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Peripheral Blood Neutrophil Extracellular Trap Production and Degradation in Chronic Periodontitis

Running title: NETs in Periodontitis

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Abstract

Aims: To investigate ex vivo peripheral neutrophil extracellular trap (NET) production and their subsequent degradation by plasma in chronic periodontitis patients, and periodontally and systemically healthy-matched controls.

Materials and methods: Chronic periodontitis patient and control (n=40 pairs) peripheral blood neutrophils were stimulated for NET quantification. A subset of patients received non-surgical periodontal therapy (n=19) and NETs were quantified 3-months later alongside controls. Blood plasma was collected from patients and controls to quantify plasma-induced NET degradation (n=19 pairs). Subsequent experiments quantified plasma concentrations of DNase-1, immunoglobulin G (IgG), free light chains (FLCs) and cystatin C.

Results: No differences were observed in NET production between patients and controls. However, NET production decreased significantly in patient's post-treatment. Plasma NET degradation was significantly lower in patients than controls, which may be due to significantly reduced DNase-1 levels as demonstrated, or potentially due to elevated IgG/FLC concentrations in patients. NET degradation post-periodontal treatment was comparable between patients and controls.

Conclusions: NET production was comparable between patients and controls; however, non-surgical therapy causes attenuated NETs. NET degradation by plasma is impaired in untreated chronic periodontitis, potentially increasing the chronic NET burden, which may enhance antimicrobial function, or conversely, increase the risk of auto-immune/inflammatory responses.

Clinical relevance

Scientific rationale for study: Periodontitis is characterised by altered neutrophilic responses to periodontal pathogens, involving hyper-reactivity in reactive oxygen species (ROS). ROS-dependent NET release enhances antimicrobial functions, but has been linked to autoimmune disease. This study aimed to characterise NET release and removal in periodontitis.

Principal findings: No difference in NET quantification was observed pre-treatment, compared with healthy controls, but successful periodontal treatment reduced NET production. However, plasma-derived NET degradation was decreased in patients compared with controls.

Practical implications: Excess NET production or retention within tissues may serve as an important antimicrobial strategy in untreated periodontitis; conversely this may contribute to the pathogenesis of periodontitis via autoimmune responses.

Introduction

Periodontitis is a chronic inflammatory disease, initiated by dysbiosis within the subgingival plaque biofilm and progressed by a damaging dysregulation of the hosts' inflammatory-immune response (Hajishengallis, 2014). This results in chronic, destructive and non-resolving inflammation of the periodontal tissues (Bascones-Martínez and Figuero-Ruiz, 2003). In addition to periodontal tissue destruction, periodontitis is associated with other inflammatory diseases of ageing, such as diabetes mellitus and rheumatoid arthritis (Chapple and Genco, 2013, de Pablo et al., 2009).

Neutrophils are the principal inflammatory cell in active periodontal lesions. Peripheral blood neutrophils (PBN) isolated from periodontitis patients are hyperactive, with regard to reactive oxygen species (ROS) generation in the absence of exogenous stimulation, and hyper-reactive, exhibiting increased ROS production when subjected to stimulation (Matthews et al., 2007a, Matthews et al., 2007b). The underlying mechanisms responsible for this neutrophil hyper-responsivity remain to be elucidated, however there are indications for both constitutive abnormalities in periodontitis neutrophils, and also evidence for neutrophil priming by serum-derived cytokines (Dias et al., 2011, Ling et al., 2015). Resultant extracellular ROS release contributes to oxidative stress and downstream host collateral tissue damage (Chapple and Matthews, 2007).

Neutrophils also have alternative defence mechanisms at their disposal, including the production of neutrophil extracellular traps (NETs). NETs constitute a highly conserved anti-microbial strategy that results from inflammatory-immune cells releasing their de-condensed nuclear chromatin (DNA) into the extracellular space, where they immobilise and kill pathogens (Brinkmann et al., 2004). NET structures are reportedly present in plaque biofilms (Hirschfeld et al., 2015), as well as in the gingival pocket epithelium and in pus from the gingival crevice (Vitkov et al., 2010). We have also demonstrated NET-like structures within periodontally inflamed tissues (Cooper et al., 2013) and proposed that in addition to NETs' initially beneficial role, abnormal NET release and/or accumulation within periodontal tissues may trigger auto-inflammatory responses and thus a destructive inflammatory-immune response (Cooper et al., 2013, de Pablo et al., 2013). Notably NET production has been

associated with the pathogenesis of several other autoimmune diseases, such as systemic lupus erythematosus (SLE) (Hakkim et al., 2010, Leffler et al., 2012).

NET release requires NADPH-oxidase activation, resulting in the sequential production of superoxide and hydrogen peroxide, which is subsequently converted by myeloperoxidase (MPO) to hypochlorous acid (HOCl) (Fuchs et al., 2007, Palmer et al., 2012). There is currently no literature investigating NET production in chronic periodontitis patients. Given that periodontal PBNs demonstrate ROS hyper-responsivity, it is important to determine NET production in periodontitis patients.

Specific mechanisms contributing to NET clearance from tissues remain to be fully elucidated; however reports indicate that NETs are degraded by host and bacterial DNases (Meng et al., 2012). Recently it has been reported that the DNase-containing sera of systemically healthy individuals are capable of degrading NETs, however this mechanism is compromised in subsets of patients with SLE (Hakkim et al., 2010, Leffler et al., 2012). Impaired NET degradation is believed to result from formation of a physical barrier, for example, from elevated levels of anti-NET immunoglobulins, which prevent enzymatic NET degradation (Hakkim et al., 2010).

Notably, immunoglobulins comprise 2 heavy and 2 light chains (either kappa or lambda), and during their normal synthesis by B cells, light chains are produced in excess and released into circulation as free light chains (FLCs) (Solomon, 1985). FLC overproduction has been reported in several chronic diseases, which may be the result of chronic immune stimulation (Brebner and Stockley, 2013). However, the association between FLCs and NET degradation has not been investigated previously.

De Pablo et al., (2009) demonstrated that periodontitis patients produce serum autoantibodies in response to citrullinated and uncitrullinated peptides. Thus, whilst increased NET production or retention within periodontal tissues may serve to prolong NET antimicrobial function, NET-derived citrullinated peptides may concomitantly facilitate autoantibody generation and collateral host periodontal tissue damage (Cooper et al., 2013, White et al., 2016).

The purpose of this study, therefore, was to determine whether NET release is altered in chronic periodontitis patients, compared with periodontally healthy age- and gender-matched controls. In a subset of patients, NET release was quantified following successful periodontal therapy and compared with the same healthy controls. Additionally, NET degradation by patient and control plasma pre- and post-treatment was investigated. Subsequent experiments sought to establish whether an immunoglobulin or immunoglobulin-FLC barrier prevents the enzymatic removal of NETs in periodontitis patients. This analysis involved the quantification of immunoglobulin G (IgG) and FLCs in patient and control plasma samples.

Materials and Methods

Study populations: Volunteers were diagnosed with chronic periodontitis at Specialist Periodontal Centres in Birmingham, UK (n=20) and Thessaloniki, Greece (n=20). Periodontitis was defined as the presence of at least two non-adjacent sites with probing pocket depths >4mm, along with radiographic bone loss $\geq$30% of the root length in accordance with the consensus criteria of the European Federation of Periodontology (Tonetti and Claffey, 2005). Healthy controls were recruited from dental staff at Birmingham, UK and Thessaloniki, Greece. All volunteers were age- ($\pm$5 yrs) and gender-matched to their corresponding patient pair. All participants were systemically healthy, had not smoked for at

least 10 years, were not pregnant and were not taking any medication that may influence periodontal health. Ethical approval for the study was obtained from West Midlands Research Ethics Committee, UK (10/H1208/48), and the School of Dentistry ethical committee at Aristotle University, Thessaloniki, Greece (317/23/1/2013).

Non-surgical periodontal therapy was provided in Birmingham, UK, which 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.

NET release was determined at both centres in periodontitis patients and controls prior to periodontal treatment using the same laboratory protocols and reagents. To ensure consistency of approach and calibration of centres DS (Thessaloniki) visited and trained with PW (Birmingham) in all NET protocols. Analyses of post-treatment NET release, NET degradation, plasma IgG, FLC and Cystatin C concentrations were only performed on samples from Birmingham volunteers for logistical reasons.

Collection of peripheral blood and neutrophil isolation: Venous blood was collected in lithium heparin vacutainers from patients and healthy controls in parallel. Neutrophils were isolated as described previously (Matthews et al., 2007a, Oh et al., 2008). In brief, neutrophils were isolated by centrifugation of venous blood over discontinuous Percoll density gradients. Following centrifugation, the neutrophil layer was aspirated and added to lysis buffer to remove unwanted erythrocytes. The cells were subjected to a second lysis buffer wash, prior to a final PBS wash. The supernatants were discarded and the cells were re-suspended in PBS prior to counting. Cell viability and purity were confirmed by trypan blue exclusion and flow cytometry, respectively, and was typically >98%.

Collection of plasma: Venous blood was collected in lithium heparin vacutainers from patients and controls. Plasma was isolated by centrifugation (1,000 rcf for 30 min at 4°C), aliquoted into cryotubes (500 µl in each) and stored at -80°C.

Quantification of NETs: NET production was quantified as previously described (Palmer et al., 2012). Briefly, neutrophils were re-suspended in RPMI-1640 and added to a pre-blocked 96-well microplate (1×10^5 in 150 µl per well). Following a 30 min baseline incubation period (37°C), selected wells were stimulated with 50 nM phorbol myristate acetate (PMA) or 0.75 mM HOCl. Cells were incubated for 3 hours (37°C, 5% CO_2) and NET DNA was subsequently digested by the addition of micrococcal nuclease (MNase) (15 µl at 1 unit/ml) for 15 min. Cells were pelleted (10 min at 1,800 rcf) and the supernatant (150 µl) transferred to a black microplate prior to the addition of Sytox green (Life Technologies; 15 µl, 10µM). Fluorescence was read in arbitrary fluorescence units (AFU) using a fluorometer (Birmingham - Twinkle LB970, Berthold Technologies; Thessaloniki - Victor X5, PerkinElmer Inc. USA). NET production by patients and controls was compared by fluorescence microscopy, whereby isolated neutrophils ($1x10^5$) were added to clear 24-well plates in 260 µl RPMI-1640 and stimulated as previously described. Following stimulation, cells were incubated for 3 hours (37°C, 5% CO_2), after which, 25 µl of 10 µM Sytox green was added to each well and NETs visualised (epi-fluorescent microscope, Nikon Eclipse TE300). A calibration curve was generated by quantifying Sytox green stained-calf thymus DNA to enable data normalisation between the centres. This approach provided interpolated values for NET production in µg/ml.

Quantification of NET degradation by plasma: Neutrophils were isolated from a systemically and periodontally healthy individual and stimulated for NETs with HOCl as previously described (Palmer et al., 2012). Post-NET release, previously isolated plasma

from periodontitis patients and matched controls were defrosted and diluted to 10% in PBS (Hakkim et al., 2010). Plasma was added to wells in 50 µl aliquots and incubated for 3 hours (37°C, 5% CO_2). Selected wells containing NETs were treated with 1 unit/ml MNase for 10 min to act as a positive control for NET degradation, and were considered as the "100% NET degradation" standard. After 3 hours incubation, plates were centrifuged (10 min at 1800 rcf) and the number of NETs liberated into the supernatant, as a result of incubation with plasma, were quantified as described previously.

Quantification of plasma DNase-1, IgG subclasses 1–4, FLCs and cystatin C
DNase-I levels were measured in previously collected patient and control plasma samples (n=19 pairs; pre-treatment only) using a fluorescence-based DNase detection kit (Jena Bioscience (Jena, Germany), in which plasma (10 µl) was incubated with a fluorescent DNase probe. After incubation (30 min at 37°C, 5% CO_2), fluorescence was detected using a fluorometer under the same conditions as for NET quantification.

Previously collected plasma samples were analysed by The Binding Site Group Ltd (Birmingham, UK). IgG subclasses 1–4 were quantified using the SPAPLUS® analyser, in addition FLC kappa and FLC lambda concentrations were quantified using the SPAPLUS® turbidimeter (Freelite, The Binding Site Group Ltd). Cystatin C levels were also uantified using the SPAPLUS® assay. Cystatin C levels were measured to determine whether excessive plasma FLCs in periodontitis patients might have resulted from impaired renal FLC clearance.

Data analysis: Statistical analyses in GraphPad Prism 5 included NET production pre- and post-treatment, NET degradation, and IgG and FLC quantification using Mann-Whitney tests (unless otherwise stated).

Results

Clinical measures including longitudinal changes following periodontal treatment. Clinical measures for the chronic periodontitis patients and matched controls recruited in the UK and Greece are presented in Table 1. All clinical measures of periodontal disease were significantly reduced at the post-treatment review ($p<0.05$), consistent with the literature and with patient levels becoming comparable to those of controls (Ling et al., 2015, Ling et al., 2016).

Periodontitis is not associated with elevated NET production by peripheral blood neutrophils. No significant difference was measured in extracellular DNA release from PBNs in patients compared with controls. This was evident in both patient populations (Birmingham, UK – n=20; Thessaloniki, Greece – n=20) for PBS-treated (negative control, *p=0.89*) as well as PMA- *(p=0.52*) and HOCl-stimulated *(p=0.73*) PBNs (Figure 1a). Comparable NET production between patients and controls was observed in both patient populations when analysed separately, as well as when pooled. Similarly, representative micrographs of sytox-stained DNA showed the characteristic network of extracellular DNA strands, with no qualitative differences in NET morphology between patients and controls (representative images n=3) (Figure 1b).

NET production significantly decreased following successful periodontal treatment. In a subset of patients from Birmingham, UK, NET assays were repeated between 2-months (minimum) and 3-months (maximum) following successful periodontal treatment and assayed in parallel with their healthy controls (n=19 pairs). To account for day-to-day

neutrophil variability pre- and post-treatment, NET production by healthy controls was expressed as a ratio with that of patients (assayed simultaneously). NET production in patients neutrophils for PBS- as well as PMA- and HOCl-stimulated cells significantly decreased relative to healthy controls as a result of treatment (Mann-Whitney ***p=0.03*, ***p=0.008*, **p*=0.*043* for PBS, PMA and HOCl, respectively) (Figure 2).

Plasma-derived NET degradation is significantly reduced in periodontitis patients, but restored following successful treatment. There was a significant difference in NET degradation between patient and control plasma (n=19 pairs; Mann-Whitney, *****p=0.0001*), with plasma derived from patients degrading significantly fewer NETs. It was noted that patients exhibited a larger distribution of NET degradation compared with controls (Figure 4a). Plasma degradation assays were repeated between 2-months (minimum) and 3-months (maximum) following successful treatment and assayed in parallel with the same healthy controls (n=19 pairs). Plasma-derived NET degradation in patients was comparable with that of their healthy counterparts (Mann-Whitney, *p=0.124*). However, the range of patient NET degradation still exhibited greater heterogeneity compared with healthy controls (Figure 3).

Comparison of plasma DNase-1, IgG, FLC and cystatin C concentrations in periodontitis patients and matched periodontally healthy controls.

DNase-I concentrations measured in pre-treatment patient and control plasma samples (n=19 pairs) were significantly lower in patient samples compared with controls (Mann-Whitney, *p=0.04*) (Figure 4).

Protein turbidimetric analysis demonstrated that plasma IgG concentrations were consistently higher in patients, compared with controls. This was statistically significant for IgG subclass 2 pre-treatment (Mann-Whitney **p=0.038*), IgG subclass 3 pre- and post-treatment (Mann-Whitney ****p=0.0007*, ***p=0.0042* pre and post-treatment, respectively), and IgG subclass 4 post-treatment (Mann-Whitney **p=0.042*) (Figure 5).

FLC concentrations were also consistently higher in patients compared with controls. This was statistically significant for FLC kappa pre- and post-treatment (Mann-Whitney **p=0.049*, **p=0.048* pre- and post-treatment, respectively), the kappa/lambda ratio pre-treatment (Mann-Whitney **p=0.047*), and summated FLC levels pre-treatment (Mann-Whitney **p=0.032* (Figure 6).

There was however no significant difference in cystatin C levels (mg/litre) between control and patient plasma samples both pre- and post-treatment (Mann-Whitney *p=0.74*, *p=0.82* pre- and post-treatment, respectively, n=19 matched pairs; data not shown), demonstrating no impairment of renal FLC clearance.

Discussion

Neutrophils play a pivotal role within the periodontal lesion in response to colonisation by pathogenic bacteria (Miyasaki, 1991, Schenkein, 2006). However, it is widely accepted that a dysregulated neutrophilic response contributes to tissue destruction in chronic periodontitis (Van Dyke et al., 1993). Neutrophil hyper-responsivity with regard to ROS production is likely to contribute to the degradation of periodontal tissues, and given the recently reported deficiencies in neutrophil chemotactic accuracy in periodontitis patients (Roberts et al., 2015), may increase neutrophil tissue transit times and consequently cause collateral tissue damage (Matthews et al., 2007a, Matthews et al., 2007b, Waddington et al., 2000).

The putative role of NETs is to immobilise and kill pathogens by co-localising with, and tethering the microorganisms. Currently, it is unknown whether NETs have the capacity to kill all bacterial species, and it is postulated that NETs may only immobilise bacteria (Beiter et al., 2006, Menegazzi et al., 2012). It is recognised that NET production relies upon ROS, specifically NADPH-oxidase activation and the subsequent production of hydrogen peroxide (Fuchs et al., 2007). Recent reports have confirmed this mechanism and also identified the involvement of MPO in NET formation (Kirchner et al., 2012, Metzler et al., 2011, Papayannopoulos et al., 2010, Palmer et al., 2012). Thus, we hypothesised that ROS hyperactivity and -reactivity in periodontitis patient neutrophils would translate to elevated NETs.

NETs have previously been shown to exist in purulent exudate from periodontal pockets (Vitkov et al., 2009, 2010), and this is corroborated by recent findings that demonstrate neutrophils infiltrate the dental plaque and subsequently release NET structures (Hirschfeld et al., 2015). Further support for the presence of NET production in periodontitis is that periodontal pockets reportedly provide an appropriate environment for ROS production, by the provision of sufficient oxygen tension and pH (Mettraux et al., 1984, Eggert et al., 1991). However, alternative NADPH-oxidase-independent pathways leading to NET production have also been reported (Parker et al., 2012, Pilsczek et al., 2010).

No differences, however, were measured in PBN NET production between patients and controls. A potential explanation could relate to a recently reported self-protective mechanism within glutathione deficient periodontitis neutrophils, involving re-siting of certain NADPH-oxidase components to outer cell membrane lipid rafts (Dias et al., 2013). This mechanism was reported in PBN from periodontitis patients following the discovery that these cells were oxidatively stressed, as evidenced by an altered redox state. Thus, when challenged by a further stimulus the periodontitis patient neutrophils release superoxide extracellularly to avoid further oxidative stress and risk of cell necrosis. It is therefore plausible that re-direction of ROS to the extracellular space may limit the intracellular ROS levels available for NET production (Dias et al., 2013, Guichard et al., 2005).

To investigate NET production further, their quantification post-treatment was determined in a sub-set of periodontitis patients' neutrophils and compared with their respective healthy controls (n=19 pairs). A significant decrease in NET release was evident in periodontitis patient neutrophils following successful therapy. Previous findings have demonstrated that successful non-surgical treatment results in a reduction in neutrophil ROS hyper-reactivity following FcγR-stimulation, unlike baseline ROS hyperactivity, which did not normalise as a result of treatment (Matthews et al., 2007a). It has been proposed that priming of PBNs by cytokines within blood plasma may contribute to the FcγR-mediated neutrophil hyper-reactive phenotype observed in periodontitis, which is reversible with effective treatment

(Dias et al., 2011). Reduced neutrophil priming and ROS production following successful treatment may represent one explanation for the reduced post-therapy NET production.

NET degradation by chronic periodontitis patients' plasma was also quantified, and demonstrated that NET degradation was significantly lower in a subset of chronic periodontitis patients pre-treatment, relative to controls. To our knowledge this is the first report evaluating NET removal in periodontitis patients. Mechanisms involved in NET clearance remain to be thoroughly elucidated; however reports indicate that host-derived DNases degrade NETs to affect their removal from tissues where high levels of citrullination may potentiate autoimmune responses against citrullinated proteins. Attenuated NET degradation has been reported in SLE, and has been suggested to result from either the presence of DNase-1 inhibitors in patient serum or from some form of physical molecular barrier that prevents DNase-1 access to cleavage sites on the NET backbone (Hakkim et al., 2010, Leffler et al., 2012).

We report, for the first time, lower plasma DNase-1 concentrations in patients compared with controls, as well as elevated Kappa FLCs, Kappa/Lambda ratio's and summated FLCs, which may individually or in combination explain the decreased NET degradation measured in patients. Notably, cystatin C levels were comparable between patients and controls, indicating that elevated FLCs are not the result of insufficient renal FLC clearance, but likely the result of increased FLC production by B cells, which are the dominant lymphocyte in active periodontitis lesions. Increased circulating IgG and FLCs in patients may translate to IgG and/or FLC binding to NET structures, given the significantly elevated serum auto-antibody levels to citrullinated histones of DNA already reported by our group in periodontitis patients (dePablo et al. 2013). Such immunoglobulin binding may either tether bacteria to NETs, facilitating their destruction, or conversely may provide a physical barrier that prevents the degradation of NETs by DNase-1, as demonstrated in SLE (Leffler et al. 2013). However, further extensive studies into the role of IgGs and FLCs in NET degradation are warranted and currently underway to further elucidate the mechanisms of decreased NET degradation in periodontitis.

Attenuated NET removal may constitute an antimicrobial host response during infection, as NETs can prevent microbial dissemination by entrapping a range of periodontal bacteria (Brinkman *et al.*, 2004). The host's ability to adjust the extracellular "life-span" of NETs in response to the microbial biofilm may also explain why no differences in PBN NET release were observed between patients and controls. Conversely, an accumulation of NETs as a result of impaired clearance may impact deleteriously upon the host, as it has been reported that NETs provide a source of autoantigens, which may trigger autoimmune responses and facilitate disease progression (Krysko et al., 2006, Lande et al., 2011, Spengler et al., 2015). It is also possible that NET production in periodontal tissues may serve as a plausible mechanism for the production of anti-citrullinated protein antibodies (ACPA) following a break in immune tolerance to NET-derived citrullinated (de Pablo et al., 2009), and this process may be exacerbated if NETs are not removed in a timely manner. Indeed this is one plausible explanation for the higher prevalence of ACPAs and higher serum ACPA titres in periodontitis patients compared with controls (de Pablo et al., 2013).

Conclusions

The reported studies have demonstrated that NET production is not significantly different between patients and controls, however patient NET production decreased following

successful periodontal therapy. Notably, in a subset of patients in which NET degradation by plasma was investigated, patients were less able to degrade NETs, compared with controls. The reported decreased plasma DNase-levels may explain, in part, the impeded NET degradation observed in patients. Another plausible mechanism may be the reported increased plasma IgG and FLC levels, potentially generating a physical barrier to prevent NET removal in patients. Further experiments are warranted to confirm these hypotheses. Notably, NET degradation assays post-treatment demonstrated comparable results between patients and controls. Taken together, the data provide new insights into the myriad of abnormal neutrophil behaviours displayed during the pathogenesis of periodontitis, specifically in relation to NET production and clearance.

References

Bascones-Martínez, A. & Figuero-Ruiz, E. (2003) Periodontal diseases as bacterial infection. *Medicina oral, patologia oral y cirugia bucal* **9,** 101-107; 192-100.

Beiter, K., Wartha, F., Albiger, B., Normark, S., Zychlinsky, A. & Henriques-Normark, B. (2006) An endonuclease allows Streptococcus pneumoniae to escape from neutrophil extracellular traps. *Current Biology* **16,** 401-407.

Brebner, J. A. & Stockley, R. A. (2013) Polyclonal free light chains: a biomarker of inflammatory disease or treatment target? *F1000 medicine reports* **5**.

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D. S., Weinrauch, Y. & Zychlinsky, A. (2004) Neutrophil extracellular traps kill bacteria. *science* **303,** 1532-1535.

Chapple, I. L. & Genco, R. (2013) Diabetes and periodontal diseases: consensus report of the Joint EFP/AAP Workshop on Periodontitis and Systemic Diseases. *Journal of clinical periodontology* **40**.

Chapple, I. L. & Matthews, J. B. (2007) The role of reactive oxygen and antioxidant species in periodontal tissue destruction. *Periodontology 2000* **43,** 160-232.

Cooper, P. R., Palmer, L. J. & Chapple, I. L. (2013) Neutrophil extracellular traps as a new paradigm in innate immunity: friend or foe? *Periodontology 2000* **63,** 165-197.

de Pablo, P., Chapple, I. L., Buckley, C. D. & Dietrich, T. (2009) Periodontitis in systemic rheumatic diseases. *Nature Reviews Rheumatology* **5,** 218-224.

de Pablo, P., Dietrich, T., Chapple, I. L., Milward, M., Chowdhury, M., Charles, P. J., Buckley, C. D. & Venables, P. J. (2013) The autoantibody repertoire in periodontitis: a role in the induction of autoimmunity to citrullinated proteins in rheumatoid arthritis? *Annals of the rheumatic diseases***,** annrheumdis-2012-202701.

Dias, I., Chapple, I., Milward, M., Grant, M. M., Hill, E., Brown, J. & Griffiths, H. R. (2013) Sulforaphane restores cellular glutathione levels and reduces chronic periodontitis neutrophil hyperactivity in vitro. *PLoS One* **8,** e66407.

Dias, I. H., Matthews, J. B., Chapple, I. L., Wright, H. J., Dunston, C. R. & Griffiths, H. R. (2011) Activation of the neutrophil respiratory burst by plasma from periodontitis patients is mediated by pro-inflammatory cytokines. *Journal of clinical periodontology* **38,** 1-7.

Fuchs, T. A., Abed, U., Goosmann, C., Hurwitz, R., Schulze, I., Wahn, V., Weinrauch, Y., Brinkmann, V. & Zychlinsky, A. (2007) Novel cell death program leads to neutrophil extracellular traps. *The Journal of cell biology* **176,** 231-241.

Guichard, C., Pedruzzi, E., Dewas, C., Fay, M., Pouzet, C., Bens, M., Vandewalle, A., Ogier-Denis, E., Gougerot-Pocidalo, M.-A. & Elbim, C. (2005) Interleukin-8-induced priming of neutrophil oxidative burst requires sequential recruitment of NADPH oxidase components into lipid rafts. *Journal of Biological Chemistry* **280,** 37021-37032.

Hajishengallis, G. (2014) Immunomicrobial pathogenesis of periodontitis: keystones, pathobionts, and host response. *Trends in immunology* **35,** 3-11.

Hakkim, A., Fürnrohr, B. G., Amann, K., Laube, B., Abed, U. A., Brinkmann, V., Herrmann, M., Voll, R. E. & Zychlinsky, A. (2010) Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis. *Proceedings of the National Academy of Sciences* **107,** 9813-9818.

Hirschfeld, J., Dommisch, H., Skora, P., Horvath, G., Latz, E., Hoerauf, A., Waller, T., Kawai, T., Jepsen, S. & Deschner, J. (2015) Neutrophil extracellular trap formation in supragingival biofilms. *International Journal of Medical Microbiology*.

Kirchner, T., Möller, S., Klinger, M., Solbach, W., Laskay, T. & Behnen, M. (2012) The impact of various reactive oxygen species on the formation of neutrophil extracellular traps. *Mediators of inflammation* **2012**.

Krysko, D. V., D'Herde, K. & Vandenabeele, P. (2006) Clearance of apoptotic and necrotic cells and its immunological consequences. *Apoptosis* **11,** 1709-1726.

Lande, R., Ganguly, D., Facchinetti, V., Frasca, L., Conrad, C., Gregorio, J., Meller, S., Chamilos, G., Sebasigari, R. & Riccieri, V. (2011) Neutrophils activate plasmacytoid dendritic cells by releasing self-DNA–peptide complexes in systemic lupus erythematosus. *Science translational medicine* **3,** 73ra19-73ra19.

Leffler, J., Martin, M., Gullstrand, B., Tydén, H., Lood, C., Truedsson, L., Bengtsson, A. A. & Blom, A. M. (2012) Neutrophil extracellular traps that are not degraded in systemic lupus erythematosus activate complement exacerbating the disease. *The Journal of Immunology* **188,** 3522-3531.

Leffler, J., Gullstrand, B., Jönsen, A., Nilsson, J-A., Martin, M., Blom, A.M., Bengtsson, A.A. (2013) Degradation of neutrophil extracellular traps co-varies with disease activity in patients with systemic lupus erythematosus. **15,** R84.

Ling, M. R., Chapple, I. L. & Matthews, J. B. (2015) Peripheral blood neutrophil cytokine hyper-reactivity in chronic periodontitis. *Innate immunity* **21,** 714-725.

Ling, M. R., Chapple, I. L. & Matthews, J. B. (2016) Neutrophil superoxide release and plasma C-reactive protein levels pre- and post-periodontal therapy. *Journal of clinical periodontology* **CPE-02-16 (in press)**.

Matthews, J., Wright, H., Roberts, A., Cooper, P. & Chapple, I. (2007a) Hyperactivity and reactivity of peripheral blood neutrophils in chronic periodontitis. *Clinical & Experimental Immunology* **147,** 255-264.

Matthews, J., Wright, H., Roberts, A., Ling-Mountford, N., Cooper, P. & Chapple, I. (2007b) Neutrophil hyper-responsiveness in periodontitis. *Journal of dental research* **86,** 718-722.

Menegazzi, R., Decleva, E. & Dri, P. (2012) Killing by neutrophil extracellular traps: fact or folklore? *Blood* **119,** 1214-1216.

Meng, W., Paunel-Gorgulu, A., Flohé, S., Hoffmann, A., Witte, I., MacKenzie, C., Baldus, S. E., Windolf, J. & Logters, T. (2012) Depletion of neutrophil extracellular traps in vivo results in hypersusceptibility to polymicrobial sepsis in mice. *Crit Care* **16,** R137.

Metzler, K. D., Fuchs, T. A., Nauseef, W. M., Reumaux, D., Roesler, J., Schulze, I., Wahn, V., Papayannopoulos, V. & Zychlinsky, A. (2011) Myeloperoxidase is required for neutrophil extracellular trap formation: implications for innate immunity. *Blood* **117,** 953-959.

Miyasaki, K. T. (1991) The neutrophil: mechanisms of controlling periodontal bacteria. *Journal of periodontology* **62,** 761-774.

Oh, H., Siano, B. & Diamond, S. (2008) Neutrophil isolation protocol. *JoVE (Journal of Visualized Experiments)***,** e745-e745.

Palmer, L., Cooper, P., Ling, M., Wright, H., Huissoon, A. & Chapple, I. (2012) Hypochlorous acid regulates neutrophil extracellular trap release in humans. *Clinical & Experimental Immunology* **167,** 261-268.

Papayannopoulos, V., Metzler, K. D., Hakkim, A. & Zychlinsky, A. (2010) Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. *The Journal of cell biology* **191,** 677-691.

Parker, H., Dragunow, M., Hampton, M. B., Kettle, A. J. & Winterbourn, C. C. (2012) Requirements for NADPH oxidase and myeloperoxidase in neutrophil extracellular trap formation differ depending on the stimulus. *Journal of leukocyte biology* **92,** 841-849.

Pilsczek, F. H., Salina, D., Poon, K. K., Fahey, C., Yipp, B. G., Sibley, C. D., Robbins, S. M., Green, F. H., Surette, M. G. & Sugai, M. (2010) A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to Staphylococcus aureus. *The Journal of Immunology* **185,** 7413-7425.

Roberts, H. M., Ling, M. R., Insall, R., Kalna, G., Spengler, J., Grant, M. M. & Chapple, I. L. (2015) Impaired neutrophil directional chemotactic accuracy in chronic periodontitis patients. *Journal of clinical periodontology*.

Schenkein, H. A. (2006) Host responses in maintaining periodontal health and determining periodontal disease. *Periodontology 2000* **40,** 77-93.

Solomon, A. (1985) Light chains of human immunoglobulins. *Methods in enzymology* **116,** 101.
Spengler, J., Lugonja, B., Jimmy Ytterberg, A., Zubarev, R. A., Creese, A. J., Pearson, M. J., Grant, M. M., Milward, M., Lundberg, K. & Buckley, C. D. (2015) Release of Active Peptidyl Arginine Deiminases by Neutrophils Can Explain Production of Extracellular Citrullinated Autoantigens in Rheumatoid Arthritis Synovial Fluid. *Arthritis & Rheumatology* **67,** 3135-3145.
Tonetti, M. & Claffey, N. (2005) Advances in the progression of periodontitis and proposal of definitions of a periodontitis case and disease progression for use in risk factor research. *Journal of clinical periodontology* **32,** 210-213.
Van Dyke, T. E., Lester, M. A. & Shapira, L. (1993) The Role of the Host Response in Periodontal Disease Progression: Implications for Future Treatment Strategies*. *Journal of periodontology* **64,** 792-806.
Vitkov, L., Klappacher, M., Hannig, M. & Krautgartner, W. D. (2010) Neutrophil fate in gingival crevicular fluid. *Ultrastructural pathology* **34,** 25-30.
Waddington, R., Moseley, R. & Embery, G. (2000) Reactive oxygen species: a potential role in the pathogenesis of periodontal diseases. *Oral diseases* **6,** 138-151.
White, P., Chicca, I., Cooper, P., Milward, M. & Chapple, I. (2016) Neutrophil Extracellular Traps in Periodontitis A Web of Intrigue. *Journal of dental research* **95,** 26-34.

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Figure Legends

Table 1: Clinical measures of chronic periodontitis patients & matched healthy controls recruited in the UK (baseline n=20; review n=19) & Greece (baseline n=20). Volunteer ages (at time of sampling) were compared by Mann-Whitney test (no significant differences within or between groups). 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 the UK volunteer cohorts have been published previously (Ling et al., 2015)

[*]probing pocket depth ≥4mm, gingival index, plaque index & post-treatment review data were collected solely from the UK cohort.
[#]comparison with corresponding controls ($p<0.05$).
[$]comparison with chronic periodontitis before treatment ($p<0.05$).
Abbreviations: NR, not reported; SD, standard deviation; UK, United Kingdom.

Figure 1: NET production in pre-treatment periodontitis patients and healthy controls
(a) Pre-treatment NET production was quantified fluorometrically by Sytox Green staining following a 3-hour incubation period after neutrophil stimulation (UK and Greek cohort). (i) PBS (unstimulated negative control), (ii) PMA (50 nM) and (iii) HOCl (0.75mM). Statistical significance calculated using Mann-Whitney tests. Data are presented as AFU (arbitrary fluorescence units) and expressed as mean ± SEM (n=20 pairs in quadruplicate). (**b)** Fluorescence microscopy of NETs from patients and controls pre-treatment. NETs were visualised at x20 magnification following Sytox green staining in response to PBS (unstimulated negative control), PMA (50 nM) and HOCl (0.75 mM) exposure for 3 hours. White arrows indicate NET strands. Images are representative of 2 experiments performed in subjects recruited to Birmingham Dental Hospital, UK, and were performed in triplicate. Scale bars represent 100µm.

Figure 2: Pre- and post-treatment NET production by periodontitis patients and healthy controls.
NET production pre- and post-treatment expressed as a ratio of patients to matched controls (y-axis). (a) PBS (unstimulated negative control), (b) PMA (50 nM) and (c) HOCl (0.75 mM). Data are presented as AFU (arbitrary fluorescence units) (n=19 pairs in quadruplicate).

Figure 3: NET degradation by plasma from periodontitis patients and controls pre- and post-periodontal treatment
HOCl-stimulated (0.75 nM) neutrophils were incubated with 10% plasma from periodontitis patients and healthy age/gender matched controls for 3 hours. NETs were quantified fluorometrically using the Sytox green assay. Percentage NET degradation was calculated based on a 1 U/ml MNase following 15 min digestion that was used to represent the 100% standard. (a) A significant difference between patient and control NET degradation was observed pre-treatment, however this difference was absent when the assay was repeated post-treatment (b). Data expressed as mean ± SEM (n=19 matched pairs).

Figure 4: Plasma DNase-1 concentrations in periodontitis patients and controls

DNase-I levels were measured from pre-treated patient plasma samples alongside healthy-matched controls. DNase-I levels were quantified fluorometrically following 30 min incubation with a DNase detection probe. Significantly reduced DNase-I levels were detected in patient samples relative to control. Data expressed as means ± SEM (n=19 matched pairs).

Figure 5: Plasma IgG concentrations in periodontitis patients and controls
IgG subclasses 1-4 concentrations were measured by protein turbidimetric analysis in plasma samples from periodontitis patients and healthy matched controls pre- and post-treatment. (a) IgG subclass 1, (b) IgG subclass 2, (c) IgG subclass 3, (d) IgG subclass 4. Data expressed as means ± SEM (n=19 matched pairs).

Figure 6: Plasma FLC concentrations in periodontitis patients and controls
(a) Free kappa light chains; (b) free lambda light chains; (c) the kappa/lambda ratio and (d) summated FLCs were measured by protein turbidimetric analysis in plasma samples from periodontitis patients and healthy matched controls pre- and post-treatment. Data expressed as means ± SEM (n=19 matched pairs).

Table 1

Clinical measures*		Chronic periodontitis patients		Controls
		Baseline	Review	
Age, years, mean±SD (range)	UK	46±8 (37-61)		46±8 (32-62)
	Greece	52±8 (36-70)	NR	50+11(35-82)
Probing pocket depth, mm, mean±SD	UK	3.0±0.8[#]	2.1±0.5[$]	1.5±0.4
	Greece	3.9±0.8[#]	NR	1.8±0.7
No. probing pocket depths >4mm, median (range)	UK	27 (5-91)[#]	7 (0-52)[$]	0 (0-4)
% sites bleeding on probing, median (range)	UK	43 (16-87)[#]	14 (3-35)[$]	2 (0-39)
	Greece	65 (50-100)[#]	NR	3 (0-23)
Gingival index, median (range)	UK	2 (1-3)[#]	1 (0-1)[$]	1 (0-1)
Plaque index, median (range)	UK	2 (1-3)[#]	1 (0-2)[$]	1 (0-2)

Volunteer ages (at time of sampling) were compared by Mann-Whitney test (no significant differences within or between groups). 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 the UK volunteer cohorts have been published previously (Ling et al., 2015)

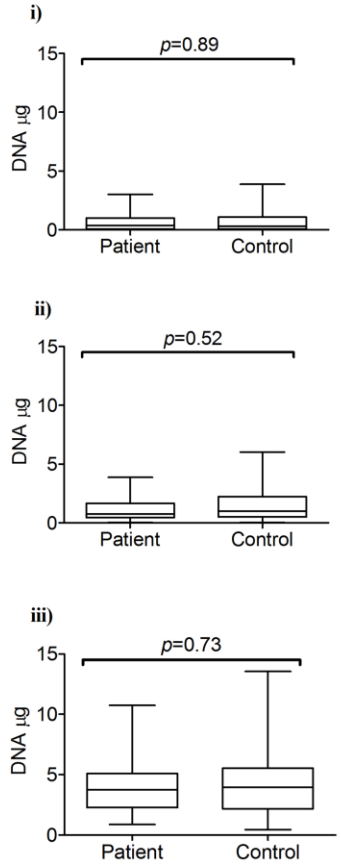
[*]probing pocket depth ≥4mm, gingival index, plaque index & post-treatment review data were collected solely from the UK cohort.

[#]comparison with corresponding controls ($p<0.05$).

[$]comparison with chronic periodontitis before treatment ($p<0.05$).

Abbreviations: NR, not reported; SD, standard deviation; UK, United Kingdom.

Fig 1 A


i)
p=0.89
15
10
5
0
DNA µg
Patient
Control
ii)
p=0.52
15
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DNA µg
Patient
Control
iii)
p=0.73
15
10
5
0
DNA µg
Patient
Control

Fig 1B

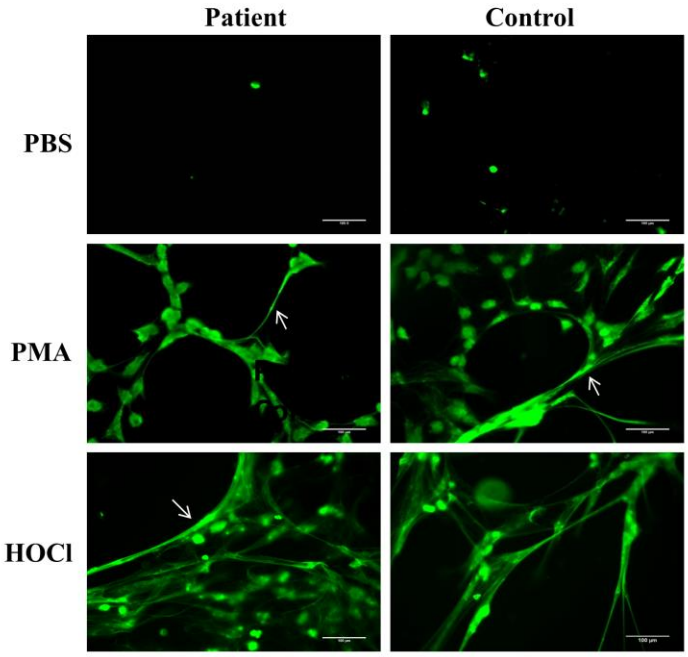


Fig 2

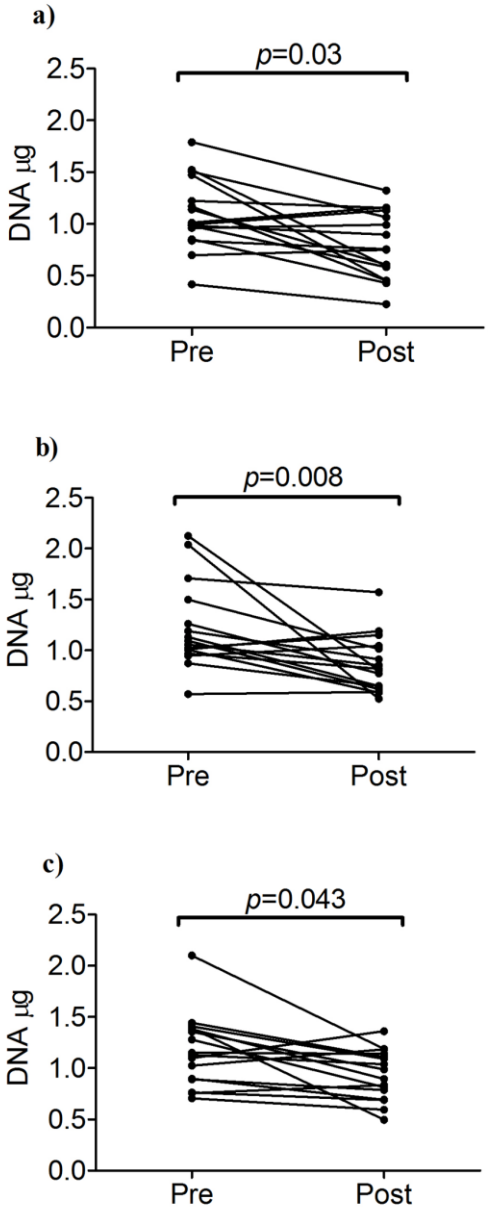

a)
p=0.03
DNA µg
2.5
2.0
1.5
1.0
0.5
0.0
Pre
Post
b)
p=0.008
DNA µg
2.5
2.0
1.5
1.0
0.5
0.0
Pre
Post
c)
p=0.043
DNA µg
2.5
2.0
1.5
1.0
0.5
0.0
Pre
Post

Fig 3

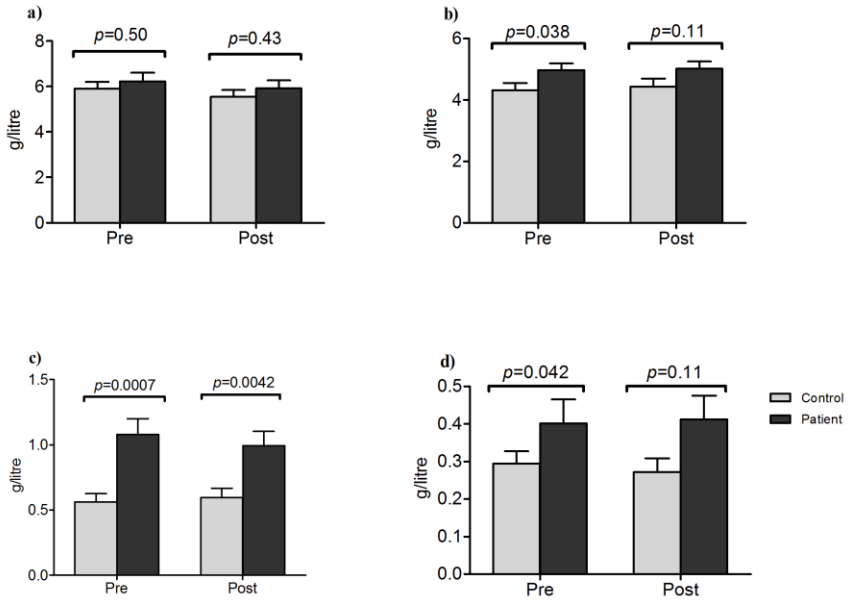
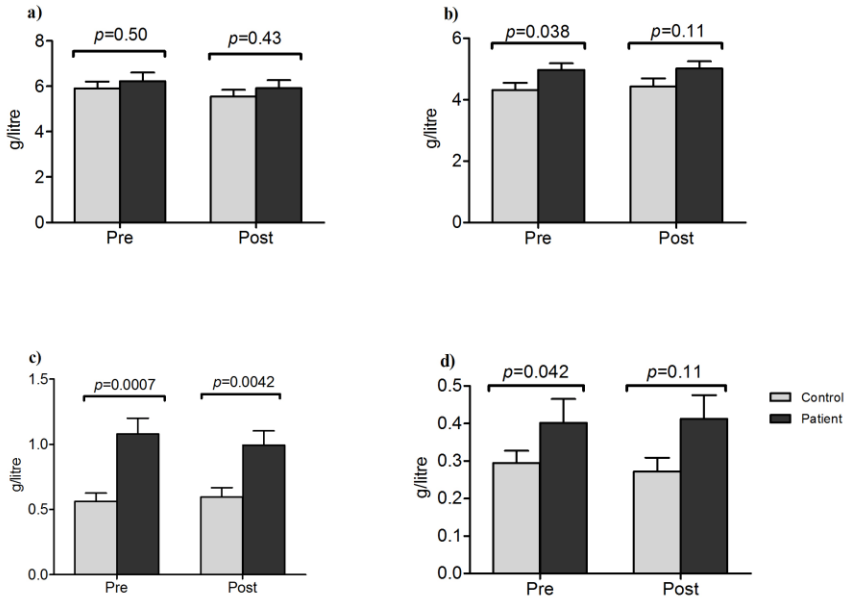

a)
p=0.50
p=0.43
g/litre
Pre
Post
b)
p=0.038
p=0.11
c)
p=0.0007
p=0.0042
d)
p=0.042
p=0.11
Control
Patient

Fig 4


a)
p=0.50
p=0.43
g/litre
Pre
Post
b)
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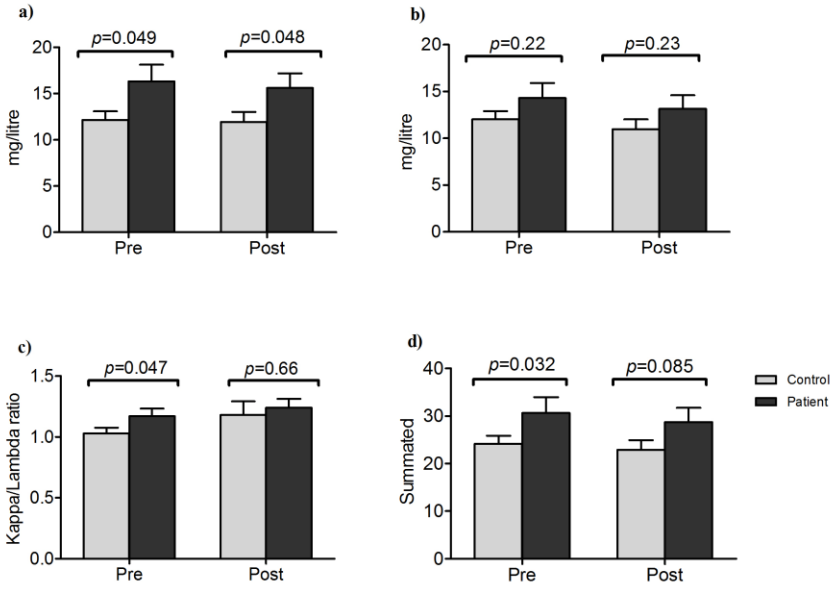
c)
p=0.0007
p=0.0042
d)
p=0.042
p=0.11
Control
Patient

Fig 5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p=0.048
mg/litre
20
15
10
5
0
Pre
Post
b)
p=0.22
p=0.23
mg/litre
20
15
10
5
0
Pre
Post
c)
p=0.047
p=0.66
Kappa/Lambda ratio
1.5
1.0
0.5
0.0
Pre
Post
d)
p=0.032
p=0.085
Summated
40
30
20
10
0
Pre
Post
Control
Patient