UNIVERSITY OF MEDICINE AND PHARMACY OF CRAIOVA
DOCTORAL SCHOOL

IMPORTANCE OF THE HISTOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY IN ASSESING THE MORPHOLOGICAL EVOLUTION OF TISULAR INTEGRATION OF DENTAL IMPLANTS

PhD THESIS

ABSTRACT

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CUPRINS

INTRODUCTION 3
STATE OF KNOWLEDGE 3
CHAPTER 1 Histology și histophisiology of the periimplant soft tissues 3
CHAPTER 2 Periimplant pathology 4
CHAPTER 3 Bone augmentation materials 4
PERSONAL CONTRIBUTIONS 5
CHAPTER 4 Histological study of the periimplant mucosa 5
CHAPTER 5 Immunohistochemical study of the periimplant mucosa 7
CHAPTER 6 Evaluation of the osteointegration of some bone augmentation materials in an in vivo study on laboratory rats 9
GENERAL CONCLUSIONS 10
BIBLIOGRAPHY 11

Key words: Periimplant mucosa, experimenta model, osteointegration, immunohistochemical markers, periimplant pathology.
INTRODUCTION

The behaviour of the periimplant mucosa depends on several factors, among which: the quality of the soft tissue, implant depth, the type of the used biomaterial and the aspect of its surface (Berglundh T, Lindhe J, Ericsson I, Marinello CP, Liljenberg B, Thomsen P. 1991; Ericsson I, Lindhe J, 1993; Linkow L, Rinaldi A, Weiss W, Smith G, 1990). Interface between soft tissue and implant is represented, at the end of the healing time, by three zones well determined: e sulcular epithelium, junctional epithelium, and the connective tissue around the implant. These structures are similar to the ones belonging to the superficial periodontium, but not identical (Friberg B, 1994).

The medical literature reports extremely different the incidence of the periimplant pathology: from a very low prevalence of 5% up to 56% (Cecchinato D, Parpaiola A, Lindhe J, 2014; Atieh MA, Alsabeeha NH, Faggion CM Jr, Duncan WJ, 2013; Klinge B, Meyle J, 2012; Albrektsson T, Buser D, Chen ST, Cochran D, DeBruyn H, Jemt T, et al, 2012; Zitzmann NU, Berglundh T, 2008; Mombelli A, Lang NP, 1998). Such a big span is justified by the existence of different definitions used in the classification of this pathology. This is why it is so difficult to compare the results of different studies, resulting also the need of even deeper and further researches that can lead to a consensus on this topic.

We were motivated in choosing this theme by many aspects: the dental implants today are more and more often used in the last decade in the aesthetic and functional rehabilitations; there is a continuous concern regarding the improving of the different type of implants, but also the appearance of new materials, with an increased degree of tolerance; not in all cases we can deliver an explanation of failures; clinical success can be influenced by different features of periimplant tissues. Certain clinical situations require bone defect reconstruction through alopastic materials.

An in-depth morphologic study, with conventional histological techniques, but also through immunohistochemistry, brings very important information that can define the morphophysiology of the gingival mucosa in the successful cases and also can contribute in explaining some of the failures. With this step, we committed for a contribution to a better understanding of the periimplant soft tissue, the morphological support being the one that can explain different clinical situations. Further more, as we know now, till now there are no studies to follow the matching between clinical, histopathological and immunohistochemical aspects at the level of periimplant soft tissue, in patients with neither mobility or radiological expression of bone resorption, after 4 months from insertion, some of them even without clinical signs of inflammation.

THE STATE OF KNOWLEDGE
CHAPTER 1
HISTOLOGY AND HISTOPHYSIOLOGY OF THE PERIIMPLANT SOFT TISSUE

Experimental studies have shown similarities between gingival and periimplant mucosa, both at the level of the epithelium and of the connective tissue, although the lack of the cement on implants leads to differences in the two structures, regarding the orientation of the collagen fibres (Sîrbu I, 2006). Other authors (Berglundh T, Lindhe J, Ericsson I et al, 1991) have compared the healthy periimplant mucosa with the one surrounding a tooth. The mucosa surrounding dental implants forms a kind of seal comparable with the one realised by the junctional epithelium. Formation of such a barrier begins after 1-2 weeks from healing and it ends after 6-8 weeks from intervention. This periimplant junction is made of three types of epithelium: Peri implant epithelium (EPI), Sulcular peri-implant epithelium (ESPI) and oral Epithelium (EO) (Cochran DL, Hermann JS, Schenk RK, Higginbottom FL, Buser D, 1987; Buser D, Bragger U, 1989). These epithelial structures are similar to the ones belonging to the superficial periodontium, but they are not identical.

CHAPTER 2
PERIIMPLANT PATHOLOGY

The pathological modifications of tissues in contact with dental implants define the periimplant pathology. Alteration of the periimplant tissues can involve the soft tissue surrounding or belonging to dental structures. An inflammatory process limited at the periimplant soft tissues will be named periimplant mucositis. The inflammatory pathology of the soft tissues, associated with progressive bone resorption surrounding implant defines the periimplantitis. The bone remodeling, evenly distributed of the alveolar bone, which originally surrounds the tooth, during the osseo integration of the implant is considered as a physiological process. If the bone loss surrounding the implant is extended and affects the whole implant surface, we don’t have the osseo integration, and the implant becomes mobile.

Initially, the inflammatory processes are limited at the level of the soft tissues in the area of emergence of the implant, the apical area remaining unaffected. Depending of the local defense, this process can extend towards the bone support of the implant, transforming in periimplantitis (Jepsen S, Berglundh T, Genco R et al, 2015, Albrektsson T, Buser D, Sennerby L, 2012). If not properly diagnosed and managed, the periimplant disease can lead to complications and th loss of the implant. Mombelli et al. (Mombelli A, Müller N, Cionca N, 2012) have described the periimplant diseases as infectious situations, having common features with a chronic periodontitis. Currently, although the hypothesis of bacterial infection related to plaque accumulation as an etiological factor is still accepted, it seems to be a multifactorial disease, in which so called combined factors (related to patient, the surgical protocol and the prosthetic component) could contribute to development and the degree of severity of pathology (Konstantinidis IK, Kotsakias GA, Gerdes S, Walter MH, 2015; Qian J, Wennerberg A, Albrektsson T, 2012).

There are two paths of the periimplant pathogenesis: classical and retrograde. The classical path (from soft tissue, towards apical, to the bone), is associated with plaque which
induces inflammatory modifications in the soft tissues surrounding dental implants, leading to the progressive remodelation of the periimplant tissue, with bone loss, resulting eventually in the loss of the (Esposito M, Grusovin MG, Worthington HV, 2012). The retrograde pathogenesis (from the bone to the soft tissues) is associated with bone loss appearing as result of micro fractures caused by overloading, premature loading or by the lateral forces related to occlusal or prosthetic factors (Fu JH, Hsu YT, Wang HL, 2012; Uribe R, Penarrocha M, Sanchis JM, Garcia O, 2004).

CHAPTER 3
BONE AUGMENTATION MATERIALS

The bone defects surrounding implants can be treated with surgical or non-surgical techniques. In the surgical techniques, the use of bone augmentation materials is almost everytime mandatory (Nisha Mahato, Xiaohong Wu, Lu Wang, 2016). There is an open dispute in this area, whether the autologous materials, despite osteoinductive and osteogenic properties can be called further as golden standard in such situations, in which slow resorbability and a proper three-dimensional stability seem more adequate (Povilas Daugela, Marco Cicciù, Nikola Saulacic, 2016).

The most important biomaterials for the routine clinical usage are divided in 5 subcategories, after their origins (Laurencin C, Khan Y, El-Amin SF, 2006):

1. Natural origin materials: are divided in bone grafts prelevated and replacements of grafts: autologous (from the same individual), allogenic (from the same species), xenogeneic (from another species) and ficogenics (marine origin) with coral, chitosanic or sponge structure.

2. Synthetic materials (alloplastic): including replacement of bone grafts based on ceramics such as the beta tricalcium phosphate, calcium sulphate and bioglasses, as well as polymers resorbable and non-resorbable, each of them being used individually or mixed with other materials.

3. Composite materials: by mixing more materials (i.e. bioactive calcium phosphates and polymers) on one hand associates the osseoconductive properties of different classes of materials, and on the other hand is improving the mechanical resistance.

4. Materials combined with growth factors: are represented by the natural growth factors and recombinated, used alone or mixed with other materials, such as TGF-beta, the growth factor platelet derived (PDGF), Fibroblastic growth factor (FGF) and the morphogenetic bone protein (BMP).

5. Live cells materials. This kind of materials operates with living cells, like for eg mesenchymal stem cells to generate new tissue alone or are sown in a matrix-based skeleton.
PERSONAL CONTRIBUTIONS
CHAPTER 4
HISTOLOGICAL STUDY OF THE PERIIMPLANT MUCOSA

The studied material has been collected from 40 non-smoker women and men, ages 32-65, at which implants were inserted between October 2014 and January 2018. The collection of fragments of mucosa, for the morphological study, was performed 4 months after insertion of the implants, they did not show mobility and no imaging signs of bone resorption. We divided the patients studied into two groups:
- group I, consisting of 28 patients with no inflammatory clinical signs;
- Group II, consisting of 12 patients with clinically evident inflammatory signs.

Processing of the material was done by the paraffin inclusion technique, the sections obtained were stained with hematoxylin-eosin and trichrome Goldner-Szekely.

Sections from patients in group I, patients with no inflammatory clinical signs, showed few changes. At the surface of soft periimplantar tissue there is an epithelium, which has been orchestrated on some sections (Figure 4.1) and parchailed on others (Figure 4.2). Also, there was a process of acanthosis (Figure 4.3), which led to an increase in epithelial thickness and accentuation of epithelial ridges, which are much elongated and broad. At the epithelial level, starting with the spinous layer, some cells showed edema, gaining a balloon appearance (Figure 4.4). The epithelium of the periimplantation 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.

Group II sections from patients with clinically evident inflammatory signs showed more intense changes, the surface of the epithelium was frequently thin and ulcerated, and the underlying chorion did not always have an intense inflammatory process (Fig 4.5, Fig. 4.6). Abundant vascularization was present in most cases of group II, especially at the level of superficial lamina and conjunctive papillae. The vessels were typical capillaries, but also young, newly formed vessels with turgescent epithelium. In the areas where the fibril collagen component was more abundant, the vascularization was lower.
Fig. 4.1 periimplant tissue. Ortokeratinized Epithelium. Col. HE X 200

Fig. 4.2 periimplant tissue. Parakeratinized Epithelium. Col. HE X 100

Fig. 4.3 Acanthosis with parakeratosis Col. HE X 100.

Fig. 4.4 Edema of superficial epithelial cells and the spinous, vacuolar aspect. Col. HE X 100

Fig. 4.5 Epithelial ulcerated zone. Inflammatory process in the lamina propria. Col. HE X 100

Fig. 4.6 Intense inflammatory infiltrate, subepithelial and perivascular. Col Tricr MassonX 200
CHAPTER 5

IMMUNOHISTOCHEMICAL STUDY OF THE PERIIMPLANT MUCOSA

The studied material consisted of 40 periimplantary mucosal blocks from the patients in which the histological study was performed. The research pursued:- IHC study of inflammatory infiltrate (IHC detection of B lymphocytes with anti CD20 antibody, IHC detection of T lymphocytes with anti CD3, anti CD4 and anti CD8 antibodies, IHC detection of neutrophil polymorphonuclear cells with CD15, highlighting IHCa of macrophages with CD68 antibody, of CD9 alpha plasma, CDR alpha, IHC mapping of mast cells with tryptase);- IHC blood vessel and angiogenesis study using the CD34 antibody.

The presence of CD3-positive lymphocytes indicates the existence of an inflammatory process. They were diffuse and less rarely grouped, especially perivascular or subepithelial, at the level of the connective papillae (Figure 5.1, Figure 5.2). 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 arranged in the form of an infiltrated diffuse or perivascular (Fig. 5.3, Fig. 5.4), while lymphocytes CD8 + cells were present in extremely small number of localized mainly perivascular and subepithelial or absent (Fig. 5.5).

In the lamina propria, at inflammatory sites, LB were some sections, the most numerous cells, having a diffuse aspect(Fig. 5.6), but sometimes have a provision grouped in a ring around capillaries typical and of angiogenesis or nodular appearance (Figure 5.7). Both CD79-alpha positive plasmids, as well as LB, 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 conjunctivae, but also in the other areas of their own lamine, especially located perivascularly (Figure 5.8).

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 had not only a subepithelial distribution, the epithelium adjacent to the papillary joints, but also the other areas of the conjunctiva. 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. Irrespective of the layout, they have usually been identified near capillaries or angiogenesis vessels, ie where blood-borne antigens come (Figure 5.9).

The microscopic examination of the sections revealed the presence of mast cells in active inflammatory areas in both groups of patients, but they were much better represented in group II patients (Figure 5.10). The angiogenesis process was of the capillary type, being located especially subepithelialy, up to the surface of the conjunctivae papiles, in the immediate vicinity of the epithelium, and in the areas with inflammatory infiltration (Figure 5.11, Fig.5.12). Angiogenesis was exclusively capillary, with pre-existing vessels starting point. The
angiogenesis process was present both subepithelial and in the areas of the corion with inflammatory infiltration.

**Fig. 5.1** Lymphocytes T, difuze disposed and perivascular. Immunomarking with antibody anti CD3 X 100.

**Fig. 5.2** Lymphocytes T în lamina propria, superficial. Immunomarking with antibody anti CD3 X 100.

**Fig. 5.3** Frequents lymphocytes T helper CD4+, difuze X 100.

**Fig. 5.4** Frequents lymphocytes T helper CD4+, difuze X 200.

**Fig. 5.5** Rare lymphocytes T citotoxic CD8+ difuze disposition X 100.

**Fig. 5.6** Lymphocytes B difuze disposed. Immunomarking with CD20 X 100.

**Fig. 5.7** Lymphocytes B CD20+ perivascular arranged X 100.

**Fig. 5.8** Plasmaocytes CD79-alfa+, subepithelial X 100.

**Fig. 5.9** Macrophage CD68+ dispuse among collagen fibres X100.
CHAPTER 6
OSSEOINTEGRATION EVALUATION FOR SOME BONE AUGMENTATION MATERIALS, IN VIVO, IN RATS

For this study, we performed three batches consisting of 12 laboratory Whistar adult rats weighing 360-400 g, kept under the same environmental conditions, at a constant temperature of 20 °C to 24 °C, receiving the same diet. Two experimental cavities with a diameter of 4 mm were made using a spherical cutter: a cavity at the level of the calvary and a cavity at the level of the maxillary bone. Cavities were treated differently for each batch of study. For the first batch, the cavities were augmented with the Alveoprotect collagen material (Bredent Medical, Senden, Germany). At the second batch we used bone augmentation material Ossceram nano (Bredent Medical, Senden, Germany). The third group was the control group, in which the cavities were left unaugmented.

Both samples were obtained from the calvaria and jaw, which had been cut with the help of micromotor, the appropriate size to accommodate both the bone healing area and the adjacent normal bone. Samples were analyzed by 3 methods: direct clinical examination (macroscopic examination), optical coherence tomography (OCT) - Fig. 6.1, Fig. 6.2, microscopic examination (conventional microscopy methods and immunohistochemistry techniques).

On histological preparations from samples collected from laboratory animals, we generally observed the filling of experimental bone defects with connective tissue repair with various bone extensions from surrounding bone tissue (Figure 6.3 - Figure 6.5).

CD68 is a highly expressed glycoprotein of monocytes and tissue macrophages and can be used as a marker for proliferation and differentiation of osteoblasts and osteoclasts for bone regeneration and remodeling (Figure 6.6). Immunohistochemical observations on the bone remodeling stage have not correlated in most cases with the
time elapsed from the augmentation of the experimental defect, which indicates a
dynamic rebalancing between the formation and bone resorption phenomena.
Lectin plays multiple roles in different stages that occur during bone tissue healing:
inflammation, reconstruction and remodeling. Blood vessel invasion brings cellular
elements necessary for structural reorganization of collagen and replacement with newly
formed tissue (Figure 6.7, Figure 6.8).

![Fig. 6.1 OCT of a maxillary sample, showing the bone defect covered with Alveoprotect, after 4 months, during the healing process](image1)

![Fig. 6.2 OCT of a sample from maxillary, showing the bone defect covered with Ossceram nano, 4 months after treatment, during healing.](image2)

![Fig. 6.3 mineralization limits of the bone defect occupied by a fibrous connective tissue Col HE X 200](image3)

![Fig. 6.4 Ossification centers inside the repairing connective tissue Col HE X 200](image4)

![Fig. 6.5 Synthetic material surrounded by connective tissue newly formed, two months after applying it. Col HE X 200](image5)
GENERAL CONCLUSIONS

Knowing the structure of the peri-implant mucosa helps the clinician to improve the clinical success of the dental implant treatment as the soft tissue surrounding the dental implant separates the implant from the oral cavity and performs a biological seal that prevents the development of the periimplantation pathology. Thus, the soft tissue around the implants ensures the conditions of osteointegration and hence the long-term survival of an implant.

In our study, chronic inflammatory infiltrate of the lymphoplasmocytic and macrophage type was found in all trials, including those from patients who did not clinically show signs of inflammation or were of low intensity. This suggests that the presence of clinical signs of inflammation, more than the degree or severity of symptoms, along with radiological signs of bone loss can serve as indicators for periimplants.

The immunohistochemical 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 periimplant soft tissue in healing and osteointegration.

Our findings should materialize in the therapeutic implications of building strategies for the possible use of drugs to inhibit and / or influence mast cell activation in order to increase osteointegration and to prevent and treat periimplant disorders, which would contribute to increasing implant longevity.

SELECTIVE BIBLIOGRAPHY