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The evaluation of the morphological evolution of the tissue integration of dental implants through conventional histology and immunohistochemistry techniques

FLORIAN OBĂDAN[1)], ŞTEFANIA CRĂIŢOIU[1)], HORIA OCTAVIAN MANOLEA[2)], MIHAELA-CEZARINA HÎNCU[3)], MONICA MIHAELA IACOV-CRĂIŢOIU[4)]

[1)]*Department of Histology, University of Medicine and Pharmacy of Craiova, Romania*
[2)]*Department of Dental Materials, University of Medicine and Pharmacy of Craiova, Romania*
[3)]*Department of Histology, "Lower Danube" University of Galaţi, Romania*
[4)]*Department of Prosthodontics, University of Medicine and Pharmacy of Craiova, Romania*

Abstract

Soft peri-implant tissues are important to ensure the integration of a dental implant, and information on their morphophysiology may explain some clinical failures. Through this study, we aim to contribute to a better understanding of the behavior of peri-implant soft tissue, the morphological support being the one that can explain the different clinical situations. Thus, we sought to reconcile clinical, histopathological and immunohistochemical (IHC) aspects of soft peri-implant tissue, in patients who did not show clinical mobility or radiological signs of bone resorption four months after insertion of implants, some of them showing no clinical signs of inflammation. Immunohistochemically, we highlighted the cellular populations participating in the inflammatory process present in the peri-implant mucosa, in the two groups of patients. The IHC identification of these types of cells and the degree to which each of them was represented by the use of monoclonal antibodies can provide additional insight into the local response of peri-implant soft tissue in healing and osseointegration. This helps the clinician to improve the clinical success of dental implant treatment because the soft tissue surrounding the dental implant separates the implant from the oral cavity and makes a biological seal that prevents the development of the peri-implant pathology. Thus, the soft tissue surrounding the dental implants ensures the conditions of osseointegration and hence the long-term survival of an implant.

Keywords: oral implantology, peri-implant soft tissue, acanthosis, acantholysis.

Introduction

Oral implantology enters the line of the most modern therapies for the restoration of edentulous patients, and the integration at the level of soft and hard tissue is a condition for the success of the dental implant. Peri-implant soft tissue must provide an effective barrier that prevents access to microorganisms and their products. The health and vitality of an integrated dental implant depends on the support of surrounding tissues, which not only anchor the implant to the bone but also have the important function of creating a protective seal. The behavior of the peri-implant mucosa depends on several factors, including soft tissue quality, depth of implant, type of biomaterial used and appearance of its surface [1–3]. Literature reports a very high maintenance rate of between 95% and 98% for a period of 10 years, which encourages the physician to consider this type of oral rehabilitation [4, 5]. Despite this high percentage, there are many patients with peri-implant disorders [6, 7]. The specialized medical literature reports very differently the incidence of peri-implant pathology: from a very low 5% to 56% prevalence [8–13]. The extent of this incidence is justified in the existence of the various definitions used in the classification of this pathology. Hence, the difficulty of comparing different studies and also the need for more research to lead to a consensus on this subject.

We have been motivated in choosing this theme in several ways: the insertion of dental implants is a method that has been more and more frequently used in the last decades in aesthetic and functional restorations; there is a continuing concern regarding the improvement of the implant types and the discovery of new materials that are best tolerated; there is not always an explanation for failure in a particular case; clinical success may be influenced by the particularities of peri-implant tissues.

Patients, Materials and Methods

The studied material consisted of peri-implant mucosa fragments collected from 30 non-smoker patients, women and men, aged 30–65 years old. Harvesting mucosal fragments for the histological study was performed four months after insertion of dental implants, with no mobility or radiological signs of bone resorption. The patients under study were divided into two groups:

- Group I, consisting of 18 patients with no inflammatory clinical signs;
- Group II, consisting of 12 patients with clinically evident inflammatory signs.

The patients in the two groups received a different number of implants, depending on the individual clinical situation. Patients who did not give informed consent for the maneuvers necessary for harvesting the peri-implant mucosa and for the use of harvested material, both for diagnostic and research purposes, were excluded from the study.

The harvested fragments were processed by the histological inclusion technique on paraffin, the sections obtained being stained with Hematoxylin–Eosin (HE) and Goldner–Szekely trichrome. The immunohistochemical (IHC) study was performed on sections obtained from the same paraffin blocks. The study was centered on investigating the following inflammatory infiltration – the antibodies used in this study are centralized in Table 1.

Table 1 – ***Antibodies used in the IHC study***

Primary antibody	Antibody	Epitope marker	Clone	Source / Code	Antigen retrieval	Dilution
CD3	Polyclonal rabbit anti-human CD3	T-lymphocytes	F7.2.38	DAKO / A0452	Citrate, pH 6	1:50
CD4	Monoclonal mouse anti-human CD4	T-helper lymphocytes	MT310	DAKO / M0716	Citrate, pH 6	1:50
CD8	Monoclonal mouse anti-human CD8	Cytotoxic T-lymphocytes	C8/144B	DAKO / M7103	EDTA, pH 6	1:100
CD20	Monoclonal mouse anti-human CD20	B-lymphocytes	1F8	DAKO / M0784	Citrate, pH 6	1:50
CD68	Monoclonal anti-human CD68	Macrophages	KP1	DAKO / M0814	Citrate, pH 6	1:100
CD79α	Monoclonal mouse anti-human CD79α	Plasma cells	jcb117	DAKO / M7050	EDTA, pH 9	1:50
CD15	Monoclonal mouse anti-human granulocyte-associated antigen	PMNs	C3D	DAKO / M0733	Citrate, pH 6	1:25

IHC: Immunohistochemical; CD: Cluster of differentiation; PMNs: Polymorphonuclear neutrophils; EDTA: Ethylenediaminetetraacetic acid.

Results

Knowledge of the structure of the peri-implant mucosa will help the clinician to improve the clinical success of the dental implant treatment. The success and survival of a dental implant does not depend only on osseointegration.

Histological aspects of the peri-implantation mucosa in the studied groups

In our study, we evaluated the histological changes of the epithelium and connective tissue around the implant, on the sections from patients belonging to the two groups, compared to normal gingival mucosal fragments. On sections from groups I and II, we noticed the presence of changes that were of interest both to the surface epithelium and to the lamina propria and whose location, extent and intensity were different, even within the same group. Sometimes, these changes were very intense.

In the two groups of patients, we followed epithelial and lamellar changes (inflammation of the participating inflammatory cell types, presence of cellular edema, nuclear changes, intensity and type of vascularization). These parameters were quantified in three grades: mild (grade 1), moderate (grade 2) and severe (grade 3).

Sections from group I, consisting of patients with no inflammatory clinical signs, showed little, grade 1 variation. An epithelium was found on the surface of the soft tissue, which was orthokeratinized on some sections and parakeratinized on others (Figure 1, A and B). Also, there was a process of acanthosis (Figure 1C), which led to an increase in epithelial thickness and accentuation of epithelial ridges, which are much elongated and broad. Sometimes, due to very elongated epithelial ridges, the epithelial surface has a mesh appearance (Figure 1D). At the epithelial level, starting with the spinous layer, some cells showed edema, gaining a balloon appearance (Figure 1E). In the structure of the epithelium, rare neutrophilic leukocytes, an indicator of an acute inflammatory process, have also been identified. The epithelium of the peri-implant mucosa showed changes that varied in appearance and intensity from one case to another, and, even in the same case, areas with different structural aspects were detected.

The peri-implant mucosal cortex showed changes that affected cell populations, the collagen fibril component and the blood vessels. Some inflammatory cells, especially between the epithelial ridges, in the immediate vicinity of the epithelium, along with fibrocytes and a collagen fibril component (Figure 1F), were present at the level of the chorion. Inflammatory infiltrate was also present away from the epithelium but most often, it was delimited, being located on narrow surfaces. The presence at this level of the lymphoplasmocytic cell infiltrate, as well as many macrophages, indicates the development of a specific, but also nonspecific, macrophage defense process. There are also numerous fibrotic cells stimulated by the local inflammatory process in the collagen fibrillogenesis process. Coronary vascularization was increased, especially in areas where the inflammatory process was more intense.

Group II sections, from patients who showed clinically evident inflammatory signs, showed more intense changes, from moderate to severe, compared to those from group I patients who clinically showed no inflammatory signs. The surface of the epithelium was frequently thin and/or ulcerated (Figure 2). The acanthosis process was more intense, resulting in a papilloma aspect of the lamina propria (Figure 3). Epithelial cells showed sometimes-pronounced intracellular edema, causing cell membrane rupture (Figure 4). At the level of the chorion, there is an inflammatory infiltrate, sometimes intense, which corresponds to a severe degree of inflammation associated with intense vascularization (Figure 5).

Sometimes, the changes were of interest to a limited area of connective tissue, other times the changes were on a considerably larger stretch of connective tissue adjacent to a regionally ulcerated epithelium. In areas of connective tissue of the soft peri-implant tissue, which had an inflammatory process, on some sections we found the presence of a well-represented collagen fibril component, which dissociated the inflammatory infiltrate, suggesting a limitation of it, thus a tendency to extinguish inflammation.

Depending on gender, we noted that, in both groups, women showed all degrees of inflammation and the most severe cellular edema, which could be explained by the physiological hormonal constellation of this gender.

IHC study of inflammatory infiltrate

We followed the clinical, histopathological (HP) and IHC aspects of soft peri-implant tissue in patients who did not show mobility or radiological signs of bone resorption four months after insertion of the dental implants, some of whom showed no inflammatory clinical signs. Immunohistochemically, we highlighted the cellular populations participating in the inflammatory process present in the peri-

implant mucosa in the two groups of patients. Inflammatory infiltrating cells were lymphocytes, macrophages, plasmocytes, mast cells, polymorphonuclear neutrophil (PMN) cells, fibrocytes. The most numerous cells were populations and sub-populations of lymphocytes and macrophages, indicating the existence of a chronic, lymphoplasmocytary and macrophage inflammatory process. Sometimes, however, we have found an aggravation of this chronic process through the presence of PMNs. The location, extent and intensity of the inflammatory infiltrate were different, for group I patients, compared to group II and, even within the same group, there were different aspects. Inflammatory infiltrates sometimes exhibited a diffuse disposition, but there were also areas with delimitation tendencies. On sections from the group II patients, the areas occupied by the inflammatory infiltrate were more numerous, larger in the surface and with higher cell density than those from the patients belonging to the group I. The IHC identification of these types of cells and of the degree to which each of them has been represented by the use of monoclonal antibodies can provide additional insight into the local response of peri-implant soft tissue in healing and osseointegration.

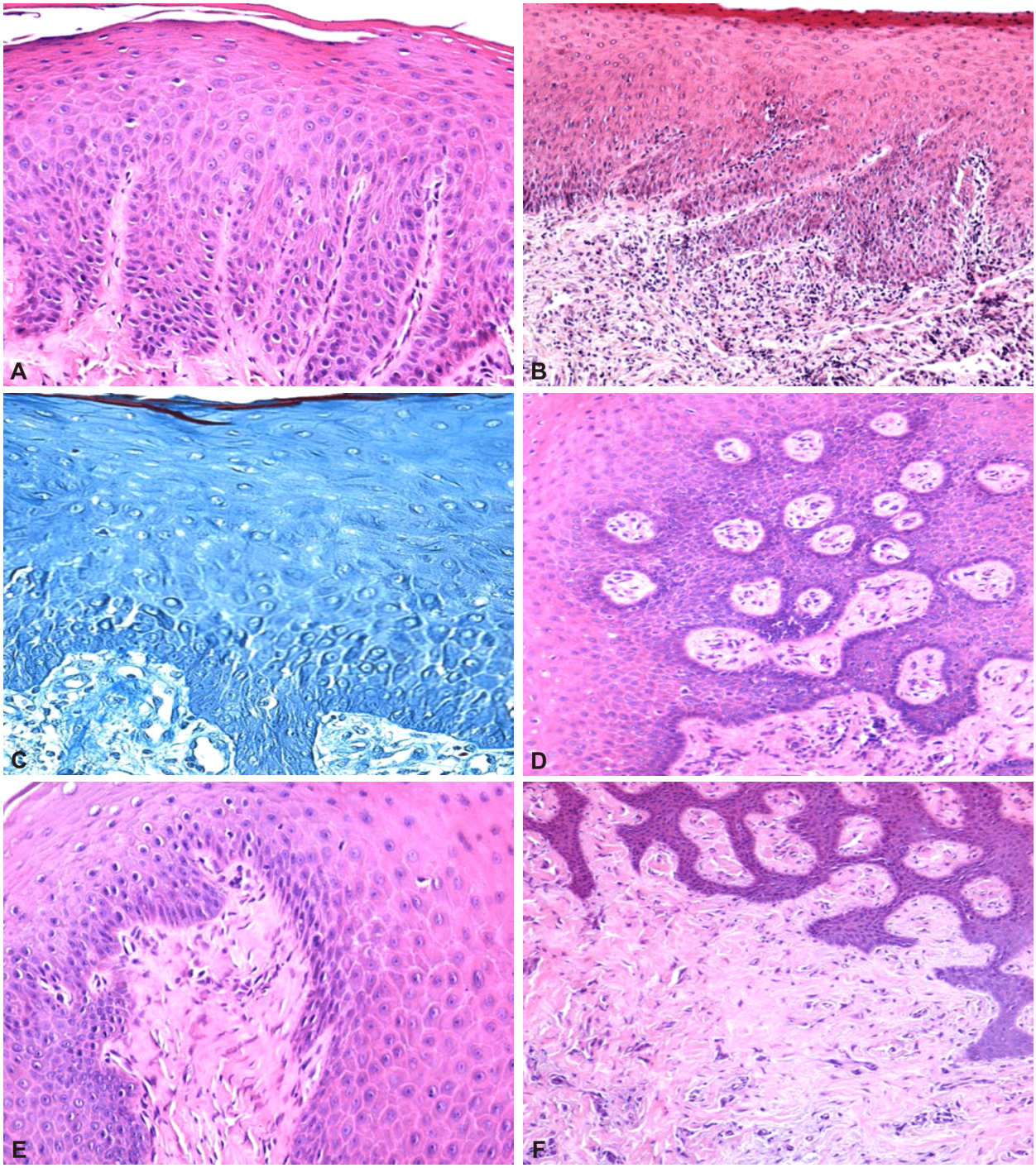

Figure 1 – ***First group of patients. Peri-implant mucosa: (A) Orthokeratinized epithelium and deep epithelial ridges; (B) Parakeratinized epithelium; (C) Acanthosis and parakeratosis; (D) Network aspect of the epithelial surface due to branched and interdigitated epithelial ridges; (E) Cellular edema at the level of the spiny and superficial layer of the epithelium – low inflammatory infiltration in the conjunctivae; (F) Inflammatory chronic infiltration reduced in the subepithelial area. HE staining: (B and F) ×100; (A, D and E) ×200. Masson's trichrome staining: (C) ×200.***

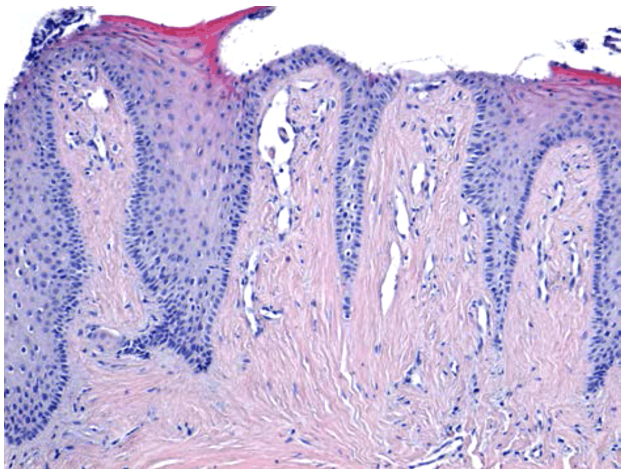

Figure 2 – ***Second group of patients. Ulcerated epithelium. Moderate inflammatory process at the lamina propria level. HE staining, ×100.***

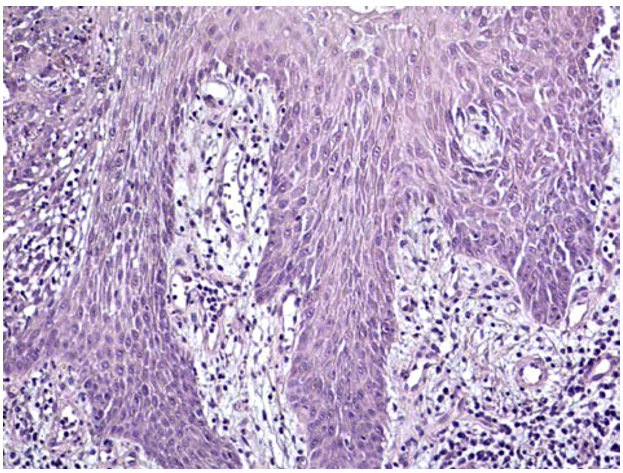

Figure 3 – ***Second group of patients. Papilloma aspect of the lamina propria, due to very elongated epithelial ridges. HE staining, ×100.***

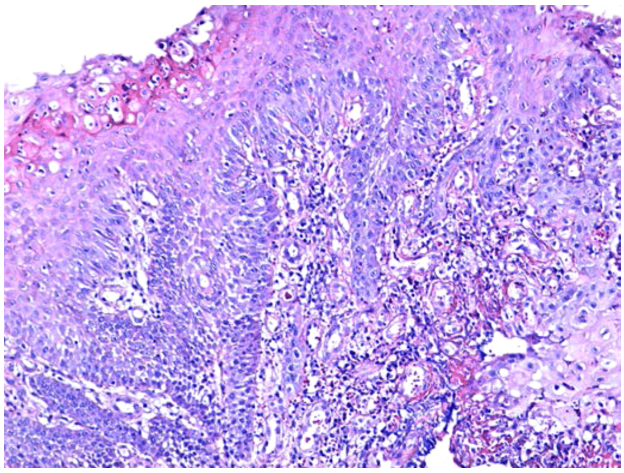

Figure 4 – ***Papillomatosis and edema of epithelial cells in the spinal and superficial layer with vacuolar appearance. Intense chronic inflammatory infiltrate at lamina propria level. HE staining, ×100.***

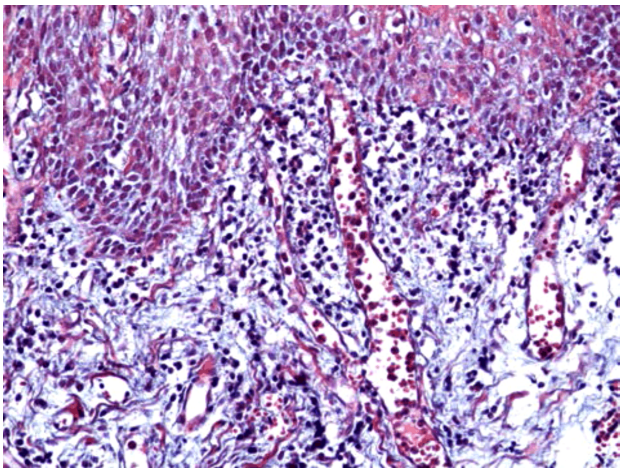

Figure 5 – ***Intense, subepithelial and perivascular inflammatory infiltrate. Masson's trichrome staining, ×200.***

IHC highlighting of T-lymphocytes

T-lymphocytes are cellular mediated immune cells. Multiple subpopulations of T-helper lymphocytes (LTh), cytotoxic or killer T-lymphocytes (LTc), suppressor T-lymphocytes (LTs), T-lymphocytes with memory (LTM) are formed by multiplication of activated T-lymphocytes. These cell types are morphologically similar, but have different functions. In our study, T-lymphocytes were detected immunohistochemically, with the anti-CD3 antibody, which revealed the presence of both LTh and LTc. The presence of CD3+ lymphocytes indicates the existence of an inflammatory process. They were diffuse and less rarely grouped, especially perivascular or sub-epithelial, at the level of the connective papillae (Figures 6 and 7).

We highlighted CD4+ (LTh) and CD8+ (LTc) lymphocytes because the CD4/CD8 lymphocyte ratio is known to be an indicator of immunomodulation status. T-helper, CD4+ lymphocytes were variable, frequent, moderate or rare, but were present in a larger number than CD8+ suppressor/cytotoxic T-lymphocytes. CD4+ lymphocytes were dissected as a diffuse or perivascular infiltrate (Figure 8), while CD8+ lymphocytes were present in extremely small numbers, especially perivascular and subepithelial or even absent (Figure 9).

IHC highlighting of B-lymphocytes

In order to highlight B-lymphocytes, pivotal cells of humoral-mediated immunity, we used the anti-CD20 antibody that did not mark the first and last stage of B-lymphocyte development. For this reason, for the detection of plasma platelets presenting with antigenic stimulation of B-lymphocytes, we used the anti-CD79α antibody, a protein present on the B-lymphocyte surface when antigenically stimulated. The presence of B-lymphocytes and plasma mutants indicates the existence of a humoral immune mechanism in the peri-implantation soft tissues. Compared to T-lymphocytes, B-lymphocytes were identified in much greater numbers, indicating a higher humoral immune response compared to the immune cell type reaction in both patient groups. Humoral immune response was more intense on sections from patients who had clinically evident signs of inflammation.

CD20+ B-lymphocytes were identified in the inflammatory infiltrate present in both groups of patients, even

though the intensity and extent of the CD20+ was significantly lower in the first group. B-lymphocytes were disposed near the epithelium in the connective tissue (Figure 10). At the level of the lamina propria, into the inflammatory areas, B-lymphocytes were, in some sections, the most numerous cells, clinging to a diffuse appearance (Figure 11), but sometimes they were grouped, in the shape of a crown, around the typical capillaries, but also around angiogenesis capillaries, or had a nodular appearance (Figure 12). CD79α+ plasma cells, as well as B-lymphocytes, were well represented numerically, although they varied from one case to another, with a non-homogeneous distribution and embedding a diffuse or localized pattern. They were identified at the same sites with B-lymphocytes, especially subepithelial, penetrating to the surface of the connective tissue, but also in the other areas of the lamina propria, especially located perivascularly (Figure 13).

IHC highlighting of macrophages and PMNs

Macrophages are not only the exponent of non-specific defense cells, acting through phagocytosis, but also participating in the specific defense process by cooperating with immune cells, performing immunomodulatory action. We highlighted the macrophages with the anti-CD68 antibody. Their distribution in peri-implant soft tissue differed from one patient group to another, but also within the same group, were numerically different from one case to another. The largest number of macrophages was identified in sections of group II patients, but they were also present on the sections of group I patients, even if there were fewer. Their distribution was different, being found alongside the other cells involved in the inflammatory process. They have been found isolated, or have a diffuse or localized appearance in the form of a cellular group. These aspects probably correlate with the intensity of the inflammatory process and the presence of antigens. Regardless of the layout, they were usually identified near capillaries or angiogenesis vessels, where the blood-borne antigens arrive (Figure 14).

Neutrophil PMNs were detected with the anti-CD15 antibody. They were only identified on some sections from patients belonging to group II, with clinically apparent inflammatory signs. Their presence on these sections, along with B-lymphocytes, indicates a greater severity of the process. They were diffused in the areas of lamina propria with inflammation, along with the other cells of the inflammatory infiltrate, and were sometimes also identified intra-epithelial (Figure 15).

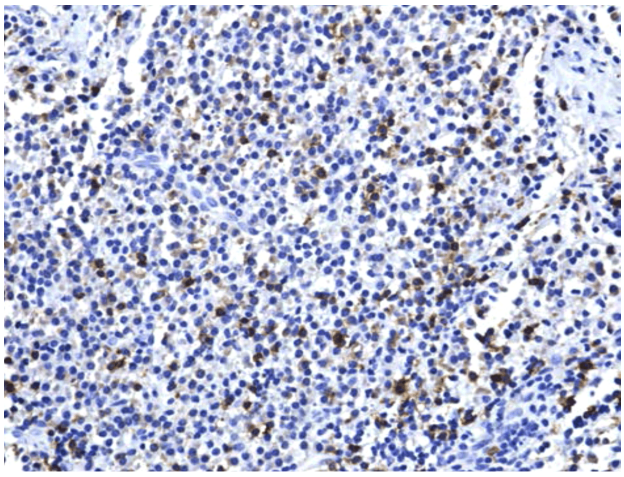

Figure 6 – ***Diffuse and perivascular CD3+ T-lymphocytes. Anti-CD3 antibody immunomarking, ×100.***

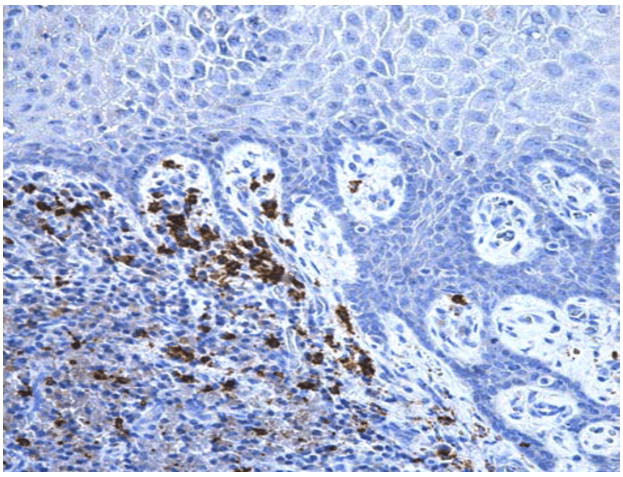

Figure 7 – ***Subepithelial CD3+ T-lymphocytes. Anti-CD3 antibody immunomarking, ×100.***

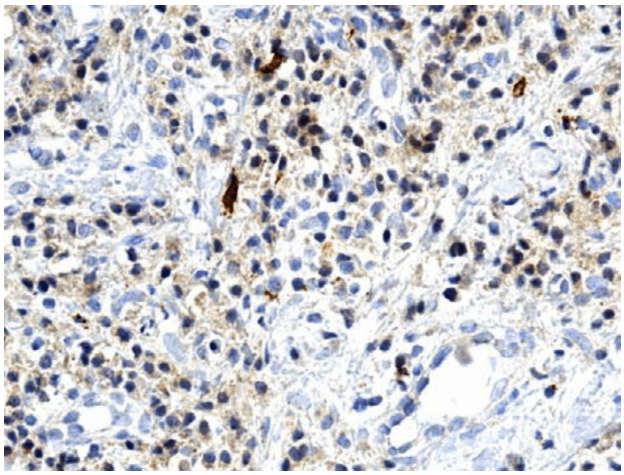

Figure 8 – ***Diffuse lymphocytic infiltrate with numerous CD4+ T-helper lymphocytes. Anti-CD4 antibody immunomarking, ×100.***

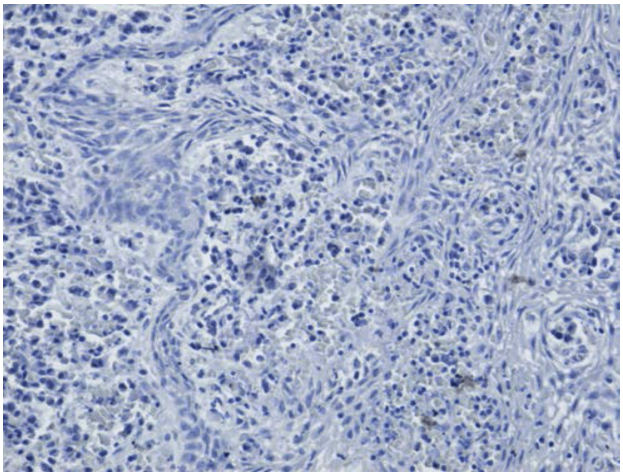

Figure 9 – ***Rare cytotoxic CD8+ T-lymphocytes disposed mostly subepithelial. Anti-CD8 antibody immunomarking, ×100.***

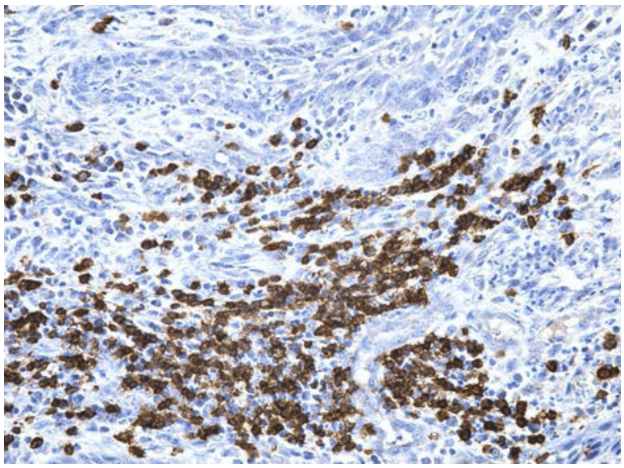

Figure 10 – ***CD20+ B-lymphocytes disposed subepithelial and intraepithelial. Anti-CD20 antibody immunomarking, ×100.***

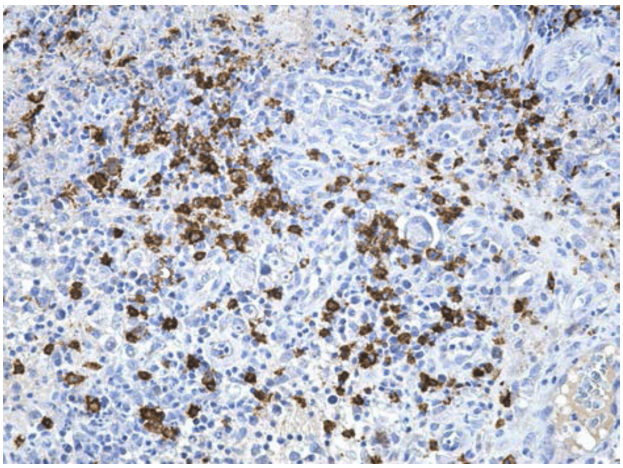

Figure 11 – ***Diffuse CD20+ B-lymphocytes at lamina propria level. Anti-CD20 antibody immunomarking, ×100.***

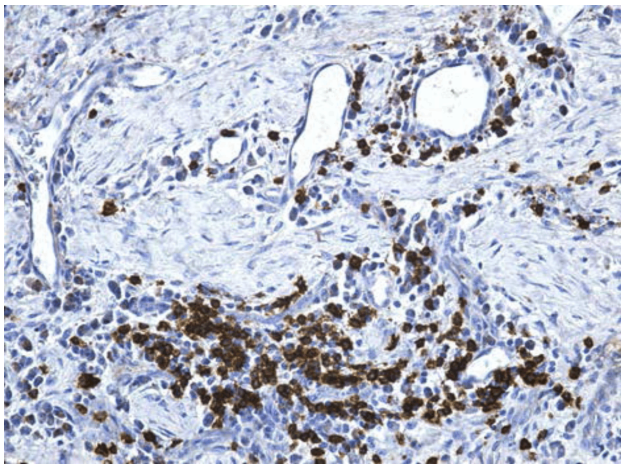

Figure 12 – ***Perivascular CD20+ B-lymphocytes. Anti-CD20 antibody immunomarking, ×100.***

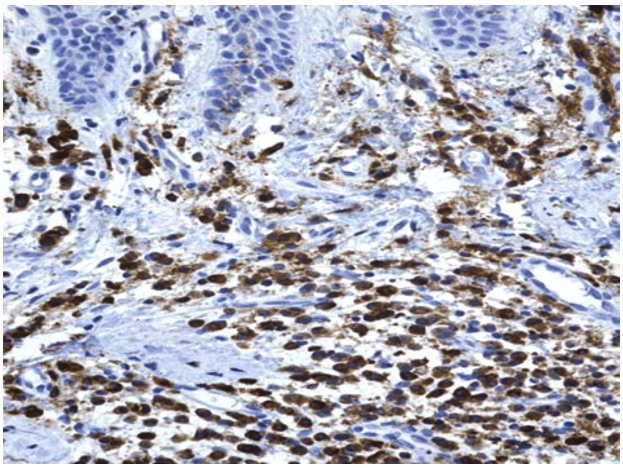

Figure 13 – ***Subepithelial CD79α+ plasma cells. Anti-CD79α antibody immunomarking, ×200.***

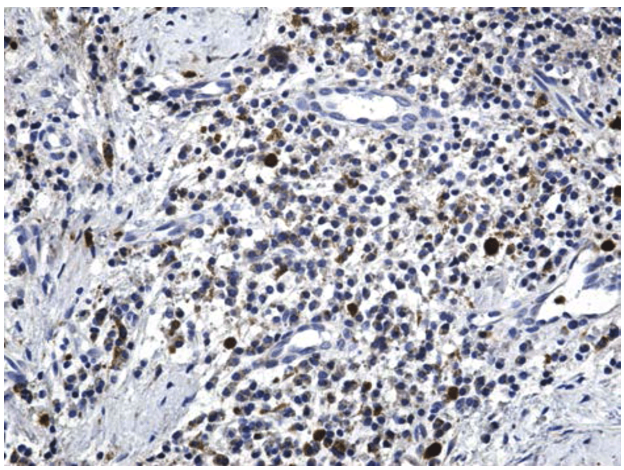

Figure 14 – ***Diffuse and perivascular CD68+ macrophages. Anti-CD68 antibody immunomarking, ×100.***

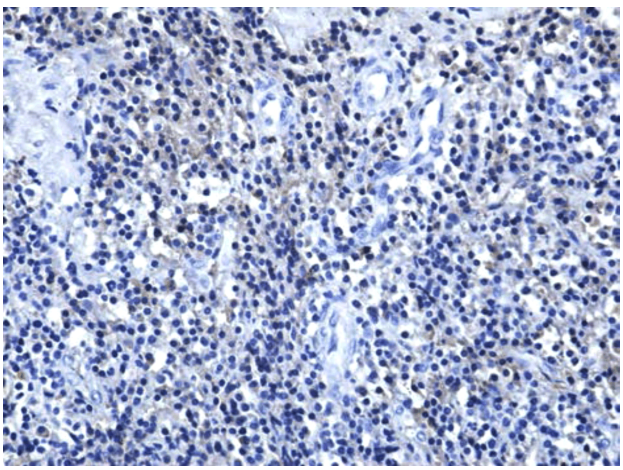

Figure 15 – ***Perivascular CD15+ PMNs. Anti-CD15 antibody immunomarking, ×100. PMNs: Polymorphonuclear neutrophils.***

Discussions

Complications in oral implantology can lead to implant loss. Tissues in contact with dental implants may present, under certain conditions, pathological changes, which is the peri-implant pathology. In these situations, the inflammatory process is either of low intensity, only affecting the soft parts around the implant (gingivitis, or mucositis) or severely, causing progressive bone resorption. Affection of the alveolar bone associated with an inflammatory pathology of the soft parts is known as peri-implant, which is considered to be the major cause of the failure of dental implants.

In our study, we tracked the HP aspects characteristic of soft peri-implant tissues, in patients who did not show inflammatory clinical signs and in those patients where these signs were obvious. The histological examination of the peri-implant mucosa, in animal models and in humans, even in the absence of the bacterial plaque, shows the presence of inflammatory cells in accordance with what we have also observed on the microscopic sections of the two groups of patients. On the sections taken from the peri-implant area from clinically healthy subjects and from patients with inflammation, the soft tissue presented inflammatory infiltration. The presence of lymphocytes signified the existence of an effective immune response, which is essential for the lasting success of osseo-integrated dental implants [14]. Sanz *et al.* [15] studied biopsies of soft tissue prelevated from a number of six patients that presented peri-implantitis and found that an inflammatory lesion occupied 65% of the connective tissue. They also concluded that inflammatory changes were of interest in both epithelium and connective tissue and found an increased number of plasma cells and other mononuclear cells.

In our research, inflammatory infiltrate was found in all samples, including those from patients who did not clinically show signs of inflammation or who were of low intensity. This suggests that the existence of inflammation, along with bone resorption, indicate peri-implantitis. The intensity and extent of the inflammatory process was of varying degrees, varying between the patients of the two groups but also within the same group, from one patient to another, indicating a different reactivity. Also, the inflammatory process varied in intensity and stretch, even in the same patient, with areas with inflammatory process and free areas.

HP characterization of peri-implant tissues in failed implants indicated the presence of inflammatory lesions in the peri-implant mucosa [15, 16], while in other reports the infiltrate inflammatory cell was virtually absent [17]. Moreover, histological analysis of peri-implant tissues was frequently limited to bone tissue despite the identification of inflammation signs at the level of the peri-implant mucosa, at the time of explantation [18]. Esposito *et al.* [17], in a research concerning the HP study of late implant failures, found only the presence of moderate inflammatory infiltrate at the level of the peri-implant tissue perimeter. In this context, it is understood that in nine out of 10 cases examined, no inflammation clinical signs were found. The "implant failure" term cannot represent a condition of the existence of a similar periodicity with other situations in which there were soft tissue inflammation and bone loss. Other authors [19] note these microscopic aspects present on the sections only from clinically ill patients, showing both signs of inflammation and bone loss. Other authors [17], mentioned above, show that most of the implants (nine out of 10) who showed mobility also lacked inflammation clinical and histological signs. The different observations indicated by these studies are because of the obvious variations of the causes of the failures.

Also, more than half of the sections of the two groups of patients showed a moderate degree of inflammation, but this, correlated with age, was much more intense in younger patients, indicating a tissue hyper-reactivity in these patients. The same aspect is confirmed by other authors [20]. Our study shows that clinical examination is useful for monitoring the status of the peri-implant mucosa, in both healthy subjects and patients, but it has its limitations. Clinical attitude should also take into account the predisposition of female patients for inflammation and edema. This result is in agreement with other authors [20].

The most important role in maintaining an implant belongs to the connective tissue. If it is of good quality, so will the fixation of the epithelium. If connective tissue is inflamed to a significant degree, it affects both denting and jaw junction [21, 22]. Thus, the extracellular matrix of gingival connective tissue plays an important role in the homeostasis of dental implants. Some authors have analyzed the presence of different leukocytes in the alveolar mucosa before and after it has become peri-implant mucosa [23]. Seymour *et al.* [24], on sections of patients who have clinically shown signs of inflammation, have shown the presence of inflammatory cell infiltrates in which the B-lymphocyte ratio was greater than that of T-lymphocytes. Other studies [25] found the presence of B-lymphocytes higher compared to T-lymphocytes, with an even greater difference between the two cell categories, namely 4.1% B-cells (CD19+) and 7.3% T-cells (CD3+). This coincides with the findings of Seymour *et al.* (1989), according to whom B-lymphocytes occupy a large proportion of total lymphocytes in the peri-implant mucosa. This is consistent with our study, which identified a much larger number of B-lymphocytes in the second group of patients, and T-lymphocytes were present in the first group of patients, but in low numbers. On the other hand, other authors [14, 26] show that T-lymphocytes predominate at the mucositis level, explained by the fact that histological analysis was done by those who found the prevalence of B-cells on sections from patients with signs and symptoms of inflammation, while the other authors, who found the presence of higher T-lymphocytes, did the IHC analysis on sections from the clinically healthy peri-implant mucosa. In our study, there was an obvious prevalence of B-lymphocytes.

Our study shows that lesions from the other two groups of patients did not differ only in their size but also in the number and density of CD79α+ plasma cells, CD68+ macrophages, CD15+ PMNs, CD3+, CD4+ and CD8+ T-lymphocytes, CD20+ B-lymphocytes, which were higher in the second group of patients. These differences indicate that the inflammatory response in patients who had obvious clinical signs of inflammation was much more intense by promoting cells that are part of both the non-specific, genetically-transmitted and non-specific genetic defense system prior to contact with the pathogen, as well as from the specific adaptive immune system, which allows not only specific recognition and elimination of antigens, but also an individualized response adapted to the type of aggression. The fact that on the sections of patients with evidence of inflammatory clinical signs B-lymphocytes were present in a significantly greater number compared to T-lymphocytes and along with them they identified CD15+ cells (PMNs) indicating a greater severity of the process. This is consistent with other specialized

studies [27]. In contrast, patients of group I showed more B-lymphocytes than T-lymphocytes, but much lower than group II. CD4+ lymphocytes were identified in a larger proportion for group I compared to CD8+ lymphocytes, which were more numerous for group II patients. Cytotoxic CD8+ T-lymphocytes, by enzymatic activity may cause peri-implantation tissue destruction and thus their presence could be an indicator of aggressive potential. The presence of CD4+ and CD8+ lymphocytes shows that at soft peri-implant tissue level originated from the two categories of patients, there is a complex immune process and the stage of the disease may alter the ratio of these populations of T-helper lymphocytes and cytotoxic T-lymphocytes. It follows that the severity of the affection correlates not only with the size of the lesions, but especially with the cellular profile (increased B-lymphocytes and plasma cells), which characterizes the second group of patients in our study.

Our results indicate the existence of a humoral immune process, but also of cellular immune reactions in the tissues examined, immunohistochemically confirmed by the presence of B-lymphocytes and plasma cells and T-lymphocytes in the inflammatory infiltrate. However, there is the possibility of transformation, of converting a stable lesion into a progressive one, which implies a change in inflammatory infiltration, the lymphocyte ratio, not only in lymphocyte populations, but also in the sub-populations of these main categories.

Conclusions

Knowledge of the structure of the peri-implant mucosa helps the clinician to improve the clinical success of dental implant treatment, because the soft tissue surrounding the dental implant separates the dental implant from the oral cavity and provides a seal that prevents the development of peri-implant pathology. Thus, the soft tissue around the implants ensures the conditions of osseointegration and hence the long-term survival of an implant. In our study, chronic inflammatory infiltrate, lymphoplasmocytary and macrophage type, was found in all samples, including those from patients who did not clinically show signs of inflammation or were of low intensity. This suggests that the existence of inflammation, along with radiological images that suggest bone loss, are reliable signs of peri-implantitis. Our study shows that clinical examination is useful for monitoring the status of the peri-implant mucosa, in both healthy subjects and patients, but it has its limitations. The IHC identification of these types of cells and the degree to which each of them was represented by the use of monoclonal antibodies can provide additional insight into the local response of peri-implant soft tissue in healing and osseointegration.

Conflict of interests

The authors declare that they have no conflict of interests.

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Corresponding author
Ştefania Crăiţoiu, Professor, MD, PhD, Department of Histology, Faculty of Medicine, University of Medicine and Pharmacy of Craiova, 2 Petru Rareş Street, 200349 Craiova, Dolj County, Romania; Phone +40723–458 081, e-mail: scraitoiu@yahoo.com

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