be the only explanation for the association between HLA and RA. There are some observations suggesting that the association between HLA and RA may be based on other mechanisms. For instance the gene-dose effect elucidated for HLA-SE alleles (75) cannot be explained only by shared epitopes, but is rather compatible with the role of HLA polymorphisms in mechanisms shaping the T cell repertoire. In patients with RA T- cell repertoire abnormalities have been described (76). Moreover, HLA-DRB1*0901 is not considered a shared epitope allele but found associated with RA in meta-analyses (71). In addition it is well known that HLA alleles may be in linkage disequilibrium. For instance HLA-DRB1*04 is in a tight linkage disequilibrium with HLA-DQB1*08 and –DQB1*07. Both alleles showed enhanced affinities for citrulline compared to arginine residues. These results suggest that HLA-DQ alleles may be contribute on binding of citrullinated peptides (77). Through genome-wide association studies (GWAS) in particular 100 non-HLA risk loci for RA have been identified. Only 20% of these loci contain coding variants, with the remaining variants occurring in non-coding regions whose functions are largely unknown (78). Further studies are needed to investigate genetic risk factors for RA (61) .

Drugs used to treat RA can have an effect on ACPA levels (79). Therefore, the current medication and its influence on the ACPA level was considered. DMARDs were found positive associated to anti-CCP positivity in univariate (Table III) and multivariate (Table VII, model 1) analyses. DMARDs include various medications such as azathioprine, sulfasalazine and methotrexate, which are prescribed to suppress autoreactive immune cells. Generally, DMARDs are prescribed more frequently to seriously diseased ACPA-positive than ACPA negative RA-patients (74) which correlates probably with the positive association between DMARDs and anti-CCP antibody positivity revealed in our study. Therefore we conclude that DMARDs *per se* do not represent a risk factor for an elevated ACPA serum level. On the contrary the application of DMARDs is known to lead to a decrease in the ACPA level (80).

Conclusion

Periodontitis is if any only a minor risk factor for anti-CCP positivity. The described association between *A. actinomycetemcomitans* and anti-CCP positivity could be interesting as this bacterium could trigger citrullination of peptides in the oral cavity. Therefore, this association should be reviewed in a large-scale study. HLA-DRB1*04 and underrepresentation of HLA-DRB1*13 do likely represent important indicative factors for anti-CCP and anti-CEP-1 antibody positivity whereas HLA-DRB1*07 is a putatively protective factor only against anti-CCP antibodies.

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