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ORIGINAL ARTICLE

Influence of IL-6 haplotypes on clinical and inflammatory response in aggressive periodontitis

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Abstract
Objectives The aim of this study was to investigate the inflammatory response in aggressive periodontitis (AgP) patients after periodontal therapy and associate these changes to subjects' interleukin-6 (IL-6) genetic variants.
Materials and methods Twelve non-smoking UK Caucasian patients with AgP were selected based on their *IL6* haplotypes (six haplotype positive and six haplotype negative based on polymorphisms rs 2069827 and rs 2069825) and underwent full mouth non-surgical periodontal therapy, followed by open flap surgery. Gingival crevicular fluid (GCF) and peripheral blood samples were taken at baseline and at six different time points after treatment. Gingival biopsy samples were harvested during surgery and underwent immunohistochemical analysis for identification of IL-6.
Results An overall improvement in clinical periodontal parameters was observed following periodontal therapy. Haplotype status was associated with clinical presentation, *Aggregatibacter actinomycetemcomitans* counts in subgingival plaque samples, white cell count, neutrophils, red cell count and haemoglobin. GCF IL-6 concentrations increased dramatically 1 day after surgery and IL-6 haplotype-positive subjects exhibited a higher magnitude in this increase.
Conclusions IL6 haplotypes may have an effect on clinical presentation and magnitude and kinetics of local and systemic inflammatory responses following non-surgical and surgical periodontal therapy in aggressive periodontitis.
Clinical relevance Detecting IL-6 haplotype-positive periodontitis patients might become helpful in identifying subjects prone to excessive inflammatory response and increased periodontal breakdown.

Keywords Periodontitis · Genetic · Inflammation · Interleukin

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Introduction

Periodontitis is a chronic low-grade local infection characterised by raised serum levels of inflammatory biomarkers [1] and a dysmetabolic state [2, 3]. Periodontal therapy results in a transient bacteraemia [4] and an increase in inflammatory markers (such as interleukin-6, IL-6) with considerable individual variability [5]. Different allelic variants (defined as 'functional') in host response genes, characterised by base pair changes or other modifications, can predispose to disease in humans by having an effect on gene activity and protein production. In periodontitis, genetic variants could potentially affect the local and systemic response to bacterial colonisation and treatment response [6, 7]. Functional *IL6* genetic promoter variants have been shown to affect IL-6 production and systemic release [8, 9] and bacterial growth [10]. An excessive release of IL-6 locally could predispose to periodontitis by an amplification of the pro-inflammatory cascade, by osteoclast-stimulating activities [11, 12] and by stimulating the growth of periodontopathogenic bacteria [7]. *IL6* genetic variants have been associated with presence of periodontitis in some populations [13] and with detection of periodontopathogenic

bacteria [14] and serum levels of IL-6 and C-reactive protein in periodontitis cases [15]. A specific *IL6* haplotype determined by combinations between loci rs2069827 and rs2069825 showed an association with aggressive periodontitis (AgP) [16]. However, the association between *IL6* genetic variants and periodontitis is still controversial [17, 18]. Limited evidence is present about the systemic inflammation in subjects diagnosed with AgP [19]. The hypothesis behind this study is that *IL6* genotypes might predispose to local and systemic inflammation and response to treatment in AgP. The aims of this study were to assess the possible effects of *IL6* genetic variants on local and systemic IL-6 levels, *Aggregatibacter actinomycetemcomitans* counts and inflammatory response and clinical outcomes in AgP patients after treatment.

Materials and methods

This was a controlled clinical trial of approximately 5 months duration.

Subject selection

From a total of 76 subjects referred to the Department of Periodontology, Eastman Dental Hospital and screened for participation from November 2007 to January 2009, a total of 12 patients were included in the study. The study had been approved by the UCL/UCLH Committees on the Ethics of Human Research (reference 07/H0713/74), and all subjects signed a written consent to take part. Inclusion criteria were: (1) diagnosis of AgP [20], (2) probing pocket depths (PPD) and clinical attachment level (CAL)≥6 mm and bleeding on probing in at least three sites on different teeth, (3) 16 to 40 years old, (4) all grandparents of UK Caucasian ethnic origin. Exclusion criteria included: (1) tobacco smoking (within the previous 3 months), (2) presence of other chronic oral conditions as assessed by the examining clinician, (3) pregnancy, (4) reported history of medical conditions or concomitant systemic medications, (5) intake of anti-inflammatory drugs or antibiotics within 1 and 3 months, respectively, (6) known infectious diseases and (7) previous periodontal therapy within 12 months.

Experimental procedures

- Clinical examination

At the baseline visit, the patients' height, weight and waist circumference were taken. A single calibrated examiner (G.P.) blind to haplotype status measured full mouth plaque score (six sites/tooth) [21], full mouth PPD, CAL (= PPD+gingival recession) and bleeding on probing (FMBS) [22] at six sites/tooth. Repeated measurements of ten non-study subjects with at least 15 min separation showed 98 % intra-examiner repeatability within 2 mm for PPD and CAL.

- Periodontal treatment and follow-up visits

The study outline is presented in Fig. 1. All treatment sessions were performed by a single qualified periodontist (L.N.) blind to haplotype status. Full mouth non-surgical periodontal therapy (NSPT) under local anaesthesia and oral hygiene instructions were provided after baseline (v1). In order to standardise the treatment provided to all subjects, following a first re-evaluation with the collection of periodontal measurements (v3), periodontal surgical treatment (open flap debridement) was performed under local anaesthesia (v4, day 70) in the worst affected quadrant if clinical indication was found to do so (residual >5 mm PPDs with bleeding on probing). Full-thickness muco-periosteal flaps were elevated, removal of granulation tissue and subgingival debridement and if needed localised removal of non-supporting bone (osteoplasty) were performed according to needs, and the flaps were readapted and sutured. The patients were then clinically assessed and had blood and gingival crevicular fluid samples taken from 1 to 90 days after the surgical procedure (visits 5, 6, 7, 8 and 9). A full set of periodontal measurements (PPD, CAL, FMPS and FMBS) was taken again in the last visit. If the need for further treatment was identified at the final assessment, subjects received further periodontal non-surgical or surgical treatment outside the study protocol. All subjects completed the study, and data from each visit were used for the analysis.

- Blood sampling and analyses

Blood was collected by venipuncture from the antecubital vein of the patients (at visit 1, 3, 5, 6, 7, 8 and 9) for full blood count and differential, glucose and lipid analysis (at

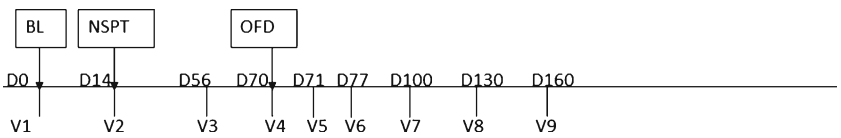

Fig. 1 Study flowchart. *V* visit, *D* day, *BL* baseline, *NSPT* non-surgical periodontal treatment, *OFD* open flap debridement (periodontal surgery). Blood and gingival crevicular fluid (GCF) samples were taken at *V1*, *V3*, *V5*, *V6*, *V7*, *V8* and *V9*

the Haematology Laboratory of the University College Hospital Trust), plasma separation and DNA extraction. All patients had been fasting (no food or drinks) for 6 h prior to blood sampling on all visits. One hundred microlitres of plasma in duplicate were used for IL-6 ELISA (Quantikine HS, R&D System, Minneapolis). The inter- and intra-assay coefficients of variation were calculated to be 7.3 and 7.8 %, respectively.

- Haplotype definition

DNA was extracted from peripheral blood cells and used for real-time polymerase chain reaction analysis as previously described [23]. Hidden duplicates were added to each plate to test error rates. However, no detection errors were observed. All genotyping was performed blindly with respect to clinical diagnosis by a single investigator (M.P.).

The study was designed to have a balanced number of haplotype positive (H+) and haplotype negative (H−) individuals. Subjects were screened for polymorphisms at position −1363 (rs 2069827) and −1480 (rs 2069825) in the *IL6* gene promoter. Homozygous subjects for −1363 G allele and −1480 C allele (no deletion) were defined as H+, while all other subjects were H−. This haplotype is in strong linkage disequilibrium with the IL6 −174 polymorphism, which has shown evidence of association with IL-6 production [8, 9, 24]. However, the effect of the haplotype, rather than of the single locus, seems to be stronger in determining IL-6 production [25].

- Gingival crevicular fluid sampling

The site with the deepest pocket depth in the mouth was selected as test site, while a site with PPD<4 mm and no bleeding on probing in the same quadrant was selected as control site for sampling procedures. After isolating the area and removing the supragingival plaque, gingival crevicular fluid (GCF) was collected (at visit 1, 3, 5, 6, 7, 8 and 9) from the two selected sites per patient (test and control) by gentle insertion of PerioPaper strips (OraFlow, Inc, Smithtown, NY, USA) in the gingival sulcus for 30 s. Each paper strip was then placed in a Periotron machine (Oraflow Inc., NY, USA) to estimate a volume reading and then stored at −70 °C. A Periotron curve was obtained by measuring triplicate readings of known volumes of plasma absorbed in the paper strips (0.1 to 2 μl). The degree of recovery of IL-6 from the paper strips was estimated by comparing IL-6 concentrations in six plasma samples with and without prior absorption to paper strips followed by elution in 100 μl phosphate-buffered saline (PBS; experiments done in duplicate) [26]. Percentage efficiency of recovery was equal to 98 %. Samples were eluted in 100 μl PBS, further diluted in 100 μl PBS and 100 μl was analysed by ELISA in duplicate as described above (plasma analysis). All GCF analysis was performed blindly with respect to clinical diagnosis by a single investigator (N.C.).

- Histological analysis

IL-6 is expressed by macrophages, neutrophils, fibroblasts, keratinocytes and endothelial cells [27]. In order to assess if functional IL6 haplotypes could affect intracellular presence of IL-6 directly in the affected area, a biopsy sample of gingival tissue from the internal aspect of the flap was harvested from the site with the deepest pocket during surgery (visit 4). The biopsies were prepared for formalin fixation and paraffin embedding [28]. Five-micrometre-thick sections were prepared from each tissue portion and exposed to immunohistochemical staining. Following antigen optimization, the most suitable method of unmasking IL-6 antigen was identified as pH 6.0 in a waterbath at 97 °C for 40 min, using the polyclonal antibody against human IL-6 (IL-6Rα (C20), Santa Cruz Biotechnology Inc., Germany). The histological measurements were performed using a microscope (Olympus BX50, Best Scientific LTD, Wroughton, UK) equipped with an imaging system (Image Pro-PLUS 4.5, Media Cybernetics, Bethesda, USA) [18]. Each slide was categorised into two zones: infiltrated connective tissue (ICT) and periphery of the ICT (non-ICT). A 400-point lattice was superimposed over the tissue area at a magnification of ×400, and the number of cross-points on positive cells was counted and expressed as a percentage of the tissue area related to the total number of points [29].

- Microbiological analysis

Samples of subgingival plaque were taken from test and control sites with sterile curettes at visit 1, 3 and 9. After a single stroke, each microbiological sample was extracted from the pocket and immediately placed into 1 ml of sterile reduced transport fluid [30] and later analysed by qPCR for quantification of *A. actinomycetemcomitans*, the periodontopathogenic bacterium which consistently showed an association with *IL6* genetic variants in different populations [14, 31].

- Sample size calculation and statistical analysis

Twelve patients (six per group) were considered enough to show a 3-pg per site difference (standard deviation 1.5) between haplotype groups, with >0.80 power [32]. The primary outcomes of this study were IL-6 concentrations in plasma and GCF. Secondary outcomes were inflammatory and lipid parameters, namely: serum white blood cell count, neutrophil, lymphocyte, eosinophil and basophil counts and glucose levels, as well as periodontal clinical parameters (PPD, LCAL, FMPS, FMBS), *A. actinomycetemcomitans* and percentage of IL-6-positive cells in gingival biopsies. All data were analysed by the use of the SPSS 17.0 package. The alpha value was set at 0.05. All the continuous and categorical data were analysed by ANOVA and Chi-square, respectively. Two-way repeated ANOVA was used to study changes of laboratory parameters over time. Relative changes in plasma and GCF IL-6 were also

computed with the formula (IL - 6 concentration , at a specific visit − baseline concentration)/baseline concentration . Age, gender, body mass index (BMI), number of deep gingival pockets (PPD >6 mm), haplotype status (H+ or H−) and time were entered as covariates for these analyses. *A. actinomycetemcomitans* counts were log-transformed for the analysis as they were not normally distributed. Back-transformed data are presented. Correlation analysis was performed with Spearman rank test.

Results

Baseline data analysis

Out of 12 participants, 9 (6 H+ and 3 H−) were diagnosed with generalised AgP, while 3 (all H−) were diagnosed with localised AgP. Table 1 shows the demographic and clinical parameters of patients divided by haplotype status. Clinically, H+ patients had increased average CAL (P=0.049), while no differences were detected for any of the studied laboratory parameters.

Longitudinal data analysis

Improvements in clinical parameters were detected in both groups following periodontal treatment. H+ subjects had statistically significant reductions in average CAL (p=0.046) and number of PPD >6 mm (p=0.028) between baseline and last visit, while only the number of PPD >6 mm was significantly less in H− subjects after treatment (p=0.027). The test sites exhibited statistically significant reductions (p<0.05) in CAL and PPD for both groups (data not presented in tables).

IL-6 concentrations in GCF increased after surgery (visit 5, day 71) in all patients (p<0.001) before decreasing again. Figure 2 shows the changes in inflammatory parameters during the course of the study for subjects divided by haplotype. GCF and plasma IL-6 concentrations showed a similar pattern between groups, with an increase after surgery (v5) for both H+ and H− subjects. Leukocyte and neutrophil numbers were constantly higher for H+ subjects throughout the study (p=0.009 and p=0.032, respectively, at multivariate analysis). Red cell count and haemoglobin were consistently higher in H+ subjects (p=0.041 and p<0.001, respectively). Only moderate associations were observed between haplotype status and absolute GCF (p=0.113) and plasma IL-6 concentrations (p=0.079). A statistically significant difference in relative changes of GCF IL-6 after therapy was noted in H+ compared to H− (p=0.041; Fig. 3).

Average *A. actinomycetemcomitans* counts between test and controls did not change significantly after treatment (data not shown). Multivariate analyses confirmed that *A. actinomycetemcomitans* counts were associated with BMI and haplotype status independent of age and gender differences. In particular, H+ individuals tended to have always higher counts of *A. actinomycetemcomitans* at each visit in test sites compared to H− individuals (p=0.017 for trend; Fig. 4). Furthermore, while *A. actinomycetemcomitans* counts decreased throughout the study in H− subjects, an initial decrease was followed by a new increase in H+ subjects at the last study visit. Individuals with higher BMI had higher counts of *A. actinomycetemcomitans* at all visits (R=0.4, Spearman rank test for correlation), while IL-6 plasma and GCF levels were not associated with bacterial counts (data not shown).

Figure 5 shows a histological section of a biopsy sample harvested during the periodontal surgical procedure and showing staining of IL-6-positive cells. No differences were detected for number of IL-6-positive cells in gingival tissues (p=0.519 in ICT and p=0.624 in non-ICT).

Table 1 Demographic and clinical parameters in relation to *IL6* haplotypes

		Haplotype positive (H+)		Haplotype negative (H−)		Comparisons (p values)
		(n=6)	%	(n=6)	%	
Age		36.7±0.7	–	33.8±1.4	–	0.106
BMI		24.1±1.2	–	26.3±2.7	–	0.457
Waist measurement (cm)		88.8±3.8	–	89.2±4.1	–	0.280
Gender	Male	2	33.3	2	33.3	1.000
	Female	4	66.7	4	66.7	
No. of teeth		29.7±2.2	–	29.2±1.8	–	0.682
FMPS		–	21.0±8.2	–	16.2±6.3	0.654
FMBS		–	34.5±13.3	–	19.6±7.9	0.357
Average PPD (mm)		4.6±0.6	–	3.1±0.3	–	0.058
Average CAL (mm)		4.8±1.6	–	3.2±0.8	–	0.049
Number of PPD >6 mm		44.5±27.4	–	15.7±8.3	–	0.065
A.a. counts (cells/sample)		8,306±13,628	–	1,139±2,645	–	0.235

Continuous values are reported as mean±standard error. The last column reports the statistical significance of the differences between the two groups (H+ and H−) (ANOVA and Chi-square for continuous and categorical variables, respectively)

BMI body mass index, *FMPS* full mouth plaque score, *FMBS* full mouth bleeding score, *PPD* probing pocket depth, *CAL* clinical attachment loss, *A.a. Aggregatibacter actinomycetemcomitans*

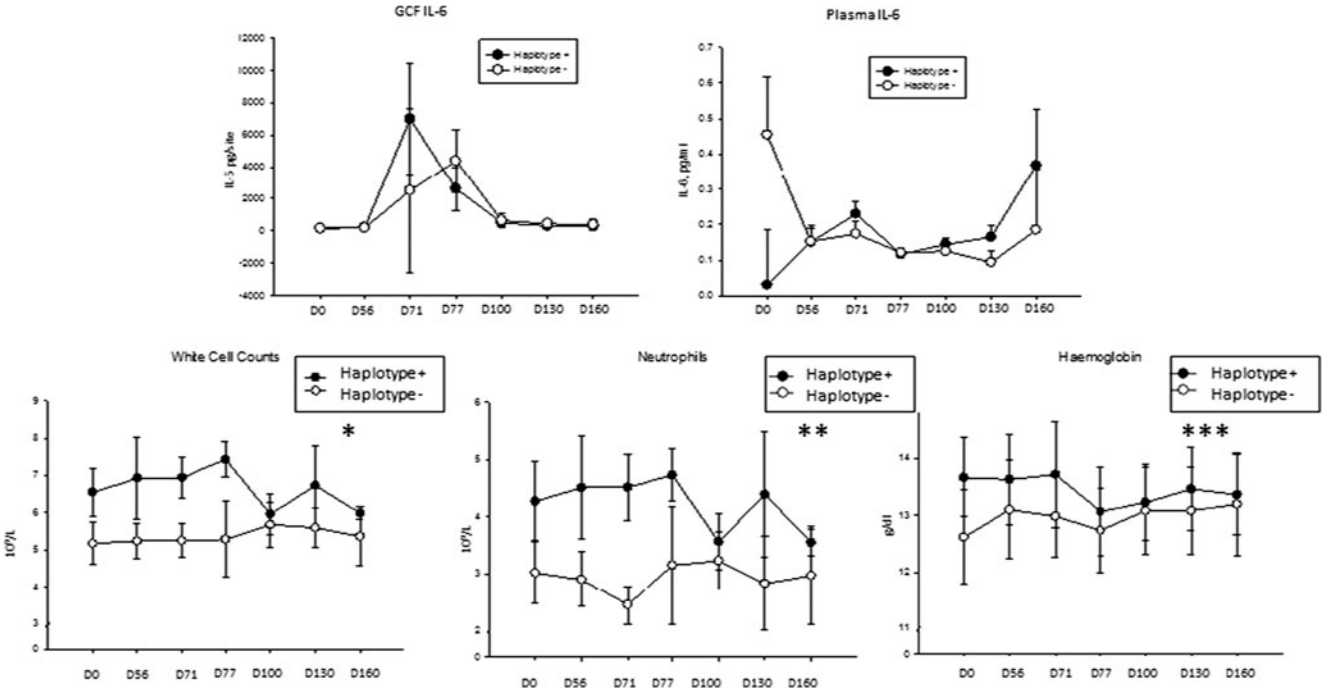

Fig. 2 Mean and standard error values of IL6 GCF concentrations, IL6 plasma concentrations, serum white cell count, neutrophil count and haemoglobins from visit 1 (baseline) to 7 (90 days post-surgery) for subjects divided by *IL6* haplotype (H+ and H−). *p=0.009, **p=0.032, ***p<0.001, respectively, at multivariate analysis

Discussion

This study showed an association between interleukin-6 (*IL6*) genetic variants and magnitude and kinetics of inflammatory responses following periodontal therapy in AgP. This is the first study, to our knowledge, where patients were selected based on their genetic profile prior to periodontal treatment. In order to reduce confounding effects, only non-smoking UK Caucasians subjects were selected. Periodontal therapy gave clinical results in line with the literature on non-surgical and surgical therapy [33, 34].

A tendency to an increase in crude inflammatory parameters (local and systemic IL-6 levels, neutrophils) was observed up to the first week post-periodontal surgery, followed by a decrease till the end of the study. The IL-6 concentration increase was sharper and faster than the leukocyte increase and was particularly evident locally, with average GCF concentrations going up 40-fold of baseline values. Although we only sampled one diseased site, no associations were detected between systemic and local IL-

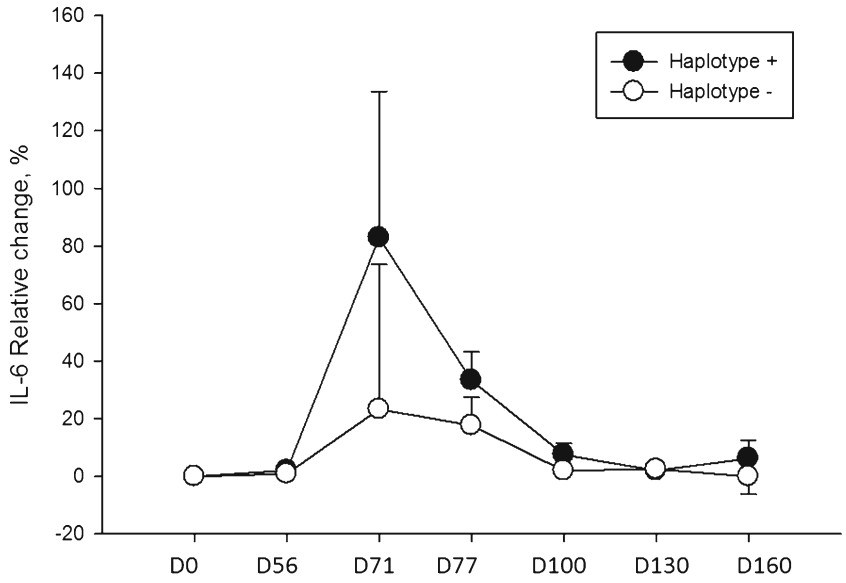

Fig. 3 Relative changes (compared to baseline) for IL-6 concentrations in gingiva crevicular fluid (GCF) from visit 1 to 7 (90 days post-surgery) for subjects divided by *IL6* haplotype (H+ and H−). p=0.041 at multivariate analysis

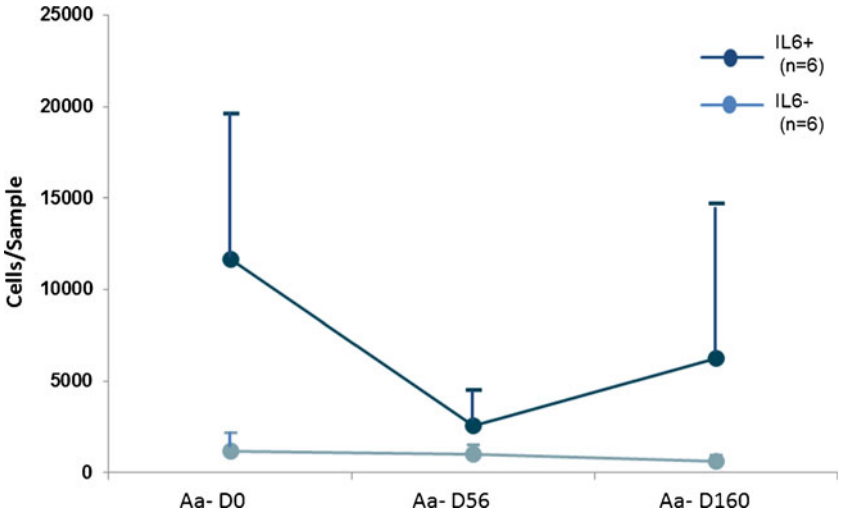

Fig. 4 *A. actinomycetemcomitans* counts in test sites in patients divided by haplotype (IL6 H+ and IL6 H−). H+ subjects consistently showed higher counts compared to H− subjects (p=0.017 for trend)

6, bringing further evidence for a local source of IL-6 production. This was further confirmed by the detection of IL-6-positive cells in gingival biopsies. These variations in serum inflammatory parameters are in line with other reports in the literature on the inflammatory response after non-surgical and surgical periodontal treatment on chronic periodontitis patients [15, 35]. The magnitude of the increase in neutrophil counts was less marked in the current study (following surgery) than in previous studies following NSPT and extractions [35], suggesting that the systemic response to the elevation of a full-thickness mucosal flap in a quadrant already treated non-surgically may be less than the effect resulting from the trauma of debridement and extractions in a very inflamed subgingival environment [36]. The systemic inflammatory effect of periodontitis and its treatment may be linked with trauma, inflammatory mediators dumped in the systemic circulation and with the release of periodontal pathogens from inflamed gingival pockets [4].

IL6 H+ subjects were selected based on their supposed increased release of IL-6, which could lead to a hyper-inflammatory status [8, 9, 24], and on their consistent association with presence of periodontopathogenic bacterium *A. actinomycetemcomitans* [14, 23, 31]. *IL6*+ subjects in this study showed higher extent of periodontitis (measured as number of gingival pockets) and higher counts of the periodontal pathogen *A. actinomycetemcomitans*, associated with a higher inflammatory response. The clinical response in both groups was comparable, although *IL6*+ subjects had a higher overall periodontal attachment level gain. The association between *IL6* −174 GG and *A. actinomycetemcomitans* is in agreement with previous studies from our group, confirming the possible infectogenomics effect of *IL6* genotypes on *A. actinomycetemcomitans*' growth [14, 23, 31]. H+ subjects also appeared to show a 'faster' inflammatory response compared with the apparently more prolonged inflammatory response in relation with H− haplotype status (longer regression time to normal values for number of peripheral neutrophils and IL-6 GCF levels). This is in agreement with previous studies highlighting a certain degree of heterogeneity across individuals with regard to magnitude and kinetics of inflammatory changes following periodontal therapy [5, 36]. Genetic variants (such as *IL6* haplotypes in this case) may partially explain such variations. Residual confounding, despite adjustment in the analysis, may be represented by disease severity between groups. However, no difference in disease severity was noted between groups in the test sites, where GCF and microbial samples were harvested (data not presented).

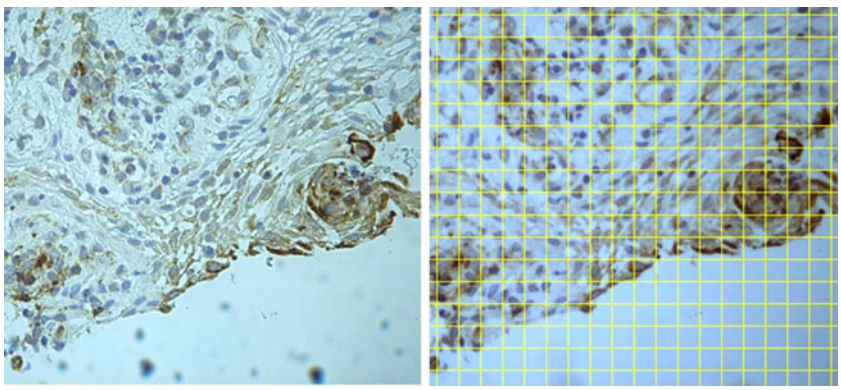

Fig. 5 Image of a gingival biopsy harvested during the surgical procedure. Part of a 400-point lattice superimposed over the inflamatory cell infiltrate at ×400 magnification. The *red arrows* point to cross-points positively identified with IL-6-positive cells

The limitations of this study include a small sample size with absence of untreated controls, inclusion of both LAgP and GAgP subjects, and surgical treatment limited to only one quadrant. The novelty lies in the selection of patients a priori based on their haplotypes and in the analysis of a relatively homogeneous group of aggressive periodontitis cases. The 'hyper-inflammatory' haplotype (H+) was associated with disease severity, bacterial (*A. Actinomycetemcomitans*) counts and higher systemic inflammation. The triad of periodontal bacteria, environmental factors and genetic variants may trigger the inflammatory response leading to AgP, and different haplotypes may influence disease progression and response to treatment. Larger studies need to be conducted to prove the effect of these *IL6* and possibly other genotypes and haplotypes in affecting local and systemic inflammation as well as clinical response in aggressive periodontitis cases.

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Conflict of interest The authors declare that they have no conflict of interest.

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