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Neutrophil superoxide release and plasma C-reactive protein levels pre- and post-periodontal therapy

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ABSTRACT

Aim: To determine peripheral blood neutrophil superoxide release and C-reactive protein (CRP) concentration in chronic periodontitis patients, before and after non-surgical periodontal treatment.

Materials & Methods: Neutrophils were isolated from patient and control volunteers (n=20) and superoxide measured by lucigenin-enhanced chemiluminescence with and without stimulation with unopsonised *P. gingivalis*, unopsonised *F. nucleatum* and phorbol 12-myristate 13-acetate (PMA) before and 2-months following non-surgical therapy. Corresponding high-sensitivity plasma CRP concentrations were also determined.

Results: At pre-treatment baseline, patient neutrophils released more superoxide in the absence ($P<0.032$) and presence of periodontal bacteria ($P<0.013$) and after PMA stimulation ($P=0.041$) compared to control cells. Post-therapy, patient neutrophil superoxide release was reduced to control cell levels. Median patient plasma CRP concentrations were non-significantly higher than control values and were reduced after therapy (1.80mg/l to

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1.36mg/l). Patient pre-treatment baseline, unstimulated neutrophil superoxide release showed a significant positive correlation with plasma CRP concentration (P=0.01).

Conclusions: Chronic periodontitis is characterised by peripheral neutrophils exhibiting superoxide hyperactivity and hyper-reactivity to periodontal pathogens that is not a constitutive feature of periodontitis patients. The positive, pre-therapy relationship between unstimulated neutrophil superoxide release and plasma CRP is consistent with a protective role for CRP in reducing oxidative stress and systemic inflammation *in vivo*.

Clinical Relevance

Scientific rationale for the study: The pathogenesis of chronic periodontitis is characterised by a dysregulated neutrophilic inflammatory-immune response and greater concentrations of systemic inflammatory markers. These are important in relation to the potential for collateral host tissue damage and also an increased risk of systemic co-morbidity.

Principal findings: Neutrophils from patients with chronic periodontitis released significantly more superoxide in the absence and presence of periodontal pathogens, which was corrected by successful non-surgical treatment.

Practical implications: Periodontal treatment may help to normalise systemic neutrophil oxidative/inflammatory activity and reduce the risk of periodontitis-associated co-morbidity.

Conflict of Interest and Source of Funding:

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INTRODUCTION

Chronic periodontitis is a prevalent inflammatory disease resulting from a dysregulated host response to the plaque biofilm that forms on tooth surfaces in the absence of adequate oral hygiene (Van Dyke 2008). The disease is characterised by neutrophil hyperactivity/-reactivity in terms of the generation of reactive oxygen species (ROS) and raised levels of systemic inflammatory markers, such as C-reactive protein (CRP; Paraskevas et al. 2008). While the former is thought to be responsible for collateral host tissue damage (reviewed by Chapple and Matthews 2007), recent studies have suggested that physiological levels of CRP may generally reduce neutrophil ROS generation (Ling et al. 2014).

There have been a variety of reports demonstrating neutrophil ROS hyper-reactivity in untreated chronic periodontitis, however, in terms of physiologically relevant stimuli, these studies have been almost exclusively limited to Fc□□receptor (Fc□R) stimulation (reviewed by Chapple and Matthews 2007). Two studies have investigated neutrophil responses to unopsonised *F. nucleatum* and demonstrated hyper-reactivity in terms of extracellular neutrophil ROS release in untreated chronic periodontitis patients (Matthews et al. 2007a, Dias et al. 2013). One of these studies also demonstrated that patient neutrophils released exaggerated amounts of ROS in the absence of overt stimulation (i.e. hyperactivity; Matthews et al. 2007a).

Neutrophils isolated from treated chronic periodontitis patients have similarly been reported to exhibit luminol-detectable (intracellular) Fc□R-stimulated ROS hyper-reactivity suggesting that this aberrant response is constitutional rather than reactive (Fredriksson et al. 2003, Gustafsson et al. 2006). However, no objective clinical data supporting the presence of periodontal health was provided. By contrast, the only longitudinal intervention study investigating the impact of clinically verified, successful therapy demonstrated loss of luminol-detectable (intracellular) Fc□R-stimulated ROS hyper-reactivity but retention of

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hyperactivity in respect of unstimulated isoluminol-detectable (extracellular) ROS release (Matthews et al. 2007b). Current data are therefore unclear in relation to the effect of successful periodontal therapy on unstimulated and stimulated neutrophil ROS generation. Furthermore, there are currently no longitudinal intervention studies performed following stimulation with periodontal pathogens.

Recently, it has been shown that physiologically-relevant concentrations of CRP significantly influence neutrophil ROS generation and release in the absence and presence of *F. nucleatum* and Fc□R stimulation (Ling et al. 2014). This has important implications since it is recognised that plasma CRP levels are elevated in patients with untreated chronic periodontitis (Paraskevas et al. 2008, Li et al. 2014). Although several studies report FcγR-stimulated ROS hyper-reactivity and higher CRP concentrations in patients compared to controls, no attempt has been made to relate these two parameters (Fredriksson et al. 1998, 1999, 2003, Gustafsson et al. 2006).

The aim of this current study was to determine neutrophil superoxide release from patients with chronic periodontitis, in the absence and presence of stimulation with unopsonised periodontal pathogens, before and 2 months after clinically verified, successful non-surgical periodontal therapy. In addition, high sensitivity plasma CRP concentrations were measured before and after therapy to determine any relationship with neutrophil superoxide release.

MATERIALS & METHODS

Ethical approval, study populations and clinical measures of disease in patients before and after therapy

Ethical approval was granted by the West Midlands Research Ethics Committee (ref. 10/H1208/48) and the Birmingham Community Healthcare NHS Trust. The study was adopted onto the National Institute of Health Research Network Portfolio (Study ID 10318)

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and registered on ClinicalTrials.gov (Identifier NCT01233765). The study cohorts consisted of volunteers diagnosed with chronic periodontitis (n=20; 12 male and 8 female; mean age = 46+8 yrs, range 37-61 yrs) along with age- and gender-matched periodontally and systemically healthy control volunteers (n=20; 12 male, 8 female; mean age = 46+8 yrs, range 32-62 yrs). The sample size was based upon our previous published studies of neutrophil ROS responses (Matthews et al. 2007a, 2007b) where, using 18-19 volunteer pairs, significant differences in unstimulated, extracellular, isoluminol-detectable ROS release were detected between patient and control cells. Analysis of these published data showed that 18-20 patient-control pairs were required for a test of equality at the 0.05 level of significance with 80% power. All volunteers were never-smokers and systemically healthy as confirmed by a detailed medical history questionnaire. Chronic periodontitis was defined as the presence of at least two non-adjacent sites with probing pocket depths >4mm, along with radiographic bone loss >30% of the root length (non-first molar or incisor sites) in accordance with the consensus criteria of the European Federation of Periodontology (Tonetti et al. 2005). All clinical measures of periodontal disease (probing pocket depth, bleeding on probing, gingival and plaque indices) were significantly greater in the patient compared to control volunteer cohort ($P<0.001$). Patient volunteers received tailored oral hygiene instruction and conventional non-surgical periodontal therapy by a single operator. This consisted of scaling and root surface debridement of all periodontal pockets >4mm performed under local anaesthesia on a quadrant-by-quadrant basis within a maximum of 4 weeks. All clinical measures of disease were significantly reduced at the 2-month post-periodontal treatment review ($P<0.05$), with patient levels becoming comparable to those of healthy controls (Ling et al. 2015).

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Collection of peripheral blood samples and isolation of neutrophils

Peripheral blood was obtained from paired volunteers with chronic periodontitis and age-/gender-matched controls before (baseline; n=20) and 2 months after conventional non-surgical periodontal therapy (review; n=19). Blood was obtained between 09:00 and 10:00 am from a patient and a matched healthy control volunteer within 30 min of each other. Blood was collected from the antecubital fossa into Vacutainers™ (Greiner, Bio-One Ltd, Stonehouse, UK; 6ml) containing lithium heparin (17 IU/ml) as an anticoagulant. Peripheral blood neutrophils were isolated using a discontinuous Percoll gradient (δ = 1.079:1.098), followed by erythrocyte lysis as previously described (Ling et al. 2014, 2015).

Determination of plasma CRP concentration

Vacutainers™ were centrifuged in sealed buckets at 1,000 RCF for 30 mins at 4^{o}C and the separated plasma removed, aliquoted into cryogenic vials (0.5 ml/vial) and stored at -80^{o}C. High-sensitivity CRP (hsCRP) analyses were subsequently performed by the UK National Protein Reference Centre, Immunology Department and Protein Reference Unit, Sheffield Teaching Hospitals NHS Foundation Trust. Laboratory analyses of plasma CRP concentration were performed blinded to the origin of the samples.

Preparation of stimuli

Fusobacterium nucleatum (*Fn*; ATCC 10953) and *Porphyromonas gingivalis* (*Pg*; ATCC 33277) were grown anaerobically at 37 ^{o}C as previously described (Roberts et al. 2002). Bacteria were washed 3 times in sterile PBS and heat-treated (100 ^{o}C for 10 min) prior to dilution with sterile PBS to give a final suspension of 8.5 x 10^9 bacteria/ml and 4.1 x 10^9 bacteria/ml for *F. nucleatum* and *P. gingivalis* respectively, and stored at -80 ^{o}C. Phorbol 12-myristate 13-acetate (PMA; P8139; Sigma-Aldrich Ltd, Gillingham, Dorset, UK) was

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reconstituted with DMSO to give a 1 mg/ml (1.62 mM) stock solution that was stored at -20 ^{o}C. Working suspensions/solutions of all stimuli were prepared by diluting stock solutions with PBS immediately prior to use to give a multiplicity of infection (MOI) of 100:1 for bacteria and a final concentration of 5 nM for PMA.

Preparation of lucigenin

A 1 mg/ml stock solution of lucigenin (N.N'-Dimethyl-9,9'biacridium dinitrate; M8010; Sigma-Aldrich Ltd, Gillingham, Dorset, UK) in PBS was prepared and stored at 4 ^{o}C. A 0.33 mg/ml working solution was prepared by diluting this stock 1:3 with PBS.

Lucigenin-enhanced chemiluminescence assay to measure extracellular superoxide anion radical production

Twenty µl of glucose-supplemented PBS (PBS containing 1 mM glucose, 1.5 mM $CaCl_2$ and 1 M $MgCl_2$; GPBS), 25 µl of PBS and 30 µl lucigenin (0.33 mg/ml; specific for detecting extracellular superoxide release; Dahlgren et al. 1985) were added to pre-blocked (PBS containing 1 % BSA, overnight, 4 ^{o}C) white microwells (Microlite 2, VWR, Lutterworth, UK). Following the addition of reagents, 100 µl of neutrophil cell suspension (1 x 10^6 cells/ml in GPBS; 1 x 10^5 cells) were then added to each well and the plate immediately transferred to the plate reader and light output recorded for 1 s per well in relative light units (Berthold microplate luminometer LB96v, Berthold Technologies UK Ltd) at 37 ^{o}C. Baseline light output was recorded for 30 mins before addition of stimuli or vehicle control (PBS; 25 µl), giving a total volume of 200 µl per well. Light output as then recorded for a further 120 mins. All samples were analysed in quadruplicate, with paired patient and control cells isolated and analysed at the same time, maintaining the specific patient/control pairing both pre- and post-treatment. Peak relative light units (RLU) were used for assessment of

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chemiluminescence kinetics, as this is considered a reliable parameter (Allen 1986) and corresponds with the area under the chemiluminescence curve (Allen 1986, Hasegawa et al. 1997). All laboratory lucigenin-enhanced chemiluminescence analyses were performed blinded to the origin of the samples.

Data handling and statistical analyses

Data were recorded and manipulated in Microsoft Excel and evaluated using GraphPad Prism 5 (version 5.04; GraphPad Software, La Jolla, CA, USA). Data distribution was determined using the Kolmogorov-Smirnov test. Differences in probing pocket depths, the only dataset found to be normally distributed, were determined by ANOVA followed by Tukey-Kramer multiple comparisons test. All other statistical comparisons were performed using non-parametric methods, as indicated in the text and/or Table/Figures. Detection of ROS by chemiluminescence methods can show considerable day-to-day variation making the inclusion of a paired, age and gender matched control, whose neutrophils are analysed simultaneously with those from the patient, important if consistent and comparable results are to be obtained (Yaffe et al. 1999). Data derived from such experiments are thus normally analysed using paired statistical analyses to determine the presence of neutrophil hyper-reactivity (Fredriksson et al. 1998, 1999, 2003, Matthews et al. 2007a, 2007b), namely Wilcoxon rank sum and Friedman followed by Dunn's multiple comparisons tests as outlined within the figure legends. Plasma hsCRP concentrations were compared using Mann-Whitney U test.

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RESULTS

Longitudinal changes in clinical measures

Non-surgical periodontal therapy resulted in improvements in all clinical measures of the disease at the 2-month post-treatment review with the percentage of sites exhibiting bleeding on probing and gingival and plaque indices being reduced to healthy control levels (Table 1).

Neutrophil superoxide release in the absence and presence of stimulation

Patient neutrophils released significantly more unstimulated superoxide compared to controls at baseline pre-treatment (P=0.005; Figure 1). After stimulation with *P. gingivalis*, *F. nucleatum* and PMA, both patient and control neutrophils released significantly greater amounts of superoxide compared with PBS vehicle control (P<0.05) and the amount of superoxide release differed significantly between stimuli (P<0.0001). Although there were no significant differences between *P. gingivalis* and *F. nucleatum* stimulated superoxide release, PMA stimulated approximately 3-6 times more superoxide compared to bacterial stimuli (P<0.001). Overall, patient neutrophils released significantly more superoxide compared to controls when comparing the combined data for all stimuli (P=0.0001) and data for individual stimuli, including PBS (vehicle control) (Figure 1).

At the 2-month post-treatment review, there were no significant differences in unstimulated superoxide release between patient and control neutrophils (Figure 1). However, in agreement with the pre-treatment data, neutrophils released significantly more superoxide after stimulation (P<0.05), with PMA eliciting a significantly higher chemiluminescence response compared to bacterial stimuli (P<0.01). Overall, there were no significant differences in stimulated superoxide release between patients and control neutrophils when comparing the combined data for all stimuli (P=0.26) or when analysing data for the individual stimuli (Figure 1).

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As a percentage of paired control, there was a significant reduction in patient superoxide release at review compared to baseline for PBS treated cells (P=0.004; vehicle control), *P. gingivalis* (P=0.004) and *F. nucleatum* (P=0.0007) stimulated cells (Figure 2). There were no significant changes in unstimulated or PMA stimulated superoxide levels between baseline and review, although median levels of both were lower post-therapy.

Plasma hsCRP and relationship with neutrophil superoxide release

There was considerable variation in plasma hsCRP concentration within both patient and control groups at baseline and review (Figure 3). Although the median patient hsCRP concentration (1.80mg/l) was higher than that of controls (0.95mg/l), and decreased following periodontal treatment (1.36mg/l, patient; 0.91mg/l, control), the differences were not statistically significant.

Regression analyses of unstimulated superoxide release and plasma CRP concentration demonstrated a moderate, significant positive correlation for patients at baseline (P=0.01; Figure 4), which was not found at post-therapy review. No such relationship was detected for the control group or when stimulated superoxide levels were investigated.

DISCUSSION

This is the first study to determine neutrophil superoxide release in a longitudinal intervention study designed to assess the effect of successful periodontal therapy on peripheral blood neutrophil hyperactivity (increased superoxide release in the absence of stimulation) and hyper–reactivity after stimulation with unopsonised periodontal pathogens. The data demonstrate that neutrophils from chronic periodontitis patients exhibit both hyperactivity, as well as hyper-reactivity to unopsonised *F. nucleatum* and *P. gingivalis*, both

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of which can be reduced or removed after successful non-surgical periodontal therapy. These findings suggest that the hyperactive/-reactive neutrophil superoxide release is secondary to inflammation rather than being a constitutional feature of periodontitis patients.

Hyper-reactive neutrophil ROS generation in response to Fc□R-stimulation is a well characterised pathological feature of chronic periodontitis (reviewed by Chapple and Matthews 2007). By contrast, hyper-reactivity to unopsonised periodontal pathogens is limited to our previous reports investigating *F. nucleatum* (Matthews et al. 2007a, Dias et al. 2013) and baseline unstimulated hyperactivity has only been described in terms of increased isoluminol-detectable ROS (detecting HOCl derived from extracellular release of both superoxide and hydrogen peroxide; (Matthews et al. 2007a, 2007b). The lucigenin-based data presented here is specific for extracellular superoxide release, and confirm and extend these reports and support the conclusion that neutrophils in chronic periodontitis release excessive superoxide in the absence and presence of unopsonised *F. nucleatum* and *P. gingivalis*. The molecular basis for this increased ROS generation has been suggested to be due to impaired activity of the antioxidant regulator nuclear factor erythroid-2-related factor 2 (Nrf2) in periodontitis neutrophils resulting in activation of the redox sensitive enzyme acid sphingomyelinase, with subsequent lipid raft formation and translocation of increased NADPH oxidase to the outer cell membrane (Dias et al. 2013).

The effect of periodontal treatment was determined in order to elucidate whether the exaggerated neutrophil ROS release in chronic periodontitis was secondary to the inflammatory reaction within the periodontal tissues during periodontal disease or the result of constitutive differences between periodontitis patients and controls. The only previous longitudinal intervention study suggested that unstimulated neutrophil extracellular ROS (isoluminol-detectable) release was constitutional but that FcγR-stimulated, luminol-detectable intracellular ROS was probably reactive in nature, being partially reduced

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following periodontal treatment and completely removed in the presence of priming doses of *P. gingivalis* and *F. nucleatum* (combined MOI = 2.25:1; Matthews et al. 2007b). However, data for FcγR-stimulated extracellular, isoluminol-detectable ROS generation was equivocal, suggesting that hyper-reactivity was unaffected by therapy but not detectable if neutrophils were primed with *P. gingivalis* and *F. nucleatum*. Our current data support a reactive basis for exaggerated superoxide release by both unstimulated patient neutrophils and those stimulated with unopsonised *F. nucleatum* and *P. gingivalis*. The discrepancy between these two studies in terms of the effect of therapy on ROS release might relate to the specificity of the chemiluminescence substrates and methods employed. Lucigenin is relatively specific for superoxide whereas isoluminol, used in the presence of horseradish peroxidase, primarily detects HOCl, which is enzymatically generated from extracellular hydrogen peroxide that may be derived from dismutation of released superoxide as well as efflux of hydrogen peroxide from the cell.

This study was limited to chronic periodontitis, which is the most prevalent form of periodontitis. In terms of ROS generation by unstimulated neutrophils from patients with aggressive disease, studies have reported higher (Gainet et al. 1998, 1999 using DCFH-DA/flow cytometry; Guentsch et al. 2009 using luminol; Iwata et al. 2009 using cytochrome c reduction), lower (Shipara et al. 1991 using cytochrome c reduction) and similar (Shapira et al. 1994 using cytochrome c reduction; Guentsch et al. 2009 using isoluminol) levels compared to those from control cells. Data from these papers on stimulated ROS generation are similarly complex with four suggesting hyper-reactivity, to histone opsonised bacteria (Shipara et al. 1994) or fMLP (Gainet et al. 1998, 1999; Iwata et al. 2009), and one showing hypo-reactivity to *P. gingivalis* and *A. actinomycetemcomitans* using luminol (predominantly intracellular ROS) but normal reactivity with isoluminol (extracellular ROS; Guentsch et al. 2009). Interestingly, neutrophils from patients with aggressive disease that were primed with

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IL-8 were hypo-reactive to fMLP (Gainet et al. 1999) whereas another study suggested that priming with *P. gingivalis* LPS was required for fMLP hyper-reactivity (Shapira et al. 1994). However, only Gainet and co-workers have investigated the effect of periodontal treatment, the success of which was not substantiated. Both studies indicated that unstimulated intracellular ROS hyperactivity was normalised post-treatment (Gainet et al. 1998, 1999). However, the effect of treatment on the hyper-reactivity of neutrophils from aggressive periodontitis patients to fMLP was not assessed but pre-treatment hypo-reactivity of IL-8-primed cells to fMLP was normalised (i.e. not detected post-therapy; Gainet et al. 1999). These data suggest that intracellular ROS generation in aggressive periodontitis may be "acquired/secondary" to the disease process rather than being "constitutive", in agreement with the data presented on extracellular release of superoxide in chronic periodontitis reported in this study, as well as those reported for intracellular, luminol-detectable ROS in chronic disease (Matthews et al. 2007b).

Interestingly, the therapy-related removal of the exaggerated superoxide release presented here contrasts with persistence of neutrophil cytokine (IL-8, TNFα, IL-1β) hyper-reactivity in response to *F. nucleatum* and *P. gingivalis*, recently demonstrated within this same volunteer cohort (Ling et al. 2015). Taken together, these data suggest that both reactive and constitutional mechanisms underpin the hyperactive/-reactive neutrophil phenotype in chronic periodontitis or that "normalisation" of excessive neutrophil cytokine production may take longer to correct than that of superoxide release.

Although there were no significant differences in plasma CRP levels between patients and controls or between sampling visits in this study, median levels were lower post- compared to pre-therapy in patients (1.80 & 1.36mg/l). In view of this apparent change, and because it has been shown that physiologically-relevant concentrations of CRP (1-10mg/l) reduce unstimulated and Fc□R-stimulated superoxide release from healthy volunteer

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neutrophils but increase their response to *F. nucleatum* (Ling et al. 2014), the relationship between superoxide release and CRP concentration was investigated. Surprisingly, analysis of the current data demonstrated a significant, moderate positive correlation between unstimulated superoxide release and plasma CRP concentrations in untreated patients that was not found post-therapy. This apparent contradiction in the data is explained by the fact that, in the present study, superoxide assays were performed on isolated and washed neutrophils in the absence of endogenous CRP. Thus, while the pre-treatment, positive relationship between unstimulated superoxide release and CRP is only a cross-sectional observation, its loss post-therapy is consistent with neutrophil superoxide "hyperactivity" actually having a reactive basis rather than being constitutional. In the light of recent studies demonstrating that physiologically relevant concentrations of CRP (1-10mg/l) reduce unstimulated intra- and extra-cellular ROS production by peripheral neutrophils (Ling et al. 2014), these data are also consistent with the concept that the increased plasma CRP seen in chronic periodontitis (reviewed by Paraskevas et al. 2008) may reduce peripheral blood neutrophil hyperactivity and offer a degree of host protection from systemic neutrophil-associated ROS release and oxidative stress *in vivo*.

In summary, these data demonstrate that peripheral blood neutrophils from untreated chronic periodontitis patients release excessive amounts of superoxide in the absence and presence of stimulation with unopsonised *F. nucleatum* and *P. gingivalis*. Successful therapy removed this exaggerated superoxide response suggesting that hyperactivity/-reactivity was not a constitutional feature of chronic periodontitis patients but secondary to periodontal inflammation. In addition, the positive relationship between unstimulated neutrophil superoxide release and plasma CRP, detected pre-therapy, is consistent with a protective role for CRP in reducing oxidative stress *in vivo*.

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TABLE LEGEND

Table 1: Clinical measures of chronic periodontitis patients and matched healthy controls. One patient (no. 13) elected to discontinue participation in the study during the treatment phase. Probing pocket depths were compared by one-way ANOVA followed by Tukey-Kramer test. All other comparisons were performed using Kruskal-Wallis test followed by Dunn's test. Clinical data for these volunteer cohorts have been published previously (Ling et al. 2015).

[*] comparison with controls

[#] comparison with chronic periodontitis before treatment

[$] comparison with chronic periodontitis after treatment; *ns* = not significant ($P>0.05$)

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Clinical measure	Chronic periodontitis patients		Controls
	Baseline (n=20)	**2 month Review (n=19)**	**Baseline (n=20)**
Probing pocket depth (mm; mean + SD)	3.0+0.8 (P<0.001)*	2.1+0.5 (P<0.001)$^{\#}$	1.5+0.4 (P<0.01)$^{\$}$
Number of probing pocket depths >4 mm (median; range)	27 (5-91) (P<0.001)*	7 (0-52) (P<0.05)$^{\#}$	0 (0-4) (P<0.01)$^{\$}$
Percentage of sites bleeding on probing (median; range)	43 (16-87) (P<0.001)*	14 (3-35) (P<0.001)$^{\#}$	2 (0-39) (ns)$^{\$}$
Gingival index (median; range)	2 (1-3) (P<0.001)*	1 (0-1) (P<0.001)$^{\#}$	1 (0-1) (ns)$^{\$}$
Plaque index (median; range)	2 (1-3) (P<0.001)*	1 (0-2) (P<0.001)$^{\#}$	1 (0-2) (ns)$^{\$}$

Table 1: Clinical measures of chronic periodontitis patients and matched healthy controls. One patient (no. 13) elected to discontinue participation in the study during the treatment phase. Probing pocket depths were compared by one-way ANOVA followed by Tukey-Kramer test. All other comparisons were performed using Kruskal-Wallis test followed by Dunn's test. Clinical data for these volunteer cohorts have been published previously (Ling et al. 2015).

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FIGURE LEGENDS:

Figure 1: *Lucigenin detectable superoxide (peak RLU) released by neutrophils from patient and healthy controls at baseline (n=20) and the 2-month review (n=19) without stimulation and in the presence of additional PBS (vehicle control), P. gingivalis (MOI 100:1), F. nucleatum (MOI 100:1) or PMA (5nM).* Box and whisker plots show median, interquartile range, maximum and minimum peak RLU. *P*-values calculated using one-tailed Wilcoxon. Stimulated levels of superoxide were significantly greater than those for PBS vehicle control for all stimuli, at baseline and review, for both patient and control groups ($P<0.05$; Friedman followed by Dunn's multiple comparisons test).

Figure 2: *Effect of periodontal treatment on unstimulated and stimulated superoxide release from patient neutrophils (n=19) without stimulation and in the presence of additional PBS (vehicle control), P. gingivalis (MOI 100:1), F. nucleatum (MOI 100:1) and PMA (5nM).* Data presented as patient RLU as a percentage of paired control RLU at baseline and the 2-month review. Box and whisker plots show median, interquartile range, maximum and minimum. *P-values* calculated using one-tailed Wilcoxon.

Figure 3: *Plasma hsCRP concentrations for patients and healthy controls at baseline and the 2-month review (n=19).* Box and whisker plots show median, interquartile range, maximum and minimum. Outlying value (calculated as >1.5x interquartile range) is shown as a solid circle (•) above the y-axis maximum.

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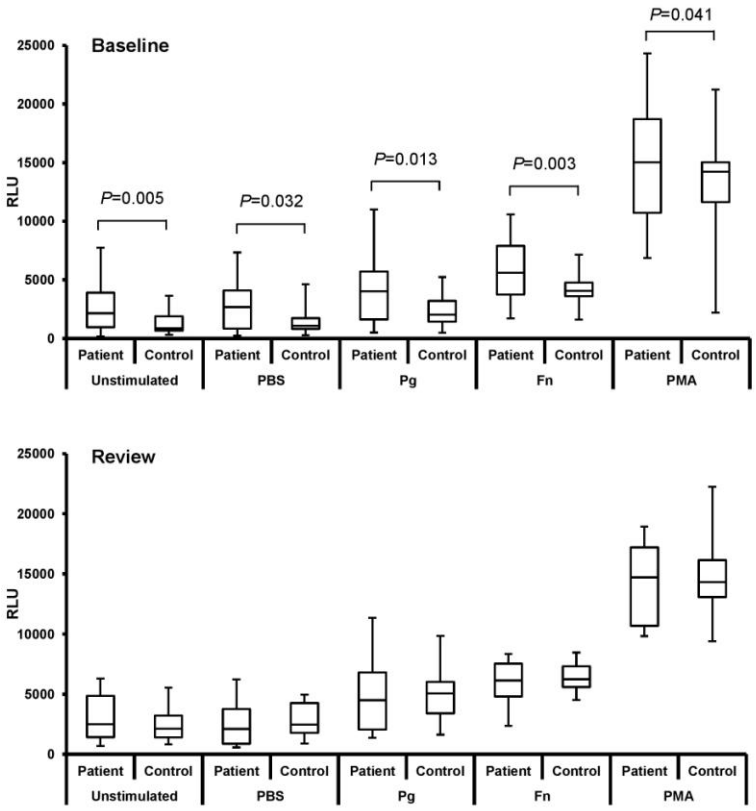
Figure 4: *Scatterplots and regression analyses of unstimulated patient (n=18) and control (n=17) neutrophil chemiluminescence and plasma hsCRP concentration (mg/l) at baseline and review. P*-values corrected to adjust for multiple comparisons.



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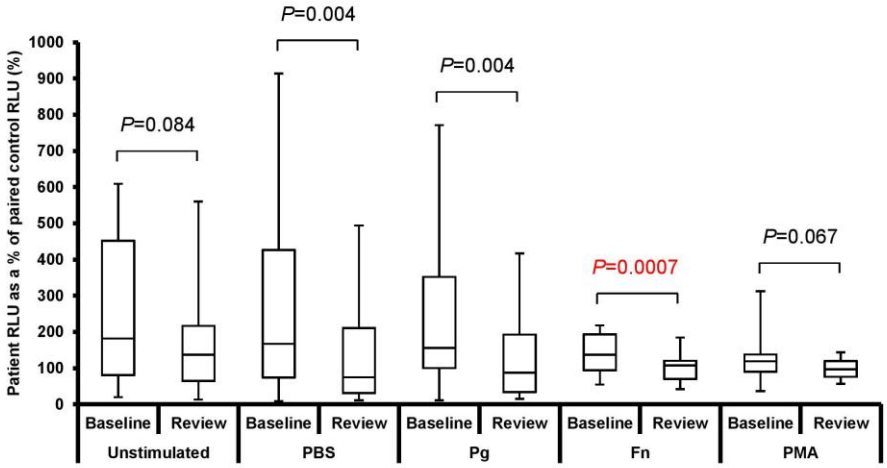

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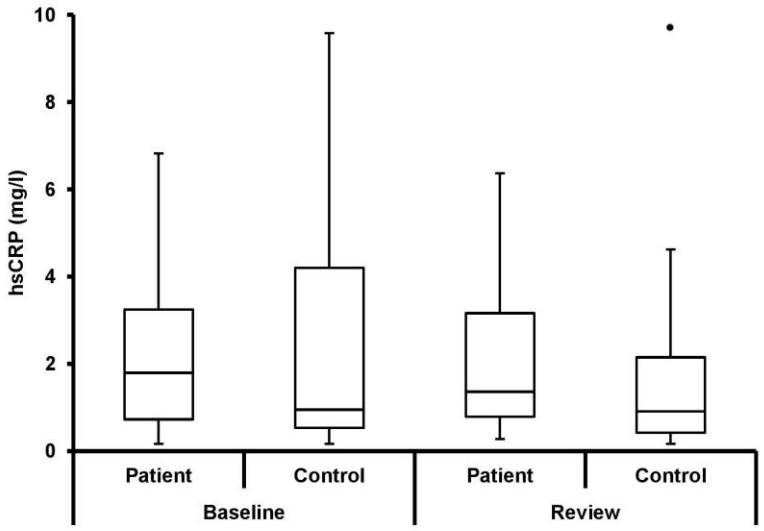

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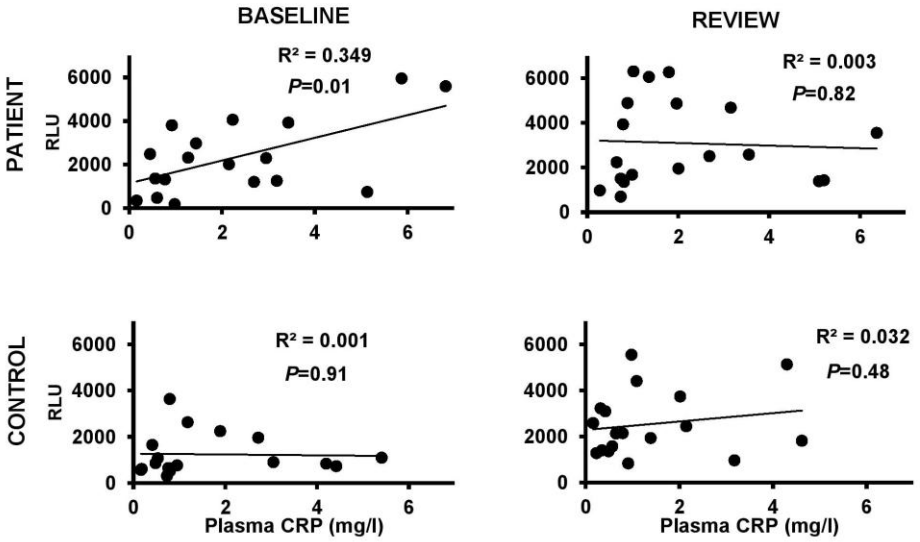

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