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# Cytokine and matrix metalloproteinase expression in fibroblasts from peri-implantitis lesions in response to viable *Porphyromonas gingivalis*

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*Background and Objective:* To assess inflammatory reactions of fibroblasts in the pathophysiology of peri-implantitis, we compared the pro-inflammatory and matrix-degrading responses of gingival and granulation tissue fibroblasts from periodontally healthy controls, peri-implantitis, and periodontitis lesions to an *in vitro* challenge with *Porphyromonas gingivalis*.

*Methods:* Fibroblasts from periodontally healthy, peri-implantitis and periodontitis donors were challenged with viable *P. gingivalis*. The inflammatory reactions of fibroblasts were analyzed before and after 6 h *P. gingivalis* challenge, and 2.5 and 18 h after removal of the challenge. Gene expression and induction of pro-inflammatory mediators, and matrix metalloproteinases (MMPs) were assessed by real-time polymerase chain reaction. Protein expression was measured by enzyme-linked immunosorbent assay.

*Results:* Non-challenged fibroblasts from peri-implantitis and periodontitis lesions expressed higher levels of *interleukin (IL)-1β*, *IL-8*, and *monocyte chemotactic protein (MCP)-1* than fibroblasts from periodontally healthy individuals. The *P. gingivalis* challenge induced expression of *IL-1β*, *IL-8*, *IL-6*, *MCP-1*, and *MMP-1* in periodontitis and peri-implantitis fibroblasts, but not in fibroblasts from periodontally healthy individuals. *MMP-8* expression was higher in non-challenged peri-implantitis fibroblasts than in fibroblasts from periodontally healthy individuals. However, the *P. gingivalis* challenge downregulated *MMP-8* gene expression in peri-implantitis fibroblasts. After removal of the *P. gingivalis* challenge, peri-implantitis fibroblasts sustained higher induction of *IL-1β, MCP-1*, and *MMP-1* compared to periodontitis fibroblasts.

*Conclusions:* Fibroblasts from peri-implantitis and periodontitis lesions gave a more pronounced inflammatory response to the *P. gingivalis* challenge than fibroblasts from healthy donors. They may therefore be involved in the development of inflammation in peri-implantitis and periodontitis. Moreover, the

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sustained upregulation of inflammatory mediators and MMP-*1* in peri-implantitis fibroblasts may play a role in the pathogenesis of peri-implantitis.

Peri-implantitis is an inflammatory disease affecting the alveolar bone and mucosal tissues surrounding the dental implant that is characterized by mucositis and loss of osseous integration (1). Peri-implantitis shares important disease characteristics with chronic periodontitis, such as presence of chronic inflammation and bacterial species associated with the initiation and progression of the disease (2,3). Moreover, patients with a history of periodontitis may be more susceptible for developing peri-implantitis (4,5). Despite these similarities, important differences also exist between peri-implantitis and periodontitis. In contrast to the often, localized horizontal bone loss around natural teeth in periodontitis, bone loss around implants shows a circumferential and angular pattern (6). Inflammatory lesions in experimental peri-implantitis have been shown to be more aggressive and extensive compared to lesions around natural teeth (7). Additionally, it has been reported that inflammation around implants does not always resolve with the removal of infection (8).

Although the etiology of peri-implantitis is multifactorial, certain microorganisms are essential for its initiation and progression (2). *Porphyromonas gingivalis* is a gram-negative, anaerobic bacterium that is strongly associated with peri-implantitis (9). It has a variety of ways to interact with host cells (10). Tissue destruction in peri-implantitis in response to bacteria such as *P. gingivalis* is a result of the persistent and ineffective inflammatory immune responses from host cells. Host cells respond to bacterial challenge by releasing certain pro- and anti-inflammatory mediators, which in turn mediate degradation of collagen and extracellular matrix, and bone resorption (11,12).

Despite the important role of bacteria in peri-implantitis, these bacteria can also be found in small numbers in apparently healthy oral cavities (13), which emphasizes the fact that bacteria are not solely responsible for causing the disease. In addition, patients who have already lost an implant are at greater risk for subsequent implant losses (14). Thus, host-related factors are also important in the development of peri-implantitis.

In peri-implantitis, fibroblasts can play an important role in the inflammatory process and in tissue homeostasis and destruction (15,16). First, upon interaction with *P. gingivalis* fibroblasts release inflammatory mediators such as interleukin (IL)-1β, IL-6, IL-8 (17), and monocyte chemotactic protein (MCP)-1 (18), which can enhance tissue damage by increasing tissue perfusion, recruiting immune cells, and amplifying overall inflammation (19). Secondly, fibroblasts challenged with *P. gingivalis* release matrix metalloproteinases (MMPs) (20), which degrade extracellular matrix, but may also cleave – and thereby activate or inactivate – certain pro-inflammatory mediators (21). Furthermore, fibroblasts produce tissue inhibitors of metalloproteinases (TIMPs) that restrict matrix breakdown by MMPs (15,22), and transforming growth factor-beta (TGFβ)-1, which regulates fibrogenesis and vascular homeostasis (23).

Even though specialized immune cells play an important role in inflammation, these cells produce inflammatory mediators only transiently, and they can develop tolerance to certain virulence factors of oral bacteria (24). Fibroblasts on the other hand do not develop such tolerance and may play an important role in sustaining inflammation (25). This is particularly relevant for peri-implantitis lesions because in contrast to the effectiveness of non-surgical treatment for periodontitis (26), peri-implantitis lesions may not respond very well to non-surgical treatment (27).

Fibroblasts isolated from distinct anatomical sites, or fibroblasts isolated from inflamed tissues, can also play distinct roles in health or inflammation (28,29). We have previously found that fibroblasts from different sites in the periodontium differed in their response to viable *P. gingivalis* even within one donor (18). We therefore hypothesized that peri-implant fibroblasts play a distinct role in the pathogenesis of peri-implantitis. As it is not known how peri-implant granulation tissue fibroblasts (PIGFs) interact with bacteria associated with peri-implantitis such as *P. gingivalis*, this study aimed to compare the pro-inflammatory and matrix-degrading responses of fibroblasts from healthy donors, and peri-implantitis and periodontitis lesions to an *in vitro P. gingivalis* challenge.

## Material and methods

### Tissue donors and fibroblast isolation

PIGFs were obtained from seven peri-implantitis patients during flap surgery as part of peri-implantitis treatment. The surgical approach consisted of an incision extending mesially and distally to the implant after the removal of prosthetic suprastructure to facilitate access. A full thickness tissue flap was reflected and the inflamed granulation tissue was carefully removed with a curette. The tissue samples were further processed on the same day. The peri-implantitis patients were free from active periodontitis and had no known history of periodontitis. Gingival fibroblasts from five periodontally healthy controls (HGF) and nine chronic periodontitis patients (PGF) were obtained from teeth that were extracted as part of periodontal treatment (PGF), or as part of treatment for other reasons than periodontitis (HGF), such as trauma or esthetical reasons. To prevent contamination with periodontal ligament/other fibroblasts, only free gingiva that was not firmly attached to the tooth surface, was dissected (18). Periodontally

healthy donors showed no signs of inflammation/periodontitis/alveolar bone loss. Patients with periodontitis displayed deepened pockets, bleeding on probing, and advanced loss of alveolar bone visible on radiographs. None of the donors had systemic diseases or was pregnant. None of the donors was a current smoker. All donors had given written informed consent, and the study was approved by the Medical Ethical Committee of the VU University Medical Center Amsterdam.

Fibroblasts were obtained by dissecting free gingiva remains from extracted teeth. To prevent contamination with periodontal ligament/other fibroblasts, only free gingiva that was not firmly attached to the tooth surface, was dissected (18). Tissue samples were washed twice in Dulbecco's modified Eagle medium (DMEM; 4.5 g/L glucose + L-glutamine + pyruvate; Invitrogen/Gibco, Paisley, UK) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), minced into smaller pieces, and 2% antibiotics/antimycotics (PSF; 100 U/mL of penicillin, 100 μg/mL streptomycin and 250 ng/mL of amphotericin B; Sigma, St Louis, MO, USA) and cultured in DMEM containing 10% FBS and 2% PSF, in a humidified atmosphere with 5% $CO_2$ at 37°C until growth of fibroblasts was seen under the phase contrast microscope (Olympus CK2; Olympus, Tokyo, Japan). Cells were expanded through passages 1–4 and stored in liquid nitrogen at passage 4. Morphologically HGF, PIGF, and PGF had similar appearance under the phase contrast microscope. Furthermore, the fibroblastic nature of HGF, PGF, and PIGF was assessed by evaluating the expression of the *FMOD* (*fibromodulin*) gene, a marker for HGF (30), which was expressed at similar levels in HGF, PGF, and PIGF (data not shown). Experiments were performed with cells from passages 5 to 7.

### Bacterial strain and culture

*Porphyromonas gingivalis* HG91 (also designated as strain 381) was cultured anaerobically (80% $N_2$, 10%$H_2$, 10% $CO_2$) until log-growth phase in brain–heart infusion broth supplemented with hemin (5 mg/L) and menadione (1 mg/L). Purity was checked with gram-staining.

Viable *P. gingivalis* were harvested by centrifugation. Bacterial pellets were washed twice in sterile phosphate-buffered salt solution (PBS; Gibco BRL, Paisley, UK) and resuspended in antibiotic/antimycotic-free DMEM with 10% FBS. The optical density was measured at 690 nm to establish the number of colony forming units (CFUs). A suspension of $2 \times 10^8$ CFU/mL was used to challenge the fibroblasts.

### *Porphyromonas gingivalis* challenge to fibroblasts

Fibroblasts were challenged with viable *P. gingivalis* as reported previously (18). In short, fibroblasts ($10^4$/well) were grown until subconfluence in 24-well plates. Medium was removed and replaced with 0.5 mL of a *P. gingivalis* HG91 suspension of $2 \times 10^8$ CFU/mL in antibiotic-free DMEM with 10% FBS. Only DMEM without PSF, supplemented with 10% FBS was added to control fibroblasts (non-challenged).

HGFs, PGFs, and PIGFs were incubated with *P. gingivalis* for 6 h. After the *P. gingivalis* challenge, fibroblast morphology was checked for abnormalities or cell detachment by phase-contrast microscopy. Subsequently fibroblasts were washed with sterile PBS and lysed in lysis buffer as supplied with RNeasy Mini Kit for RNA extraction (Buffer RLT; Qiagen, Hilden, Germany), supplemented with β-mercaptoethanol. Experiments were performed in quadruplicate.

### Priming of fibroblasts by *Porphyromonas gingivalis* and subsequent removal of challenge

PIGFs from seven peri-implantitis donors and PGFs from seven periodontitis donors were used. Fibroblasts ($10^4$/well) were grown until subconfluence in 24-well plates. Medium was removed and replaced with 0.5 mL of a *P. gingivalis* suspension in antibiotic-free DMEM with 10% FBS and incubated for 6 h as described above. At the end of the 6 h challenge, *P. gingivalis* was removed by washing three times with sterile PBS. Cells were then incubated in DMEM supplemented with 10% FBS and 2% PSF for 30 min to kill the remaining *P. gingivalis*. Susceptibility of *P. gingivalis* to penicillin *in vitro* is known in the literature (31) and in our preliminary *in vitro* experiments (data not shown), this protocol proved to completely kill $2 \times 10^8$ CFU/mL of *P. gingivalis* HG91. After 30 min, cells were washed three times with sterile PBS and the medium was replaced with DMEM without antibiotics. Supernatants and cell lysates were collected at 6 h after the *P. gingivalis* challenge (baseline), and 2.5 and 18 h after removing *P. gingivalis*. At each time point, the cells were washed three times with sterile PBS before lysing them. The non-challenged fibroblasts underwent the same treatments and acted as controls for different time points. All experiments were performed in quadruplicate.

### mRNA expression

Fibroblast RNA was isolated using the Qiagen RNeasy Mini Kit according to the manufacturer's protocol. The RNA concentration was measured using a Nanodrop spectrophotometer (NanoDrop Technologies; Thermo-Fischer Scientific, Wilmington, DE, USA). mRNA was reverse-transcribed to cDNA using the MBI Fermentas RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania), using both the Oligo(dT)18 and the D(N)6 primers according to the manufacturer's protocol.

Real-time polymerase chain reaction (PCR) primers for *IL-1β*, *IL-6*, *IL-8*, *MCP-1*, *TIMP-1*, *MMP-1*, *MMP-2*, and *MMP-8* were used as published previously (18,32). Primers for *TGFβ-1* were designed using PRIMER EXPRESS software, version 2.0 (Applied Biosystems, Foster City, CA, USA). All primers were ordered

from Sigma-Aldrich Co. LLC, (Haverhill, UK). Sequences of the relevant forward and reverse primers for the respective genes are given in Table 1. We used a serial dilution of human reference total RNA (Stratagene, La Jolla, CA, USA) to create a standard curve to check the PCR efficiency. Real-time PCR was performed on Roche LightCycler 480 (F. Hoffmann-La Roche AG, Basel, Switzerland). Reactions were performed with 2 ng of cDNA in a total volume of 11 μL containing the LightCycler® 480 SYBR Green I Master Mix (F. Hoffmann-La Roche AG), consisting of DNA double-strand-specific SYBR Green I dye for product detection and characterization, FastStart Taq DNA Polymerase and 0.91 pM/μL of each primer. After an activation step with the FastStart Taq DNA Polymerase for 5 min at 95°C, 40 cycles were run of a two-step PCR consisting of a denaturation step at 95°C for 10 s, annealing and extension steps of 60°C for 5 s, 72°C for 10 s, and 78°C for 5 s. Subsequently, the PCR products were subjected to melting curve analysis to test if any non-specific PCR products were generated. Samples were normalized for the expression of the housekeeping gene *β2-microglobulin*, which was not affected by the experimental conditions. Relative gene expression was calculated by the method proposed by Livak and Schmittgen (33), by calculating the $\Delta Ct$ ($Ct_{\text{gene of interest}} - Ct_{\text{housekeeping gene}}$), and the expression of different genes was expressed as $2^{-(\Delta Ct)}$. Fold increase in gene expression (induction) was expressed as $2^{-(\Delta\Delta Ct)}$, wherein $\Delta\Delta Ct = \Delta Ct_{\text{challenged}} -$ average $\Delta Ct$-value$_{\text{non-challenged}}$.

*Table 2*. Patient characteristics and clinical parameters of fibroblast donors and target teeth/implants

| | Periodontally healthy controls | Peri-implantitis | Periodontitis |
|---|---|---|---|
| *n* donors | 5 | 7 | 9[a] |
| Gender (male/female) | 1/4 | 6/1 | 3/6 |
| Mean age (±SD) in years | 54.4 (±18.7) | 57.8 (±12.4) | 54.4(±9.2) |
| BOP (*n*) | 1 | 7 * | 5 |
| Mean PD (±SD) | 2.7 (±0.8) | 5.1 (±0.9) ** | 6.6 (±2.8) *** |

BOP, number of target teeth/implants showing bleeding on probing; mean PD, mean probing depth of pocket in millimeters around target teeth/implants.
[a]Fibroblasts from five or seven patients with periodontitis were used in a different experiment. *$p = 0.008$, **$p = 0.005$, ***$p = 0.01$ (significant difference compared to periodontally healthy controls).

### Protein production

Cell culture supernatants from five peri-implantitis and five periodontitis patients challenged with *P. gingivalis* for 6 h were used for determining the protein production of IL-8, MMP-8, and TIMP-1. The protein levels of IL-8 were determined by enzyme-linked immunosorbent assays (ELISA; PeliKine ELISA kits, Sanquin Blood Supply Foundation, Amsterdam, the Netherlands), according to the manufacturer's protocol. The sample quantities were normalized against a serial dilution of a known concentration of the relevant protein provided by the manufacturers. Sensitivity of the IL-8 assay was 1–3 pg/mL. MMP-8 and TIMP-1 concentrations in 10 × diluted culture supernatants were measured by ELISA (Biotrak MMP-8 human ELISA system; Amersham Pharmacia Biotech, Buckinghamshire, UK; Quantikine human TIMP-1 immunoassay; R&D Systems, Minneapolis, MN, USA) according to the manufacturers' protocols. The average lower limits of detection were 0.032 and 0.08 ng/mL, respectively. All ELISA experiments were performed in duplicate.

### Statistical analysis

Comparisons between the total mRNA expression, induction of gene expression and protein production between groups were tested with Student's *t* test when data were normally distributed. Wilcoxon matched pairs and Mann–Whitney tests were used to analyze the non-parametric data. If variances in groups were not equal, Welch's correction for unequal variances was applied. Differences were regarded statistically significant at a value of $p < 0.05$. Tests were performed with GRAPHPAD PRISM

*Table 1*. Real-time polymerase chain reaction primer sequences

| | Primer sequences | |
|---|---|---|
| Gene | 5′-3′ Fw | 5′-3′ Rev |
| *IL-1β* | CTTTGAAGCTGATGGCCCTAAA | AGTGGTGGTCGGAGATTCGT |
| *IL-6* | GGCACTGGCAGAAAACAACC | GGCAAGTCTCCTCATTGAATCC |
| *IL-8* | GGCAGCCTTCCTGATTTCTG | CTGACATCTAAGTTCTTTAGCACTCCTT |
| *MCP-1* | CAGCCAGATGCAATCAATGC | TGCTGCTGGTGATTCTTCTATAGCT |
| *TGFβ-1* | CTCGGAGCTCTGATGTGTTGAA | CACCCGCGTGCTAATGGT |
| *TIMP-1* | CCTTCTGCAATTCCGACCTCGTC | CGGGCAGGATTCAGGCTATCTGG |
| *MMP-1* | GAAGTTGATGAAGCAGCCCAGATGT | CAGTTGTGGCCAGAAAACAGAAGTGAAA |
| *MMP-2* | ATCCGTGGTGAGATCTTCTTCTT | AGCCAGGATCCATTTTCTTCTT |
| *MMP-8* | GCTGCTTATGAAGATTTTGACAGAG | ACAGCCACATTTGATTTTGCTTCAG |
| *β2-microglobulin* | AAGATTCAGGTTTACTCACGTC | TGATGCTGCTTACATGTCTCG |

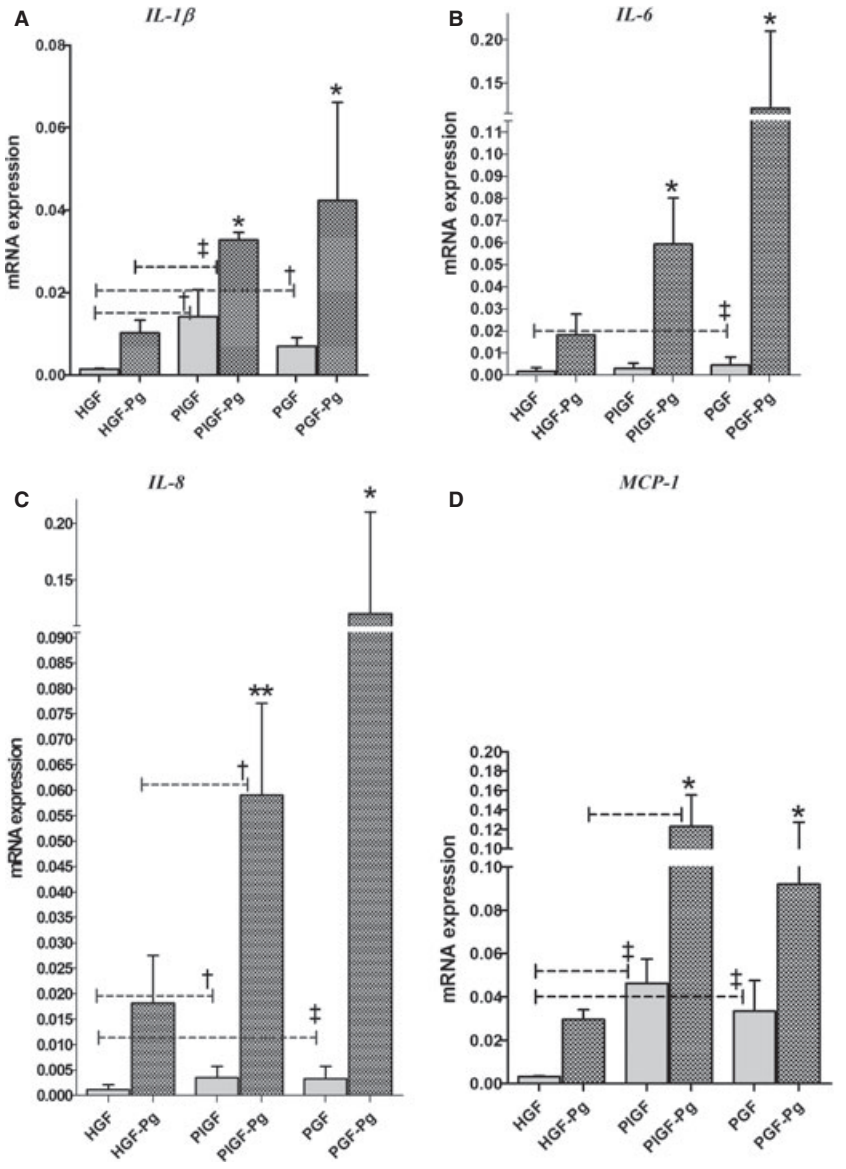


*Fig. 1.* mRNA expression levels of pro-inflammatory mediators before and after a 6 h *Pg* challenge. mRNA expression levels of *IL-1β* (A), *IL-6* (B), *IL-8* (C), and *MCP-1* (D) normalized for the expression of housekeeping gene *β2-microglobulin* (as calculated by the $\Delta Ct$ method) in HGF ($n = 5$), PIGF ($n = 7$) and PGF ($n = 7$) before and after a 6 h challenge with viable *Pg*. Bars represent the average ± SEM of mRNA expression levels from the non-challenged and challenged fibroblasts from experiments performed in quadruplicate. $*p < 0.05$, $**p < 0.01$ represent significant difference between the non-challenged and challenged cells of the same group. $\dagger p < 0.05$, $\ddagger p < 0.01$ represent significant differences between different groups. IL, interleukin; MCP, monocyte chemotactic protein; HGF, fibroblasts from periodontally healthy controls; *Pg*, *P. gingivalis*; PGF, fibroblasts from chronic periodontitis patients; PIGF, peri-implant granulation tissue fibroblasts.

software (version 5, by MacKiev Software™, San Diego, CA, USA).

## Results

General and clinical characteristics of the donors included in this study are shown in Table 2. No significant differences were found between the ages of different groups of donors. Bleeding on probing (BOP) was more frequently detected around target implants in peri-implantitis patients compared to periodontally healthy controls ($p = 0.008$). Compared to target teeth in periodontally healthy controls, higher values for probing depths were found around target implants ($p = 0.005$) in peri-implantitis and teeth ($p = 0.01$) in patients with periodontitis.

### mRNA expression of interleukin (IL)-1β, IL-6, IL-8 and monocyte chemotactic protein-1 before and after the *Porphyromonas gingivalis* challenge

mRNA expression of *IL-1β*, *IL-6*, *IL-8*, and *MCP-1* was significantly upregulated in response to the *P. gingivalis* challenge in PIGFs ($p = 0.031$, 0.015, 0.0078, 0.023 respectively) and PGFs ($p = 0.016$, 0.015, 0.015, 0.03 respectively), but not in HGFs (Fig. 1A–D). mRNA expression levels of *IL-1β*, *IL-8*, and *MCP-1* in PIGFs were higher compared to HGFs both in the non-challenged ($p = 0.030$, 0.045, 0.0016 respectively) as well as challenged cells ($p = 0.0025$, 0.045, 0.029 respectively; Fig. 1A, C, D). mRNA expression levels of *IL1-β*, *IL-6*, *IL-8*, and *MCP-1* were higher ($p = 0.048$, 0.0025, 0.002, 0.002 respectively) in PGFs than HGFs in the non-challenged but not in the challenged cells (Fig. 1A–D). Differences in mRNA expression of *IL-1β*, *IL-6*, *IL-8*, and *MCP-1* between PIGFs and PGFs did not reach statistical significance in the non-challenged as well as challenged cells.

### Differential expression of matrix metalloproteinasesin response to the *Porphyromonas gingivalis* challenge

Compared to the non-challenged cells, the *P. gingivalis* challenge upregulated expression of *MMP-1* in PIGFs ($p = 0.039$) and PGFs ($p = 0.047$), but not in HGFs (Fig. 2A). In the non-challenged cells, mRNA expression levels of *MMP-8* in PIGFs were higher than in HGFs ($p = 0.029$, Fig. 2B). Interestingly, mRNA expression of *MMP-8* was downregulated in the challenged PIGFs ($p = 0.039$) compared to the non-challenged PIGFs (Fig. 2B). A similar trend was observed in the mRNA expression of

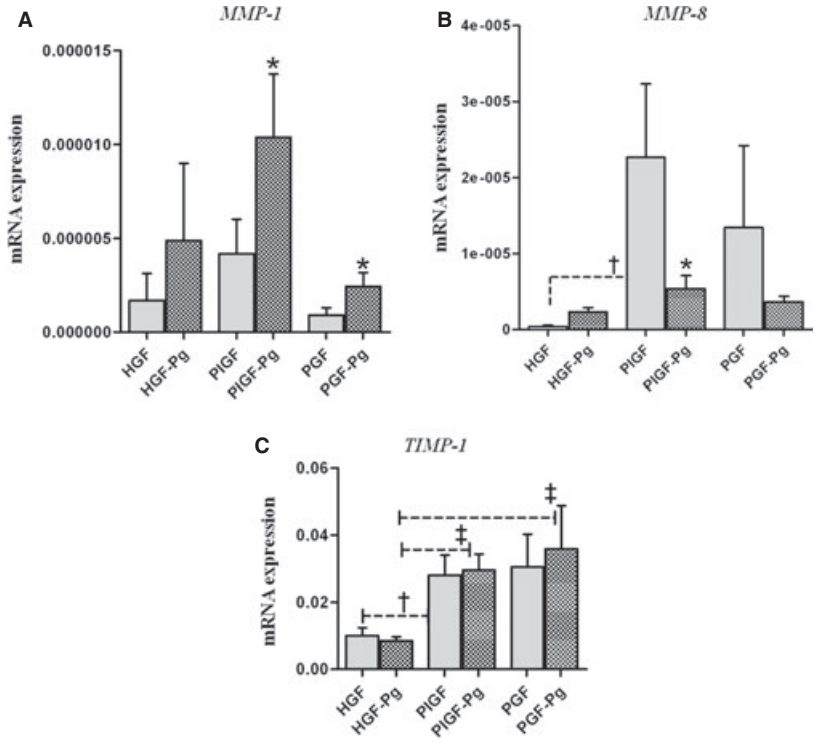


*Fig. 2.* mRNA expression levels of *MMP-1*, *MMP-8*, and *TIMP-1* before and after a 6 h *Pg* challenge. mRNA expression levels of *MMP-1* (A), *MMP-8* (B), and *TIMP-1* (C) normalized for the expression of housekeeping gene *β2-microglobulin* (as calculated by the $\Delta Ct$ method) in HGF ($n = 5$), PIGF ($n = 7$), and PGF ($n = 7$) before and after a 6 h challenge with viable *Pg*. Bars represent the average ± SEM of mRNA expression levels from the non-challenged and challenged fibroblasts from experiments performed in quadruplicate. $*p < 0.05$, $**p < 0.01$ represent significant difference between the non-challenged and challenged cells of the same group. $\dagger p < 0.05$, $\ddagger p < 0.01$ represent significant differences between different groups. HGF, fibroblasts from periodontally healthy controls; MMP, matrix metalloproteinase; *Pg*, *P. gingivalis*; PGF, fibroblasts from chronic periodontitis patients; PIGF, peri-implant granulation tissue fibroblasts; TIMP, tissue inhibitors of metalloproteinases.

*MMP-8* in PGFs, but the decrease in mRNA expression was not significant. mRNA expression of *TIMP-1* was not significantly changed in response to *P. gingivalis* challenge in any group of fibroblasts (Fig. 2C), although compared to HGFs the expression of *TIMP-1* was higher in PIGFs in the non-challenged ($p = 0.048$) as well as the challenged cells ($p = 0.0051$). *TIMP-1* mRNA expression was also significantly higher in PGFs challenged with *P. gingivalis* compared to the challenged HGFs ($p = 0.0025$, Fig. 2C).

No significant differences were observed in mRNA expression levels of *TGFβ-1* and *MMP-2* either between the non-challenged vs. the challenged fibroblasts of the same group or among different groups of fibroblasts (data not shown).

### Protein production levels of interleukin-8, matrix metalloproteinase-8 and tissue inhibitors of metalloproteinases-1 correspond with mRNA expression

To confirm whether the statistically significant changes observed in mRNA expression levels also lead to similar changes in protein production, we measured the protein production of IL-8, MMP-8, and TIMP-1 by ELISA. As the only difference between challenged and non-challenged HGFs was found for MMP-8 mRNA expression, protein productions were only determined for PIGFs and PGFs. Moreover, *P. gingivalis* proteases have been reported to cleave inflammatory mediators such as IL-1β and IL-6 (18,34); therefore, we chose to measure protein production levels of IL-8, MMP-8, and TIMP-1. Consistent with the increased mRNA expression, IL-8 protein production was also significantly increased in PIGFs ($p = 0.002$) and PGFs ($p = 0.031$) challenged with *P. gingivalis*, compared to the non-challenged cells (Fig. 3A). Protein production of MMP-8 and TIMP-1 from PIGFs and PGFs before and after the *P. gingivalis* challenge showed similar trends to the mRNA expression although the differences were not significant (Fig. 3B and C). Protein production of TIMP-1 by PIGFs was significantly higher ($p = 0.008$) compared to PGFs in the non-challenged cells (Fig. 3C).

### Gene induction of interleukin-1β, monocyte chemotactic protein-1, and matrix metalloproteinase-1 differ between fibroblasts from peri-implant granulation tissue and patients with periodontitis after removal of the *Porphyromonas gingivalis* challenge

As there was no significant upregulation of pro-inflammatory mediators by HGFs in response to *P. gingivalis*, only PIGFs and PGFs were used in further experiments. To assess differences in the dynamics of pro-inflammatory and matrix degrading responses between PIGFs and PGFs, we removed *P. gingivalis* from fibroblast cultures after the 6 h challenge and measured mRNA expression at 2.5 and 18 h after the removal. Induction of mRNA expression for *IL-1β*, *IL-6*, *IL-8*, *MCP-1*, and *MMP-1* in PIGFs and PGFs were compared. Significantly higher gene induction of *IL-1β*, *MCP-1*, and *MMP-1* was observed in PIGFs compared to PGFs after removal of *P. gingivalis*. No significant differences were found in the gene induction of any other mediators studied (data not shown). Gene induction of *IL-1β* was higher in PIGFs

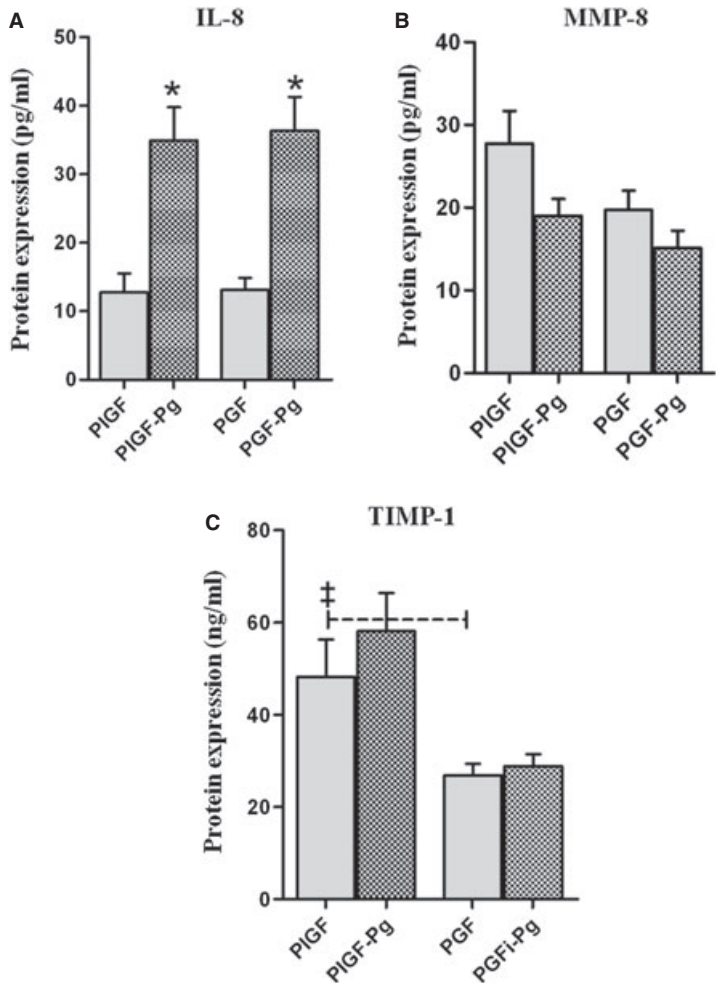


*Fig. 3.* Protein expression of IL-8, MMP-8, and TIMP-1 at before and after a 6 h *Pg* challenge. Protein expression levels of IL-8 (pg/mL) (A), MMP-8 (ng/mL) (B) and TIMP-1 (ng/mL) (C) in cell culture supernatant from the non-challenged and challenged fibroblasts (enzyme-linked immunosorbent assay) from peri-implantitis patients (PIGF, $n = 5$) and periodontitis patients (PGF, $n = 5$) before and after a 6 h challenge with viable *Pg*. Bars represent the average ± SEM of protein expression levels from the non-challenged and challenged fibroblasts from experiments performed in quadruplicate. $*p < 0.05$, $**p < 0.01$ represent significant difference between the non-challenged and challenged cells of the same group. $\dagger p < 0.05$, $\ddagger p < 0.01$ represent significant differences between different groups. HGF, fibroblasts from periodontally healthy controls; MMP, matrix metalloproteinase; *Pg*, *P. gingivalis*; PGF, fibroblasts from chronic periodontitis patients; PIGF, peri-implant granulation tissue fibroblasts; TIMP, tissue inhibitors of metalloproteinases.

compared to PGFs 2.5 h ($p = 0.0003$) as well as 18 h after the *in vitro* removal of *P. gingivalis* ($p = 0.008$; Fig. 4A). Gene induction of *MCP-1* was higher in PIGFs ($p = 0.01$) compared to PGFs 2.5 h, but not 18 h after removal of *P. gingivalis* (Fig. 4B). *MMP-1* gene induction was also higher in PIGFs 2.5 h ($p = 0.02$) and 18 h ($p = 0.016$) after removal of *P. gingivalis* (Fig. 4C).

## Discussion

In the current study, the role of fibroblasts in peri-implant inflammation and tissue breakdown in peri-implantitis was assessed. Studies focusing on the role of bacterial interaction with fibroblasts in the pathogenesis of peri-implantitis are scarce. Important differences between fibroblasts from peri-implantitis patients, patients with periodontitis and healthy individuals have been reported (15,16).

In the present study, we found that PIGFs and PGFs were in a more pro-inflammatory state compared to fibroblasts from HGFs before a *P. gingivalis* challenge, which is consistent with an earlier report (16). This implies that PIGFs and PGFs are in an activated state and maintain their activated state for several passages in culture. When challenged with viable *P. gingivalis*, in contrast to HGFs, PIGFs and PGFs up-regulated their *IL-1β*, *IL-6*, *IL-8*, and *MCP-1* gene expression. This more pronounced response from PIGFs and PGFs to *P. gingivalis* may reflect their hyper-reactivity to bacterial challenge. *MMP-1* expression was also upregulated in response to *P. gingivalis* in PIGFs and PGFs, but not in HGFs. Interestingly, this upregulation of *MMP-1* was not accompanied by a significant change in expression of *TIMP-1* and the fibrogenic factor *TGFβ-1*, which indicates that *P. gingivalis* plays a role in the matrix breakdown through fibroblasts. It has been reported earlier though that fibroblasts from peri-implantitis patients play a role in matrix breakdown by both an increased production of MMP-1 as well as decreased production of TIMP-1 and TGFβ-1 (15).

We also found that non-challenged PIGFs expressed more *MMP-8* compared to fibroblasts from HGFs, which is consistent with earlier reports that demonstrated increased MMP-8 levels in the peri-implant sulcular fluid of peri-implantitis patients (35). MMP-8 is produced in relatively large quantities by neutrophils (36) and production of MMP-8 by neutrophils in peri-implant sulcus may contribute towards the higher expression in peri-implant sulcular fluid. In the current study, PIGFs showed a downregulation of *MMP-8* in response to the *P. gingivalis* challenge. This downregulation of MMP-8 in response to the *P. gingivalis* challenge is interesting as a protective role for MMP-8 against site-specific alveolar bone loss in response to *P. gingivalis* has been suggested (37). This might be due to the potential of MMP-8 to breakdown

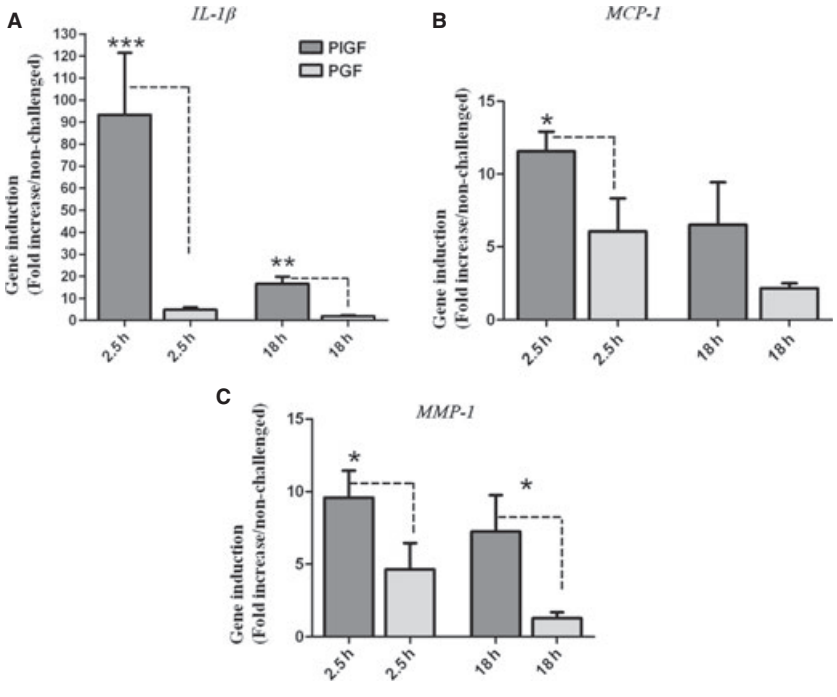


*Fig. 4*. Gene induction of *IL-1β*, *MCP-1*, and *MMP-1*, 2.5 and 18 h after removal of the *P. gingivalis* challenge. Gene induction (fold increase in mRNA expression in challenged compared with non-challenged cells) of *IL-1β* (A), *MCP-1* (B), and *MMP-1* (C) in PIGF ($n = 7$) and PGF ($n = 7$). Bars represent the mean induction level ± SEM at 2.5 and 18 h after the removal of *P. gingivalis* challenge from experiments performed in quadruplicate (Owing to undetectable levels of mRNA in the non-challenged cells of some donors, the bars for *IL-1β* represent data from five PIGF donors at 18 h; six and four PGF donors at 2.5 and 18 h respectively. For *MMP-1* the bars represent data from five PIGF and five PGF donors at 18 h). $*p < 0.05$, $**p < 0.01$, $p < 0.001$ represent significant differences between the groups. MMP, matrix metalloproteinase; PGF, fibroblasts from chronic periodontitis patients; PIGF, peri-implant granulation tissue fibroblasts; TIMP, tissue inhibitors of metalloproteinases.

and deactivate inflammatory mediators that are involved in periodontal bone resorption (38). The down-regulation of MMP-8 in the current study may indicate that *P. gingivalis* decreases protection by MMP-8 against inflammation in peri-implantitis. However, fibroblasts are not the primary cells responsible for MMP-8 production. It should be noted that we measured total MMP-8, the expression of which could be different from active forms of MMP-8.

Interestingly, we observed that after removal of *P. gingivalis*, the gene induction of *IL-1β*, *MCP-1*, and *MMP-1* remained significantly elevated in PIGFs. A recent study indicated that interaction of *P. gingivalis* lipopolysaccharide with fibroblasts might play a role in the persistence of inflammation in periodontal diseases (25). Such continuous production of inflammatory mediators by fibroblasts in response to *P. gingivalis* may play a significant role in sustaining the chronic inflammation and tissue damage by promoting migration of primary inflammatory leukocytes (39). IL-1β is an important mediator in periodontal inflammatory diseases as it stimulates the production of other cytokines, chemokines, cyclooxygenase products, and MMPs, which enhance the inflammatory process and tissue damage (40,41). Sustained induction of *IL-1β* and *MMP-1* expression by PIGFs after removal of the *P. gingivalis* suggests a more aggressive and persistent nature of inflammatory lesions around implants compared to similar lesions around natural teeth (7,8). As we have used a non-capsular *P. gingivalis* strain in our experiments, which can efficiently internalize into gingival fibroblasts (42), the internalized *P. gingivalis* may play a role in this persistent inflammatory response. Nevertheless, our results represent *in vitro* experiments and should be interpreted cautiously to explain the inflammatory response of PIGFs to *in vivo* infection in peri-implantitis. Moreover, in contrast to gingival or periodontal ligament fibroblasts, it is possible that PIGFs represent a collection of different fibroblast subpopulations in which case inflammatory responses from HGFs, PGFs, and PIGFs may not be directly comparable.

*Porphyromonas gingivalis* plays an important role in the pathogenesis of peri-implant and periodontal diseases by compromising the host immune system and interacting with host cells (9,43). Peri-implantitis is a multifactorial disease and besides bacteria, the inflammatory process around implants may be affected by the altered anatomy resulting from the presence of a foreign body and absence of a physical barrier around implants in the form of periodontal ligament. Furthermore, the difficulty to decontaminate dental implant surface and the possible interaction between peri-implant tissues and implant material (44) may also play a role in the pathogenesis of peri-implantitis.

In conclusion, granulation tissue fibroblasts from peri-implantitis and gingival fibroblasts from periodontitis lesions have a higher pro-inflammatory and matrix degrading properties and give a more pronounced pro-inflammatory response to an *in vitro* *P. gingivalis* challenge when compared to gingival fibroblasts from periodontally healthy individuals. In addition, fibroblasts from peri-implantitis lesions differ from fibroblasts from periodontitis lesions by the persistent upregulation of *IL-1β* and *MMP-1*, which may play a role in the more aggressive and persistent behavior of peri-implantitis inflammatory lesions.

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