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GENE EXPRESSION PROFILES OF MITOCHONDRIA-ENDOPLASMIC RETICULUM TETHERING IN HUMAN GINGIVAL FIBROBLASTS IN RESPONSE TO PERIODONTAL PATHOGENS

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Running Title: mitochondria and endoplasmic reticulum associated gene expression in periodontal disease

Summary Sentence: Mitochondria and endoplasmic reticulum contact may be affected negatively in the pathogenesis of periodontal disease.

FOOTNOTES

MFN1: Mitofusion 1; MFN2: Mitofusion 2; IP3R: Inositol 1,4,5-trisphosphate receptor; GRP75: Chaperone glucose-regulated protein 75; SIGMAR1: sigma non-opioid intracellular receptor 1; PINK1: phosphate and tensin homolog induced putative kinase 1; *F. nucleatum:*

Fusobacterium nucleatum; P. gingivalis: Porphyromonas gingivalis; MOI: Multiplicity of infection

ABSTRACT

Objective: The current study aimed to elucidate the potential involvement of mitochondria-endoplasmic reticulum contact genes in the pathogenesis of periodontal disease by monitoring levels of contact associated genes including Mitofusion 1 (MFN1) and MFN2, inositol 1,4,5-trisphosphate receptor (IP3R), chaperone glucose-regulated protein 75 (GRP75), sigma non-opioid intracellular receptor 1 (SIGMAR1) and phosphate and tensin homolog induced putative kinase 1 (PINK1) in human gingival fibroblasts in response to periodontal pathogens *Fusobacterium nucleatum* (*F. nucleatum*) and *Porphyromonas gingivalis* (*P. gingivalis*) *in vitro*.

Design: Primary human gingival fibroblasts were exposed to live cultures of *P. gingivalis* (W83; ATCC BAA-308) and *F. nucleatum* (subsp. Polymorphum; ATCC 10953) alone or in combination for 4 hours at a 50 or 200 multiplicity of infection. *Escherichia coli* lipopolysaccharide (10 μg/ml) exposure was used as a positive control. Gene expression levels of contact genes (MFN1, MFN2, IP3R, GRP75, SIGMAR1 and PINK1) as well as a proinflammatory cytokine, Tumor necrosis factor-α (TNF-α), and the apoptosis associated gene, Immediate early response 3 (IER3), were evaluated by reverse transcription polymerase chain reaction analysis.

Results: MFN1, GRP75, IP3R and PINK1 were significantly upregulated by *P. gingivalis* with or without *F. nucleatum*. Only *P. gingivalis* with *F. nucleatum* caused a significant upregulation of SIGMAR1. TNF-α and IER3 gene expression positively correlated with the contact-associated gene expression changes.

Conclusion: *F. nucleatum* and *P. gingivalis* alone or in combination may differentially dysregulate the gene expression levels of contact-associated genes in human gingival fibroblasts. These host-microbiome interactions may mechanistically be important in the pathogenesis of periodontal disease.

Keywords: Periodontal disease, endoplasmic reticulum, *Porphyromonas gingivalis, Fusobacterium nucleatum*, mitochondria, gingival fibroblasts.

INTRODUCTION

Endoplasmic reticulum and mitochondria are key organelles which play significant roles during energy metabolism and protein synthesis in cells. In addition, these organelles are tethered to each other to form a mitochondria-endoplasmic reticulum contact by the juxtaposition of mitochondrial surface structures including mitochondria outer membrane to specific domains of endoplasmic reticulum including mitochondria-associated membrane. This tethering is used to modulate several significant intracellular events including calcium and lipid metabolism, mitochondrial fusion, apoptosis and inflammasome formation (Liu & Zhu, 2017). Mitochondria-endoplasmic reticulum has been proposed to be due to the functional localisation of key proteins on the juxtaposed membranes (Csordas et al., 2006). For instance, Mitofusion 2 (MFN2) is located both in mitochondria-associated membrane and mitochondria outer membrane and tethers with Mitofusion 1 (MFN1) to modulate calcium transfer between mitochondria and endoplasmic reticulum (de Brito & Scorrano, 2008). Calcium transfer is also modulated by the interaction between the voltage-dependent anion channel of mitochondria outer membrane and the inositol 1,4,5-trisphosphate receptor (IP3R) of mitochondria-associated membrane. The bridge between voltage-dependent anion channel and IP3R is created by chaperone glucose-regulated protein 75 (GRP75) (Szabadkai et al., 2006) and this signalling by the IP3R-GRP75-voltage-dependent anion channel complex is prolonged by sigma non-opioid intracellular receptor 1 (SIGMAR1) in mitochondria-associated membrane by stabilizing IP3R (Hayashi & Su, 2007). The homeostasis of mitochondria during these processes is regulated by phosphate and tensin homolog induced putative kinase 1 (PINK1) which is a mainly located on mitochondria outer membrane and or in the cytosol adjacent to the mitochondrial surface (Figure 1) (Matsuda, Kitagishi, & Kobayashi, 2013).

Periodontitis is a chronic inflammatory disease initiated by host-pathogen interactions (Kinane & Lappin, 2002; Song, Zhou, Yang, Liu, & Shao, 2017). In response to the dental

plaque biofilm local tissue inflammation occurs, and the microbiome subsequently shifts to become predominantly a Gram-negative anaerobic biofilm resulting in dysbiosis (Van Dyke, Bartold, & Reynolds 2020). Certain bacteria, including *Fusobacterium nucleatum* (*F. nucleatum*) and *Porphyromonas gingivalis* (*P. gingivalis*), play a central role in the pathogenesis of the disease. *P. gingivalis* is a Gram-negative, anaerobic bacteria which has been described as a keystone pathogen and a master regulator of dysbiosis in periodontal disease (Chopra, Bhat, & Sivaraman, 2020). It has been reported to modulate the inflammatory response by modulating several host-bacterial interactions. In addition, it has been suggested to be able to orchestrate the growth and maturation of other bacteria involved in the dental plaque biofilm (Chopra, Bhat, & Sivaraman, 2020). *F. nucleatum* is a Gram-negative anaerobic bacterium, it is one of the initial Gram-negative colonizers of the dental plaque biofilm. It has been reported to play several significant roles in the pathogenesis of periodontal disease, including bridging the colonisation between Gram-negatives and Gram-positives, which is essential for biofilm formation as well as orienting environmental conditions for obligate anaerobic bacteria, such as *P. gingivalis* (Kolenbrander et al., 2002). Notably, *P. gingivalis* and *F.nucleatum* have been reported to exhibit a synergistic relation (Chopra, Bhat, & Sivaraman, 2020). *F. nucleatum* enhances the invasion of *P. gingivalis* into gingival epithelial cells (up to 20 folds) as well as augmenting its osteoclastic capacity and immunomodulatory properties, particularly in terms of cytokine production (Chopra, Bhat, & Sivaraman, 2020).

Previously, some biological effects of *P. gingivalis* on mitochondria and endoplasmic reticulum in periodontium cells have been reported (Napa et al., 2017; Bai et al., 2016; Domon et al., 2009; Yamada, Nakajima, Domon, Honda, & Yamazaki, 2015). *P. gingivalis* lipopolysaccharide has been reported to negatively alter the functions of mitochondria including their reactive oxygen species release, as well as reductions in adenosine triphosphate production in human gingival fibroblasts (Napa et al., 2017). In addition, alterations in

endoplasmic reticulum function including the stress response were detected in human periodontal ligament cells in response to *P. gingivalis* lipopolysaccharide exposure (Bai et al., 2016). In addition, these alterations of endoplasmic reticulum stress were also reported in gingiva from periodontitis patients (Domon et al., 2009) and in mice with experimentally induced periodontitis. (Yamada, Nakajima, Domon, Honda, & Yamazaki, 2015). However, so far, no study has evaluated the potential involvement of mitochondria-endoplasmic reticulum contact-associated gene expression in the pathogenesis of periodontal disease. To better understand the mechanistic pathway involved in the host-microbiome interaction in periodontal disease we analyzed the modulation of contact genes in response to exposure to the key pathogens, *P. gingivalis* and *F. nucleatum*.

MATERIALS AND METHODS

Bacterial Culture

P. gingivalis (W83; ATCC BAA-308) and *F. nucleatum* (subsp. Polymorphum; ATCC 10953) were grown on horse blood agar plates (Oxoid, Basingstoke, UK) in an anaerobic condition which includes 80% nitrogen, 10% carbon dioxide and 10% hydrogen at 37 °C. Overnight bacterial cell cultures in Scheadler's broth were prepared for both bacteria. Bacteria cells were pelleted. Then cells were washed 3 times with sterile PBS and diluted with sterile cell culture media (DMEM/Hams12, Sigma Aldrich, UK). *Escherichia coli* (*E.coli)* lipopolysaccharide (10 µg/ml) (Sigma Aldrich, UK) was also prepared by dilution in sterile cell culture media (DMEM/Hams12, Sigma Aldrich, UK) (Aral, Milward, Gupta, & Cooper, 2020).

Cell culture

Primary in human gingival fibroblasts were kindly gifted by Dr Richard Shelton, School of Dentistry, University of Birmingham (IRAS project ID 171283 and REC reference

15/NW/0079). Gingival fibroblast cells from a periodontally and systemically healthy female donor of 21 years of age were cultured in aerobic conditions of 95% air and 5 % carbon dioxide at 37°C in culture media (DMEM/F12, 10% fetal bovine serum (FBS), 1% Penicillin and Streptomycin and 1% Amphotericin B (Sigma-Aldrich, UK)) and transferred into 6-well plates ($4x10^5$ cell in 1 ml per well) and allowed to attach to culture overnight. A low (50) and high multiplicity of infection (MOI) (200) were chosen for infection. Human gingival fibroblasts in an antibiotic and antifungal-free media were then exposed to live cultures of *F. nucleatum* or *P. gingivalis* or to the combination of both at 50 or 200 MOI for 4 hours. *E. coli* lipopolysaccharide (10 µg/ml) challenge was used as positive control. For negative control group cells were only incubated with cell culture media for 4 hours (Aral, Milward, Gupta, et al., 2020). All essays were performed and repeated 3 times and in triplicate.

Reverse transcription polymerase chain reaction (RT-PCR) analysis

To examine gene expression levels, total RNA was obtained from cell cultures with a kit (RNeasy Plus Mini Kit, Qiagen, UK) according to the manufacturer's instructions. The amount of total RNA amount (ng/µl) was measured by a spectrophotometer (Genova Nano, Jenway, UK). Then, cDNA was synthesised by reverse transcription of total RNA (Tetro kit, Bioline, UK) according to the manufacturer's instruction. Reverse transcription polymerase chain reaction (RT-PCR) was used to detect the relative quantification of genes by using a system a (LightCycler® 480 system, Roche, Switzerland) with a master mix (SYBR Green I Master mix , Roche, Switzerland). After cycling (95°C for 10 min, 45 cycles of 95°C 20 sec, 60°C 20 sec and 72°C 30 sec) melt curve analysis was generated. Primers specific to target genes were used during the assays. (Table 1) The efficiency of primers was controlled by sample dilutions of cDNAs (1:1-1:1000). Then CT values were assessed for evaluated genes. $2^{-\Delta\Delta CT}$ method was used to determine the fold change differences (Aral, Milward, Gupta, et al., 2020).

Statistical analysis

For statistical analysis were performed by using a program (SPSS 20.0, IBM, Chicago, USA). Data were presented with mean and ± standard deviations for study groups. Normal distribution of the data was determined via the Kolmogorov-Smirnov test. 2-way ANOVA with Bonferroni posthoc comparisons were used due to the normally distributed data. For correlation analysis Pearson correlation coefficient was used. A p value < 0.05 was considered statistically significant.

RESULTS

Gene expression analysis demonstrated significant differences in levels for the genes evaluated in each group (Figure 2 & Table 2). MFN1 was significantly upregulated in response to all evaluated *P. gingivalis* exposure groups however it was downregulated by *F. nucleatum* at the low and high MOI used (Figure 2A).

MFN2 was differently regulated by the exposure groups. Low dose *F. nucleatum* (50) did not alter MFN2 gene expression levels however the high MOI (200) exposure resulted in a significant downregulation. *P. gingivalis* treatment at low doses significantly downregulated MFN2 levels, however at high doses significantly upregulation of expression was detected. The presence of *F. nucleatum* and *P. gingivalis* together significantly increased gene expression levels of MFN2 in human gingival fibroblasts (Figure 2B).

GRP75 expression was affected in all groups studied and an upregulation trend detected, indeed, all exposure groups significantly increased the gene expression levels of GRP75. The presence of the two bacteria in combination caused a dramatic increase in expression levels (Figure 2C).

IP3R was not affected by *F. nucleatum* at the relatively low doses used however with the increased exposure dose *F. nucleatum* significantly caused a downregulation in levels.

Interestingly, the presence of *P. gingivalis* with or without *F. nucleatum* significantly increased gene expression levels of IP3R in gingival fibroblast cells (Figure 2D).

PINK1 was not affected by *F. nucleatum* treatment alone at both high and low doses. However *P. gingivalis* with or without *F. nucleatum* exposure resulted in statistically significant upregulation of PINK1 gene expression levels (Figure 2E).

For SIGMA1R gene expression levels, both *F. nucleatum* an *P. gingivalis* alone did not cause any affect in human gingival fibroblasts however the combined infection of *F. nucleatum* and *P. gingivalis* statistically significantly increased SIGMA1R expression (Figure 2F).

Gene expression levels of the apoptosis associated transcript, IER3 and the key pro-inflammatory cytokine, TNF-α, were statistically significantly upregulated in response to exposure to *F. nucleatum* and *P. gingivalis* alone or combination (see Figures 3 and 4, respectively).

Multiple comparisons were also performed between groups for all genes evaluated (Table 2). MFN1 and GRP75 expression showed statistically significant difference in all multiple comparisons. For MFN2 except for Control vs *F. nucleatum* 50, *F. nucleatum* vs *F. nucleatum* +*P. gingivalis* 50, *F. nucleatum* 200 vs *F. nucleatum* +*P. gingivalis* 200, *P. gingivalis* 200 vs *F. nucleatum* +*P. gingivalis* 50 and *P. gingivalis* 200 vs *F. nucleatum* +*P. gingivalis* 200, all multiple comparisons were statistically significantly different. For IP3R also all groups rather than Control vs *F. nucleatum* 50 and *P. gingivalis* 200 vs *F. nucleatum* +*P. gingivalis* 50 showed statistically significance differences. In terms of SIGMA1R and PINK1 gene expression, more multiple comparisons failed to reach significance. The increase of MOI for *F. nucleatum* and *P. gingivalis* alone or combined resulted in significant upregulation of gene expression levels for both IER3 and TNF-α (Table 2).

Correlation analysis between IER3 and TNF-α and the contact-associated genes demonstrated a significant and positive correlation with mitochondria-endoplasmic reticulum

contact-associated genes, including MFN1, MFN2, IP3R, GRP75, SIGMAR1 and PINK1 (see Table 3 and 4, respectively).

DISCUSSION

The modulatory role of mitochondria-endoplasmic reticulum contact in several significant cellular functions including the regulation of calcium metabolism, inflammasome formation and apoptotic processes has been described in the literature (Liu & Zhu, 2017). Notably, the disruption of mitochondria-endoplasmic reticulum contact has been reported to adversely result in significant health conditions, including neurodegenerative diseases (Liu & Zhu, 2017). Previously a member of these contact, mitochondria, has been found to be negatively affected, and results in reactive oxygen species overproduction, adenosine triphosphate reduction and fission in human gingival fibroblasts in response to *P. gingivalis* lipopolysaccharide exposure (Napa et al., 2017). Dysregulation of endoplasmic reticulum including stress response has also been reported in *in vitro* (Bai et al., 2016), *in vivo* (Yamada et al., 2015) and clinical studies (Domon et al., 2009) in periodontal disease. However, no study has evaluated the gene expression profiles of mitochondria-endoplasmic reticulum contact in the pathogenesis of periodontal disease. According to the results presented in the current study, now for the first time, mitochondria-endoplasmic reticulum contact involved genes MFN1, MFN2, IP3R, GRP75, SIGMA1 and PINK have been shown to be significantly dysregulated in human gingival fibroblasts in response to periodontal pathogens *F. nucleatum* and *P. gingivalis.* Consequently, it is reasonably to hypothesis that the role of mitochondria-endoplasmic reticulum contact-associated genes may be disrupted in response to oral pathogens. This mechanism therefore may be associated with host-microbiome interactions during the pathogenic progression of periodontal disease.

MFN1 and MFN2 have been proposed to be involved in the modulation of calcium metabolism during mitochondria-endoplasmic reticulum contact. MFN2 knockdown was

reported to cause an increase of endoplasmic reticulum-mitochondria tethering and calcium transfer from endoplasmic reticulum to mitochondria (Leal et al., 2016). In addition, MFN1 and MFN2 in mitochondria-endoplasmic reticulum contact have been shown to be associated with processes of mitochondrial fusion (Marchi, Patergnani, & Pinton, 2014) and fission (Youle & van der Bliek, 2012) which modulate the shape, size number and physiological function of mitochondria. In the current study, MFN1 was upregulated in response to *P. gingivalis* with or without *F. nucleatum* however it was downregulated by *F. nucleatum* exposure alone. MFN2 was downregulated by a relatively high MOI of *F. nucleatum* and the low MOI of *P. gingivalis* used here however it was significantly upregulated in response to the high MOI exposure of *P. gingivalis* and also the combination of *P. gingivalis* and *F. nucleatum*. Therefore, it may be suggested that *F. nucleatum* and *P. gingivalis* may play different roles in calcium metabolism in endoplasmic reticulum and mitochondria, as well as disrupting fusion and fission of mitochondria by dysregulating MFN1 and MFN2 expression levels.

IP3R encodes a mitochondria-associated membrane located protein regulating the release of calcium from endoplasmic reticulum which provides the main storage for calcium intracellularly (Marchi et al., 2014). IP3R interacts with voltage-dependent anion channel located in mitochondria outer membrane for the efflux of calcium from endoplasmic reticulum to mitochondria with the aid of the coupling protein GRP75. Dysregulation of calcium metabolism causes higher calcium efflux from endoplasmic reticulum to the cytosol and mitochondria (Marchi et al., 2014) and this negatively affects mitochondrial calcium and subsequently mitochondrial dynamics. In the current study the IP3R transcript was significantly upregulated by *P. gingivalis* in the presence or absence of *F. nucleatum.* In addition, the high MOI exposure of *F. nucleatum* significantly downregulated IP3R expression. GRP75 was also significantly upregulated in response to both high and low MOI of *F. nucleatum* or *P. gingivalis* or in response to the combined exposure to both. Therefore, it may

be suggested that periodontal pathogens may affect calcium transfer from endoplasmic reticulum to mitochondria by dysregulating IP3R and GRP75.

SIGMAR1 is a mitochondria-associated membrane located protein which stimulates calcium from endoplasmic reticulum to Mitochondria via the IP3R receptor (Hayashi & Su, 2007). An increase in SIGMAR1 expression has been reported to reduce the endoplasmic reticulum stress response however a decrease in levels promotes apoptosis (Hayashi & Su, 2007). In the current study both the relatively low and high MOI exposures of *P. gingivalis* or *F. nucleatum* did not alter the gene expression levels of SIGMAR1. However, *P. gingivalis* and *F. nucleatum* exposure combined at both high and low MOI significantly upregulated SIGMAR1 transcript levels. Therefore, it may be proposed that a mix infection of the pathogens which occurs in the dental plaque may dysregulate SIGMAR1 and subsequently calcium metabolism contributing to the pathogenesis of periodontal disease.

PINK1 is a mitochondria outer membrane gene and a cytosol located serine/threonine kinase which controls the quality of mitochondria, by playing a role in mitochondrial stabilization, phosphorylation of chaperones, and regulation of mitophagy. The silencing of PINK1 causes reactive oxygen species accumulation in mitochondria resulting in oxidative damage. (Matsuda et al., 2013; Wang et al., 2011) In the current study the gene expression levels of PINK1 upregulated in response to *P. gingivalis* +/- *F. nucleatum* however were unchanged by *F. nucleatum* alone. Therefore, it may be suggested that the quality of mitochondria regulated by PINK1 may be disrupted by periodontal pathogens in the pathogenesis of periodontal disease.

Mitochondria (Shahid et al., 2018) and endoplasmic reticulum (Pizzo & Pozzan, 2007) have been reported to play significant roles in regulating apoptosis. Mitochondria can initiate the intrinsic pathway of apoptosis in response to several stimuli, including excessive ROS (Aral, Aral, & Kapila, 2019). The presence of the unbalanced endoplasmic reticulum stress has

also been reported to initiate apoptosis (Szegezdi, Logue, Gorman, & Samali, 2006). Mitochondrial oxidative phosphorylation (Shahid et al., 2018) (Shen, Guo, Santos-Berrios, & Wu, 2006) has previously been shown to be regulated by Immediate early response 3 (IER3) gene, which is an early response gene expressed, in response to several stimuli including inflammatory cytokines such as TNF-α and lipopolysaccharide, in a variety of cell types including macrophages and epithelial cells (Feldmann, Pittelkow, Roche, Kumar, & Grande, 2001; Shahid et al., 2018). IER3 is known to control ROS production by targeting ATPase inhibitory factor 1 which reduces ROS production (Shen, Guo, Santos-Berrios, & Wu, 2006). Overexpression of IER3 prevents apoptotic stimuli-induced ROS production in different cell types and therefore IER3 may play a significant role in the modulation of cellular reduction-oxidation during homeostasis (Shen, Guo, Santos-Berrios, & Wu, 2006). Notably, IER3 has been reported to contain an endoplasmic reticulum membrane-associated domain (Wu, 2003). Studies have shown that it plays a significant role in the pathogenesis of several chronic diseases, including arthritis, and that it has both pro- and anti-apoptotic actions which are reportedly mediated due to its relatively high expression in mitochondria (Shahid et al., 2018). Interestingly, the anti-apoptotic activity of IER3 has also been strongly correlated with its ability to suppress intracellular ROS production (Shen, Guo, Santos-Berrios, & Wu, 2006). In tumor cells IER3 has been reported to induce apoptosis and consequently act as a tumor suppressor (Shahid et al., 2018). In the current study, to evaluate the potential effect of *P. gingivalis* and *F. nucleatum* on the gene expression profiles involved in both mitochondria endoplasmic reticulum contact and apoptosis, IER3 gene expression was also monitored. Such as this study, to our knowledge, has never previously been performed. Interestingly, all bacteria exposure groups showed a statistically significant upregulation of IER3 expression in gingival fibroblasts (Figure 3). The increase in the amount of bacterial exposure also increased the gene expression level dose-dependently (Table 2). In addition, all evaluated contact genes were

found to be positively correlated with IER3 expression (Table 3). Consequently, it may be suggested that the periodontal bacteria, *F. nucleatum* and *P. gingivalis,* trigger contact associated and apoptosis-related gene expression, as well as mitochondria and endoplasmic reticulum connected IER3 expression in human gingival fibroblasts.

Mitochondria-endoplasmic reticulum contact has been also suggested to play a role in calcium dependent apoptosis. The disrupted calcium efflux from the endoplasmic reticulum to cytosol and mitochondria (Marchi et al., 2014), subsequently disorganizes adenosine triphosphate synthesis (Bonora et al., 2012) and apoptosis (Orrenius, Zhivotovsky, & Nicotera, 2003). This efflux from the endoplasmic reticulum triggers apoptosis due to the mitochondrial permeability transition pore which regulates the release of cytochrome C and other apoptotic factors (Marchi et al., 2014). Previously cytochrome c was found to be significantly upregulated in response to *P. gingivalis* in human periodontal ligament cells (Seo, Cha, Kim, Lee, & Woo, 2012). In addition, caspase-9 which is involved in the mitochondria regulated apoptotic pathway was also found to be elevated in periodontal disease (Aral, Aral, & Kapila, 2019). Therefore, dysregulation of apoptosis in periodontal disease may be a result of the dysregulation of calcium metabolism intracellularly. Notably *P. gingivalis* has been reported to increase the release of calcium in both human neutrophils (Jayaprakash, Demirel, Khalaf, & Bengtsson, 2015) and gingival epithelial cells (Belton et al., 2004). *F. nucleatum* has been also demonstrated to enhance intracellular calcium oscillation (Krisanaprakornkit, Jotikasthira, & Dale, 2003). A limitation of the current study is that the calcium levels of cells were not evaluated, and therefore further studies are needed to elucidate the potential effect of contact-associated genes on calcium dependent apoptosis in the pathogenesis of periodontal disease.

Mitochondria and endoplasmic reticulum have previously been reported to be associated with inflammatory processes. Gene expression levels of MFN2 in human periodontal ligament stem cells have been shown to be significantly upregulated in response to

TNF-α challenge which is a key proinflammatory cytokine that regulates the immune response (Zhai et al., 2018) In the current study, both *F. nucleatum* and *P. gingivalis* alone or combined at both high and low exposure levels significantly upregulated the TNF-α transcript (Figure 4). Levels were also enhanced significantly with increased exposure (Table 2). Notably, the expression levels of TNF-α were significantly correlated with all contact-associated genes evaluated (Table 4). Consequently, it may be proposed that both the periodontal pathogens, *F. nucleatum* and *P. gingivalis,* can dysregulate gene expression levels involved in both mitochondria-endoplasmic reticulum contact and inflammation. Thus, a potential regulatory link between TNF-α expression and contact gene expression could be considered. However, further confirmatory studies are required. Interestingly, mitochondria endoplasmic reticulum contact has been also reported to be involved in inflammasome modulation (Zhou, Yazdi, Menu, & Tschopp, 2011). These multiprotein complexes are central to regulating inflammatory events, in particular the release of interleukin-1β (IL-1β) during the immune response (Aral, Milward, Kapila, Berdeli, & Cooper, 2020). Furthermore, mitochondria are a major source of reactive oxygen species (Marchi et al., 2012) which are known to promote Nod-like receptor 3 (NLRP3) inflammasome activation (Zhou et al., 2011). Notably, NLRP3 is subsequently reported to relocate from the endoplasmic reticulum to mitochondria-endoplasmic reticulum contact during inflammation (Zhou et al., 2011). In addition, induction of the inflammasome results in the translocation of another inflammasome component, apoptosis-associated speck-like protein containing a CARD, from the cytosol to mitochondria-associated membrane (Zhou et al., 2011). The inhibition of IP3R was found to decrease calcium efflux from the endoplasmic reticulum and this reduced Interleukin-1β secretion in bone marrow-derived macrophages. Thus, mitochondria-endoplasmic reticulum contact and mitochondria-associated membrane have been proposed to be a significant site for inflammasome activation and formation (Marchi et al., 2014). In the current study expression levels of IP3R were significantly upregulated in

response to *P. gingivalis* with or without *F. nucleatum.* In addition, we have previously shown that infection of *P. gingivalis* +/- *F. nucleatum* at a MOI of 100 for 24h significantly dysregulated the gene expression levels of NLRP3, ASC, IL-1β in human gingival fibroblasts (Aral, Milward, & Cooper, 2020). Therefore, it may be suggested that both dysregulation of inflammasomes and mitochondria-endoplasmic reticulum contact genes in human gingival fibroblasts in response to *P. gingivalis* and *F. nucleatum* may be associated with the pathogenesis of periodontitis. However, further studies are needed.

In the current study two different MOIs were used for both pathogens to investigate the dose dependent relationship involved in mitochondria-endoplasmic reticulum contact transcript regulation. Notably, MFN2 and IP3R were significantly downregulated with the increase of MOI of *F. nucleatum*. All evaluated genes except for PINK1 were significantly upregulated in response to the increasing MOI levels of *P. gingivalis*. MOI increase of *F. nucleatum* and *P. gingivalis* further upregulated MFN2, IP3R and SIGMAR1 however they significantly downregulated MFN1, GRP75, PINK1. Therefore, levels of these bacteria within the dental biofilm *in vivo* may affect mitochondria-endoplasmic reticulum contact and therefore influence the progression of periodontal disease.

Exposure of the anaerobes, *P. gingivalis* and *F. nucleatum,* to human gingival fibroblasts has been performed under aerobic conditions, which includes 95% air and 5 % carbon dioxide at 37°C. Notably, *F. nucleatum* has been reported to be able to survive in conditions which include oxygen even higher than those present in air (Diaz, Zilm, &Rogers, 2000). However, *P. gingivalis* has only been suggested to be able to survive and grow in the presence of relatively low oxygen concentrations, i.e. 6% (Lewis, Iyer, & Anaya-Bergman, 2009). This difference in viability has been reported to be explicable due to the different capacity of the two organisms to tolerate oxidative stress, i.e. *F. nucleatum* has been reported to be 20 times more tolerant compared with *P. gingivalis*. This characteristic property of *F.*

nucleatum has been reported to be necessary to provide support for the growth and survival of *P. gingivalis* under aerobic conditions in co-culture models (Diaz, Zilm, & Rogers, 2002). Consequently, it may be suggested that the exposure groups of *F. nucleatum* +/- *P. gingivalis* might reflect their pathogenicity even under aerobic conditions used in the current study. The presence of *P. gingivalis* in supragingival plaque, saliva and mucosa in which an aerobic atmosphere exists, has also been reported (Diaz et al., 2012). Therefore, some literature has suggested that *P. gingivalis* could also be classified as being aerotolerant (Diaz & Rogers, 2004). In the current study, *P. gingivalis* exposure alone also triggered changes in all gene expression levels studied (except for SIGMAR1) notably, even under aerobic conditions. However further studies are needed to confirm this hypothesis.

In the current study, for the first time, gene expression of mitochondria-endoplasmic reticulum contact associated genes in human gingival fibroblast has been demonstrated. These molecular events may provide a mechanistic insight into novel host-microbiome interactions which occur during the pathogenesis of periodontal disease. In addition, the potential modulation of these gene expression changes in response to different exposure levels of *P. gingivalis* and F. *nucleatum* alone or due to the combination of both has also now been reported. Notably, the possible correlation of the apoptosis-associated gene, IER3, and the inflammatory cytokine, TNF-α, with contact-associated genes has also now been reported. Therefore, the expression of contact genes in human gingival fibroblasts and the dysregulation of genes in response to major periodontal pathogens, *P. gingivalis* and F. *nucleatum,* can now be proposed as being involved in the pathogenesis of periodontal disease.

The current study concentrated on contact-associated gene expression to provide a better understanding of host-microbiome interactions that may occur during the pathogenesis of periodontal disease. Contact protein levels were not monitored, however, future studies of levels and localisations should be performed to build on novel findings. Furthermore, our data

now support the initiation of other studies, such as knockdown analyses of contact-related molecules, to expand our knowledge of their role in the host-microbiome interactions which occur in the pathogenesis of periodontal disease.

CONCLUSION

The mitochondria-endoplasmic reticulum contact involved structural genes of MFN1, MFN2, IP3R, SIGMAR1 and PINK1 were dysregulated in human gingival fibroblasts in response to exposure to the major periodontal pathogens *F. nucleatum* and *P. gingivalis* alone in combination. *P. gingivalis* alone may alter the contact gene expression levels more negatively compared with *F. nucleatum* alone. Consequently, the periodontal bacteria *F. nucleatum* and *P. gingivalis* may disturb mitochondria-endoplasmic reticulum contact-associated genes during host-microbiome interactions during the pathogenesis of periodontal disease.

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FIGURE LEGENDS

Figure 1. Schematic illustration of mitochondria-endoplasmic reticulum contact-associated proteins. (MAM: Mitochondria associated membrane, ER: endoplasmic reticulum, IMM: Inner mitochondrial membrane, MOM: mitochondria outer membrane, MERC: mitochondria-endoplasmic reticulum contact).

Figure 2. Gene expression analysis in human gingival fibroblasts in the exposure groups. Gene expression levels of MFN1 (A), MFN2 (B), GRP75 (C), IP3R (D), PINK1 (E), SIGMAR1 (F) were differentially regulated in response to *P. gingivalis* and *F. nucleatum* or to the combination of both in human gingival fibroblasts. Data is presented as mean±standard deviations. Columns show means and error bars represent standard deviations. * describes statistically significant compared with the control group (p<0,05). (Fn50: *F. nucleatum* at 50 MOI, Fn 200: *F. nucleatum* at 200 MOI, Pg 50 : *P. gingivalis* at 50 MOI, Pg 200: *P. gingivalis* at 200 MOI, Fn+Pg 50: *F. nucleatum* and *P. gingivalis* combined at 50 MOI, Fn+Pg 200: *F. nucleatum* and *P. gingivalis* combined at 200 MOI)

Figure 3. Gene expression levels of IER3 in the different exposure groups. All exposure groups demonstrated statistically significantly upregulated IER3 expression compared with the control group. Data is shown as mean±standard deviations. * shows statistically significance (p<0,05). (Fn50: *F. nucleatum* at 50 MOI, Fn 200: *F. nucleatum* at 200 MOI, Pg 50: *P. gingivalis* at 50 MOI, Pg 200: P. gingivalis at 200 MOI, Fn+Pg 50: *F. nucleatum* and *P. gingivalis* combined at 50 MOI, Fn+Pg 200: *F. nucleatum* and *P. gingivalis* combined at 200 MOI).

Figure 4. Gene expression levels of TNF-α in the different exposure groups. *F. nucleatum* and *P. gingivalis* alone or combined demonstrated statistically significantly upregulated TNF- α transcript levels compared with control group. Data is shown as mean±standard deviations. *

describes statistically significance ($p<0,05$). (Fn50: *F. nucleatum* at 50 MOI, Fn 200: *F. nucleatum* at 200 MOI, Pg 50: *P. gingivalis* at 50 MOI, Pg 200: *P. gingivalis* at 200 MOI, Fn+Pg 50: *F. nucleatum* and *P. gingivalis* combined at 50 MOI, Fn+Pg 200: *F. nucleatum* and *P. gingivalis* combined at 200 MOI).