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Biomarker levels in peri-implant crevicular fluid of healthy implants, untreated and non-surgically treated implants with peri-implantitis

Diederik F. M. Hentenaar¹ | Yvonne C. M. De Waal² | Arjan Vissink¹ |
Arie Jan Van Winkelhoff² | Henny J. A. Meijer^{1,2} | Sylvia C. Liefers³ |
Frans G. M. Kroese³ | Gerry M. Raghoobar¹

¹Department of Oral and Maxillofacial Surgery, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands

²Center for Dentistry and Oral Hygiene, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands

³Department of Rheumatology and Clinical Immunology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands

Correspondence

Gerry M. Raghoobar, Department of Oral and Maxillofacial Surgery, University of Groningen, University Medical Center Groningen, PO Box 30.001, 9700 RB Groningen, The Netherlands.
Email: g.m.raghoobar@umcg.nl

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Abstract

Aim: To compare biomarker levels in peri-implant crevicular fluid (PICF) of healthy implants with levels in PICF of implants with peri-implantitis (before and after non-surgical treatment).

Materials and methods: Samples were taken from 20 healthy implants ($n = 17$ patients) and from 20 implants with peri-implantitis ($n = 19$ patients) before and 3 months after non-surgical treatment using the Airflow Master Piezon[®] (EMS). A Luminex[™] assay was used to evaluate pro-inflammatory and anti-inflammatory cytokines IL-1 β , TNF- α , IL-6 and G-CSF, collagen degradation enzyme MMP-8, chemokines MCP-1 & MIP-1 α /CCL3, bone markers OPG and sRANKL and interferon- γ . Clinical and radiographical characteristics were assessed. A Mann-Whitney U and Wilcoxon signed-rank test analysed between- and within-group differences.

Results: IL-1 β and MMP-8 levels were found significantly elevated in implants with peri-implantitis ($p = .007$; $p = <.001$, respectively). No difference in levels of TNF- α , IL-6, MCP-1 and MIP-1 α /CCL3, OPG and G-CSF between healthy and diseased implants was found. Levels of sRANKL and INF- γ were under the level of detection. None of the biomarker levels improved after non-surgical therapy, and levels of IL-1 β and MMP-8 remained high.

Conclusion: Implants diagnosed with peri-implantitis have higher levels of IL-1 β and MMP-8 in PICF compared to healthy implants. Non-surgical therapy did not influence the inflammatory immune response.

KEYWORDS

biomarkers, crevicular fluid, multiplex immunoassay, non-surgical treatment, peri-implantitis

1 | INTRODUCTION AND RATIONALE

Peri-implant infections are characterized by an inflammatory response to a bacterial imbalance leading to soft tissue inflammation with or without progressive loss of supporting bone (i.e.,

peri-implantitis or peri-implant mucositis, respectively; Schwarz et al., 2018). Objective methods to differentiate between peri-implant health and disease, and to evaluate the effect of therapeutic intervention are lacking since traditional clinical diagnostic methods, such as pocket probing, bleeding on probing and radiographic assessment,

exert a weak sensitivity/specificity (Heitz-Mayfield, 2008; Hashim et al., 2018; Alassy et al. 2019). It is thought that immunological host-derived molecules, such as cytokines, chemokines, proteolytic and tissue breakdown enzymes, could serve as adjunctive parameters to ameliorate the diagnosis, prediction and management of peri-implant disease (Ramseier et al., 2009; Sexton et al., 2011; Syndergaard et al., 2014; Kinney et al., 2014; Carinci et al., 2019).

Since 1989, when Apse et al. (1989) demonstrated the presence of fluid in the osseointegrated implant pocket (i.e., peri-implant crevicular fluid [PICF]), a broad research effort has been spent to analyse host-derived immunological biomarkers present in this fluid (Apse et al., 1989; Armitage, 2004; Lamster & Ahlo, 2007). Ideally with the aim to develop PICF diagnostics (e.g., point-of-care testing), to flank clinical and radiographical examination in the monitoring of peri-implant tissue health (Carinci et al., 2019). To date, most studies have focused only on a limited number of biomarkers, mainly including only 1 or 2 classical pro-inflammatory cytokines (i.e., IL-1 β , TNF- α and/or IL-6) (Dursun et al. 2016). These markers are commonly chosen since they exert synergistic properties in the initiation of inflammatory marker cascade and are produced at local sites with inflammation. In addition, they probably play an important role in osteoclast formation and, hence, resorption of bone structures (Hirano et al., 1990; Dinarello, 2000; Tanaka et al., 2014). Studies on several important anti-inflammatory biomarkers, collagen degradation enzymes, osteoclastogenesis-related cytokines and chemokines (e.g., granulocyte colony-stimulating factor [G-CSF], matrix metalloproteinase-8 [MMP-8], monocyte chemoattractant protein [MCP-1], macrophage inflammatory protein [MIP-1 α /CCL3], bone markers [OPG and sRANKL] and interferon- γ) have, however, never been performed properly (Duarte et al., 2016). Also, whether these biomarker levels in PICF could be helpful to evaluate the outcome of peri-implantitis therapy has scarcely been evaluated in previous studies (Basseti et al., 2014; Renvert et al., 2016). Considering that cytokines are involved in broad networks, which to a large extent orchestrate the immuno-inflammatory process, an expanded approach of biomarker evaluation to increase the chance to find biomarkers that could help to distinguish between healthy and diseased implants is necessary (Feghali & Wright, 1997; Duarte et al., 2016).

Therefore, the aim of the present study was to assess scarcely investigated or priorly not investigated biomarkers next to the commonly studied classical pro-inflammatory biomarkers in PICF of healthy implants and implants with peri-implantitis (before and after non-surgical treatment).

2 | MATERIALS AND METHODS

2.1 | Study design

A combined cross-sectional and intervention study was conducted in accordance with the Declaration of Helsinki on human studies (World Medical Association, 2013). The SPIRIT guidelines for reporting a clinical trial were followed (Chan et al., 2013).

Clinical Relevance

Scientific rationale for study: To assess whether peri-implant health and disease are accompanied by different bio-marker levels in PICF as well as to evaluate the effect of non-surgical peri-implantitis treatment on these levels.

Principal findings: Peri-implantitis implants showed higher levels of IL-1 β and MMP-8 in PICF compared to healthy implants. Immune response seemed not to change by a single non-surgical peri-implantitis intervention. sRANKL and INF- γ appeared under level of detection using a customized Luminex™ assay.

Practical implications: PICF collection, in addition to clinical and radiographical examination, helps to understand peri-implant health. Evaluation of non-surgical peri-implantitis therapy outcome using PICF diagnostics did not show to be helpful.

2.2 | Study population

Patients with implants with peri-implantitis from the Center of Dentistry and Oral Hygiene and the Department of Oral and Maxillofacial Surgery of the University Medical Center Groningen (UMCG), Groningen, the Netherlands, were consecutively recruited to participate in the study, according to specific inclusion and exclusion criteria (see Table 1). The study was executed at the Department of Oral and Maxillofacial Surgery of the University Medical Center Groningen between September 2017 and November 2019. The study was conducted in accordance with the Medical Research Involving Human Subjects Act (WMO) and in full accordance with the principles of the Declaration of Helsinki as stated in 64th WMA General Assembly, Fortaleza, Brazil, October 2013. Before the start of the study, all patients signed an informed consent. All patients were scheduled for a single non-surgical intervention using the Airflow Master Piezon® (Electro Medical Systems, EMS). This therapy was applied on 6 sites around the implant (mesial/mid/distal buccal, mesio/mid/disto lingual) for 5 s per site. All treatments were performed by one experienced dental hygienist (SS). Patients received oral hygiene instructions with emphasis on the daily use of interdental brushes with application of 0.12% chlorhexidine gel (Dentaid Benelux).

Seventeen adult patients scheduled for routine dental/implant check-up at the Center for Dentistry and Oral Hygiene and the Department of Oral and Maxillofacial Surgery of the University Medical Center Groningen, with only healthy implants and free from periodontal inflammation (probing pocket depth [PPD] $\leq$ 3 mm, no bleeding on probing [BoP] and no anatomical loss of periodontal structures) were consecutively asked to participate as control subjects. Healthy implants (HI) were defined as: PPD $<$ 5 mm, no bleeding/suppurate on probing (BoP/SoP) and no marginal bone loss (MBL). Control group patients did not undergo a therapeutic

TABLE 1 Inclusion and exclusion criteria

Inclusion criteria

- The patient was ≥ 18 years of age;
- The patient had at least one endosseous implant in the oral cavity with clinical and radiographical signs of peri-implantitis. Peri-implantitis was defined as progressive loss of marginal bone ≥ 2 mm, as compared to the baseline radiograph (after placement of the definitive restoration) in combination with bleeding and/or suppuration on probing (de Waal et al., 2015);
- The implants had been in function for at least 2 years;
- The patient was capable of understanding and giving informed consent.

Exclusion criteria

- Medical and general contraindications for intervention;
- A history of local radiotherapy to the head and neck region;
- Pregnancy and lactation;
- Uncontrolled diabetes mellitus ($\text{HbA1c} > 7\%$ or > 53 mmol/mol);
- Use of antibiotics during the last 3 months;
- Known allergy to chlorhexidine;
- Long-term use of anti-inflammatory drugs;
- Incapability of performing basal oral hygiene measures as a result of physical or mental disorders;
- Implants with bone loss exceeding 2/3 of the length of the implant or implants with bone loss beyond the transverse openings in hollow implants;
- Implant mobility;
- Implants at which no position can be identified where proper probing measurements can be performed;
- Previous surgical treatment of the peri-implantitis lesions;
- Previous non-surgical treatment of the peri-implantitis lesions during the last 3 months (scaling or curettage)
- Chronic bronchitis and asthma.

intervention. The Medical Ethics Review Board of the University Medical Center Groningen (METc UMCG) has discussed and considered whether or not the sampling the study protocol falls within the scope of the Medical Research Involving Human Subjects Act (WMO) and decided that no ethics committee approval was needed for assessment of these patients (METc 2018.537).

In both groups, all eligible implants present were included until 20 implants per group (according to our sample size calculation) were sampled.

2.3 | Peri-Implant crevicular fluid (PICF)

2.3.1 | Biomarker sampling and volume quantification

A 30-s sampling protocol following Wassall and Preshaw (2016) was applied. In brief, before sampling the sample site was isolated with cotton rolls and dried with a gentle stream of air. Two PICF samples were collected from the same implant pocket per included implant in the healthy and diseased group using Periopaper® strips (Oraflow Inc.; Stewart et al., 1993). These presterilized filter paper strips were placed 1–2 mm into the sulcus/pocket and absorbed fluids up to 1.2 μL . To minimize evaporation, volume quantification was performed immediately after sampling, using a Periotron 8000 device

(Oraflow Inc.). Volumes were used to calculate modified concentration levels. The Periotron 8000 was calibrated before commencing the study and recalibrated periodically, following the manufacturer's recommendations. A calibration curve was generated accordingly. Directly after quantification, the two Periopaper® strips per implant were pooled in a dry Eppendorf tube® (Eppendorf AG). The tubes were placed on ice for transport to the laboratory and stored at -80°C until antibody array quantification took place. Implants with peri-implantitis (PI) were sampled at baseline (T0) and additionally at 3 months after therapy (T3). Health implants were only sampled at baseline.

2.3.2 | Biomarkers of interest, determination and analysis (using Luminex™ xMAP multi-analyte profiling technology)

Periopaper® strips were thawed at the day of analysis after being stored dry at -80°C . To extract PICF from the strips, Luminex™ assay buffer (23 μL) was added to each vial after which all the samples were vortexed for 30 min. Before centrifugation, the Periopaper® strips were fixed in the tube's cap. The samples were then centrifuged for 60 min at 300 rpm (8.7 g) at 4°C , followed by another 2 min of centrifugation at 12,000 rpm (13,800 g) at 4°C . All samples were washed three times to yield a total elution volume of 70 μL . The samples were processed according to the manufacturer's recommendations (ThermoFisher Scientific Inc.), in duplicate on a 96-well plate, including a standard line on all runs. The results were analysed using the MAGPIX® (with xPONENT® software) fluorescent detection system. Total biomarker concentration levels (Luminex™ output) were determined in the elution buffer as pg/mL.

A customized, highly sensitive bead-based multiplex immunoassay (Invitrogen ProcartaPlex Human 10-plex Luminex™ panel) was used to simultaneously analyse the following 10 biomarkers: interleukin 1 β (IL-1 β), interleukin 6 (IL-6), tumour necrosis factor alpha (TNF- α), monocyte chemoattractant protein 1 (MCP-1/CCL2), macrophage inflammatory protein 1 alpha (MIP-1 α /CCL3), interferon gamma (IFN- γ), matrix metalloproteinase 8 (MMP-8), soluble receptor activator of nuclear factor kappa-B ligand (sRANKL), osteoprotegerin (OPG) and granulocyte colony-stimulating factor (G-CSF). According to the manufacturer's instructions, the selected biomarkers' lower limits of detection were as follows: 1.62 pg/mL for IL-1 β ; 8.01 pg/mL for IL-6; 9.96 pg/mL for TNF- α ; 3.56 pg/mL for MCP-1; 1.87 pg/mL for MIP-1 α /CCL3; 14.40 pg/mL for IFN- γ ; 35.91 pg/mL for MMP-8; 9.11 pg/mL for OPG; 7.40 pg/mL for sRANKL; and 12.72 pg/mL for G-CSF.

2.4 | Clinical and radiographic examination

Peri-implant and full-mouth clinical parameters were assessed, including bleeding on probing (BoP), suppuration on probing (SoP), probing pocket depth (PPD) and plaque index (PI). Peri-implantitis

implants were assessed at baseline (T0) and 3 months (T3) after therapy. Healthy implants were only assessed at baseline (T0). All examinations were undertaken by the same researcher (DFMH).

Peri-apical radiographs were taken (Planmeca Intra X-ray unit; Planmeca) using a paralleling technique and an individualized X-ray holder (Meijndert et al., 2004). Peri-implant bone loss was assessed at the mesial and distal implant site using DICOM software (DicomWorks 1.5, UMG) by two examiners (DFMH and HJAM) showing an almost perfect observer agreement (Viera & Garrett, 2005). Within-group (peri-implantitis before and after treatment) bone loss differences were examined.

2.5 | Sample size calculation and statistical analysis

The findings of priorly performed recoverability experiments were used to perform a sample size calculation. A group size of 18 implants per group was determined with an average effect size (Cohen's *D*) of 0.9 and a power of 80%. To correct for a possible 10% drop-out (of implants), a total of 20 implants per group were required as sufficient amount to reach a reliable statistical significant difference using a significant (α) level of 0.05. Data analysis was performed using IBM SPSS Statistics (Version 23.0 for Windows; IBM Corp) and GraphPad software (GraphPad Prism version 7.02 for Windows). The outcomes were tested for normality using the Shapiro–Wilk test. A chi-square and Fisher's exact test were used to analyse the categorical baseline characteristics between the healthy control subjects and peri-implantitis patients. A Mann–Whitney *U*-test was used to evaluate group differences between healthy and diseased implants. A Wilcoxon signed-rank test was applied to analyse within-group differences (untreated vs. treated peri-implantitis implants). A *p* value of <.05 was considered to be statistically significant for all the parameters.

3 | RESULTS

In this study, a total of 60 samples (healthy implants at baseline [*n* = 20], peri-implantitis implants at baseline [*n* = 20] and 3 months after treatment [*n* = 20]) were collected for further evaluation. The mean age in the peri-implantitis group [10 males, nine females] and control group [12 males, five females] was 56.5 ($\pm$ 11.5) and 63.9 ($\pm$ 17.6) years, respectively (Table 2). No significant differences in the patient and implant characteristics were found between healthy and diseased implants at baseline. However, a greater variety of implant brands was seen in the peri-implantitis group (see Table 2).

Luminex™ concentration biomarker levels (in pg/ml per 30 s and pg per 30-s) as well as modified concentration levels [in pg/ μ l] are presented using the equation described by Wassall and Preshaw (2016) [(Luminex™ $\times$ 0.2)/PICF volume] to show that correction of biomarker concentration levels for low crevicular fluid volumes creates artificial elevated biomarker levels and therefore a potential source of error for analysis (see Tables 2a and 2b). Especially in healthy implants, correction shows unreliable significantly different outcomes (see Tables 3a

TABLE 2 Characteristics of the healthy implants in the control group and the peri-implantitis implants in the test group

	Healthy	Peri-implantitis
Total implants/total patients	20/17	20/19
Mean age ($\pm$ SD)	63.9 ($\pm$ 17.6)	56.5 ($\pm$ 11.5)
Sex [M/F] (<i>n</i>)	12/5	10/9
Smokers	3 (17%)	5 (26%)
Implants brand, implant surface; <i>n</i> implants		
Nobel		
Porous anodized surface, TiUnite®	10 (50%)	6 (30%)
Straumann		
Sandblasted large grit acid-etched, SLActive®	10 (50%)	8 (40%)
Other	0	6 (30%)

and 3b; the modConc column). Therefore, Luminex™ concentration outcomes in pg/ml per 30 s (see Figure 1) were used for the assessment of biomarker levels in this study. At last, quantitative analysis of PICF showed a significant higher amount of PICF in diseased implants compared to healthy implants at baseline (Table 4).

Regarding the clinical parameters, no significant change in mean peri-implant BoP, PPD and PI was seen in peri-implantitis patients after treatment (Table 4). However, a significant reduction in mean periodontal full-mouth plaque scores (%) was observed (17.3 ($\pm$ 22.0) vs. 6.6 ($\pm$ 9.9) (*p* = .021)).

3.1 | Biomarker levels in healthy versus diseased

Significant higher median levels of classical pro-inflammatory enzyme IL-1 β (390.5 [87.0;555.5 pg/ml per 30 s] vs. 783.5 [414.0;2607.3 pg/ml per 30 s]) and extracellular matrix (ECM) degradation enzyme MMP-8 (20,590.2 [13,512.4;26,929.4] pg/ml per 30 s vs. 34,829.5 [24,145.0;41,791.5]) pg/ml per 30 s (*p* = .007; *p* < .001, respectively) were found in the PICF of peri-implantitis implants compared to healthy implants (Table 3a). Pro-inflammatory levels of TNF- α and IL-6, anti-inflammatory levels of G-CSF, chemokine levels of MIP-1 α /CCL3 and MCP-1 and bone remodelling levels of OPG showed comparable amounts in the PICF of healthy and diseased implants (*p* = .402, *p* = .680, *p* = .109, *p* = .829, respectively). Levels of sRANKL and INF- γ were under the limit of detection (levels under 7.40 pg/ml for sRANKL and under 14.40 pg/ml for INF- γ).

3.2 | Biomarker levels in diseased implants before and after non-surgical therapy

Biomarker levels of untreated and treated peri-implantitis implants are presented in Table 3b. The majority of biomarkers did not change

TABLE 3A Median (IQR) biomarker levels in the healthy implants (HI) and diseased implants at baseline (PI TO)

Biomarker (median; [IQR])	Implant status	Lumines-30 s (pg/ml) HI		Lumines-30 s (pg) HI vs PI TO		modConc [pg/μl] HI vs PI TO	
		Luminex-30 s (pg/ml)	p value	Luminex-30 s (pg)	p value	modConc [pg/μl]	p value
IL-1β	HI	390.5 [87.0;555.5]	0.007	29.29 [6.5;41.7]	0.007	45.1 [26.3;121.2]	0.267
	PI TO	783.5 [414.0;2607.3]		58.8 [31.1;195.5]		57.9 [38.0;158.9]	
IL-6	HI	20.3 [10.3;48.9]	0.402	1.54 [0.8;3.7]	0.394	2.2 [1.1;3.0]	0.002
	PI TO	30.6 [10.6;67.4]		2.3 [0.8;5.1]		1.7 [0.8;1.3]	
TNF-α	HI	11.3 [7.8;16.6]	0.133	0.86 [0.6;1.2]	0.162	2.15 [1.1;2.96]	0.002
	PI TO	13.0 [10.5;20.4]		1.0 [0.8;1.5]		1.1 [0.8;1.33]	
MCP-1	HI	48.13 [26.9;72.9]	0.136	3.64 [2.0;5.5]	0.133	7.4 [1.9;12.0]	0.136
	PI TO	58.2 [40.3;92.8]		4.4 [3.0;7.0]		5.2 [2.8;6.6]	
MIP-1α	HI	15.63 [8.8;31.8]	0.109	1.2 [0.7;2.5]	0.081	3.0 [1.8;5.2]	0.001
	PI TO	10.8 [7.2;17.9]		0.8 [0.5;1.4]		0.8 [0.5;1.5]	
MMP-8	HI	20590.2 [13512.4;26929.4]	0.001	1544.25 [1013.4;2019.7]	0.001	3224.6 [1773.3;5627.2]	0.094
	PI TO	34829.5 [24145.0;41791.5]		2612.2 [1810.8;3134.4]		1974.7 [1292.1;3895.6]	
OPG	HI	34.3 [19.3;53.0]	0.829	2.55 [1.4;4.0]	0.839	5.5 [3.3;9.2]	0.001
	PI TO	33.9 [20.3;66.2]		2.5 [1.6;5.0]		2.2 [1.3;4.1]	
G-CSF	HI	0.0 [0.0;16.7]	0.680	0.0 [0.0;1.2]	0.667	0 [0.0;1.7]	0.989
	PI TO	0.0 [0.0;24.0]		0.0 [0.0;1.8]		0.0 [0.0;1.33]	

Note: Outcomes presented in absolute Luminex™ concentration per 30-s, in pg/ml (picogram per milliliter), Luminex™ concentration in pg (picogram) per 30-s (corrected for 75 μl (microliter) elution) and as modified concentration [in pg/μl]; corrected for 75 μl elution and PICFvolume.

Mann-Whitney U test significant outcome $p < 0.05$.

Abbreviation: IQR, interquartile range.

TABLE 3B Median (IQR) biomarker levels in the peri-implantitis group at baseline (PI T0) and 3 months after treatment (PI T3)

Biomarker (median; [IQR])	Implant status	Luminex-30 s (pg/ml) PI T0 vs PI T3		Luminex-30 s (pg) PI T0 vs PI T3		Lum-30 s (pg) PI T0 vs PI T3		modConc [pg/μl] PI T0 vs PI T3	
		Luminex-30 s (pg/ml)	p value	Luminex-30 s (pg)	p value	Lum-30 s (pg)	p value	modConc [pg/μl]	p value
IL-1β	PI T0	783.5 [414.0;2607.3]	0.156	58.8 [31.1;195.5]	0.156	57.9 [38.0;158.9]	0.145		
	PI T3	738.5 [358.3;1516.8]		55.4 [26.9;113.8]		47.1 [21.5;123.4]			
IL-6	PI T0	30.6 [10.6;67.4]	0.247	2.3 [0.8;5.1]	0.227	1.7 [0.8;1.3]	0.003		
	PI T3	38.5 [11.3;76.4]		2.9 [0.9;5.7]		2.1 [1.1;6.2]			
TNF-α	PI T0	13.0 [10.5;20.4]	0.247	1.0 [0.8;1.5]	0.219	1.1 [0.8;1.3]	1.000		
	PI T3	11.5 [9.0;16.8]		0.9 [0.7;1.3]		0.9 [0.7;1.4]			
MCP-1	PI T0	58.2 [40.3;92.8]	0.526	4.4 [3.0;7.0]	0.514	5.2 [2.8;6.6]	0.709		
	PI T3	57.6 [40.0;120.3]		4.31 [3.2;9.0]		4.1 [2.4;6.6]			
MIP-1α	PI T0	10.8 [7.2;17.9]	0.001	0.8 [0.5;1.4]	0.001	0.8 [0.5;1.5]	0.012		
	PI T3	14.9 [9.4;30.0]		1.13 [0.7;2.2]		0.9 [0.8;2.7]			
MMP-8	PI T0	34829.5 [24145.0;41791.5]	0.167	2612.2 [1810.8;3134.4]	0.167	1974.7 [1292.1;3895.6]	0.028		
	PI T3	36775.0 [23351.5;65973.3]		2758.1 [1751.4;4947.9]		3288.0 [1582.9;5359.8]			
OPG	PI T0	33.9 [20.3;66.2]	0.526	2.5 [1.6;5.0]	0.588	2.2 [1.3;4.1]	0.411		
	PI T3	32.4 [25.0;59.0]		2.4 [1.9;4.4]		2.89 [1.6;4.2]			
G-CSF	PI T0	0.0 [0.0;24.0]	<0.001	0.0 [0.0;1.8]	<0.001	0.0 [0.0;1.3]	<0.001		
	PI T3	32.3 [26.8;41.6]		2.4 [2.0;3.1]		2.39 [1.8;3.6]			

Note: Outcomes presented in absolute Luminex™ concentration per 30-s in pg/ml (picogram per milliliter), Luminex™ concentration in pg (picogram) per 30-s (corrected for 75 μl (microliter) elution) and as modified concentration [in pg/μl]; corrected for 75 μl elution and P1CF volume.

Wilcoxon signed-rank test significant outcome $p < 0.05$.

Abbreviation: IQR, interquartile range.

FIGURE 1 Indicating changes in biomarker levels per 30-s in pg/ml of IL-1 β , IL6, TNF- α , MCP-1, MIP-1 α /CCL3, MMP-8, OPG and G-CSF between healthy control implants and diseased implants before (PI T0) and after (PI T3) non-surgical peri-implantitis therapy. Significant differences between groups are represented as * (p value $\leq .05$)

at 3 months after therapy; levels of IL-1 β and MMP-8 remained high. Moreover, a significant increase in median levels of chemokine MIP-1 α /CCL3 (10.8 [7.2;17.9] pg/ml per 30 s vs. 14.9 [9.4;30.0] pg/ml per 30 s, $p < .001$) and anti-inflammatory growth factor G-CSF (0.0 [0.0;24.0] pg/ml per 30 s vs. 32.3 [26.8;41.6] pg/ml per 30 s, $p < .001$) was seen at 3 months after treatment.

IL-1 β and MMP-8 compared to healthy implants whereas no difference in levels of IL-6, TNF- α , MIP1 α /CCL3, MCP-1, OPG and G-CSF was found between both groups. Levels of sRANKL and INF- γ appeared to be under using the customized Luminex™ panel in this study. The effect of therapy on these biomarkers, as well as on peri-implant clinical and radiographical outcomes, appeared low.

4 | DISCUSSION

In this study, 10 host-derived biomarkers were assessed in PICF of healthy implants and compared with biomarkers in PICF of implants with peri-implantitis using a customized Luminex™ multiplex panel. Additionally, the effect of non-surgical peri-implantitis therapy on the 10 host-derived biomarkers was evaluated. Outcomes showed that implants with peri-implantitis had significantly higher levels of

4.1 | Healthy versus diseased biomarker levels in PICF

Classical pro-inflammatory cytokines (IL-1 β , TNF- α and IL-6), alone or in combination, belong to the most frequent investigated immunological markers in relation to peri-implant disease. Recent systematic reviews and meta-analyses have indicated moderate evidence in the literature to support that pro-inflammatory cytokines could

TABLE 4 Mean (SD) clinical peri-implant parameters in the healthy implant group (HI), peri-implantitis group at baseline (PI T0) and peri-implantitis group 3 months after non-surgical treatment (PI T3)

Clinical parameters [mean $\pm$ SD]	Healthy	PI T0	p value (healthy vs. PI T0)	PI T3	p value (PI T0 vs. PI T3)
PPD (mm)	1.9 ($\pm$ 0.6)	5.0 ($\pm$ 1.1)	<.001	4.7 ($\pm$ 1.3)	.140
BoP (%)	3.4 ($\pm$ 7.0)	58.4 ($\pm$ 27.8)	<.001	47.5 ($\pm$ 32.5)	.108
SoP (%)	0.0 ($\pm$ 0.0)	19.2 ($\pm$ 23.7)	<.001	20.8 ($\pm$ 28.0)	.944
PI (%)	0.0 ($\pm$ 0.0)	15.0 ($\pm$ 23.4)	.002	8.4 ($\pm$ 16.7)	.256
Full mouth PPD (mm)	NA	1.48 ($\pm$ 1.01)	NA	1.42 ($\pm$ 1.08)	.844
Full mouth BoP (%)	NA	9.7 (1 $\pm$ 4.4)	NA	7.2 ($\pm$ 8.2)	.875
Full mouth SoP (%)	NA	0.0 ($\pm$ 0.0)	NA	0.0 ($\pm$ 0.0)	1.000
Full mouth PI (%)	NA	17.3 ($\pm$ 22.0)	NA	6.6 ($\pm$ 9.9)	.021
MBL (mm)	0.56 ($\pm$ 0.5)	4.17 ($\pm$ 1.75)	<.001	4.24 ($\pm$ 1.84)	.737
Mean PICF volume (μ l)	0.14 ($\pm$ 0.11)	0.42 ($\pm$ 0.25)	<.001	0.39 $\pm$ (0.28)	.588
Mean Periotron value	52.93 ($\pm$ 24.05)	101.3 ($\pm$ 34.88)	<.001	96.2 ($\pm$ 33.3)	.467

Abbreviations: BoP, bleeding on probing; MBL, marginal bone loss; NA, not applicable; PI, Plaque index; PICF, peri-implant crevicular fluid; PPD, probing pocket depth; SoP, suppuration on probing.

differentiate between peri-implant health and peri-implant disease, especially regarding levels of IL-1 β and TNF- α (Faot et al., 2015; Duarte et al., 2016; Ghassib et al., 2019). This study seems to be in accordance with this finding for levels of IL-1 β , underlining the potential adjunctive role for this marker in the diagnosis of peri-implantitis. In contrast to IL-1 β , we were not able to find a difference between health and disease for levels of TNF- α and IL-6 (Fonseca et al. 2014). Although it is hypothesized in the literature that TNF- α or IL-6, next to IL-1 β , are potential diagnostic markers, a recent meta-analysis by Ghassib et al. (2019) has shown that the literature on these markers is still scarce and with a high level of heterogeneity. Especially for levels for IL-6, limited evidence has been found to discriminate between peri-implantitis and healthy implants. In accordance with our study, two previous studies did not find a difference between implants with peri-implantitis and healthy implants regarding levels of IL-6 (Melo et al., 2012; Severino et al., 2016). Therefore, whether IL-6 and TNF- α exert the same diagnostic potential as IL-1 β remains inconclusive.

In addition to the classical pro-inflammatory markers, the extracellular matrix degradation enzyme major matrix metalloproteinase 8 (i.e., MMP-8) is another frequently evaluated marker in PICF (Kivelä-Rjamäki et al. 2003; Xu et al. 2008; Basegmez et al. 2012; Thierbach et al., 2016;). Comparable to what is found in patients with periodontal disease, there seems moderate evidence in the literature showing upregulated levels of MMP-8 in PICF of implants with peri-implant disease (Salvi et al., 2012; Alassy et al., 2019; Ghassib et al., 2019). However, a true comparison between peri-implant health and peri-implantitis for this marker was only sparsely studied (Arakawa et al., 2012; Janska et al., 2016; Wang et al., 2016). In line with the studies by Janska et al. (2016) and Arakawa et al. (2012), our study is one of the few studies who reported elevated levels for peri-implantitis implants compared to healthy implants. Therefore, the present study seems to enhance the moderate evidence of upregulated levels of MMP-8 in PICF of implants with peri-implantitis. It therefore might

be hypothesized that MMP-8 may serve a promising role, in addition to IL-1 β , to differentiate between peri-implant health and disease (Thierbach et al., 2016; Al-Majid et al., 2018; Alassy et al., 2019).

Alongside with pro-inflammatory markers and MMP-8, MIP-1 α /CCL3 is a protein with chemotactic (stimulation of cell migration) properties which plays an important role in inflammation and increased activation of bone resorption cells (osteoclasts). In a study by Petković et al. (2010), increased levels of MIP-1 α /CCL3 in PICF of diseased implants were found when compared to healthy implants, whereas no difference between healthy and diseased sites was found for levels of MIP-1 α /CCL3 in a more recent study by Bhavsar et al. (2019). In addition to this latter study, our findings also indicate that this marker does not seem to differentiate between peri-implant health and disease. To date, it therefore does not seem likely to expect a diagnostic potential role for MIP-1 α /CCL3 in peri-implant disease; however, more studies evaluating this marker are needed to confirm this finding.

Another important chemotactic protein is MCP-1. This protein is considered the first discovered human chemokine and is a well-known chemoattractant for monocytes (Rollins, 1996; Deshmane et al., 2009; Mulholland et al., 2019). To the best of our knowledge, MCP-1 has not been previously evaluated in PICF of peri-implantitis patients. So far, we have only found two in vitro studies on MCP-1 in the current literature reporting inconsistent outcomes (Bordin et al., 2009; Irshad et al., 2013). Our study seems the first to report on this marker in a clinical setting with no difference in the concentration levels of MCP-1 in the PICF between healthy and diseased implants. However, there was a trend towards increased levels in diseased implants ($p = .136$). To what extent this marker plays a role in peri-implant disease remains to be found.

As part of the RANK/sRANKL/OPG system, the markers OPG and sRANKL play a pivotal role in bone biology (i.e., regulation of osteoblast and osteoclast activities). OPG protects the bone from excessive resorption by binding to sRANKL. Hereby, sRANKL is

prevented from binding to RANK (a receptor bound to osteoclasts) which in turn prevents activation of osteoclast cells (Rakic et al., 2013). It therefore seems likely that both markers are involved in alveolar bone destruction in peri-implantitis (Arikan et al., 2011). However, to date, conflicting results regarding both markers have been reported in the literature. In a study of Monov et al. (2006), subjects with increased peri-implant bone loss and clinical signs of inflammation did not show any increased levels of sRANKL. Additionally, significantly lower sRANKL concentrations, OPG total amounts and OPG concentrations in peri-implantitis implants were reported by Arikan et al. (2011) when compared to healthy implants. Our study seems in line with the results on OPG reporting no difference in levels between both groups. On the other hand, significantly higher levels were found in peri-implantitis sites by Rakic et al. (2013), but without a difference in OPG/RANKL ratio. Therefore, although it seems reasonable to believe that both markers are important in peri-implant sites with bone loss, as of yet, the literature does not seem to support this thought.

At last, a biomarker which previously not seemed to be evaluated in peri-implant fluid is G-CSF (Panopoulos & Watowich, 2008). This cytokine is known as a type of growth factor that stimulates bone marrow to produce white blood cells (e.g., neutrophil granulocytes). Although relatively few of the samples in this study (in both groups) had G-CSF levels above the level of detection, we believe with our study to be the first to show no difference in G-CSF between healthy and diseased implants. Considering that a recent study, which focused on a close relative of G-CSF (i.e., macrophage-CSF) in the PICF of peri-implantitis patients, found higher levels of macrophage-CSF when compared to peri-implant mucositis patients (Lira-Junior et al., 2020), interpretation of our outcomes should be done cautiously. However, one might suggest that colony stimulating factors might play a role in the pathogenesis of peri-implant disease.

Altogether, the research effort spent thus far on markers around implants with and without signs of inflammation has identified one potential biomarker (IL-1 β) which could reliably be used in PICF diagnostics, to flank clinical and radiographical examination in differentiating between both groups. In addition, our study shows a promising role for the association between the expression of MMP-8 and the pathophysiology of peri-implantitis. However, this needs to be rigorously confirmed in future studies together with data on other potential markers (e.g., TNF- α , MCP-1 and G-CSF).

4.2 | Biomarker levels in PICF before and after therapy

As far as we know, only two studies assessed the influence of non-surgical peri-implantitis therapy on markers in the PICF of peri-implantitis sites (Basseti et al., 2014; Renvert et al., 2016). Our study seems in accordance with study by Renvert et al. (2016) who neither found any differences in the majority of the studied cytokines (6 out of 9). A clinically stable treatment outcome was found in their study

in only 22% of the cases at 6 months after therapy, using a single intervention with either an air-abrasive device or Er:YAG laser. Our study noticed a similar limited clinical effect, with persisting signs of inflammation ($\pm 50\%$ of patients showing BoP and unchanged levels of SoP) 3 months after non-surgical peri-implantitis treatment and unchanged levels for the majority of biomarkers. In contrast, Basseti et al. (2014) found lower levels of IL-1 β and MMP-8 at 3 months after therapy. However, additional delivery of local minocycline microspheres to the mechanical debridement with titanium curettes and glycine powder air polishing was applied in their study. Hence, this could have led to a suppressed immune response with subsequently lower biomarker levels after therapy. Considering that the non-surgical therapy seemed unsuccessful, it might be speculated that clinical and radiographical parameters after non-surgical therapy are immunologically underlined. However, with our study, it seems not possible to truly support or deny the potential use of a change in biomarker as a monitor to assess the effectiveness of a peri-implantitis treatment with PICF analysis.

4.3 | Limitations of the study and future recommendations

Interpreting the findings of this study, the following limitations should be kept in mind.

Due to the limited sample size, no sub-analyses could be performed for several possible confounding factors (e.g., smoking, age and sex). Therefore, interpretation of our results with previous studies should be done with caution. Although an association between elevated inflammatory biomarkers levels (such as interleukin-1 β , interleukin-6, interleukin-10 and tumour necrosis factor- α) in the PICF and smoking is described (Tatli et al., 2013; Ata-Ali et al., 2016), no differences in smoking prevalence between the healthy control and peri-implantitis subjects in this study were seen. Therefore, interference of smoking with our analysis was not assumed.

A minor drawback of the study might be the difference in therapies applied. We used the Airflow Master Piezon[®] to either apply air polishing or ultrasonic therapy. Considering the limited effect of non-surgical peri-implantitis interventions in general, as well as the limited effect observed in our study, the influence of therapy difference on immunological markers was considered rather low.

At last, for future research groups with interest in sRANKL and INF- γ , recoverability experiments seem recommended when using a ThermoFisher Luminex assay plate, in order to obtain accurate and reliable outcomes.

5 | CONCLUSION

Peri-implant crevicular fluid diagnostics of implants diagnosed with peri-implantitis showed higher levels of IL-1 β and MMP-8 compared to healthy implants. Non-surgical therapy did not seem to influence the inflammatory immune response. Hence, evaluation of

non-surgical peri-implantitis therapy outcome using PICF diagnostics does not seem helpful.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

All authors were involved conceiving the study; D.H. collected and processed the data. D.H. and S.L. analysed the data. D.H. led the writing; Y.d.W., A.J.v.W., G.R., H.M., F.K. and A.V. critically revised the manuscript. All the authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

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

ORCID

Diederik F. M. Hentenaar <https://orcid.org/0000-0002-7904-5548>

[org/0000-0002-7904-5548](https://orcid.org/0000-0002-7904-5548)

Yvonne C. M. De Waal <https://orcid.org/0000-0001-5638-462X>

Henny J. A. Meijer <https://orcid.org/0000-0003-1702-6031>

Gerry M. Raghoobar <https://orcid.org/0000-0003-3578-7141>

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