Pertinent cell population to characterize periodontal disease

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ABSTRACT

The purpose of this *in situ* study is to quantify the inflammatory cell subsets and the area fraction (AA%) occupied by collagen fibers in human healthy and diseased (four different stages) gingival connective tissue in order to establish a possible correlation between periodontal disease resulting in collagen breakdown and specific inflammatory cell subsets.

Paraffin gingival tissue sections from eight healthy controls (group 0), 10 patients with gingivitis (group 1), 10 patients with moderate periodontitis (group 2) and 10 patients with severe periodontitis (group 3) were immunohistochemically investigated using antibodies against CD-45+, CD-3+, CD-8+, CD-20+, CD-68+, and EMA+ (plasma cells).

The AA% occupied by gingival collagen fibers significantly decreased from 54.12% in group (0) to 38.58% in group (1), to 31.87% in group (2), and to 25.46% in group (3). In progressive lesions of periodontal disease, CD-3+ and CD-8+ cell numbers were increased in early stages within the connective tissue, while CD-20+ cell numbers were increased only in late stages. On the other hand, EMA+, CD-68+ and CD-45+ cell numbers were progressively increased from group (0) to group (3). We demonstrated that CD-68+ monocyte/macrophages, CD-45+ leukocyte common antigen and notably EMA+ plasma cells are pertinently correlated with the severity of periodontal disease and related collagen breakdown.

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1. Introduction

The periodontal disease is an inflammatory process initiated and maintained by bacterial plaque and its metabolic products that trigger local infiltration of inflammatory cells associated with breakdown of the extracellular matrix macromolecules (Page and Schroeder, 1976). Among these, collagen quantitatively constitutes the major component of the gingival connective tissue and plays a key role in its architecture and is therefore implicated in pathological states such as periodontitis (Séguier et al., 2000). The composition of the inflammatory cells which infiltrate different tissue compartments in periodontal lesions may indicate which arm of the immune system most effectively deploys locally to protect the periodontal tissues. Bandtzaeg and Kraus (1965) showed the presence of immunoglobulin producing plasma cells in the gingival tissues of patients with periodontal disease. This was the first evidence which demonstrated that adaptive immune mechanisms play a role in the pathogenesis of periodontal inflammation. In 1970, Ivanyi and Lehner (1970) using peripheral blood lymphocyte transformation assays, highlighted a role for cell-mediated immunity in periodontal disease. Since then, immunohistological studies have provided evidence for the immunological nature of the response to plaque bacteria (Listgarten, 1986).

Thus, in the gingival connective tissue, many studies have shown important changes in cellular populations during the periodontal disease. It is clear from histological studies that the predominant cell type of the chronic gingival lesion is the lymphocyte (Page and Schroeder, 1976; Seymour et al., 1979a,b). In the early stages of gingival inflammation, pro-inflammatory cytokines secreted by activated monocytes, macrophages and other cells (e.g. fibroblasts, epithelial and endothelial cells) predominate (Page, 1986). The macrophages are key cells of the innate immune system involved in the progression of periodontal disease from an acute inflam-

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matory condition to a chronic pathology (Grenier and Grignon, 2006; Zappa et al., 1991) and which are present in higher numbers in active periodontal lesions than in inactive sites (Zappa et al., 1991). The macrophages are also an important source of pro-inflammatory cytokines, including interleukin-1 β (IL-1β) and tumor necrosis factor-α (TNF-α), which stimulate the activation of cellular immunity, thereby amplifying the inflammatory cascade, and contributing to host tissue destruction (Grenier and Grignon, 2006; Lappin et al., 1999).

Moreover, it has been recently shown that during periodontitis, gingival collagen loss was significantly correlated with an increase in the number of inflammatory cells, such as CD-3+ CD-8+ and CD-45+ (Séguier et al., 2000; Ejeil et al., 2003).

Seymour et al. (1979a,b) described the frequent association of plasma cells with fibroblasts suggesting that they are important in the pathogenesis of periodontal disease. However, few reports are available in the literature on the *in situ* quantification of infiltrating inflammatory cells (Lappin et al., 1999; Séguier et al., 1999, 2000).

To our knowledge, no previous studies have been carried out to quantify a panel of inflammatory cell subsets including plasma cells in gingival tissue compartments reflecting four different stages of the periodontal disease (control, gingivitis, moderate and severe periodontitis). Therefore, the purpose of the present study is to investigate, through in vitro tissue immunolocalisation, which cell population correlates most with the different periodontally diseased study groups. Moreover, since collagen is the main macromolecular component of gingival connective tissue, we evaluated whether the collagen fibers breakdown (occurring in periodontal lesions) could be correlated with a particular inflammatory cell population and with the different diseased study groups.

2. Materials and methods

2.1. Patient's selection and clinical evaluation

With patient's written informed consent (in accordance with the principles outlined in the Helsinki declaration, and approved by the local ethics committee[*]), gingival biopsies were collected* from 38 patients aged between 18 and 79 years old (21 males and 17 females) undergoing tooth extraction (for orthodontic reason, periodontal disease etc.), and showing various stages of periodontal disease.

The patients included in this study: (i) had no oral or major systemic diseases; (ii) did not suffer from hypertension or any other vascular diseases (which may affect extracellular matrix integrity), diabetes mellitus or acute necrotizing ulcerative gingivitis; (iii) had not taken any medications or undergone periodontal therapy within the preceding 6 months; (iv) were not pregnant. Moreover, partially edentulous patients (with less than 20 teeth), those undergoing orthodontic treatments, and those wearing ill-fitting prostheses were excluded from this study.

Full mouth periodontal examination chart was done for all the patients included in this study. The diagnosis of periodontal disease was based on five clinical and radiographic criteria (vertical bone resorption): gingival index (GI) of Loe and Silness (1963), plaque index (PI) of Silness and Löe (1964), bleeding on probing (SBI) (Muhlemann and Son, 1971), pocket depth (PD), with accompanying clinical attachment loss (CAL) measured using a calibrated Michigan probe with William's markings, and radiographic vertical bone loss (peri-apical X-ray using paralleling technique). The per-

centage of bone loss was assessed in all radiographs by digital image processing analysis using UTHSCSA ImageTool program (University of Texas Health Science Center at San Antonio, TX), and based on the calculated linear distances (from the cemento-enamel junction CEJ) of the Root length and the residual bone.

Measurements were carried out by the same investigator, minimizing variability. Based on the foregoing indices and criteria, and according to the classification system for periodontal diseases and conditions (Armitage, 1999), each gingival sample was included in one of the four following experimental groups.

- Group (0) (control): Eight healthy controls (four males and four females, aged between 18 and 55 years old, with a mean age of 26 years) showing clinically healthy gingiva without bleeding and no evidence of vertical bone resorption or periodontal pockets.
- Group (1): Ten patients (six males and four females, aged between 22 and 79 years old, with a mean age of 49.9 years), presenting clinical evidence of gingivitis, such as red color, swelling of the gingival margin and bleeding corresponding to gingival indices 2 and 3 (Löe and Silness) without vertical bone resorption or periodontal pocketing.
- Group (2): Ten patients (four males and six females, aged between 41 and 68 years old, with a mean age of 57.9 years): moderate chronic periodontitis 3–4 mm CAL; (4–6 mm pockets, <50% bone loss).
- Group (3): Ten patients (six males and four females, aged between 35 and 76 years old, with a mean age of 54.4 years): severe chronic periodontitis ≥5 mm CAL; (>6 mm pockets, >50% bone loss).

2.2. Gingival biopsies

The gingival biopsies (0.2 cm × 0.4 cm) were harvested from the buccal keratinized gingiva before tooth extraction under local anesthesia while avoiding infiltration into the biopsy site, and deformation or compression of the samples. One biopsy was taken per patient, and tissues on tooth surfaces were incised to include the entire soft tissue walls of the investigated pockets. The entire pocket and junctional epithelia as well as the adjacent connective tissue were carefully removed with a surgical blade. Immediately after harvesting, each tissue specimen was immediately washed with saline water and then fixed for 48 h in 10% neutral buffered formalin.

After paraffin embedding, 6 μm thick serial tissue sections were prepared with a manual microtome and stained routinely with hematoxylin and eosin in order to evaluate the structural integrity of the epithelia and the severity of the inflammation. Collagen fibers were stained with Sirius red F3Ba according to Junqueira et al. (1979) from 8 μm thick sections.

2.3. Immunohistochemistry

Paraffin tissue sections were deparaffinized and hydrated through xylene and a graded series of alcohol. Sections were rinsed for 5 min in distilled water. Endogenous peroxidase activity was blocked through incubation in 3% (v/v) H_2O_2 for 5 min. Tissue sections were washed in PBS for 5 min, incubated for 10 min in non-fat dried milk and washed twice for 5 min in PBS. Tissue sections were incubated for 1 h with a panel of primary monoclonal and polyclonal antibodies with well-defined specificities, such as anti-CD45 (leukocyte common antigen; dilution (1:100)[†], anti-CD3 (cortical and medullary thymocytes and peripheral T

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lymphocytes; dilution 1:100)[†], anti-CD8 (suppressor/cytotoxic T lymphocytes; dilution 1:50)[†], anti-CD20 (B lymphocytes; dilution 1:200)[†], anti-CD68 (monocytes/macrophages; dilution 1:50)[†], and anti-EMA (plasma cells; dilution: 1:100)[†]. For unmasking antigens, tissue sections were placed in a microwave oven in citrate buffer (0.1 mol/l, pH 6.0) for 3×5 min (at 650 W), after rehydration in phosphate buffered saline (PBS) pH 7.2. The sections were first incubated with one of the primary antibodies mentioned above, then with bionylated appropriate secondary antibodies (anti-mouse and anti-rabbit immunoglobulins), and finally with streptavidin peroxidase conjuguate. Each incubation was carried out for 30 min at room temperature, followed by three baths of 10 min each in PBS. The peroxidase was revealed in a dark chamber using 3′3-diaminobenzidine tetrahydrochloride with hydrogen peroxide in buffer solution for 12 min. The sections were counterstained with hematoxylin. No specific reactivity of the antibodies was noticed by omitting the primary antibody or by using an irrelevant primary antibody presenting isotype-matched primary antibody.

The different cell populations were observed in all samples, and positively stained cells were differentiated from unstained cells by a well-defined brownish ring at the periphery of the cells or within the cells themselves.

2.4. Quantitative determination of collagen fibers

The evaluation of the area fraction (AA%) of collagen fibers in the connective tissue was determined in sections stained with Sirius red F3Ba. Histological sections were observed in polarized light under a standard microscope[‡] equipped with a video camera. Digital image processing analysis was performed using UTHSCSA ImageTool program[§]. The colored images generated by the video camera were converted into 256 different gray levels. The gray scaled image was then transformed into a binary black and white image by thresholding and isolating collagen fibers versus non-collagenous background. The area fraction (AA%) occupied by collagen fibers was then calculated. For all the gingival histological images, a polygonal area selection tool has enabled us to select all the connective tissue area excluding the epithelium from the sections. For each gingival sample, three tissue sections were stained on each slide, and so the area fraction values represent the mean of three measurements.

2.5. Quantitative determination of cell populations

The quantitative evaluation of immunolabeled cells was performed by morphometric analysis in the connective tissue excluding the epithelium. The determination of the number of immunolabeled cells per unit area (number of cells/mm^2) was performed for each reagent with a microscope[‡] using a $100 \times$ objective. The microscope was equipped with a video camera and each microscopic field has been analyzed semi-automatically using a software program[¶]. For each gingival sample, three tissue sections were stained on each slide. Immunolabeled cells were counted in six randomly selected microscopic fields per tissue section so that a total of 18 microscopic fields were evaluated in each case for the evaluation of cell numbers.

2.6. Statistical analysis

Statistical analysis was performed using non-parametric tests and the subject was used as the measurement unit. Means and standard deviations of all the studied variables were calculated in the different stages of periodontal disease and in the control group. Comparisons of the different variables (collagen area fraction and inflammatory cell subsets) between the study groups were performed using the Kruskal–Wallis test. If significant differences were found, post-hoc two-group comparisons were assessed with the Mann–Whitney U test to evidence significant differences among the different groups of periodontal disease: 0 = control, 1 = gingivitis, 2 = moderate periodontitis, 3 = severe periodontitis.

In order to establish correlations between the AA% of collagen fibers and the number of each inflammatory cell subset in the four groups included in this study, groups of coefficients of correlation and p value were determined (applying the Kruskal–Wallis test and then the Mann–Whitney U test). Spearman rank correlation analysis was also used to analyze the correlations between the collagen area fraction and the inflammatory cells quantity as well as the correlations between the inflammatory cells quantity and the clinical stages that were recoded into quantitative variables (0, 1, 2, 3). p values <0.05 were considered to be statistically significant for all statistical tests. All data analysis was performed using a statistical package [SPSS 11.5 (SPSS Inc., Chicago, IL, USA)].

3. Results

The results are given as mean ± standard deviation (mean ± SD) to describe the dispersion of the data.

3.1. Area fraction (AA/%) of collagen fibers

In group (0) (control), the area fraction (AA%) (mean ± SD) occupied by collagen fibers in the connective tissue was 54% (±6%). Additionally, collagen bundles underlying the epithelium appeared normal in size and orientation, and strongly stained (Fig. 1A). In group (1) (gingivitis), the area fraction decreased to 38% (±6%), and collagen fibers of the connective tissue were sparse and disrupted (Fig. 1B). In group (2) (moderate periodontitis) AA% of collagen fibers was 31.8% (±6%), collagen fibers were sparse compared with group (1) (gingivitis) (Fig. 1C). In group (3) (severe periodontitis), AA% of collagen fibers was significantly decreased and rarified reaching 25% (±5%). Furthermore, collagen fibers of the connective tissue were mostly destroyed and discarded (Fig. 1D). When compared to the control group, the area fraction occupied by collagen fibers was significantly and continuously decreased in group (1) (gingivitis) (Mann–Whitney: $p < 0.001$); in group (2) (moderate periodontitis) (Mann–Whitney: $p < 0.001$) and in group (3) (Mann–Whitney: $p < 0.005$).

3.2. Inflammatory cell populations

Inflammatory cell populations of the gingival connective tissue were observed in all samples but varied in number, and positively stained cells were differentiated from non-stained cells with a well-defined brown ring on the periphery of the cell or in the cells themselves. Figs. 2–4 illustrate the indirect immunodetection of CD-68+ (macrophages), EMA+ (plasma cells), and CD-45+ (leukocyte common antigen) respectively in gingival tissue sections from control healthy patients (A), gingivitis patients (B), moderate periodontitis (C), and severe periodontitis patients (D).

The mean numbers of the immunolabeled cells reacting with each antibody per mm^2 are given for the four groups of patients

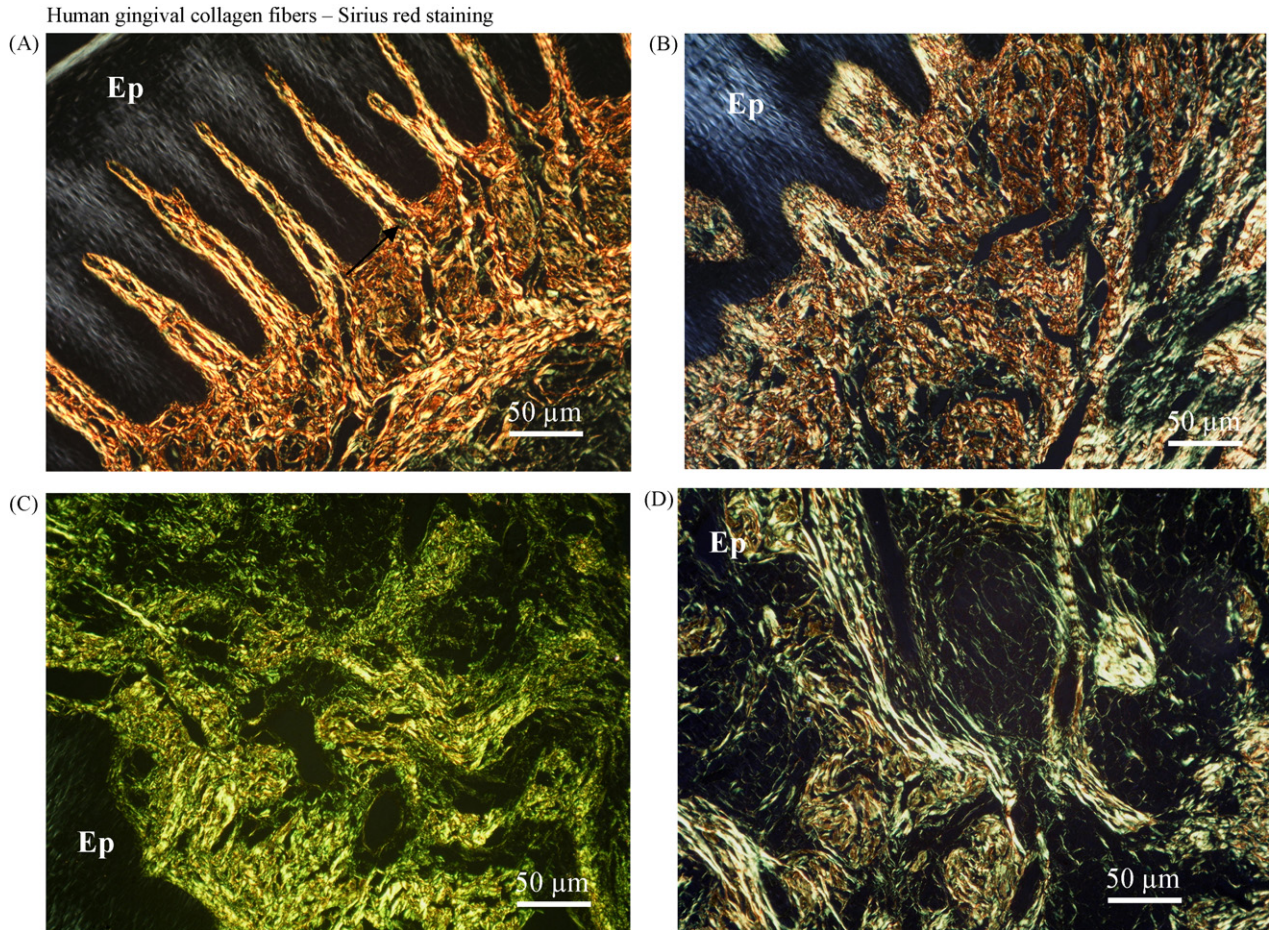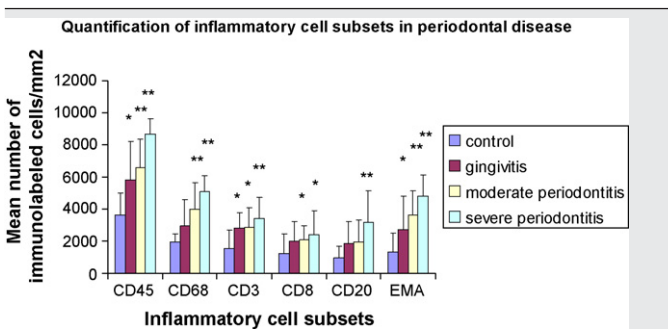Human gingival collagen fibers – Sirius red staining

Fig. 1. (A–D) Histological documents which illustrate collagen component of gingiva in the different groups included in this study: controls (A), gingivitis (B), moderate periodontitis (C) and severe periodontitis (D). (A) Control healthy patient, group 0; (B) gingivitis patient, group 1; (C) moderate periodontitis patient, group 2 and (D) severe periodontitis patient, group 3. Tissue sections were stained with Sirius red F3Ba. With healthy patient (A) collagen bundles filled the gingival digitations, appearing thin and regularly positioned. At the lower connective tissue part, collagen bundles were progressively more and more dispersed and rarefied from gingivitis (B), to moderate (D) and severe periodontitis (D). Tissue sections were observed with polarized light. Ep: epithelium; scale: 50 μm.

in Table 1 together with the standard deviation and significance of differences between the groups.

In gingivitis group (1), a significant increase of CD-45+ ($p < 0.05$), CD-3+ ($p < 0.02$), EMA+ ($p < 0.05$) was noted when compared to control group (0). Although the numbers of CD-8+, CD-20+ and CD-68+ cells were increased, differences were not statistically significant from those of controls. In group 2 (moderate periodontitis), when compared with group (1), a significant increase was noted for CD-

Table 1
Diagrammatic representation of the mean number and standard deviation of inflammatory cells (cells/mm^2) in connective tissue of different clinical periodontal stages specimens.

45+ ($p < 0.05$), CD-68+ ($p < 0.03$) and EMA+ ($p < 0.04$). The increasing of CD-3+ is significant in this group (2) only when compared with controls (0) ($p = 0.021$) but not when compared with gingivitis group (1) ($p < 0.78$). On the other hand the increasing of CD-8+ ($p = 0.549$) and CD-20+ ($p = 0.842$) was not statistically significant when compared with control (0) and gingivitis (1) groups. In group (3) (severe periodontitis) we noted a marked significant increase of CD-45+ ($p < 0.008$), CD-68+ ($p < 0.03$), and EMA+ ($p < 0.04$) when compared with previous moderate periodontitis group (2). It is only in this group with severe periodontitis that we noted a significant increase of CD-20+, when compared with controls ($p = 0.009$). Regarding CD-8+ and CD-3+, no significant differences were noted when compared with gingivitis group (1) and with moderate periodontitis group (2).

3.3. Correlations between collagen AA% and inflammatory cell population number

Statistical analysis results illustrated in Tables 2a and 2b clearly singled out the correlations between the AA% of collagen fibers and the number of each inflammatory cell subset in the four groups included in this study. Spearman rank correlation analysis (Table 3) also demonstrated the significant correlations between quantitative variables of collagen area fraction and the inflammatory cells' quantity. All inflammatory cell subsets except CD-8+ were sig-
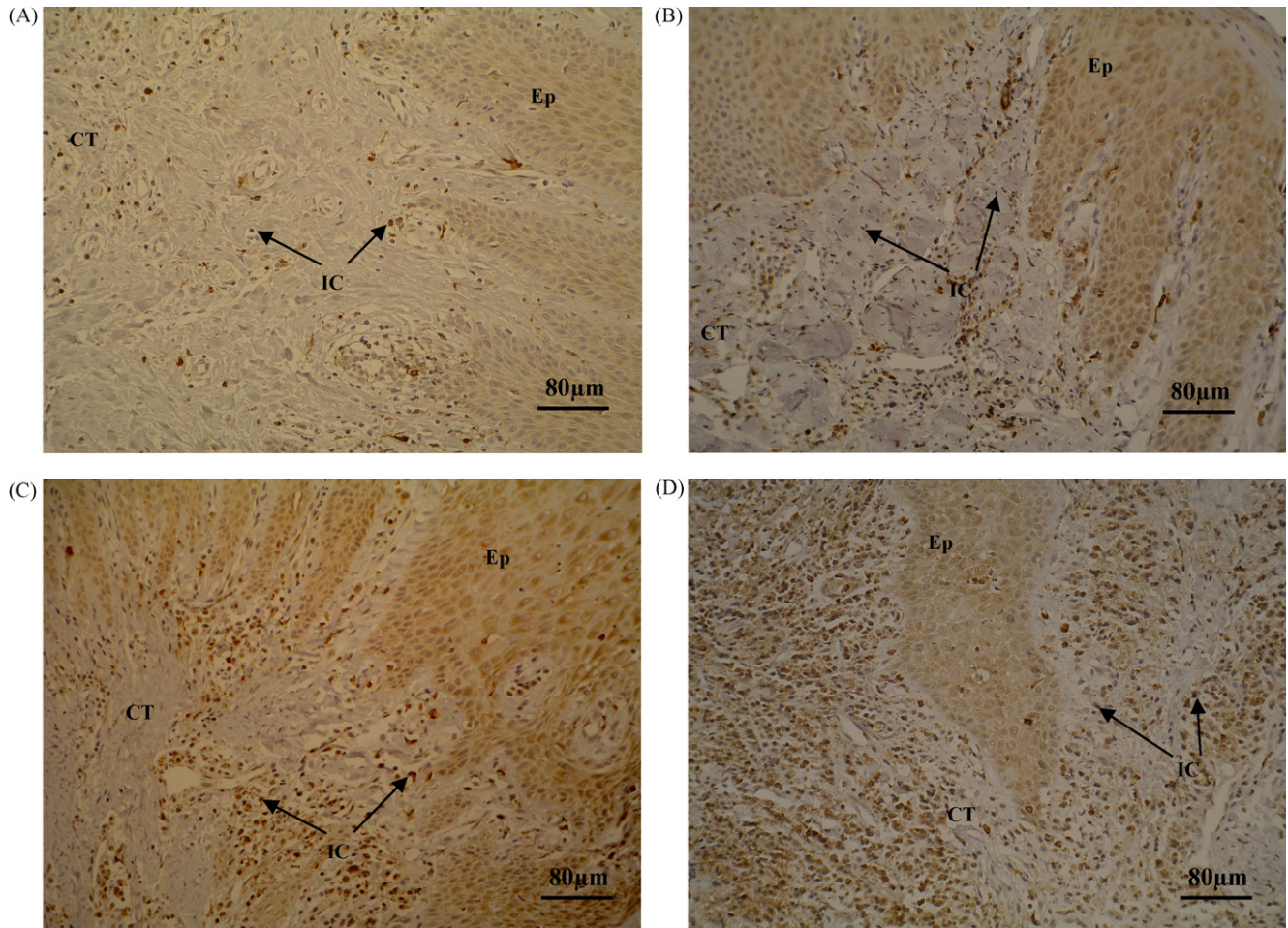
Fig. 2. Indirect immunostaining of CD-68+ macrophages in healthy and diseased gingival connective tissue. Indirect immunodetection of CD-68+ macrophages and counterstaining with hematoxylin. Gingival section from control healthy patient (A), gingivitis patient (B), moderate periodontitis (C) and severe periodontitis (D). With control patient few CD-68+ cells were evidenced in gingival connective tissue. Gingival sections of a patient in gingivitis group (B), a patient in moderate periodontitis group (C) and a patient in severe periodontitis group (D) showed continuous increase in the number of CD-68+ macrophages from gingivitis group to severe periodontitis group. Ep: epithelium; CT: connective tissue; scale: 80 μm; IC: inflammatory cells.

Table 2a
Correlation between inflammatory cells and periodontal disease groups: Kruskal–Wallis non-parametric test.

Inflammatory cells	CD-3+	CD-8+	CD-20+	CD-45+	EMA+	CD-68+
Kruskal–Wallis test p value	0.016	0.065	0.038	0	0.001	0.001

Significant correlation between each inflammatory cell subset and pooled groups of periodontal disease were evidenced.

Table 2b
Correlations between the different study groups and inflammatory cell subsets.

Mann–Whitney test	CD-3+	CD-8+	CD-20+	CD-45+	EMA+	CD-68+
p values 01	**0.015**	0.074	0.167	**0.049**	**0.049**	0.236
p values 02	**0.021**	**0.034**	0.122	**0.003**	**0.003**	**0.009**
p values 03	**0.006**	**0.027**	**0.009**	**0**	**0**	**0**
p values 12	0.78	0.549	0.842	**0.044**	**0.039**	**0.023**
p values 13	0.447	0.497	0.095	**0.008**	**0.028**	**0.008**
p values 23	0.393	0.796	0.143	**0.007**	**0.039**	**0.023**

Mann–Whitney non-parametric test showing correlations among different groups of periodontal disease and inflammatory cell subsets (significant p values are in bold). CD-45+, CD-68+ and EMA+ were significantly correlated with the different stages of periodontal disease. CD-3+, CD-8+ and CD-20+ were irregularly revealed to be significantly correlated.

nificantly and inversely correlated with the area fraction (AA%) occupied by collagen fibers.

Non-parametric Mann–Whitney test demonstrated that CD-45+, CD-68+ and EMA+ were significantly correlated with the different stages of the periodontal disease. On the contrary, CD-3+, CD-8+ and CD-20+ were irregularly noted to be significantly correlated with the different stages of the periodontal disease (Table 2b).

Significant correlations were noted between all inflammatory cell subsets and the area fraction (AA%) occupied by collagen fibers in the different groups included in this study except for CD-8+ cells (Table 3). This was confirmed by scatter plots as shown in Fig. 5a–f illustrating correlations between AA% of collagen fibers and the mean number of each immunolabeled cell subset. On the other hand, significant correlations were noted between all cell subsets and clinical stages (Table 3).

When clinical criteria (index scores) were taken into consideration – gingival index, plaque index, bleeding on probing, bone
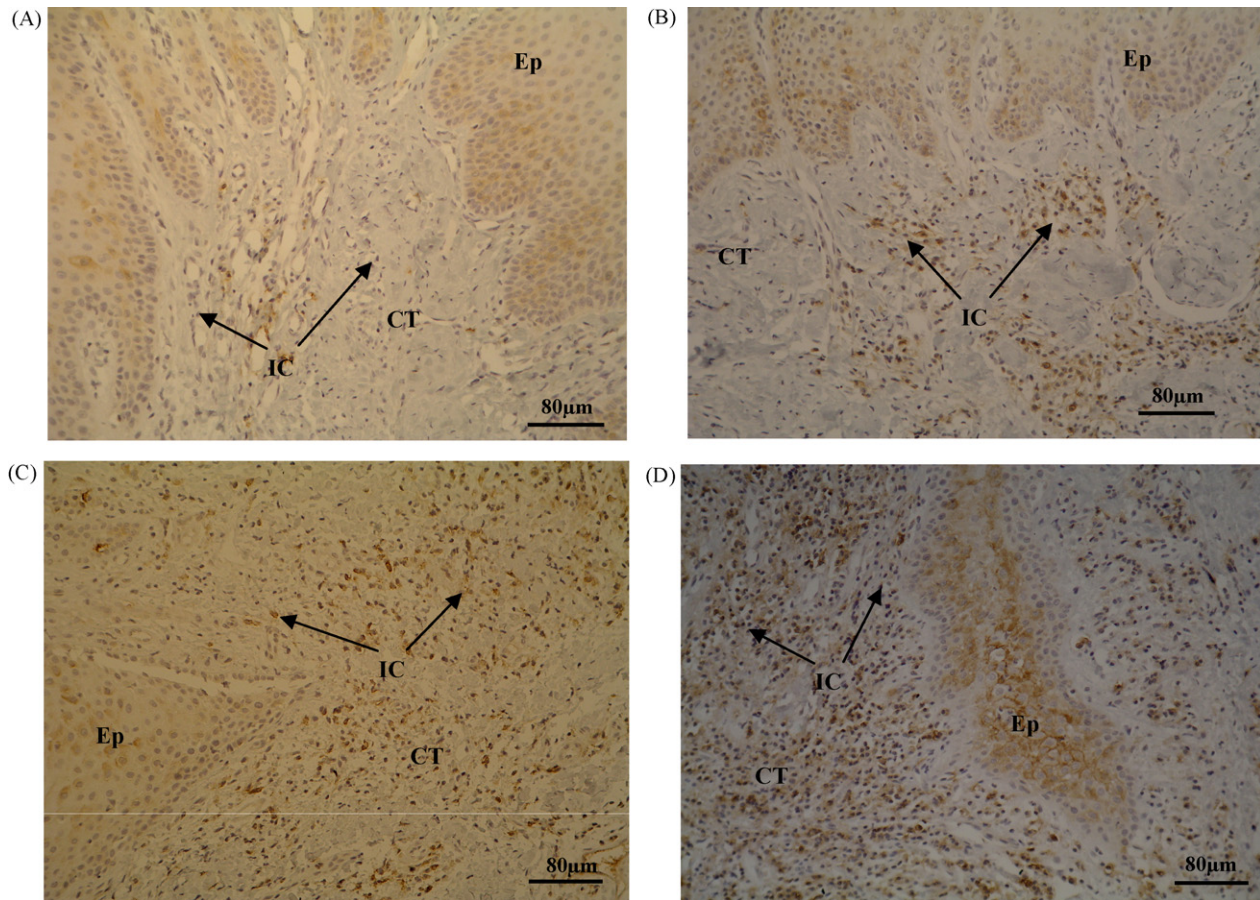
Fig. 3. Indirect immunostaining of EMA+ plasma cells in healthy and diseased gingival connective tissue. Indirect immunodetection of EMA+ plasma cells and counterstaining with hematoxylin. Gingival section from control healthy patient (A), gingivitis patient (B), moderate periodontitis (C) and severe periodontitis (D). (A) With control healthy patient rare EMA+ cells were shown in gingival connective tissue. From gingivitis patient (B) to moderate (C) and severe periodontitis (D) continuous increased number of plasma cells were evidenced in connective tissue. Ep: epithelium; CT: connective tissue; scale: 80 μm; IC: inflammatory cells.

Table 3
Spearman correlations between the mean number of each inflammatory cell subset and AA% of collagen fibers represented in the first row. Spearman correlations between the mean number of each inflammatory cell subset and recoded clinical periodontal stages (0, 1,2, 3) represented in the second row.

Correlations inflammatory cells and AA and clinical stages (0, 1, 2, 3)	CD45 +	CD68 +	EMA+	CD3+	CD8+	CD20+
Spearman correlation with AA% collagen patients	−0.67	−0.55	−0.58	−0.43	−0.28	−0.38
p value	<0.0001	0.0006	0.0002	0.0091	0.0991	0.0214
Spearman correlation with clinical periodontal stage	0.737(**)	0.678(**)	0.664(**)	0.455(**)	0.399(*)	0.468(**)
p value	<0.0001	<0.0001	<0.0001	0.005	0.015	0.003

Only CD-8+ were not significantly correlated with AA% occupied by collagen fibers in the different groups included in this study (first row). Significant correlations were evidenced between all cell subsets and clinical stages (second row).

loss, pocket probing depth and clinical attachment loss, from which patients were diagnosed as controls, gingivitis, moderate periodontitis, and severe periodontitis patients – significant correlations were established between each cell subset and each clinical criteria (index score) (Table 4).

4. Discussion

The present quantitative analysis, by means of morphometric and automated image analysis associated with immunohistochemistry, investigated infiltrating inflammatory cell subsets and the loss of collagen fibers in different clinical groups of periodontal disease (based on gingival index scores and pocket measurements) in order to demonstrate correlations, if any, between collagen break-

down, periodontal disease stages, and a particular inflammatory cell population.

The quantification of the gingival collagenic component in the control group (0) revealed that the area fraction occupied by collagen bundles (AA%; mean (±SD)) was 54.1% (±6.2%), in accordance with previous studies (Séguier et al., 2000; Lappin et al., 1999; Ejeil et al., 2003; Gogly et al., 1997; Junqueira et al., 1979; Stouffi et al., 1987). A significant decrease of AA% was also noted (p < 0.05) in the gingivitis group (38.5% (±6.6%)) when compared with group (0). In the moderate periodontitis group (2) a larger decrease of AA% was observed, when compared with the previous group (1). Moreover, a statistically significant difference was evidenced, for the first time, between the moderate (31.8% (±6.5%)) and the severe periodontitis (25.4% (±5.6%)) groups (groups 2 and 3). These findings revealed a
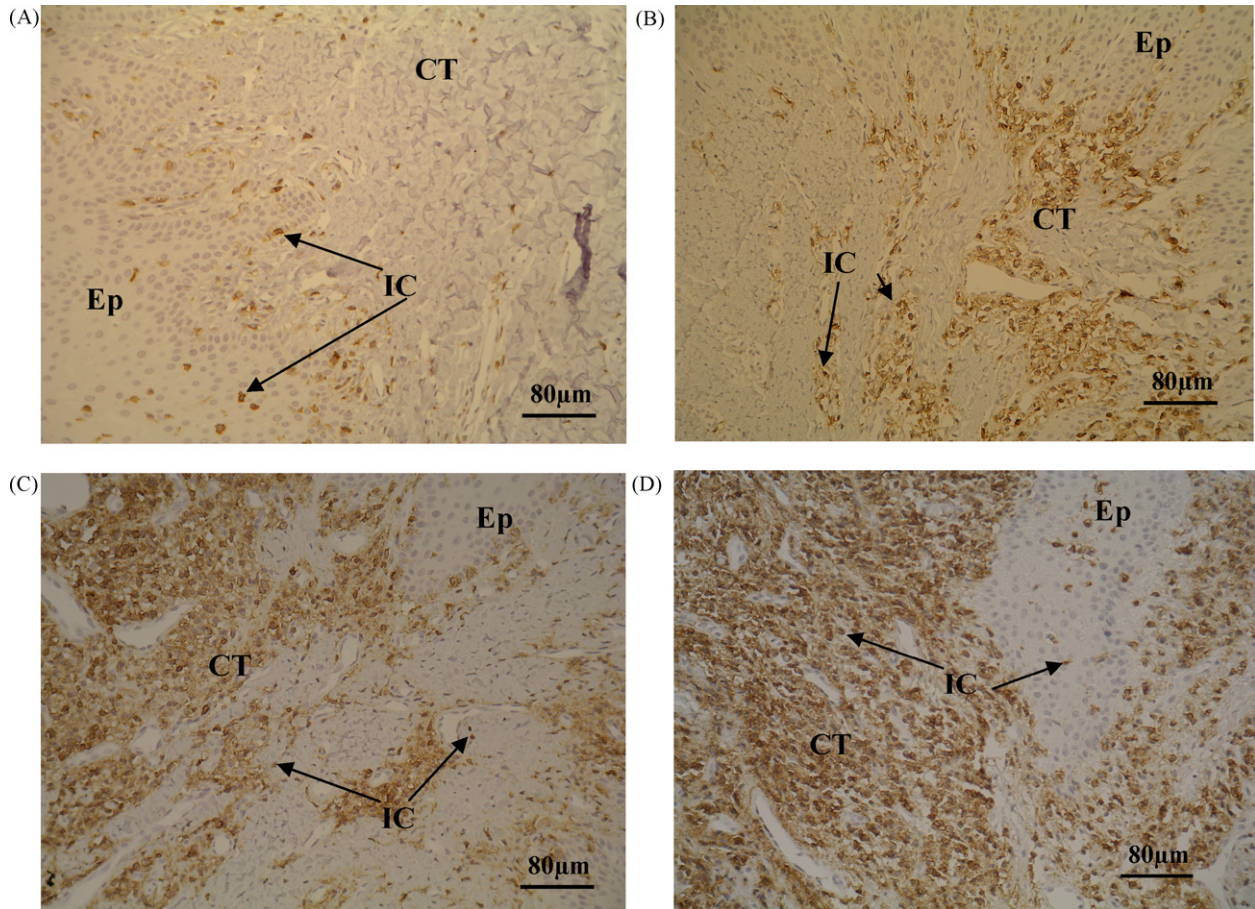
Fig. 4. Indirect immunostaining of CD-45+ (leukocyte common antigen) in healthy and diseased connective tissue. Indirect immunodetection of CD-45+ (leukocyte common antigen) and counterstaining with hematoxylin. Gingival section from control healthy patient (A), gingivitis patient (B), moderate periodontitis (C) and severe periodontitis (D). In control healthy patient (A) few CD-45+ cells were detected in connective tissue. The number of CD-45+ cells increased continuously from control healthy patient (A) to moderate (C) and severe periodontitis (D). Ep: epithelium; CT: connective tissue; scale: 80 μm; IC: inflammatory cells.

clear difference pertaining to the loss of connective tissue integrity between the study groups (healthy, gingivitis, moderate and severe periodontitis).

The quantitative analysis of the different cell subsets present in the gingiva of the study groups clearly implicated all inflammatory cells in periodontal disease. However, it should be noted that the prevalence of these cell subsets varies between different groups according to the severity of periodontal disease.

Several studies evaluated the role and topography of inflammatory cells in relation with the periodontal disease. They were based on a visual estimation or an approximation (percentage) of the different inflammatory cell subsets; Lindhe et al. (1980) applied stereological techniques and morphological criteria of cells to assess the composition of inflammatory infiltrates within gingival tissue with advanced periodontitis. They reported that the plasma cells occupied 31% of the lesions while the proportion of the lymphocytes

Table 4
Correlations between each inflammatory cell subset and the different periodontal disease indices.

Correlations		Stages 0, 1, 2, 3	CD3	CD8	CD20	CD45 LCA	EMA	CD68
Clinical stages 0, 1, 2, 3	Correlation coefficient			0.455(**)	0.399(*)	0.468(**)	0.737(**)	0.664(**)	0.678(**)
	Significance (2-tailed)			0.005	0.015	0.003	0	0	0
Gingival index	Correlation coefficient		0.855(**)	0.532(**)	0.496(**)	0.438(**)	0.752(**)	0.654(**)	0.641(**)
	Significance (2-tailed)		0	0.001	0.002	0.007	0	0	0
Plaque index	Correlation coefficient		0.674(**)	0.360(*)	0.294	0.32	0.546(**)	0.443(**)	0.548(**)
	Significance (2-tailed)		0	0.029	0.077	0.053	0	0.006	0
Bleeding on probing	Correlation coefficient		0.823(**)	0.449(**)	0.400(*)	0.366(*)	0.657(**)	0.480(**)	0.544(**)
	Significance (2-tailed)		0	0.006	0.016	0.028	0	0.003	0.001
Bone loss	Correlation coefficient		0.957(**)	0.357(*)	0.332(*)	0.425(**)	0.684(**)	0.613(**)	0.648(**)
	Significance (2-tailed)		0	0.03	0.044	0.009	0	0	0
CAL-Pocket probing	Correlation coefficient		0.747(**)	0.370(*)	0.418(*)	0.434(**)	0.639(**)	0.510(**)	0.567(**)
	Significance (2-tailed)		0	0.024	0.01	0.007	0	0.001	0

Significant correlations were evidenced between each cell subset and each clinical criterion (index scores).

varied between 5% and 10%. Macrophages and polymorphonuclear (PMN) were found in densities of 1–2%. Thus, the volume occupied by plasma cells was at least three times higher than the proportion of lymphocytes. Other inflammatory cells occurred only in small numbers. Similar findings were reported in studies in which morphological/stereological techniques were used (Zappa et al., 1991; Liljenberg et al., 1994; Passo et al., 1988; Berglundh et al., 1998). Immunological studies provided evidence for T-cell domination in stable lesions and an increase in the number of B cells in progressive lesions (Gemmell et al., 2007; Ishikawa, 2007).

With respect to the control group (0), the quantification of the inflammatory cell subsets highlighted the fact that the clinical diagnosis as healthy was not corroborated by the histological findings. In this group (0), the T-cell population quantity was more important than B cell population (B cells and plasma B cells), in accordance with previous studies showing that T-cell lymphocytes are predominant in stable and healthy sites (Gemmell et al., 2007; Reinhardt et al., 1988).

In gingivitis group (1), a significant increase of EMA+, CD-45+ cells as well as CD-3+ T-cells was noted. These results are in accor-
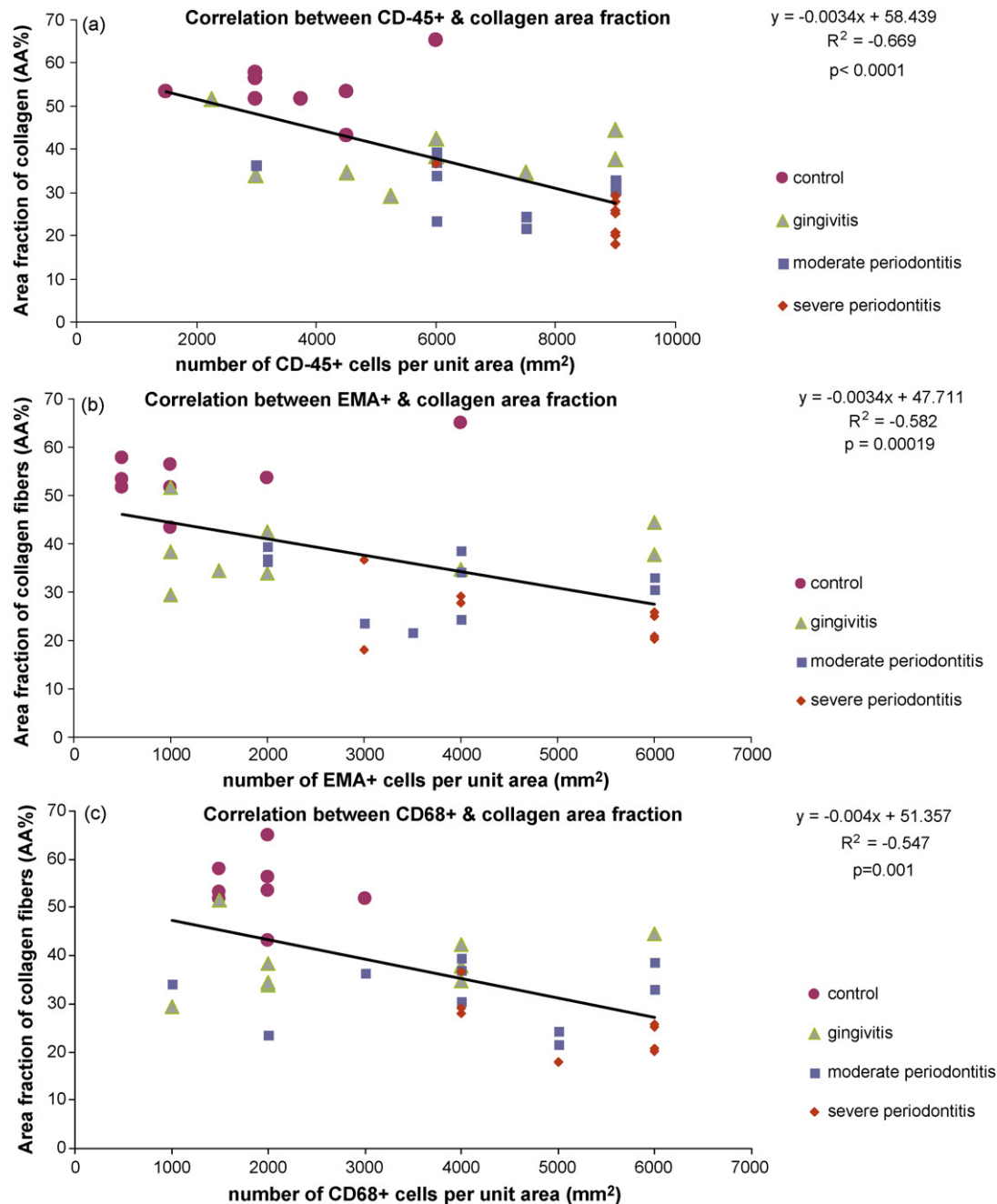

Fig. 5. Correlation between the area fraction (AA%) of collagen fibers and each cell subset. (a) Significant correlations were noted between AA% of collagen fibers and the mean number of CD-45+ (leukocyte common antigen) cells. $R^2 = -0.669$ and $p < 0.0001$. (b) Significant correlations were noted between AA% of collagen fibers and the mean number of EMA+ (plasma cells) cells. $R^2 = -0.582$ and $p < 0.0001$. (c) Significant correlations were noted between AA% of collagen fibers and the mean number of CD-68+ (macrophages) cells. $R^2 = -0.547$ and $p = 0.001$. (d) Significant correlations were noted between AA% of collagen fibers and the mean number of CD-3+ (T-cells) cells. $R^2 = -0.429$ and $p = 0.009$. (e) Significant correlations were noted between AA% of collagen fibers and the mean number of CD-20+ (B cells) cells. $R^2 = -0.382$ and $p = 0.021$. (f) CD-8+ (T cytotoxic cells) were not significantly correlated with the AA% of collagen fibers. $R^2 = -0.279$ and $p = 0.099$.
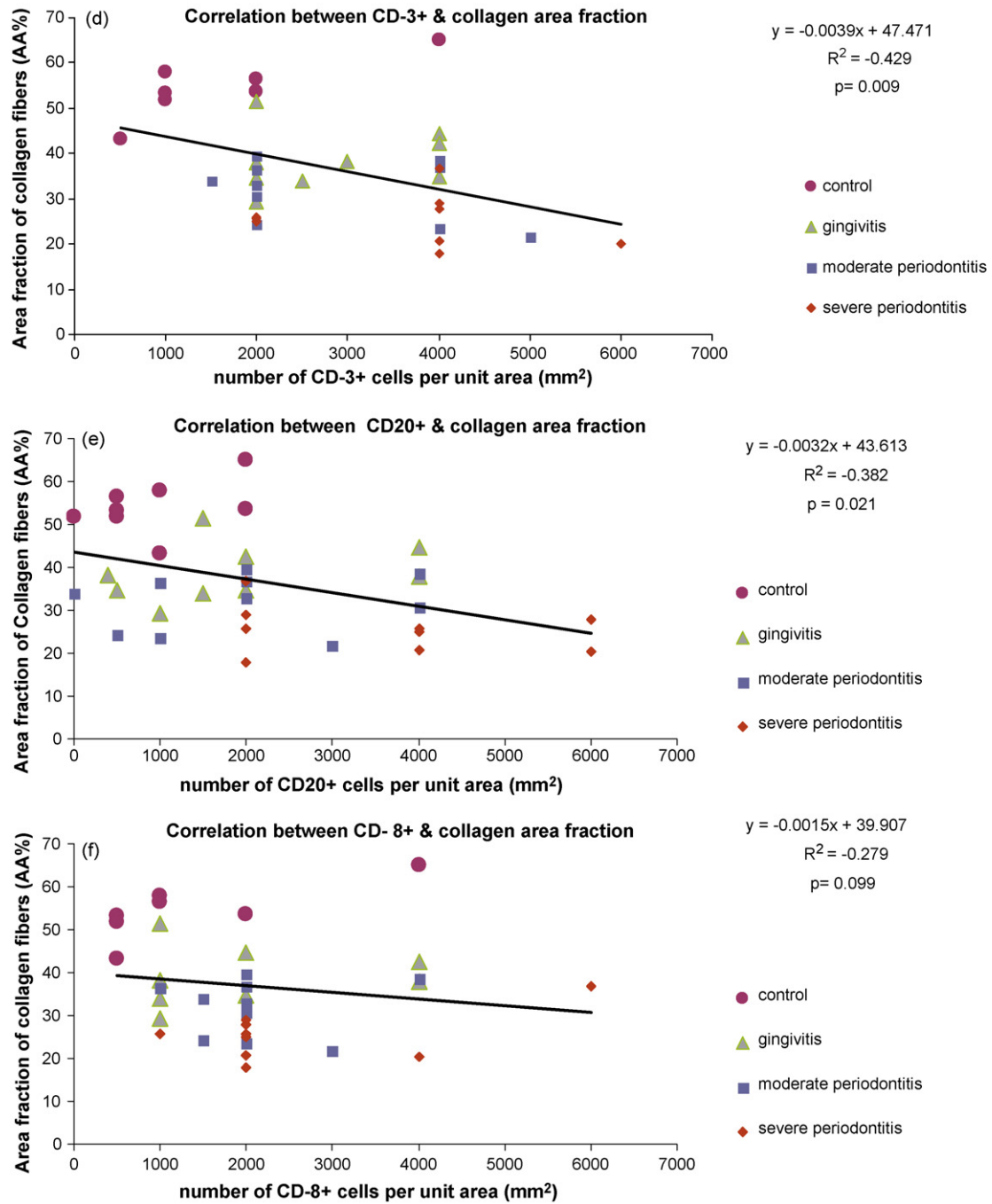
Correlation between CD-3+ & collagen area fraction

$y = -0.0039x + 47.471$
$R^2 = -0.429$
$p = 0.009$

- control
- gingivitis
- moderate periodontitis
- severe periodontitis

Correlation between CD20+ & collagen area fraction

$y = -0.0032x + 43.613$
$R^2 = -0.382$
$p = 0.021$

- control
- gingivitis
- moderate periodontitis
- severe periodontitis

Correlation between CD-8+ & collagen area fraction

$y = -0.0015x + 39.907$
$R^2 = -0.279$
$p = 0.099$

- control
- gingivitis
- moderate periodontitis
- severe periodontitis

Fig. 5. (*Continued*).

dance with those of Seymour et al. (1983) and Séguier et al. (2000), implicating the T-cells in the earlier stages of inflammation. T-to-B cell ratio was still positive despite a clear increase of plasma cells at this stage.

While the comparison of group (2) (moderate periodontitis) to group (0) revealed an increase of all inflammatory subsets, only a significant increase of CD-45+, EMA+, and CD-68+ was observed when compared with gingivitis group (1). Neither T-cells, nor B cells showed any significant increase.

In fact, the only significant increase of B cells was observed in group three when compared with previous groups, whereas CD-45+, CD-68+, and EMA+ progressively (and statistically) increased across groups, and B-plasma to T-cell ratio shifted to positive

for periodontitis groups. Such finding with respect to the B cell population is in accordance with immunohistochemical studies suggesting an increase in the B-to-T cell ratio in adult periodontitis (Lappin et al., 1999), and a shift from T-cell domination in stable lesions to an increase in the number of B cells and plasma cells in progressive lesions (Seymour et al., 1979a,b).

The number of monocytes/macrophages (CD-68+) constantly increased in the different pathological stages, suggesting that these cells were particularly implicated in the course of periodontal disease (Taubman et al., 1984; Seymour, 1991). Other investigations showed a significant increase in the macrophage to T-cell ratio in adult periodontitis groups (Lappin et al., 1999; Taubman et al., 1984). Macrophages, through their production of a variety of

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cytokines (Beklen et al., 2007), proteinases (Wallace et al., 2008), reactive oxygen species (Persson et al., 2008) can also lead to connective tissue breakdown (Taubman et al., 1984). This is consistent with the progressive loss of collagen component demonstrated in this study and correlated with CD-68+ cells.

We also noted a constant increase of plasma cells (EMA+) through study groups reflecting the severity of periodontal disease. Moreover, the correlation between collagen loss and EMA+ cells was clearly illustrated in this study through scatter plot. This is in accordance with studies which highlighted the fact that the increase in collagen breakdown was associated with an increase of plasma cells density (Joachim et al., 1989, 1990). Furthermore, it was demonstrated that in periodontal disease, collagen types I and III are recognized by locally produced antibodies, such as IgM, IgG, and IgA plasma cells, leading to collagen breakdown by means of different anti-collagen antibodies (Takahashi et al., 1997; Hirsch et al., 1988, 1989).

In conclusion, periodontitis lesions are characterized by large proportions of inflammatory cells playing a crucial role in the gingival extracellular matrix degradation. This study showed that the cells which correlated most with inflammatory periodontal lesions were plasma cells (EMA+), leukocyte common antigen (CD-45+) and monocytes/macrophages (CD-68+). T-cells (CD-3+, CD-8+) and B-cells (CD-20+) were only noted to be active at early or late stages of periodontal disease. This study underscores the fact that plasma cells are key cells, strongly correlated with periodontal disease and related collagen breakdown.

Conflict of interest

The authors declare that they have no conflict of interests.

Acknowledgements

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