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The influence of apical periodontitis on circulatory inflammatory mediators in peripheral blood: A prospective case–control study

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Abstract

Aim: To explore the influence of apical periodontitis (AP) on inflammatory markers in blood of otherwise healthy individuals and to depict the inflammatory profile of the healing after dental extraction.

Methodology: This is a prospective case–control intervention study, during which, individuals with a diagnosis of AP of one affected tooth were included, along with a control group matched for age and gender. A broad panel of blood inflammatory mediators was examined longitudinally in all subjects during six visits. In the case of the AP subjects, the tooth with AP was extracted at the third visit. Results were analysed by linear regression analyses and linear mixed-model analyses.

Results: A total of 53 subjects were included in the study, 27 with AP and 26 without. Fifteen females and 12 males were included in the AP group, and 14 females and 12 males in the control group. At baseline, granulocyte colony-stimulating factor ($p < .001$), interleukin (IL)-1β ($p = .03$) and IL-4 ($p = .01$) were significantly lower in AP subjects than in controls. Comparison of the differences between baseline and the last visit, i.e. 3 months after the tooth extraction, showed a significant reduction in IL-10 ($p = .03$) and IL-12p70 ($p = .01$).

Conclusions: The immunologic profile of chronic AP in one tooth and its healing profile reveals a systemic low-grade inflammation through compensatory immunosuppression. A larger lesion or multiple lesions could disrupt the balance that the system is trying to maintain, resulting in loss of homeostasis.

KEYWORDS

apical periodontitis, endodontics, low-grade inflammation, systemic health

INTRODUCTION

Apical periodontitis (AP) is an inflammatory reaction of the periapical tissues of a tooth after pulp necrosis and subsequent infection of the root canal system (Kakehashi et al., 1965). Pulp necrosis can occur after the invasion of microorganisms into the tooth and is usually the result of an irreversible inflammatory response in the pulp. After

pulp necrosis, the microorganisms invade the root canal system until they eventually reach the root apex (Moller et al., 1981). To confine the infection a complex inflammatory response starts in the area where the root canal meets the periapical tissues. Locally, the jaw bone is resorbed, in order to make space for inflammatory tissue.

Bone remodelling is one of the characteristics of AP as a response to the bacteria and their byproducts. It is the result of the induction and differentiation of osteoclasts that are modulated by inflammatory markers that are activated during the inflammatory process (Troen, 2003). Some of the pro- and anti-cytokines that are involved in this activation are interleukin (IL)-1β, IL-6, interferon (IFN)-γ and tumour necrosis factor (TNF)-α, transforming growth factor (TGF)-β and IL-4. Since then, these cytokines are produced as a response to the endodontic infection, the extent of the bone damage, the AP lesion depends on the virulence of the involved microorganisms (Dessaune Neto et al., 2018) In a chronic inflammatory state, such as AP, there is a constant fluctuation in the balance between pro- and anti-inflammatory cytokines of the host response to the antigen stimulation (Dessaune Neto et al., 2018; Toledo et al., 2019).

This chronic local inflammation around the root tip may not stop there. The inflammatory and proinflammatory cytokines that keep the local inflammation going may also result in systemic low-grade inflammation. Locally produced mediators recruit inflammatory cells from elsewhere in the body, and they find their way to the bloodstream. Inflammation is an essential component of immune surveillance and host defence, yet a chronic low-grade inflammatory state is a pathological feature of a wide range of chronic conditions, such as metabolic syndrome, nonalcoholic fatty liver disease, type 2 diabetes mellitus and cardiovascular disease (Minihane et al., 2015).

In recent years, a popular topic in endodontics' research has been the examination of the systemic consequences of these inflammatory mediators (IM). Many studies have been published about circulating IM in the blood of people with AP (Abdolsamadi et al., 2008; Cotti et al., 2011, 2015; Garrido et al., 2019; Harjunmaa et al., 2018). A recent systematic review and meta-analysis showed that systemically healthy individuals with AP presented higher concentrations of some IM in blood than in healthy individuals without AP and that multiple lesions were associated with more circulatory IM (Georgiou et al., 2019). However, questions about the validity of some study results were raised by inconsistencies in study designs, treatments, included population and possible bias of unpublished research because of 'negative' outcomes. Additionally, as every study examines a limited number of IMs, no complete image of the inflammatory response of the subjects was formed.

In order to eliminate these inconsistencies we designed a study in which the subjects would be followed for a longer time before and after the treatment was designed. The treatment of AP with the extraction of the infected tooth has been compared with healthy controls. The purpose of the several examination stages before the treatment was to form a baseline of naturally occurring fluctuations and random fluctuations at the different time points. The overall objective was to answer the questions of the case–control and intervention studies, after eliminating the design inconsistencies. The individual objectives were thus: (i) To compare the plasma concentrations of IM of AP subjects to controls before the extraction (baseline), (ii) to evaluate the difference between the baseline and the final time points within AP cases and (iii) compare the difference between cases and controls at the different time points after the tooth extraction and, i.e. the healing period.

MATERIALS AND METHODS

This was a multicentre prospective case–control intervention study that was conducted at several private dental practices in the Netherlands and at the Academic centre of dentistry in Amsterdam (ACTA). The study is reported in compliance with the Reporting Items for study Designs in Endodontology (PRIDE) (http://pride-endodontic guidelines.org/). The study was registered prospectively with Clinical Trial Registry in the Netherlands (www.trial register.nl) with registration number NL6081, and its detailed protocol has been published (Georgiou et al., 2021). All procedures were performed in accordance with good clinical practice guidelines and declarations of Helsinki and have received the approval of the Medical Ethical Committee of the VU medical Centre, Amsterdam, the Netherlands (registration number 2016.187).

The study objective, procedures and associated risk and benefits were explained to the eligible individuals. A signed informed consent form was obtained from all the individuals willing to participate in the study. The subjects could quit participation at any time during the study without having to give a reason.

All procedures were performed in accordance with good clinical practice guidelines and declarations of Helsinki. Sample size calculations were performed while designing the study. In order to answer the main question, which is to compare cases versus controls, a sample size has been calculated with G*Power software (G*Power 3.1.9.2 software Franz Faul; Universitaet Kiel). Data retrieved from previous scientific papers where the concentrations of chosen mediators were determined in serum were used: for C-reactive protein (CRP) (Marton &

Kiss, 1992) with values 6.6 ± 4.2 mg/L for subjects with AP and 3.9 ± 1.8 mg/L for subjects without AP, for IL-1β with values 225.12 ± 43.72 pg/ml for cases and 77.52 ± 31.05 pg/ml for controls (Zhu et al., 2015) and for IL-17A with values 12.48 ± 2.00 pg/ml for cases and 4.43 ± 0.54 pg/ml for controls (Arababadi et al., 2010). The power was set at 80% with an alpha <0.05. Calculated sample sizes were $n = 17$ (CRP), $n = 17$ (IL 1β) and $n = 15$ (IL 17a). These group sizes will also exceed 80% statistical power in the dependent statistical tests, namely the longitudinal data analysis. Considering possible nonattendance or late exclusion, the group sizes were set at $n = 30$ for the intervention group and $n = 30$ for the controls without AP. The aim was to acquire complete datasets for at least 25 AP plus 25 control subjects.

The subjects were recruited from different locations: a referral dental practice limited to endodontics, from a referral dental practice limited to implantology and from six general dental practices in the Netherlands. The inclusion took place from January 2017 to September 2019. Individuals 18–80 years of age with AP, confirmed with an intra-oral radiograph were included. The AP was asymptomatic or had only minor symptoms and appeared as a radiolucent area around one or more root tips of the affected tooth. AP was diagnosed when in the periapical region, the periodontal ligament space (on the radiograph) was at least twice as wide as in the mid-root regions. The patient would rather have the affected tooth extracted because a root canal treatment would have a poor prognosis. Furthermore, the rest of the dentition, not included in the study, should not have AP and also no marginal periodontitis. To confirm this, teeth were clinically examined. Discoloured teeth or teeth with restorations that did not respond to cold testing or that were tender to percussion or palpation were additionally examined with an intra-oral radiograph. The control group was recruited from the same practices on a voluntary basis with the perquisite that they were systemically healthy, during their appointment for the regular half-year dental check. This was assessed after the completion of a medical history questionnaire. Also, the controls did not have marginal or AP assessed with oral examination by one of the investigators. All subjects, in both groups, underwent screening according to a list of strict criteria in oral and general health levels, and their family doctor was informed about their participation in this study (Georgiou et al., 2021). A potential subject who met any of the following criteria on a systemic level was excluded: smoking, pregnancy or lactation, diabetes mellitus, chronic inflammatory diseases such as Crohn's disease or hepatitis, use of antibiotics 1 month prior, use of corticosteroids or NSAIDs, chemotherapy or previous head/neck irradiation, any surgery 6 months prior, extra-oral swelling preoperatively, malaise, colds or influenza. On an oral level potential subjects were excluded in case of stomatitis dental prosthesis carriers, absence of periapical radiolucency, the presence of tenderness to percussion., the absence of a positive sensibility test of the dental pulp, previous surgery on the considered tooth or root fracture. Late exclusion would take place if the healing would be compromised (e.g. alveolitis). Other reasons for late exclusion were: other infection-related inflammations, the use of antibiotics or corticoids for any reason, dental hygienist visit during the course of the study, in case of changes in health/surgery of any kind, in case of taking up smoking or other changes in lifestyle. The subject volunteered to participate and consented to six blood draws at six different time points. The subjects of the AP group also had to donate the extracted tooth.

Clinical procedures for the collection of blood samples

After inclusion, within 4 weeks in order to eliminate the influence of any seasonal fluctuations, cases were matched to controls for age and sex, with an exact matching procedure. All subjects visited six times for the blood draw and an update on their health status. During the first visit, first, the informed consent form was signed by the subject and the investigator. Then, a health questionnaire was filled in and a detailed intra-oral examination was performed. During the examination, the number of decayed, missing and filled teeth was recorded and periodontal health was registered with the Dutch Periodontal Screening Index. If recent radiographs were not available, the oral examination would be completed with a radiographic examination. If the inclusion criteria were met and the subject was included, blood was drawn. Subsequent visits were planned preferably 6 weeks and 3 weeks before the planned tooth extraction of the AP tooth, on the day of the tooth extraction and 1, 6 and 13 weeks after the tooth had been extracted. The blood draw of the matched control subject took place in parallel and no tooth extraction was performed. All the appointments were planned at the same time of the day for every individual in order to avoid differences in the mediators attributed to the circadian rhythm of the subjects.

Blood was collected by venipuncture of an antecubital vein using the Vacutainer system (Greiner Bio-One), in a 9 ml containing EDTA blood tube. Immediately thereafter, onsite the tube with the blood was centrifuged for 10 min × 1000 G (Heraeus Labofuge 300; Heraeus Med GmbH), and the supernatant plasma (~4 ml) was carefully collected. The plasma was cooled to 4°C and transported to ACTA. Then, the plasma was aliquoted in 0.5-ml aliquots in 1.5-ml microcap centrifuge tubes and stored

frozen at -80°C. Transportation and storage of the plasma occurred within 24 h of the blood draw.

Quantitative protein assessment

All analyses were performed by Arcadia, a translational research platform (UMC Utrecht, Utrecht, the Netherlands). Concentrations of most of the selected proteins were determined using MSD electro-chemiluminescence immunoassay technology (Meso Scale Diagnostics).

Granulocyte colony-stimulating factor (G-CSF), IL-1α, IL-17A and vascular endothelial growth factor (VEGF) were measured using a custom-made multi-analyte immunoassay kit (customized V–plex cytokine panel 1) according to the manufacturer's protocol. INF-γ, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12p70 and TNF-α were measured using a custom-made multi-analyte immunoassay kit (customized V–plex Proinflammatory panel 1) according to the manufacturer's protocol. Accordingly, CRP and MIP-1α were measured using a custom-made V–plex Vascular Injury panel 2 and a Chemokine panel 1 single-analyte immunoassay kit, respectively. All measurements were performed in duplicates.

Osteoprotegerin (OPG) validation in this sample set was performed using the MSD R-Plex human OPG antibody set in combination with MSD GOLD Small Spot Streptavidine plates on a 2-fold diluted plasma sample according to the manufacturers' instructions.

All measurements were performed using the Quickplex SQ120 (Meso Scale Discovery, MSD). Protein concentrations were measured as pg/ml. Data analysis was performed using MSD Discovery Workbench 4.0 software (Meso Scale Diagnostics). sRANKL measurement was performed using a magnetic bead-based singleplex assay (# LXSAHM; R&D Systems). Two-fold diluted samples were added to these assays and run according to the manufacturers' protocol on a Bio-Plex Multiplex System (Bio-Rad). Protein concentrations were measured as pg/ml. Data analysis was performed using Bio-Plex Manager 6.1 (Bio-Rad).

Statistical analysis

Statistical analyses were performed using the Stata 15.0 (Stata Corp LP) and R (v.4.1.3). The continuous data were presented as mean (± SD)/median (min–max). The level of confidence was determined at 95%, $\alpha = .05$. In order to assess the normality of the data, we visually inspected the Normal Q-Q Plots of the residuals after analysis. The differences between the AP and control group at baseline comparing sex and age category distributions were tested with a chi-squared test (sex) or *t*-test (age). The first two visits before extraction were averaged and that value was used as a baseline. The following analyses were performed: (1) Linear regression analyses to analyse the difference between cases and controls at baseline. (2) Linear mixed model analyses to analyse the difference between the baseline and the last measurement within the AP cases to analyse the difference before and after treatment. (3) linear mixed model analysis to analyse the difference in development over time between cases and controls after treatment (i.e. the healing period). In addition, regarding the difference in development over time between cases and controls, effect modification with age and sex was investigated with an interaction term in the mixed effects model. Regarding age, the subjects were divided into three groups: 18–35, 36–49 and 50–80 years. For both mixed model analyses, only a random intercept on the patient level was added to the model to take into account the dependency of the repeated observations within the patient.

RESULTS

A total of 53 subjects were included in the study, 27 with AP and 26 without (Figure 1). The average age of the AP group was 52.7 years (SD: 14.5) and of the control group 51.5 (SD: 13.17) with no statistically significant difference between the groups ($p > .05$). Fifteen females and 12 males were included in the AP group, and 14 females and 12 males in the control group ($p = 1$ after drop-outs). Two individuals dropped out of the AP group; one on visit four and one on the second visit, for personal reasons, unrelated to this study. In the control group, three subjects dropped out in the fourth, fifth and sixth visit because they had to use antibiotics for a nondental reason. All available data were used. The descriptive data of the concentrations of mediators included in our analyses are summarized in Table A1 of the appendix. One mediator, IL-1α, was under the detection limit for most individuals. Whenever the value of a mediator was under the detection limit, the lower limit of the assay was used as the value.

Table 1 shows the results of the linear regression analyses regarding the difference between cases and controls at baseline. Significant differences were found for GM-CSF, IL-1β and IL-4, with concentrations in the control subjects being higher than in the AP subjects.

Table 2 shows the results of the linear mixed model analyses regarding the within-group changes between the baseline measurement and the last measurement in the AP cases. Concentrations of IL-10 and IL-12p70 were significantly lower at the last measurement compared with the baseline. Although there were also noteworthy

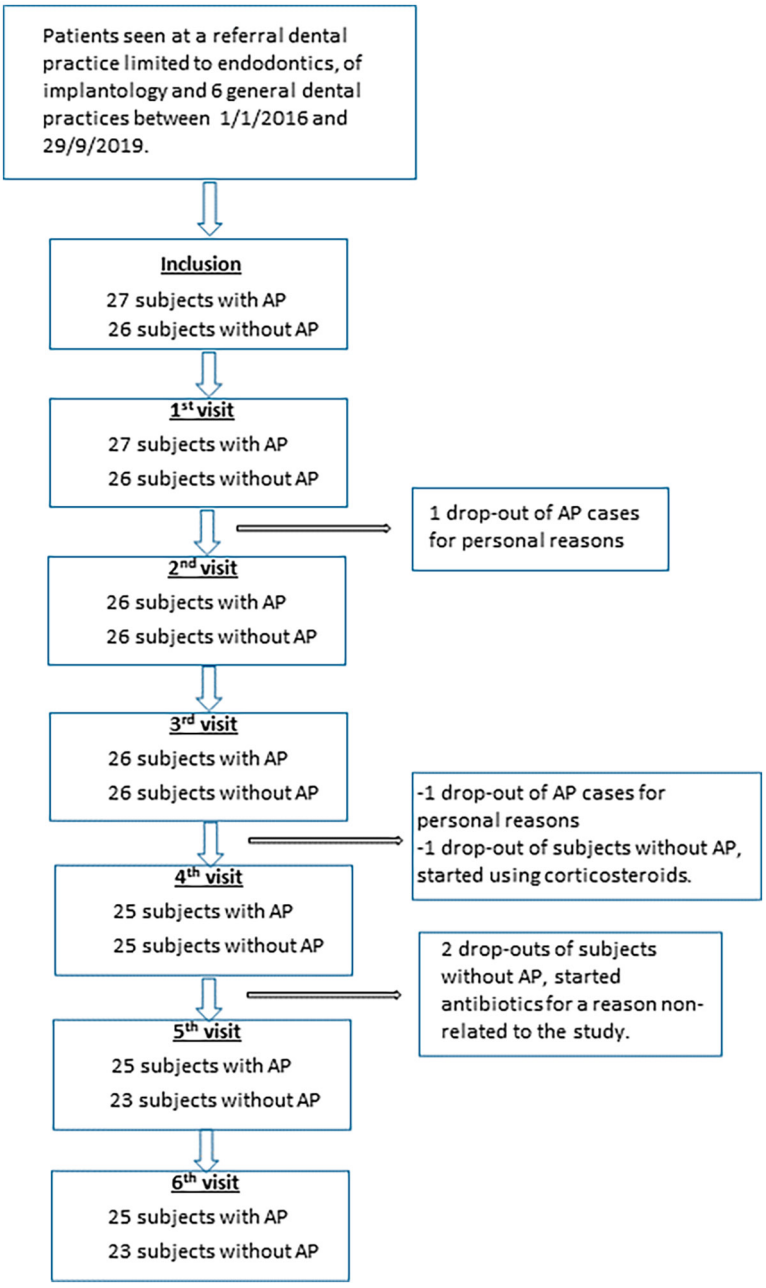

FIGURE 1 Flowchart with the inclusion of subjects

changes in the concentrations of TNF-α, RANKL and CRP, they were not statistically significant. There was also a noteworthy difference (reduction) between baseline and visit 3 in the concentration of TNF-α of −.20 with $p = .04$ CI (−0.38 to −0.01) (Figure 2).

Table 3 shows the results of the linear mixed model analyses comparing the AP cases and the controls regarding the three measurements after tooth extraction and per follow-up. The concentrations of G-CSF, IL-4 and IL-1β were significantly higher after tooth extraction in the AP cases. The concentration of inflammatory markers in the time is shown in Figures 2–4.

Finally, it was investigated whether the differences between AP cases and controls were different for males and females and whether they were different for different age groups. For males and females, no significant differences were found, while for age significant interactions were demonstrated for IL-17A (stronger differences for the third age group) and IFN-γ (stronger differences for the second and strongest for the third age group).

DISCUSSION

The possible systemic consequences of AP have often been addressed in the literature; however, there were always limitations in study designs (Georgiou et al., 2019). This study was designed in which, for the first time, a very broad panel of IM in blood was examined longitudinally in AP and control subjects (Georgiou et al., 2021). In the case

TABLE 1 Results of linear regression analyses comparing the AP cases and the controls at baseline

Blood biomarker	Coefficient (difference) (pg/ml)	*p*-Value (95% CI) (pg/ml)
VEGF	10.15	.24 (−6.59 to 26.88)
CRP	1 134 250	.28 (−909 793.6 to 3 178 293)
G-CSF	**0.04**	**.000** (0.02 to 0.05)
IL-1α	0.06	.18 (−0.03 to 0.15)
IL-1β	**0.02**	**.03 (0.00 to 0.05)**
IL-4	**0.01**	**.01 (0 to 0.02)**
IL-6	−0.05	.63 (−0.24 to 0.15)
IL-8	−0.10	.84 (−1.17 to 0.96)
IL-10	−0.53	.31 (−1.56 to 0.49)
IL-12p70	0	.89 (−0.04 to 0.04)
IL-17A	−0.03	.84 (−0.31 to 0.26)
IFN-γ	2.30	.32 (−2.2 to 6.8)
MIP-1α	−35.16	.28 (−101.23 to 30.92)
OPG	30.86	.46 (−50.94 to 112.66)
TNF-α	0	.98 (−0.41 to 0.43)
RANKL	−2.08	.33 (−6.34 to 2.18)

Note: The values in bold are the statistical significant results.

Abbreviations: AP, apical periodontitis; CRP, C-reactive protein; G-CSF, granulocyte colony-stimulating factor; IFN, interferon; IL, interleukin; MIP-1α, macrophage inflammatory protein-1α; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor kappa-B ligand; TNF, tumour necrosis factor; VEGF, vascular endothelial growth factor.

TABLE 2 The comparison of blood concentration of inflammatory mediators between baseline (average visit 1 and 2) and visit 6 of AP cases

Blood biomarker	Coefficient (difference) (pg/ml)	*p*-Value (95% CI) (pg/ml)
VEGF	1.78	.65 (−5.93 to 9.50)
CRP	−1 313 800	.09 (−2 849 021 to 221 420.4)
G-CSF	−0.001	.89 (−0.02 to 0.02)
IL_1α	0.03	.55 (−0.06 to 0.12)
IL-1β	−0.002	.839 (−0.02 to 0.02)
IL-4	−0.001	.196 (−0.003 to 0.0007)
IL-6	−0.04	.763 (−0.32 to 0.23)
IL-8	−0.46	.270 (−1.29 to 0.36)
IL-10	**−0.25**	**.03 (−0.48 to −0.02)**
IL-12p70	**−0.039**	**.012 (−0.04 to −0.01)**
IL-17A	−0.06	.65 (−0.30 to 0.19)
IFN-γ	−0.15	.966 (−7.06 to 6.77)
MIP-1α	4.02	.271 (−3.14 to 11.19)
OPG	−2.61	.839 (−27.75 to 22.53)
TNF-α	−0.18	.07 (−0.37 to 0.01)
RANKL	2.42	.06 (−0.11 to 4.95)

Note: The values in bold are the statistical significant results.

Abbreviations: AP, apical periodontitis; CRP, C-reactive protein; IFN, interferon; IL, interleukin; MIP-1α, macrophage inflammatory protein-1α; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor kappa-B ligand; TNF, tumour necrosis factor; VEGF, vascular endothelial growth factor.

of the AP subjects, the tooth with AP was extracted at the third visit. At baseline, G-CSF, IL-1β and IL-4 were significantly lower in AP subjects than in controls. Comparison of the differences between the baseline and the last visit, i.e. 3 months after the tooth extraction, showed a significant reduction in IL-10 and IL-12p70. Tooth extraction implies trauma to tooth-surrounding tissues and its healing also had an effect on the fluctuation of IMs. At first glance when inspecting the results, it seems that AP has no effect on systemic IM and that the choice of the control group was unfortunate, since, at baseline, the concentrations of all the IMs except IL-10 and MIP-1α were always higher in controls than in AP subjects. Evoking an inflammatory response in itself, the healing of the tooth extraction resulted in some changes in most IMs within the AP subjects. Even though the concentrations of the IMs in AP subjects were the highest after tooth extraction, they were still lower than those in the control group. However, the controls were carefully selected on the basis of their good health and they matched with the sex and age and were included within 4 weeks of inclusion of the AP subjects. This series of results can be explained by compensatory immunosuppression, whereby a chronic inflammatory state such as AP evokes compensatory immunosuppression, which inhibits proinflammatory processes by impairing the functions of effector immune cells, e.g. macrophages, T-cells and natural killer (NK) cells (Salminen, 2021a). The result of immunosuppression is homeostasis, which is the state of steady chemical and physical conditions maintained by living systems to function optimally. The ability of the system to reach homeostasis can be seen as characteristic of a healthy resilient organism (Figure 5) (Huber et al., 2011). The common three characteristics of the compensatory anti-inflammatory state, which involve (i) the induction of myeloid-biased emergency myelopoiesis, (ii) the expansion and activation of immunosuppressive cells and (iii) the increased expression of anti-inflammatory cytokines TGF-β and IL-10 (Ahn et al., 2017; Mira et al., 2017). All in all, the effect of immunosuppression is a hamper immune response to intruders.

Macrophage inflammatory protein-1α (MIP-1α) is produced by the bone marrow and is crucial for the recruitment of macrophages and T lymphocytes from the circulation to sites of infection or injury. It also acts as a

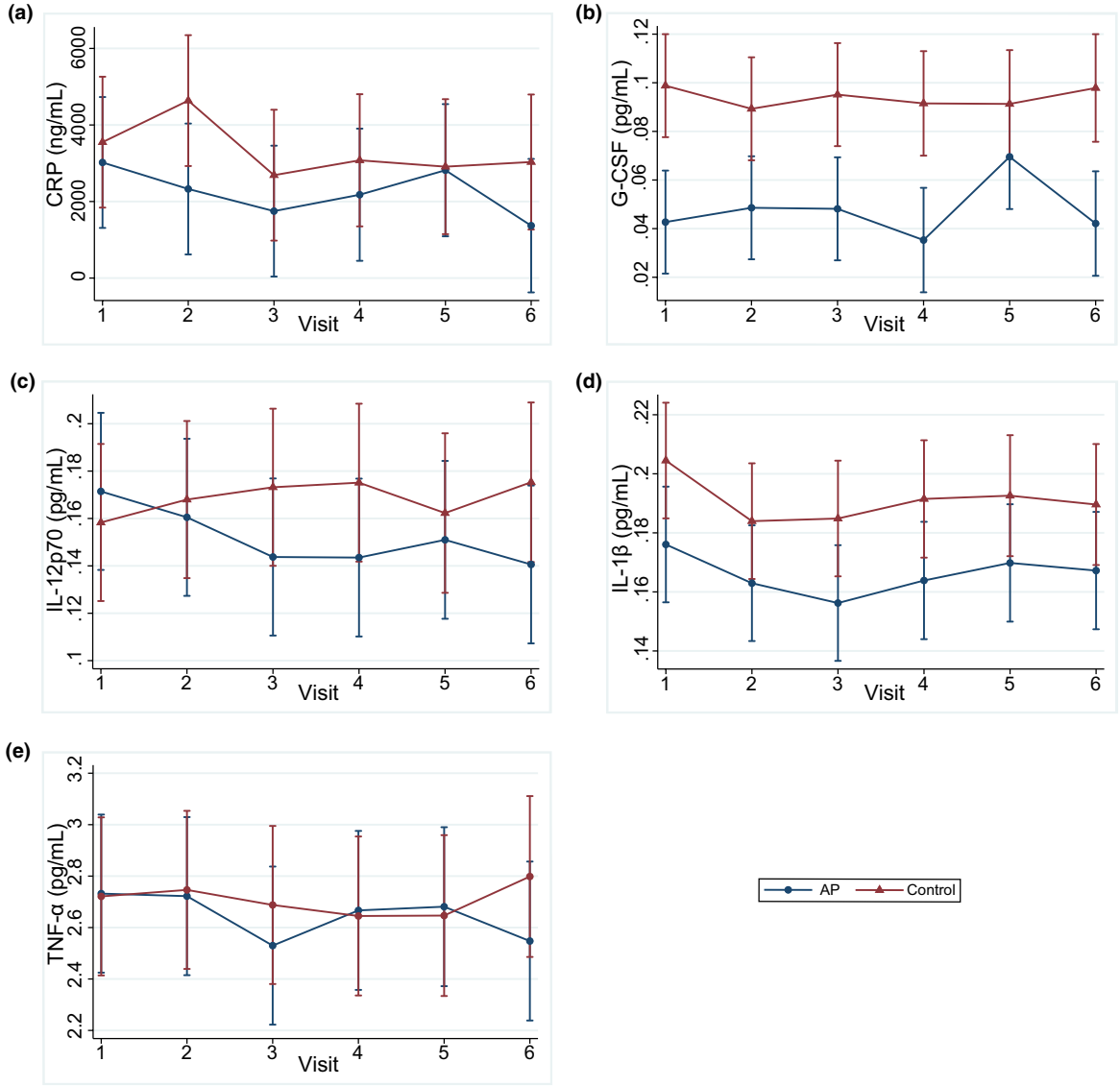

FIGURE 2 The concentration of the proinflammatory markers longitudinally during the six visits (a) CRP, (b) G-CSF, (c) IL-12p70, (d) IL-1β, (e) TNF-α. CRP, C-reactive protein; G-CSF, granulocyte colony-stimulating factor; IL, interleukin; TNF, tumour necrosis factor

potent osteoclast activator and induces bone resorption (Bhavsar & Al-Sabbagh, 2015; Broxmeyer et al., 1999). When comparing the level of MIP-1alpa in the plasma of AP subjects and control subjects, it was observed that there were higher serum concentrations of MIP-1α/CCL3 in the AP group than in the healthy control group but not statistically significant. These findings correspond with studies examining the systemic effect of marginal periodontitis (de Queiroz et al., 2008), while there has been no study about the levels of MIP-1α in AP subjects. Considering that MIP-1α is produced by the bone marrow and there is increased myelopoiesis in a state of low-grade inflammation, the fact that MIP-1α concentration is constantly higher in AP subjects and does not reduce 3 months after the tooth extraction, can be explained.

Our finding was that the concentrations of G-CSF, IL-1β and IL-4 in the peripheral blood of the subjects were significantly higher in the control group than in the AP subjects. G-CSF and IL-4 produced by Natural Killer T-cells (NKT) inversely regulate IL-1β production by macrophages (Ahn et al., 2017). NKT cells are a distinct T-cell subset that links innate and adaptive immune responses. NKT cells regulate macrophage production of IL-1β via cytokine production. Among cytokines secreted by NKT cells, G-CSF and IL-4 inversely influence IL-1β production. G-CSF regulates LPS-mediated pro-IL-1β expression and inflammasome activation. IL-4 enhances the M2 polarization of macrophages, which as a consequence inhibits IL-1β production. These mediators are therefore involved in the pathway that is regulated by NKT cells and is inhibited while in the state of compensatory immunosuppression.

TABLE 3 The differences between AP subjects and controls during all the follow-up measurements and per follow-up

Blood biomarker	Overall effect, *p*-value (95% CI) (pg/ml)	1st follow-up effect, *p*-value (95% CI) (pg/ml)	2nd follow-up effect, *p*-value (95% CI) (pg/ml)	3rd follow-up effect, *p*-value, (95% CI) (pg/ml)
VEGF	7.48, .44 (−11.40 to 26.35)	9.93, .25 (−6.91 to 26.77)	5.35, .56 (−12.80 to 23.50)	9.42, .373 (−11.30 to 30.15)
CRP	885 726.8, .41 (−1 213 881 to 2 985 335)	912 934, .275 (−726 151.2 to 2 552 019)	−509 214.1, .63 (−1 560 459 to 2 578 887)	1 009 196, .405 (−1 367 011 to 3 385 402)
IL-17A	0.06, .62 (−0.16 to 0.28)	0.12, .350 (−0.13 to 0.38)	0.06, .61 (−0.18 to 0.31)	0.05, .71 (−0.21 to 0.31)
G-CSF	0.03, **.01** (0.01 to 0.06)	0.04, **.00** (0.02 to 0.06)	0.03, .06 (0.00 to 0.06)	0.03, **.04** (0.00 to 0.06)
INF-γ	4.33, .26 (−3.27 to 11.94)	3, .23 (−1.88 to 0.87)	4.19, .228 (−3.40 to 11.79	6.39, .27 (−4.83 to 17.62)
IL-8	0.24, .69 (−0.93 to 1.40)	−0.07, .90 (−1.19 to 1.04)	0.09, .887 (−1.10 to 1.27)	0.18, .79(−1.15 to 1.50)
IL-10	−0.40, .35 (−1.23 to 0.44)	−0.47, .32 (−1.40 to 0.45)	−0.44, .325 (−1.33 to 0.44)	−0.29, .50 (−1.13 to 0.55)
IL-12p70	0.02, .28 (−0.02 to 0.06)	0.03, .19 (−0.02 to 0.09)	0.02, .401 (−.02 to 0.06)	0.02, .31 (−0.02 to 0.06)
IL-4	0.01, **.02** (0 to 0.02)	0.01, **.02** (0.0 to 0.01	0.01, **.04** (0.00 to 0.02)	0.01, **.05** (0.00 to 0.02)
IL-1β	0.02, **.04**, (0 to 0.05)	0.03, **.007** (0.01 to 0.05)	0.03, **.05** (0.00 to 0.05)	0.02, .11(−0.00 to 0.05)
IL-6	0.09, .63, (−0.27 to 0.45)	−0.05, .574 (−0.22 to 0.12)	−0.02, .89 (−0.25 to 0.22)	0.21, .42 (−0.31 to 0.74)
TNF-α	0.08, .7, (−0.31 to 0.47)	0.08, .68(−0.31 to 0.48)	−0.02, .931 (−0.42 to 0.39)	0.15, .48 (−0.26 to 0.57)
OPG	39.17, .33 (−39.91 to 118.26)	22.41, .575 (−56.01 to 100.85)	36.11, .354 (−40.22 to 112.43)	41,06, .34 (−43.87 to 125.99)
MIP-1α	42.20, .30 (−122.54 to 38.14)	−39.94, .31 (−117.08 to 37.19)	−43.41, .30 (−125.87 to 39.05)	−42.33, .324 (−126.46 to 41.79)
RANKL	−2, .34 (−6.1 to 2.10)	−0.88, .65 (−4.71 to 2.95)	−1.14, .59 (−5.29 to 3.01)	−2.83, .20 (−7.19 to 1.52)

Note: The values in bold are the statistical significant results.

Abbreviations: AP, apical periodontitis; CRP, C-reactive protein; IFN, interferon; IL, interleukin; MIP-1α, macrophage inflammatory protein-1α; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor kappa-B ligand; TNF, tumour necrosis factor; VEGF, vascular endothelial growth factor.

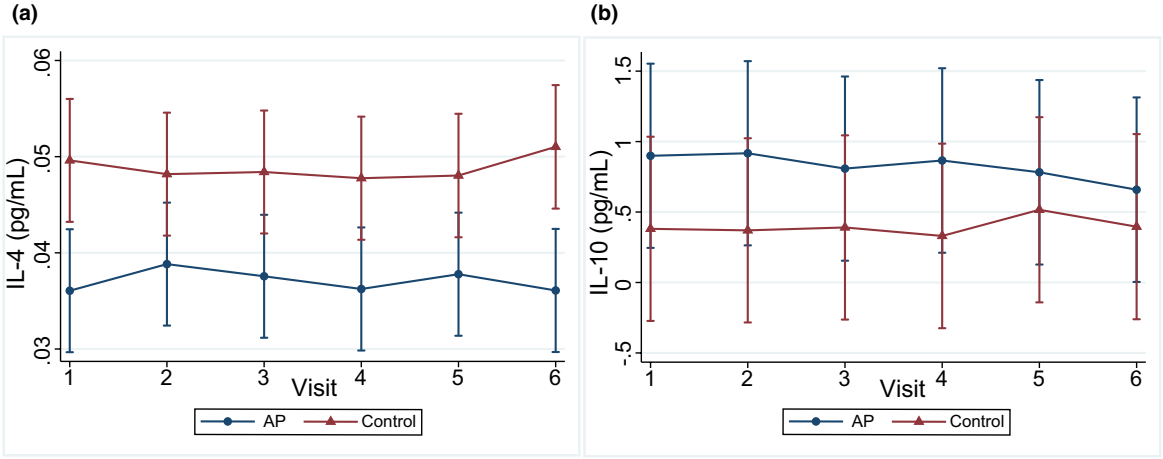

FIGURE 3 The concentration of the anti-inflammatory markers longitudinally during the six visits (a) IL-4, (b) IL-10. IL, interleukin

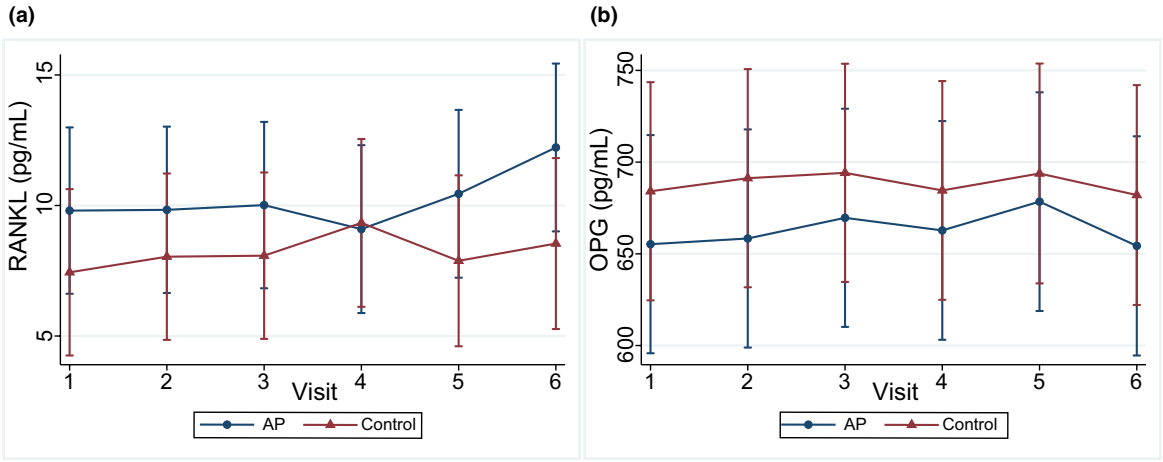

FIGURE 4 The concentration of bone metabolism markers longitudinally during six visits (a) RANKL (b) OPG. OPG, Osteoprotegerin; RANKL, receptor activator of nuclear factor kappa-B ligand

During the healing of the tooth extraction, there were also significant treatment effects when measuring the concentration of G-CSF, IL-1β and IL-4 in the circulatory blood of the AP subjects. It should be noted that G-CSF increases IL-1β production by macrophages at one-tenth the concentration of IL-4, suggesting that G-CSF might provide a stronger signal than IL-4 for IL-1β production according to a study published recently (Ahn et al., 2017). This can be seen in the first follow-up visit when there was a very significant increase in G-CSF ($p = 0$) while for IL-4 and Il-1 β the changes were significant but more moderate.

After the tooth extraction of the AP subjects, the system tries to recover and exit the state of low-grade inflammation. Anti-inflammatory cytokines, with IL-10 being one of the main, secreted by regulatory immune cells are the key messengers, which induce the immunosuppressive responses in inflamed tissues of AP (Salminen, 2021b). After the AP is alleviated and 3 months have passed since the extraction of the teeth, there is a significant difference in the concentration of IL-10, being significantly lower at the last follow-up. In the existing literature, one study examined the IL-10 in AP subjects in a case–control study (Garrido et al., 2019). In that study, the levels of the concentrations of IL-10 were the same in AP and control subjects, but also subjects with marginal periodontitis and smokers were included. Therefore, the validity of these results is questionable, since potentially confounding factors were not taken into consideration. In our study, the levels of IL-10 were higher in the AP subjects compared with controls but not significantly. On the other hand, after the tooth extraction, there was a significant reduction. This information would have been missed if it was not for the longitudinal design of our study.

The concentration of IL-12p70 at the last follow-up visit after the extraction of the teeth was significantly lower than at baseline. IL-12p70 is a bioactive member of the IL-12 family of cytokines, which are key players

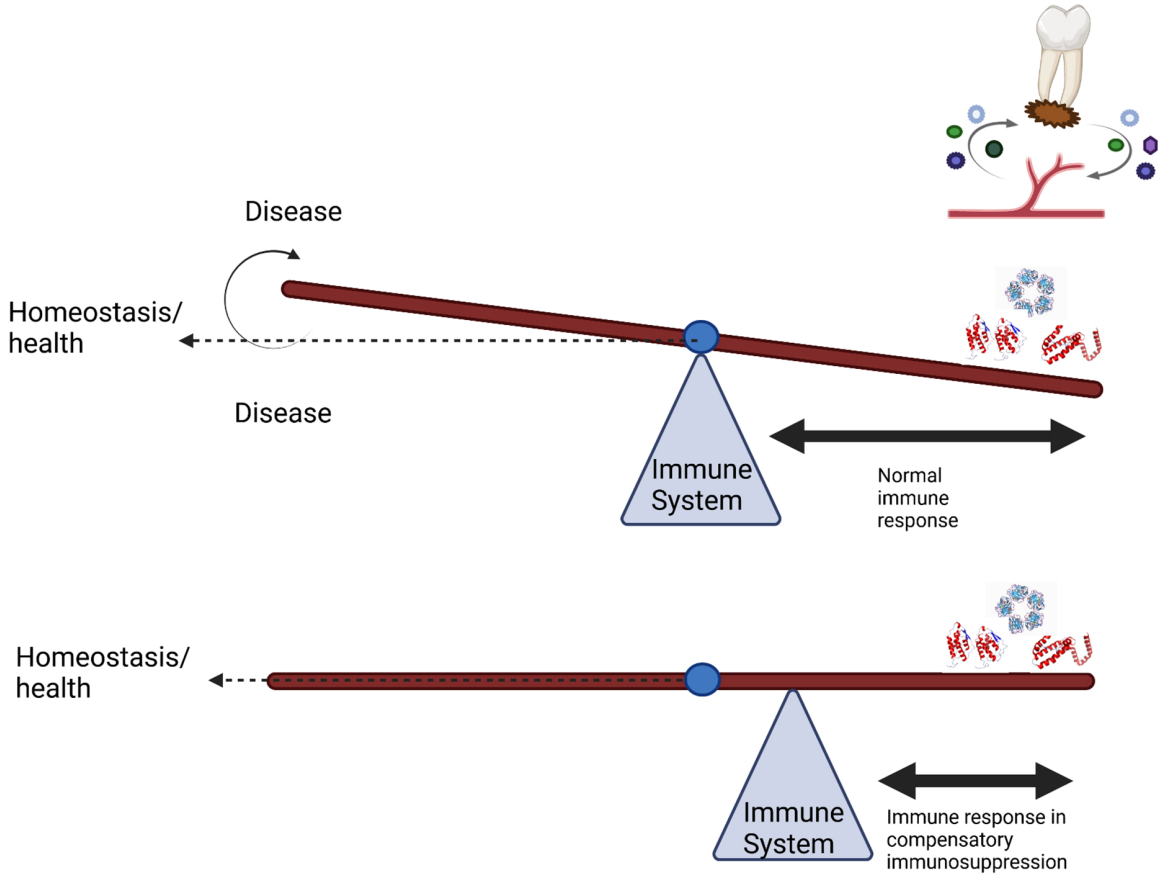

FIGURE 5 In chronic low-grade inflammation, the immune response is decreased in order to maintain homeostasis.

in the regulation of T-cell responses. IL-12 is composed of p35 and p40 subunits, which, when combined, form the IL-12p70 (Gee et al., 2009). To our knowledge, no other study has looked into the levels of IL-12p70 longitudinally, although, one case–control study (Garrido et al., 2019) found no significant differences between cases and controls. Considering the immunologic profile of low-grade inflammation, comparisons within the individuals are more appropriate for the purposes of making conclusions about the effect of AP on the IL-12p70 levels.

Several studies exploring the systemic effect of AP have examined concentrations of CRP. Various case–control studies (Garrido et al., 2019; Harjunmaa et al., 2018; Ren & Malmstrom, 2007; Sirin et al., 2019), animal studies (Buttke et al., 2005) and interventional studies with one follow-up (Poornima et al., 2021; Ren & Malmstrom, 2007) were performed. All these case–control studies found a 'dose-dependent' relationship between the concentration of CRP, the severity of the inflammation (symptomatic, with fistulas and/or swelling, bigger lesions) and the number of AP teeth (Georgiou et al., 2019). Sirin et al. (2019) concluded that patients' CRP levels were reliable predictive indicators for AP severity. They also found no statistically significant difference in the levels of CRP between the control group and AP subjects with one asymptomatic tooth, in whom the levels of CRP were even slightly lower. Since we also included subjects with one asymptomatic AP tooth: the minimum burden, Sirin Ozcelik's results are consistent with our own. Also, longitudinally, there was a statistically significant difference between the first visit and the visit 3 months after tooth extraction ($p = .04$); this corresponded to the results of previous studies (Poornima et al., 2021). However, this difference becomes nonsignificant when we use the average of visits 1 and 2 as a baseline ($p = .09$), which was performed in the current study to eliminate normal fluctuations. Up to today all the studies performed used only a single baseline measurement.

In the design of the study, blood plasma values from visits 1–3 were meant to be averaged to serve as a baseline. However, at visit 3 blood had been drawn minutes before local anaesthesia and tooth extraction and later analysis showed that the concentration of TNF-α was lower than in blood drawn at visit 1 ($p = .05$). This drop in TNF-α may have resulted from the psychological stress caused by the extraction that took place at the same visit. There is evidence that stress promotes an immunosuppressive cytokine phenotype. In a recent

animal study, it was found that, despite an *in-vitro* challenge with bacterial LPS, the concentration of TNF-α and IL-1beta of rats was lower, when they experienced severe stress. The levels of IL-10 remained unaffected (Connor et al., 2005). These results correspond to the findings of this study. Therefore, it was decided to use only visits 1 and 2 as the baseline. For future longitudinal studies, it is important not to combine an intervention with a baseline measurement as subjects can be nervous about undergoing the intervention.

In the present study, bone-remodelling IM OPG and receptor activator of nuclear factor kappa-B ligand (RANKL) were also examined, and the analytical data confirmed that remodelling procedures were taking place during ongoing AP. The concentration of OPG in AP subjects at all visits was lower than that in controls, although not significantly, and was even lower in visit 6 (Figure 4b). The RANKL concentration was slightly higher in AP subjects than in controls and increased even more after the tooth extraction (Figure 4a). Although all the changes were not significant, some trends were obvious and they correspond with previous findings from animal studies (Koth et al., 2021; Ribeiro et al., 2020). This is the first time that OPG and RANKL were measured in peripheral blood in subjects with AP and also the first time the levels after dental extraction were examined.

This study design was designed to alleviate any bias and doubts in previously published work on this subject. Out of a need for carefully planned clinical studies that would resolve the inconsistencies in terms of designs and the choice of IMs examined, the case and control groups were carefully chosen and matched and the health of all participants was monitored during the different visits. Diagnosis (chronic AP) and treatment (extraction of the tooth involved) were the same for all participants. In the profile of IMs of peripheral blood, we were thus able to examine the minimum burden that AP can have on the healthy human body. In sick individuals, with disrupted homeostasis, the burden and effect of AP on their health may be greater. This is an important topic for future research. Finally, after consultation with an expert in longitudinal data analysis, the most suitable method was used to analyse the data.

Despite these strengths, there are also some limitations that should be considered in future study design. All subjects were followed for a period of 3 months after tooth extraction. We assumed that the case-group values of these biomarkers after healing would be at the same levels as those in the control group. However, it was observed that not all mediators returned to the control levels. It is possible that this is attributed, not only to systemic reasons but also local. In some cases there are periapical bacteria in biofilms within the lesion, that may take longer than 3 months to resolve completely (Riool et al., 2014). It is, therefore, proposed that, in order to understand how long it takes for the system to fully recover, future studies should include more than six visits, 6 months and possibly 1 year after tooth extraction.

In addition, a very broad panel of IMs was examined, which was costly. Due to the exploratory nature of this study, a wide array of proteins has been selected. However, some of these mediators were not very indicative of the situation or were involved in the inflammatory process later on in the inflammatory pathway. We propose that future studies examine CRP, GM-CSF, IL-4, IL-1β, IL-10, IL-12p70 and OPG / RANKL. Additionally, even though it was not examined in this study, TGF-β would also be an indicator of low-grade inflammation (Jonsjo et al., 2020). Finally, since some IM, such as TNF-alpha, are also affected by other factors such as the stress described above, a stressful therapeutic intervention should not take place at the same time as blood is drawn.

CONCLUSIONS

To our knowledge, this is the first longitudinal evaluation of the effects of AP and dental extraction in such a broad selection of IM in peripheral blood. The immunologic profile of chronic AP in one tooth seems to reveal a systemic low-grade inflammation through compensatory immunosuppression—which, on the basis of other studies in this field, seems to be dose-dependent. A larger lesion or multiple lesions could disrupt the balance that the system is trying to maintain, resulting in loss of homeostasis.

AUTHOR CONTRIBUTIONS

A. C. Georgiou, S.V. van der Waal and W. Crielaard involved in conceptualization. A. C. Georgiou and S. V. van der Waal performed the study. A. C. Georgiou and J. W. R. Twisk analysed the data. A. C. Georgiou drafted the manuscript. A. C. Georgiou, J. W. R. Twisk, P. Ouwerling, A. H. Schoneveld and S. V. van der Waal reviewed and edited the final manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data will be available on request.

ETHICS STATEMENT

Approval was sought and received by the Medical Ethical Committee of the VU medical Centre, Amsterdam, the Netherlands (registration number 2016.187).

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APPENDIX 1

TABLE A1 An overview of the median and range values of case and control subjects of every inflammatory mediator in every visit.

Var	Case/control		V1	V2	V3	V4	V5	V6
VEGF	Case	Median	42.35	48.23	40.62	42.46	46.01	45.22
		Range	14.63–128.35	12.85–133.04	10.79–165.06	13.52–161.89	13.21–183.54	11.35–158.69
	Control	Median	65.70	57.53	57.04	55.67	61.99	57.35
		Range	21.75–166.52	25.93–116.26	19.68–134.62	25.09–131.25	21.17–143.04	25.29–196.06
CRP	Case	Median	1 141 515.80	1 082 270.87	1 099 067.26	1 349 565.36	1 538 493.27	1 039 188.84
		Range	76 016.90–26 902 071.08	89 091.29–8 074 830.23	159 853.31–7 079 752.56	202 348.30–7 242 890.66	73 865.66–23 489 705.81	70 619.27–4 433 403.91
	Control	Median	1 400 557.56	1 804 507.65	1 356 567.75	1 092 261.92	1 299 373.85	1 206 144.37
		Range	76 335.26–29 493 987.2	75 561.5–30 501 988.67	77 509.82–16 554 069.96	74 615.54–18 041 859.42	158 350.33–22 765 521.65	95 935.69–29 524 901.44
GM-CSF	Case	Median	0.06	0.05	0.07	0.06	0.04	0.06
		Range	0.01–0.15	0.00–0.22	0.02–0.16	0.01–0.14	0.01–0.65	0.01–0.16
	Control	Median	0.09	0.11	0.09	0.10	0.09	0.11
		Range	0.04–0.21	0.03–0.17	0.02–0.18	0.01–0.18	0.03–0.17	0.05–0.18
IL1alpha	Case	Median	0.10	0.26	0.49	0.29	0.14	0.25
		Range	0.02–0.50	0.26–0.50	0.10–1.57	0.01–0.44	0.05–0.39	0.06–0.49
	Control	Median	0.10	0.24	0.18	0.17	0.07	0.12
		Range	0.02–1.59	0.02–0.47	0.04–0.59	0.02–0.3	0.04–0.17	0.03–0.40
IL-1beta	Case	Median	0.18	0.17	0.16	0.17	0.17	0.16
		Range	0.04–0.29	0.04–0.24	0.02–0.22	0.06–0.26	0.06–0.32	0.05–0.36
	Control	Median	0.19	0.19	0.20	0.19	0.19	0.18
		Range	0.13–0.44	0.07–0.26	0.10–0.25	0.13–0.29	0.11–0.42	0.10–0.32
IL-4	Case	Median	0.04	0.04	0.04	0.04	0.04	0.04
		Range	0.01–0.10	0.01–0.10	0.01–0.01	0.00–0.10	0.01–0.10	0.00–0.10
	Control	Median	0.05	0.05	0.04	0.04	0.04	0.05
		Range	0.03–0.09	0.02–0.09	0.03–0.09	0.03–0.09	0.03–0.09	0.03–0.09
IL-6	Case	Median	0.65	0.53	0.58	0.61	0.54	0.59
		Range	0.16–2.05	0.13–1.79	0.16–1.81	0.32–2.08	0.23–1.72	0.19–1.72
	Control	Median	0.51	0.61	0.55	0.46	0.47	0.54
		Range	0.22–1.19	0.21–2.13	0.28–1.58	0.22–1.53	0.20–4.6	0.22–8.82

TABLE A1 (Continued)

Var	Case/control		V1	V2	V3	V4	V5	V6
IL-8	Case	Median	6.80	7.52	6.85	6.84	7.06	6.56
		Range	4.03–14.37	3.19–16.87	4.32–16.97	3.76–13.82	3.79–14.09	0.05–15.55
	Control	Median	7.21	6.61	6.78	7.34	6.32	7.30
		Range	4.00–14.55	4.45–10.49	4.24–10.93	4.27–11.88	3.82–15.72	4.10–13.59
IL-10	Case	Median	0.31	0.30	0.28	0.29	0.28	0.26
		Range	0.19–14	0.14–14.41	0.15–13.34	0.15–12.21	0.16–10.72	0.04–10.05
	Control	Median	0.36	0.33	0.34	0.29	0.27	0.39
		Range	0.21–0.99	0.20–0.84	0.19–0.98	0.20–0.55	0.17–5.00	0.17–1.13
IL-12p70	Case	Median	0.14	0.14	0.13	0.12	0.13	0.13
		Range	0.08–0.41	0.07–0.32	0.05–0.32	0.02–0.36	0.07–0.37	0.02–0.38
	Control	Median	0.14	0.14	0.14	0.14	0.13	0.14
		Range	0.04–0.49	0.07–0.47	0.07–0.60	0.06–0.44	0.05–0.42	0.06–0.40
IL-17A	Case	Median	0.38	0.58	0.38	0.34	0.43	0.41
		Range	0.03–3.99	0.13–3.44	0.05–1.64	0.08–1.22	0.13–2.31	0.03–2.09
	Control	Median	0.47	0.39	0.41	0.43	0.42	0.57
		Range	0.09–2.10	0.06–2.00	0.07–3.78	0.08–1.72	0.06–3.42	0.10–2.01
IFN-gamma	Case	Median	7.57	6.69	4.70	5.28	5.36	5.36
		Range	2.58–55.69	2.11–21.08	2.12–31.17	2.33–68.76	3.27–42.37	1.85–33.68
	Control	Median	7.94	7.68	7.98	7.61	6.83	7.89
		Range		3.32–77.48	3.28–61.89	3.53–88.71	3.47–28.06	3.73–182.15
MIP-1alpha	Case	Median	12.03	10.93	10.62	11.85	12.13	11.21
		Range	6.47–929.33	5.13–880.03	5.56–1060.02	4.88–1046.63	3.18–1120.31	6.85–1001.32
	Control	Median	10.93	11.25	10.55	10.54	10.30	11.03
		Range	3.15–17.13	3.07–17.82	3.82–17.81	4.45–18.00	4.93–14.70	6.34–19.02
OPG	Case	Median	620.07	615.43	669.79	638.94	685.35	629.32
		Range	347.87–925.48	355.65–1021.67	393.90–941.20	400.57–884.60	398.13–881.50	441.79–926.52
	Control	Median	664.94	685.38	676.71	688.20	715.12	679.06
		Range	421.67–1019.50	418.66–985.30	459.82–1104.28	471.91–1042.91	458.49–1074.59	439.18–1216.87

TABLE A1 (Continued)

Var	Case/control		V1	V2	V3	V4	V5	V6
TNF-alpha	Case	Median	2.58	2.59	2.52	2.50	2.48	2.31
		Range	0.82–4.75	0.81–4.72	0.89–3.73	0.84–4.63	0.84–4.78	0.03–4.36
	Control	Median	2.69	2.57	2.52	2.33	2.59	2.70
		Range	1.59–4.14	1.54–4.73	1.51–4.41	1.53–4.18	1.63–3.94	1.56–4.75
RANKL	Case	Median	10.10	8.53	10.60	10.00	11.09	14.02
		Range	0.87–36.8	2.75–31.15	2.15–26.94	0.3–24.95	2.82–29.91	1.61–44.84
	Control	Median	8.99	11.36	8.15	11.36	10.84	11.36
		Range	0.36–25.01	0.84–23.06	0.3–30.85	0.84–27.48	0.36–25.43	2.93–22.9

Abbreviations: CRP, C-reactive protein; IFN, interferon; IL, interleukin; MIP-1α, macrophage inflammatory protein-1α; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor kappa-B ligand; TNF, tumour necrosis factor; VEGF, vascular endothelial growth factor.