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ORIGINAL ARTICLE

VEGF, Microvessel Density, and CD44 as Inflammation Markers in Peri-implant Healthy Mucosa, Peri-implant Mucositis, and Peri-implantitis: Impact of Age, Smoking, PPD, and Obesity

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Abstract—Several biologic processes affect the supporting peri-implant tissue leading to implant failure and complications, mainly referred to inflammation that is still poorly investigated in the peri-implant soft tissues. Our aim was to investigate in peri-implant healthy mucosa, peri-implant mucositis, and peri-implantitis the expression of some angiogenesis markers highly associated with inflammation, and evaluate its relationships with age, smoking, peri-implant pocket depth (PPD), and body max index (BMI). Moreover, we wanted to study the impact of these clinical parameters in the disease pathogenesis. Forty-eight total patients were recruited. Sixteen had at least one successfully osteointegrated dental implant (group A) and 32 had at least one osseointegrated implant in need of a peri-implant treatment for inflammatory/infectiveous reasons: precisely 16 for mucositis (group B) and 16 for peri-implantitis (group C). VEGF, CD34, and CD44 immunohistochemical expression was evaluated in the interproximal biopsies of marginal peri-implant tissue and correlated with the clinical parameters. A significant difference between groups in mean PPD was found, while the distribution by age, gender, smoking, and BMI resulted similar. Group C had significantly higher levels of VEGF, CD34, and CD44 expression compared to the other groups. VEGF, CD34, CD44, and peri-implant pocket depth were all positively correlated. Our study revealed that peri-implantitis is a condition characterized by unique and distinctive features. Our results supported that PPD has a great impact on the peri-implantitis and it is closely related to the inflammation marker expression. The identification of specific biomarkers might help in choosing distinct treatment approaches for target individuals.

KEY WORDS: inflammation markers; VEGF; CD44; peri-implantitis; mucositis; peri-implant pocket depth.

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INTRODUCTION

Generally, implant rehabilitations achieve high success rates, but some factors can lead to biological implant failure [1]. In osseointegrated implant, for example, an imbalance in the host-parasite equilibrium leads to a series of inflammatory changes causing the mucositis, a reversible inflammatory lesion in the superficial tissue of the peri-implant mucosa, or, if such an inflammation is combined with loss of bone, the peri-implantitis thus in a late failure [2].

Angiogenesis plays a key role in inflammation and healing, and seems to be important in the preservation of healthy periodontal tissues and in the pathogenesis and progression of periodontal lesions [3]. It also plays an important role in osseointegration process by contributing to inflammatory and regenerative phases of surrounding alveolar bone [4].

Vascular endothelial growth factor (VEGF) is one of key regulators of physiologic angiogenesis. It induces endothelial cell growth, increases vascular permeability, promotes cell migration, and inhibits apoptosis as well as the permeabilization of blood vessels which leads to inflammation [5]. Several studies showed that it is involved in the regulation of inflammation in periodontal disease [3, 5, 6] and increases in the peri-implantitis-affected implants [7].

The hematopoietic progenitor cell antigen CD34 is usually used to identify vascular endothelial cells and hematopoietic stem and progenitor cells. The assessment of microvessel densities (MVD), detected by antibody to CD34, is frequently used to quantify angiogenesis in archival tissue. MVD evaluation is commonly applied for the estimation of angiogenesis level and is widely accepted to play a role in the pathogenesis of some inflammatory conditions. Studies on CD34 expression showed an increasing vascularity in the peri-implantitis [8].

The CD44, an integral membrane glycoprotein, also seems to be take part in the *in vivo* formation of blood vessels and in the organization and stability of endothelial tubular networks during the angiogenesis. CD44 together with its ligand hyaluronan (HA) can modulate the inflammatory reaction and local immune responses *via* the T-lymphocytes [9]. CD44-HA complex is implicated in several inflammatory diseases including periodontitis [3, 10].

The involvement of such factors in periodontitis has been widely studied; on the contrary, little is still known in the progression of peri-implantitis. It is well established that there are links between angiogenesis and inflammation and that they can be considered inflammatory markers *per se*.

Therefore, our aim was to conduct a comparative immunohistochemical evaluation of CD44, VEGF, and CD34 in healthy peri-implant soft tissues and in peri-implant soft tissues affected by mucositis and peri-implantitis, investigating the relationship with age, smoking, peri-implant pocket depth (PPD), and body max index (BMI), and the impact of these clinical parameters in the disease pathogenesis.

MATERIAL AND METHODS

Patient Selection

Forty-eight subjects who have at least one osseointegrated implant were selected in a pool of patients of Dental Clinics in Pesaro and Osimo, Italy.

Exclusion criteria were as follows: (i) presence of an important disease (HIV+, HCV+, allergies, etc.); (ii) inadequate plaque control judged by > 20 using the O'Leary plaque index [11]; (iii) administration of antibiotics, corticosteroids, or nonsteroidal anti-inflammatory drugs within the 6 months prior to treatment; (iv) being affected by chronic periodontitis.

The subjects were divided as follow: 16 had at least one successfully osteointegrated dental implant (group A), 32 had at least one osseointegrated implant in need of a peri-implant treatment for inflammatory/infectiveous reasons: precisely 16 for mucositis (group B) and 16 for peri-implantitis (group C).

Selection criteria for peri-implant gingival samples were as follows: (i) peri-implant gingival tissues around successfully osteointegrated dental implant with no clinically visible plaque accumulation, no peri-implant pocket depth (PPD) more than 2 mm, and no bleeding on probing (BOP) (*healthy samples, group A*); (ii) peri-implant gingival tissues around osseointegrated implant in need of peri-implant treatment with BOP without radiological evidence of bone loss (BL) (*mucositis samples, group B*); (iii) peri-implant gingival tissues around osseointegrated implant in need of peri-implant treatment with swelling tissue with BOP, PPD larger than 5 mm with evidence of BL [12] (*peri-implantitis samples, group C*). Four PPD measurements were taken around each implant: mesial, distal, buccal, and lingual/palatal, by a 15-UNC periodontal probe; their values were measured to the nearest millimeter from the gingival margin to the bottom of the pocket. For all gingival samples, an interproximal biopsy of marginal peri-implant tissue was performed. In the healthy and the mucositis subjects, the biopsies were taken in esthetical gingival remodeling interventions or mucogingival plastic surgery around dental implant. In peri-implantitis subject, the biopsy was obtained during surgical treatment of peri-implantitis.

The study protocol was approved by the local human ethic committee and all patients were, on an individual basis, informed about the aim of the investigation and gave their written consent. The

investigation was conducted according to the tenets of Helsinki Declaration of 1975, as revised in 2000.

Histological and Immunohistochemical Procedures

All biopsy specimens were fixed in 10% neutral buffered formalin and embedded in paraffin. Some sections (4–5 μm) were stained with hematoxylin–eosin for morphological evaluations while some sections were immunostained with anti-CD34 (clone HPCA1, Beckton Dickinson, Erembodegem, Belgium), anti-VEGF (sc-7269, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti CD44 (Signet Laboratories Inc., Dedham, MA, USA) according to the procedures of Lucarini et al. [3]. Immunohistochemical evaluation was performed under a light microscope and independently by two observers (G.L. and A.Z.) who were blinded to the patient group. To evaluate intra- and inter-observer variability, each slide was observed three times by each examiner. Only the mean count value was calculated for each case and used for statistical analysis. The k value was >0.80, showing a substantial agreement between the two observers.

CD34 staining was detected in endothelial cells, VEGF in epithelial, endothelial and dermal infiltrate cells, and CD44 in dermal cells. Images were captured with a digital camera (Nikon DS-Vi1, Nikon Instruments, Europe BV, Kingston, Surrey, England.) connected to a computer.

VEGF-positive vessels, CD34-positive microvessels (Microvessel Density, MVD), and CD44-positive dermal cells were evaluated in at least 10 fields/samples (area of each field 0.07 mm^2; imaging software NIS Elements BR 3.22, Nikon Instruments) at × 400 magnification and quantified as a percentage of the total counted cells or vessels [3]. The fields were randomly selected evaluating the most positive, moderately, and less positive areas.

Statistical Analysis

Univariate analyses were performed by two-tailed Student's t test at baseline between test and control groups; Pearson's correlation was used to analyze the relationship between marker expressions and clinicopathological data; chi-squares test was used to compare nominal data (statistical software SPSS ver17.0, SPSS Inc., Chicago, IL, USA). The results were expressed as means ± standard deviation (SD). We considered $p < 0.05$ to indicate statistical significance.

RESULTS

Clinical Results

Forty-eight total patients (24 males and 24 females, average age of 48.40 years) who had at least one osseointegrated implant were recruited. Descriptions of the clinical parameters and statistical analysis are shown in Table 1. Statistical analysis revealed a significant difference between groups in mean PPD (3.31-mm group A and 3.88-mm group B *vs* 6.44 group C; $p < 0.001$). Distribution by age, gender, smoking, and BMI was similar in all the groups.

Histological and Immunohistochemical Evaluation

The comparison between the slices from biopsy samples of the healthy peri-implant tissue (Group A), peri-implant mucositis (Group B), and peri-implantitis tissue (Group C) is represented in Fig. 1.

Healthy peri-implant tissue showed the mucosa covered by stratified squamous epithelium; in addition, a layer of vascular fibrous connective tissue was evident. A few stromal inflammatory cells and rarely some lymphoid cells in the basal layer were observed.

Peri-implant mucositis displayed an inflammatory infiltrate at the level of the connective tissue lateral to the barrier epithelium.

In peri-implantitis tissue, adjacent to an ulcerated pocket epithelium, a great inflammatory lesion showing an evident granulation tissue and a dense inflammatory infiltrate was detected.

The mean levels of the immunohistochemical marker expression in healthy, mucositis and peri-implantitis groups are summarized in Table 2. Antibodies against CD34 and VEGF were used to determine the vascular proliferation.

VEGF staining was localized at basal layer of the squamous epithelium, in stromal cells, and mostly in the vessels. We focused our interest on the vessel expression (Fig. 2). VEGF was differently expressed in the three examined groups. In group C, endothelial cells presenting high VEGF expression were prevalent (72.44 ± 3.44) with statistically significant differences ($p < 0.001$) with respect to group A (37.44 ± 4.80) and group B (53.44 ± 3.79).

Many microvessels were stained with CD34 (MVD 22.69 ± 1.81) in group C; they were mostly localized in the submucosa where we observed a wide inflammatory infiltrate. In group B, we found a slight MVD increase (15.88 ± 1.86) while in group A only

Table 1. Description of the Clinical Parameters and Statistical Analysis

Parameters	Group A	Group B	Group C	*p* value
Age (mean ± SD)	48.31 ± 5.04	47.50 ± 4.59	49.38 ± 6.14	$p = 0.608$
Gender (♂/♀)	8/8	9/7	7/9	$p = 0.779$
Smoking (not/yes)	8/8	8/8	9/7	$p = 0.920$
PPD (mean ± SD)	3.31 ± 0.60	3.88 ± 1.31	6.44 ± 1.67	$p < 0.001$
BMI (mean ± SD)	29.44 ± 4.34	32.56 ± 5.88	33.94 ± 5.71	$p = 0.61$

Group A, healthy peri-implant tissues; Group B, mucositis; Group C, peri-implantitis

a few CD34-positive microvessels were present in the submucosa (MVD 11.19 ± 1.1), (Fig. 2). Evident significant differences between group C and the other groups were found ($p = 0.001$).

A significantly greater CD44 positivity was found in connective tissue cells in group C compared with groups A and B ($p < 0.001$), (Fig. 2).

When the relationship between the markers was analyzed, Pearson's correlation revealed that CD34, VEGF, CD44, and PPD were all positively correlated (Table 3).

DISCUSSION

Few researchers have investigated the effects of the inflammatory process in the peri-implant soft tissues. In this study, we evaluated some markers of inflammation such as VEGF, CD34, and CD44 in peri-implant healthy mucosa, peri-implant mucositis, and peri-implantitis.

The bacterial infection in the peri-implant region is considered a first stage of peri-implant mucositis and peri-implantitis [13] as well as the presence of an appropriate amount of keratinized gingiva is required to prevent the risk of the increase of gingival index, plaque index, pocket depth, and bleeding on probing/modified bleeding index [14].

In peri-implantitis, the bacterial-induced inflammatory reaction leads to loss of supporting bone and in many cases it seems to respond poorly to the traditional therapies [15]. Several authors found greater infiltration of inflammatory cells in the peri-implant mucosa [16, 17] and enhanced expression of inflammatory factors, many of which can promote angiogenesis contributing to the severity of the inflammation [18].

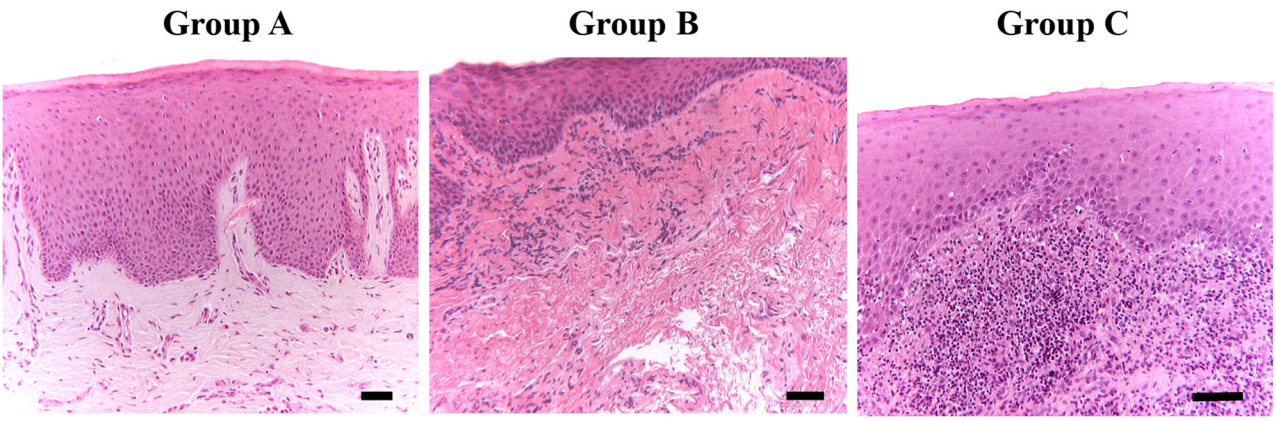

Fig. 1. Representative pictures of histological sections of healthy peri-implant tissue (group A), peri-implant soft tissues affected by mucositis (group B), and peri-implantitis (group C). In group A, a few stromal inflammatory cells were present; in group C, an evident granulation tissue and a dense inflammatory infiltrate were evident with respect to group B. (hematoxylin & eosin, bars 50 μm).

Table 2. Results of the Statistical Analysis of Immunohistochemical Expression of VEGF, CD34, and CD44. Data Shown as Mean ± SD

	Group A	Group B	Group C	*p* value
VEGF	37.44 ± 4.80	53.44 ± 3.79	72.44 ± 3.44	$p < 0.001$
CD34	11.19 ± 1.17	15.88 ± 1.86	22.69 ± 1.81	$p < 0.001$
CD44	31.81 ± 3.27	42.19 ± 5.26	67.88 ± 4.98	$p < 0.001$

Group A, healthy peri-implant tissues; Group B, mucositis; Group C, peri-implantitis

A number of studies have discussed the effect of titanium alloys, dental implant surface characteristic, and treatments on angiogenesis process. However, some aspects concerning the mechanism of dental implants, in particular their surface characteristics or action in angiogenesis mechanism, remain little known [4].

Our results demonstrated that mucosa affected by peri-implantitis had significantly higher levels of VEGF, MVD, and CD44 expression compared to healthy mucosa and mucositis. Our data are in accord with the findings of Bullon et al. [8], Johnson et al. [19], and Wang et al. [7], that found increased vascular proliferation and VEGF in the peri-implantitis patients.

VEGF plays an important role in the regulation of inflammatory periodontal disease [5, 6] and it is overexpressed in the majority of inflamed tissues and correlated with poor prognosis [18]. VEGF expands the vascular network, increases tissue edema, and

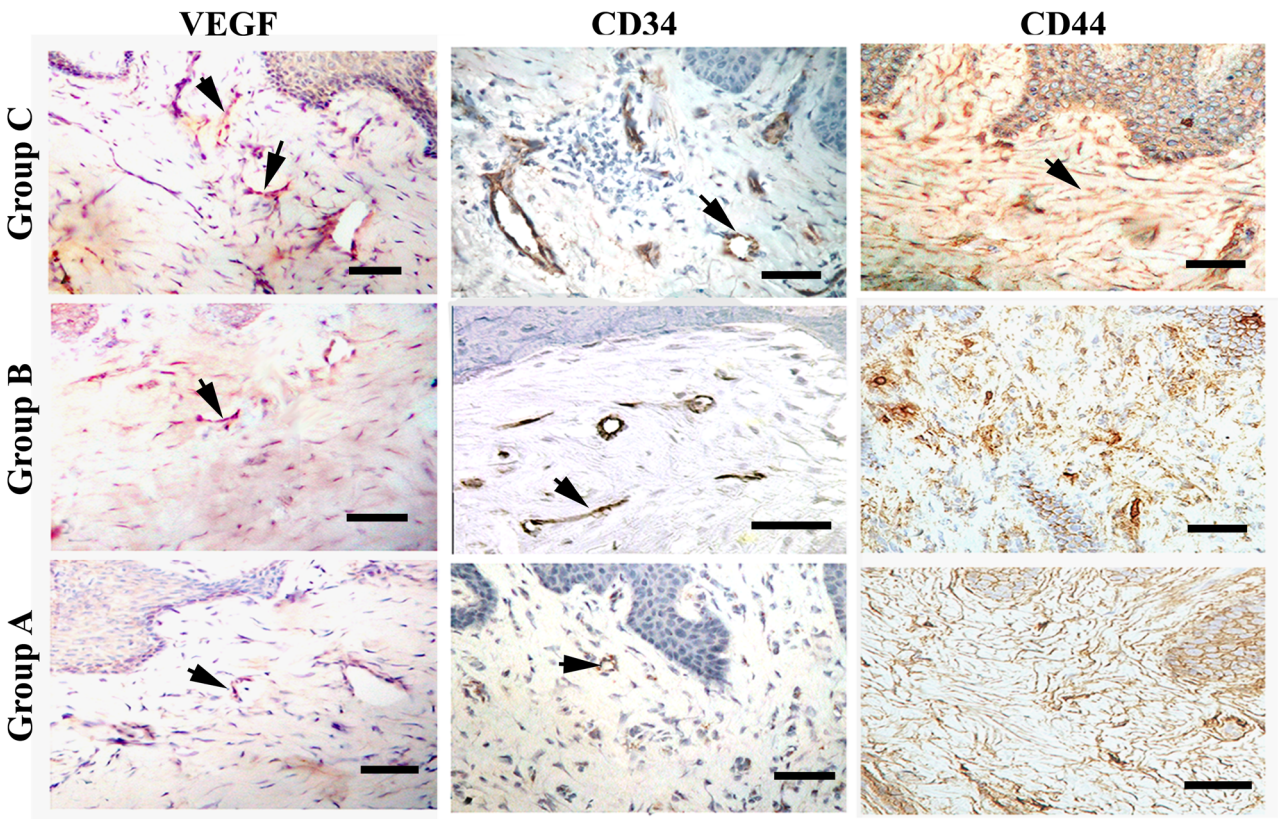

Fig. 2. Immunohistochemical expression of VEGF, CD34, and CD44 in peri-implantitis (group C), in peri-implant soft tissues affected by mucositis (group B), and in healthy gingiva (group A). VEGF expression was localized in endothelial cells of the vessels at the submucosal level (↑). CD34 stained microvessels (↑) while CD44 expression was detected on surfaces of gingival dermal cells (↑). (Immunoperoxidase reaction, bars 20 μm).

Table 3. Pearson's Correlations

		CD34	VEGF	CD44	BMI	PPD
CD34	Pearson correlation	1	.922**	.922**	.320*	.714**
	P (statistical significance)		.000	.000	.026	.000
	N	48	48	48	48	48
VEGF	Pearson correlation	.922**	1	.970**	.489**	.811**
	P (statistical significance)	.000		.000	.000	.000
	N	48	48	48	48	48
CD44	Pearson correlation	.922**	.970**	1	.480**	.851**
	P (statistical significance)	.000	.000		.001	.000
	N	48	48	48	48	48
PPD	Correlazione di Pearson	.714**	.811**	.851**	.692**	1
	Sig. (2-code)	.000	.000	.000	.000	
	N	48	48	48	48	48

decreases the rate of blood flow, probably supporting the etiology of gingivitis and its progression to severe diseases [5]. Verardi et al. [20] hypothesized in peri-implantitis an interaction of the host's inflammatory factor C1q that promotes secretion of pro-inflammatory cytokines in human periodontal fibroblasts and VEGF. He found a C1q increase in peri-implantitis compared to healthy gingiva and a sevenfold increase over baseline of VEGF in the peri-implantitis cultures upon C1q stimulation. These data suggested that the C1q might activate multiple, synthetic signaling pathways, leading to VEGF release, and enhance formation of new blood vessels exacerbating progression of periodontal disease.

CD44 is involved in the angiogenesis too [21] and in multiple physiologic functions such as cell to cell adhesion, cell matrix interaction, and above all lymphocyte recruitment to inflammatory sites through adhesion to HA, the principal ligand. In periodontitis, soluble CD44 was found elevated [10, 22] and HA/CD44-positive cells were observed increased in the connective tissue [3, 23]. During inflammation, HA stimulates immune cells and enhances regulator cytokines of the inflammatory process through an interaction with CD44 and TLR4 receptors [24]. According to our knowledge, at present, there is no literature available on CD44 in peri-implantitis. In this study, we report an increase of CD44 expression in peri-implantitis with respect to mucositis, supporting its association with the inflammation and its involvement also in the peri-implantitis disease.

Analyzing the relationships between the markers, we found that VEGF, CD34, and CD44 are all closely related each other both in healthy mucosa and in diseased one, suggesting a synergic participation in the periodontal status.

Our three clinical groups were also well balanced for potential confounding factors such as smoking, age, gender, BMI, and PPD.

Some systematic reviews have reported an increased risk for peri-implantitis in smokers [25] resulting in a higher implant failures [26]. On the contrary, according to the findings of Koldsland [27] and Roos-Jansåker [28], in our study, the presence of smoking was similar in all the groups and it most likely did not interfere with our analyses.

Regarding obesity, an association between this factor and severity of periodontal disease has been previously demonstrated [29, 30]. To the best of our knowledge, ours is the first evaluation of BMI and relation with peri-implantitis. Our results suggested that obesity could play a similar role in modulating the initiation and progression of periodontal disease both in mucositis and peri-implantitis.

We found that only PPD values showed a statistically significant increase in the peri-implantitis group and they resulted in correlation with the inflammation marker expression in all the groups.

In conclusion, our findings suggest that peri-implantitis is a condition with unique and distinctive features that need to be thoroughly investigated. The peri-implant pocket depth has revealed a great impact on the disease and it is closely related to the inflammatory marker expression.

A greater knowledge of this disease, also through our findings, will allow the clinician to better understand and

prevent its occurrence and arrest its progression. The identification of specific biomarkers as inflammation mediators involved in the onset and progression of peri-implant disease might help in choosing distinct treatment approaches for target individuals.

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COMPLIANCE WITH ETHICAL STANDARDS

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

Ethical Approval. All procedure performed in studies involving human participants were in accordance with the ethical standards of the Institutional Research Committee and of Helsinki Declaration of 1975, as revised in 2000, as well as its later amendments or comparable ethical standards.

Informed Consent. Informed consent was obtained from all individual participants included in the study.

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