

ORIGINAL ARTICLE

Hypercitrullination and anti-citrullinated protein antibodies in chronic apical periodontitis, a laboratory investigation. Does autoimmunity contribute to the pathogenesis?

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Abstract

Aim: The presence of *Gram-negative* anaerobic bacteria, in particular, *Porphyromonas gingivalis* (*P. gingivalis*) in periapical granulomas predicts the generation of citrullinated proteins in the lesion. Citrullination of proteins may lead to the formation of anti-citrullinated autoantibodies (ACPA-s) initiating the formation of an autoimmune loop which may contribute to the perpetuation of inflammatory reactions and tissue damage in chronic apical periodontitis. The objective of this study was to demonstrate the formation of citrullinated proteins in chronic apical periodontitis and whether they can act as autoantigens.

Methodology: Twenty-five periapical granulomas ($n = 25$) were investigated in the study. Healthy periodontal tissue samples served as normal control tissue ($n = 6$). The peptidyl-citrulline level was determined with the dot blot method. ACPA levels were analysed using anti-citrullinated cyclic peptide (anti-CCP) EDIA kit. Differences between periapical granuloma and control samples were assessed using Mann-Whitney U tests. p Values $< .05$ were considered as statistically significant.

Results: Protein concentrations, peptidyl-citrulline levels and anti-CCP ratios were compared between periapical granuloma and healthy control groups. Multiple linear regression analysis revealed significant ($p = .042$) hypercitrullination in periapical granuloma samples. Moreover, there was a significant difference in the ACPA ratios between periapical granuloma (2.03 ± 0.30) and healthy control (0.63 ± 0.17) groups ($p = .01$). Seventeen of 25 periapical granuloma samples (17/25; 68%), whereas one out of six control samples (1/6; 17%) were shown to be positive for the presence of ACPA.

Renáta Martos and Ildikó Tar contributed equally to this work.

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Conclusions: This is the first study detecting the presence of citrullinated peptides and APCA in periapical granuloma, suggesting the contribution of autoimmune reactions in the pathogenesis and perpetuation of chronic apical periodontitis.

KEYWORDS

anti-citrullinated protein antibody, chronic apical periodontitis, citrullination, periapical granuloma

INTRODUCTION

The presence of Gram-negative anaerobic infection is a predisposing factor for a posttranslational protein modification, citrullination, catalysed by prokaryotic (bacterial) peptidylarginine deaminases (PPADs) enzymes (Mangat et al., 2010). Citrullination has been demonstrated to contribute to the inflammatory process ongoing in periodontal disease (Tar et al., 2021; Wegner et al., 2010). Proteins that can serve as substrates of PPAD enzymes are normally not citrullinated. The citrullinated forms of these proteins are immunogenic resulting in the formation of anti-citrullinated autoantibodies (ACPA-s), such as anti-citrullinated cyclic protein antibodies (anti-CCP), anti-citrullinated enolase antibodies (anti-CEP), and anti-citrullinated vimentin antibodies (anti-CMV) (Sofat et al., 2014).

In patients with periodontal disease, citrullinated proteins were detected in gingiva tissue samples and in gingival crevicular fluid (Harvey et al., 2013). Citrullination and production of anti-CCPs were related to the presence of *Porphyromonas gingivalis* (*P. gingivalis*). Periodontal disease and chronic apical periodontitis have important similarities, such as sharing common microbiota, association with elevated local and systemic levels of proinflammatory mediators and tissue samples signs of multiple forms of active immune reactions (Caplan, 2004; Sundqvist, 1994).

Apical periodontitis is initiated by Gram-negative anaerobic bacteria located inside the pulp chamber and through the apical and lateral foramina may reach the periodontal ligaments and periapical tissues (Siqueira & Rôças, 2007). These bacteria include *Prevotella* spp., *Porphyromonas* spp., *Treponema* spp., and *Fusobacterium* spp which may play a dominant role in pulpal infections (Chu et al., 2006; Gomes et al., 2004; Montagner et al., 2010; Rôças & Siqueira, 2010). In the course of the ongoing endodontic infection, microbes, their antigens and toxins reach the apical periodontal ligament and surrounding tissues through the apical foramen and trigger immunological defence mechanisms (Georgiou et al., 2019; Martinho et al., 2014). A delicate balance between protective and destructive reactions ultimately

results in the formation of granulation tissue around the apical foramen, consisting of necrotic, exudative, granulomatous and fibrous zones (Márton & Kiss, 2000, 2014; Morse, 1977; Petean et al., 2022; Yanagisawa, 1980). In the necrotic and exudative zones, abundant neutrophils eliminate the majority of microbes rendering the granulomatous and fibrous zones sterile (Márton & Kiss, 2014).

In this study, the aim was to show that citrullinated proteins may also be formed in chronic apical periodontitis and may act as autoantigens inducing the production of anti-CCP, as in periodontal disease. Therefore, the presence of citrullinated proteins and anti-CCP in chronic apical periodontitis lesions was examined.

MATERIALS AND METHODS

Study patients and sample collection

The study was performed according to the requirements of the Institutional Review Board of the University of Debrecen (No 5035-2018 DE RKEB/IKEB) as well as the Code of Ethics of the World Medical Association and was carried out in accordance with the ethical standards of the Helsinki Declaration (Revision 2000). Informed consent was obtained from study patients.

Forty periapical lesions were surgically removed from patients attending the Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, University of Debrecen. Fifteen samples were excluded from the study due to impaired general health of patients and/or drug treatment on a regular daily basis which influenced inflammatory reactions. General health status was determined based on the data of medical history and on patients' medical records.

Patients with complaints suggesting the presence of a disease, with known medical conditions, and taking medication on a regular basis were not considered "healthy" and were not included in the study. No further physical, laboratory and imaging investigations were initiated in patients with a good general health condition, as defined above, in line with the rules of the General Data Protection Regulation of the European Union. Exclusion criteria

were patients with ongoing medical problems and/or drug taking on a regular basis, interfering with inflammatory reactions, and refusing the signature of informed consent. Finally, 25 samples were included in the study. The sample size of both periapical granuloma and control groups was restricted due to the limited availability of patients fulfilling enrolment criteria. Healthy Caucasian patients with chronic apical periodontitis were included if conservative (endodontic) treatment was refused or the teeth could not be saved. Fifteen specimens were obtained by tooth extraction, nine specimens were removed by surgical root extraction and one specimen was obtained by apicectomy, according to the surgical protocol of Barnes (Barnes, 1984). Tissue specimens were dissected into two parts. One part was processed conventionally for light microscopy and the diagnosis was confirmed by histology as described previously (Márton et al., 1990).

Control non-inflamed periodontal tissue samples ($n = 6$) were obtained from healthy patients attending for retained or impacted third molar removal from the jaw at the Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, University of Debrecen. None of the retained or impacted third molars was associated with inflammatory reactions either in the hard (bone tissue) or in the soft tissues. Patients undergoing tooth extraction or apicectomy because of chronic apical periodontitis were not a suitable source for obtaining control tissue samples because soft tissues surrounding the involved teeth were inflamed and patients did not require additional dental surgical interventions at the time of removal of periapical granuloma.

All samples were snap-frozen and stored in liquid nitrogen till examination.

Protein extraction

Frozen tissue samples were pulverized and then homogenized in lysis buffer (50 mmol/L Tris-HCl, 1 mM EDTA, 17 mmol/L beta-mercaptoethanol and 0.5% Triton-X100™). The protein content was measured using the Bradford method (Bradford, 1976).

Peptidyl-citrulline level determination

The peptidyl-citrulline levels in periapical granuloma samples originating from patients and controls were examined using a dot-blot. This technique can be used in quantitative settings (Aznar, 2017; Tian et al., 2017) however, in this study we have utilized it in a semi-quantitative manner. Our aim was to examine the presence of citrullination and to determine the relative differences in

citrullination levels between the samples originating from diseased or control groups. The dot blot analysis for the determination of peptidyl-citrulline levels was designed based on the widely used protein microarrays, such as the ProteomeProfiler (https://www.rndsystems.com/products/proteome-profiler-antibody-arrays?gclid=CjwKCAiAzp6eBhByEiwA_gGq5NWfCEZPCTrvY2iPHWO5qgXnvlabpJatIKiUT03tp66OAFVZfQYIIRoCeOsQAvD_BwE&gclsrc=aw.ds), but in an inverse manner. In the ProteomeProfiler the different antibodies are spotted to the membrane and one sample is added, in our setup, the different samples were spotted to the nitrocellulose membrane (VWR International Ltd., Radnor, Pennsylvania, USA) and one antibody (anti-citrulline antibody) was added. The tissue lysates samples corresponding to 20 µg total protein were spotted to the membrane in duplicate by applying vacuum suction. After drying, the unbound areas of the membrane were blocked with 1% PVP (polyvinylpyrrolidone) in TTBS (10 mmol/L Tris, 150 mmol/L NaCl, pH 8.0) for 60 min at room temperature (RT) to prevent nonspecific antibody binding. In order to examine the peptidyl-citrulline levels, the membrane was incubated with 1:15 000 rabbit anti-citrulline polyclonal antibody (PAB0672, Covalab UK Ltd) at 4°C overnight, followed by incubation with 1:5000 biotinylated anti-rabbit IgG antibody (Covalab) for 60 min at RT. Then, 1:15 000 streptavidin conjugated to horse radish peroxidase (Sav-HRP, Biolegend) was added and incubated for 30 min at RT. The membrane was visualized using an ECL substrate detection kit and Super Signal (Thermo Fisher Scientific Inc.) and the chemiluminescence of the spots was recorded with FluorChem Q System (Protein Simple Inc.) for quantitative Western blot imaging controlled by AlfaView (Protein Simple Inc.) software. Densitometry was carried out by using Quantity One 4.6.7 (Bio-Rad Ltd.) software and individual background subtraction was applied. For normalization, a fixed concentration of oxidized haemoglobin was used. Density data were calculated (detected intensity/mm²) in the case of all samples and normalized to the density values detected for haemoglobin. Based on the protein concentration the volume of the sample containing 20 µg proteins was calculated and the results were given as mean AU/L value ± SD. The relative quantification of peptidyl-citrulline amounts was done based on the normalized intensity of the spots containing 20 µg protein (Figure S1).

Determination of anti-CCP

The level of anti-CCP antibodies was analysed with the help of an anti-CCP EDIA kit (Eurodiagnostica, Malmö, Sweden) according to the manufacturer's instructions. Briefly, a

sandwich enzyme-linked immunosorbent assay (ELISA) has been carried out and the amount of anti-CCP present in the 100 μ L sample was determined. The reaction scheme was the following: in the wells of CCP-coated microplate provided in the kit either 100 μ L of reference solutions (reference control, positive control, negative control) or 100 μ L of tissue lysates were incubated for 1 h at room temperature, followed by 1 h incubation with a conjugate solution and 30 min incubation with the substrate solution. The absorbance was read at 450 nm and the absorbance ratio was calculated by dividing the blank-subtracted absorbance of each sample by the absorbance of the reference control. The ratios were calculated according to the manufacturer's instructions against positive and negative test controls. With a ratio equal or higher than 1, samples were considered positive for the presence of anti-CCP antibody (AU/L).

Statistical analysis

The sample size was statistically unpredictable, as no previous study on the citrullinated peptides and antibodies generated against them in chronic apical periodontitis was available. It was difficult to pre-determine the optimal sample size to be investigated, since previous studies examined 23 patients with periodontal diseases in association with rheumatoid arthritis (Tar et al., 2021), whilst studies examining the pathology of chronic periapical lesions included 13–36 patients (Abou-Rass & Bogen, 1998; Márton & Kiss, 1992; Minczykowski et al., 2001; Nesse et al., 2012). Statistical analysis was carried out with SPSS 25.0 (IBM Inc.). The restricted availability and the inequality in the number of periapical granuloma and healthy control tissue samples required that the groups with different sample sizes and at least one of them containing less than 10 samples were compared with the non-parametric Mann–Whitney *U* test. Normality was evaluated by Shapiro–Wilk test. Pearson's chi-squared test was used to check the associations between categorical variables.

p Values <.05 were considered as statistically significant. Spearman correlation coefficient was counted for age and peptidyl-citrulline levels to decide their age dependence. Potential influencing factors of peptidyl-citrulline levels were identified using multiple linear regression. Robust regression was used to identify the potential influencing factors of anti-CCP antibody levels.

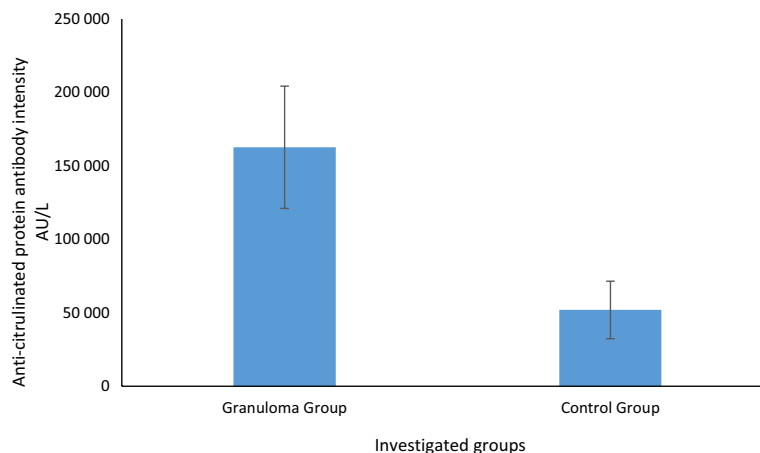
RESULTS

There were no significant differences in age (19 [17–22], median [IQR] vs. 26.5 [20–39], *p* = .113) and gender (male/female 28.57%/71.43% vs. 33.33%/66.67%, *p* = .813) distribution of the periapical granuloma and the control groups. The protein concentrations of the chronic apical periodontitis group and the control group were statistically similar (*p* = .419) with 1.91 ± 0.37 mg/mL in the chronic apical periodontitis group, and 1.17 ± 0.45 mg/mL in the control samples.

Peptidyl-citrulline levels in chronic apical periodontitis samples were more than three times higher than in controls with $161\,597 \pm 146\,532$ AU/L and $47\,889 \pm 55\,611$ AU/L, respectively (Figure 1). Multiple linear regression analysis showed a significant association (*p* = .042) between the presence of periapical granuloma (data points of the chronic apical periodontitis group vs. data points of the control group) and peptidyl-citrulline levels (95% confidence interval: 6237 and 317 248), as based on the adjusted (for age and gender) coefficient (coeff. = 161 742).

Levels of anti-CCP antibodies were statistically significantly higher in the chronic apical periodontitis group (1.72 [0.82–2.69] AU/L) than in the control group (0.52 [0.37–1.43] AU/L) (*p* = .008) (Figure 2). In the chronic apical periodontitis group, most samples (17/25; 68%) were anti-CCP positive, whilst only one case out of 6 (1/6; 17%) was anti-CCP positive in the control group (Figure 3). There were no correlations between peptidyl-citrulline levels and age (*R* = .04, *p* = .81) and anti-CCP levels (*R* = .43, *p* = .1). Being

FIGURE 1 Examination of the level of citrullinated proteins in chronic apical periodontitis and control group. The protein-bound citrulline levels (y axis) in form of anti-citrullinated protein antibody intensity is plotted in the periapical granuloma and control groups, respectively (x axis).



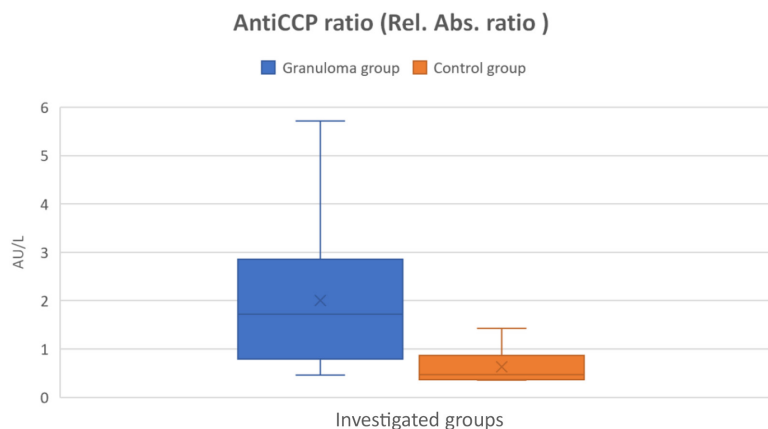


FIGURE 2 Examination of the level of ACPA in chronic apical periodontitis and control groups. The anti-CCP ratio as relative absorbance ratio (y axis) was plotted in the chronic apical periodontitis and control groups, respectively (x axis).

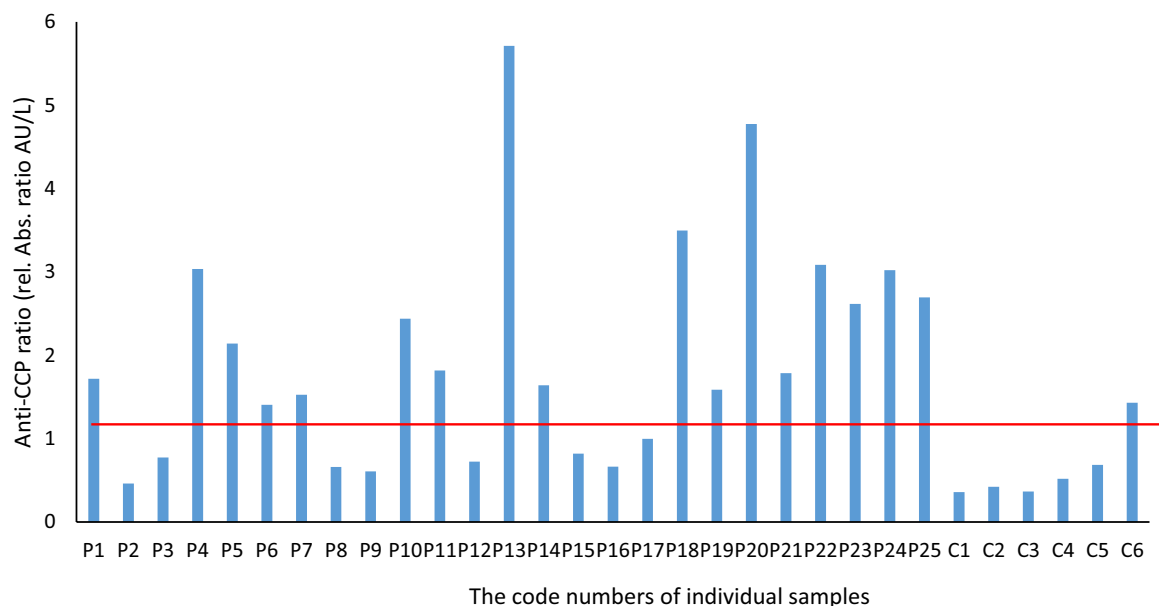


FIGURE 3 Distribution of ACPA level in individual samples. The anti-CCP ratios (y axis) were plotted in the case of each tested sample. P1-P25 represent patients with periapical granuloma, whilst the C1–C6 represent the controls. The red line represents the threshold; below 1 the ratio represents a negative value, and above 1 it is considered positive.

in the chronic apical periodontitis group seemed to be a significant risk factor (coeff. = +0.29, $p = .005$) for the levels of anti-CCP antibodies. Based on the adjusted (for age, gender, peptidyl-citrulline level, anti-CCP absorbance) coefficient, it increased the anti-CCP ratio level by 0.29 AU/L.

DISCUSSION

In order to elucidate immune-inflammatory mechanisms active in chronic apical periodontitis, for the first time, the levels of citrullinated proteins and ACPA in human periapical granuloma samples were investigated. A positive correlation may suggest that there could be an autoimmune pathway in chronic apical periodontitis via peptidylarginine deaminases (PAD) or PPAD activities (Čolić et al., 2007; Niazi & Bakhsh, 2022).

Chronic apical periodontitis mimics many characteristics of periodontal disease. They share the bacterial flora (*Prevotella* spp., *Porphyromonas* spp., *Treponema* spp., and *Fusobacterium* spp), proinflammatory cytokines, and in both cases, there is tissue destruction. In order to elucidate immune-inflammatory mechanisms active in apical periodontitis, the levels of citrullinated proteins and ACPA in human periapical granuloma samples were investigated. A positive correlation may suggest that there could be an autoimmune pathway in chronic apical periodontitis via peptidylarginine deaminases (PAD) or PPAD activities (Čolić et al., 2007).

Citrullination is a posttranslational protein modification, controlled by PADs and resulting in a new 3D structure with a possible capability of acting as autoantigens in several diseases. In humans, five tissue-specific PAD isozymes can be responsible for inducing hypercitrullination

in immune cells, such as macrophages, neutrophils and eosinophils (Hill et al., 2003; Vossenaar et al., 2003). Citrullination under physiological conditions may have a regulatory role by modifying the function or half-life of the proteins which is not associated with ACPA production (Baka et al., 2012). The presence of Gram-negative anaerobic infections, specifically *P. gingivalis* is a predisposing factor for citrullination (Engström et al., 2018; Mangat et al., 2010; Wegner et al., 2010). During inflammatory reactions, PAD enzymes represent a non-specific, but potent defence mechanism against microbes (Engström et al., 2018; Li et al., 2010). Foreign pathogens and bacteria, such as *P. gingivalis* can be marked by neutrophil extracellular traps (NETs) and present a signal for an autoimmune response, ultimately leading to 'NET-osis' (Georgiou et al., 2021; Yang et al., 2016). The PPAD and arginine gingipains are possessed by *P. gingivalis*. These enzymes are associated with the citrullination of arginine residues in human proteins (Wegner et al., 2010). The altered protein structure may activate an immune reaction against host molecules and induce the production of ACPA-s in parallel with the activation of autoreactive T-cells (Baka et al., 2012; Sakkas et al., 2017) as it was shown in periodontal disease earlier (Detert et al., 2010). Periodontal disease was suggested as an etiological factor in patients with rheumatoid arthritis (RA) and the presence of anti-CCP increased the risk of autoimmune reaction (Nakken et al., 2017; Wu et al., 2020). Citrullinated histone H3, which was also detected in inflamed periodontal tissues, was suggested as a target for ACPA in patients with RA (Janssen et al., 2017). In RA, the main ACPA is the anti-CCP, which autoantibody was related to the presence of *P. gingivalis*. Hypercitrullination and leakage of citrullinated proteins from neutrophils can be induced also by a leukotoxin of the oral periodontopathogen *Aggregatibacter actinomycetemcomitans* (Konig et al., 2016). Furthermore, viral infections may induce the generation of ACPAs also with a possibility to cross-react with host citrullinated proteins (Pratesi et al., 2006).

Periapical granuloma as a demarcated tissue injury with a combination of specific and non-specific immune reactions shows an excellent example of cell-mediated defence mechanisms with the dominance of neutrophils and macrophages. In periapical granuloma, macrophages may present citrullinated proteins for the T cells provoking the production of antibodies against citrullinated peptides, giving rise to an autoimmune reaction (Georgiou et al., 2022; Nesse et al., 2012). Anti-citrullinated protein/peptide antibodies may contribute to destructive processes. It was suggested that deposition of ACPA-s may lead to increased osteoclastogenesis and bone loss. Moreover, hypercitrullination was observed during processes such as apoptosis and autophagy associated with autoimmunity.

Genetic background, different environmental factors, amongst them smoking have been suggested to play a role in ACPA production, however, the exact pathogenic role has not yet been elucidated and has not yet been demonstrated in chronic apical periodontitis before our observation (Forslind et al., 2004; Mangat et al., 2010; Valesini et al., 2015).

In this study, the presence of significantly elevated peptidyl-citrulline levels and anti-CCP antibody levels (demonstrated by multiple linear regression and robust regression analyses, respectively) supported the presence of an autoimmune loop of stimulation contributing to the development and perpetuation of inflammation in the periapical area. The presence of citrullinated proteins in healthy tissue samples is no surprise, as human PAD enzymes can catalyse this ubiquitous posttranslational modification (Lugli et al., 2015). Nevertheless, the presence of ACPA-s was observed in one single control tissue sample, whereas in the rest of control samples no ACPA-s were detected (Wu et al., 2020). There was also a tendency of higher manifestation of citrullinated proteins suggested by observed higher levels of peptidyl-citrulline in chronic apical periodontitis vs. control samples.

The limited size of both periapical granuloma and control samples and the difference between these two types of sample sizes is a major limitation of the present study. However, suitable regression analysis models provided statistical power to support the clinical relevance of hypercitrullination in chronic apical periodontitis despite the lack of a linear relationship between the levels of citrullinated proteins and ACPA-s. Validation of an autoimmune nature of chronic apical periodontitis requires further investigations with a higher sample size, both periapical granuloma and control groups. However, the results suggest the presence of a possible autoimmune component in chronic apical periodontitis.

CONCLUSIONS

This is the first study detecting the presence of citrullinated peptides and ACPA in chronic apical periodontitis, suggesting the contribution of autoimmune reactions in the pathogenesis and perpetuation of chronic apical periodontitis.

AUTHOR CONTRIBUTIONS

Renáta Martos: drafted the manuscript, participated in the analysis and the interpretation of data. Ildikó Tar: drafted the manuscript, participated in the analysis and the interpretation of data. Attila Csaba Nagy: participated in statistical analysis and interpretation of data. Éva Csósz: performed laboratory analysis and participated in the

interpretation of data. Csongor Kiss: conceived the study and design the research work, critically revised the manuscript for important intellectual content, responsible for the approval of the final version. Ildikó Márton: conceived the study and design the research work, critically revised the manuscript for important intellectual content, responsible for the approval of the final version.

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CONFLICT OF INTEREST STATEMENT

All authors declare they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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