

JOURNAL OF BIOLOGICAL REGULATORS & HOMEOSTATIC AGENTS

Volume 31, No. 2 (Supplement), April - June, 2017

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EVALUATION OF CHEMICAL-PHYSICAL PROPERTIES AND CYTOCOMPATIBILITY OF THERACAL LC

A. BUONAVOGLIA¹, D. LAURITANO², D. PERRONE¹, F. ARDITO¹,
G. TROIANO¹, M. DIOGUARDI¹, V. CANDOTTO³, F.J. SILVESTRE⁴ and L. LO MUZIO¹

¹*Department of Clinical and Experimental Medicine, Foggia University, Foggia, Italy;* ²*Department of Medicine and Surgery, University of Milano Bicocca, Monza, Italy;* ³*Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, Italy;* ⁴*Stomatology & Oral Surgery, Dr Peset University Hospital, Department of Stomatology, University of Valencia, Spain*

TheraCal LC (TLC, Bisco Inc., Schaumburg, IL, USA) is a light-cured, resin-modified, calcium silicate-filled base/liner material designed for direct and indirect pulp-capping. In this study the result of the evaluation in vitro of the biocompatibility and chemical-physical properties of TLC are reported. TLC specimens were prepared under aseptic conditions in strict compliance with the manufacturer's instructions and sterilized. Osteoblast-like cells (MG63) were used. For different time points, solubility, water uptake, alkalinizing activity and cytotoxicity were evaluated. In ddH₂O and in DMEM+FBS, TLC showed a loss of material increasing simultaneously with the absorption capacity. The increase of water uptake of the material promoting the solubilization of mineral ions in medium is a requisite for a bioactive material. The alkalinizing activity is correlated to antimicrobial/bacteriostatic activity and to the ability to favor the formation of apatite deposits. The pH values for water absorption after immersion of the disks ranged between 8 and 9 at each times of evaluation. Cytotoxicity was not observed in MG63 cells treated with TLC and after 5 days, the cells were organized to form a confluent monolayer as demonstrated by fluorescence microscopy observation. TLC showed biocompatibility on MG63 cells allowing a physiologic cell growth and differentiation. The chemical-physical properties and biocompatibility of TLC observed in vitro in the present study, allows considering this cement as an innovative pulp-capping material for the vital pulp therapy.

One of the objectives of operative dentistry is to maintain the pulp health in compromised teeth, thus reducing the need for endodontic treatment or tooth extraction.

One method used for this purpose is the pulp-capping technique that consists in the placement of biocompatible materials on the exposed pulp tissue to preserve its health and stimulate repair by mineralized tissue formation (1). Fundamental feature for these materials is biocompatibility,

which includes antibacterial properties, sealing capabilities and bioactivity (2-7).

The pulp capping material is useful to seal the pulp against bacterial penetration, to encourage the pulp to wall off the exposure site by initiating a dentine bridge and to maintain the pulp tissue healthy (8).

Calcium hydroxide is the most popular agent for direct and indirect pulp capping (9, 10). However, calcium hydroxide has defects such as poor bonding

Key words: biocompatibility, calcium silicate-based endodontic sealers, cytotoxicity, mineral trioxide aggregate, periodontal ligament stem cells

Mailing address:

Dorina Lauritano, DDS,
Department of Medicine and Surgery,
University of Milano Bicocca, Via Cadore 48, 20052 Monza, Italy
Tel.: +39.0392332301 - Fax: +39. 03923329892
e-mail: dorina.lauritano@unimib.it

0393-974X (2016)

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to dentin, material reabsorption, high solubility, mechanical instability and poor bonding with composite resins. With these restorative materials, an intermediate layer of resin-modified glass ionomer is necessary.

Initially, Mineral trioxide aggregate (MTA) was proposed in endodontics to seal off all the pathways of communication between the root canal system and the external surface of the tooth (11).

For the first time, Ford et al, reported the evaluation of MTA performance for pulp capping in monkey teeth, showing higher performance of MTA in comparison to calcium hydroxide (12). MTA cements have the potential to be used as pulp-capping materials.

Significant disadvantages of self-cure MTA for the restorative dentistry include the slow-setting times, from several minutes to hours and the consequent risk of fast dissolution and removal of the cement, wash-out of the fresh (not-set) cement (13) and difficulties in stratification of restorative materials over a fresh liner. As a result, two-step procedures are frequently requested for interim restorations. The incorporation of light-curable resins has been proposed for many materials to improve mechanical properties and reduce setting time. The reduced setting time of MTA materials may extend their clinical use and make their application advisable in extremely wet and blood-contaminated surgical sites, and in pulp cap performed in a single treatment appointment.

TLC (Bisco Inc., Schaumburg, IL, USA) is a new light-cured, resin-modified, calcium silicate-filled base/liner material designed for direct and indirect pulp-capping. It contains polymerizable methacrylate monomers, Portland cement type III, polyethylene glycol dimethacrylate, and barium zirconate, WHITE CEM 1 52.5–1°, $\text{CaSO}_4 \cdot 1/2\text{H}_2\text{O}$, CaCl_2 , Bi_2O_3 , Montmorillonite, NaF.

In this study the evaluation of the biocompatibility and chemical-physical properties of TLC is proposed.

MATERIALS AND METHODS

Pulp-capping Materials

TLC (Bisco Inc, Schaumburg, IL, USA) specimens for the experiments were prepared under aseptic

conditions in strict compliance with the manufacturer's instructions. After 120 sec of light-curing, the surface of the cement was hard enough to support the experiments (WOODPECKER, LED.B, Wavelength 420-480 nm, Power Density 1000-1200 mW/cm²). The cylindrical specimens obtained (4mm in diameter, 2mm thick and 3g of weight) were allowed to set for 24 h in a humid atmosphere and aseptic conditions. For cytocompatibility assays, samples were sterilized by incubation for 2 h in 200U/mL penicillin, 200µg/mL streptomycin, 250µg/mL fungizon and 50mg/L gentamycin (14, 15). These were added to the culture wells using sterile forceps. All the samples were subsequently incubated at 37°C with 5%CO₂ in DMEM following a 1 h ultraviolet exposure. The eluates of materials were prepared separately and were incubated in DMEM-F12 at 37°C in a 5% CO₂ for 1, 2, 3 and 7 days (16).

Chemical-physical properties

Solubility. The solubility of the materials was determined using the method described in ISO 6876 (17). The mass of the cements was measured gravimetrically using an analytical balance (KERM-Bel100-2) with an accuracy of 0.001g after 1, 14 and 28 days of soaking in deionized water or in DMEM+FBS, i.e. each weight measurement was repeated three times.

Water uptake. Three specimens were placed in 5 ml of deionized water and maintained in a cabinet at 37°C. The wet weight was recorded after pre-determined intervals (1, 6, 24 and 48h). After incubation, the specimens were dried at 37°C for 24 h and then weighed using an analytical balance (KERM-Bel100-2). For every time point three samples were used and each measurement was repeated three times.

Alkalinizing properties. TLC possesses antibacterial properties, correlated with alkalinizing activity and this is shown through an increase the local pH during their hydration reaction (18, 19). pH value of the deionized water was evaluated in the presence of TLC disks (n=3) for different storage times (3 h and 1, 3, 7, 14 and 28 days). After incubation, the specimens were dried at 37°C for 24 h and then potentiometrically measured using an analytical pHmeter (Eutech). For every time point, three samples were used and each measurement was repeated 3 times.

Cytocompatibility evaluation

Cell line culture. MG63 osteoblast-like cells, originally isolated from a human osteosarcoma, were used. Cells were cultured in DMEM medium (Sigma Aldrich) supplemented with 10% fetal bovine serum (FBS), 2% glutamine, and 1% (w/v) streptomycin/penicillin at 37°C in 5% CO₂ atmosphere. Confluent MG63 were detached using a trypsin-EDTA solution and counted using a Juli FL(TwinHelix) and 4x10⁴ cells seeded onto 96 well plates and incubated with the supernatant of each cements and for each time-points.

Cytotoxicity tests. Cytotoxicity tests were performed with the Transwell insert (Sigma-Aldrich) methodology (19, 20). The cytotoxicity of TLC was assessed with cells grown in the lower chamber of a 24-mm diameter Transwell plate with a 3.0 µm pore size polycarbonate membrane (PC). The Transwell membrane of the inner chamber containing the pulp capping materials were placed into the lower chamber of the 24-well culture plate containing at the bottom 4x10⁴ cells/well and incubated at 37°C in 5%CO₂ atmosphere for 3, 24, 48, 72h and 7 days respectively. Some wells were incubated with only cells in the medium (negative control). At the end of each incubation time, the cell viability was performed with the MTT assay [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)] (Molecular Probes) at 24, 48, 72 h and 7 days (20). The results were presented as percentage of cell viability referred at cells incubated in the absence of pulp capping materials set at 100%. The specimens used for each experiment were performed in triplicate. The reagent was added in a ratio of 1:10 to the cell culture and then the cells were kept in the incubator for 4 h at 37°C. The degree of fluorescence and the relative values of absorbance were then acquired by reading in a spectrophotometer (Multiskan Go, Thermo Fisher Scientific) at a wavelength of 570 nm.

Eluate extractions. Eluates were prepared similarly to a method previously described (21) by placing 1 sample cylinder into each well of a plastic 24-well tissue culture plate (Greiner, bio-one) and adding 2 mL DMEM to completely cover the cylinders (22, 23). Plates were placed on a plated rocker (25cpm). Eluate from each sample were collected at 3, 24, 48, 72 h and 7 days and then they were used as medium for xCelligence Tests.

xCELLigence Tests. Fifty microliters of cell culture medium were added to each well for the impedance

background measurement. 4x10⁴ cells and extract were seeded to each well. The E-Plates were incubated at 37°C with 5% CO₂ and monitored on the RTCA system at 2-minute time intervals, for up to 86 h, with or without treatment.

Morphological evaluation. Once performed, the cytotoxicity test of the different materials, after washing every Transwell with PBS, fluorescent dyes (Ready probes red/blue Hoechst 33342) were added per well, in order to detect the cell proliferation. After incubation time, the images were acquired using Fluorescence Microscopy (EVOS FL, Thermo Fisher Scientific).

Statistical Analysis. Statistical analysis was performed using Prism 6.00 (GraphPad software, La Jolla, CA, USA) using the Student's t-test with the level of significance at p<0.05. Cell index (CI) for real-time dynamic cytotoxicity assessment (N=3) and slope calculations for the migration assessments were calculated automatically by the RTCA Software Package 1.2 of the RTCA system. Normalizations were performed using the RTCA Software Package 1.2. Numerical data were expressed as a mean ± standard deviation.

RESULTS

Solubility

TLC showed a loss of material increasing over soaking time in both media (Fig. 1A). For every time of incubation, the results displayed a lower weight loss in deionized water than in DMEM+FBS.

Water Uptake (WU)

The results of water adsorption are shown in Fig. 1B. An increase of water uptake from 1 h to 24 h can be observed. After 24 h, the WU% is not more significant.

Alkalinizing properties

Fig. 1C shows the pH value of soaking water after immersion of the disks. The pH values range between 8 and 9 for each time of analysis. Alkalinizing power was in agreement with previous studies. Increase of environmental pH is related to release of Calcium ions (24).

Cytotoxicity tests

The results of the MTT assays over all the time

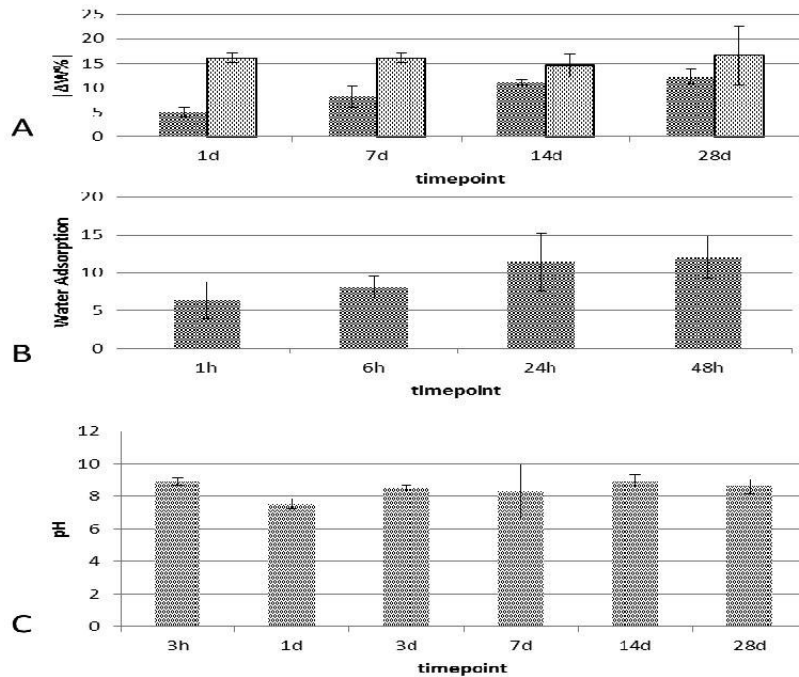


Fig. 1. A): Solubility (mean $\pm$ standard deviation, triplicate) expressed as absolute value of percentage weight variation ($|\Delta W\%|$) after different time point of soaking at 37°C in (a) deionized water and (b) DMEM+FBS. For the time points, the $|\Delta W\%|$ values are not significantly different ($P > 0.05$); **B):** Water adsorption (mean $\pm$ standard deviation, triplicate) after different times of soaking in deionized water: For the time points, the Water Adsorption values are not significantly different ($P > 0.05$); **C):** pH (mean $\pm$ standard deviation, triplicate) of the soaking water in presence of the TLC disks showing the alkalinizing activity after different storage times. For the time points, the pH values are not significantly different ($P > 0.05$).

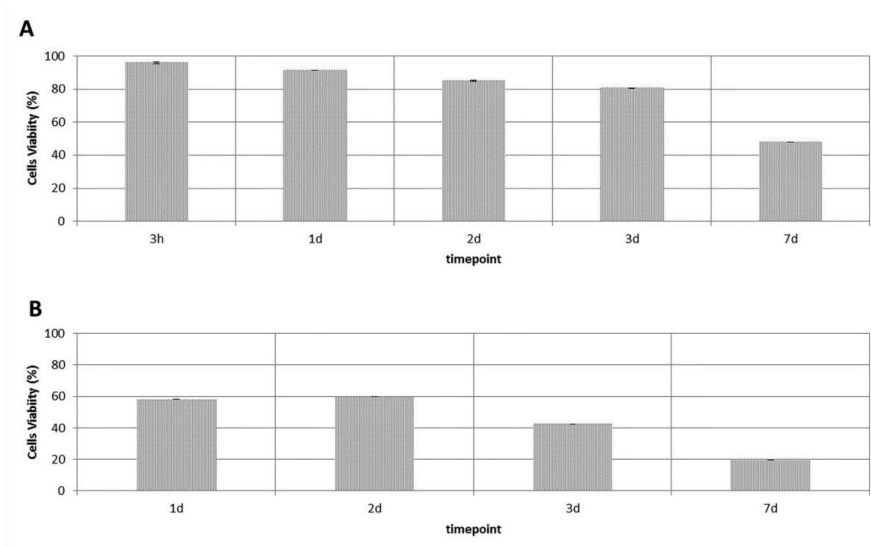


Fig. 2. MG63 cells viability by using Transwell Method with MTT assay in indirect (A) and direct (B) cytocompatibility tests. Y-axis-percentage of cell viability referred at cells incubated in absence of pulp capping material set at 100%. For every time points, the Cell Viability (%) values are significantly different ($P < 0.05$).

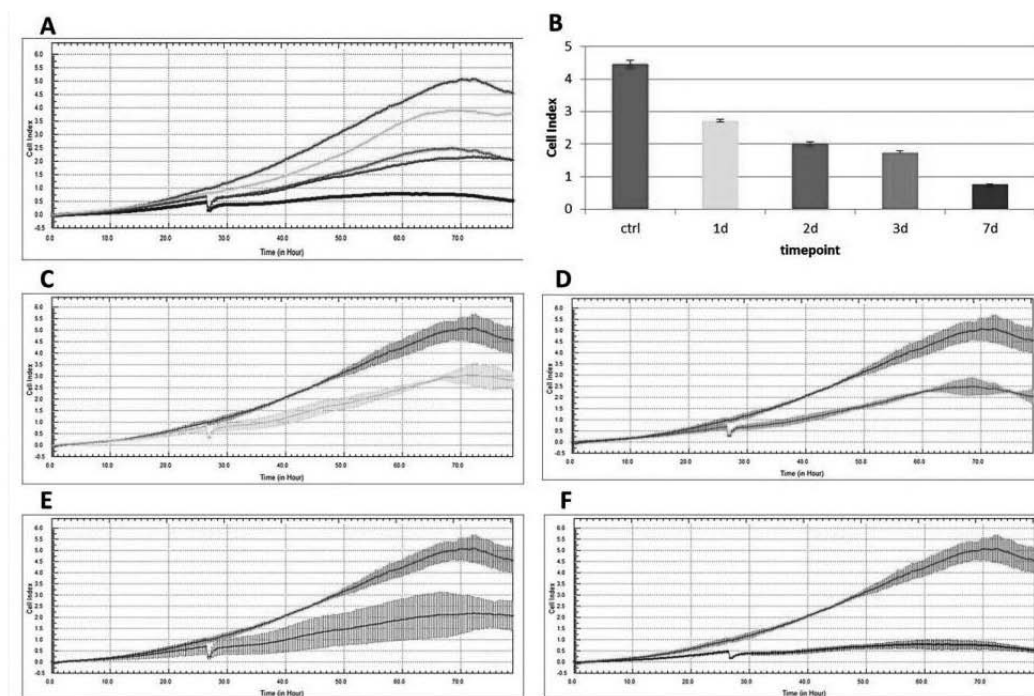


Fig. 3. Cells viability with RTCA analysis. Effect of TLC eluates (• CTRL, • 1d, • 2d, • 3d, • 7d) on MG63, for every time point (A, B), • CTRL vs • 1d (C), • CTRL vs • 2d (D), • CTRL vs • 3d (E), • CTRL vs • 7d (F). Lines represent mean CI ± standard deviation. For every time points, the CI values are significantly different ($P < 0.05$).

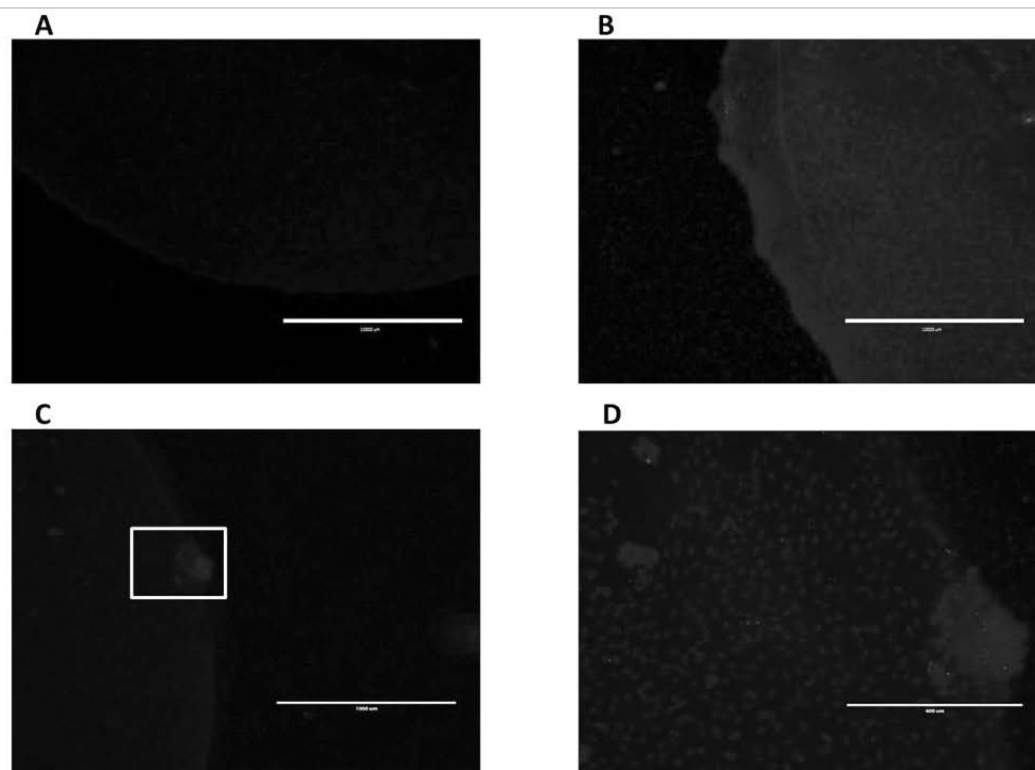


Fig. 4. Fluorescence images of vitality assay. MG63 cells were cultured in contact with TLC disks after 3 days (A) and after 5 days (B, C). Image D is a zoom of Image C.

periods are shown in Fig. 2. TLC did not induce any compromising cytotoxicity on cells in all time points. Presence of monomer resins seem not reduce the viability and number of cells.

At all time-points of incubation, a statistically significant high viability was observed both for direct (Fig. 2A) and for indirect (Fig. 2B) MTT assay. To confirm the results above mentioned, we performed an eluates analysis using xCELLigence® system that evaluated the kinetics of cell adhesion and the cell index, parameter proportional to cell proliferation. MTT results, according to xCelligence® analysis (Fig. 3), revealed that the eluates did not have cytotoxic effects.

Morphology Evaluation

Fluorescence microscopy images of vitality assay are presented in Fig. 4. It is interesting to observe cell proliferation on samples surface in all the pictures. The Fluorescence images obtained after 3 and 5 days incubation confirmed the MTT assay results: TLC did not have cytotoxic effects.

DISCUSSION

Calcium Silicate hydraulic cements, conventionally defined MTA cements, were initially proposed as root-end materials (25) and to repair iatrogenic endodontic perforations.

Iatrogenic and pathologic root perforations repair, the positioning of apical barriers in immature teeth, and retrograde filling of root apices involve the placement of a material in contact with the periodontal ligament and/or periradicular bone. The ideal material for these purposes should be biocompatible, able to seal the root canal system, dimensionally stable, unaffected by the presence of moisture, and easy to manipulate. These properties are verifiable in MTA.

MTA preparations are considered calcium containing silicate cements with a composition similar to that of Portland cement (PC). Both are composed of calcium phosphate, calcium, and silicon oxide. MTA, in addition, contains bismuth oxide, which provides radiopacity. MTA is a powder consisting of fine hydrophilic particles that sets in

the presence of moisture to form a hard structure, unaffected in dimensional stability by the presence of body fluids. This important property allows to form a stable seal (11). MTA calcium silicates in contact with water generate calcium hydroxide, that increase pH and make it antibacterial, biocompatible and aid in the release of bioactive dentin matrix proteins (26). Hydroxyapatite crystals form over MTA in contact with tissue fluid (27). Moreover, MTA cements exhibit calcified tissue-conductive activity and facilitate the differentiation of human orofacial mesenchymal stem cells and the mineralization process in human dental pulp cells (28).

These properties of MTA can be used in vital pulp therapy as pulp capping material in substitution of calcium hydroxide (12). In literature Calcium hydroxide is described as the most popular agent for direct and indirect pulp capping (9). However, calcium hydroxide has several defects, such as poor bonding to dentin, material reabsorption, high solubility, mechanical instability and poor bonding with composite resins; with these restorative materials it is necessary an intermediate layer of resin-modified glass ionomer.

In addition, the formation of reparative dentine may not be due to the bioinductive capacity of the material but due to a defense mechanism of the pulp induced by the irritant nature of calcium hydroxide. The high pH of calcium hydroxide suspensions causes liquefaction necrosis at the surface of the pulp tissue with the formation of a necrotic layer at the material-pulp interface (26).

MTA is more effective than calcium hydroxide materials, MTA has an enhanced interaction with dental pulp tissue, limited pulp tissue necrosis (less caustic effect) shortly after its application and reduced pulp inflammation (20). MTA facilitated the proliferation/differentiation of human dental pulp cells (29) and exhibited calcified tissue-conductive activity with the ability to stimulate faster complete dentine bridge formation and new hard tissue formation (30). However blood contamination and humidity may change the physical properties of MTA cements increasing their setting time (31).

Normally setting time of self-cure MTA cements is 30-70 min but is longer in the presence of serum

proteins and/or blood, clinical conditions of a pulpar exposure where the material is placed on a connective soft tissue. Also, a fresh liner does not allow a correct stratification of the restorative material at the base of dental cavity. Using self-cure MTA as a pulp capping material, a two-step procedure is necessary: firstly, the removal of infected dentin and positioning calcium silicate and secondly, to control the occurred setting of MTA and stratification of definitive restorative material.

The incorporation of light curable resins reduces setting time (120 sec) and improve mechanical properties as demonstrated by this study.

TLC showed a lower weight loss in deionized water than in DMEM+FBS. This trend may be explained by a precipitation reaction between calcium ions released by the cement and phosphate ions originating from DMEM+FBS (17, 32).

An increase of water uptake from 1 to 24 h, that promotes the solubilization of mineral ions (Ca) in medium can be observed, a requisite for a material to be bioactive (17). The concept of bioactivity is related to the ability to exchange information within a biological system and an essential requirement is the formation of a biologically active bone-like apatite layer on its surface in a biological environment (33).

The pH values range between 8 and 9 for each time of analysis, in agreement with previous studies. Release of calcium ions and the consequent increase of pH favor the ability to form apatite deposits and accelerate the apatite nucleation (34).

TLC did not induce any compromising cytotoxicity on cells in all time points. Presence of monomer resins do not seem to reduce the viability and number of cells. Previous studies demonstrated that self-cure MTA cements are good substrates for osteoblast growth and are able to induce cell proliferation (35). TLC showed similar biocompatibility. In fact, the Fluorescence images obtained after 5 days incubation showed the cells from a planctonic phase formed aggregates, colonizing the entire discs.

An immediate stratification with restorative materials which reduce the number of appointments and the risk of bacterial penetration after sealing loss of temporary restorative material in classic two-step procedure, is possible.

All this represents an innovative approach to ensure clinical success, which is of paramount importance especially in patients with oral disease (36-45).

This study demonstrated that chemical-physical properties and biocompatibility of TLC make it an innovative pulp capping material, able to use in vital pulp therapy.

ACKNOWLEDGEMENTS

We thank Bisco Inc. for the materials provided. The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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EVALUATION OF THE INTRAEPITHELIAL PAPILLARY CAPILLARY LOOPS IN BENIGN AND MALIGNANT ORAL LESIONS BY *IN VIVO* VIRTUAL CHROMOENDOSCOPIC MAGNIFICATION: A PRELIMINARY STUDY

M. CONTALDO¹, A. LUCCHESI¹, E. GENTILE¹, C. ZULLI², M. PETRUZZI³,
D. LAURITANO⁴, M. R. AMATO², P. ESPOSITO², G. RIEGLER² and R. SERPICO¹

¹University of Campania-Luigi Vanvitelli, Multidisciplinary Department of Medical-Surgical and Odontostomatological Specialties, Naples, Italy; ²University of Campania-Luigi Vanvitelli, Department of Clinical and Experimental Medicine - Gastroenterology, Naples, Italy; ³University of Bari "Aldo Moro", Dipartimento Interdisciplinare di Medicina, Sezione Malattie Odontostomatologiche-Policlinico di Bari, Bari, Italy; ⁴Department of Medicine and Surgery, University of Milano Bicocca, Monza, Italy

This preliminary study aims to establish the Virtual Chromoendoscopic Magnification (VCM) feasibility to visualize and distinguish the Intraepithelial Papillary Capillary Loops (IPCL) patterns of benign oral pathologies from malignant ones. Thirty-one consecutive subjects affected by oral lesions/pathologies underwent white light examination and VCM imaging by the Narrow Band Imaging System (Olympus Medical Systems Corp., Tokyo, Japan). A class system of four IPCL types corresponding to progressive vessel disarray was adopted. IPCL type IV were considered criterion of "malignancy". A histopathological exam completed the diagnosis: sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy were calculated. IPCL patterns of benign oral entities ranged from types I to III. IPCL type IV was associated with malignancy in 4 out of 6 cases. Sensitivity, specificity, PPV, and NPV were 100%, 93%, 67% and 100%, respectively. This study preliminarily describes IPCL patterns of different oral mucosal diseases and confirms the association between IPCL IV and oral cancer.

Vascular homeostasis is regulated by the balance between pro-angiogenic and anti-angiogenic factors (1).

Several conditions such as metabolic stresses, inflammation, angiogenic factors up-regulation and anti-angiogenic factors inactivation during tumor progression promote neoangiogenesis, a process where endothelial cells proliferate and vessel fenestrations increase (2-8). Generally, cancerous blood vessels differ from non-cancerous ones. Squamous cell carcinoma (SCC) of the oropharynx may present microvascular irregularities (9); cancerous vessels are tortuous, fenestrated,

finely ramified and dilated. Vascular permeability, increased fragility, and a tendency to hemorrhagic phenomena may also occur, resulting in ineffective tissue perfusion and hypoxia (10). Angiogenic activity may be present in pre-neoplastic lesions in the bladder (11), mammary gland ducts (12), bronchi (13) and oral cavity (14) but also in some inflammatory diseases affecting oral mucosae, such as oral lichen planus (3, 15).

Microvessels density (MVD) analysis quantifies angiogenesis *ex vivo* on histologic sections and immunohistochemically determines topographical

Key words: virtual chromoendoscopic magnification, narrow band imaging, oral cancer, oral pathology, non-invasive imaging

Mailing address:

Maria Contaldo,
Multidisciplinary Department of Medical-Surgical and Odontostomatological Specialties, University of Campania-Luigi Vanvitelli,
Via L. De Crecchio 6, 80138 Naples, Italy
Tel.: +39 081 566 7675 - Fax +39 081 5665477
e-mail: maria.contaldo@gmail.com

0393-974X (2016)

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distribution of capillary blood vessels. MVD shows the presence of intraepithelial entrapped blood vessels (IEBVs) twofold in carcinoma in situ (CIS) than in normal and dysplastic epithelia (16).

Neo-angiogenesis is also a prerequisite for developing oral squamous cell carcinoma (OSCC). OSCC prognosis, therapeutic approach, and outcome vary according to several factors, such as tumor size at the time of diagnosis, adhesion molecule down-regulation (17), and neoangiogenesis, which are all co-responsible for the development of distant metastases and/or second primary malignancies (18, 19).

To date, about 60-70% of OSCCs are diagnosed at advanced stages (Stage III and IV); this diagnostic delay is responsible for a high mortality ratio (18). In addition, literature reports upper aerodigestive tract second primary cancers in 17-30% of patients due to the “field cancerization” phenomenon (20). For these reasons, patients with a history of OSCC must be diagnosed early and followed up carefully.

Clinical examination is the first approach in investigating oral mucosa alterations, but it may be affected by operator-related errors and is often inadequate in distinguishing among non-neoplastic lesions that may clinically look like a tumor one. Consequently, histologic examination remains the gold standard for assessing a diagnosis and establishing prognosis (21).

In recent decades, adjunctive techniques, such as vital staining with toluidine blue (22) and Lugol's iodine (23), tissue autofluorescence visualization (24) and *in vivo* confocal microscopy (25, 26), have emerged to enhance *in vivo* recognition and distinction among benign, premalignant and malignant lesions (27). Virtual Chromoendoscopic Magnification (VCM) is an *in vivo* imaging technique able to visualize microvascular architecture in suspicious, pre-cancerous, and early cancer oral lesions in order to noninvasively establish tumor neo-angiogenesis presence (28).

VCM, also known as Narrow Band Imaging (NBI), was developed in Japan (29). Unlike the standard broadband white light spectrum, VCM produces a blue/green light that penetrates less deeply into tissue, thus giving the operator a higher resolution of superficial structures (30). Indeed, the

depth of penetration of light through tissues depends on its wavelength. The filters used in VCM select blue and green lights with wavelengths of 415 and 540 nm respectively, which corresponds to the peaks of absorption of hemoglobin (28).

VCM has been widely investigated and applied to image gastric and intestinal pre-neoplastic vessel alterations (29, 30), and its effectiveness has been greatly demonstrated in the pharynx (31, 32) and esophagus (33). Under physiological conditions, the 540 nm wavelength light displays subepithelial regular-branching vessels as cyan or green in color, while the 415 nm wavelength light displays superficial blood vessels as brown in color (29, 30). However, abnormal vasculature within the subepithelial layers loses its regular branching arrangement and brownish dots and vessels with extensions, dilatations, weaving and differing shapes (30) are displayed. VCM findings such as brownish dots, tortuous and non-tortuous intraepithelial papillary capillary loops (IPCL), calibre changes and varying shapes in IPCL and brownish mucosae are shown to be suggestive of squamous mucosal neoplasia; these patterns are always detectable by VCM whereas they are rarely visible in conventional observation (13).

Recently, several authors have well-defined differences between normal and abnormal vasculatures in head and neck cancers (34-36). VCM revealed its major usefulness in detecting precancerous lesions and carcinoma *in situ* as significantly superior to white light mode (31) and in supporting the preoperative and intraoperative diagnostic workup and follow-up of patients with a history of OSCC as being able to detect early recurrences or second primary tumors (35). Literature lacks works focusing on the use of VCM for IPCL description in non-tumor conditions. Starting from the mentioned evidence, this pilot study was performed to establish the VCM analysis feasibility in similar pathologies and lesions.

MATERIALS AND METHODS

Patients

This is a preliminary descriptive study approved by

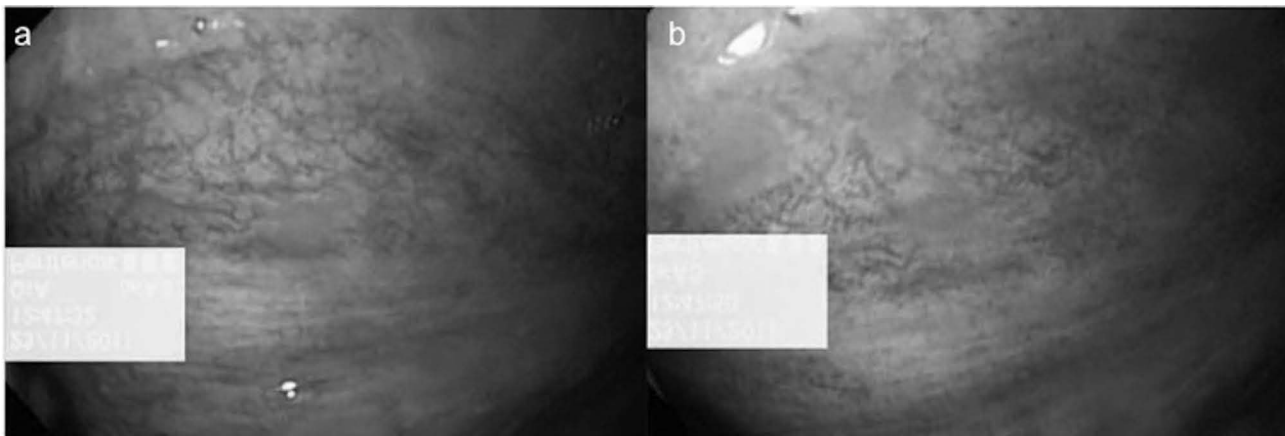


Fig. 1. White light mode vs. VCM. The vessels visualization is clearly improved in VCM lighted tissues (b) when compared with white lighted ones (a).

the Biomedical Institutional Review Board of the authors' institution, and is in accordance with the Declaration of Helsinki and its later amendments. In this study, 31 consecutive patients with oral lesions and pathologies from different origins were observed in oral pathology and medicine outpatient settings. The patients were enrolled at the Oral Pathology Unit at the Second University of Naples, Italy, after informed and written consent. All patients (15 women and 16 men, age ranged from 23 to 88 years-old, mean age 59.61 ± 14.46), underwent VCM examination at the Department of Internal and Experimental Medicine and Surgery "F. Magrassi and A. Lanzara" at the same University. The clinical data of the patients are shown in Table I.

Mucosal Examination

After routine clinical assessment, all subjects underwent the following procedures:

1. Conventional white light examination (Fig. 1a). Examinations were first conducted with the traditional white-light broadband endoscopy (white light mode) to magnify the entire lesion and the surrounding mucosa.

2. Virtual Chromoendoscopic Magnification (VCM) imaging (Fig. 1b). The Narrow Band Imaging System (Olympus Medical Systems Corp., Tokyo, Japan) (35) allowed for the analysis of microvascular anatomy in detail. In order to describe the vascular architecture, the authors adopted the IPCL classification system developed by Inoue and modified by Takano (35).

The system consists of four classes corresponding to the increasing IPCL disarrangement related to cancer

progression (Fig. 2):

- Type I – normal mucosa and regular loops;
- Type II – dilatation and presence of intersections of the loops;
- Type III – elongated and tortuous loops;
- Type IV – disruption of the microvascular pattern and neovascularization (plus brownish area in the context of abnormal IPCL).

IPCL types was established by an experienced expert in VCM, which were blind for the clinical features and the diagnosis of the examined area.

Two different grouping criteria was considered to define VCM positivity. In the first one, lesions were clinically considered "malignant" and hence "VCM positive" for IPCL when classified as type III or IV, in accordance with previous works (22, 35, 37-45). In the second one, the lesions were considered "malignant", and hence "VCM positive" for IPCL when classified as type IV, which allows to establish if the cut off for malignancy identification was solely the IPCL IV or not. The time required to perform the VCM imaging was about 5 min (as per the recorded data).

Histologic analysis and treatment of lesions

After taking an incisional or excisional biopsy, the lesions underwent histopathological assessment according to the degree of extension and severity in order to confirm the clinical provisional diagnosis. The samples were sent to the Department of Pathological Anatomy at the Second

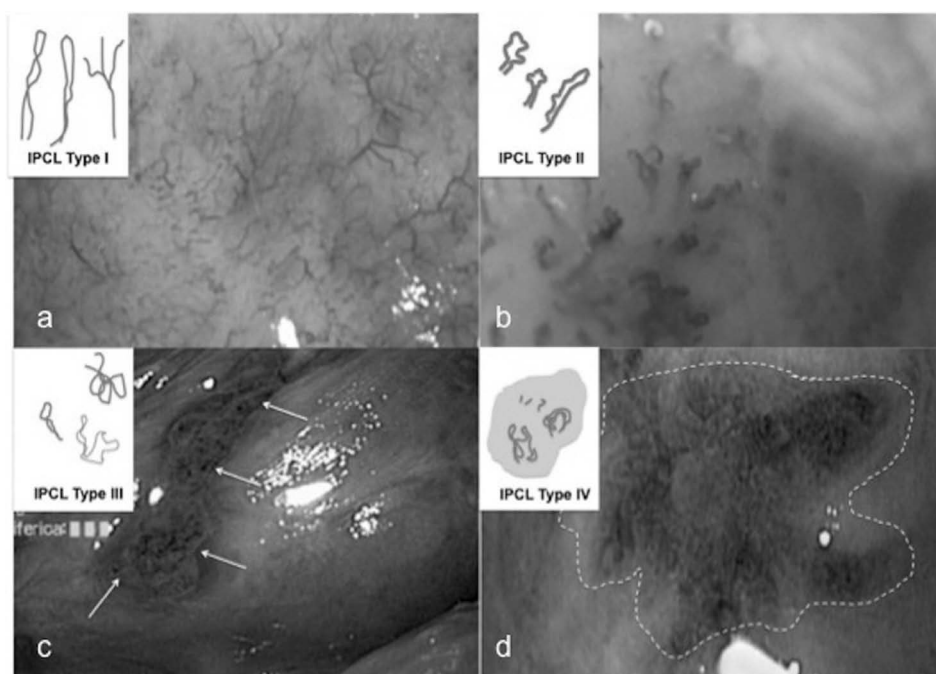


Fig. 2. Four-types IPCL classification. Vascular loops were classified into types I to IV according to progressive vessels dilation and disarrangement.

University of Naples, Italy, and were histopathologically analyzed.

Data Analysis and Statistics

VCM results were compared with histologic reports (that were considered the gold standard) to evaluate sensitivity, specificity, and positive and negative predictive values when VCM criterion of malignancy was limited to IPCL type IV.

RESULTS

Clinical features, VCM outputs, and histopathological reports regarding the 31 cases considered are summarized in Table I. The 31 consecutive patients have been grouped according to their IPCL type (Fig. 3). VCM imaging revealed 15 cases reporting IPCL I, 6 IPCL II, 4 IPCL III, and 6 IPCL IV. IPCL types according to the clinical suspect and the respective histopathological result are summarized in Table II. As reported in Table II, 5 cases of mixed OLP have been assessed both at the boundaries of the white areas (all showing IPCL type

I) and at the centre of the atrophic/erosive lesion (2 out of 5 showed IPCL type I, 3 out of 5 showed IPCL type II).

All lesions reporting IPCL I-III were not diagnosed as malignant (Fig. 4). All cases of IPCL type I were related to benign lesions, and no false negatives were found. IPCL II was present in precancerous lesions and areas of inflammation and/or hyperkeratosis, such as hyperplastic candidiasis, leukoplakia with low dysplasia, and at the erosive areas in mixed oral lichen. In addition, 1 out of the 2 cases of oral pemphigus vulgaris showed IPCL II. IPCL III was found in 1 case of white oral lichen planus, in one geographic tongue, in one erosive oral lichen planus, and in one ulcerative lesion histologically reporting mild dysplasia. Of the 6 cases reporting IPCL IV, 1 referred to type IV hypersensitivity reaction, 1 to an ulcerative lesion with mild dysplasia, and the remaining 4 cases were OSCC.

Based on this data, the calculated sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of what was considered “VCM positive” for both IPCL type III

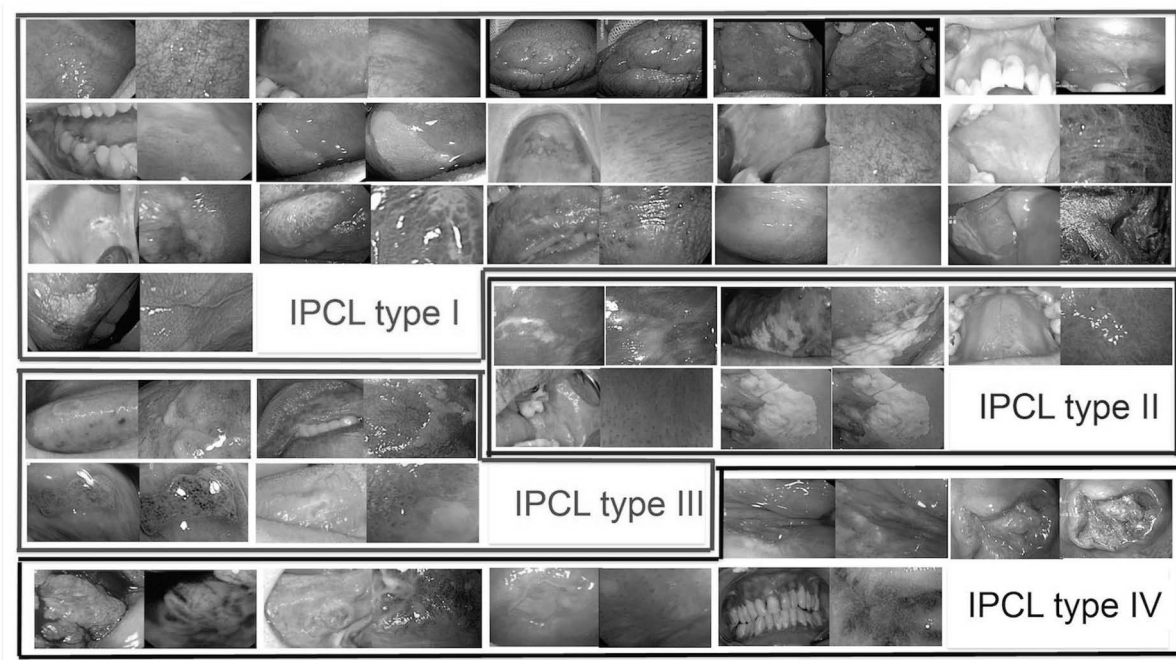


Fig. 3. Patient breakdowns by IPLC type. Each patient is represented by 2 pictures: the first is in white light, the second is under VCM light.

and IV were 100%, 78%, 40% and 100%, respectively. While the calculated sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of what was considered “VCM positive” for IPCL type IV were 100%, 93%, 67% and 100%, respectively. When IPCL type IV was found, the evidence of OSCC was strengthened by other clinical tests, such as toluidine blue and Lugol’s vital staining (21, 22) and was confirmed by histologic reports (Fig. 5).

DISCUSSION

In oral medicine, clinical examination is the first approach to evaluate both tumor and non-neoplastic mucosal lesions. However, the gold standard for diagnosis and follow-up of chronic diseases, precancerous lesions, and suspicious lesions remains the histopathological assessment after biopsy. Although advanced cancers are clearly identifiable by clinical evaluation, a great variety of non-neoplastic lesions may be clinically confused with early cancers, and many others may be associated with tumor changes not clinically recognizable.

During the past few decades, further clinical imaging systems have been developed in order to refine clinical diagnosis, thus reducing both the request for biopsy in unnecessary cases (overtreatments) and the diagnostic delay of early-cancers and small neoplastic lesions (under treatments).

Virtual Chromoendoscopic Magnification (VCM) is an *in vivo* imaging technique used to visualize microvascular architecture in suspicious, pre-cancerous, and early cancer oral lesions in order to noninvasively assess tumor neo-angiogenesis presence.

Literature has already reported the diagnostic advantage of applying VCM imaging to patients with a history of cancer because of its higher sensitivity, accuracy, and negative predictive values when compared with white light examination to discover signs of malignancy. However, little data has been reported about its application to non-neoplastic entities, and VCM has never been systematically applied to evaluate the IPCL class system of non-neoplastic condition and pathologies. Since there are a large number of entities different in etiology,

Table I. Summary of clinical, histopathological and VCE report in the patients considered.

Table 1 Summary of clinical, histopathological and VCE report in the patients considered					
Patient	Clinical appearance	Site	Histologic report	VCM IPCL	Histopathological output [#]
1	Ulcer	lip	Exfoliative Cheilitis	I	neg
2	Desquamative gingivitis	gingiva	Pemphigus vulgaris	I	neg
3	Erosion	gingiva	Type IV Hypersensitivity	IV	pos
4	Erosion, Atrophy, Hyperkeratinisation**	cheek	OLP	II	neg
5	Atrophy & thickening	tongue, dorsum	Scrotal tongue	I	neg
6	Atrophy & thickening	tongue, dorsum	Geographic tongue	III	neg
7	Hyperkeratinisation *	gingiva	OLP	I	neg
8	Normal	cheek	Healthy mucosa	I	neg
9	Normal	tongue, dorsum	Healthy mucosa	I	neg
10	Normal	tongue, margin	Healthy	I	neg
11	Nodular*	palate	Hyperplastic candidiasis	II	neg
12	Erosion, Atrophy, Hyperkeratinisation**	cheeks	OLP	I	neg
13	Erosion, White patches**	tongue, margin	OLP	III	neg
14	Omogeneous Leukoplakia*	tongue, margin	Hyperkeratosis	I	neg
15	Omogeneous Leukoplakia*	tongue, ventral	Oral dysplasia, low grade	II	neg
16	Omogeneous Leukoplakia*	palate	PVL	I	neg
17	White patches*	cheek, gingiva	OLP	I	neg
18	Ulcer with peripheral thickening**	cheek	Chronic ulcer without dysplasia	I	neg
19	Omogeneous Leukoplakia*	gingiva	Hyperkeratosis	I	neg
20	Erosion, Atrophy, Hyperkeratinisation**	cheeks	OLP	II	neg
21	Ulcer	cheek	Oral dysplasia, mild grade	III	pos
22	Erosion	tongue, margin	OLP	III	neg
23	Ulcer	cheek	OSCC, G2	IV	pos
24	Esophitic lesion with ulcer	cheek	OSCC, G2	IV	pos
25	Ulcer	cheek, fornix	OSCC, G3	IV	pos
26	Speckled ulcer	tongue, ventral	OSCC, G2	IV	pos
27	Erosion	cheeks, tongue	Pemphigus vulgaris	II	neg
28	Erosion & white patches**	cheeks, gingiva	OLP	II	neg
29	Erosion, Atrophy, White patches*	palate	OLP	I	neg
30	White patches*	cheeks, gingiva	OLP	I	neg
31	Ulcer with peripheral thickening*	tongue, margin	Oral dysplasia, mild grade	IV	neg

[#]: Histopathological output. (**pos**), positive: histological diagnosis of OSCC; (**neg**), negative: histological diagnosis of lesions different from OSCC. **OLP**: Oral Lichen Planus; **PVL**: Proliferative Verrucous Leukoplakia. *In thickened epithelia the IPCL value was considered at the perilesional areas due to high keratinization obscuring the VCE imaging. **In lesions showing both hyperkeratinized and erosive areas, IPCL reported is referred to the erosive intralesional area, while the VCE assessment at the boundaries of the lesion (perilesional areas) were always scored IPCL I.

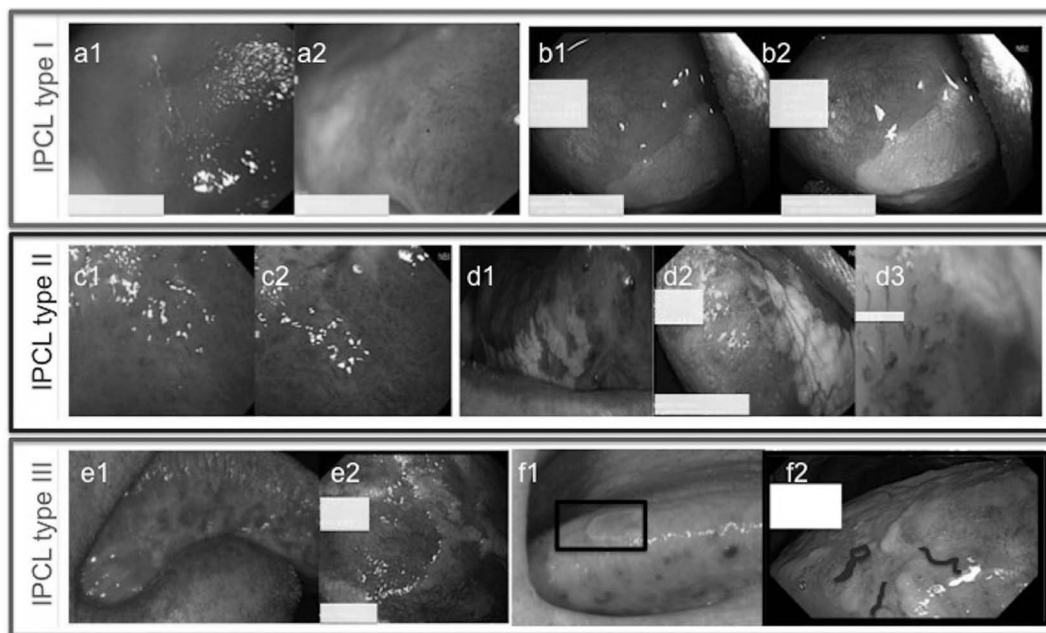


Fig. 4. Two representative cases for each IPCL types I-III. **a)** Patient #19, IG, male; **a1)** The gingival leukoplakia under white light; **a2)** The VCM at the perilesional area of the lesion revealed IPCL type I; **b)** Patient #17, IB, female; **b1)** The strong white patches at the cheek, visible under white light, did not allow the VCM light to penetrate (**b2**) and the VCM assessment performed at the perilesional area, (white arrows) revealed IPCL type I (**b2**); **c)** Patient #20, MV, male; **c1)** The atrophic/erosive area of the lesion, diagnosed as oral lichen planus, under white light; **c2)** The same area, under VCM light, showed IPCL type II; **d)** Patient #15, GA, female; **d1)** Leukoplakia with histologic report of low-grade dysplasia under white light; **d2)** VCM light did not pass through the hyperkeratinized central area of the lesion, thus preventing the visualization of the IPCL (original magnification X60); **d3)** At higher magnification (X120), the perilesional area showed IPCL type II; **e)** Patient #13, FD, male; **e1)** This mixed OLP clinically showed reticular striae alternating with atrophic/erosive areas; **e2)** VCM light revealed IPCL type III at level of the erosive/atrophic areas (original magnification X90). **(f)** Patient #6, MV, male; **f1)** Clinical picture of the geographic tongue; **f2)** Under VCM light, IPCL III were found, according to the histologic findings of inflammatory infiltrate and dilated and disarranged vessels (original magnification X90).

clinical appearance, and behavior, this preliminary descriptive analysis was performed on 31 consecutive patients in order to establish the feasibility of VCM to define their IPCL and to distinguish them from cancers on the basis of the IPCL class system.

OSCCs and cases of glossitis, candidiasis, and other clearly benign entities were considered. In all the examined conditions, VCM was manifestly not essential to determine the diagnosis. However, VCM analysis was confirmed to be strongly reliably in distinguishing between benign and malignant lesions on the basis of the IPCL class system.

Many of these benign conditions, such as geographic tongue and OLP reported IPCL III and hence were considered “positive” for the previously

adopted criteria of IPCL positivity. For this reason, in the present study an alternative definition of “VCM positivity” was restricted solely to the IPCL type IV in order to exclude these conditions and reduce “false positives”. By these novel inclusion/exclusion criteria, specificity and positive predictive value (PPV) increased from 78% to 93% and from 40% to 67% respectively when the IPCL type IV were solely considered malignancy indicators, thus increasing the capacity and the probability to discover “real positive to malignancy” by the VCM assessment.

The lack of false negatives confirmed the high sensitivity and negative predictive value of VCM, thus avoiding undiagnosed OSCCs. The presence of IPCL type III in some benign conditions may

Table II. *VCM outcome according to clinical and histopathological report.*

Clinical features	Histopathological report	IPCL I	IPCL II	IPCL III	IPCL IV
Healthy mucosa	-	3			
Hyperkeratotic lesions	Hyperplastic candidiasis of palate (n=1)		1		
	Leukoplakia without dysplasia (n=2)	2			
	Leukoplakia with Low dysplasia (n=1)		1		
	PVL (n=1)	1			
	White areas in mixed OLP* (n=5)	5			
	White OLP (n=4)	3		1	
Tongue pseudolesions	Geographic tongue (n=1)			1	
	Scrotal tongue (n=1)	1			
Erosive lesions	Cheilitis (n=1)	1			
	Type IV Hypersensitivity reaction (n=1)				1
	Erosive OLP (n=1)			1	
	Atrophic/Erosive areas in mixed OLP# (n=5)	2	3		
	Oral Pemphigus vulgaris (n=2)	1	1		
Ulcerative lesions	Without dysplasia (n=1)	1			
	Mild dysplasia (n=2)			1	1
	OSCC (n=4)				4

Due to their clinical appearance, the five mixed OLP showed both hyperkeratosis and atrophy/erosion. In these cases VCE assessments were performed both at level of the white areas (), and at the atrophic/erosive ones (#).*

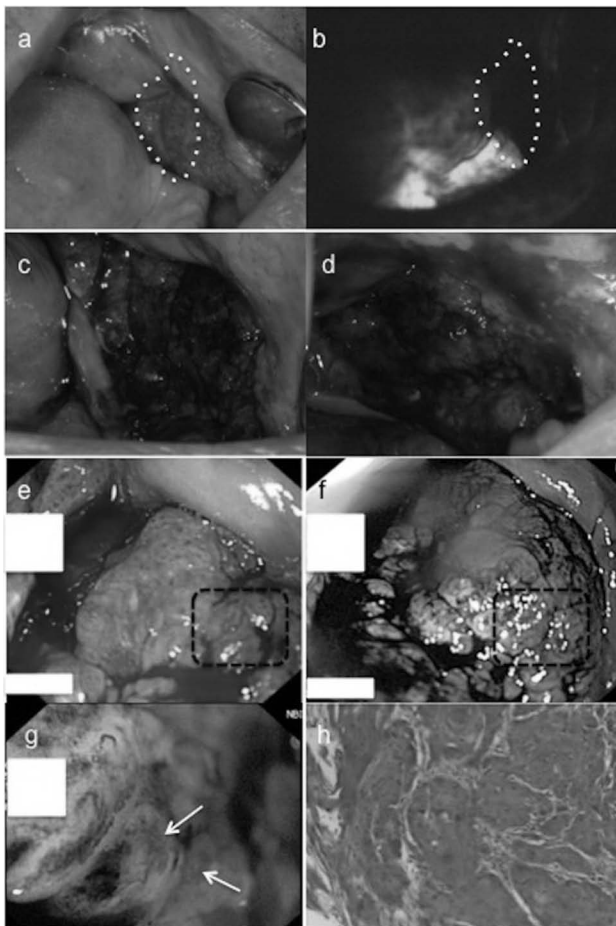


Fig. 5. IPCL type IV in patient #26, MA, male. Speckled ulcer. **a)**: Clinical appearance; **b)**: The lesion, as seen by the autofluorescence system, reported loss of fluorescence compatible for malignancies. The dotted roundish areas in **(a)** and **(b)** corresponded with the main region interested by loss of fluorescence. The malignant nature of the lesion was also supported by the toluidine blue **(c)** and Lugol's iodine **(d)** vital staining results. The massive bleeding of the lesion, visible in white light mode **(e)**, was visually amplified under VCM light **(f)**, corresponding with the dark areas. **(g)**. The squared dotted area in **(e)** and **(f)** correspond with picture in **(g)** under VCM examination: abnormal neovascularization, characterized by dilated and tortuous IPCL, in the context of brownish areas (white arrows), was clearly ascribable to IPCL type IV, (original magnification X120); **h)**: Haematoxylin eosin staining of histologic sections confirmed the tumoral nature of the ulcer, with the presence of nests of tumoral cells in the context of cancer-related blood vessels (original magnification X80).

be justified by chronic inflammation and/or neovascularization which cause vessel abnormalities classified into IPCL type III, as in the case of erosive oral lichen planus, where vascular disarrangement has been previously proven by several authors (16,17).

In hyperkeratotic lesions, the whitish plaque obstructed the penetration of broadband white light and the spectrum of the filtered light of the VCM; hence, in these cases, VCM observation was conducted at the perilesional edges, and the findings were 11 of 13 cases with IPLC I and 2 of 13 with IPCL II. Where small erosive areas were present in the context of keratinization (2 of 13 cases), the VCM examination was conducted both at the perilesional areas, where IPCL I were always found, and on the lesional erosive areas, where IPCL II were found in one case and IPCL III in the other.

These evidences represent limits of the VCM assessment in those hyperkeratinized lesions needing further investigations and still requiring histopathological assessment for diagnosis. A further limitation to VCM imaging, was found in bleeding lesions, where the rich hemoglobin content absorb the entire light and is expressed by dark areas (Fig. 5f).

Despite these limitations, VCM may be considered a valid adjunctive tool for oral mucosa non-invasive examination and to follow-up the at-risk lesions and conditions, such as OLP and chronic candidiasis, where a malignant transformation may be highlighted by the switch from IPCL III to IV.

Further extended studies with a larger cohort of patients are needed in order to better understand the potentiality of VCM to evaluate if non-neoplastic oral pathologies can be correlated with a specific IPCL type and to establish if IPCL could be considered as potential prognostic indicators in chronic inflammatory/dysimmune oral diseases.

ACKNOWLEDGEMENTS

The authors thank Sarah Keyes, from the BC University, Vancouver, Canada, for her linguistic revision of the manuscript.

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ULTRASOUND IMAGING OF ORAL FIBROMA: A CASE REPORT

D. DI STASIO¹, D. LAURITANO², R. PAPARELLA¹, R. FRANCO³,
M. MONTELLA³, R. SERPICO¹ and A. LUCCHESI¹

¹*Multidisciplinary Department of Medical and Dental Specialties, University of Campania- Luigi Vanvitelli, Naples, Italy;* ²*Department of Medicine and Surgery, University of Milan-Bicocca, Monza, Italy;* ³*Department of Mental and Physical Health and Preventive Medicine, University of Campania-Luigi Vanvitelli, Naples, Italy*

Oral mucosa is constantly under the influence of various internal and external stimuli, therefore it is interested by a range of developmental disorders, irritation, inflammation, and neoplastic conditions. Traumatic fibroma is one of these lesions. The aim of this work is to obtain an ultrasound characterization of a lip fibroma with a high frequency probe (8-18 MHz) as feasibility and experimental model of this non-invasive technique in the oral mucosa compared with its histo-pathological section. Ultrasound characterization of a fibroma in a 50-year-old patient in the right retro commissural area of mucosa was performed. The ultrasound images were compared with its histological image. Fibroma is a traumatic lesion clinically recognizable and with a complex and well-defined histopathology structure. Although further investigations are necessary to determine the potential of ultrasonographic images as an aid in the diagnosis of oral lesions, this case report might be a model for ultrasound imaging compared with its histological section.

Oral mucosa is constantly under the influence of various internal and external stimuli, so it is interested by a range of developmental disorders, irritation, inflammation, and neoplastic conditions (1-5). Reactive lesions show a correlation to a low-grade irritation or injury, such as chewing, food impaction, calculus, or iatrogenic injuries such as broken teeth, overhanging dental restorations and extended flanges of denture (6). Traumatic fibroma is a localized hyperplastic lesion, determined in response to irritation or trauma which is commonly seen in the buccal mucosa (7). These lesions are most prevalent in the 4–6th decade of life; they are very common and totally benign and the female-to-male ratio is almost 2:1 (6). They present a smooth surface, nodular appearance, hard consistency and a sessile base. Color is similar to the mucosa or may

be bluish, measuring up to 2 cm in diameter, and displaying slow growth due to low mitotic index. Lesions are often characterized by a capsule, usually well delimited and do not produce metastasis. Microscopically, the fibromas appear as a nodular mass of fibrous connective tissue with collagen fibers mixed to fibroblasts and covered by a keratinized squamous epithelium (8). Recent advances in the field of sonography have extended the spectrum of applications to soft tissues, adding skin layers among the possibilities for study. Among all of the imaging modalities, sonography has several advantages for studying the skin and the mucosa, in addition to an optimal penetration/resolution balance that permits good discrimination of the different cutaneous layers (9, 10). Importantly, this technique is safe; in fact, it lacks the secondary effects of radiation as computed

Key words: mucosa, cavity, soft, tissue, connective

Mailing address:

Alberta Lucchese, DMD, PhD
Multidisciplinary Department of Medical and Dental Specialties,
Second University of Naples, Via L. De Crecchio 6, 80138 Naples, Italy
Tel.: +39 0815667670 - Fax: +39 0815667674
e-mail: alberta.lucchese@unina2.it

23(S)

0393-974X (2016)

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tomography and is comfortable because it does not confine the patient in a reduced space as magnetic resonance imaging. Moreover, dermatologic sonographic studies do not usually require the injection of a contrast medium (9). Buccal mucosa is involved in almost all cases of oral sub-mucous fibrosis and also lends itself well to ultrasonographic examination because of its superficial location and absence of underlying bone. Despite these facts, very few studies have reported the use of ultrasonography in the evaluation of oral mucous and submucous lesions (11). Normally, in other areas, such as breast or uterus, during an ultrasonography examination a similar lesion appears as a heterogeneously hyperechoic mass with an indistinct margin and posterior acoustic shadowing (12).

The aim of this work is to obtain an ultrasound characterization of a lip fibroma with a high frequency probe (8-18 MHz) as feasibility and experimental model of this non-invasive technique in oral mucosa.

Case report

The case of a 50-year-old female is presented. The patient showed a small nodular lesion, of about 0.5 cm in diameter, in the right retro commissural area. On palpation, it was ovoid and firm in consistency. The lesion was in correspondence to a dental

malocclusion (cross bite), so a traumatic etiology was hypothesized (Fig. 1).

The examination was performed with a portable GE Logiq-e R7 ultrasound machine. The monitor was a 15" LCD, with a resolution of 1024x768. The transducer was a hockey stick transducer 8-18 MHz. The axial field of view was from 0.4 mm to 2 cm and the lateral from 0.7 mm to 2 cm.

The ultrasound image shows a lesion about 5.46 mm long and 3.14 mm deep. The lesion appears as a heterogeneously hyper-echoic nodule, with distinct margin and posterior acoustic shadowing: this detail gives information on the fibrous nature of the lesion. On the top of the nodule, a layer of 0.3 mm is visible and it represents the epithelial layer. Even more in the surface, it is possible to notice a slightly hypo-echoic area that seems to represent the hyper- para keratinized layer as shown in the histological image, expression of the epithelial hyperkeratosis that is also clinically visible. The deeper layers seem to be separated from the epithelial tissue by an anechoic area that should represent the dermal-epidermal junction. The area below the epithelial tissue is distinctly recognizable by a lower echogenicity compared to that seen in the most superficial tissues; this could be the sub-epithelial stromal component below. Finally, an anechoic area of circular shape with



Fig. 1. *Clinical image of the fibroma.*

well defined margins is present and may correspond to the vascular structures in the connective tissue also visible in the histological image.

The image shows no pattern of infiltration, secondary to the benignity of the lesion, since the limits separating the fibroma from the surrounding tissues seem clearly visible. A surgical excision was performed and the bioptic sample was analyzed. The histological image, enlarged 5x, shows a polypoid lesion appearance and a stromal proliferation. This sample shows: hyperkeratosis; a pattern of parakeratosis, with small nuclei in stratum corneum cells; acantosis; papillomatosis; a continue basal membrane; sub-epithelial vascular proliferation; and a mild chronic inflammatory infiltrate. The correlation between the histological sample and the high-resolution ultrasound image is shown in Fig. 2.

DISCUSSION

This case report allows us to compare an ultrasound image of the surface, high-resolution, obtained *in vivo* and in real time, with histo-pathological findings.

Fibroma is a traumatic lesion clinically recognizable and with a complex and well-defined structure histopathology, therefore it may be a

good model for ultrasound imaging compared with its histological section. This method should be extended to other lesions of the oral mucosa to define sensitivity and specificity of this technique.

High res-Ultrasound is already being used successfully in other fields of medicine, for example in dermatology (10). Introducing the use of this technique in the oral cavity may be used in the early detection of neoplastic and non-neoplastic lesions (13). In addition, this technique can be helpful in the pre-surgical evaluation of other oral lesions. Indeed, the clinical observation can define only lateral and longitudinal extension of a lesion within a safety margin, but not depth and infiltration of deeper structures.

Although an ultrasonographic examination can not replace a biopsy to make the diagnosis of a lesion, this technique presents many advantages:

1. it could be a valid support in diagnosis as it is a non-invasive, real time technique that provides data in conformity to the reality;
2. it could be a valid support in pre-surgical evaluation of the lesion;
3. other imaging techniques, such as confocal microscopy (14, 15) and optical coherence tomography (16), provide high-resolution

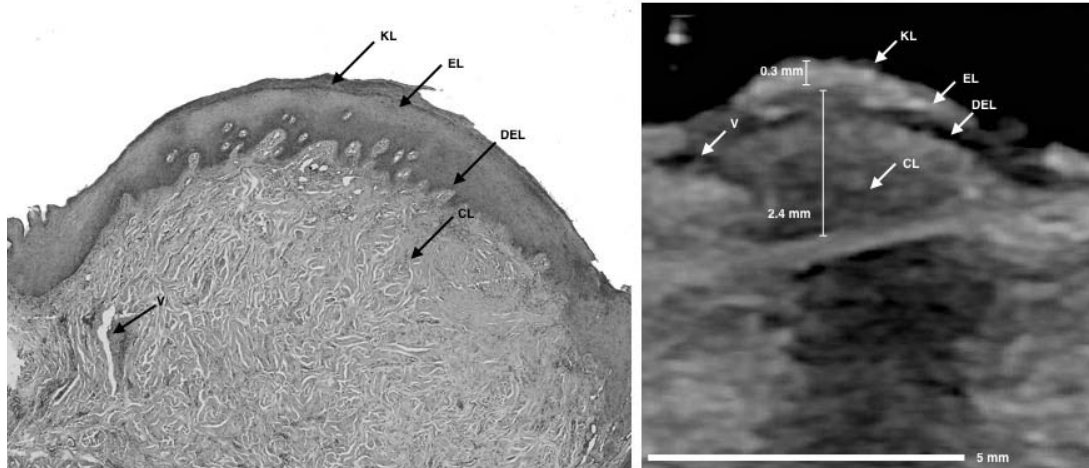


Fig. 2. The image represents a histological section of the fibroma compared with the ultrasound image of the same: at the top a hyper-para keratinized layer (keratinocyte layer; KL) is represented. The epithelial layer is shown as a uniform hyperechoic area (epithelial layer; EL). The stromal-connective component appears relatively hypoechoic (connective layer; CL). Between these two layers there is a well-defined anechoic line that could be attributed to the dermo-epidermal zone (dermal-epidermal layer; DEL). An anechoic area of circular shape with well defined margins could correspond to vessels in the connective component (vessels; V).

images but, at the same time, very low penetration (≤ 0.5 mm); in contrast, sonography, that allows the use of variable-frequency probes, has a reasonable balance between high penetration and resolution without loss of definition when changing the depth (9);

4. high-frequency ultrasonography is an excellent tool for the diagnosis of different oral mucosa conditions but could presents many critical issues;
5. this method is operator-dependent;
6. it is crucial to understand the normal mucosa ecographic structure as well as the correlation between histological and sonographic findings.

Further investigations are necessary to determine the potential of ultrasonographic images as an aid in the diagnosis of oral lesions.

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SURVEY RESULTS OF CURRENTLY USED TECHNIQUES AND MATERIALS BY DENTISTS FOR FABRICATION OF COMPLETE REMOVABLE DENTAL PROSTHESES

A. MEIROWITZ¹, R. NOWOTNY KLEIN², D. LAURITANO³, N. HAREL⁴,
J. BLOCK⁴, S. MATALON⁵, F. CARINCI⁶ AND Z. ORMIANER⁵

¹*Maurice and Gabriela Goldschleger School of Dental Medicine, Tel Aviv University, Tel Aviv, Israel;* ²*Department of Oral Rehabilitation, The Maurice and Gabriela Goldschleger School of Dental Medicine, Tel Aviv University, Tel Aviv, Israel;* ³*Department of Medicine and Surgery, University of Milan-Bicocca, Monza, Italy;* ⁴*Department of Oral Rehabilitation, The Maurice and Gabriela Goldschleger School of Dental Medicine, Tel Aviv University, Tel Aviv, Israel;* ⁵*Department of Oral Rehabilitation, The Maurice and Gabriela Goldschleger School of Dental Medicine, Tel Aviv University, Tel Aviv, Israel;* ⁶*Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, Italy*

The present study was based on the screening of procedures and materials used by dentists in constructing complete removable dental prostheses, considering their professional background and years of experience. Questions arose regarding differences and similarities within the local dental society, of which approximately 75% were trained in European countries with differences in academic background, and compared to international dentistry. A questionnaire web site was used in order to anonymously collect answers to the survey by general practicing and specialist dentists. The survey contained 28 multiple choice questions that were based on 5 parameters: years of experience, location of dental studies, specialization, location and field of specialization. One hundred one survey sheets were collected. The results show statistical significance ($p < 0.05$) for the following comparisons: practicing time had an impact on practitioner-technician interaction, while the level of qualification had an effect on articulator and face-bow usage. Location of dental studies, specialization and residency had impact on final impression materials, teeth set-up as well as tooth shape and color selection. The differences found between dentists' working techniques set the ground for future research that should focus on rating the success of the different methods towards a goal of creating recommendations for an ideal set of working techniques.

Complete removable dental prostheses are an inseparable part of dental therapy for edentulous patients (1-3). Even though dental implant-supported restorations serve a stable and more comfortable artificial dentition and therefore seem to have taken over the field of partial and complete removable dental prostheses, there are still indications for this type of therapy. The correct construction of a

complete removable dental prosthesis, as well as a fixed prosthesis, requires cooperation and trust between the dentist and the dental technician (4-6). Knowledge of mechanical, biological and physical factors is essential in order to incorporate retention, stability and support in the denture design (1, 7-9). Retention, stability and support are the three pillars that support a successful prosthesis. They

Key words: complete denture, survey, fabrication technique, specialization, dental technicians

Mailing address:

Prof. Francesco Carinci, MD
Department of Morphology, Surgery and Experimental Medicine,
University of Ferrara, Via Luigi Borsari 46, 44100 Ferrara, Italy
Tel.: +39 0532 455582 - Fax: +39 0532 455582
e-mail: crc@unife.it - web: www.carinci.org

influence the relationship between the tissue surface of the denture base and the mucosal surface of the edentulous ridges (9). Various clinical and laboratory techniques are utilized to succeed in achieving these objectives.

The sequence of impression making, preliminary and final (10-14), working models and mounting in articulator with face bow (15-17) are subject to various clinical methods. Receiving a good final esthetic result, when fabricating a complete removable dental prosthesis is challenging. The choice of color, shape, angle and position of acrylic teeth forming the dentition can be complicated. The dental market offers a large variety of choices, which is dependent on the patient's variables such as: sex, age, type of natural dentition before tooth loss, influence of the patient's surroundings, as well as on the experience of the dentist and technician (1, 8, 9, 15, 18). Positioning of the teeth has to follow customized criteria of shape and occlusal schemes.

Every dentist must have the knowledge and expertise to design a complete removable dental prosthesis. Nevertheless, numerous issues can affect the clinical stages and outcome achieved, including the level and location of dental education, clinical experience and specialization in prosthodontics or in other dental fields. In Israel, dental professionals can be divided into two groups, according to education level: general dental practitioners, who graduated from university dental education programs, 25% of whom graduated from Israeli Universities, who adopt the same learning criteria as in most of the Universities in USA. Seventy-five% were trained overseas, mainly in Russia and Romania. The second group are specialists primarily in clinical training, such as oral rehabilitation, endodontics, periodontics, oral surgery and orthodontics. In USA, UK, Scandinavian countries and Israel there are post graduate specialty training programs (19) which emphasize an evidence-based critical approach to the literature, with independent and enduring learning attitudes. These two facts may have an impact on the skill of complete removable dental prosthesis construction.

The purpose of this study was to collect, through a specific questionnaire, the information

needed to point out the way in which Israeli dental professionals, with different training backgrounds, use similar or different working techniques and materials. This was examined according to 5 parameters regarding years of experience, location of dental studies, specialization, location of studies of the specialization and field of specialization. The survey was built to cover all important stages and commonly known clinical sequence of complete removable dental prosthesis construction. This sequence is expected to be widely acknowledged and practiced among dentists in Israel.

MATERIALS AND METHODS

This study was exempted by the TAU Ethics Committee.

A questionnaire web site (<http://tfasim.org.il/tofes/zhvmq>) was used in order to anonymously collect answers to the survey filled in by general practitioners and specialist dentists. The questionnaire contained 28 multiple choice questions regarding the procedures and materials used in the construction of complete dentures (Table I).

For each question, only one answer could be marked. The first 5 questions categorize the dentists according to years of experience, location of dental studies, specialization, location of specialization studies, and field of specialization. The following 23 questions regarded the most important stages in full denture construction. In order to ensure the reliability of the survey, submission of the questionnaire was limited to one for each IP address, ensuring that each responder could submit the questionnaire only once. The questionnaire was sent to over 300 dentists practicing in Israel. Overall, 101 replies were received.

The question regarding years of experience was phrased using the choices: 0-5 years, 5-10 years, 10-15 and over 15 years. Due to the fact that only 5 dentists had 10-15 years of experience, the results of this group were merged with the results of the 5-10 years' group. The analysis was therefore conducted based on: 0-5years, 5-15 years and over 15 years.

The question regarding field of specialization was phrased using the choices: prosthodontics, periodontics, maxillofacial surgery and other. Due to a small number

Table I. *The Questionnaire.*

1. How many years have you been practicing dentistry?
 - a. Less than 5 years
 - b. 5-10 years
 - c. 10-15 years
 - d. More than 15 years
2. Where did you study dentistry?
 - a. Israel
 - b. Abroad
3. What is the level of your dental education?
 - a. General practitioner
 - b. Resident
 - c. Specialist
4. Where did you specialize (for residents or specialists)?
 - a. Israel
 - b. Abroad
5. In which field did you specialize?
 - a. Prosthodontics
 - b. Periodontics
 - c. Oral and Maxillofacial surgery
 - d. Other
6. Which impression tray do you use for the primary impression?
 - a. Regular impression tray
 - b. Anatomical impression tray (for edentulous ridges)
7. When fabricating the custom tray do you perform spacing with wax?
 - a. Yes, in the entire tray
 - b. Yes, in undercut areas
 - c. Yes, along the ridges
 - d. No, I don't perform spacing with wax
8. What custom tray do you prefer using?
 - a. With retention holes
 - b. Without retention holes
9. In the custom tray, where do you make retention holes?
 - a. In the entire tray
 - b. Only along the edentulous ridges
 - c. Only on the palate area
10. Do you use a face-bow?
 - a. Always
 - b. Frequently
 - c. Sometimes
 - d. Seldom
 - e. Never
11. Which articulator do you use?
 - a. Fully adjustable
 - b. Semi adjustable
 - c. Hinge type
12. Which make of articulator do you use?
 - a. Whipmix
 - b. Hanau
 - c. Quickperfect
 - d. Other
13. In your opinion, how necessary is it to use a semi-adjustable articulator?
 - a. In every case
 - b. In most cases
 - c. Sometimes
 - d. Never
14. Do you think it is possible to achieve identical results with a Hinge articulator?
 - a. Always
 - b. Mostly
 - c. Sometimes
 - d. Never
15. What in your opinion is of higher importance – working with a highly skilled dental technician, or using a semi or fully adjustable articulator as well as a face-bow?
 - a. Highly skilled technician
 - b. Semi or fully adjustable articulator as well as a face-bow
 - c. Both are equally important
16. What material do you use for taking the final impression?
 - a. Polyether
 - b. Polysulfide
 - c. Silicones
 - d. Other

17. What material do you use for border molding the custom tray?
 - a. Silicone
 - b. Thermoplastic material
18. Do you perform posterior damming (post dam)?
 - a. Yes
 - b. No
19. Do you mark the post dam?
 - a. Yes
 - b. No
20. When you mark the post dam – where do you mark it?
 - a. In the mouth
 - b. On the model
 - c. On the margins
21. When you mark the post dam – who marks it?
 - a. Dental technician
 - b. Dentist
 - c. Both
22. What teeth set-up technique do you use?
 - a. Balanced
 - b. Lingualized
 - c. Other
23. Teeth with which cusp inclination do you usually choose?
 - a. 20 degrees
 - b. 30 degrees
 - c. Other
24. What tooth form do you usually choose?
 - a. Synoform (partial anatomy)
 - b. Cuspiform (full anatomy)
 - c. Non-anatomy (flat morphology)
25. According to which criterion do you choose the shape of the teeth?
 - a. Pictures from a younger age
 - b. Shape of the face
 - c. Shape of the residual ridges
 - d. Other
26. Who chooses the shade of the teeth?
 - a. Dental technician
 - b. Dentist
 - c. Patient
27. Do you try-in the front 6 teeth (on the occlusal rim) before mounting the remaining teeth?
 - a. Always
 - b. Frequently
 - c. Sometimes
 - d. Never
28. Do you perform a remount in the articulator after finishing the denture?
 - a. Always
 - b. Frequently
 - c. Sometimes
 - d. Never

Table II. Significant results by years of experience.

		11. Which articulator do you use?				15. What in your opinion is of greater importance – working with a highly skilled dental technician, or using a semi or fully adjustable articulator as well as a face-bow?			
		a. Fully adjustable	b. Semi adjustable	c. Hinge type	Total	a. Highly skilled technician	b. Semi or fully adjustable articulator as well as a face-bow	c. Both are equally important	Total
How many years have you been practicing dentistry?	a. Less than 5 years	0	19	1	20	8	4	8	20
		0%	95%	5%	100%	40%	20%	40%	100%
	b. 5-15 years	0	14	1	15	7	0	8	15
		0%	93.3%	6.7%	100%	46.7%	0%	53.3%	100%
	d. More than 15 years	6	41	19	66	37	1	28	66
		9.1%	62.1%	28.8%	100%	56.1%	1.5%	42.4%	100%
Total		6	74	21	101	52	5	44	101
		5.9%	73.3%	20.8%	100%	51.5%	5%	43.6%	100%

of responses from periodontists (3), and maxillofacial surgeons (3), the analysis was conducted based on the parameter: Prosthodontics and Other.

A Chi-squared test and Fisher's exact test were carried out to assess the analysis based on the following 5 parameters:

- 1) Years of experience: 0 to 5 years; 5 to 15 years; Over 15 years.
- 2) Location of dental studies: Israel; Abroad.
- 3) Specialization: General practitioner; Resident; Specialist.
- 4) Location of studies of the specialization: Israel; Abroad.
- 5) Field of specialization: Prosthodontics; Others.

RESULTS

Statistical significance ($p < 0.05$) was found for the comparisons shown below.

Years of experience (Table II)

No connection was found between years of experience and the type of articulator used. Most of the dentists use semi-adjustable articulators.

Dentists practicing over 15 years believe that the well-trained technician is of more importance than

the articulator or the articulator and well-trained technician equally.

Dentists practicing less than 5 years and dentists practicing 5-15 years believe that either the well trained technician or the articulator and well trained technician equally are more important than the articulator itself ($P=0.013$).

Location of dental studies (Tables III and IV)

Location of dental studies, Israel or worldwide, had an impact on use of materials, choice of tooth shape, choice of tooth color and on teeth set-up. Dentists who studied in Israel prefer to use silicones, polyether or polysulfide as a final impression material, and not any other material, while dentists who studied overseas may use other materials ($P = 0.045$).

Dentists who studied in Israel tend to use pictures of patients from a younger age to choose the shape of the teeth more than dentists who studied overseas, while dentists who studied out of the country tend to choose the shape of the teeth according to the residual ridges more than dentists who studied in Israel ($p=0.021$). Dentists who studied abroad let the patients choose the teeth color more frequently

Table III. Significant results by place of dental education.

		16. What material do you use for taking the final impression?					25. According to which criterion do you choose the shape of the teeth?				
		a. Polyether	b. Polysulfide	c. Silicones	d. other	Total	a. Pictures from a younger age	b. Shape of the face	c. Shape of the residual ridges	d. Other	Total
Where did you study dentistry?	a. Israel	21	20	31	0	72	10	42	6	14	72
		29.2%	27.8%	43.1%	0%	100%	13.9%	58.3%	8.3%	19.4%	100%
	b. Abroad	7	6	13	3	29	1	15	9	4	29
		24.1%	20.7%	44.8%	10.3%	100%	3.4%	51.7%	31%	13.8%	100%
	Total	28	26	44	3	101	11	57	15	18	101
		27.7%	25.7%	43.6%	3%	100%	10.9%	56.4%	14.9%	17.8%	100%

Table IV. Significant results by place of dental education.

		25. Who chooses the shade of the teeth?				26. Do you try-in the front 6 teeth (on the occlusal rim) before mounting the remaining teeth?				
		a. Dental technician	b. Dentist	c. Patient	Total	a. Always	b. Frequently	c. Some times	d. Never	Total
Where did you study dentistry?	a. Israel	2	64	6	72	24	16	26	6	72
		2.8%	88.9%	8.3%	100%	33.3%	22.2%	36.1%	8.3%	100%
	b. Abroad	3	20	6	29	7	10	5	7	29
		10.3%	69%	20.7%	100%	24.1%	34.5%	17.2%	24.1%	100%
	Total	5	84	12	101	31	26	31	13	101
		5%	83.2%	11.9%	100%	30.7%	25.7%	30.7%	12.9%	100%

Table V. Significant results by level of dental education.

		29. Do you use a face-bow?						12. Which articulator do you use?			
		a. Always	b. Frequently	c. Sometimes	d. Seldom	e. Never	Total	a. Fully adjustable	b. Semi adjustable	c. Hinge type	Total
What is the level of your dental education?	a. General Practitioner	2	6	19	15	25	67	6	42	19	67
		3%	9%	28.4%	22.4%	37.3%	100%	9%	62.7%	28.4%	100%
	b. Resident	6	2	5	4	1	18	0	18	0	18
		33.3%	11.1%	27.8%	22.2%	5.6%	100%	0.0%	100%	0%	100%
	d. Specialist	2	2	3	7	2	16	0	14	2	16
		12.5%	12.5%	18.8%	43.8%	12.5%	100%	0%	87.5%	12.5%	100%
	Total	10	10	27	26	28	101	6	74	21	101
		9.9%	9.9%	26.7%	25.7%	27.7%	100%	5.9%	73.3%	20.8%	100%

Table VI. Significant results by level of dental education.

		26. Which make of articulator do you use?					25. According to which criteria do you choose the shape of the teeth?				
		a. Whipmix	b. Hanau	c. Quickperfect	d. Other	Total	a. Pictures from a younger age	b. Shape of the face	c. Shape of the residual ridges	d. Other	Total
What is the level of your dental education?	a. General Practitioner	3	28	4	32	67	7	42	11	7	67
		4.5%	41.8%	6%	47.8%	100%	10.4%	62.7%	16.4%	10.4%	100%
	b. Resident	0	4	6	8	18	4	9	0	5	18
		0%	22.2%	33.3%	44.4%	100%	22.2%	50%	0%	27.8%	100%
	d. Specialist	3	0	10	3	16	0	6	4	6	16
		18.8%	0%	62.5%	18.8%	100%	0%	37.5%	25%	37.5%	100%
	Total	6	32	20	43	101	11	57	15	18	101
		5.9%	31.7%	19.8%	42.6%	100%	10.9%	56.4%	14.9%	17.8%	100%

Table VII. Significant results by field of specialization.

		13. What teeth set-up technique do you use?				24. What tooth form do you usually choose?			
		a. Balanced	b. Lingualized	c. Other	Total	a. Synoform (partial anatomy)	b. Cuspiform (full anatomy)	c. Non-anatomy (flat morphology)	Total
In which field did you specialize?	a. Prosthodontics	25	3	0	28	19	8	1	28
		89.3%	10.7%	0%	100%	67.9%	28.6%	3.6%	100%
	b. Other	10	0	3	13	5	3	5	13
		76.9%	0%	23.1%	100%	38.5%	23.1%	38.5%	100%
	Total	35	3	3	41	24	11	6	41
		85.4%	7.3%	7.3%	100%	58.5%	26.8%	14.6%	100%

($p=0.049$). Most of the dentists choose the shape of the teeth according to the shape of the patient's face ($p=0.014$) and choose the tooth color themselves ($p=0.049$). Dentists who studied in Israel tend to perform tooth try-in of only the front teeth before proceeding to the full tooth arrangement more than dentists who learned overseas ($p=0.042$).

Specialization – general dental practitioners versus residents and specialists (Tables V and VI)

The level of qualification had an influence on the usage of an articulator and face-bow as well as on tooth shape selection. Residents and specialists use the face-bow more than general practitioners.

Residents use the face-bow more than specialists ($p=0.003$). Residents only use semi-adjustable articulators whereas specialists use Hinge type or semi-adjustable articulators ($p=0.005$). Most of the general practitioners use the Hanau articulator. Specialists and residents mainly use the Quickperfect articulator ($p=0.005$). However, most dentists use a semi-adjustable articulator regardless of their further education. The most common criteria for choosing the tooth shape by general practitioners and residents is the shape of the patient's face. None of the residents choose the tooth shape according to the residual ridges and none of the specialist choose the tooth shape according to pictures from the past ($p=0.014$).

Location of studies of the specialization

No statistically significant results were found.

Field of specialization - prosthodontics compared to other (Table VII)

The Balanced Articulation tooth positioning technique is more common than the Lingualized Occlusion technique with no connection to specialization ($p=0.018$). Prosthodontists use either Balanced Articulation or Lingualized Occlusion tooth positioning, while other specialists might use other tooth positioning techniques. Prosthodontists mostly use the synoform tooth shape ($p=0.012$) and other specialists tend to use the non-anatomy tooth shape.

DISCUSSION

Some main issues result from this survey. Practicing time had an impact on practitioner-technician interaction, while the level of qualification had an effect on articulator and face-bow usage. Final impression materials, teeth set-up, tooth shape and color selection are influenced by location of dental studies. Dentists who studied overseas differ in certain working techniques compared to Israeli-trained dentists, while the latter, prosthodontists and residents tend to follow the recommended workflow as suggested in the literature.

According to dental literature, the currently used materials are elastomeric impression materials such as polyether, silicones and polysulphide, where each

material type has its specifications for use (1, 10, 15, 20). The survey results indicate that dentists who studied in Israel prefer to use silicones, polyether or polysulfide as a final impression material and no other material. Nevertheless, dentists who studied overseas may use other materials. It is also interesting that of the different types of impression materials, 43.6% of the dentists who participated in the survey preferred silicones.

Semi-adjustable articulators serve the dentist's needs for complete denture occlusal recording and eccentric movement options, and can be categorized in the range between the extremes of the hinge articulator and the fully adjustable articulator (1, 9). Most dentists who participated in the survey are using a semi-adjustable articulator. Residents are using only the semi-adjustable articulator, whereas specialists are using either the hinge type articulator or the semi adjustable articulator.

Disagreement exists in dental literature regarding the efficiency of the usage of the face-bow in full denture therapy (12, 21, 22). The majority of dentists who participated in the survey rarely or never use a face-bow. Residents use the face-bow more than specialists.

Tooth try-in of only the front teeth is an essential part of denture construction. The majority of the dentists who participated in the survey do perform this stage before proceeding with the teeth positioning.

Most of the dentists who participated in the survey choose the shape of the teeth according to the shape of the patient's face. None of the specialists choose the tooth shape according to pictures from the past. None of the residents choose the tooth shape according to the residual ridges. The teeth color was mostly chosen by the dentists, nevertheless the results show that dentists who studied overseas tend to let the patient choose the teeth color more often than dentists who studied in Israel.

The occlusal concept of posterior teeth shape is controversial (23). In the absence of clear evidence of the benefits of one kind of tooth anatomy, the literature suggests that dentists should use the least complicated shape that will satisfy their concepts of occlusion (24, 25). Nevertheless, as a guide line, it can be said that bilateral balanced articulation is

usually connected with anatomical teeth, while non-anatomical teeth are usually used for the monoplane articulation and for the lingualized occlusal concept which is a combination of upper anatomical and lower non-anatomical teeth (26). Among the dentists who participated in the survey, the balanced tooth positioning technique is more common than the lingualized technique. Prosthodontists might use either the balanced or lingualized tooth positioning, while other specialists use the balanced or other tooth positioning techniques.

CONCLUSIONS

The results of this survey present differences in the techniques practiced among Israeli dentists according to the parameters chosen and in comparison to current dental literature. It can be summarized that prosthodontists follow the literature suggestions more than general practitioners but less than residents. Dentists who studied overseas, as compared to dentists who studied in Israel, differ in certain working techniques, but most concepts in Israeli dental practices are uniform. Years of experience did not have an impact on the use of materials or working techniques in full denture therapy, but did have a significant effect by increasing the importance of working with a well-trained dental technician.

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BILATERAL SYMMETRY OF ANTERIOR MAXILLARY INCISORS: EVALUATION OF A COMMUNITY-BASED POPULATION

Z. ORMIANER¹, A.L. SOLODUKHIN¹, D. LAURITANO², P. SEGAL¹, D. LAVI¹,
F. CARINCI³ AND J. BLOCK¹

¹*Department of Oral Rehabilitation, The Maurice and Gabriela Goldschleger School of Dental Medicine, Tel Aviv University, Tel Aviv, Israel;* ²*Department of Medicine and Surgery, University of Milan-Bicocca, Monza, Italy;* ³*Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, Italy*

The final outcome of dental treatment needs to be not only clinically sufficient, but also esthetically pleasing. Bilateral symmetry in the maxillary incisor teeth is of significant importance in esthetic dentistry. In restorative dentistry, symmetry refers to the appearance of balance around the dental midline. The purpose of this study was to measure and compare the maxillary incisor teeth dimensions from both sides of the dental midline, in order to assess if this symmetry occurs naturally. From the student community population at Tel Aviv University, 66 students between the ages of 20-35 (35 males, 31 females) were enrolled and gave consent. The inclusion criteria for this study were: upper maxillary incisors that have never undergone restorative or rehabilitative treatment, and no history of orthodontic treatment. Standardized digital photographs were taken, and the length and width of the maxillary central and lateral incisors were measured and proportions were calculated. SPSS was used to compare the measured differences between teeth on the left versus right of the midline. Tooth proportions were not significantly different between the left and right sides. Asymmetry was found only between the lengths of the maxillary lateral incisors ($p=0.009$); the width for these teeth was symmetrical. A significant statistical difference was not found on most parameters when evaluating symmetry of the upper incisors. Therefore, when treating the esthetically important anterior of the mouth, care must be taken to ensure bilateral symmetry to mirror the natural symmetry found in most patients.

Esthetics is one of the primary reasons patients turn to restorative dentistry. The final outcome of dental treatment needs to be not only clinically sufficient, but also esthetically pleasing. Esthetics in natural settings has been traditionally defined using four main parameters: frame and reference, proportion and idealism, perspective and illusion, and symmetry (1).

Symmetry is the first parameter detectable to an observer. Symmetry infers a correspondence in size, shape, and relative position of parts on opposite sides of a dividing line or median plane or about a center

or axis (2-10). In the context of restorative dentistry, symmetry refers to the appearance of balance around the dental midline (the line between the upper central incisors).

Past research on dental symmetry has indicated that a smile considered esthetic was one with symmetrical teeth adjacent to the dental midline, and with less symmetry of teeth that were further from the midline (11). Mavroskoufis and Ritchie found that a small amount of asymmetry of the upper central incisors occurs and is esthetically pleasing, as long as the deviation is between 0.3-0.4 mm in the

Mailing address:

Prof. Francesco Carinci, MD
Department of Morphology, Surgery and Experimental Medicine,
University of Ferrara, Via Luigi Borsari 46, 44100 Ferrara, Italy
Tel.: +39 0532 455582 - Fax: +39 0532 455582
e-mail: crc@unife.it - web: www.carinci.org

mesio-distal plane of the tooth (12). This assumption has been further verified in the literature (13, 14). In other studies, artificial changes in proportion and symmetry were made to the teeth in order to enhance esthetic effects, irrespective to the natural condition of the teeth (15, 16).

In Bishara's review of symmetry (11), he indicates that perfect symmetry of the body is a theoretical concept and is actually very rare in nature. However, he notes that teeth are more symmetrical respective to other organs, possibly as physiologic compensation for the asymmetric jawbones (15, 16).

One of the accepted contemporary tools for assessing esthetics is with the use of standard facial photography (17, 18). The photographs may be full-faced or lower-faced and may include use of a lip retractor (a tool used to retract the lips and cheeks in order to better view the teeth and mouth). In past research, photographs have been given to groups of independent reviewers for evaluation of esthetics and other clinical features (15, 19) to ensure a more reliable assessment of these factors (15, 16, 20, 21).

Analysis of natural dental symmetry has not been well-studied, and has not been quantified in a representative sample of the population. The purpose of this study was to assess the prevalence of natural dental symmetry in a sample from the general population, and to compare the dimensions of the upper incisors on either side of the midline. We hypothesize that, like other organs in the body, there will be a lack of symmetry in the maxillary teeth in the esthetic portion of the mouth.

MATERIALS AND METHODS

After receiving approval from the ethics committee of Tel Aviv University, 66 students from Tel Aviv and Bar Ilan University were recruited and enrolled for the study. Each subject signed an informed consent document, and photographs were taken of each subject's face. Patients with teeth that had anterior restorations or crowns were not included, or if they had undergone orthodontic treatment or had extreme (unnatural) asymmetry.

Standardized digital photographs were taken using a Canon 450D SLR (Single Lens Reflex) camera, with a standard lens (18-55mm) and a tripod. The photographs

included all of the subject's face, so that the four upper incisors could be fully seen, but the eyes were hidden to maintain privacy. The photographs were taken with the dentition fully closed (inter-contact position) and with a lip retractor.

Photography was carried out as follows: the patient sat in a chair with his/her back adjacent to the backrest. The camera was placed on a tripod adjacent to the edge of the chair. The camera was raised so that the lens was perpendicular to the upper central incisors, and the center of the lens was between the ends of the incisal edges. The lip retractor was pulled to the side and forward (towards the camera). Photographs had to yield clear images from which precise measurements could be taken, with no blurring, dirt or any other blemishes. The photographs did not undergo any digital manipulations.

Using computer software with image processing features, the images were enlarged, zoomed, and cropped to create a fold-to-fold image. On the fold-to-fold photographs, mesio-distal and occlusal-gingival measurements were taken of the tooth crown on the central and lateral upper incisors (Fig. 1). Measurements of length and width ratios between the maxillary central and lateral incisors were obtained. The cropping and measurements were done using Microsoft Word 2016. SPSS was used to compare the measured differences between teeth on the left versus right of the midline.

In order to ensure accurate results, four independent reviewers completed the measurements and calculations. The average of those four measurements for each sample were taken and an ANOVA test with repeated measures was performed to check the reliability between the measurements. Ratios between the right and left teeth of each subject were compared to a value of 1, assuming perfect symmetry would produce a ratio of exactly 1. Comparisons of tooth symmetry were evaluated using a two-way paired T-test.

RESULTS

Assessment of the reliability of results between the four reviewers showed a high correlation (0.9) between the reviewers' results (Table I). After analyzing the photographs of the 66 subjects, no statistical difference in symmetry was found between most of the measurements (Table II).



Fig. 1. *Example of Photographic Processing and Measurement Technique.*

Only the length of the maxillary lateral incisors was statistically different, with a difference of 0.02288 (2.29%) and significance of 0.009 (Table III, Fig. 2). Further looking at differences by sex, no statistically significant difference was found between men and women regarding asymmetry of the central and lateral incisors ($p=0.50$, data not shown).

DISCUSSION

Teeth have a significant role in the esthetic appearance of the mouth and face, and one of the critical parameters of esthetics is symmetry. Our analysis showed that there is symmetry between the anterior maxillary teeth on the right side and left sides in most cases. This result is not necessarily consistent with what is known in the literature today. The human body, and the cranium region in particular, is not symmetrical (11). However, other studies (22, 23) have shown that dento-alveolar structures, including teeth, are relatively symmetrical.

We did find that there is significant peripheral asymmetry in the length between the lateral incisors. This is consistent with the fact that as you move away from the midline, incidence of asymmetry increases (1). However, further research is warranted to more fully explore this phenomenon.

Our research also found that no statistically significant difference was found between men and women regarding the lack of symmetry in the anterior maxillary region. This conclusion is consistent with other studies, which also found no significant difference between men and women within the same ethnic group (24,25).

Table I. *Correlation between the Examiners' Measurements.*

Paired Samples Correlations				
Significance	Correlation	=N		
000.	987.	66	Length of Central Incisors	Pair 1
000.	976.	66	Length of Lateral Incisors	Pair 2
000.	986.	66	Width of Central Incisors	Pair 3
000.	929.	66	Width of Lateral Incisors	Pair 4

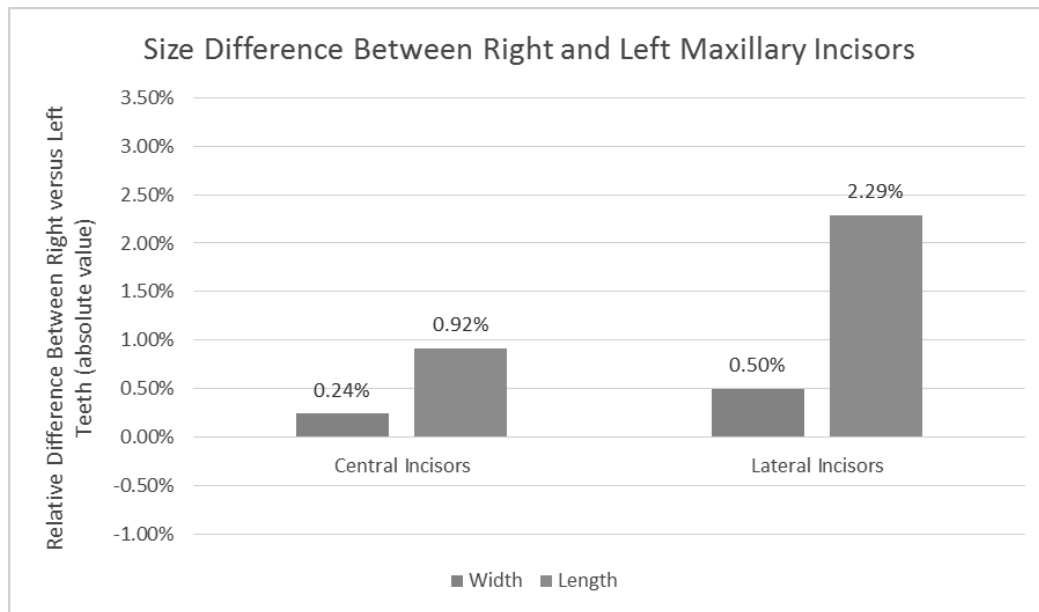


Fig. 2. Relative Difference in Symmetry Between Left and Right Maxillary Incisors. This figure shows the relative difference when comparing the right versus left incisors. Only the length of the lateral incisors showed asymmetry between the right and left sides.

Table II. Summary of ANOVA Results Comparing Examiner's Measurements.

ANOVA Results	Paired Samples Statistics						
Significance	F	Standard Error of Mean	Standard Deviation	Mean	N		
464.	858.	04739.	38499.	1.3459	66	Length of Right Central Incisor	Pair 1
107.	2.060	04714.	38300.	1.3367	66	Length of Left Central Incisor	
192.	1.595	03566.	28971.	1.1033	66	Length of Right Lateral Incisor	Pair 2
636.	570.	03824.	31065.	1.1261	66	Length of Left Lateral Incisor	
393.	1.002	03508.	28501.	1.1116	66	Width of Right Central Incisor	Pair 3
748.	407.	03541.	28767.	1.1139	66	Width of Left Central Incisor	
697.	479.	02369.	19247.	7277.	66	Width of Right Lateral Incisor	Pair 4
540.	722.	02570.	20876.	7327.	66	Width of Left Lateral Incisor	

Table III. *T-test to Evaluate Differences in Left versus Right Maxillary Incisors.*

Test Paired Samples									
		Paired Differences					t	df	Significance
					95% Confidence Interval of the Difference				
		Mean	Standard Deviation	Std Error of Mean	Lower	Upper			
Pair 1	Length of right central incisor – Length of left central incisor	.00917	.06090	.00750	-.00580	.02414	1.223	65	.226
Pair 2	Length of right lateral incisor – Length of left lateral incisor	-.02288	.06857	.00844	-.03974	-.00602	-2.710	65	.009
Pair 3	Width of right central incisor – width of left central incisor	-.00239	.04800	.00591	-.01419	.00941	-.404	65	.688
Pair 4	Width of right lateral incisor – width of left lateral incisor	-.00499	.07743	.00953	-.02402	.01405	-.523	65	.603

Limitations

This work had several limitations. The first limitation was the method of photography. Although our photographic method was acceptable and has been used in similar works (21, 26-31), the suggested method is to use a 90-105 mm lens or a macro lens, as these lenses capture natural anatomy more easily (18). We used a standard lens and to compensate for any potential lack of precision, we were diligent in ensuring a precise camera position in order to avoid distortion.

An additional limitation and potential confounder to the research is related to the morphology of the maxillary dental arch. Not everyone has the same shape of arch, and different types of occlusions have different curvatures (30, 31). This affects the position and angle of the teeth, and how they look and are measured through the lens of the camera.

It has been recommended that template molds can be another way to assess symmetry, in addition to using facial photography (27-29). In this study, we chose to perform computerized clinical measurements using photography alone, as computerized measurements can yield more accurate results than what can be obtained than

with other measurements, particularly since the measurement interval of interest is less than 1 mm (20, 21).

CONCLUSIONS

Our research evaluated dental symmetry in a population of 66 research subjects and confirmed that there is a natural symmetry between the maxillary central incisors. This is an important finding for restorative dentistry professionals, as restorations should be symmetrical for this area to reflect natural dentition and natural dental anatomy.

Additionally, we found that there is a natural asymmetry with lateral incisors in the dimension of length, likely due to their position further away from the midline of the dental arch. This finding is also of value to dental professionals who create restorations for this area, and wish to mirror natural anatomy.

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THE EFFECT OF ENRICHING DENTURE ADHESIVES WITH CHLORHEXIDINE DIACETATE ON THE PROLIFERATION OF *CANDIDA ALBICANS*: AN *IN VITRO* ANALYSIS

S. MATALON¹, G. FRYDMAN², D. LAURITANO³, H. SLUTZKY², E. SHLOMO²,
S. LEVARTOVSKY², F. CARINCI⁴ and Z. ORMIANER¹

¹Senior Lecturer, Dept. of Oral Rehabilitation, The Maurice and Gabriela Goldschleger School of Dental Medicine, Tel Aviv University, Tel Aviv, Israel; ²Instructor, Dept. of Oral Rehabilitation, The Maurice and Gabriela Goldschleger School of Dental Medicine, Tel Aviv University, Tel Aviv, Israel;

³Department of Medicine and Surgery, University of Milan-Bicocca, Monza, Italy; ⁴Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, Italy

Denture adhesives are widely used in dentistry. The growth of *Candida albicans* on the denture base is common on both jaws, particularly in elderly patients. A strong correlation has been found between the growth of *Candida albicans* and dentures, potentially leading to denture stomatitis. Recent studies show that the addition of chlorhexidine diacetate (CHDA) to denture adhesives can have a protective effect against denture stomatitis. The aim of this research is to examine the antifungal properties of three denture adhesives when CHDA is added. We hypothesize that the addition of CHDA to denture adhesives will result in antifungal activity against *Candida albicans*. Three denture adhesive materials (Corega, Protefix and Kukident) were prepared with and without the addition of CHDA in order to measure whether CHDA increased the resistance of the adhesives to the growth of *Candida albicans*. Dynamic and static intercellular changes were assessed through direct contact test (DCT) and confocal microscopy. The tested materials were evaluated immediately after setting and at 14 days post-setting. When combined with CHDA, all three denture adhesive materials decreased *Candida albicans* growth compared to the control ($p < 0.0001$). This inhibition of growth was more prominent in the first 24 h. The confocal microscopy results supported the DCT findings. The addition of CHDA to denture adhesive pastes inhibits growth of *Candida albicans*, and may potentially reduce the incidence of denture stomatitis. Further studies are warranted.

Denture adhesives are widely used in dentistry. Adhesive materials improve the retention of dentures by liquid absorption, filling the gaps between the denture and the soft tissue and changing the coefficient of the liquid surface tension between the denture and the tissue (1). These materials improve the patient's chewing quality, their comfort and self-esteem (2). It has been estimated that 30% of patients are using or have used adhesive materials for dentures

(3). Denture stomatitis occurs in 11-67% of patients with dentures (4-6), mostly in elderly patients and under the maxillary dentures (4, 7, 8). The strong correlation between *Candida albicans* growth and dentures has been well-described in literature (8-11).

Adhesive materials often contain antibacterial components such as hexachlorophene, sodium borate, sodium tetraborate and ethanol (12), though

Key words: denture stomatitis, denture adhesives, Candida albicans, chlorhexidine diacetate

Mailing address:

Prof. Francesco Carinci, MD,
Department of Morphology, Surgery and Experimental Medicine,
University of Ferrara, Via Luigi Borsari 46, 44100 Ferrara, Italy
Tel.: +39.0532.455582 - Fax: +39.0532.455582
e-mail: crc@unife.it - web: www.carinci.org

0393-974X (2016)

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these agents have limited defense against *Candida albicans* (13) even if they are effective against bacteria (14-22). Gates et al. (23) demonstrated bacterial growth on denture adhesive materials after a two week incubation, with fungal domination. Ekstrand et al. (24) examined 19 adhesive materials for toxicity and fungal growth, and found fungal growth on most materials. Scher (25) studied the addition of antibacterial and antifungal materials to denture adhesives in order to treat denture stomatitis, but had inconclusive results.

Recent studies have shown that the addition of chlorhexidine diacetate (CHDA), even in low concentrations, has a positive effect against denture stomatitis. This effect might be due to reduction in phospholipase production by *Candida albicans* (26, 27).

The aim of this study is to examine the antifungal properties of three denture adhesives when CHDA is added and to assess the dynamic and static intercellular changes through direct contact test (DCT) and confocal microscopy. We hypothesized that the addition of CHDA to denture adhesives will result in antifungal activity against *Candida albicans*.

MATERIALS AND METHODS

Test materials

Three denture adhesive materials were tested in this analysis: Protefix (Queisser Pharma), Kukident (Procter & Gamble) and Corega (Glaxo SmithKline Pharmaceuticals). The adhesives were handled in strict compliance with the manufacturers' instructions, with the addition of 7.7% (weight) powdered CHDA (28). *Candida albicans* 58919 were grown from frozen stock in Brain Heart Infusion (BHI) (Difco Laboratories).

Experimental design

The test modified protocol called for 78 of 96 well microtiter plate to consist of experimental wells, control wells and calibration wells, arranged as follows: 1): 3 sets of 8 wells served as experimental wells, containing BHI plus the tested adhesive, the tested organism and CHDA; 2): 3 sets of 8 wells containing BHI plus the tested adhesive and the tested organism; 3): 3 sets of 8 wells served as

calibration wells. An additional set of 3 wells served as the positive control, i.e. identical microbial inoculum was placed on the sidewall of uncoated wells and processed as the experimental wells. The negative control consisted of a set of three wells coated with the tested materials (as in the experimental wells) with the addition of growth medium only.

The experiment was set up as follows: a 96-well flat bottom microtiter plate was held vertically, i.e. the surface of the plate wall was perpendicular to the floor and the side walls of 24 wells were coated evenly with an equal amount of the tested adhesives (surface area of 21mm²), which were prepared in accordance with manufacturers' instructions. Another 24 wells were coated with the tested adhesives mixed with 7.7% weight CHDA. Special care was taken to avoid the material flow to the bottom of the well, which would interfere with the light path through the micro plate well and result in false readings. 30_{μl} of *Candida albicans* suspension in BHI was placed on the tested materials while the plate remained in the vertical position. The plate was incubated for 45 min at 37°C until the suspension evaporated, thus ensuring direct contact between the tested microorganism and the surface of the denture adhesive materials. The plate was then positioned horizontally and 220_{μl} of BHI were added to each well including the negative control wells. The positive control wells were filled with 275_{μl} BHI. A calibration test was performed for each plate to obtain a comparative qualitative scale. From the three positive control wells, 55_{μl} were taken after 2 min of gentle vortex agitation and transferred to an adjacent set of wells that contained 220_{μl} of fresh BHI. This five-fold dilution was repeated 6 consecutive times.

The calibration curves served as a scale to measure the relative number of *Candia albicans* as described. Based on these calibration curves we were able to calculate 2 parameters which were indicative of the linear material-fungal interactions: (i) The slope of the linear portion of the growth curve expressed changes in fungal growth rate, and (ii) The distance of the ascending portion of the growth rate from the Y-axis correlates with the number of viable microorganisms at time zero. The growth curves for each well were calculated and a regression line was performed on the linear segment of each curve. Each experimental microtiter plate processed throughout the study contained all its controls as well as a calibration set of wells. Similar

experiments were carried out where the tested adhesives were allowed to age for one and 14 days, respectively. Aging was performed by adding phosphonate buffered saline which was replaced every 24 h.

The quantitative direct contact test (DCT) was used to evaluate the antimicrobial properties. The DCT is based on turbidometric determination of microbial growth in a 96 well-microtiter plate (96 well flat bottom nunclon, Nunc), while the evaluated material is in direct contact with tested micro-organisms in suspension. The DCT and its advantages have been described by Weiss et al, and include reproducibility of results and its independence of the solubility and molecular size of the tested materials (29).

The kinetics of the outgrowth in each well was recorded by measuring the optical density at 650_{nm} every 30 min for a period of 24 h using a temperature-controlled spectrophotometer (Versamax, molecular devices corporation) set at 37°C.

Confocal laser microscopy was used to observe microorganism activity and to monitor static and dynamic intra and extra-cellular changes (30-32).

Statistical analysis

The values of the negative control wells were considered as baseline and were subtracted from the respective experimental data and plotted as growth curves. The curves for each well were analyzed and a regression

line was calculated on the linear ascending segment of the curve, expressed by the equation $y=ax+b$. Two-way ANOVA and Tukey multiple comparisons were applied to the slope of the linear equation.

Ethical Requirements

This study was deemed exempt by the TAU Ethics Committee.

RESULTS

The combined interaction of material, microorganism and time was assessed by two-way ANOVA and found to be statistically significant ($p<0.0001$).

There was a significant difference between the tested adhesives after 24 h (Fig. 1). All tested materials combined with CHDA decreased *Candida albicans* growth compared to the control. Among the denture adhesives without CHDA added, Protefix showed similar growth as the control, while Kukident and Corega enhanced microbial growth.

There was a significant difference between all the tested materials after 14 days of aging (Fig. 2). All CHDA-containing denture adhesives decreased *Candida albicans* growth compared to the control groups. This decrease was greater after 24 h, yet the adhesives without addition of CHDA increased

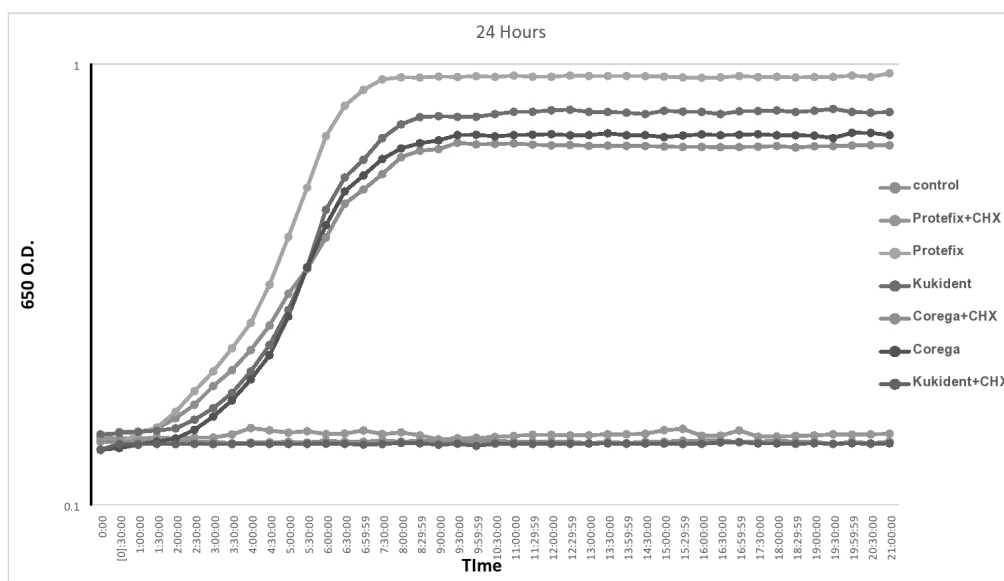


Fig 1. Growth of *Candida albicans* Among the Tested Materials at 24 h. All tested materials combined with Chlorhexidine diacetate decreased *Candida albicans* growth compared to the control ($p<0.0001$).

Candida albicans growth similarly to the control group (Table I).

Confocal microscopy results

The confocal microscopy results supported the Direct Contact Test findings (Fig. 3, 4). Protefix, Kukident and Corega with CHDA demonstrated inhibition of *Candida* growth. Correspondingly, *Candida* growth was observed on the adhesives that did not have the addition of CHDA.

DISCUSSION

The purpose of this study was to test the antifungal properties of three types of denture adhesives with and without the addition of CHDA. Dynamic and static intercellular changes were assessed through direct contact test (DCT) and confocal microscopy. Denture adhesive materials (Corega, Protefix and Kukident) were prepared with and without the addition of CHDA in order to test whether CHDA inhibited the growth of *Candida albicans*.

The results were consistent with the study hypothesis that assumed that the addition of CHDA to denture adhesives inhibits fungal growth, but as expected, this inhibition decreased with time. After 24 h, the

denture adhesives mixed with CHDA constricted the *Candida* growth compared to the control group. Protefix without CHDA showed the same growth as control, while Corega and Kukident were found to enhance fungal growth.

Two weeks following the start of the experiment, the denture adhesives mixed with CHDA still had a detaining effect on *Candida* growth, but to a lesser degree compared with the 24-h mark. It is possible that the materials decompose and dissolves and therefore, the effect of CHDA decreases. Alternatively, the denture adhesives without added CHDA were found to increase fungal growth similarly to the control group, but again, to a lesser degree compared with the 24-h mark. It is also possible that the increase in the adhesives solubility with time causes decomposition of the organic component of adhesives and therefore, the *Candida* growth rate decreases. Analysis of confocal microscopy shows that in the presence of CHDA, the number of non-vital cells (red) is greater compared to vital cells (green). In contrast, the number of vital cells in the samples without CHDA is greater compared to the same material with the addition of CHDA.

The results of this study show that fungal growth on denture adhesive materials is consistent with

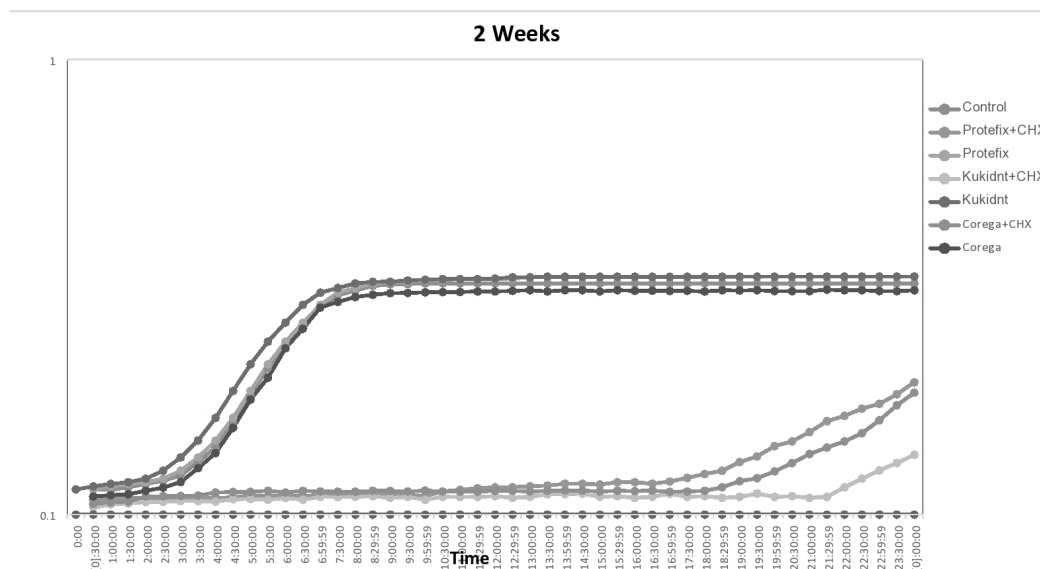


Fig 2. Growth of *Candida albicans* Among the Tested Materials at 14 days. There was a significant difference between all the tested materials after 14 days of aging. This decrease was greater after 24 h.

Table I: *Candida albicans* growth rate after 24 h and 14 days

Time Material	24 hours	14 Days
Control	0.0354±0.017★	0.0082±0.0002«
Protafix + Chlorhexidine	0.0011±0.001◆	0.0038±0.002t
Protafix	0.0396±0.024★	0.033±0.0039v
Kukident + Chlorhexidine	0.0008±0.0002◆	0.0004±0.00009t
Kukident	0.0619±0.0049❖	0.026±0.007v
Corega + Chlorhexidine	0.00006±0.00003◆	0.0025±0.0014t
Corega	0.0754±0.01❖	0.025±0.0059v
Significance	P<0.0001	P<0.0001

Candida albicans growth rate as described by the gradient in the linear part of growth curve. Each value represents the average of optical density ± standard deviation of fungal growth curve in eight different wells. Similar symbols at the same columns indicates values with no significant difference.

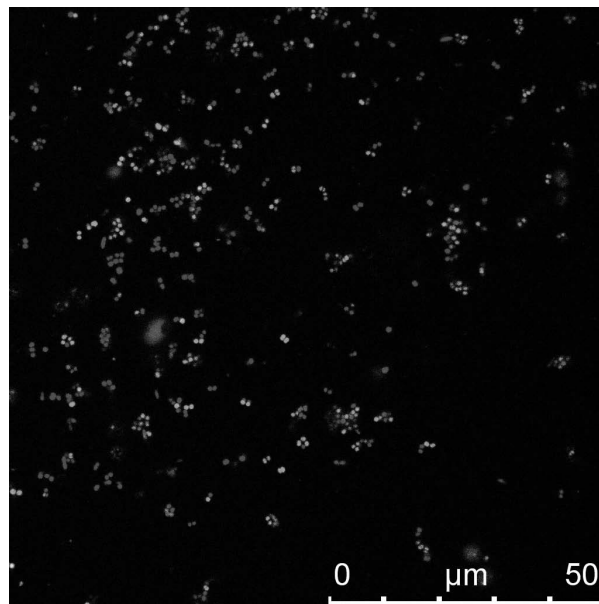


Fig 3. A candidal growth was observed on those materials without the addition of CHDA. Red dots mean dead candida; Green dots mean live candida.

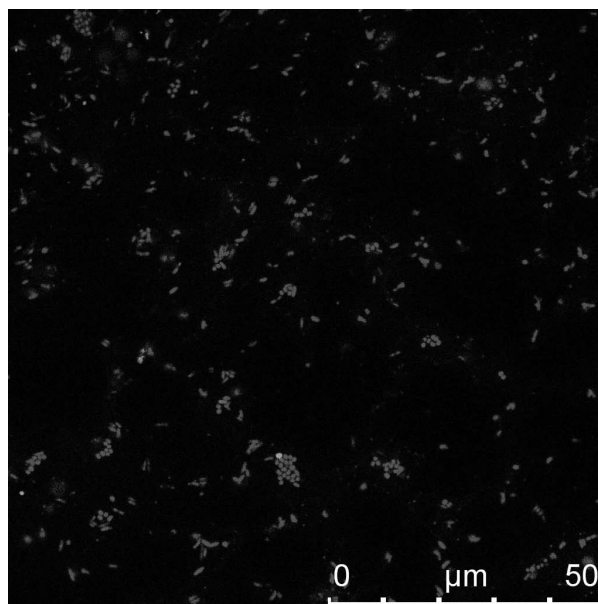


Fig 4. *Protefix with CHDA demonstrated inhibition of Candidal growth. Red dots mean dead candida; Green dots mean live candida.*

previous studies, which demonstrated that fungal growth was 2-fold greater when compared to other microorganisms (23, 24). Chlorhexidine has been found to be a growth inhibitor of several species of *Candida*, including *Candida albicans*, hence there are recommendations for mouthwash containing 0.12% chlorhexidine diglucanate to treat oral candida (33, 34). Chlorhexidine has a fungicidal effect as well as an inhibitory effect on the ability of *Candida* to adhere to epithelial cells (35, 36) which may be useful in the prevention of denture stomatitis.

CONCLUSIONS

This study shows that addition of 7.7% weight powdered CHDA to denture adhesive pastes may inhibit growth of *Candida albicans*. Use of CHDA combined with denture adhesive pastes to prevent or mitigate oral denture stomatitis warrants further study.

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TEMPERATURE CHANGES OF ONE-PIECE IMPLANTS DURING THE SETTING OF ACRYLIC RESIN TEMPORARY CROWN. THE EFFECT OF IMPLANT DIAMETER. AN *IN VITRO* STUDY

S. SLUTZKEY¹, O. COHEN¹, D. LAURITANO², O. MOSES¹, Z. ORMIANER³, H. TAL¹, R. KOLERMAN¹, F. CARINCI⁴ and S. MATALON³

¹*Department of Periodontology and Oral Implantology, School of Dental Medicine, Tel-Aviv University, Tel-Aviv, Israel;* ²*Department of Medicine and Surgery, University of Milan-Bicocca, Monza, Italy;* ³*Department of Oral Rehabilitation, School of Dental Medicine, Tel-Aviv University, Tel-Aviv, Israel;* ⁴*Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, Italy*

The first 2 authors contributed equally to this work.

The purpose of this work is to evaluate changes in temperature of one-piece titanium implant surface during the setting of acrylic resin temporary crowns and to correlate thermal changes to implant diameter. Thirty-three one-piece implants (ARRP, Alpha-Biotec) were divided into 3 groups according to diameter size (G1=3 mm, G2=3.3 mm, G3=3.6 mm). Implants were mounted on an acrylic glass apparatus. Thermocouples were positioned at the most coronal thread. Lower incisor temporary polycarbonate crowns were filled with 80 µL of self-curing acrylic resin and positioned immediately on the implant abutment. Thermal changes of the implant surface were recorded continuously for 10 min. Data were statistically analyzed using one-way analysis of variance. The mean initial temperature (C0) of groups G1, G2 and G3 was similar (24.79±0.78°C, 25.26±0.63°C, 24.97±1.06°C, respectively). The setting of the acrylic resin temporary crown resulted in a significant increase in the implant surface temperature of all groups. The mean thermal amplitude (ΔC) for groups G1, G2 and G3 were 6.79±1.02°C, 6.61±0.94°C, 6.65±1.26°C, respectively. The mean time to maximum temperature (Tmax) for groups G1, G2 and G3 were 337.38±42.91 sec, 324.69±41.46 sec and 317.98±37.91 sec respectively (P>0.05). Direct application of auto-polymerizing resin to the titanium abutment of one-piece implants significantly increased the cervical implant surface temperature. Implant diameter did not influence the temperature changes.

One-piece implants have been designed to minimize marginal bone resorption by eliminating the submucosal microgap (1-11). For immediate function of one-piece implants with interim prosthesis, an insertion torque of 35 to 45 N-cm is recommended (12, 13). Unlike two-piece implants, one-piece implants lack complications associated with microleakage, micro-movement and screw-loosening (14). Furthermore, the structure and

strength of one-piece implants enable the safe use of relatively small diameters. One-piece implants are entirely suitable for the aesthetic zone, allowing sufficient distance from adjacent teeth and preservation of the interproximal bone and the gingival papillae (15, 16).

Several studies have reported favorable results with one-piece implants and an immediate loading treatment approach in terms of implant stability,

Key words: implants, temperature, provisional, restoration

Mailing address:

Prof. Francesco Carinci, MD
Department of Morphology, Surgery and Experimental Medicine,
University of Ferrara, Via Luigi Borsari 46, 44100 Ferrara, Italy
Tel.: +39.0532.455582 - Fax: +39.0532.455582
e-mail: crc@unife.it - web: www.carinci.org

overall survival rate and bone changes over time (17-27). However evidence suggests that one-piece implants have low success rates, with cumulative survival rates of 89.3% (average 1-year follow-up for 550 implants), 94.8% (2-year follow up for 115 implants) and 94.9% (average 10.2 month follow up for 117 implants reported in 3 studies (19, 20).

One limitation of one-piece implants is that they must be prepared *in situ* to achieve proper emergence profile and angulation and be restored with a crown or partial denture (20). This intraoral procedure, which is performed immediately after implant placement, might compromise osseointegration via heat production and by impairing initial stability (28, 29).

Primary implant stability is a prerequisite for achieving osseointegration and is related to the implants micromotion (30). Animal studies indicated that micromotion above a threshold of 50-100 μm result in fibrous union instead of osseointegration (31, 32). Primary stability depends on several factors; these include bone density and dimension, the implant geometry and the surgical technique. Primary stability following abutment preparation of one-piece dental implant was previously investigated by our group with a special "external fixation device" (7). Implants inserted with insertion torque of 15 N-cm lost primary stability after abutment preparation even though they had ISQ value of 57.3 (70.5 adjusted) while implant inserted at a torque of 30 N-cm did not lose primary stability during abutment preparation. It was concluded that insertion torque seems to be a more important factor than ISQ value in predicting primary stability of one-piece implant system (7).

Heat production during provisionalization of one-piece implants stem from the implant abutment preparation and during the setting of the interim restoration material. It has been shown that osteoblast cultures exposed to heat shock at 42°C, induced activation of apoptosis mechanisms (33). Serial studies of intravital microscopy of heated bone have shown that rabbit tibiae heated to 50°C for 1 min and to 47°C for 5 min showed up to 40% bone resorption and replacement by fat cells at the heated site after 40 days (33-35). Furthermore, even the heating of

bone to 47°C for 1 min resulted in fat cell injury and inconsistent bone injury.

Recently it was reported that abutment preparation of one-piece implant with turbine water spray irrigation of 15 ml/min and 30 ml/min were associated with mean increase in crestal bone temperature of $8 \pm 1.5^\circ\text{C}$ and $4 \pm 1.1^\circ\text{C}$ respectively; while abutment preparation without water irrigation resulted in extensive heat generation exceeding 56°C in few seconds (36). Similar results were reported by Gronkiewicz et al who found that critical temperature threshold of 47°C was always surpassed during abutment preparation of one-piece implants when only air coolant was used (37).

Limited number of studies investigated heat production during provisional restoration of implant abutment, and in those studies, the temperature rise was observed on two-piece implant systems (38, 39). In one study, the implants were embedded in an acrylic resin model of a human mandible and the mean temperature rise reported was 4°C to 5°C at the cervical region (38). In a second study, the implants were embedded in a sheep iliac bone in a 37°C water bath and the mean temperature rise found was only 0.5°C to 1.04°C, depending on the type of acrylic resin (39). To the best of our knowledge, heat production during provisional restoration of one-piece implants has never been studied.

The aim of this study is to evaluate thermal changes of one-piece implant neck during the setting of acrylic resin temporary crown and to investigate the effect of implant diameter.

MATERIALS AND METHODS

Experimental design

Thirty-three implants (ARRP, Alpha-Biotec) were equally divided into 3 groups according to implant diameter: G1 (3 mm implant), G2 (3.3 mm implant) and G3 (3.6 mm implant). The supracrestal abutment of all 3 groups of implants had the same dimensions: a width of 3.25 mm at bone level, a width of 2.5 mm at the coronal end, and a length of 9.5 mm.

Temperature measurement

The implants were mounted and fixed into machined

slots in a custom-made acrylic glass apparatus (Fig. 1). K-type copper-constantan (Pico Technology, St Neots, Cambridgeshire, UK) 30-gauge wire thermocouples were positioned at the most coronal thread. The thermocouples were tightened against the implant threads with plastic screws (Fig. 1). Thermal changes on the implant surface were recorded continuously at 100-ms sampling intervals using a TC-08 thermocouple data logger (Pico Technology, St Neots, Cambridgeshire, UK) and data acquisition software (Picolog, Pico Technology, St Neots, Cambridgeshire, UK). All measurements were recorded at room temperature.

Provisional crown preparation

Lower incisor temporary polycarbonate crowns (#62, 3MTMESPETM) were filled with 80 μ L of self-curing acrylic resin (UNIFASTTM Trad, GC). The acrylic resin was mixed thoroughly for 10-15 seconds in a powder/liquid ratio of 0.4 gr/400 μ L. The crowns were filled after the acrylic resin reached a dough-state (after approximately 20-30 sec, according to manufacturer instructions), and were then put on the implant abutment while ensuring that no resin flowed on the implant surface (Fig. 2). The acrylic resin was left to set on the implant abutment for 10 min.

Data processing and statistical analysis

Initial (C0) and maximum (Cmax) temperature measurements were extracted for the 3 groups (Fig. 3). The time elapsed until Cmax was reached (Tmax) was measured. The thermal amplitude ($\Delta C = C_{\text{max}} - C_0$) was calculated, and temperature differences (C0 and Cmax), time differences (Tmax) and amplitude differences (ΔC) were analyzed for the 3 groups using the one way analysis of variance at a 95% confidence level (IBM SPSS Statistics 22.0, Armonk, NY, USA).

RESULTS

The mean initial temperature (C0) for groups G1, G2 and G3 was $24.79 \pm 0.78^\circ\text{C}$, $25.26 \pm 0.63^\circ\text{C}$ and $24.97 \pm 1.06^\circ\text{C}$, respectively (Table I), with no significant difference between the groups ($P = .420$). During the setting of the acrylic resin temporary crown there was a significant increase from baseline temperature (C0) in all groups ($P < 0.05$) (Fig. 4). Mean maximal temperature (Cmax) for groups G1, G2 and G3 was $31.58 \pm 1.39^\circ\text{C}$, $31.88 \pm 1.25^\circ\text{C}$ and $31.63 \pm 1.93^\circ\text{C}$, respectively (Table I),

with no significant difference between them ($P = .893$). The mean amplitude of thermal changes (ΔC) for groups G1, G2 and G3 was $6.79 \pm 1.02^\circ\text{C}$, $6.61 \pm 0.94^\circ\text{C}$ and $6.65 \pm 1.26^\circ\text{C}$, respectively. The mean Tmax (time to maximum temperature) for groups G1, G2 and G3 was 337.38 ± 42.91 sec, 324.69 ± 41.46 sec and 317.98 ± 37.91 sec, respectively (Table I), with no significant difference between them ($P = .534$).

DISCUSSION

The results of the present study demonstrated that direct application of a polycarbonate crown filled with self-polymerizing resin on a one-piece implant abutment resulted in pronounced heating of the cervical implant area, with a mean temperature increase of 6.6°C .

In a study by Li et al. (33), a heat shock of 42°C induced activation of apoptosis mechanisms in osteoblast cultures, while heat shock of 48°C resulted in irreversible damage. Taking into consideration a baseline temperature of 37°C , a change of 6.6°C found in our study may induce *in vivo* damage to the surrounding bone resulting in impaired osseointegration.

Our results are comparable with those of Ormianer et al (38) who used an intermediate volume of acrylic resin and reported a mean temperature rise of 4°C to 5°C at the cervical region. However Kazemi et al (39), who used implants embedded in fresh sheep iliac bone in a 37°C water bath, found a mean temperature rise of only 0.5°C to 1.04°C , depending on the type of acrylic resin. These differences in mean temperature rise between the studies may be explained by the location of the thermocouple that was used to measure the temperature in the cervical area of the implant.

Preparation Results from different studies suggest that implants have a limited heat conduction capacity and that temperature conduction decreases markedly from the abutment apically along the implant (38-40). In the current study, the thermocouple was placed approximately 0.5 mm apical to the abutment-implant junction, compared with 2 mm apically described by Kazemi et al (39). Another explanation for the differences between the studies may be due

Table I. *Temperatures and time frames of heat production during acrylic resin setting.*

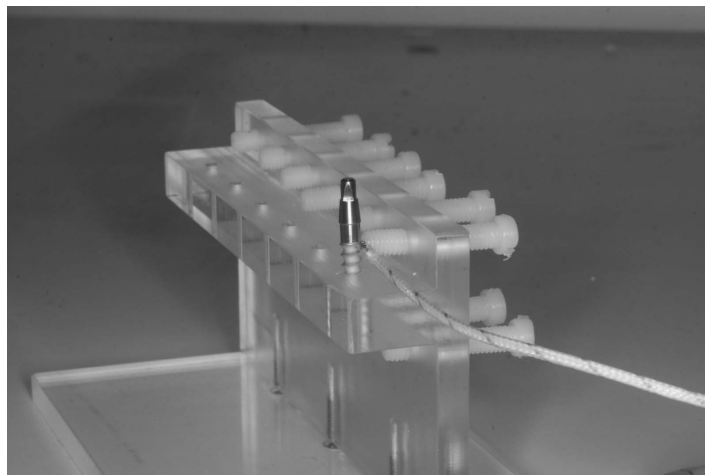
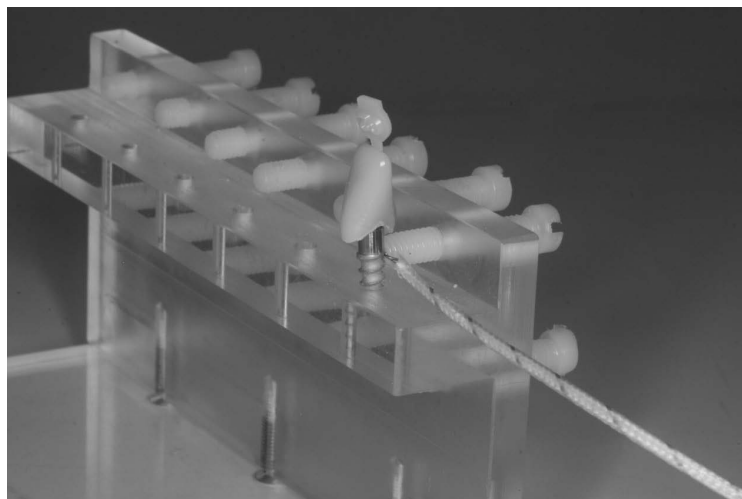
Implant Group	C0 (°C)	Cmax (°C)	ΔC (°C)	Tmax (sec)
(n= 11 each)				
G1 ^a	24.79±0.78	31.58±1.39	6.79±1.02	337.38±42.91
G2 ^b	25.26±0.63	31.88±1.25	6.61±0.94	324.69±41.46
G3 ^c	24.97±1.06	31.63±1.93	6.65±1.26	317.98±37.91

Values are presented as mean±standard deviation.

C0: Initial temperature measurement; **Cmax-** maximum temperature measurement;

ΔC: Thermal amplitude; **Tmax:** - time elapsed until Cmax; **sec:** seconds.

^a:3 mm diameter; ^b:3.3 mm diameter; ^c:3.6 mm diameter.

**Fig. 1.** *Mounting and fixing of an implant into machined slots in a custom-made acrylic glass apparatus.***Fig. 2.** *Placement of the filled crown on the implant abutment while ensuring that no resin flows on the implant surface.*

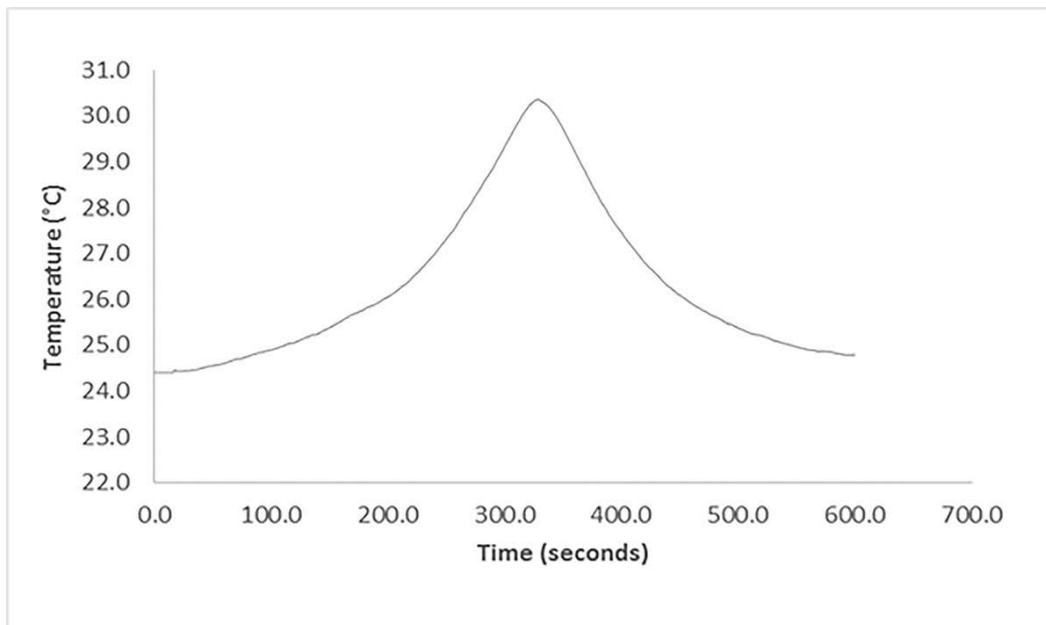


Fig. 3. An example of recorded temperature over time. The thermal amplitude (ΔC) was calculated by subtracting the initial change (C_0) from the maximum change (C_{max}).

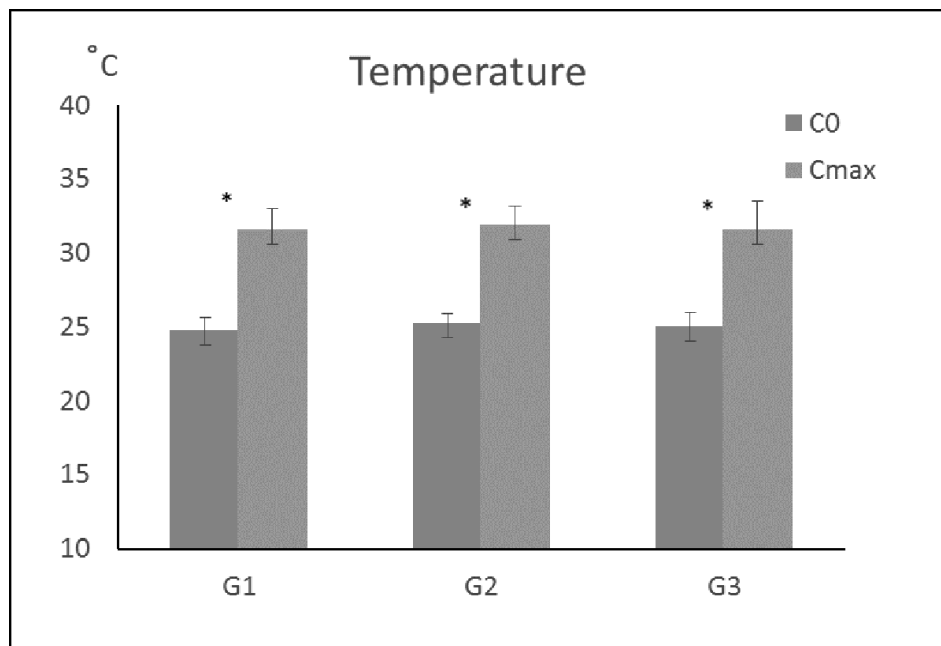


Fig. 4. Baseline (C_0) and maximal temperature (C_{max}) recorded in groups. **G1:** (3.0 mm-implants); **G2:** (3.3 mm- implants); **G3:** (3.6 mm-implants). Data presented in Celsius degrees as mean value and standard deviation. *: denotes $p < 0.05$.

different jaw models. An acrylic glass apparatus which held only a small part of the implant body was used in this work, and most of the implant was in contact with air, which has low thermal conductivity (0.02 W/km), while Ormianer et al. (38) and Kazemi et al. (39) used an implant body that was embedded in a jaw model which probably absorbed a significant portion of the heat, having a 30 times higher thermal conductivity than air (0.54 to 0.58 W/km).

Thermal changes create a considerable thermal increase at the implant-cortical bone interface. On the other hand, previous studies demonstrated that implants have a limited heat conduction capacity, and that this is influenced by implant length, width and the physical characteristics of the implant specific to type of titanium alloy (41-43). This study is believed to be the first one to assess the correlation between implant diameter and temperature increase during provisional crown preparation in a reproducible experimental setup, and no influence of the 3 different evaluated implant diameters was found on any of the tested parameters.

One of the limiting factors of this study was that the acrylic resin was left to set exothermally on the implant abutments with no attempt to cool the abutment or provisional crown. Even so, the mean temperature increase, which was 6.6°C, may induce osteoblast apoptosis *in vivo* (29). In addition, in the present study a small standard volume of 80 µL of acrylic resin was used in the polycarbonate crowns. In the clinical setting, however, the amount of self-polymerizing acrylic used in: impression abutment connection, the soldering index connection, the lining of provisional restorations and provisionalization of large temporary crowns, is expected to be higher than the contents of the small polycarbonate crown used in the current study and therefore may result in a greater temperature rise.

Within the limitations of this *in vitro* study, direct application of auto-polymerizing resin to the abutment of one-piece implants resulted in increased cervical implant temperature. Implant diameter did not influence the temperature changes. To avoid thermal injury to the surrounding bone, it is recommended to constantly cool the implant with water spray, during the setting of the provisional

restoration. Further studies using other restoration materials and implant systems should be conducted to confirm these results.

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ADULT SYNDROME: DENTAL FEATURES OF A VERY RARE CONDITION

L. AZZI¹, V. MAURINO¹, R. VINCI², F. CROVERI¹, A. BOGGIO¹, A. TAGLIABUE¹,
J. SILVESTRE-RANGIL³ and L. TETTAMANTI¹

¹Department of Medicine and Surgery, Research Centre into Paediatric Dentistry and Rare Syndromes, University of Insubria, ASST dei Sette Laghi, Dental Clinic, Varese, Italy; ²Oral Surgery, Università Vita-Salute San Raffaele, Milan Italy; ³Stomatology & Oral Surgery, Dr Peset University Hospital, Department of Stomatology, University of Valencia, Spain

The Acro-Dermato-Ungual-Lacrimal-Tooth syndrome (ADULT syndrome) is one of the rarest ectodermal dysplasias and it is associated with several malformations involving especially the limbs. The most clinical features are the presence of ectrodactyly, syndactyly, hypermelanosis or multiple lentigines, onychodysplasia, abnormalities in the lacrimal duct, recurrent conjunctivitis, photophobia, mammarian hypoplasia, hypotrichosis and frontal alopecia, hypohydrosis, cutaneous photosensitivity, nasal bridge prominence, exfoliative dermatitis and xerosis. The ectodermal dysfunction expresses itself with conoid teeth, enamel hypoplasia, dentinal dysplasia and especially hypodontia, with following functional and aesthetic defects. We report the case of an 11-year-old Caucasian girl affected by ADULT syndrome.

The Acro-Dermato-Ungual-Lacrimal-Tooth syndrome (ADULT syndrome) is one of the rarest ectodermal dysplasias and it is associated with several malformations, especially involving the limbs (1).

The ADULT syndrome is classified among the Group 1 Ectodermal Dysplasias, which includes all the forms involving abnormalities of the p63 gene, such as the EEC syndrome (2), AEC syndrome, limb-mammary syndrome, Rapp-Hodgkin syndrome, *Tricho dento osseous* syndrome, Witkop's disease, Ellis-van Creveld syndrome, Weyers acrodental dysostosis.

It was first described by Propping and Zerres in 1993 (1), in a family including seven cases with various expression, but it is only in 2007 that the ADULT syndrome was introduced among the rarest syndromes (0.05% of frequency). Only a few cases are known all over the world. In Italy, a case-report has been published by Rinne, Spadoni and Kjaer in 2006 (3). Most of the cases are described in Caucasian

individuals, but recently a few new cases have been reported among individuals belonging to different races (4).

The most distinguishing clinical features are the presence of ectrodactyly, syndactyly, hypermelanosis or multiple lentigines, onychodysplasia, abnormalities in the lacrimal duct, recurrent conjunctivitis, photophobia, mammarian hypoplasia, hypotrichosis and frontal alopecia, hypohydrosis, cutaneous photosensitivity, nasal bridge prominence, exfoliative dermatitis and xerosis (5).

All the reported clinical features are referred to an abnormal development of the ectodermal tissues due to a specific missense mutation involving the p63 gene located on chromosome 3 (3q27). It is an autosomal dominant disorder involving a family group, but several ex-novo mutations could also occur (6-12).

The most common mutation is R298Q, related to Arginine 298 aminoacid of the p63 binding domain (3, 13).

Key words: ADULT syndrome, ectodermal dysplasia, dental agenesis, dental malformation, rare disease

Mailing address:

Lorenzo Azzi, DMD
Department of Medicine and Surgery,
University of Insubria, Via Piatti, 10, 2110 Varese, Italy
Tel.: +39.0332-825625 - Fax: +39.0332-825655
e-mail: lorenzoazzi86@hotmail.com - web: www.carinci.org

0393-974X (2016)

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The p63 is also an oncosuppressor gene. Even if Rinne, Spadoni and Kjare (3) have found a great quantity of p63 deposits in an undifferentiated squamous cell carcinoma in a Finnish patient affected by ADULT syndrome, nowadays it's not possible to establish a causal relationship between a p63 mutation and cancer.

Among the craniofacial deformities, one distinguishing characteristic is the total absence of labiopalatal clefts, being this a useful feature to distinguish the ADULT syndrome from the other ectodermal dysplasia.

The ectodermal dysfunction expresses itself with conoid teeth, enamel hypoplasia, dentinal dysplasia and especially hypodontia, with following functional and aesthetic defects.

The developmental defect of the dentition contributes to render a hypoplastic third medium of the face. The absence of the permanent teeth determines an alteration of the physiologic bite augmentation with a consequent loss in the vertical dimension of the third medium of the face.

The ADULT syndrome can be diagnosed by observing the clinical features, but the final diagnosis

is made by genetics, due to the presence of different cases with an incomplete penetrance.

The prognosis of the syndrome is good. No systemic involvement or mental retard have been reported. Patients show a normal IQ. The only complications are of aesthetic nature, with the exception of ectrodactyly and syndactyly which require a surgical intervention.

Case report

We report the case of an 11-years-old Caucasian girl affected by ADULT syndrome. The first visit occurred in 2010.

The orthopantomogram (Fig. 1A) and lateral (Fig. 1B) radiographs, with the following cephalometric study, revealed the presence of a skeletal deep-bite, a brachicephalic subtype, a missed vertical development due to the absence of the permanent molars and due to a collapse of the premaxilla following the absence of permanent incisors.

A thorough dental hygiene treatment was performed every two months because the patient was unable to wash her teeth correctly since the presence of ectrodactyly and syndactyly (Fig. 2).

Conservative and endodontic treatments were

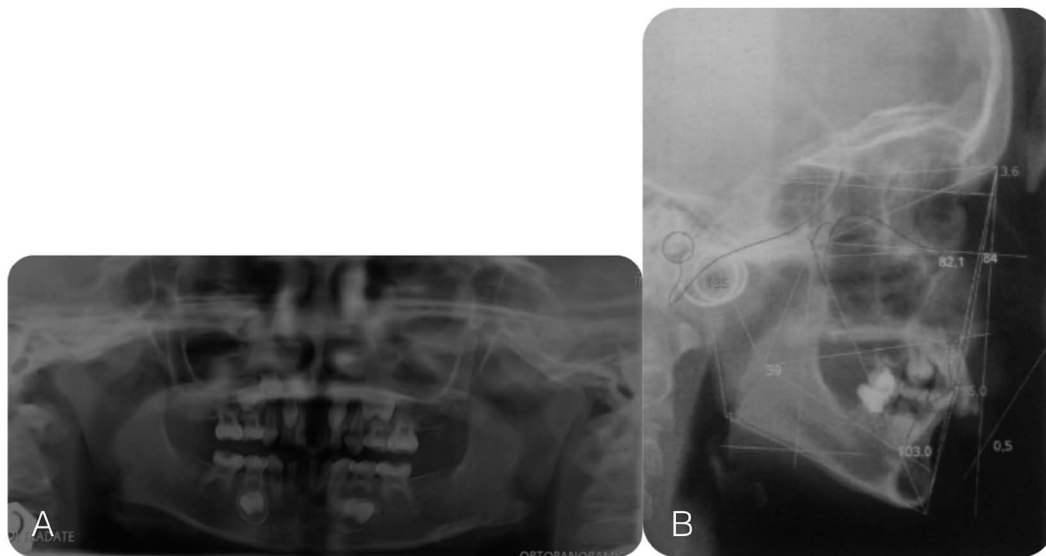


Fig. 1. A): the panoramic radiograph shows multiple agenesis with a particular attention focused on the absence of the permanent molars and permanent incisors; **B):** the cephalometric analysis reveals the presence of a reduced vertical dimension of the third medium of the face due to the absence of permanent molars and the missed bite augmentation.

executed where necessary (Fig. 3 A-C). A prosthetic plan was performed in order to re-establish a correct vertical dimension. Composite inlays were applied upon the dental elements 7.4, 7.5, 8.4 and 8.5. A fixed prosthesis was also applied preparing dental elements 1.2, 5.1, 6.1, 2.2 and 7.2, 7.1 and 8.2 (Fig. 3D-F). By using prosthetic treatment, it has been possible to re-establish the vertical dimension and the anterior guide in order to help the physiologic maxillo-mandibular growth.

DISCUSSION

The ADULT syndrome (acro-dermato-ungual-lacrimal-tooth syndrome) is a rare condition which affects the normal development of most of the ectoderm-derived structures, such as hands, nails, lacrimal ducts, hair, melanocytes and cranio-facial bones. No labiopalatal clefts have been reported in the international literature, and this feature is useful to distinguish the ADULT syndrome from the other



Fig. 2. A): the clinical appearance of the facial tissues: hair dryness, xerosis, nose-bridge prominence and a reduced third-medium face dimension are evident; B): the clinical appearance of ectrodactyly; C): radiographic aspects of ectrodactyly.

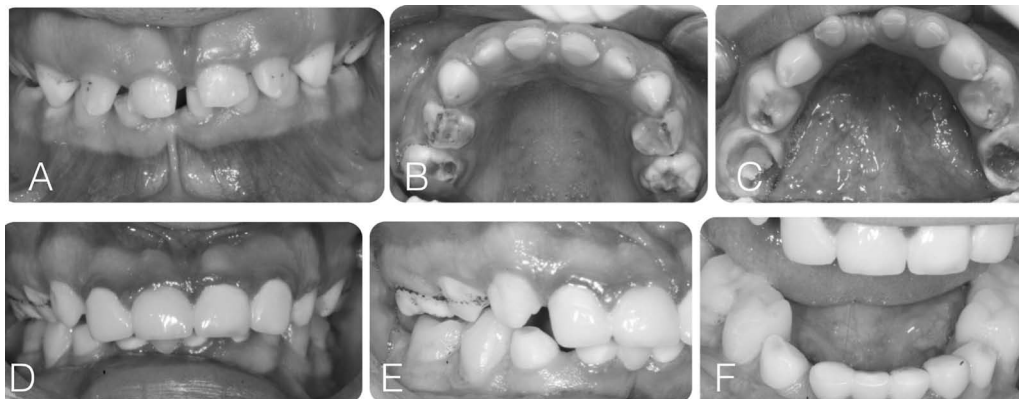


Fig. 3. A-C): Clinical intraoral overview. It is possible to notice a bad-keeping dental hygiene, multiple caries, and deep-bite due to the absence of a posterior occlusion and due to a premaxilla collapse; D-F): the oral clinical situation after treatment with posterior inlays and anterior fixed prosthesis. This treatment lead to re-establishing a correct vertical dimension and an anterior mandibular guide in order to help the physiologic maxillo-mandibular growth.

ectodermal dysplasia (14-26).

In the ADULT syndrome, it is possible to detect a high-graded hypodontia often involving the permanent molars which are responsible for a correct bite augmentation and vertical dimension of the third medium of the face.

During dentition change, the use of inlays or fixed prosthesis could provide a general improvement to re-establish a correct maxillo-mandibular growth.

At the end of the growth, once sex maturity has been reached, it becomes possible to execute a definitive prosthetic intervention through the use of dental implants and the application of single or multiple crowns, following the distinctive clinical situation of every patient.

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GENETIC CORRELATION BETWEEN RHEUMATOID ARTHRITIS AND PERIODONTAL DISEASE: THE ROLE OF SEX AND IL-10

L. AZZI¹, S. RANIA², F. SPADARI³, R. VINCI⁴, M. MANFREDINI¹,
F. CROVERI¹, A. BOGGIO¹, L. TETTAMANTI¹, A. TAGLIABUE¹,
J. SILVESTRE-RANGIL⁵ and C. BELLINTANI⁶

¹Department of Medicine and Surgery, University of Insubria, ASST dei Sette Laghi, Dental Clinic, Unit of Oral Pathology, Varese, Italy; ²ASST Valle Olona, Dental Clinic, Unit of Oral Surgery and Medicine; ³Department of Biomedical, Surgical and Dental Sciences, University of Milan, Ospedale Maggiore Policlinico, Unit of Oral Pathology and Medicine, Milan; ⁴Oral Surgery, Università Vita-Salute San Raffaele, Milan Italy; ⁵Stomatology and Oral Surgery, Dr Peset University Hospital, Department of Stomatology, University of Valencia, Spain; ⁶ASST Valle Olona, Unit of Rheumatology and Immunopathology

The chronic stimulation of the immune system due to the presence of bacterial antigens within periodontal tissues has been associated with several autoimmune diseases, like diabetes mellitus, infective endocarditis or cardiovascular atherosclerosis. The current study aims at evaluating the correlation between Rheumatoid Arthritis (RA) and Periodontal Disease (PD) with special attention to genetic polymorphisms in cytokine expression. A total number of 34 patients affected by RA were recruited. Each of them underwent haematochemical analysis and data were collected for Rheumatoid Factor (RF), Anti-Citrullinated Protein's Antibody (CCP) and HLA-BDR1. DAS-28 questionnaire for disease activity was fulfilled by the rheumatologist, while a periodontal examination was carried out by the dental clinician and crevicular fluid samples were collected to evaluate the IL-6, IL-10 and VDR polymorphisms. A connection between CCP and IL-10 polymorphisms was found, with IL-10 expressing protecting tendency against periodontal disease when CCP are found in the bloodstream ($p=0.0017$). Finally, males mainly expressed IL-10 predisposing genes ($p=0.046$), while females showed a greater tendency to express RF ($p=0.014$) and CCP ($p=0.050$). This paper corroborates the idea of a correlation between sex, IL-10 polymorphisms and RA, which should be studied in depth, since recent papers have shown that IL-10 injected into joints seems to decrease inflammation.

Rheumatoid Arthritis (RA) is a systemic, chronic disease, which has a multifactorial aetiology and an immunomediated pathogenesis. It affects several tissues and organs, such as the skin, blood vessels, heart, lungs and muscles, but joints are the mainly affected site. At this level the disease is associated with the onset of a non-suppurative proliferative synovitis. Joints are typically affected bilaterally in a

symmetric pattern. The disease, if untreated, leads to the destruction of joint cartilages, bone erosion and ankylosis (1).

Disease prevalence in western countries has only recently risen, as described by paleoanthropological studies (2). About 1% of the worldwide population is affected by RA. Sub Saharan and Caribbean black people appear to be less involved. The female to male

Key-words: rheumatoid arthritis IL-10, rheumatoid arthritis and periodontal disease, IL-10 genetics

Mailing address:

Lorenzo Azzi, DMD
Department of Medicine and Surgery,
University of Insubria, Via Piatti, 10, 2110 Varese Italy
Tel.: +39.0332-825625 - Fax: +39.0332-825655
e-mail: lorenzoazzi86@hotmail.com

0393-974X (2016)

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ratio is 3:1, even though this difference is reduced among the elderly. The mean age ranges between 40 and 70 years, with two peaks in young adults and in pre-menopausal women (3).

The disease is idiopathic. RA is a disease which can be explained by an autoimmune response after exposition to an unknown antigen in a genetically predisposed individual (4). It seems that the molecular mimicry caused by infective agents could be the underlying cause of the disease, likewise in other autoimmune diseases. Therefore, HLA-BDR1 haplotypes are associated with the disease (5).

The autoimmune response, which leads to the activation of T-helper cells and other lymphocytes and the production of cytokines, destroys joint architecture.

The first phase is characterized by the presence of monocytic-macrophagic cells just below the basal layer of the synovial membrane. At this time, IL-1, IL-6 and TNF cytokines are increased. Activation of T-helper cells positive for CD154 leads to the polyclonal proliferation of B-lymphocytic cells and the production of autoantibodies (6).

The clinical course of the disease is variable. RA usually involves the small distant joints, most frequently those of the hands and feet, with a symmetrical pattern. Joints appear swollen and painful. Stiffness, especially in the morning, is evident. The clinical progress of the disease can be rapid or slow and patients usually go through alternate periods of partial/complete remission and of flare-up. Over time, this chronic condition leads to the impairment of function and deformity, especially of the hands (7).

Extra articular manifestations include several tissues and organs and are usually linked to the intensity of the disease activity (8) such as, rheumatoid nodules, rheumatoid vasculitis, pleuropulmonary manifestations, neurological symptoms, ocular signs, Felty's syndrome, osteoporosis and lymphoma.

Oral symptoms and signs are xerostomia, temporo-mandibular joint dysfunction, ulcers due to the administration of methotrexate, increased predisposition to periodontal disease. Sometimes Sjögren's Syndrome is associated to RA. Several studies suggest the presence of intrafamilial genetic

predisposition, especially within direct line (9).

Among cytokines, Interleukine-6 plays an important role, since an uncontrolled overproduction may lead to the onset of severe chronic disease with autoimmune response. Some papers showed that the use of a monoclonal antibody directed against IL-6 receptor is effective in the treatment of RA (10). Vitamin D receptor gene is also involved, especially in accompanying osteoporosis and pathological fractures (11).

In 1987 the American College of Rheumatology developed Revised Criteria for the classification of RA (12) (Fig. 1). These criteria provide a sensibility rate of 94% and a specificity rate of 89%. The final diagnosis is made considering clinical features, radiographic imaging, blood chemistry and synovial liquid examination. Serum values are important indicators for disease activity and appear as prognostic elements to distinguish between aggressive and non-aggressive forms and to determine the response to treatment.

High levels of ESR and CRP indicate the presence of inflammation. CRP is especially connected with disease severity and appearance of lesions (13).

RF is an autoantibody directed against the FC portion of IgG antibodies and is expressed in 60-70% of patients with RA. It is not a highly specific antibody, since it can also be found in Cryoglobulinemia, Sjögren's Syndrome and chronic hepatopathies. However, it is a valuable prognostic factor, since it is high when RA appears aggressive and with an erosive clinical evolution (14).

Anti-CCP, on the contrary, are highly specific and sensitive. They are characteristically expressed in RA, so their value is both diagnostic and prognostic (15).

The DAS28 is a measure of disease activity in RA. DAS stands for 'Disease Activity Score' and the number 28 refers to the 28 joints examined in this assessment (16).

There is a wide range of measures to assess disease activity in RA including examination of joints for swelling and tenderness, global scores of pain and overall status, blood markers of inflammation (e.g. ESR and CRP), questionnaires (e.g. the HAQ), X-rays, and newer imaging techniques such as

ultrasound and MRI. These results are then fed into a complex mathematical formula to produce the overall disease activity score. A DAS28 of more than 3.2 implies active disease, less than 3.2 low disease activity, and less than 2.6 remission.

Genetic predisposition to periodontal disease

With tooth decay, PD is one of the two most common diseases of the oral cavity. It affects about 80% of the world population. The disease involves the supporting periodontal tissues which include the gums, periodontal ligament and alveolar bone. The final evolution of the disease is the loss by exfoliation of dental elements. Genetic predisposition may play an important role in determining whether an individual can be affected, but dental plaque is the causal agent. PD may have several clinical forms, but chronic periodontitis is the most widespread among adults. It can affect individuals of any age, but prevalence increases with age. It can be confined to a single group of teeth or affect the entire mouth (17).

The hypothesis of a genetic predisposition in PD was firstly formulated in 1935 (18). The discovery that genetics influences the development of PD dates back to 30 years ago (19). This new evidence has shown that PD, like other diseases (diabetes, cancer, etc.), requires a genetic susceptibility to develop.

Individuals respond differently to the attack of the oral microflora according to their genetic predisposition. Furthermore, genetics determines how each person interacts with environmental factors (such as oral biofilm) in the onset of PD. The relationship between genetic and environmental factors determines the onset of PD. Lifestyle (smoke and poor oral hygiene) affects the onset and progression of PD, but alone it is not able to cause it (20). The probability of developing PD related to heredity is about 50% (21).

Genetic susceptibility to multifactorial diseases is usually due to several gene polymorphism instead of a single, or few, gene mutations. Common variation in the genetic code may result in an altered expression or in functional changes of the encoded proteins, therefore in an increased disease severity.

Recently, research into susceptibility factors

of periodontitis has mainly focused on genes that modulate immune regulation, such as cytokines, chemokines, cell-surface receptors, enzymes and proteins related to antigen recognition. Cytokines, such as IL-10 and IL-6, are key factors which mediate the inflammatory process during periodontitis progression. They have a role in activation, proliferation and differentiation of B cells which are the majority of infiltrating cells in advanced periodontitis lesions. Therefore, common variations in the genetic code can alter the progression of disease because they may be responsible for the repeated cycles of tissue inflammation (22).

In PD, the microbiota accumulated in the subgingival region is the environmental factor, which influences the inflammatory response in periodontal tissues. However, cytokines contribute to connective tissue destruction and bone resorption (23-30).

Another factor associated with bone resorption in PD is Vitamin D Receptor (VDR), which has been regarded as a periodontitis susceptibility factor. Recent articles have reported a review of scientific literature as to the genetic association analysis between common polymorphism of candidate genes and periodontitis (31). VDR expression may be linked to bone pathology following periodontal destruction.

IL-1 genes are pro-inflammatory agents, since IL-1 is associated with inflammation, bone resorption. TNF- α is also linked to an increased degree of inflammation within periodontal tissues. IL-1 and TNF- α are also associated with RA, thus these two diseases can be linked on the basis of the role played by pro-inflammatory cytokines could be formulated. IL-10 is considered an anti-inflammatory cytokine, which modulates the activity of IL-1 and TNF- α . A recent paper has underlined the presence of Interleukin-6 gene methylation both in RA and PD (32).

The current study aims at evaluating the correlation between RA, with special attention to rheumatoid parameters, and PD genetic predisposition.

MATERIALS AND METHODS

Thirty-four patients with RA were recruited into

the current study. The study was conducted in the Unit of Rheumatology, Ospedale di Gallarate, VA, Italy. The mean age was 52.85 $\pm$ 15.64. Inclusion criteria were the diagnosis of RA and more than 30-years-old, while the exclusion criteria were the absence of concomitant autoimmune diseases, especially Sjögren syndrome, and partial or completely edentulism. An informed consent was read, understood and signed by all patients.

The rheumatologist collected data about disease severity and laboratory findings. Among haematochemical analysis, RF, anti-citrullinated protein antibody (CCP) and HLA-BDR1 were prescribed. The DAS28 questionnaire was completed by the physician in order to measure the clinical scores of disease activity. A dental clinician carried out a periodontal examination, collecting data from periodontal probing with special attention to depth probing.

Afterwards, a paper cone was inserted for 30 sec into the gingival sulcus near the dental element with the deepest probing value registered for every quadrant and then sent to the laboratory for evaluation in a sterile test tube. Samples for periodontal tissues were also used to assess genetic polymorphism in IL-6, IL-10 and VDR.

The following three polymorphisms were investigated: at IL6 the XR_108749.1:n.50-321G >C (rs1800795); at IL10 the NG_012088.1:g.4433A >C (rs1800872); and at VDR the NM_000376.2:c.1056T >C (rs731236). Single nucleotide polymorphism (SNP) assays were selected using the Applied Biosystems SNP browser Software

(Applied Biosystems, Foster City, CA, USA). Genotyping were performed using ABI PRISM 7500 Sequence Detection System and TaqMan chemistry according to the manufacturers' protocols (Applied Biosystems). Genotypes were scored by researchers in a blind system (biologists had no information about the clinical disease classification of the samples).

RESULTS

A total number of 34 patients affected by RA were recruited in the current study, 24 females and 10 males.

Periodontal genetic analysis regarded the expression of polymorphisms in IL-6, IL-10 and VDR genotypes (Table Ia). IL-6 was regarded as predisposing to periodontal disease when genotype GG of the polymorphism rs1800795 (G/C) was expressed, while genotypes GC and CC were considered to be protective against periodontal disease. Three patients expressed the protecting genotype, while 31 expressed the predisposing one.

IL-10 was considered predisposing to periodontal disease when genotypes AA and AC of the polymorphism rs1800872 (A/C) were expressed, while genotype CC was considered to be protective against the disease. Twelve patients expressed the protecting genotype, while 22 expressed the predisposing one.

Table I a	PREDISPOSING	PROTECTING
INTERLEUKIN-6 GENOTYPE	31	3
INTERLEUKIN-10 GENOTYPE	22	12
VIT D RECEPTOR GENOTYPE	28	6

Table I b	RF	CCP	HLA-BDR1	DAS-28
POSITIVE	21	19	10	17
NEGATIVE	13	15	24	17

VDR was considered as predisposing to periodontal disease when genotype TC of the polymorphism rs731236 (T/C) was expressed, while genotypes TC and CC were considered protective against the disease. Six patients expressed the protecting genotype, while 28 expressed the predisposing one.

Rheumatoid parameters collected from haematochemical analysis were the RF, the CCP and the presence of HLA-BDR (Table Ib).

Table I. a): values distribution of IL-6, IL-10 and VDR genotypes; b): rheumatic parameters collection.

The rheumatologist fulfilled the DAS-28 questionnaire to gather a total score of disease for every patient (Table Ib). RF was positive in 21 patients and negative in 13; CCP was positive in 19 patients and negative in 15; HLA-BDR1 was registered in 10 patients while it did not appear in the remaining 24.

DAS-28 indicated 17 patients with a score of 1 ($x < 2.6$), 9 with a score of 2 ($2.6 < x < 3.2$) and 8 with a score of 3 ($x > 3.2$); class 2 and 3 were united in a unique class so that the division was between 17 patients with absent disease activity and 17 with higher scores of disease.

To test the concordance of results between periodontal genetic indices a Chi-square analysis was performed. There was not a significant result between IL-10 and VDR ($p=0.406$), while, when the

individual is genetically predisposed to periodontal disease in IL-6 genes, IL-10 ($p=0.014$) and VDR ($p=0.020$) also seem to be correlated and the cytokines express predisposing genes.

To test the concordance of results between rheumatic parameters, a Chi-square analysis was performed. Rheumatic factors among them showed strict correlations within the population, according to data from the international literature (RF vs. CCP $p=0.000$; RF vs HLA $p=0.003$; CCP vs HLA $p=0.001$).

DAS-28 is a parameter that results from both laboratory findings and clinical evaluation of the disease. Thus it is less associated with rheumatic parameters than the others (DAS-28 vs RF $p=0.078$; DAS-28 vs CCP $p=0.016$; DAS-28 vs HLA $p=0.452$). Therefore, CCP appears as the parameter that is more associated to other factors.

Chi-square analysis was performed to compare predisposing and protecting genotypes of IL-6 with rheumatic parameters (Table II). There was no statistically significant difference, even though it should be considered that most patients expressed a predisposing genotype and the two groups were not balanced (IL-6 vs RF $p=0.154$; IL-6 vs CCP $p=0.107$; IL-6 vs HLA $p=0.876$; IL-6 vs DAS-28 $p=0.07$).

The same analysis was performed to compare genotypes of IL-10 with rheumatic parameters. There were no significant differences (IL-10 vs RF

Table II. a): Chi-square statistical analysis between rheumatic parameters and periodontal genetic factors; only CCP and IL-10 appear to be correlated in a opposite way; b): Chi-square statistical analysis between sex on one side and periodontal genotypes and rheumatic parameters on the other. Males express IL-10 predisposing genotype to periodontal disease more often than women, who on the contrary express RF and CCP with higher frequency.

Table II a	RF	CCP	HLA-BDR1	DAS-28
IL-6	0,154	0,107	0,876	0,07
IL-10	0,056	0,017	0,247	0,473
VDR	0,231	0,558	0,644	0,368

Table II b	IL-6	IL-10	VDR	RF	CCP	DAS-28	HLA
SEX	0,242	0,046	0,816	0,014	0,050	0,452	0,109

CRITERION	DEFINITION
1. Morning stiffness	Morning stiffness in and around the joints, lasting at least 1 hour before maximal improvement
2. . Arthritis of 3 or more joint areas	At least 3 joint areas simultaneously have had soft tissue swelling or fluid (not bony overgrowth alone) observed by a physician. The 14 possible area are right or left PIP, MCP, wrist, elbow, knee, ankle, and MTP joints
3. Arthritis of hand joints	At least 1 area swollen (as defined above) in a wrist, MCP or PIP joint
4. Symmetric arthritis	Simultaneous involvement of the same joint areas (as defined in 2) on both sides of the body (bilateral involvement of PIPs, MCPs, or MTPs is acceptable without absolute symmetry)
5. Rheumatoid nodules	Subcutaneous nodules, over bony prominence, or extensor surfaces, or in juxtaarticular regions, observed by a physician
6. Serum rheumatoid factor	Demonstration of abnormal amounts of serum rheumatoid factor by any method for which the result has been positive in <5% of normal control subjects
7. Radiographic changes	Radiographic changes typical of rheumatoid arthritis on posteroanterior hand and wrist radiographs, which must include erosions or unequivocal decalcification localized in or most marked adjacent to the involved joints (osteoarthritis changes alone do not qualify)

Fig. 1. Revised Criteria for the classification of Rheumatoid Arthritis.

$p=0.056$; IL-10 vs HLA $p=0.247$; IL-10 vs DAS-28 $p=0.473$), even though there is a predominance of positive RF when IL-10 expresses a protecting genotype in an opposite way, thus explaining the almost significant result in the statistical analysis.

On the contrary, IL-10 and CCP are associated in an opposite pattern, confirming the data mentioned above; when IL-10 expresses protecting genotypes against periodontitis, CCP is increased, while when IL-10 predisposing genes are expressed, CCP is decreased (IL-10 vs CCP $p=0.017$).

VDR results were compared with rheumatic parameters by Chi-square analysis, which ruled out any statistically significant correlation (VDR vs RF $p=0.231$; VDR vs CCP $p=0.558$; VDR vs HLA $p=0.644$; VDR vs DAS-28 $p=0.368$).

Finally, sex was compared to periodontal genetic predisposition and rheumatic parameters (sex vs IL-6 $p=0.242$; sex vs IL-10 $p=0.046$; sex vs VDR

$p=0.816$; sex vs RF $p=0.014$; sex vs CCP $p=0.050$; sex vs HLA $p=0.109$; sex vs DAS-28 $p=0.452$). IL-10 predisposing gene is expressed more in males than females ($p=0.046$), while RF ($p=0.014$) and CCP ($p=0.050$) are more common in females than males.

DISCUSSION

Rheumatoid Arthritis (RA) is a systemic, chronic disease that is characterized by a multifactorial etiology and an immunomediated pathogenesis. Even though the disease is idiopathic, since it is an autoimmune response without an apparent causal agent, recent findings in the literature have tried to explain how genetic predisposition and environmental factors may play a role in the maintenance of chronic inflammation in the joints and other organs.

The chronic stimulation of the immune system due to the presence of bacterial antigens within periodontal tissues has been associated with several systemic autoimmune diseases, like diabetes mellitus (33) or other pathologies, infective endocarditis (34) or cardiovascular atherosclerosis (35).

Rheumatoid Arthritis and Periodontal Disease (PD) share some common features, in particular many of the cytokines which maintain inflammation (i.e. IL-6, IL-10, TNF- α).

A total number of 34 patients affected by RA were recruited into the current study, 24 females and 10 males. Among rheumatic parameters RF, CCP and HLA-BDR1 were collected by blood samples, while DAS-28 questionnaire was fulfilled by the rheumatologist in order to establish a recognized score of the disease activity.

Genetic predisposition to periodontal disease is mainly due to the presence of several polymorphisms of the genes expressing IL-6, IL-10 and VDR.

GG genotype of the rs1800795 polymorphism in IL-6 genes triggers periodontal tissue destruction, while on the contrary genotypes GC and CC seem to act as protective agents. In IL-10 genes polymorphism rs1800872 CC genotype is protective against PD, while genotypes AA and AC are predisposing factors. In VDR TT genotype of the polymorphism rs731236 is a predisposing agent, while genotypes TC and CC are protective.

Statistical analysis was performed to compare genetic predisposition to PD in each patient with rheumatic parameters, in order to check any interaction between host susceptibility to periodontitis and genetic predisposition and clinical features of the concurrent RA. IL-6, IL-10 and VDR genes polymorphisms were compared with collected data on RF, CCP, HLA-BDR1 and DAS-28.

IL-6 did not turn out to be significantly associated with any of the rheumatic parameters (IL-6 vs RF $p=0.154$; IL-6 vs CCP $p=0.107$; IL-6 vs HLA $p=0.876$; IL-6 vs DAS-28 $p=0.07$). However, it should be considered that only 3 patients expressed the protective polymorphism of the gene, while 31 showed a predisposing polymorphism. The two groups were not balanced, so it was impossible to establish or deny any connection between IL-6 and

RA.

The same consideration should be done for VDR (VDR vs RF $p=0.231$; VDR vs CCP $p=0.558$; VDR vs HLA $p=0.644$; VDR vs DAS-28 $p=0.368$), but also in this case, 28 individuals expressed a predisposing polymorphism of the gene while only 6 showed the protective variant, so the two subgroups were quantitatively not homogeneous.

In addition, while there was no link between IL-10 and RF ($p=0.056$), HLA ($p=0.247$) and DAS-28 ($p=0.473$), a statistical significant correlation was detected between IL-10 and the CCP. The presence of CCP in the bloodstream was related to the presence of protective polymorphism CC in IL-10 genes, showing an opposite tendency in this case ($p=0.017$).

This connection should be further investigated, since recent papers have shown that IL-10 injected into joints affected by RA seems to decrease inflammation and thus is linked to biopharmaceuticals to strengthen drug efficacy (36). Sex represented an important feature of statistical analysis.

RA is known to be associated more with females than males (3:1 ratio). While there was not any correlation with IL-6 and VDR periodontal polymorphisms ($p=0.242$ and $p=0.816$ respectively), a correlation was found with IL-10 ($p=0.046$), being the predisposing polymorphisms AC and AA expressed more in males than in females.

Considering that females expressed RF ($p=0.014$) and CCP ($p=0.050$) to a major content, these data confirm those reported before between IL-10, RF and CCP. It seems that males express more IL-10 and less RF and CCP, while females express more RF and CCP and less IL-10, being IL-10 an interesting agent to be used for systemic therapy against RA. Furthermore, IL-10 periodontal polymorphism appears to be useful in this association. At the end of the study, different conclusions can be drawn.

Firstly, a connection between Anti-citrullinated protein antibody and periodontal expression of IL-10 genes' polymorphisms, with IL-10 protecting tendency against periodontal disease increasing when CCP are found in bloodstream. This correlation suggests a possible role of IL-10 in RA.

Finally, males express IL-10 predisposing genes to

periodontal disease more than females, while females show a greater tendency to express RF and anti-citrullinated protein antibody. This opposite behavior corroborates the idea of a correlation between IL-10 and RA which should be studied in depth.

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HUMAN β 2-DEFENSIN IN ORAL LICHEN PLANUS EXPRESSES THE DEGREE OF INFLAMMATION

L. AZZI¹, P. MORETTO², R. VINCI³, F. CROVERI¹, A. BOGGIO¹,
J. SILVESTRE-RANGIL⁴, L. TETTAMANTI¹, A. TAGLIABUE¹ and A. PASSI²

¹Department of Medicine and Surgery, University of Insubria, ASST dei Sette Laghi, Dental Clinic, Unit of Oral Pathology, Varese, Italy; ²Department of Medicine and Surgery, University of Insubria, School of Medicine, Unit of Biochemistry, Varese, Italy; ³Oral Surgery, Università Vita-Salute San Raffaele, Milan Italy; ⁴Stomatology & Oral Surgery, Dr Peset University Hospital, Department of Stomatology, University of Valencia, Spain

In this work, it was decided to study the role of HBD-2, an inducible and not constitutionally expressed defensin, in the pathogenesis of Oral Lichen Planus (OLP) and of Burning Mouth Syndrome (BMS). Seventeen OLP patients, with a positive histopathologic diagnosis of the disease, were recruited into this study in order to measure the relative quantity of HBD-2 in their saliva and crevicular fluid. The values were compared with those collected from a group of 9 patients affected by the Burning Mouth Syndrome (BMS) and with a control group (CTRL) of 9 patients. There was no statistically significant difference between the groups ($p=0.523$; $p=0.897$). However, patients affected by OLP showed a dycotomic distribution of values: while 10 of them showed similar values to those found out in the other two groups, 7 patients expressed high levels of HBD-2, and 3500 pg/ml was the threshold to distinguish the subgroups. During the dental visit the clinician classified OLP patients into two groups according to the clinical presentation of the disease: reticular and hyperplastic (white OLP), atrophic and erosive forms (red OLP). There was a statistical significant correlation between the clinical and numeric classification of the patients ($p=0.004$; $p=0.001$), and the expression of HBD-2 was higher in the red OLP group than in the white OLP group ($p=0.000$; $p=0.000$). In conclusion, this study shows that HBD-2 represents an index to assess active inflammation and it is probably linked to the presence of the typical band-like CD8+ infiltrate in Oral Lichen Planus.

Antimicrobial peptides (AMPs) are polypeptides of less than 100 amino acids that are found in host defense settings and have antimicrobial activity at physiological concentrations under conditions prevailing in the tissues of origin (1).

They represent an ancient arm of the innate immune system whose role is to neutralize invading microbes directly. Over 1500 AMPs have been described from different living beings, including

plants, birds, insects, mammals and humans.

Even though the number of AMPs in nature is large, some features in the general structure are shared among them. They are usually small peptides, commonly made up of about 30 amino acids, and are generally positive charged.

They show an amphipathic structure which is crucial for their antimicrobial properties: it seems that the positive charged molecules interact with

Key-words: rheumatoid arthritis IL-10, rheumatoid arthritis and periodontal disease, IL-10 genetics

Mailing address:

Lorenzo Azzi, DMD,
Department of Medicine and Surgery,
University of Insubria, Via Piatti 10, 2110 Varese, Italy
Tel.: +39 0332 825625 - Fax: +39 0332 825655
e-mail: lorenzoazzi86@hotmail.com

0393-974X (2016)

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negatively-charged phospholipids of microbial membranes, while their hydrophobic residues, which are 40-50% on average, aid integration into the microbial cell membrane and lead to its disruption (2).

First named as AMPs due to their antimicrobial properties, other biological effects of AMPs have been described recently: chemotaxis, immunomodulating activity, angiogenesis and wound repair. This is the reason why the ancestral AMPs represent an interesting and challenging field of research (3). Among AMPs described in humans, defensins occupy a strategic position.

Defensins are a family of small-sized β -chained peptides whose molecular weight ranges from 3.5 to 4.5 kDa and include 6 cysteine residues which can create disulfide bridges. There are three different families of defensins according to the position of the disulfide bridges: α , β and θ -defensins (4). α -defensins are composed of 29 to 35 aminoacids and are characterized by 1-6, 2-4 and 3-5 cystein disulfide bridges. There are 6 Human α -defensins. Four of them are expressed in neutrophil granules, and are also referred to as human neutrophil peptides HNP-1 through -4. The α -defensins 5 and 6 (HD5 and HD6) are found in Paneth cells of the small intestine and in the epithelial cells of the female urogenital tract (5).

β -defensins are characterized by 1-5, 2-4, 3-6 cystein disulfide bridges. There are six human β -defensins, but it is believed that others could be discovered. They are produced by epithelial cells (3).

HBD-1 is constitutively expressed in the respiratory and urinary epithelia while HBD-2 is expressed in psoriatic skin but also in urogenital, gastrointestinal and respiratory epithelia. HBD-3, described for the first time in psoriasis, has been found in all epithelia and particularly in saliva and cervicovaginal fluid. HBD-4 has been detected in testicles, stomach and uterus, while HBD-5 and HBD-6 have been mainly found in epididimus. α - and β -defensins are codified on chromosome 8. θ -defensins are not found in humans and new world primates. As to β -defensins, while HBD-1 is constitutively expressed, HBD-2 can be induced by IL-1 α , interleukin-1 β , TNF- α , interferon- γ , Gram-

positive and Gram-negative bacteria, *C. albicans*, *M. tuberculosis*, LPS. The activity of HBD-2 is increased by several bacterial products through the interaction with toll-like receptors (TLRs) 2 or 4 (6). They are also able to modulate the activity of the dendritic cells and the expression of their epitopes. Defensins can induce the maturation of the dendritic cells towards TLR-4. Thus AMPs represent a bridge between the innate immune system and the adaptive immune system.

In humans it is debated whether functional impairment or enhancement of AMPs can influence the clinical evolution of several diseases, among which infective and chronic inflammatory diseases. Defensins are known for their activity against certain viruses, like for example the Human Immunodeficiency Virus (HIV), the Human Papilloma Virus (HPV), Cytomegalovirus (CMV) or against certain bacteria, like *Mycobacterium tuberculosis* or *Pseudomonas aeruginosa* (7).

For antibacterial activities, β -defensins have proved to be effective in fighting *S.aureus*, *E.coli* and *M. tuberculosis* (8). For antifungal activities, β -defensins have turned out to be especially active against *C.albicans* and other *Candida* species, with the exception of *C. glabrata* (9). For antiviral activities α -defensins are capable of inhibiting Human Immunodeficiency Virus (HIV) and Herpes Simplex Virus (HSV) (10). For anti-parasite activities, several studies have reported that defensins are active against *Toxoplasma gondii* and *Trypanosoma cruzi* (11).

A decrease in number or function of defensins could increase the host's susceptibility to infections. On the contrary, in autoimmune or dysregulated immune responses the AMPs' activity seem to be altered, as it has been shown in psoriasis (12) and atopic dermatitis, fibrosis cystica, idiopathic pulmonary fibrosis, alveolar proteinosis, acute respiratory distress syndrome, lung transplantation, allergic rhinitis.

It seems that functional and epigenetic defects of defensins' expression could be a determining factor in the pathogenesis of some autoimmune diseases, including diabetes mellitus and Crohn's disease (13). Recently the international literature has

been focusing on the role of defensins in the oral cavity and their association with several mucosal and gingival diseases.

Common mucosal oral lesions include *Candidiasis*, recurrent *Herpes labialis*, *erythema migrans*, hairy tongue, Recurrent *Aphthous Stomatitis* and Oral Lichen Planus. The oral cavity is an environment in which antimicrobial peptides fulfil the important function of maintaining balance between health and disease. A lot of AMPs have been cited in the oral literature, being the α -defensins mainly expressed in neutrophils, the β -defensins in the epithelium and the LL-37 cathelicidin in both epithelium and neutrophils.

Defensins have been found in oral tissues, salivary glands, salivary secretions, crevicular fluid. In the oral cavity HBD-1 was described for the first time in 1998 (14). Up to now, only a small group of studies has investigated HBD-1 or HBD-2 in the saliva of patients with OLP or other mucosal conditions (15,16).

Oral Lichen Planus (OLP)

Lichen Planus is a common, chronic cutaneous disorder which often affects the oral mucosa. Since skin lesions resemble the lichens growing on rocks, Wilson was the first who named the disease after these vegetable organisms. Even though the word Lichen Planus can suggest a fungal condition, it is now accepted that it is a chronic autoimmune disease. Most patients with lichen planus are middle-aged adults and the ratio of women to men is 3:2. It is thought that about 1% of the population may have this condition (17). In the oral cavity, it is typically characterized by lesions consisting of radiating white or gray, velvety, thread-like papules in a linear, annular or retiform arrangement forming classic lacy, reticular patches, rings and streaks over the buccal mucosa and, to a lesser extent, on the lips, tongue and palate.

These lesions do not cause significant symptoms in the majority of patients, while in others there may be a complaint of a burning sensation in the involved area (18).

Reticular lichen planus is the most frequent form of the disease. This variant usually does not trigger

any symptom and involves the posterior buccal mucosa bilaterally. Other oral mucosal surfaces may also be involved concurrently, such as the lateral and dorsal tongue, the gingivae, palate, and vermillion border. Reticular lichen planus is thus named because of its characteristic pattern of interlacing white lines (Wickman's striae). These lesions are typically variable, they wax and wane over weeks or months.

A hyperplastic form of lichen planus may also occur on the oral mucosa, generally appearing as well-circumscribed, elevated white lesion resembling leukoplakia. In such cases, biopsy is usually necessary to make a diagnosis. An atrophic form of lichen planus occurs with some frequency and clinically appears as smooth, red, poorly defined areas, often but not always with peripheral striae evident.

Erosive lichen planus, although not as common as the reticular form, is more significant for the patient because the lesions are usually symptomatic. Clinically, there are atrophic, erythematous areas with central ulceration of varying degrees. If the erosive feature is severe, epithelial separation from the underlying connective tissue may occur. This results in the relatively rare presentation of bullous lichen planus.

The clinical forms can be grouped into two families: the so-called "white" lichen planus, which includes the papular, the reticular and the hyperplastic form, and the "red" lichen planus, which comprises the atrophic, the erosive and the bullous forms (Fig. 1). The red forms are those in which inflammation is more represented, they are often symptomatic and may undergo malignant transformation, even though it is uncommon. The histopathologic features of lichen planus are characteristic but may be not specific, because other conditions, such as lichenoid drug reaction, lichenoid amalgam reaction, oral-versus-graft disease (GVHD), lupus erythematosus (LE), chronic ulcerative stomatitis and oral mucosal cinnamon reaction may also show a similar histopathologic pattern (Fig. 2).

There is some controversy surrounding its malignant potential. There seems to be a slightly higher incidence of oral squamous cell carcinoma in patients with oral lichen planus than in the general

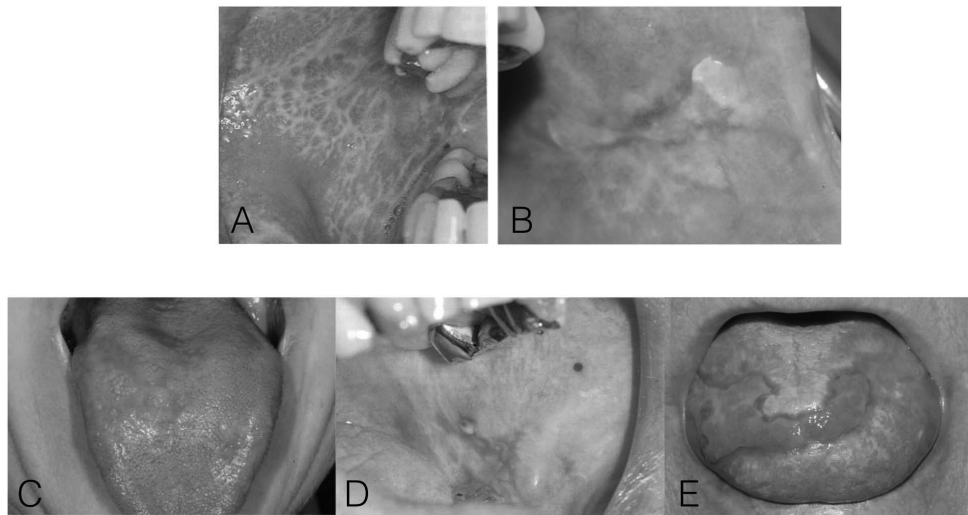


Fig. 1. Clinical features of Oral Lichen Planus. **A)**: Reticular OLP. Interlacing white lines involving the posterior buccal mucosa, the most common site of oral involvement; **B)**: Hyperplastic OLP. Smooth white plaques which may be confused with oral leukoplakia; **C)**: Atrophic OLP. Atrophic areas with irregular borders replacing the normal papillary surface of the tongue; **D)**: Erosive OLP. Ulceration of the buccal mucosa showing peripheral radiating keratotic striae; **E)**: Bullous OLP. This form is very rare. A bullous lesion is circumscribed by radiating keratotic striae on the dorsum of the tongue.

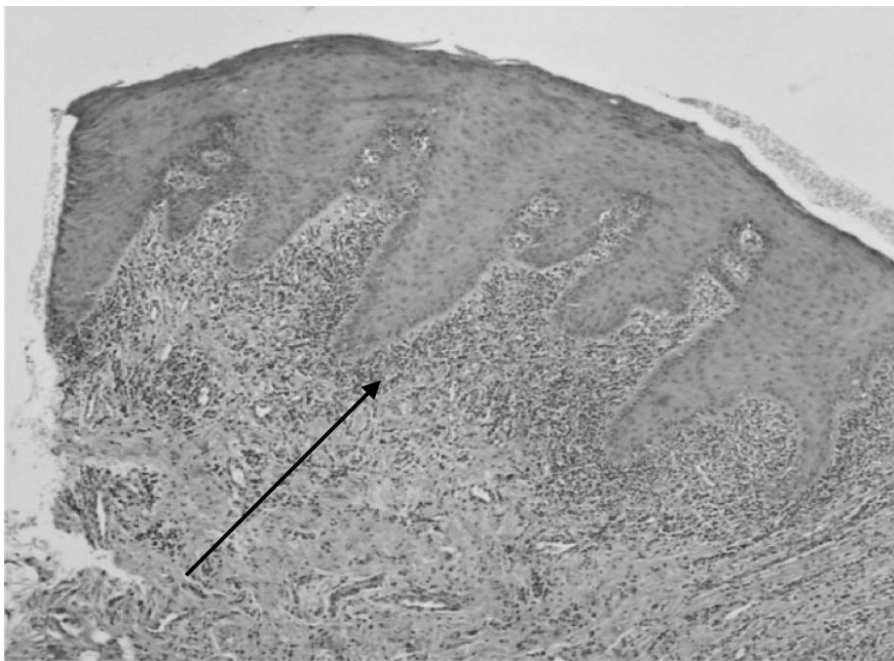


Fig. 2. Histopathological aspect of OLP. Hyperkeratosis, saw-toothed rete ridges and a band-like infiltrate of lymphocytes (arrow) immediately subjacent to the epithelium.

population. The actual overall frequency of malignant transformation is low, ranging from 0.3 to 3%. The forms that more commonly undergo malignant transformation are the erosive and atrophic ones (19).

At present, there is no cure, although various agents have been tried. Medical treatment of OLP is essential for the management of painful, erythematous, erosive, or bullous lesions. The main aim of current OLP therapy is to relieve painful symptoms, heal oral mucosal lesions, reduce the risk of oral cancer, and maintain good oral hygiene. As it is an autoimmune mediated condition, corticosteroids are recommended. Patients should be followed up periodically, particularly those affected by the erosive or atrophic forms and those who also have a history of alcohol and tobacco abuse, because of the risk of malignant transformation (20).

Although *in vitro* studies into HBDs in oral epithelium cells are well documented, oral epithelium has only scarcely been the subject of *in*

vivo investigations. Published studies have shown that HBD-2 peptide is expressed mainly in granular and keratinized layers, but is not easily seen in the non-keratinized oral epithelium, including buccal and junctional epithelia. Abiko et al (21) found that in 43% of cases of lichen planus, there was a positive immunohistochemical staining for HBD-2 in the spinous layer. Staining was marked even in lichen planus with non-keratinized epithelium. The border of the area with transition of the non-keratinized normal epithelium into lichen planus separated the positively stained areas from those showing no staining. The immunohistochemical localization of HBD-2 peptide in lichen planus was stronger and wider than in normal epithelium. The site of HBD-2 expression was faint and restricted to the keratinized area and to part of the granular layers in normal oral epithelium. Intense staining of HBD-2 in a lower spinous layer of lichen planus was often observed. The presence of HBD-2 is linked to the

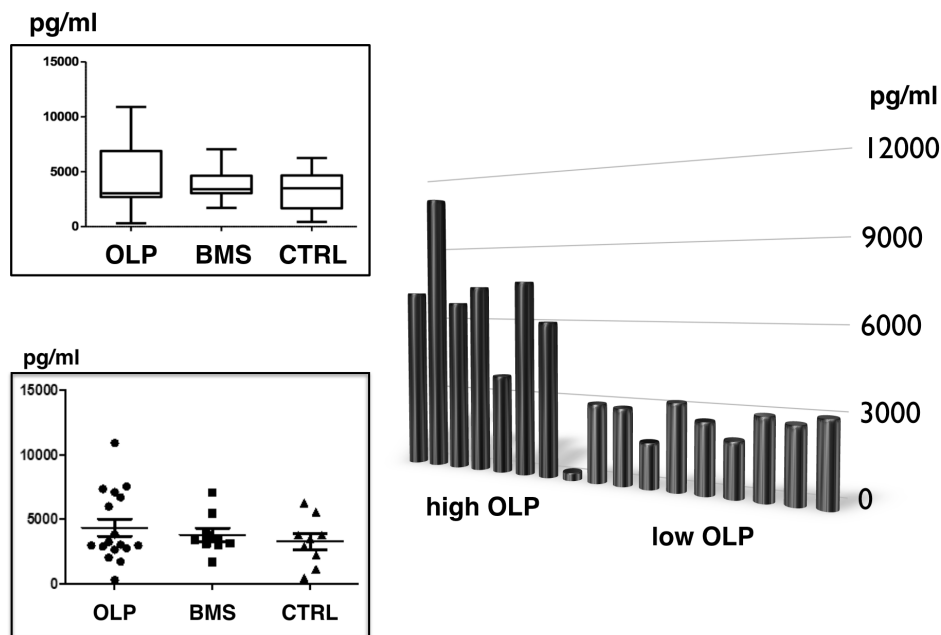


Fig. 3. There was not any significant difference between the OLP, BMS and CTRL groups. However, patients affected by OLP showed a dycotomic distribution of values: while 10 of them showed similar values to those found out in the other two groups, 7 patients expressed high levels of HBD-2, and 3500 pg/ml was the threshold to distinguish the subgroups.

presence of inflammatory infiltration. Specific inflammatory conditions related to lichen planus may be involved in the upregulated expression of HBD-2. The inflammatory infiltrate in lichen planus is composed of lymphocytes, predominantly T-cells. These T-cells produce many types of cytokines, which, in turn, stimulate the production of other cytokines in epithelial cells. Expression of HBD-2 is upregulated by stimulation of cytokines, including TNF- α and IL-1. In lichen planus, TNF- α is increasingly produced in both sub-epithelial inflammatory infiltrate and epithelial cells. Upregulated expression of HBD-2 in lichen planus may be involved in increased production of TNF- α .

In a following paper, Nishimura, Abiko et al (22) reported that the localization pattern of HBD-3 mRNA in oral tissues was almost the same as that of HBD-2. Previous papers had shown that HBD-2 expression was involved in keratinocyte differentiation. HBD-3 mRNA was localized in the upper spinous and granular layers in normal oral epithelium. These results may indicate that HBD-3 expression is also involved in keratinocyte differentiation.

Although both HBD-2 and HBD-3 are upregulated by inflammatory stimulation such as the one triggered by cytokines and bacteria, cytokines that stimulate upregulation are different. In contrast to HBD-2 that was induced by TNF- α , no regulation of HBD-3 was found after stimulation, but HBD-3 was induced by IFN- γ . IFN- γ production has been also confirmed in recent papers about lichen planus. The upregulated expression of HBD-3 in lichen planus in the present study may be involved in the production of IFN- γ .

Burning Mouth Syndrome (BMS)

Burning Mouth Syndrome (BMS) is a condition characterized by a sensation described by patients as stinging and burning that affects the oral mucosa in total absence of clinical or laboratory data to justify these symptoms (23).

This condition principally affects women at a ratio of approximately 4:1; this difference between the sexes might be explained by biological, psychological and sociocultural factors; however, such factors have not yet been defined (24).

The pathophysiology of idiopathic burning pain

is unclear and has generated controversy over the years. The aetiopathogenesis seems to be complex, and in a large number of patients, it most likely involves interactions among local, systemic and/or psychogenic factors (25). The role of the peripheral nervous system, particularly pertaining to concurrent dysgeusia has been a topic of study. The results in the international literature suggest that BMS oral burning may be a disorder of peripheral pain pathways, accompanied by a variety of positive and negative neurological findings in some patients (26).

Lauria and co-workers showed that BMS patients had a significantly lower density of epithelial nerve fibers in the biopsy specimens of the anterior two thirds of the tongue than controls. This feature was more evident with a longer duration of symptoms. Epithelia and sub-papillary nerve fibers showed diffuse morphological changes indicating axonal degeneration (27).

Particular emphasis has been placed on concurrent symptom of xerostomia and some papers showed the presence of a very low basal salivary flow in BMS patients, even though the stimulated salivary flow remains within a normal range of values (28). In the international literature, there is not any study on a possible role of defensins in the pathogenesis of the syndrome.

MATERIALS AND METHODS

The current study was conducted in the Department of Surgical and Morphological Sciences, University of Insubria, ASST dei Sette Laghi, Unit of Oral Pathology, Dental Clinic, Varese, Italy. A total number of 35 patients were recruited for this study. Among them, 17 were affected by Oral Lichen Planus (OLP group), 9 by Burning Mouth Syndrome (BMS group) and 9 were included as a control group (CTRL group).

Inclusion criteria for the OLP group were the presence of a positive histopathologic diagnosis of the disease, age greater than 30, while patients undergoing topical or systemic therapies were excluded from the recruitment. Inclusion criteria for the BMS group were the final diagnosis of a primary idiopathic burning pain according to the guidelines of the international literature, age greater than 45 and female sex, whereas the presence of other

concomitant mucosal pathologies, such as a secondary burning pain linked to systemic conditions or other neuropathies, were exclusion criteria.

The control group included healthy patients with demographic similarities to the other two groups, especially considering age and F:M ratio. Each patient underwent a dental examination by a clinician who was the same throughout the whole study.

Firstly, a sample of saliva was collected through the spitting method for about 1 min in a sterile test tube. Secondly, a sterile paper cone was inserted for at least 30 sec into the gingival sulcus nearby a dental element chosen through randomized criteria. The procedure was carried out 4 times per patients, each time near a different dental element, in order to collect a sufficient quantity of crevicular fluid. Furthermore, each OLP patients was also evaluated for the clinical appearance of the lesions, subdividing the population into 2 subgroups: red OLP, when patients showed an atrophic or erosive variant of the disease; white OLP, when reticular and hyperplastic forms were detected. Collected specimens were sent to the Biochemistry Laboratory to be analysed. The samples were stored at a temperature of -20°C before examination.

Human β -Defensin 2 Elisa Kit protocol was used for this study (EK-072-37 Phoenix Pharmaceuticals, Inc.). The immunoplate was precoated with anti-Human β -Defensin 2 Capture Antibody and the nonspecific binding sites were blocked. Human β -defensin 2 in the standard solution or in the sample could bind to the capture antibodies immobilized in the wells.

After washing procedure, the Biotinylated anti-Human β -defensin 2 Detection Antibody, which could bind to Human β -defensin 2 trapped in the wells was added. After washing, the Streptavidin-Horse-radish Peroxidase (SA-HRP) which catalyzes the Substrate Solution (TMD) was added. The enzyme-substrate reaction was terminated by the addition of a stop solution. The intensity of the colour was directly proportional to the amount of Human β -Defensin 2 in the standard solutions or samples. A standard curve of Human β -Defensin 2 with known concentration could be generated accordingly.

Comparative statistics among the different groups was performed with *T*-Student independent variables test for parametric values and the *Chi*-square test for non parametric values by using the IBM SPSS version 20.0, SPSS Inc, Chicago, IL, USA.

RESULTS

A total number of 35 saliva and crevicular liquid samples were analysed with ELISA protocol. Seventeen patients (11 females and 6 males), were affected by Oral Lichen Planus (OLP group). Burning Mouth Syndrome (BMS group) affected nine patients, all female. Nine patients, 7 females and 2 males, patients were included in the control group (CTRL group).

The OLP group ELISA test for detection of HBD-2 in saliva showed a mean value of 4341.85 $\pm$ 2748.68 pg/ml, with a registered minimum value of 304.28 pg/ml and a maximum of 10890.56 pg/ml. The mean value of HBD-2 reported among the OLP group in the crevicular fluid was 4157.37 $\pm$ 2620.59 pg/ml with a registered minimum value of 444.65 pg/ml and a registered maximum value of 8552.84 pg/ml.

The *BMS group* ELISA test for detection of HBD-2 in saliva showed a mean value of 3789.05 $\pm$ 1565.83 pg/ml, with a registered minimum value of 1703.43 pg/ml and a registered maximum value of 7047.26 pg/ml. The mean value of HBD-2 reported in the BMS group in the crevicular fluid was 3314.94 $\pm$ 1064.27 pg/ml with a registered minimum value of 1703.43 pg/ml and a registered maximum value of 5066.56 pg/ml.

The CTRL group ELISA test for detection of HBD-2 in saliva showed a mean value of 3268.95 $\pm$ 1886.90 pg/ml, with a registered minimum value of 428.01 pg/ml and a registered maximum value of 6242.46 pg/ml. The mean value of HBD-2 reported in the CTRL group in the crevicular fluid was 4399.70 $\pm$ 3889.23 pg/ml with a registered minimum value of 530.23 pg/ml and a registered maximum value of 11767.84 pg/ml.

One-way ANOVA test was used to compare the values of salivary HBD-2 registered among the three groups. There was not any statistically significant difference among the groups with regards to the production of HBD-2 ($p=0.523$). The same test was performed to compare the values of gingival HBD-2 registered among the 3 groups. There was no statistically significant difference among the groups with regards to the production of HBD-2 ($p=0.897$). However, the distribution of values

registered in saliva and crevicular fluid was strictly correlated. Paired sample correlation test was used to compare the associated variance of salivary and gingival values within patients ($p=0.000$). Therefore, the presence of corresponding values in saliva and crevicular fluid showed how the ELISA detection method was correct and that saliva and crevicular fluid contained an equivalent quantity of HBD-2 in the same patient.

Nevertheless, observing the scatter plot and the box plot (Fig. 3), it was possible to notice that, while the mean values were similar among the groups, even if registered values were slightly higher in OLP and BMS than in the CTRL group, in OLP there was a dycotomic pattern of distribution between a subgroup showing very high values (>3500 pg/ml) and another subgroup with values similar to those detected in the other two groups (<3500 pg/ml).

The clinical evaluation of OLP revealed the presence of 10 white OLP and 7 red OLP.

A statistical non-parametric comparison was made between the classification of OLP based upon the clinical evaluation (*white vs red*) and the results of the ELISA test defined as *low* (<3500 pg/ml) or *high* (>3500 pg/ml). There was a strong statistical correspondance ($p=0.002$). The same analysis was made for the crevicular fluid and proved to be statistically significant ($p=0.000$), confirming the association between salivary and gingival values.

ANOVA analysis was performed by comparing the salivary values in relation to the dycotomic clinical classification. There was a statistically significant difference between salivary HBD-2 values registered in the red OLP subgroup and those found in the white OLP subgroup ($p=0.000$). The same result was obtained performing an ANOVA test to compare crevicular fluid values in relation to the clinical classification ($p=0.000$).

DISCUSSION

Antimicrobial Peptides (AMPs) represent a recent interesting field of research in immunobiology and medicine, even though they are an evolutionary ancient means of defense by the host through the innate immune system (29).

In recent years the international literature has shown that not only do they play a special role in fighting off infective agents (30), but also in immunomodulatory functions and wound healing (31). A reduced production or function of these small peptides imply the onset of infective disease, wound superinfection or cancer, while on the contrary an excess in their presence or activity seems to be linked to autoimmune diseases (32).

β -defensins are mainly expressed in the mucosal barriers throughout the human body, like in the bowel, the gut, the genitourinary tract and oral mucosa (4). Several studies tried to establish the role of β -defensins in the pathogenesis of periodontal disease and other oral disorders, but only a few papers can be found in literature (14, 33). Periodontal disease is characterized by chronic inflammation as well as in peri-tumoral region (34-38, 39-45).

In this study the choice was to study the role of HBD-2, which is an inducible and not constitutionally expressed defensin, in the pathogenesis of the most frequent autoimmune disease of the oral cavity, Oral Lichen Planus (OLP) and of the most diffuse neuropathic disorder, Burning Mouth Syndrome (BMS).

Seventeen OLP patients, with a positive histopathologic diagnosis of the disease, were recruited into this study in order to measure the relative quantity of HBD-2 in their saliva and crevicular fluid. The values were compared with those collected from a group of 9 patients affected by the Burning Mouth Syndrome (BMS) and with a control group (CTRL) of 9 patients.

There was no statistically significant difference between the BMS and OLP groups ($p=0.523$; $p=0.897$), however, the latter seemed to show increased mean levels of HBD-2. Besides, the study highlighted the role of HBD-2 in the maintenance and intensity of the inflammatory component in Oral Lichen Planus.

Patients affected by OLP showed a dycotomic distribution of values: while 10 of them showed similar values to those found out in the other two groups, 7 patients expressed high levels of HBD-2, and 3500 pg/ml was the threshold to distinguish the subgroups.

During the dental visit the clinician classified OLP patients into two groups according to the clinical presentation of the disease: reticular and hyperplastic OLP forms were considered to be associated with a low level of disease and inflammation (white OLP), whereas atrophic and erosive forms were related to a high degree of inflammation (red OLP).

There was a statistical significant correlation between the clinical and numeric classification of the patients ($p=0.004$; $p=0.001$) and the expression of HBD-2 was higher in the red OLP group than in the white OLP group ($p=0.000$; $p=0.000$).

In conclusion, this study shows that HBD-2, which is an inducible molecule, represents an index to assess active inflammation and is probably linked to the presence of the typical band-like CD8⁺ infiltrate in Oral Lichen Planus.

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A “BURNING” THERAPY FOR BURNING MOUTH SYNDROME: PRELIMINARY RESULTS WITH THE ADMINISTRATION OF TOPICAL CAPSAICIN

L. AZZI¹, F. CROVERI¹, L. PASINA², M. PORRINI³, R. VINCI⁴, M. MANFREDINI¹,
L. TETTAMANTI¹, A. TAGLIABUE¹, J. SILVESTRE-RANGIL⁵ and F. SPADARI³

¹Department of Medicine and Surgery, University of Insubria, ASST dei Sette Laghi, Dental Clinic, Unit of Oral Pathology, Varese, Italy; ²Laboratory for Quality Assessment of Geriatric Therapies and Services, Department of Neuroscience, IRCCS-Istituto di Ricerche Farmacologiche Mario Negri, Milan; ³Department of Biomedical, Surgical and Dental Sciences, University of Milan, Ospedale Maggiore Policlinico Ca' Granda Foundation, Unit of Oral Pathology and Medicine, Milan; ⁴Oral Surgery, Università Vita-Salute San Raffaele, Milan Italy; ⁵Stomatology & Oral Surgery, Dr Peset University Hospital, Department of Stomatology, University of Valencia, Spain

Burning mouth syndrome is defined as an intraoral burning sensation for which no medical or dental cause can be found. Recently, researchers have demonstrated an altered trophism of the small nerve fibres and alterations in the numbers of TRPV-1 vanilloid receptors. Capsaicin is a molecule that is contained in hot peppers and is specifically detected by TRPV-1 vanilloid receptors that are distributed in the oral mucosae. We aimed at verifying if topical capsaicin could prove to be an effective treatment of Burning Mouth Syndrome. A group of 99 BMS patients were recruited. We subdivided the BMS patients into two groups: the collaborative patients, who expressed a predominantly neuropathic pattern of symptoms, and the non-collaborative patients, who were characterised by stronger psychogenic patterns of the syndrome. Both groups underwent topical therapy with capsaicin in the form of a mouth rinse 3 times a day for a long period. After 1 year of treatment, the final overall success rate was approximately 78%, but with a significant difference in the success rates of the two groups of patients (87% and 20% among the collaborative and non-collaborative patients, respectively; $p=0.000$). The use of topical capsaicin can improve the oral discomfort of BMS patients, especially during the first month of therapy, but it is more effective for those patients in which the neuropathic component of the syndrome is predominant. Our hypothesis is that chronic stimulation with capsaicin leads to decreases in burning symptoms. This phenomenon is called desensitisation and is accompanied by substantial improvements in oral symptoms.

Burning mouth syndrome is defined as an intraoral burning sensation for which no medical or dental cause can be found (I.A.S.P.) (1). BMS patients often refer to sensations of xerostomia, dysgeusia, intolerance to dentures and other “bizarre” sensations (2). These conditions can coexist or occur alternately.

It is widely accepted that BMS is not a rare disease, and several studies have reported estimated incidences between 0.7% and 15% (3-6). BMS

occurs more frequently in females (5:1 ratio) and more frequently in the 6th or 7th decades of life (7). The burning sensation can be perceived bilaterally in any oral region; however, the tongue and the lips are the most frequently reported locations (8). Many theories have been proposed to explain the aetiopathogenesis of this complex pathology.

Two schools of thought are present in the international literature. The first focuses on the main

Key words: burning mouth syndrome, topical capsaicin, burning mouth therapy, BMS capsaicin, BMS therapy

Mailing address:

Lorenzo Azzi, DMD,
Department of Medicine and Surgery,
University of Insubria, Via Piatti, 10, 2110 Varese, Italy
Tel.: +39 0332 825625 - Fax: +39 0332 825655
e-mail: lorenzoazzi86@hotmail.com

0393-974X (2016)

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role of psychogenic factors. BMS patients often exhibit an anxious-depressed psychic pattern and often have been taking anxiolytics or antidepressant drugs in the years prior to presentation. Consequently, based on the psychogenic theory, there may be a type of somatisation of the psychic distress on the peripheral nervous system, which results in diminished pain thresholds and burning sensations (9, 10).

The second theory considers BMS to be a neuropathic disorder caused by alterations in the trigeminal small myelinated C fibres that are responsible for chronic burning and tingling sensations in the mouth.

Recently, researchers have demonstrated altered trophism of the small nerve fibres and alterations in the numbers of TRPV-1 vanilloid receptors (11, 12). Based on the neuropathic theory, the chronic pain may induce psychic distress that results in anxiety and depression.

The confusion surrounding BMS is due to the chicken-and-egg nature of this disease. We have noticed that improving the peripheral symptoms can help to reduce anxiety and depression and that appropriate psychotherapy can result in augmentations of pain thresholds and reductions in oral burning symptoms (13). Thus, a psychoanalytic approach is always recommended for BMS patients.

Many topical therapies have been tested in recent past decades including the following: salivary substitutes, antimycotics, antihistamines (14), alpha-lipoic acid (15), clonazepam (16), tongue protectors (17), lidocaine, menthol chewing-gum, electrostimulation, acupuncture (18, 19), laser-acupuncture (20), etc. However, none of these therapies has proven to be sufficiently effective. The data reported in the literature appear to be contradictory.

The use of capsaicin for the treatment of burning pain is a well-known approach for post-herpetic neuralgia (21) and peripheral diabetes-linked neuropathies (22). Capsaicin is a molecule that is contained in hot peppers and is specifically detected by TRPV-1 vanilloid receptors (23) that are distributed in the oral mucosae.

In this retrospective analysis, we wanted to verify

the true effectiveness of topical capsaicin in the treatment of oral burning complaints and whether it could be useful to better understand the neuropathic component of Burning Mouth Syndrome.

MATERIALS AND METHODS

A group of 99 BMS patients were recruited. The final diagnosis of primary burning mouth syndrome was made after all local, systemic, haematochemical, microbiological and allergenic factors had excluded according to a thorough protocol (24).

Informed consent was obtained from all patients, and the patients' pain perceptions and quality of life were then evaluated with psychological multi-dimensional scale questionnaires.

Subsequently, we subdivided the BMS patients into two subgroups: the collaborative patients, who were characterized by a predominantly neuropathic pattern of symptoms and the non-collaborative patients, who on the contrary were related to a stronger psychogenic pattern of the syndrome and expressed concurrent speech and comprehension disturbances.

Both groups underwent topical therapy with capsaicin in the form of a mouth rinse. The patients mixed red hot pepper powder containing 3.54 µg/mL of capsaicin with 100 mL of water, rinsed their mouths with the mixture for at least one minute, spat the mixture out and avoided drinking or eating for at least 30 minutes. Patients were instructed to carry out the mouth rinses 3 times a day after eating and were informed that treatment should be continued until the next control visit. Meanwhile, no other topical or systemic drugs were administered to the patients, to avoid polluting the results.

Repeated control visits took place after 1, 3, 6 and 12 months of treatment. During the control visits, we registered the outcome asking the patients if they reported a subjective improvement in oral burning sensations compared with prior consultation. Some patients underwent VAS scale questionnaire to assess subjective improvement. Chi-square analysis was performed to compare the results between the two groups and *T*-Student paired samples analysis was used to compare the subjective improvement within patients reported during the repeated control visits (IBM SPSS version 20.0, SPSS Inc, Chicago, IL, USA).

RESULTS

A total number of 99 patients were recruited in the current study, 81 females and 18 males. The mean age was 64.79 ± 9.96 (min 36, max 83). Seventy-six patients resulted collaborative after undergoing multidimensional scale questionnaires (mean age 64) and 23 were considered as non-collaborative (mean age 68.39). There was no statistical difference between the two groups in regards to age ($p=0.088$). After 1 month of topical therapy with capsaicin, the success rate was 67% among the recruited 99 patients, but there was a significant difference in the success rates of the two groups of patients, i.e. 74% among the 76 collaborative patients and 44% among the 23 non-collaborative ones (Fig. 1a). *Chi-square* analysis resulted statistically significant between the two groups ($p=0.000$).

After 3 months of treatment, we experienced patient drop-out rates that were equal between the two groups (Fig. 2), however, we were still able to confirm the high rate of success of 71% among the remaining 75 patients. Again, the success rates

were different between the groups (80% of the 59 collaborative and 38% of the 16 non-collaborative patients) (*Chi-square* $p=0.000$) (Fig. 1b).

After 6 months, the success rate was approximately 72% among 54 patients and the difference between the groups was stronger primarily due to the high drop-out rate among the non-collaborative patients (the success rates were 79% and 50% among the 42 collaborative and the 12 non-collaborative patients, respectively) (*Chi-square* $p=0.000$) (Fig. 1c).

After 12 months of treatment, the success rates were similar (91% among the 24 collaborative and 40% among the 5 non-collaborative patients) (*Chi-square* $p=0.000$), and the final overall success rate was approximately 76% among the still remaining 29 patients (Fig. 1d).

In our research, we determined as successful only a treatment whose outcome led to a decrease or a disappearance in patient oral symptoms and whose effects lasted over time after interruption. Twelve patients underwent VAS scale questionnaire during the first visit and were visited again after 1 month. VAS mean value T0 was 7.42 ± 1.98 with 3 as the

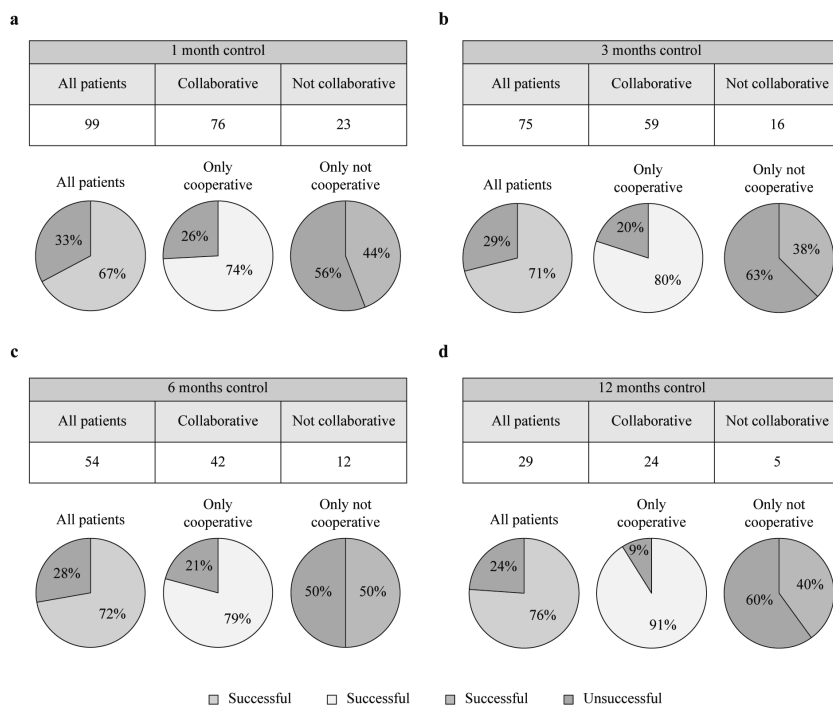


Fig. 1. Success and failure rates of treatment for the entire group and the 2 subgroups (cooperative and non-cooperative) of patients. **a):** after 1 month of treatment; **b):** after 3 month of treatment; **c):** after 6 month of treatment; **d):** after 12 month of treatment.

lowest registered value and 10 as the highest one. After 1 month, the mean score was 4.33 ± 1.92 with 2 as the lowest registered value and 7 as the highest one. The mean difference between T0 and T1 was 3.08 ± 1.83 with 1 as the lowest calculated value and 6 as the highest one.

Ten patients underwent VAS scale questionnaire also after 3 months. In this group, VAS T0 mean value was 7.4 ± 2.17 (min 3, max 10); VAS T1 mean value was 4.1 ± 2.02 (min 2, max 7) and VAS T3 mean value was 3.5 ± 1.96 (min 1, max 7). The mean difference between T0 and T1 was 3.3 ± 1.95 , while the mean difference between T1 and T3 was 0.60 ± 0.69 . *T*-Student paired samples test was used to compare the two differences and it was statistically significant ($p=0.005$), being the main improvement reported during the first month of topical capsaicin therapy and then maintained over time without larger improvement rates.

Eight patients also underwent VAS scale questionnaire after 6 months. In this group, VAS T0 mean value was 7.86 ± 2.17 (min 3, max 10); VAS T1 mean value was 4.25 ± 2.12 (min 2, max 7); VAS T3 mean value was 3.75 ± 1.98 (min 1, max 7) and VAS T6 mean value was 3.25 ± 2.38 (min 0, max 7). The mean difference between T0 and T1 was $3.63 \pm$

2.00 (min 1; max 6), the mean difference between T1 and T3 was 0.5 ± 0.76 (min 0; max 2) and the mean difference between T3 and T6 was 0.5 ± 1.41 (min 0; max 4).

T-Student paired samples test was used to compare the differences between the same patients and it was statistically significant only between the T0/T1 and T1/T3 differences ($p=0.007$), while it was not statistically significant between the T1/T3 and T3/T6 differences ($p=1$). These data confirm that the main improvement is reported after 1 month of topical therapy with capsaicin, whereas the result is maintained over time without worsening (Fig. 3).

Only 2 patients underwent VAS scale questionnaire after 12 months, therefore they were not considered for statistical analysis.

DISCUSSION

Burning mouth syndrome has a complex pathology whose aetiopathogenesis has not yet been clarified. However, it is certain that, in addition to the psychogenic factors, a neuropathic component is always present. In our experience, we believe that both the neuropathic and the psychogenic theories are correct because 90% of BMS patients have been suffering anxiety or depression for many years before the beginning of oral symptoms; however, these patients also describe the symptoms in the same manner, and their complaints are often improved by topical therapies, which suggest the presence of peripheral neuropathic involvement (13).

Thus, the use of topical therapies appears to be justified for the treatment of oral symptoms. Here, we reported our clinical experience with the use of topical capsaicin mouth rinses to achieve desensitisation of the small nerve fibres that are responsible for oral burning sensations. Burning sensation can be also related to inflammatory condition (25-36).

Our study suggests that the success rates of various topical therapies depend on the relative contributions of the neuropathic and psychogenic components of the syndrome. When a predominant neuropathic component of the syndrome is recognizable, topical capsaicin proved to be more effective, since oral burning is probably related to a small amyelinic

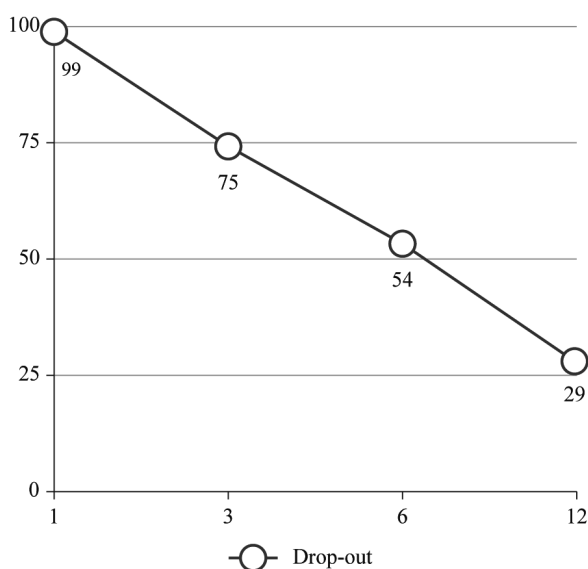


Fig. 2. The drop-out curve over the course of the observational period.

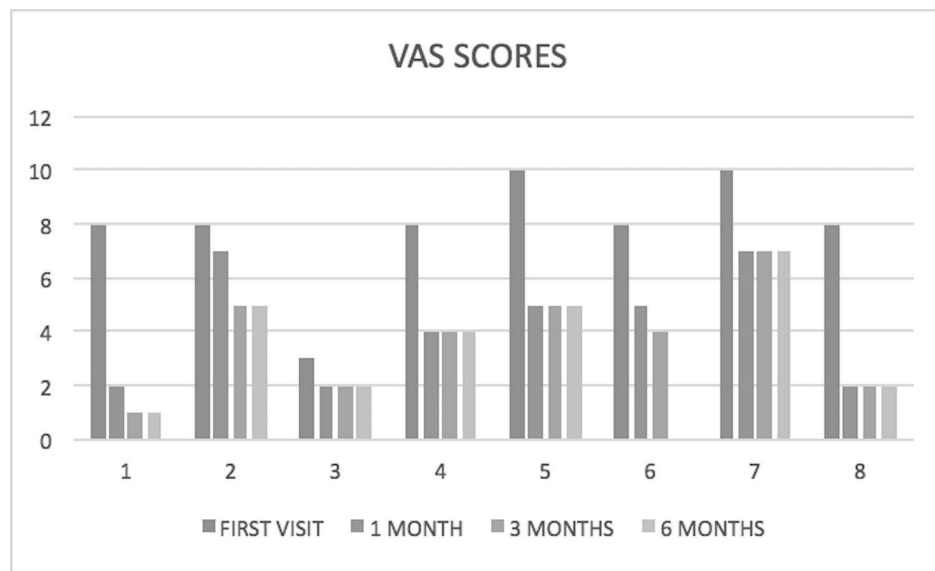


Fig. 3. The histograms show how the main improvement was reached after 1 month of topical therapy and maintained over time as registered during the following control visit.

c fibers dysfunction. On the contrary, when we deal with a predominant psychogenic origin of the syndrome, only a thorough psychiatric therapy could improve oral symptoms, while topical therapies will never be effective.

The limitations of our study include the following: there was no placebo treatment, and there were no quantitative measurements of the improvements in the symptoms in all the 99 patients. Thus, an additional randomised double-blind placebo-controlled trial is necessary to validate our hypothesis. Furthermore, a more thorough protocol to determine the inclusion and exclusion criteria is missing, especially in the strict subdivision among collaborative and non-collaborative patients.

However, based on our clinical experience, topical capsaicin was found to be much more effective than other topical or systemic remedies, such as alpha-lipoic acid or clonazepam drops, which have success rates of approximately 30% (13, 15-19) and the effects of these treatments are short-lived.

One difficulty encountered during the study was the management of the physiologic dropout of patients during the observational period. Patients who experienced a very quick improvement in their oral symptoms interrupted the prosecution of the

therapeutic protocol, while on the contrary those patients with a strong psychogenic manifestation of the syndrome lost their compliance because they became frustrated for the failure of all treatment tested by different specialists.

In conclusion, the use of topical capsaicin can improve the oral discomfort of BMS patients, but it is more effective for those patients in which the neuropathic component of the syndrome is predominant, as revealed by specific psychological multi-dimensional scale questionnaires. Psychotherapy and psychiatry remain the key therapies that will aid our understanding and treatment of this complex disease.

The purpose of this retrospective analysis was to invite other clinicians to test topical capsaicin and to report their findings to provide new perspectives on the treatment of burning mouth syndrome.

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PERIODONTAL MICROBIOMA AND RHEUMATOID ARTHRITIS: THE ROLE OF PORPHYROMONAS GINGIVALIS

L. AZZI¹, S. RANIA², R. VINCI³, F. SPADARI⁴, F. CROVERI¹, C. SCOGNAMIGLIO¹,
D. FARRONATO¹, L. TETTAMANTI¹, A. TAGLIABUE¹,
J. SILVESTRE-RANGIL⁵ and C. BELLINTANI⁶

¹Department of Medicine and Surgery, University of Insubria, ASST dei Sette Laghi, Dental Clinic, Unit of Oral Pathology, Varese, Italy; ²ASST Valle Olona, Dental Clinic, Unit of Oral Surgery and Medicine, Gallarate, Varese, Italy; ³Oral Surgery, Università Vita-Salute San Raffaele, Milan Italy; ⁴Department of Biomedical, Surgical and Dental Sciences, University of Milan, Ospedale Maggiore Policlinico, Unit of Oral Pathology and Medicine, Milan; ⁵Stomatology & Oral Surgery, Dr Peset University Hospital, Department of Stomatology, University of Valencia, Spain; ⁶ASST Valle Olona, Unit of Rheumatology and Immunopathology, Gallarate, Varese, Italy

Rheumatoid Arthritis is a disease, which can be described as an autoimmune response after molecular mimicry caused by infective agents. The current study aims at evaluating the correlation between Rheumatoid Arthritis (RA) and Periodontal Disease (PD), with special attention to the microbioma detected in the gums. Thirty-four patients with RD were recruited into the current study. Among rheumatic parameters, Rheumatoid Factor (RF), anti-citrullinated protein antibody (CCP), HLA-BDR1 and DAS28 were collected. A dental clinician evaluated the periodontal screening record (PSR). Afterwards, 1 paper cone was inserted for 30 seconds into the gingival sulcus then sent to the laboratory for evaluation. Quantitative PCR of 16S rRNA genes was performed with the hydrolysis probes method to identify and evaluate the amount *Porphyromonas gingivalis*, *Tannerella forsythensis*, *Treponema denticola*, *Fusobacterium nucleatum* and *Campylobacter rectus*. There were no statistical differences in the composition of oral microbioma between PSR groups. There were no statistical significant differences between bacterial loads and serum values. On the contrary, a positive correlation was found between the presence of *Porphyromonas gingivalis* in periodontal pockets on one side and RF and CCP on the other. Therefore, the presence of *Porphyromonas gingivalis* in periodontal pockets is associated to RA inflammatory indices.

Rheumatoid Arthritis: epidemiology and diagnosis

Rheumatoid Arthritis (RA) is a systemic, chronic disease which has a multifactorial aetiology and an immunomediated pathogenesis. It affects several tissues and organs, such as the skin, blood vessels, heart, lungs and muscles, but joints are the mainly affected site. At this level, the disease is associated with the onset of a non-suppurative proliferative synovitis. Joints are typically affected bilaterally in a

symmetric pattern. The disease, if untreated, leads to the destruction of joint cartilages, bone erosion and ankylosis (1).

Disease prevalence in western countries has only recently risen, as described by paleoanthropological studies (2). RA affects about 1% of the worldwide population. The female to male ratio is 3:1, even though this difference is reduced among the elderly. The mean age ranges between 40 and 70 years, with

Key words: Porphyromonas gingivalis, rheumatoid arthritis, periodontal disease and rheumatoid arthritis

Mailing address:

Lorenzo Azzi, DMD,
Department of Medicine and Surgery,
University of Insubria, Via Piatti 10, 2110 Varese, Italy
Tel.: +39.0332-825625 - Fax: +39.0332-825655
e-mail: lorenzoazzi86@hotmail.com

0393-974X (2016)

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two peaks in young adults and in pre-menopausal women (3).

The disease is idiopathic. RA is a disease which can be explained by an autoimmune response after exposition to an unknown antigen in a genetically predisposed individual (3). It seems that the molecular mimicry caused by infective agents could be the underlying cause of the disease, likewise in other autoimmune diseases (4).

The clinical course of the disease is variable. RA usually involves the small distant joints, most frequently those of the hands and feet, with a symmetrical pattern. In 1987 the American College of Rheumatology developed Revised Criteria for the classification of RA (5). These criteria provide a sensibility rate of 94% and a specificity rate of 89%. The final diagnosis is made considering clinical features, radiographic imaging, blood chemistry and synovial liquid examination.

Serum values are important indicators for disease activity and appear as prognostic elements to distinguish between aggressive and non-aggressive forms and to determine the response to treatment.

Rheumatoid Factor (RF) is an autoantibody directed against the Fc portion of IgG antibodies and is expressed in 60-70% of patients with RA. It is not a highly specific antibody, since it can also be found in Cryoglobulinemia, Sjögren's Syndrome and chronic hepatopathies (6). However, it is a valuable prognostic factor, since it is high when RA appears aggressive and with an erosive clinical evolution.

Anti-citrullinated protein antibodies (CCP), on the contrary, are highly specific and sensible. They are characteristically expressed in RA, so their value is both diagnostic and prognostic (7).

The DAS28 is a measure of disease activity in RA. DAS stands for 'Disease Activity Score' and the number 28 refers to the 28 joints examined in this assessment (8).

There is a wide range of measures to assess disease activity in RA including examination of joints for swelling and tenderness, global scores of pain and overall status, blood markers of inflammation (e.g. ESR and CRP), questionnaires (e.g. the HAQ), X-rays and newer imaging techniques such as ultrasound and MRI. These results are then fed

into a complex mathematical formula to produce the overall disease activity score. A DAS28 of more than 3.2 implies active disease, less than 3.2 low disease activity, and less than 2.6 remission.

Periodontal Disease and pathological microbioma

With tooth decay, periodontal disease (PD) is one of the two most common diseases of the oral cavity (9-15). It affects about 80% of the world population (16). The disease involves the supporting periodontal tissues which include the gums, periodontal ligament and alveolar bone. The final evolution of the disease is the loss by exfoliation of dental elements. It can affect individuals of any age, but prevalence increases with age. It can be confined to a single group of teeth or affect the entire mouth (17). Sick gingiva appears as erythematous, edematous and painful; the presence of periodontal pockets represents a reservoir of infection, which leads to dental mobility and loss. The prerequisite for the onset of the disease is the presence of a supragingival and then subgingival bacterial biofilm around the dental element and inside the gingival sulcus (18).

It should be underlined that dental plaque contains more than 300 different bacterial species, but only a few have been associated with a high risk of developing periodontal disease (19).

The link between biofilm bacteria and periodontitis is not random. Six species have been found to be strictly involved and responsible for the clinical course of the disease: *Aggregatibacter actinomycetemcomitans* (20), *Porphyromonas gingivalis* (21), *Tannerella forsythensis* (22), *Treponema denticola* (23) which belong to the so-called "red-complex" and *Fusobacterium nucleatum* (24) and *Campylobacter rectus* (25), which belongs to the "orange-complex".

Diagnosis requires clinical, instrumental and radiographic evaluation. Clinical and instrumental criteria are bleeding on probing, depth probing, clinical attachment level, furcation involvement, teeth looseness. The different parameters are grouped into several recognized indices, which can determine the presence or absence of periodontal disease. Once a thorough periodontal examination

and diagnosis have been performed, aetiological therapy is imperative.

Firstly, professional dental cleaning (scaling) removes dental plaque from the tooth-sites and gingival sulcus. Afterwards, when periodontal pockets are present, debridement of subgingival plaque is performed through curettage, known as root planning (26). If aetiological therapy is not sufficient, the dental clinician can remove infection surgically from those areas too deep to be reached during root planning procedures.

The current study aims at evaluating the correlation between RA and PD, with special attention to the microbioma detected in the oral cavity and gums.

MATERIALS AND METHODS

Thirty-four patients with RA were recruited into the current study, 24 females and 10 males. The study was conducted in the Unit of Rheumatology, Ospedale di Gallarate, VA, Italy. The mean age was 52.85 $\pm$ 15.64.

Inclusion criteria were the diagnosis of RA and age more than 30, while the exclusion criteria were the absence of concomitant autoimmune diseases, especially Sjögren syndrome, and partial or completely edentulism. An informed consent was read, understood and signed by all patients. The rheumatologist collected data about disease severity and laboratory findings.

Among haematochemical analysis, RF, CCP and HLA-BDR1 were prescribed. The DAS28 questionnaire was completed by the physician in order to measure the clinical scores of disease activity. A dental clinician carried out a periodontal examination, collecting data from periodontal probing with special attention to depth probing.

PSR index was calculated for every patient. PSR is a clinical parameter which allows to distinguish between the presence and absence of periodontitis and its clinical severity. Afterwards, a paper cone was inserted for 30 s into the gingival sulcus near the dental element with the deepest probing value registered for every quadrant and then sent to the laboratory for evaluation in a sterile test tube.

Specimens were processed to extract and purify DNA by using a method that includes two consecutive incubations with lysozyme and proteinase K, in order to ensure an indiscriminate Gram positive and negative bacterial lysis.

Once extracted, DNA was purified through a silica spin-column (Sigma-Aldrich, St. Louis, MO, USA).

Quantitative PCR of 16S rRNA genes was performed with the hydrolysis probes method to identify and evaluate the amount of 5 bacterial species: *Porphyromonas gingivalis*, *Tannerella forsythensis*, *Treponema denticola*, *Fusobacterium nucleatum* and *Campylobacter rectus*.

The 845 sequences of 16S rRNA gene of the Human Oral Microbiome Database (HOMD 16S rRNA RefSeq Version 10.1) were aligned to find either a consensus sequence or less preserved spots, useful to optimize the specificity of primers and dual labelled hydrolysis probes. PCR oligonucleotide sequences were designed by Primer3web (<http://primer3.ut.ee/>) and Primer Express (Life Technologies) software. The specificity of PCR assays was also checked by Primer-Blast (<http://www.ncbi.nlm.gov/tools/primer-blast/>). Absolute quantification assays were performed by using a 7500 Sequence detection System (Applied Biosystems). The thermal cycle included 10 min incubation at 95°C to activate polymerase, followed by a two-step amplification of 15 s at 95°C and 60 s at 57°C for 40 cycles. Each experiment included non-template controls to exclude reagents contamination and serial dilutions of the specific synthetic template (Eurofin MWG Operon, Ebersberg Germany). These positive controls were used to plot standard curves, i.e. threshold cycle values against the log of the copy number, that were used either to check amplification efficiency and for quantification of targets in each sample.

Data from quantitative PCR included the amount of each of the 5 investigated bacterial species and a measurement of the bacterial load from up to 34 patients. These amounts matched the number of DNA molecules detected in the real time PCR tubes that was directly related to the number of bacteria in the specimens. In order to enhance data analysis with noise removal, statistical analysis was performed on relative amounts, calculated as ratios between the amount of each species and the total bacterial load. This was able to reduce variability due to random factors such as specimen conservation, DNA extraction efficiency, and purification yield, as well as variability due to systematic factors, for instance the higher amount of bacteria expected in specimens from deeper pockets characterizing periodontitis. Comparative statistics among the different groups was performed using the IBM SPSS version 20.0, SPSS Inc, Chicago, IL, USA.

RESULTS

A total number of 34 patients affected by RA were recruited in the current study, 24 females and 10 males. The mean age was 52.85 $\pm$ 15.64.

Rheumatoid parameters collected from haematochemical analysis were the RF, the CCP and the presence of HLA-BDR1. The rheumatologist fulfilled the DAS-28 questionnaire to gather a total score of disease for every patient.

RF was positive in 21 patients and negative in 13 ones. CCP was positive in 19 patients and negative in 15 patients. HLA-BDR1 was registered in 10 patients while it did not appear in the remaining 24. DAS-28 ruled out 17 patients with a score of 1 ($x < 2.6$), 9 with a score of 2 ($2.6 < x < 3.2$) and 8 with a score of 3 ($x > 3.2$); class 2 and 3 were united in a unique class so that the division was between 17 patients with absent disease activity and 17 with higher scores of disease.

To test the concordance of results between rheumatic parameters a *Chi-square* analysis was performed. Rheumatic factors among them showed strict correlations within the population, according to data from the international literature (RF vs CCP $p=0.000$; RF vs HLA $p=0.003$; CCP vs HLA $p=0.001$).

DAS-28 is a parameter which results from both laboratory findings and clinical evaluation of the disease. Thus it is less associated with rheumatic parameters than the others do (DAS-28 vs RF $p=0.078$; DAS-28 vs CCP $p=0.016$; DAS-28 vs HLA $p=0.452$). Periodontal screening record (PSR) was calculated for every patient by the dental clinician. Twenty-two patients show a PSR score below 3, while on the contrary 12 reported a PSR greater than 3.

Microbiological analysis was performed comparing the mean load of every bacterium with PSR and with Rheumatoid Arthritis parameters using

the *T*-student parametric test for independent values.

Porphyromonas gingivalis (PG), *Tannerella forsythensis* (TF), *Treponema denticola* (TD), *Fusobacterium nucleatum* (FN) and *Campylobacter rectus* (CR) mean loads were compared to the groups of patients individuated after PSR. There were not any statistical difference in the composition of oral microbioma between the two groups (PSR vs PG $p=0.512$; PSR vs TF $p=0.434$; PSR vs TD $p=0.663$; PSR vs FN $p=0.230$; PSR vs CR $p=0.874$), being the bacterial infection influenced by other factors and not by clinical scoring of periodontal disease. Afterwards, bacterial mean loads were compared with Rheumatic parameters to check whether rheumatoid disease itself or the activity of the disease may represent a risk factor of aggressive pathogens in periodontitis.

There were no statistically significant differences (RF vs PG $p=0.295$; RF vs TF $p=0.115$; RF vs TD $p=0.113$; RF vs FN $p=0.848$; RF vs CR $p=0.367$; CCP vs PG $p=0.167$; CCP vs TF $p=0.066$; CCP vs TD $p=0.094$; CCP vs FN $p=0.560$; CCP vs CR $p=0.244$; HLA vs PG $p=0.757$; HLA vs TF $p=0.529$; HLA vs TD $p=0.691$; HLA vs FN $p=0.863$; HLA vs CR $p=0.957$; DAS-28 vs PG $p=0.160$; DAS-28 vs TF $p=0.111$; DAS-28 vs TD $p=0.144$; DAS-28 vs FN $p=0.055$; DAS-28 vs CR $p=0.060$). Only the orange-group bacteria were almost significantly associated to CCP and DAS-28.

On the contrary, a positive correlation was found between the presence of *Porphyromonas gingivalis* in periodontal pockets on one side and RF and Anti-citrullinated protein antibody on the other by performing a *Chi-square* analysis (PG vs RF $p=0.01$; PG vs CCP $p=0.007$; PG vs HLA $p=0.228$; PG vs DAS-28 $p=0.151$). Therefore, the presence of *Porphyromonas gingivalis* in periodontal pockets is associated to RA inflammatory indices, even though the total amount is not quantitatively significant.

Table I. *T*-Student test for comparison between bacterial mean load and PSR subgroups.

	PG	TF	TD	FN	CR
PSR	0.512	0.434	0.663	0.230	0.874

Table II. *T-Student test for comparison between bacterial mean loads and rheumatic parameters.*

	PG	TF	TD	FN	CR
RF	0.295	0.115	0.113	0.848	0.367
CCP	0.167	0.066	0.094	0.560	0.244
HLA	0.757	0.529	0.691	0.863	0.957
DAS-28	0.160	0.111	0.144	0.055	0.060

Table III. *Chi-square analysis for comparison between Porhyromonas gingivalis and rheumatic parameters.*

a

	PG POSITIVE	PG NEGATIVE
RF POSITIVE	18	3
RF NEGATIVE	4	9

Chi-square p=0.001

b

	PG POSITIVE	PG NEGATIVE
CCP POSITIVE	16	3
CCP NEGATIVE	6	9

Chi-square p=0.007

c

	PG POSITIVE	PG NEGATIVE
HLA POSITIVE	8	2
HLA NEGATIVE	14	10

Chi-square p=0.228

d

	PG POSITIVE	PG NEGATIVE
DAS-28 HIGH	13	4
DAS-28 LOW	9	8

Chi-square p=0.151

DISCUSSION

RA is a systemic, chronic disease which is characterized by a multifactorial aetiology and an immunomediated pathogenesis. About 1% of the world population is affected, especially women aged between 40 and 70 years (27).

Even though the disease is idiopathic, since it is an autoimmune response without an apparent causal agent, recent findings in the literature have tried to explain how genetic predisposition and environmental factors may play a role in the maintenance of chronic inflammation in the joints and other organs (1).

The oral cavity represents a reservoir of more than 300 bacterial species which colonize gingival and periodontal tissues leading to a chronic inflammatory state, where bacterial virulence is thwarted by host immunologic defenses (19).

The chronic stimulation of the immune system due to the presence of bacterial antigens within periodontal tissues has been associated with several systemic autoimmune diseases, like diabetes mellitus

(28) or other pathologies, infective endocarditis (29) or cardiovascular atherosclerosis (30).

A total group of 34 patients were recruited in the current study, 24 females and 10 males. Among rheumatic parameters RF (7), CCP (7) and HLA-BDR1 (31) were collected by blood samples, while DAS-28 questionnaire was fulfilled by the rheumatologist in order to establish a recognized score of the disease activity. The statistical analysis focused on the role of periodontal inflammation and microbioma and their role in RA.

PSR was registered for every patient and PSR=3 was considered the threshold according to which patients were subdivided into two groups: individuals with periodontitis and patients with healthy conditions.

Microbiological analysis was performed to establish the mean load of *Porhyromonas gingivalis* (PG), *Tannerella forsythensis* (TF), *Treponema denticola* (TD), *Fusobacterium nucleatum* (FN) and *Campylobacter rectus* (CR) in periodontal pockets and gingival sulcus of recruited patients.

There was no statistically significant difference between the microbioma composition in patients with PSR <3 and PSR >3. It means that the clinical status of gingival tissues is not directly correlated to which bacteria colonize periodontal pockets. Afterwards, the comparison between the bacterial mean load and rheumatic parameters demonstrated no correlation with the exception of *Porphyromonas gingivalis*, whose presence in periodontal pockets was associated with the presence of rheumatic inflammatory indices, even though independently from the mean load (PG vs RF $p=0.001$; PG vs CCP $p=0.007$).

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THE “ELFIN FACE”: CRANIOFACIAL AND DENTAL ASPECTS OF THE WILLIAMS-BEUREN SYNDROME

V. MAURINO¹, L. AZZI¹, R. VINCI², F. CROVERI¹, A. BOGGIO¹,
J. SILVESTRE-RANGIL³, L. TETTAMANTI¹ and A. TAGLIABUE¹

¹*Department of Medicine and Surgery, Research Centre into Paediatric Dentistry and Rare Syndromes, University of Insubria, ASST dei Sette Laghi, Dental Clinic, Varese, Italy;* ²*Oral Surgery, Università Vita-Salute San Raffaele, Milan Italy;* ³*Stomatology & Oral Surgery, Dr Peset University Hospital, Department of Stomatology, University of Valencia, Spain*

Williams Syndrome is a rare congenital disorder characterized by supraaortic stenosis, peripheral pulmonary artery stenosis, mental retard and dysmorphic facial features. As regards the dental aspects of the syndrome, the deletion of the elastin gene induced clinicians to suspect periodontal alterations with a greater frequency of gingivo-periodontitis, but on the contrary no association between the syndrome and periodontal diseases have been found. Furthermore, patients show a higher frequency of teeth hypoplasia, an abnormal tooth morphology during primary dentition (12.5%) and during permanent dentition. We present a case report of a 12-year-old Caucasian boy affected by Williams-Beuren Syndrome who visited our hospital for a dental and orthodontic evaluation.

Williams Syndrome was first described in 1961 by Williams et al. (1) as a congenital disorder characterized by supraaortic stenosis, peripheral pulmonary artery stenosis, mental retard and dysmorphic facial features. In 1962 Beuren (2) expanded the characteristics of the syndrome by describing the behavioral appearance of patients, who look happy and friendly. For this reason, the disease was named Williams-Beuren Syndrome. This disorder is also known as Idiopathic hypercalcemia (3) for the occasional idiopathic infantile hypercalcemia.

Williams-Beuren syndrome has an estimated incidence between 1:20.000 and 1:50.000 among live births and is less frequent than cleft lip and palate (4-12). It usually occurs as a sporadic new dominant condition although parent-child transmission has been reported. It affects both sexes equally, in all ethnic groups around the world. Usually, diagnosis

is made during adolescence when the classic manifestations of the syndrome come into evidence.

Williams-Beuren syndrome is a genetic disease caused by a heterozygous microdeletion of approximately 1.6 Mb on the long arm of Chromosome 7 (7q11.23). Since the karyotype analysis is not enough, FISH examination becomes necessary, which permits the diagnosis in 98% of cases. Thirty genes have been identified (13) within the deleted region and among them the elastin gene (ELN) is responsible for the typical connective tissue features as hernias, lax joints, deep voice and soft skin. Recent studies (13) showed some family genes TF II-I in the region of 7q11.23 as responsible for the craniofacial and dental abnormalities since they are widely expressed during the odontogenic process.

Most patients show a mental retard with IQ values between 41 and 80 (14), but their cognitive

Key words: Syndrome, face, malformation, tooth, occlusion

Mailing address:

Lorenzo Azzi, DMD,
Department of Medicine and Surgery,
University of Insubria, Via Piatti 10,
2110 Varese, Italy
Tel.: +39.0332-825625 - Fax: +39.0332-825655
e-mail: lorenzoazzi86@hotmail.com

0393-974X (2016)

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profile is very particular, since they achieve better results in verbal expression rather than in visual-spatial recognition.

Their behavior appears to be happy and friendly; the subjects are affectionate and sociable and seek contact with adults, even with strangers. They have a good memory for faces and sounds referring an oversensitivity to the latter (hyperacusia), show difficulties in concentrating and express a generalized anxiety disorder.

Among the major clinical problems, congenital heart problems occupy the first place (3, 14), such as supravulvar aortic stenosis (75%) and peripheral pulmonary artery stenosis (50%). Less common are ventricular septal defect, atrial septal defect and mitral valve prolapse.

Consequently, individuals affected by this syndrome develop hypertension more frequently at a younger age. Cardiac diseases are caused by a genetic-determined deficiency of elastine, which is an important component of the vessel walls.

Hypercalcemia was initially described as a distinguishing feature of the syndrome but recent studies found an incidence of about 15% in the first months of life with a following tendency to disappear during the second year (15).

Facial features (1, 2, 14) of these subjects are almost characteristic and begin to emerge after the second year of life, accentuating with age. They exhibit a flattened midface, depressed nasal bridge, broad forehead, prominent ears, broad nasal tip, anteverted nostrils, long philtrum, broad flattened lips, wide intercommissural distance, thick lips, wide smile and open mouth. The ear setting is low and associated with a bitemporal narrowing. Ocular findings are medial eyebrow flaring, short palpebral fissures, hypotelorism, epicanth folds, periorbital fullness and strabismus. Over 60% of patients have blue or hazel eyes with a satellite iris pattern. Usually the chin is small and not pronounced. The facial description of these subjects gives reason to the introduction of the term "Elfin face" to indicate their characteristic aspect.

Regarding the dental aspects of the syndrome, the deletion of the elastin gene has induced to suspect periodontal alterations with a greater frequency

of gingivo-periodontitis, but on the contrary no association between the syndrome and periodontal diseases have been found, probably due to an adaptive mechanism which permits the absence of loss of attack (16). However, the increase of the plaque index is caused by a poor oral hygiene in these subjects which induces a higher frequency of gingival inflammation (17). Also a greater thickening of gingiva, the presence of strong secondary labial frenula and an increased salivation have been observed (18).

Furthermore, patients show a higher frequency of teeth hypoplasia, an abnormal tooth morphology during primary dentition (12.5%) and during permanent dentition (40.7%), peg-shaped maxillary lateral incisors, bud-shaped molars, small roots, interdental spacing and microdontia (19). Hypodontia is present in 40.5% (20). The missing teeth include maxillary first and second premolars and lateral incisors, mandibular first and second premolars and central incisors without prevalence between upper jaw and mandible or between right and left side. There is a smaller crowns' dimension following the mesio-distal and the buccal-lingual directions and there is an excessive interdental spacing even if no correlation between microdontia and hypodontia seems to exist. General observation and parental history indicated that patients were roughly within normal limits for dental eruption timing and sequence.

Tongue thrusting is present in 67.7% and this could be responsible for the presence of excessive interdental spacing.

Findings also included malocclusion (85%) with a higher prevalence of Class II and Class III, open and deep bites (36.4%) and anterior crossbites.

Only few authors (20) have described the skeletal morphology of the face and four were the main characteristics described by them. A shorter length of anterior and posterior cranial base with a normal cranial base angle, an increased mandibular plane angle, a disproportion between anterior and posterior facial height and between upper and lower anterior facial height, chin deficiency and anterior inclination of the maxilla.

To date, the number of studies carried out on

patients with Williams-Beuren Syndrome regarding dental and orthodontic treatments is very low and most of them are case-reports (21-25), due to the low frequency with which this syndrome occurs. Only a few clinical trials have been published (20, 26, 27).

The main features individuated by Mass et al. (27) were: small size of anterior cranial base, deficient bony chin, increased mandibular plane angle, unusual proportion of upper to lower anterior facial height and posterior to anterior facial height. Axelsson (20) also detected an unusual shape of *sella turcica*. Tarjan (26) underlined the prominent location of the lips which are in front of the aesthetic line.

Case report

M.D.M., a 12-year-old Caucasian boy, came at our hospital for a dental and orthodontic visit. We prescribed a panoramic radiograph, a lateral radiograph, a posterior-anterior radiograph and realized dental casts. We also took several photos of the face by different angulations.

The most important alteration showed by panoramic radiography (Fig. 1A) was the presence

of multiple agenesis of teeth 1.4, 1.5, 2.5, 3.4 and 3.5 with the corresponding primary teeth persistence. There were also agenesis of 1.8-2.8 and 4.8 since their dental buds were not yet evident.

Following cephalometric analysis (Fig. 1B and Table I), we could pose the diagnosis of a skeletal Class I with a correct vertical dimension and a eugnathic forecast growth. The posterior-anterior X-ray examination (Fig. 1C) revealed a skeletal symmetry within normal values.

During the dental cast analysis (Fig. 1 D-F), a bilateral I molar and canine class with an anterior cross-bite from 1.2 to 2.2 was found. Dental midlines were coincident, overjet was negative (3 mm) and overbite was reduced (1 mm). There was a general reduction of mesiodistal dimensions of upper and lower incisors with an increased interdental space.

Photographs of the face (Fig. 2) show a good facial symmetry with a correspondence of the facial axis with the midlines of upper and lower teeth. The biotype was short-face with the upper anterior facial height decreased when compared to the lower; the ear attachment was lower but symmetrical. The



Fig. 1. A): the panoramic radiograph showed the absence of dental elements 1.4, 1.5, 2.5, 3.4, 1.8, 2.8 and 4.8; B): the lateral radiograph and the following cephalometric analysis; C): the posterior-anterior X-ray examination revealed a skeletal symmetry within normal values; D, E, F): dental casts analysis revealed a bilateral molar and canine class, an anterior cross-bite, coincident midlines, -3 mm OJ, 1mm OB and a general reduction of mesiodistal dimensions of upper and lower incisors with an increased interdental space.

Table I: *The cephalometric analysis of the clinical case.*

Skeletal sagittal dimension analysis	Mean values	12yrs values
Maxillary Position: S.N/A	82.0°± 3.5°	89.0°
Mandibular position: S.N/Pg	80.0°± 3.5°	89.0°
Intermaxillary relation: A.N/Pg	2.0° ± 2.5°	0.0°

Skeletal vertical dimension analysis	Mean values	12yrs values
Maxillary inclination: S.N/ANS.PNS	8.0°± 3.0°	1.0°
Mandibular Position: S.N/Go.Gn	33.0°± 2.5°	30.0°
Vertical intermaxillary relation: ANS.PNS/ Go.Gn	25.0°± 6.0°	29.0°

Dento-osseous analysis	Mean values	12yrs values
Upper incisor inclination: +1/ANS.PNS	100.0°± 6.0°	126.0°
Lower incisor inclination: -1/Go.Gn	94.0° ± 7.0°	102.0°
Lower incisor compensation: -1/A.Pg	2.0 ± 2.0 mm	12.0 mm

Dental analysis	Mean values	12yrs values
Overjet	3.5 ± 2.5 mm	-3.0 mm
Overbite	2.5 ± 2.5 mm	1.0 mm
Interincisal Angle	132.0° ± 6.0°	105.0°

Growth Prediction analysis	Mean values	12yrs values
Gonial Angle	125.0° ± 5.0°	132.0°
Lower Gonial Angle	72.5° ± 2.5°	71.0°
Upper Gonial Angle	52.5° ± 2.5°	61.0°
Sella Turcica Angle	122.0° ± 5.0°	119.0°
Joint Angle	143.0° ± 6.0°	140.0°
Σ1-2-3	396.0° ± 6.0°	391.0°
Anterior Cranial Base (S-N)	65.2 mm ± 3.0 mm	71.0 mm
Mandibular Body Length (Goc-Me)	59.3 mm± 5.0 mm	78.0 mm
Posterior Cranial Base (S-Ar)	34.0 mm ± 3.0 mm	34.0 mm
Mandibular Ramus Height (Ar-Goc)	47.9 mm ± 5.0 mm	41.0 mm
Posterior Facial Height PFH (S-Goc)	77.5 mm ± 7.5 mm	69.0 mm
Anterior Facial Height AFH(N-Me)	112.5 mm± 7.5 mm	108.0 mm
PFH/AFH	63.5 ± 1.5%	64.4%

lower lip was more prominent than the upper and it is in front of the aesthetic line, while the chin button was deficient, although the mandible was longer than normal.

By overlaying published data to those found in our case report, we could note some correlation.

Sure enough, the patient presented lack of several teeth: 3 second premolars, 2 first premolars and 3 third molars. There also were wide spaces between the teeth both in upper and in lower arches due to the reduced mesio-distal dimensions of the dental crowns and to a more buccal position of the upper

(126°) and lower (102°) incisors, probably caused by tongue thrusting. Even the presence of an anterior cross-bite turns out to be a common feature in these subjects.

In regards to the craniofacial aspects, in accordance with the literature, the altered values we were searching for were anterior and posterior cranial base length (with consequent maxillary deficiency), mandibular plane angle, bony chin, upper and lower anterior facial height and lips in front of the aesthetic line. The examination of the patient in this case did not detect a good correlation with the data reported in the literature as the cranial base was not small (SN=71mm), the angle of the mandibular plane was slightly decreased (S-N(Go-Gn=30° and ANS/Go-Gn=29°), whilst in most of these patients it had increased. The upper (47 mm) and lower (60 mm) facial heights were normal while the projection of the chin was reduced (N-B / Pog=-1mm) as reported by the literature.

DISCUSSION

From the analysis of our outcomes, we could conclude that the studies carried out about the dental aspects of Williams syndrome are very few due to the low frequency of disease incidence.

Often these papers aimed at proposing a simple and cheap screening method especially for the diagnosis of those patients who referred a reduced clinical symptomatology. However, in our case-report both the cephalometric and the dental analysis did not show particular characteristics that could be used for diagnosis since we found the absence of correlation of some data with those reported in the literature.

On the contrary, what our case-report showed is the increased frequency of some abnormalities, such as multiple agenesis, excessive interdental spaces, abnormal dental shape and size which need a careful follow-up in the early years of life. These features confirmed the data collected from the international literature.

The main clinical problem in carrying out a good therapy in these patients, is the lack of collaboration which is necessary during every conservative,

orthodontic or surgical approach. Patients often have severe malocclusions supported by skeletal problems and only a combined surgical-orthodontic treatment could solve them, but their severe systemic conditions, especially the cardiovascular diseases, often make it impracticable. This is the reason why in literature, we could find only one paper that describes a surgical-orthodontic treatment of a patient with Williams syndrome. Besides, although a good result could be obtained from an aesthetic and dental point of view, a very small number of patients may undergo such a type of therapy. In most cases, there is the possibility to undergo only limited orthodontic treatments to improve function and to prepare the patient for the subsequent implanto-prosthetic rehabilitation. In these circumstances, good cooperation is the key factor for successful treatment, nevertheless paying a certain attention to the difficulty of the single clinical cases and to the efforts required in treating these particular patients.

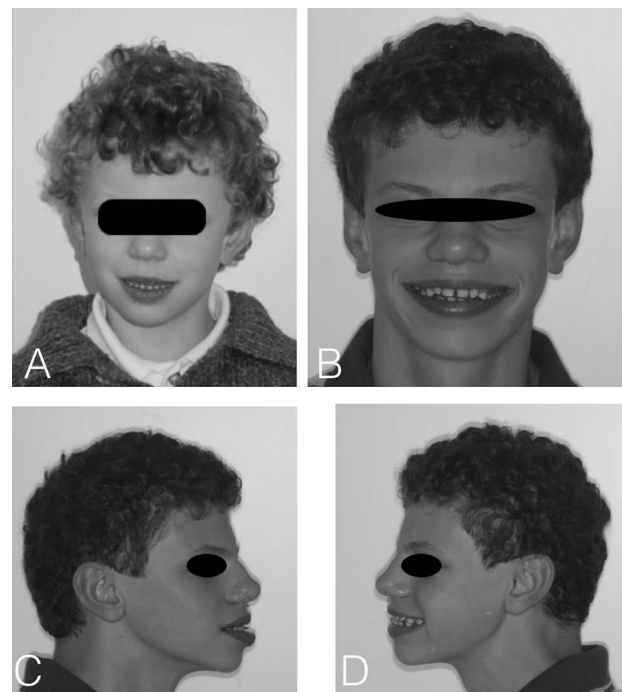


Fig. 2. *A): the patient when he was 5 years old; B, D): the patient at the age of 12, the typical “elfin-face” aspect is clearly evident.*

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ORAL MANIFESTATIONS OF SELECTIVE IGA-DEFICIENCY: REVIEW AND CASE-REPORT

L. AZZI¹, F. CROVERI¹, R. VINCI², V. MAURINO³, A. BOGGIO¹, D. MANTEGAZZA¹,
D. FARRONATO¹, A. TAGLIABUE¹, J. SILVESTRE-RANGIL⁴ and L. TETTAMANTI¹

¹Department of Surgical and Morphological Sciences, University of Insubria, Ospedale di Circolo Macchi Foundation, Unit of Oral Pathology, Varese, Italy; ²Oral Surgery, Università Vita-Salute San Raffaele, Milan Italy; ³Department of Surgical and Morphological Sciences, University of Insubria, Ospedale di Circolo Macchi Foundation, Unit of Paediatric Dentistry, Varese, Italy; ⁴Stomatology & Oral Surgery, Dr Peset University Hospital, Department of Stomatology, University of Valencia, Spain

Immunoglobulin A deficiency is the most common primary immunodeficiency defined as decreased serum level of IgA (less than 7 mg/dl) in the presence of normal levels of other immunoglobulin isotypes. Most individuals with IgA deficiency are asymptomatic and identified coincidentally. However, some patients may present with recurrent infections, allergic disorders and autoimmune manifestations, such as diabetes mellitus, Graves disease and celiac disease. The international literature has not produced any kind of review yet about intra-oral manifestations of selective IgA-deficiency. L.S., a 7-year-old Caucasian girl, was examined at our hospital. After she had undergone a professional dental cleaning, a symmetric, bilateral ulcerative gingivitis developed nearby the upper second primary molars. The gingival ulcers were persistent and did not disappear in the following 3 weeks. In the meantime, the young patient reported the presence of gastrointestinal symptoms. IgA serum level was 4.5 mg/dl, while the other isotypes levels were in the common range. The diagnosis of selective IgA-deficiency was formulated and the girl underwent further examination for the specific IgG autoantibodies in celiac disease, which were not present. Consequently, a full prevention program was planned. This case report emphasizes the role of the paediatric dentist in the early detection of systemic disorder, such as the immunological diseases. The oral cavity often reveals to be the first site of manifestation of important systemic diseases.

Immunoglobulin A (IgA) deficiency is the most common primary immunodeficiency and is defined as a decrease in serum IgA levels in the presence of normal levels of other immunoglobulin isotypes (1). Serum IgA deficiency was first described in children with ataxia-telangiectasia (2) and has since been identified in other patients, including normal patients. The prevalence of IgA deficiency ranges from 1:223 to 1:1000 in community studies and from 1:400 to 1:3000 in healthy blood donors (3).

Immunoglobulin A (IgA) was first recognised in human serum approximately 50 years ago as the second-most abundant serum immunoglobulin after IgG. Shortly thereafter, IgA, in a novel form, was found to be the primary immunoglobulin in human

secretions (4). While its role in systemic immunity is still not understood, secretory IgA antibodies can neutralise viruses, bind toxins, agglutinate bacteria, prevent bacteria from binding to mucosal epithelial cells and bind to various food antigens, preventing

Key words: IgA, IgA deficiency, selective IgA, oral IgA

Mailing address:

Lorenzo Azzi, DMD,
Department of Medicine and Surgery,
University of Insubria,
Via Piatti 10, 2110 Varese, Italy
Tel.: +39.0332-825625 - Fax: +39.0332-825655
e-mail: lorenzoazzi86@hotmail.com

0393-974X (2016)

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entry into the general circulation (5). Although secretory IgA has many known functions, the role of serum IgA is uncertain. Plasma cells producing serum IgA are located predominately in the bone marrow and to a lesser extent, in the spleen. Because serum IgA does not fix through the classical pathway, although it can do so as an aggregate by the alternative pathway, it has been suggested that IgA acts as a “discrete housekeeper”, in which foreign antigens are bound by IgA into complexes and removed by the phagocytic system, with little or no resultant inflammation (4).

Clinicians usually define selective IgA deficiency as a serum level of less than 7 mg/dl because this is the lowest level detectable in many laboratories. However, some individuals diagnosed as deficient may produce enough secretory IgA to remain healthy; some IgA-deficient individuals have normal numbers of IgA-bearing plasma cells in their intestines and produce normal levels of secretory IgA. Because secretory IgA is known to protect mucosal surfaces, it is a mystery as to why most IgA-deficient subjects remain healthy. The lack of a disease in IgA-deficient individuals is often attributed to a compensatory increase in secretory IgM. Despite the fact that most IgA-deficient subjects are not ill, IgA deficiency has been associated with an astonishing number of specific disorders. Recurrent sinopulmonary infections are the most frequent illnesses associated with IgA deficiency. The frequency of these infections in IgA-deficient subjects varies considerably (6). Patients with IgA deficiency have an increased frequency of gastrointestinal diseases, with giardiasis, nodular lymphoid hyperplasia, celiac disease, and inflammatory bowel disease as the most common (7).

A number of autoimmune diseases are associated with IgA deficiency. Among the most frequently reported are rheumatoid arthritis, haemolytic anaemia, pernicious anaemia, systemic lupus erythematosus, Still's disease, transfusion reactions due to anti-IgA antibody, dermatomyositis, vitiligo, Sjögren's syndrome, Henoch-Schönlein purpura, primary biliary cirrhosis, autoimmune hepatitis and autoimmune thyroiditis (8). Furthermore, many papers suggest that IgA deficiency can be associated

with allergic conditions. The most commonly reported disorders are allergic conjunctivitis, rhinitis, urticaria, atopic eczema, food allergy and bronchial asthma (9).

In the international literature, there are no reports of oral manifestations linked to selective IgA-deficiency. IgA is the predominant immunoglobulin isotype participating in mucosal humoral immune responses and it is the product of committed B cells that have undergone either Th cell-dependent or Th cell-independent class switching to IgA. By undergoing such class-switch recombination, while retaining the same idiotype, mature B cells diversify their repertoire of immunoglobulin genes. Class switching brought about by Th cell-dependent mechanisms gives rise to IgA with high affinity for pathogenic microorganisms and their toxins, thereby neutralising them, whereas Th cell-independent mechanisms give rise to IgA with low affinity for communal microorganisms, thereby limiting their growth (10).

Numerous studies have attempted to relate salivary IgA levels to a variety of oral diseases, such as dental caries and periodontitis (11-17). Several reviews of such reports have been published, but all available data have been, thus far, inconclusive. Distinct results may be masked because the immune defence operates in multiple layers, therefore, various compensatory activities may play a role. For example, it is well known that salivary SIgM is increased when SIgA is deficient and that compensatory IgM reacts with commensal oral bacteria, such as *S. mutans*. Here, we present a case-report of a young girl with persistent gingival ulcerations who subsequently led us to suspect and ultimately diagnose a primary selective IgA deficiency.

Case report

L.S., a 7-year-old Caucasian girl, was examined at our hospital during a first dental visit for the presence of several carious lesions. After she had undergone a professional dental cleaning, a symmetric, bilateral and ulcerative gingivitis developed near her upper second primary molars (Fig. 1). The gingival ulcers persisted for the next three weeks. Recurrent aphthous stomatitis was excluded because only the

adherent gingiva was involved. Herpetic stomatitis was also excluded because the lesions were not preceded by vesicles and they were symmetrically bilateral, non-burning, non-necrotising and not cluster-organised. In the meantime, L.S. complained of gastrointestinal symptoms, including diarrhoea and pain. We obtained a complete haematological analysis, including serum immunoglobulin isotypes, to be thorough. Surprisingly, her IgA serum level was 4.5 mg/dl, while her other isotype levels were in the normal range (Table I). A diagnosis was made of selective IgA-deficiency.

Subsequently, L.S. underwent further examination to establish if there was evidence of IgA-deficiency-linked pathologies. Given her gastrointestinal complaints, we tested for anti-endomysium, anti-gliadin and anti-transglutaminase antibodies to exclude celiac disease. Fortunately, the specific antibodies were absent, but an error was made. The anti-transglutaminase antibodies were reported as negative because the laboratory routinely searches for IgA antibodies, which are not detectable in IgA-deficient states (Table II). Therefore, we had to repeat the examination and specify anti-

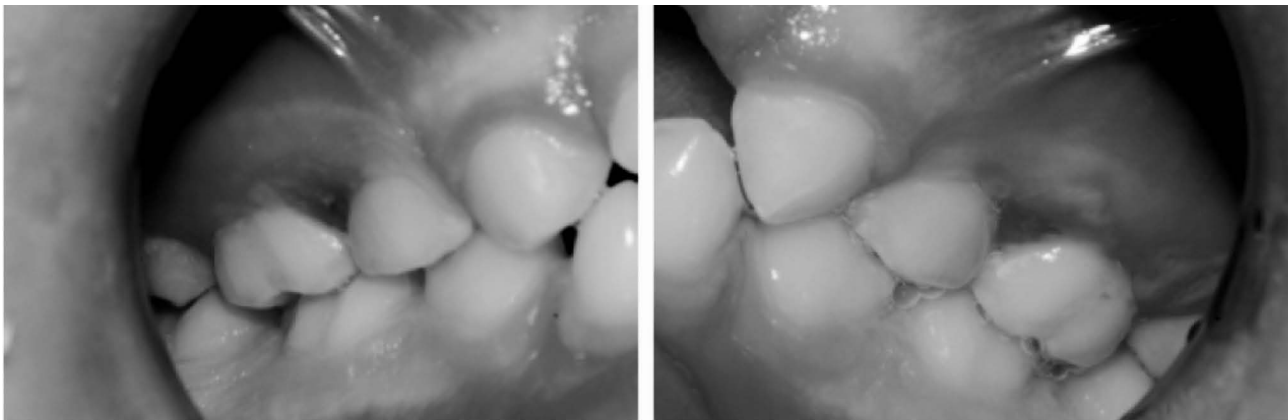


Fig. 1. Persistent gingival necrotising ulcers near the second upper primary molars. The ulcers were completely asymptomatic.

Immunoglobulin subtypes	Values (mg/dl)	Reference values (mg/dl)
IgG	270	200-360
IgM	158	45-230
IgA	4.5	70-390

Table I. Serum immunoglobulin isotype values. The diagnosis of selective Ig-A deficiency was made.

Celiac disease antibodies	Values (U/ml)	Reference values (U/ml)
Anti-gliadin (IgG)	< 11	< 11
Anti-endomysium	Negative	< 1:5 title
Anti-transglutaminase (IgA)	< 3	< 5

Table II. The specific celiac disease antibodies were potentially negative, because in IgA-deficiency, IgA anti-transglutaminase isotypes are not detected.

transglutaminase IgM antibodies to exclude celiac disease. Consequently, the patient was referred to a paediatric immunologist to rule out the presence of allergies to food or to environmental antigens, including the SIDAPA test for dental materials. These test results were negative.

Next, laboratory results for autoimmune antibodies such as ANA and ENA were obtained to exclude diseases such as idiopathic juvenile arthritis, and a complete gnathologic examination of the Temporo-mandibular Joint was made to confirm the absence of arthritis. Furthermore, genetic tests were performed to assess whether there was a familiar predisposition to autoimmune and allergic diseases. The girl's parents, who were healthy, underwent genetic testing, which was negative and fortunately both had normal serum levels of IgA immunoglobulins. Finally, L.S. entered a full dental-hygiene prevention program to minimise her risk of subsequent gingival events. Specific mouth rinses containing salivary substitutes and salivary enzymes were recommended to supplement her daily oral hygiene regimen. The gingival ulcers disappeared after 3 weeks; however, they subsequently reappeared every 2-4 weeks.

DISCUSSION

This case report highlights the importance of the role of the paediatric dentist in the diagnosis of immunologic diseases affecting children and the necessity of following a thorough protocol to prevent the main systemic manifestations of these diseases. In this case report, the presence of recurrent persistent

gingival ulcerations led us to diagnose a selective IgA-deficiency and to recommend a specific work-up to rule out the possibility of celiac disease, multiple allergies, cancer, autoimmune diseases and recurrent infections. Caution should be taken to avoid potential false-negative results by ordering tests involving the IgA antibodies, as was the case in our patient when searching for the anti-transglutaminase IgA isotype observed in celiac disease. Ultimately, we concluded that the collaboration between the paediatric dentist and the paediatric immunologist is crucial in the prevention of the evolution of a selective IgA-deficiency into a symptomatic Common Variable Immunodeficiency (CVID), which has more severe clinical manifestations.

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ODONTOMAS: REVIEW OF THE LITERATURE AND CASE REPORTS

C. GERVASONI¹, A. TRONCHET¹, S. SPOTTI¹, S. VALSECCHI¹, V. PALAZZOLO¹,
S. RICCIO¹, A. D'AIUTO², L. AZZI² and A. DI FRANCESCO¹

¹*ASST Lariana, Unit of Maxillo-facial surgery, San Fermo della Battaglia, Como, Italy;*

²*Department of Medicine and Surgery, University of Insubria, ASST dei Sette Laghi, Dental Clinic,
Unit of Oral pathology, Varese, Italy*

Odontomas are one of the most common Odontogenic Tumors of the jaw. The exact etiology of odontomas is unknown. Histologically they are composed of various formations of dental tissue (enamel, dentin, cementum and sometimes pulp). In the WHO classification, they are divided into complex odontoma and compound odontoma. Clinically, odontomas are generally asymptomatic and only in rare cases cause swelling, pain, suppuration or bony expansion. Radiologically, the tumor is initially lucent, but with time, it develops small calcifications, which eventually coalesce to form a radiodense lesion with a lucent rim. Surgical resection is the treatment of choice and there is no recurrence. The aim of this paper is to define the principal characteristics and the treatment of these lesions, based on literature and personal experience.

Odontomas are one of the most common odontogenic tumors of the jaw accounting for 22-67% of all such lesions (1, 2). They are composed of various formations of dental tissue (enamel, dentin, cementum and sometimes pulp).

The WHO classifies the odontomas into compound and complex. Differentiation between the two types is usually made through comparison of the degree of morpho-differentiation and histo-differentiation of the hard tissue.

The exact etiology of odontomas is unknown. Odontomas are generally asymptomatic, slow-growing lesions that radiologically manifest as dense radiopaque lesions surrounded by a thin radiotransparent halo. Management usually consists of either serial surveillance or surgical exeresis.

The aim of this paper is to define the principal characteristics and the treatment of these lesions, based on literature and personal experience.

Clinical, radiographic and histopathological features

Odontomas are generally asymptomatic but in rare cases cause swelling, pain, suppuration or bony expansion. Growth stops when they are histologically fully matured (1, 3-5). Many odontomas are located close to the incisal/occlusal part of an impacted tooth, thus impeding eruption. Some occur in a site where the permanent tooth is missing. Adjacent teeth may be displaced or devitalized.

Multiple-composite odontomas have been reported (6). Odontomas can also manifest as part of syndromes, such as basal cell nevus syndrome, Gardner syndrome, familial colonic adenomatosis, Tangier disease or Hermann syndrome (7, 8).

Several cases have been reported where odontomas occurred in the maxillary sinus; pain, distortion of the maxilla and chronic sinus congestion were signs

Key words: dental, tumor, jaw, tooth, benign

Mailing address:

Lorenzo Azzi, DMD,
Department of Medicine and Surgery,
University of Insubria,
Via Piatti 10, 2110 Varese, Italy
Tel.: +39.0332-825625 - Fax: +39.0332-825655
e-mail: lorenzoazzi86@hotmail.com

0393-974X (2016)

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and symptoms in almost all cases (9, 10). Infection or regional adenopathies may be observed (1).

Odontomas are generally discovered through routine radiographic examination either in dental treatment, for delays in the eruption of some permanent teeth (associated or not to persistence of the temporal tooth), or also for the presence of diastemas (5, 8, 11-13). The appearance of a palpable tumor lesion is much less common. It is important to note that these two symptoms represent almost all the clinical manifestations of odontomas (14).

Odontomas mainly occur in the permanent dentition and are rarely associated with the primary teeth (5, 13). Undiagnosed odontomas may remain in the mouth in an intrabony location for years, without causing symptoms of any kind (7, 15).

Most odontomas are small and non-aggressive, but occasionally they can reach large proportions, (up to 6 cm or more) producing bony expansion with intra- and extraoral swelling and deformity (10).

Although the etiology is unknown, they may be caused by different factors such as traumatism, infections, growth pressure, odontoblastic hyperactivity or alterations of the genetic components responsible for controlling dental development (1, 8, 11, 16).

Three different development stages can be identified radiographically (OPT and CT), depending on the degree of odontoma calcification. A first stage in which the lesion appears radiotransparent (due to the lack of calcification of the dental tissues), an intermediate stage characterized by partial calcification and a final stage in which the odontoma appears radiodense and is surrounded by a fine radiotransparent halo (2).

Compound odontomas appear as well-delimited pseudocystic lesions with a radiotransparent halo and contain radiodense zones. Multiple follicular formations are seen, these are separated by fibrous septae and the denticles are clearly distinguished (2, 12). Adjacent teeth may be displaced but are never resorbed. The complex type lesion shows irregularly shaped oval radiopacity, usually surrounded by a well-defined thin radiotransparent zone (1, 7).

Differentiation between the 2 types may be difficult. In the case of a compound odontoma, where extremely small conglomerated malformed teeth or tooth-like structures are numerous, the radiographic image is

similar to that of a complex odontoma.

The odontoma may present radiographic features similar to cementoblastoma, osteoma, ossifying fibroma, antrolith and osteoblastoma. However, in such cases the degree of opacity, the well-defined border, age of occurrence and zone of radiotransparency around the lesion should differentiate the complex odontoma from other opaque lesions (17). From a histological point of view odontomas are further sub-classified in:

- compound odontomas comprising of multiple, tooth-like structures.
- complex odontomas consisting of variable amounts of enamel and dentin without any resemblance to a tooth. The two previous aspects may coexist.

In the compound odontoma, loose fibrous matrix contains multiple tooth-like structures sometimes mixed with enamel matrix (the mature enamel cap is usually lost during decalcification procedures).

In the complex odontoma, mature tubular dentin with hollow structures are remnants of mature enamel (removed during decalcification procedures) and are sometimes filled with enamel matrix of immature enamel. Occasionally ghost cells are present, representing remnants of odontogenic epithelium that have undergone keratinization and/or cell death from local anoxia. Some cementum may circumscribe the mass. A dentigerous cyst may arise from the epithelial lining of fibrous capsula (18, 19). Management usually consists either of serial surveillance or of surgical extirpation and curettage followed by histopathological study to confirm the diagnosis (1, 2, 7).

Surgical removal of the odontoma with preservation of the impacted teeth (with or without denudation) is usual. Extraction should be carried out if the tooth is ectopic or heterotopic, with morphological alteration or presenting cystic lesion (20-22).

When the odontoma is small or average sized, the acceptable treatment is enucleation. Small and medium- size odontomas can usually be removed without difficulty depending on their proximity to neighboring structures. Problems arise with large odontomas, when their removal involves the sacrifice of large amounts of bone.

Different techniques for the approach to odontomas, depending on their anatomical position and size, are described in literature. If the odontoma involves the maxillary bone, the technique to approach it will be:

- Conventional transoral approach with removal of the vestibular cortex;
- Le Fort I downe fracture approach (Cheever's operation 1867) (23);
- Calwell Luc approach (24);
- Maxillary lateral antrotomy approach with bone flap repositioning and fixation (25);
- Fibroptique endoscopic surgical technique (for small size odontomas) (26).

If the odontomas involve the mandibular bone, different surgical approaches are described:

- Intraoral approach with removal the buccal cortex;
- Lingual approach with removal the lingual cortex (27);
- Segmental osteotomy with extraoral approach (28);
- Unilateral sagittal splint osteotomy.

Conservative treatment is always recommended to preserve the integrity of the maxillary/mandible bone. The surgical extirpation of the odontoma can be either "en bloc", or fractionate. In some cases, the large residual bony wall defect may need repairing with a bone graft.

The prognosis after treatment is very favorable, with scant relapse. In this context, the tendency towards relapse is greater when the lesion is removed in the non-calcified tissue stage of the developing odontomas (7, 21, 29, 30).

Eleven cases of giant odontoma (5 male and 6 females), were referred to the Oral and Maxillo-Facial Surgery Department at our Hospital in the last 5 years. The average age of the patients was 17-years-old at the time of the diagnosis. Seven lesions were located in the posterior region of the maxilla and the other in the posterior region of the inferior jaw. In total, 5 were complex lesions and 6 were compound.

Case 1

A 12-year-old boy without systemic disease was referred to our department for a slowly growing

swelling on the right region of the maxilla. There was no associated pain and no difficulty in opening the mouth.

The intraoral examination revealed swelling on the right side of the maxillary oral mucosa associated with an unerupted upper right first molar. No edema or inflammatory reaction were present.

An OPT radiograph revealed a well-delimited pseudocystic lesion with a radiotransparent halo and containing radiodense zones (Fig. 1A). A CT scan established the precise location and extent of the lesion, as well as its relation to the nearby lying anatomical structure; the result was a 5 cm diameter mass closely related to the maxillary sinus and the unerupted first molar and second molar (Fig. 1B).

The patient was taken up for surgery under general

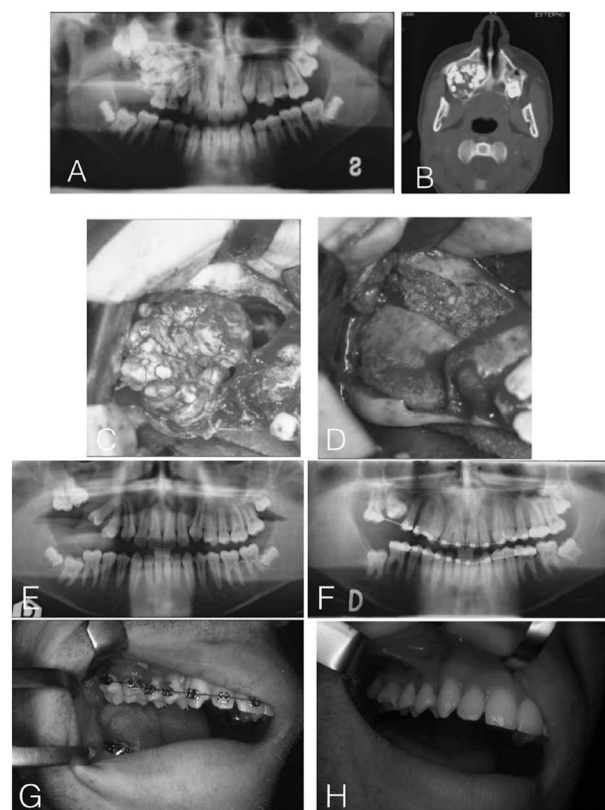


Fig. 1. *A, B): OPT radiograph and CT scan images display pseudocystic lesion with a radiotransparent halo and containing radiodense zones; C, D): intraoperative view of transoral approach by lesion removal and filling the gap with iliac bone graft and bone chips; E): radiographic view after spontaneous, but disorderly, eruption of the teeth; F, G): radiographic and clinical view during orthodontic treatment; H): final result.*

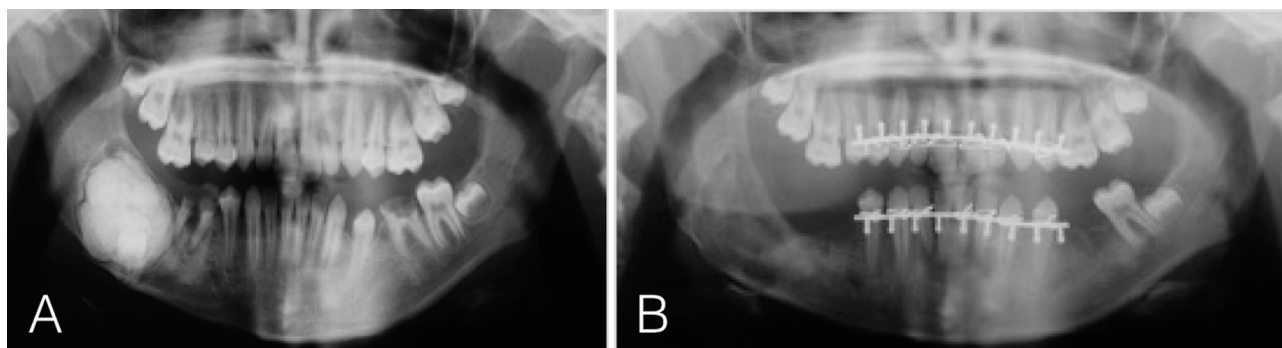


Fig. 2. *A): OPT radiograph displays a calcified mass with a radiotransparent halo in the right angle of the mandible; B): immediate post-operative OPT radiograph.*

anesthesia and a conventional transoral approach with removal of the vestibular maxillary cortex was used (Fig. 1C). The lesion was removed and the gap was filled with iliac bone graft and bone chips, to promote spontaneous eruption of the impacted teeth (Fig. 1D).

The specimen was fixed in 4% formalin solution and routinely sampled. The final diagnosis was compound odontoma. Histopathological examination revealed many small denticles with a greater degree of morphologic differentiation, so that the relationship of dentin, cementum and the enamel matrix was comparable to that of normal tissue.

The orthodontic treatment started after spontaneous eruption of premolars and molars, with a good final clinical and radiological result (Fig. 1. E-H).

Case 2

A 12 year-old girl was seen in our department with a painful swelling localized in the right part of the mandible.

Clinical intraoral examination revealed a hyperemic and aching area on the right angle and body mandible. The 46 was seriously rotten and the 47 was not detected in the oral cavity.

An OPT was done, which showed an irregularly shaped oval radiopacity, surrounded by a well-defined thin radiolucent zone with the second molar located in the distal part of the calcified mass (Fig. 2A).

A CT scan showed thinning of the buccal and lingual cortical plates, and the alveolar nerve

depressed inferiorly and buccally close to the lower border of the mandible.

The patient underwent surgery under general anesthesia and a unilateral right-side intraoral approach with removal of the buccal cortex was used. The lesion was removed with the impacted lower second molar; the rotten first molar was also taken away (Fig. 2B).

The specimen was fixed in 4% formalin solution and routinely sampled. Histopathological examination revealed many small denticles with a greater degree of morphologic differentiation, so that the relationship of dentin, cementum and the enamel matrix was comparable to that of normal tissue. The histopathological diagnosis was compound odontoma. Clinical and radiographic follow-up for two years after surgery showed good results without complication.

Case 3

A 30 year-old man without systemic disease was referred to our department for a slowly growing swelling on the left mandibular angle. No pain or difficulty in opening the mouth was associated with it.

The intraoral examination revealed swelling on the left angle of the mandible; no edema or inflammatory reaction was present.

An OPT radiograph revealed a well delimited pseudocystic lesion with a radiotransparent halo containing radiodense zones on the left angle of mandible. The radiograph demonstrated a 30 mm diameter mass with the third molar located in the infero-distal part of the calcified lesion and a dental nerve

depressed inferiorly and close to the 38 (Fig. 3A).

The treatment plan involved mass extraction with the impacted lower third molar. The patient underwent surgery with general anesthesia and a unilateral left-side sagittal split osteotomy approach was used. The lesion was removed with the impacted lower third molar. Osteosynthesis was achieved by using a miniplate, the postoperative course was uneventful (Fig. 3B).

The specimen was fixed in 4% formalin solution and routinely sampled. The final diagnosis was complex odontoma; histopathological examination revealed an irregular arrangement of dentine, enamel matrix, cementum and pulp-like connective tissue forming an irregular mass enclosed in a fibrous capsule. The patient exhibited no clinical and radiographic evidence of recurrence during the 4 years of follow-up.

DISCUSSION

The term odontoma was originally used as a general description for all mixed benign lesions of odontogenic origin composed of dental epithelial and mesenchymal cells. When classification was restricted to only those neoplastic proliferations of odontogenic cells, two types were recognized: complex and compound odontomas. Clinically, compound odontomas are approximately twice as common as complex ones.

Odontomas can be identified at any age, though many studies have reported that odontoma occurs most frequently in the second decade of life. There are no significant differences between males and females. In

relation to tumor location, most studies report a clear predominance of the upper maxilla with respect to the mandible, the most common location was the anterior portion (incisor and canine region) of the maxilla followed by the anterior portion of the mandible. Some authors also reported that the percentage of odontomas in the molar region gradually increased with each successive decade of life; other authors reported that odontomas were apparently age and location related.

Odontomas are benign tumors that usually produce no symptoms. As a result, they normally constitute casual findings in X-ray studies carried out for other reasons.

Radiologically, an odontoma manifests as a dense radiopaque lesion surrounded by a thin radiotransparent halo. In the case of a compound odontoma, the radiographic image shows a comparatively well-organized radiopaque mass of malformed teeth or tooth-like structures, usually in a radiolucent cyst-like lesion. The complex type lesion shows irregularly shaped oval radiopacity, usually surrounded by a well-defined thin radiolucent zone.

Odontomas situated in close proximity to the maxillary sinus can grow to a large size, filling the antral cavity, yet remaining asymptomatic. The most common symptom is the retention of permanent teeth, in which the odontoma impedes eruption. In such cases, the upper incisors and canines are the most commonly affected teeth. The appearance of a palpable tumor lesion is much less common. Other manifestations, although very sporadic, can include

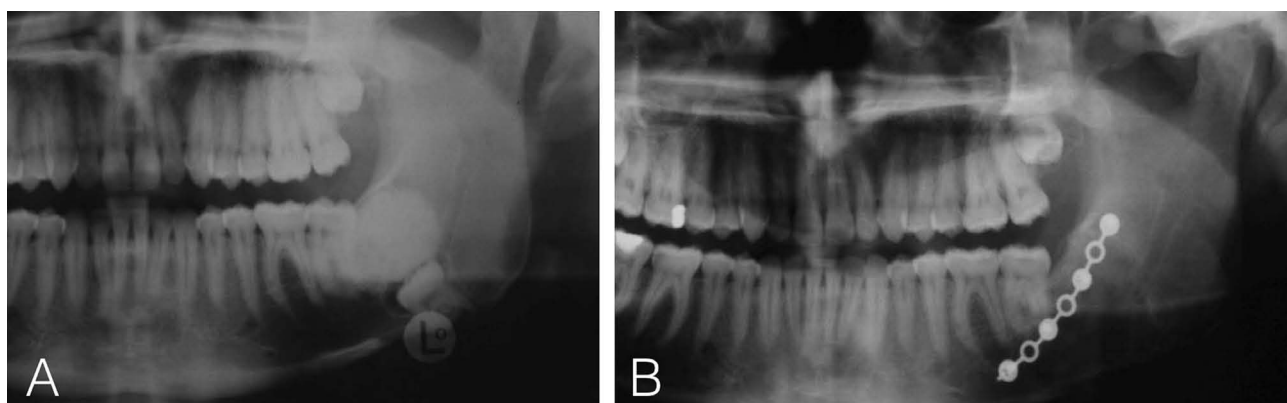


Fig. 3. *A): OPT radiograph displays an irregularly shaped oval radiopacity surrounded by a well-defined thin radiotransparent zone in the left angle of the mandible; B): OPT radiograph at 4 years follow-up.*

the agenesis of permanent teeth, pain, inflammation or infection. If not diagnosed, they may remain in the mouth in an intrabony location for years without causing symptoms of any kind.

Several cases have been reported where odontomas occurring in the maxillary sinus cause pain, distortion of the maxilla and chronic sinus congestion.

The treatment of choice recommended by the most cited authors is surgical extraction that consists of complete enucleation and curettage of the lesion and surrounding area. Careful histologic and radiographic examination of these tumors is necessary because these lesions may resemble a much more aggressive neoplasm. In general, the prognosis of these tumors is very favorable, with a scant tendency towards relapse.

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USE OF THE ORBITAL FAT PAD IN EYELID RECONSTRUCTION

A. BAJ^{1,2}, O. D'ORTO^{1,2}, M. ROMANO^{1,2}, G. A. BELTRAMINI^{1,2},
F. J. SILVESTRE³ and A. B. GIANNI^{1,2}

¹Maxillofacial and Dental Unit, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico di Milano, Milan, Italy; ²Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy; ³Stomatology & Oral Surgery, Dr Peset University Hospita, Department of Stomatology, University of Valencia, Spain

Eyelid reconstruction, though challenging, can produce a functional, esthetically pleasing lid. This should preferably be accomplished with a one-stage procedure, using robust, well-vascularized tissues similar to those being replaced, with minimal associated donor morbidity. According to the experience with Bichat pedicled flap in oral reconstruction, the authors propose the use of pedicle infraorbital fat pad for repair of conjunctival defect. Seven patients underwent eyelid reconstruction of full-thickness oncological or post-traumatic defects. In all cases, the reconstruction involved an orbital fat pad flap for the posterior lamella in addition to local flaps for the anterior lamella. All of the flaps survived. The transferred fat flap had a complete epithelization, histologically documented. No tumor relapse occurred in oncological cases. The results have been satisfactory both for clinical evaluation and for the patients. Reconstruction of the palpebral inner lamella with the orbital fat pad is a simple and low-morbidity procedure that has good morphological and functional results.

The challenge of eyelid reconstruction revolves around the restoration of complex eyelid anatomy and function, as well as recreating a natural aesthetic appearance (1-8).

The anatomy of the eyelid is made up of four constituent components: 1) the anterior lamella including skin and orbicularis oculi muscle; 2) the posterior lamella, which includes the tarsal plates and conjunctiva; 3) the medial canthal mechanism; and 4) the lateral canthus. The fundamental principle of full-thickness eyelid reconstruction (9) is the restoration of these subunits and layers, the anterior and posterior lamellae and tarsoligamentous sling. The reconstructive strategy depends on appropriate analysis and characterization of the defect. Size, composition and thickness of a palpebral defect

dictate the most appropriate surgical technique and the correct composition of the flaps or grafts used for reconstruction. Failure to reconstruct all layers results in collapse or retraction. Surgical options ranges from direct closure to local flaps in combination with grafts for bilamellar defects or lid-sharing procedures (10). Particularly, posterior lamellar reconstruction is performed to restore adequate lining and support to the eyelids such as in cicatricial scarring of the lid, or more commonly as part of the reconstruction of a full-thickness defect. The robust vascular supply of the face allows many reconstructive strategy using local flaps; a single-stage repair should be the goal.

The authors introduce a pedicled orbital fat pad for conjunctival reconstruction in full-thickness eyelid resections based on the similar use of the buccal

Key words: eye, orbit, tumors, reconstruction, soft-tissues

Mailing address:

Giada Anna Beltramini,
Maxillofacial and Dental Unit, Fondazione IRCCS Ca' Granda,
Ospedale Maggiore Policlinico di Milano,
Via Francesco Sforza 35, 20122 Milan, Italy
Tel.: +39 0255032532 - Fax: +39 0255032558
e-mail: giada.beltramini@policlinico.mi.it

0393-974X (2016)

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fat pad for reconstructing oral mucous defects. The pedicled orbital fat pad based its vascularization on terminal branches of infraorbital artery.

MATERIALS AND METHODS

The orbital pad pedicle flap was used for conjunctival repair in seven cases after eyelid tumor resection or trauma. In oncological cases the reconstruction was performed simultaneously with surgical treatment of the primary tumor; four of them had the lid removed for a basal cell carcinoma, while one suffered for a sebaceous adenocarcinoma. Patients are five women and two men, ranging in age from 35 to 83 years (mean 61.57 years).

The surgical procedures was performed in five lower eyelids and two upper eyelids; all patients had full-thickness resection up to 50% of the lid (Table I). In all cases, the palpebral repair involved the orbital fat pad flap for the posterior lamella in combination with local flaps for the anterior lamella, and a chondral graft for tarsal restoration, when necessary; we performed 3 chondral graft, one from septal and 2 from conchal cartilage.

Surgical procedure

The surgical procedure is performed under general anesthesia. After removing the tumor or scar tissues, the conjunctival fornix is horizontally incised to expose the fat compartment. The incision is made approximately 1mm below the caudal margin of the tarsal plate. The orbital fat pad is dissected and mobilized and provides up to 3 x 2 cm of pedicled tissue. The flap must be harvested slightly longer than the defect, in order to contrast healing scar contraction. The pedicled fat pad is secured to the remnants of the conjunctiva at the resection borders with resorbable sutures. In all patients the fat pad flap was left uncovered to heal spontaneously.

In our series, as usual, the anterior lamella is reconstructed with local flaps: a rotation-advancement cheek flap in lower lid reconstruction and a paramedian forehead flap or forehead galeal flap for the upper lid. To reconstruct the tarsal plate we used a chondral graft using septal or conchal cartilage.

RESULTS

In our series, all orbital fat flaps completely

survived and healed without complications. We observed that the uncovered eyelid fat pad show an epithelialized surface in 10 days, and was replaced completely by healthy-looking conjunctival tissue within 6-8 weeks after surgery.

At 6 months, all patients have received a clinical and histological evaluation. They underwent a biopsy of the inner layer of the reconstruction, which confirmed the transformation of the fat into epithelium (Fig. 1). In view of the anatomical site, we have not scheduled a series of histological samples, to avoid patient discomfort and possible scar retraction.

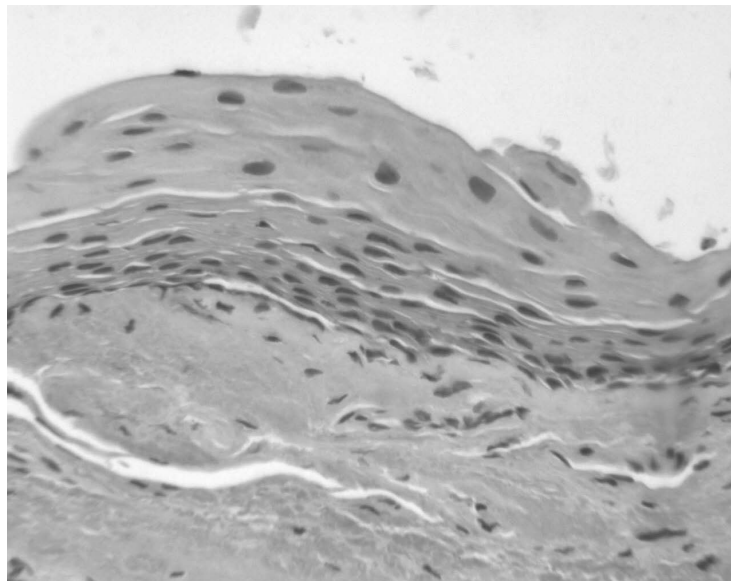
The oncological patients were followed every 3 months for 2 years and then every 6 months, according to our divisional guidelines. The total follow-up ranged from 15 to 38 months. No tumor relapse occurred. All patients had satisfactory functional and morphologic outcomes in the short, medium and long-term. None of the patients suffered from lagophthalmos, ectropion/entropion, epifora or ptosis.

DISCUSSION

The goal of eyelid reconstruction is to restore the normal anatomy and function, requiring adequate skin cover, support, and lining for the newly constructed eyelid. For the upper and lower eyelids, the anterior and posterior lamella and tarsoligamentous sling must often be reconstructed separately for optimal function (9). Most single-stage reconstructions use a combination of a flap and a graft to reconstruct the upper and lower eyelids (11-14). Any graft relies on revascularization to ensure its survival. If the graft survival is incomplete, secondary healing takes place, resulting in fibrosis and distortion. This explains the occurrence of entropion, ectropion, loss of fornix depth, and epiphora. In an attempt to overcome problems related to distortion, semi-rigid grafts can be used, such as chondro-mucosal or palatal mucosa grafts, which are often thick (15). Tarsoconjunctival grafts are an excellent choice for posterior lamellar reconstruction, since they possess the features of a normal eyelid (10). Hard palate mucoperiosteal grafts can be used to replace the posterior lamella because of their ability to provide

Table I. *Patterns of our cases.*

Patients	Age	Gender	Diagnosis	Reconstruction site
HF	38 yrs	M	Trauma, secondary reconstruction	Lower lid
CF	83 yrs	F	Basal cell carcinoma	Lower lid,
MC	67 yrs	F	Basal cell carcinoma	Lower lid,
LL	69 yrs	F	Basal cell carcinoma	Lower lid
SV	74 yrs	F	Basal cell carcinoma	Lower lid
ET	35 yrs	F	Sebaceus adenocarcinoma	Upper lid
VG	65 yrs	M	Basal cell carcinoma	Upper Lid

**Fig. 1.** *Histological examination confirmed the metaplasia of fat into epithelium. Magnification 40X, Hematoxylin and Eosin stain: stratified squamous epithelium, parakeratosis and mild chronic inflammation of the underlying stroma.*

structural support and mucosal lining (16). Buccal mucosa is another good lining option, although it lacks structural integrity and should be used in combination with a cartilage graft.

Based on our experience in the use of a buccal fat pad to reconstruct medium-sized surgical defects of the oral soft and hard tissues, we introduce a pedicled fat pad for conjunctival repair, as it is a

well-vascularized, pliable tissue with lower donor site morbidity. This flap is vascularized on terminal branches of infraorbital artery. Egyied (17) first reported the use of a Bichat fat pad in surgery; Tideman et al. (18) first described the anatomy of the buccal fat pad and reported the concept of using of this tissue as a pedicle flap and its complete epithelization without the use of a skin graft. This

technique has now been adopted widely (19). The transferred fat flap starts to epithelize within one week and is epithelized completely within 6-8 weeks; this technique does not require therefore the use of an additional mucous graft. Clinical findings indicate regenerated epithelia and mucosa, which appeared similar to normal oral mucosa; histology in healed reconstruction site show fibrous replacement of the fat tissue, and epithelization of its oral surface (20).

Similarly, the uncovered orbital fat pad flap is a simple procedure; it allows avoiding grafts and the consequent risk of scar distortion. The use of the infraorbital fat pad provides good esthetic and functional results in reconstruction of the palpebral posterior lamella.

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PARRY ROMBERG SYNDROME AND LINEAR FACIAL SCLERODERMA: MANAGEMENT IN PEDIATRIC POPULATION

E. SEGNA^{1,2}, V. PUCCIARELLI³, G.A. BELTRAMINI^{1,2},
C. SFORZA³, F. J. SILVESTRE⁴, A. B. GIANNI^{1,2} and A. BAJ^{1,2}

¹Maxillofacial and Dental Unit, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico di Milano, Milan, Italy; ²Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy; ³Functional Anatomy Research Center (FARC), Department of Biomedical Sciences for Health, University of Milan, Milan, Italy; ⁴Stomatology & Oral Surgery, Dr Peset University Hospital, Department of Stomatology, University of Valencia, Spain

Among all different types of cutaneous scleroderma, Parry Romberg syndrome and linear scleroderma “en coup de sabre” typically involve the visage. Gradual degeneration of the tissues, from the skin up to the bone, is the stigmata of the diseases and the range of clinical manifestations is wide. They typically start during childhood and slowly progress before stabilizing. Considering the gravity of the associated deformity and its impact on facial function and appearance, we strongly advocate a prompt intervention that however must be tailored on paediatric patients. There is not a general consensus on hemifacial atrophy treatment, but autologous fat grafting has been proved to be a suitable technique, due to its low morbidity, repeatability and efficacy in correcting volume defects and in improving skin texture. Following the same concept of safeness and non-invasiveness we propose 3D stereophotogrammetric acquisitions as a possible tool for the pre- and post-surgical follow up, indispensable to evaluate the surgical results. We present our case series composed of 8 paediatric patients, aged between 11 and 17 years, who underwent several lipofilling procedures, from 2012 to 2016. Starting from 2015, 3D stereophotogrammetric data has been obtained.

Linear scleroderma is characterized by localized lesions involving the extremities or the visage (1); in this second case, clinical manifestations could be assigned to two different forms, scleroderma “en coup de sabre” (LScs) and progressive hemifacial atrophy, known also as Parry-Romberg syndrome (PRS). The first one refers to a lesion of linear morphea generally located in the fronto-parietal scalp and/or in the paramedian forehead. PRS refers to the hemifacial atrophy of the skin, soft tissues, muscles,

and underlying bony structure that may be preceded by cutaneous induration, even if the skin quality is usually preserved. Sometimes these conditions co-exist in a “mixed form” (2). When the sclerotic process involves also muscles and bones, not only the morphology, but also the function is compromised. In PRS, even the central nervous system can be affected, with neurological complications, such as epilepsy and migraine (3). Other findings frequently associated are ophthalmological (enophtalmos,

Key words: linear scleroderma, three dimensional imaging, facial hemiatrophy, fat graft

Mailing address:

Eleonora Segna, MD,
Maxillofacial and Dental Unit,
Fondazione I.R.C.C.S. Cà Granda,
Ospedale Maggiore Policlinico di Milano, 20122 Milan, Italy
Tel.: +39 0255032532 - Fax: +39 0255032558
e-mail: e.segna@studenti.unimib.it

0393-974X (2016)

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uveitis, impairment of the ocular muscles) and odontostomatological (dental eruption alterations, gingival resorption and malocclusion) (4).

The etiology is still unknown, different factors are supposed to be involved: genetic factors, trauma, infections, immune-mediated processes with auto-antibodies, sympathetic and neuronal dysfunction (5). It has also been hypothesized that the *primum movens* of the disease is an endothelial damage, that leads to inflammation, ending in pro-fibrotic activity (6).

The course of the disease can be influenced by local or systemic medical therapy with immune-modulant drugs (7) and, once the final stage is reached, plastic surgery is required to restore facial morphology.

Patients have to be monitored throughout the years, in order to follow the progression of the disease and to evaluate the efficacy of treatment, both medical and surgical.

Clinical manifestations usually start during infancy, so special attention must be paid to patient care. In the present paper, we present our protocol for the treatment and follow-up of pediatric patients, which is based on low invasiveness strategies such as multiple procedures of autologous fat graft transfer, whose results are evaluated through three-dimensional (3D) stereophotogrammetric acquisitions.

MATERIALS AND METHODS

From 2012 until now, 8 children, 3 males and 5 females, aged between 11 and 17 years, were surgically treated (Table I). Every patient underwent a dermatological and rheumatologic follow up, with regularly clinical and telethermographical assessment. Overall, 18 procedures of autologous fat grafting were performed. Considering the young age, every procedure was executed under general anesthesia and the fat graft was harvested according to the Coleman technique (8), from the abdomen or from the thighs.

At first, the harvesting site was infiltrated with a saline solution enriched of epinephrine (3 mL of adrenaline in 500 mL of saline), the fat was then aspirated using a blunt cannula of 3 mm diameter and 20 cm in length attached to

a 10ml Luer-lock syringe. A light negative pressure was created by slowly withdrawing the plunger in a gradual manner and finally the fat was processed by centrifugation at 3000 rpm for 3 min. The adipose layer was purified by removing the residual oil and the blood. The average quantity of the graft injected in all procedures was 21.6 mL (min: 17 mL, max: 33 mL), according to the patients' needs and the adipose tissue availability.

Post-operatively, antibiotic therapy started in the operating room and was continued for 7 days; antalgic prophylaxis was regularly administered for the first 48 h after the surgery; a compressive bandage was maintained on the donor site for 7 days.

On average, patients' hospitalization was two-nights long, until the complete re-assessment of physiological functions. Pain was controlled by oral therapy and all complications were reported. Standard pre- and post-operative photographs of all patients were collected.

Additionally, from 2015, 3D stereophotogrammetric acquisitions of the patients were also performed, using the stereophotogrammetric system VECTRA-3D® M3 (Canfield Scientific, Inc., Fairfield, NJ). This is an optical instrument, made of a series of coordinate cameras that can simultaneously acquire facial images from different points of view. Acquired images are immediately combined and 3D facial reconstructions, with a geometrical accuracy of 1.2 mm, are obtained in few minutes (9).

To facilitate subsequent evaluations on the 3D reconstructions of the patients' facial morphology, a set of anthropometric landmarks was identified and marked on their faces, before stereophotogrammetric acquisition. Landmarks were located both visually and by gentle skin palpation and were marked using black make-up eyeliner. Land-marking procedure, performed by an expert operator, was standardized according to a previously described protocol (10). The patients sat in front of the imaging system and were asked to keep their mouth closed, teeth in loose contact and a neutral facial expression. All these procedures were not dangerous and were performed after the parents had read, comprised and signed an informed consent. Furthermore, they respected the Declaration of Helsinki and were previously approved by the local ethic committee (Università degli Studi di Milano, 27 June 2014, no.266 230 92/2014).

Subsequently, the stereophotogrammetric acquisitions were evaluated using VAM®, the VECTRA-3D® M3

associated software. The software allows to immediately appreciate, in a qualitative manner, the variations (such as volume increase or decrease), between the pre and post-surgical acquisitions. Such variations can be successively quantified, through dedicated stereophotogrammetric tools (11). Further dedicated tools allow an enhanced visualization of the skin pigmentation and vascularization.

Two of the eight children were discharged after the conclusion of their treatment. For the others six, the stereophotogrammetric data will be used for pre- and post-operative quantitative evaluations.

To illustrate our treatment protocol, we report images from two patients, thus suggesting the possible applications of this technology in the objective assessment of the results of the surgical treatments.

Case 1

Patient number 2 (Table I) arrived at our Clinic from the Rheumatologic Unit in January 2012, when she was 12-years-old. The diagnosis was linear scleroderma, subtype PRS, with involvement of the right facial side. She presented severe atrophy of the subcutaneous tissues, extended from the chin and the submandibular region to the temporal region. At clinical inspection, the affected side appeared markedly hypoplastic with a deep depression of the right mandibular angle (Fig. 1). The skin was thin, fibrotic and hyperpigmented.

The disease had started when she was 9-years-old and has been progressive for a period of 24 months, during which she underwent medical therapy with 2 bolus of metilprednisolone and 15 sessions of UVA1

Table I. Pediatric patients treated with the proposed protocol.

Patient #	Age at first treatment	Sex	Pathology	Procedures		Volume of injected fat (mL)	Operative time (min)	# of follow-up months
#1	11y	M	PRS (R)	3		21	110	65
						28	70	
						27	45	
					mean±SD	25.3±3.8	75±32.8	
#2	12y	F	PRS (L)	4		18	45	60
						21	85	
						23	80	
						24	60	
					mean±SD	21.5±2.6	67.5±18.5	
#3	14y	M	PRS (L)	3		20	95	36
						15	80	
						16	75	
					mean±SD	17±2.6	83.3±10.4	
#4	10y	F	LS (L)	3		17	65	29
						17	65	
						20	40	
					mean±SD	18±1.7	56.6±14.4	
#5	14y	F	PRS + LScs(R)	1		25	75	12
#6	17y	F	LS + lichen sclerosis (R)	2		33	85	7
						24	40	
					mean±SD	28.5±6.4	52.5±31.8	
#7	16y	M	PRS (L)	1		21	100	7
#8	14y	F	PRS (L)	1		19	45	3

PRS: Parry Romberg Syndrome; **LS:** linear scleroderma; **LScs:** linear scleroderma en coup de sabre; **R:** right side; **L:** left side; **F:** female, **M:** male.



Fig. 1: Patient n. 2 at 12 years-old, pre-operative 2D photograph.

phototherapy. Once the atrophy progression had stopped, an autologous fat grafting was proposed as reconstructive surgery. The patient and the parents were informed that more than one procedure would be needed. Fat grafts were performed every 12 months and considering the gravity of the clinical appearance, 4 surgical interventions were executed. A total quantity of 86 mL of fat was injected, ensuring very satisfying results for the surgical staff, the parents and for the young girl (Fig. 2). Telethermography confirmed a facial improvement, showing a reduction in heat dispersion after every lipofilling.

3D stereophotogrammetric acquisitions were performed before and after the last procedure, in order to better appreciate the quality of aesthetic improvement achieved after treatment (Fig. 3).

Case 2

Patient number 4 came to clinical observation when she was 10-years-old because of mild atrophy of the left mandibular region which appeared three years prior, after receiving a previous dermatologic diagnosis of linear scleroderma, which was confirmed by our group. Clinically, an area of about 4x2 cm in the right submental region was involved. Three surgical procedures were executed at 1-year distance. On average, 18 mL of fat, collected from the abdominal fat pad were injected in each surgical session. In addition to two-dimensional standard photographs, a 3D stereophotogrammetric reconstruction was acquired after the last surgery, thus also allowing a qualitative evaluation of the vascularization pattern of the involved area, through a dedicated tool (Fig. 4 a, b, c).

Furthermore, the impairment of the skin quality of the affected area was visually appreciated on the stereophotogrammetric reconstruction, through another stereophotogrammetric tool that allows a better visualization of the skin pigmentation, using brown spots (Fig. 4d).

DISCUSSION

Facial scleroderma is a very disfiguring pathology that can significantly compromise the quality of life as a consequence of the facial deformation. The visage has indeed a central role for human interactions as it hosts feelings, identity and beauty (12). The central role of the face is even more important considering the growing age: during childhood and adolescence, people start to establish relationships with others and learn how to interact therefore, malformations of the face may compromise psychological growth of the affected.

En coup de sabre scleroderma and Parry Romberg syndrome usually manifest in young age and slowly progress before stabilizing. Since the phase of active disease can last from 2 to 20 years, their psychological impact could be very important on young patients (13). For this reason, it is extremely important to correct the deformation as early as possible, in order to permit a physiological facial growth, so as not to affect psychological or social relationships of the patients.

Currently, medical therapy can help to stop the

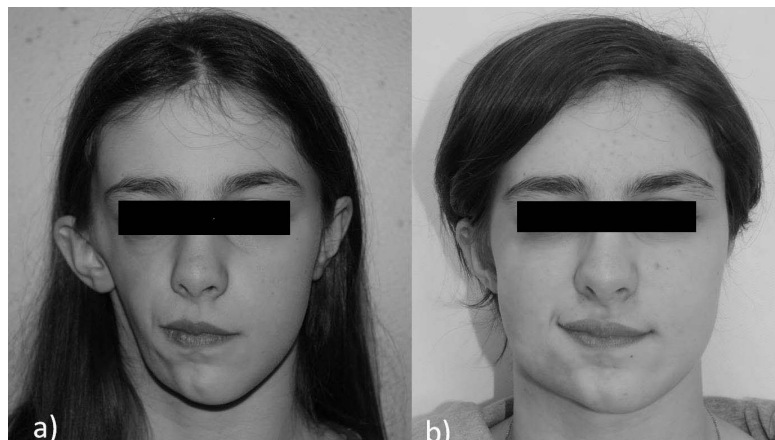


Fig. 2: Patient n. 2: *a)* pre-operative 2D frontal view, *b)* at 16 years-old, after 4 procedures of lipofilling.

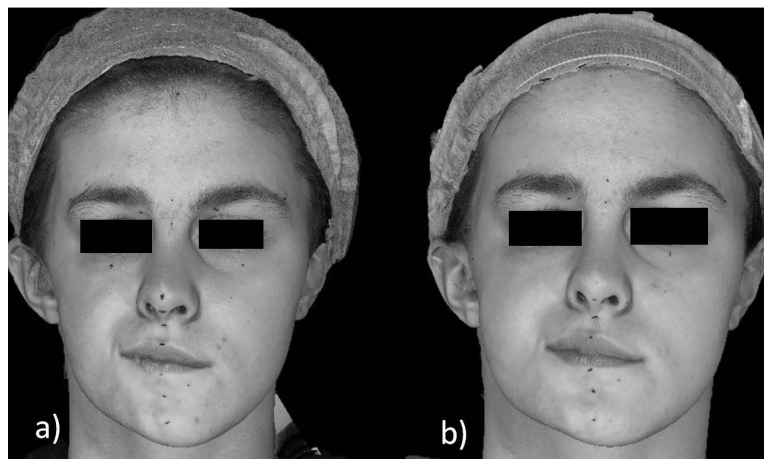


Fig. 3: Patient n. 2: 3D stereophotogrammetric reconstructions: *a)* pre-operative, *b)* post-operative.

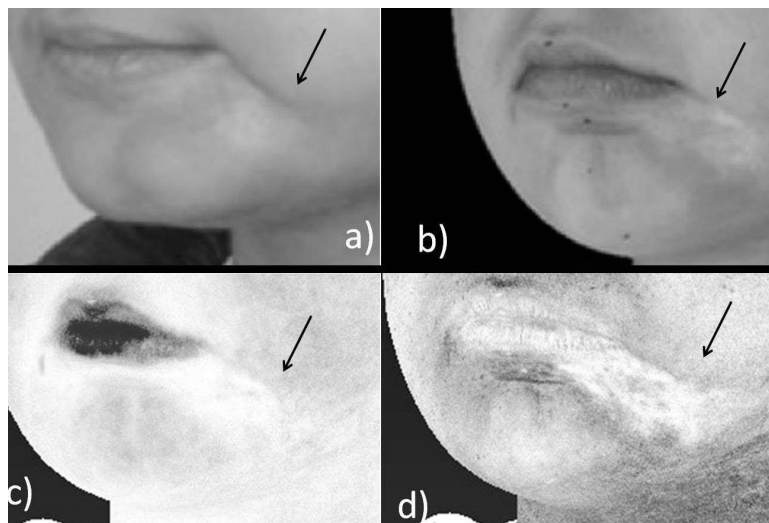


Fig. 4: Patient n. 4, details of the involved area. *a)* 2D photograph; *b)* stereophotogrammetric reconstruction; *c)* stereophotogrammetric reconstruction, using red scale; *d)* stereophotogrammetric reconstruction, using brown scale.

progression of the disease, but the facial restoration is only possible with surgery, aimed to give back the proper volume to the affected side, normalize facial symmetry, and improve skin appearance (14-22).

Different surgical techniques are employed, varying in morbidity and efficacy: local flap, such as temporalis muscle flap or galeal flap, free dermis graft, osteotomic procedure for skeletal correction, free microvascular flap (23) or filler with autologous or alloplastic materials.

Complex reconstructive techniques, with local or free flaps, are suitable options, but they require a good training in microsurgery and sometimes they are not decisive, as the flap growth may proceed not as fast as the facial one (24). Moreover, the use of heterologous prosthesis in young patients should be discouraged for risk of reactions (25) and the possible interference in facial growth (26).

On the contrary, autologous fat injection is a well-known procedure: it is easy, repeatable, has low risks and low morbidity. The efficacy of the procedure in the management of hemifacial atrophy is already well-established (27), but there are few descriptions of the method in sclerodermic pediatric patients, even if this procedure is appropriate for the treatment of young patients (28).

On the other hand, for the clinical pre and post-operative assessment, different radiological instruments are currently utilised. Among them, computed tomography (CT) scanners proved to be excellent for bone measurements, but not enough for soft tissues evaluation. Furthermore, considering the remarkable exposition to the radiations, their use must be reserved for the assessment of osseous malignancies or trauma.

Magnetic Resonance Imaging (MRI) avoids radiation exposure and is very accurate for soft tissues evaluation, but has some limits: it is expensive and has poor resolution for the dermal layer. Moreover, the exam has a long duration, and the difficulty for children to remain in the same position for all the acquisition often requires sedation mandatorily. For MRI, as for CT scanners, we retain that the price to pay is too high for a benign pathology.

Skin ultrasonography with Doppler ultrasound study is surely without risks and contraindications

and it can provide various information, such as dermal thickness and echogenicity (29): the only limit is a possible operator-dependency of the results. Like ultrasonography, thermography is non-invasive and it helps to discriminate disease activity (30), but it requires a temperature-controlled environment and is not really specific and does not provide anatomic details.

In our experience, stereophotogrammetry seems to be an adequate technique to capture the facial images of pediatric patients, in order to visualize and successively quantify the surgical treatment results (10). The reliability of stereophotogrammetric systems has already been assessed (9), together with their accuracy. Furthermore, they do not use X-rays and are fast both for image acquisition and 3d reconstruction. For these reasons, acquisition can be easily performed on pediatric patients (24, 31).

In particular, the ability to evaluate volumetric differences (32) and consequently lipofilling efficacy during the different treatment phases, offers useful information for surgical planning and for follow-up evaluation. The possibility to enhance the visualisation of skin pigmentation and vascularisation can provide additional value to clinical examination of the skin, without requiring extra examinations. Nonetheless, the chance for the patients to directly observe their facial image in three dimensions ensure a better self-evaluation of the surgical results, probably improving treatment compliance (31).

CONCLUSION

Linear localized scleroderma is the more frequent morphea in the juvenile population and the management of the affected people must take the peculiarities of the growing patients into account. A prompt intervention is useful in order to limit the developmental interferences caused by these pathologies, avoiding functional, esthetical and psychological problems and maybe making unnecessary future complex surgeries.

For the paediatric population it is mandatory to choose the less invasive surgical technique. Among all the available ones, autologous fat grafting provides good aesthetically results, improving the volume and

the skin quality, with very low morbidity; it is easy to perform and repeatable and it represents the current preferred choice.

Considering the long lasting activity and the permanency of the defect, follow up evaluations must be performed through not invasive methods. Clinical examination is absolutely the principal tool to verify the efficacy procedure, but stereophotogrammetry could help in verifying the improvement obtained by the fat graft transfer. The system is rapid, easy and does not implicate any risks. Our experience is still at the early stages but we intend to improve the use of stereophotogrammetric utilities in order to obtain not only qualitative but also quantitative data, in order to exploit all the possibilities offered by the system.

Young patients have special needs and limits, therefore we think that the best protocol for their treatment and follow-up is based on autologous fat graft transfer, together with stereophotogrammetric evaluation, in order to avoid radiation exposure (CT scanner), long lasting exams (MRI) and operator-dependent procedures (ecography and telethermography).

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BILATERAL CORONOID HYPERPLASIA: A REPORT OF SIX CASES

M. ROMANO^{1,2}, G. PORCELLINI³, D. ROSSI^{1,2},
A. BOLZONI^{1,2}, A. B. GIANNI^{1,2}, F. J. SILVESTRE⁴ and A. BAJ^{1,2}

¹Maxillofacial and Dental Unit, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico di Milano, Milan, Italy; ²Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy; ³Division of Odontostomatology, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico di Milano, Milan, Italy; ⁴Stomatology & Oral Surgery, Dr Peset University Hospital, Department of Stomatology, University of Valencia, Spain

Bilateral coronoid process hyperplasia is a rare condition defined as an abnormal elongation of the mandibular coronoid process, formed of histologically normal bone. Coronoid process hyperplasia usually develops progressively, and the clinical symptoms are often similar to those of temporomandibular joint disorders (TMD). Therefore, it is often misdiagnosed as TMD initially. From 2013 to 2016, six patients were referred to our maxillofacial surgery unit by their dentist, to evaluate mouth opening with suspected TMJ disorder. Average age was 30 years. No hypertrophy of the masseter muscles was found and the patient had no history of pain and/or dysfunction of the temporomandibular joint. Average maximal incisal opening was 16.3 mm. (Range 13-20 mm). At the end of surgery the average mouth opening achieved was of 40.3 mm. After 6 months from surgery an average mouth opening of 41 mm was obtained, with no recidivism in the coronoid process growth or decrease in the mouth opening. The only successful treatment to restore the mouth opening caused by coronoid process hyperplasia is surgical correction of coronoid-malar interference by coronoidectomy or coronoidotomy. Commencement of physiotherapy is recommended to begin between three days and one week after surgery. We also recommend the use of the TeraBite®, a simple manual physiotherapy device.

Bilateral coronoid process hyperplasia is a rare condition defined as an abnormal elongation of the mandibular coronoid process, formed of histologically normal bone. The main clinical finding is a progressive, painless difficulty in opening the mouth, due to coronoid-malar interference (1).

As it is an unusual and infrequent entity, hyperplasia of the coronoid process is often overlooked clinically. In 1853, Von Langenbeck described a situation called coronoid process hyperplasia for the first time. (2).

Coronoid hyperplasia largely affects male with a male:female ratio of 5:1 and with bilateral:unilateral

ratio of 4.7:1 (1). Many postulations have been forwarded for the pathogenesis of coronoid hyperplasia like developmental causes (3), activity of the temporal muscle (4), trauma (5, 6), endocrine influences (7), association with syndromes like trismus-pseudocamptodactyly (8) and nevus basal cell carcinoma (9). The most consistent feature of this condition is a reduction of mouth opening, although not all patients give this as their primary complaint.

No facial asymmetry is noted in most of bilateral cases but may be present in unilateral

Key words: mandible, temporalis, bone, jaw, Jacob's Disease

Mailing address:

Prof. Aldo Bruno Gianni,
Maxillofacial and Dental Unit,
Fondazione IRCCS Cà Granda Ospedale Maggiore Policlinico,
Via Francesco Sforza 35, 20122 Milan, Italy
Tel.: +39.02.55032510 - Fax: +39.02.55032815
e-mail: aldo.gianni@unimi.it

0393-974X (2016)

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coronoid enlargements (10). Coronoid process hyperplasia usually develops progressively and the clinical symptoms are often similar to those of temporomandibular joint disorders (TMD). Therefore, it is often misdiagnosed as TMD initially. For further examination, panoramic radiography can show whether the coronoid process is hyperplastic. Coronoid hyperplasia is often confused with other entities like osteoma, exostoses, osteochondroma and temporomandibular joint disorders (11).

This condition may occur as a unilateral or bilateral hyperplasia although creal-cut difference between them is obscure. This is because a unilateral coronoid hyperplasia may often have some abnormalities on the contra lateral side (1). Three-dimensional computerized tomography demonstrates coronoid process and the relation with zygomatic arch distinctly.

MATERIALS AND METHODS

From 2013 to 2016, six patients were referred to our maxillofacial surgery unit by their dentist to evaluate mouth opening with suspected TMJ disorder. All patients denied pain, click, bruxism, or trauma of the TMJ. Average age was 30 years. No hypertrophy of the masseter muscles was found and the patient had no history of pain and/or dysfunction of the temporomandibular joint. Average maximal incisal

opening was 16.3 mm. (Range 13-20 mm) (Table I).

A panoramic radiograph showed that the bilateral coronoid processes extruded above the zygomatic arch but there were no apparent changes in the mandibular condyle (Fig. 1). For all of the patients, MRI scan of TMJ performed with mouth closed and open showed no intra-articular pathology, with normal disc morphology and position, and at the TC scan, there was no evidence of bony or fibrous ankylosis of TMJ.

Hyperplasia of the coronoid process was found on both sides: the 3D reconstructions of the TC scan showed the hypertrophic bilateral coronoid process that contacted the inner surface of the zygomatic bone. (Fig. 2)

The TC scan of 3 patients showed an exostosis of bony and cartilage density tissue from the lateral aspect of the coronoid process that contacted and remodeled the inner surface of zygomatic arch, compatible with Jacob disease.

RESULTS

The surgical procedure was carried out via an intraoral approach for 3 patients and via extraoral approach by preauricular incision for the other three patients. With sagittal application of a titanium mini screw like Yoshida Technique (12) the removal of the coronoid process was easily achieved. (Fig. 3).

Immediately after surgery, the average mouth

Table I. *Patients treated between 2013 and 2016.*

Patient	Sex	Age	Pre-surgical interincisal opening (mm)	Immediate post-surgical interincisal opening (mm)	Interincisal opening after 1 year from surgery (mm)
I.O.	Male	37	16	39	39
V.B.	Male	25	20	38	40
B.M.	Male	43	13	41	39
C.G.	Male	27	19	47	45
S.N.	Male	29	13	38	43
D.M	Male	23	17	39	40



Fig. 1. *Orthopantomography showing bilateral hyperplasia of the coronoid process.*

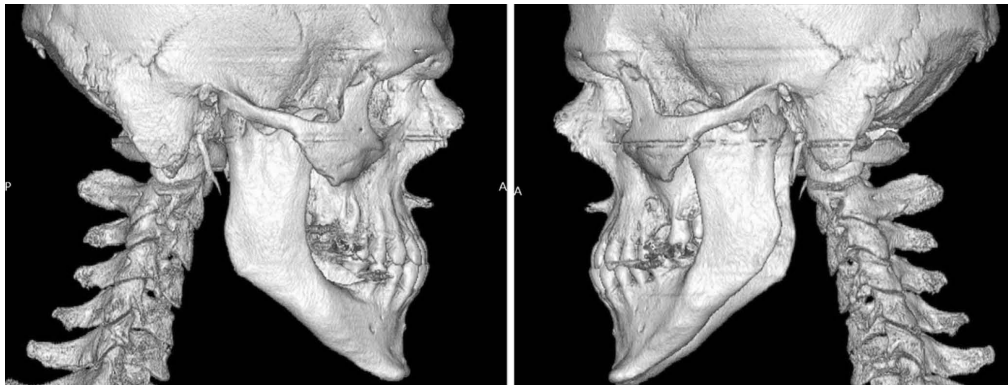


Fig. 2. *3D-CT image of bilateral coronoid process hyperplasia.*

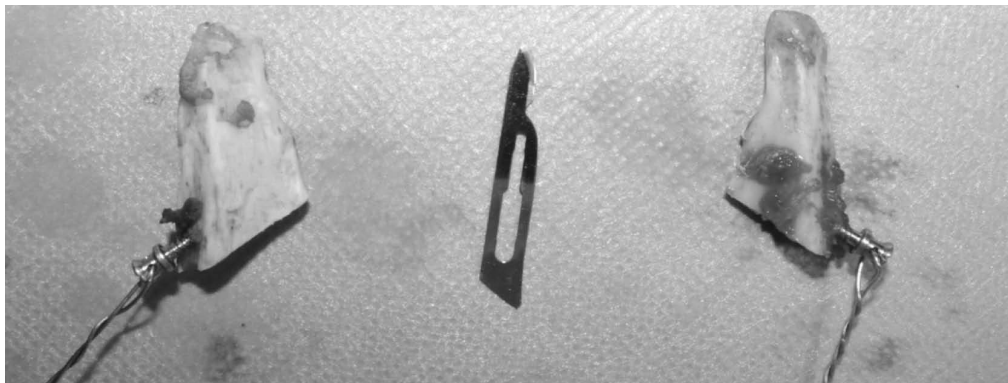


Fig. 3. *Right and left mandibular coronoid process.*

opening achieved was of 40.3 mm. (Fig. 4). Three days after surgery rehabilitation began using a Terabite®, 4-5 times a day for 15 min, over a period of 3 months. After 6 months from surgery an average mouth opening of 41 mm was obtained, with no recidivism in the coronoid process growth or decrease in the mouth opening. (Fig. 5).

DISCUSSION

Elongation of the coronoid process of the mandible was described for the first time in 1853 by Von Langebeck (2). In 1899 Jacob (13) described a synovial joint formation between an elongated coronoid process and the homolateral zygomatic bone. Since then several cases have been described, creating a certain confusion between hyperplasia of the coronoid process, Jacob's disease and even lesions such as osteoma and osteochondroma, which may also cause elongation of the coronoid process (8, 11, 14).

Unilateral and bilateral cases have been described in both men and women, at a ratio of 5:1, although bilateral cases are more frequent in men. The median age is 25 (1). The aetiology of the complaint is unknown, although several theories have been postulated, including hyperactivity of the temporal muscles, causing reactive elongation of the coronoid process (15), dysfunction of the temporo-mandibular joint caused by chronic disc displacement, which would be related with cases of unilateral hyperplasia and is mentioned as one of the causes of Jacob's

disease (16), endocrine stimuli (5), traumatism (17) and even genetic and family factors (17, 18). Trauma is thought to be a factor of coronoid process hyperplasia in some cases.

Mc Loughlin et al. (1) analyzed 31 cases of hyperplasia of the mandibular coronoid process. They discovered that many patients who suffered the disease had experienced a trauma before, especially zygomatic arch bone trauma. Some authors have suggested increased activity of the temporal muscles as a pathogenic factor (19-21).

The temporal muscle is attached to the coronoid process, to which it pulls to move the mandible. It has been reported that the osteoma responsible for constriction may be considered to be a localization of neurogenic paraosteoarthropathies (22). Endocrine abnormality may result in coronoid process hyperplasia. Bennett V. York and Steve Cockerham (3) have reported a case occurring in 2 members of 1 family, indicating that heredity may play a role in coronoid process hyperplasia.

Ferrar and Mc-Carty (9) considered TMD to be related to coronoid process hyperplasia, and they also found through an experimental study that coronoid process hyperplasia was one of the consequences of disk displacement. Isberg and Eliasson have reported 8 cases of TMD with coronoid process hyperplasia, so they thought that coronoid process hyperplasia is directly related to TMD (23).

Bilateral hyperplasia of the coronoid processes may also derive from unilateral hyperplasia. There may be a synergistic effect. Jacob's disease



Fig. 4. *Maximum mouth opening pre-surgery.*



Fig. 5. *Maximum mouth opening 1 year after surgery.*

produces restricted mouth opening, which results in bilateral temporal muscles contracting strongly. Thus, pathologic distraction osteogenesis is formed in the other coronoid process. Nonetheless, the exact pathogeny of the coronoid process hyperplasia is still controversial.

The primary complaint and clinical feature of coronoid process hyperplasia is an opening restriction (17, 24). Chronic temporomandibular joint disk displacement has been proposed as an etiologic factor of coronoid process enlargement. Consequently, the clinician easily neglects coronoid process hyperplasia when TMD exists in the same patient, in addition to painless and progressive limitation of the mouth opening, possibly evolving over years. The disorder can be diagnosed by means of radiology: panoramic radiography can reflect the morphologic change of the coronoid process. Computerized tomography shows the relationship between coronoid process and surrounding tissues, and 3-dimensional reconstructions from computerized tomography are helpful. It gives us exact information for interpretation and guidance,

especially in case of Jacob's disease (9, 23).

The main aim of treatment is to restore the mouth opening, in a stable and symmetric manner since symmetry is of paramount importance in facial aesthetic (25-32). As the problem is essentially a mechanical one, most authors (up to 90% of all cases) (1, 20) agree on intraoral coronoidectomy as the treatment of choice. This gives access to the coronoid process, decreases morbidity to the facial nerve and avoids unsightly scarring. Other authors use the submandibular approach in case of zygomatic-coronoid ankylosis, and the coronal approach has also been described in cases of very elongated coronoid process or associated lesions, such as osteochondroma (11).

It can be difficult to establish the best time to administer treatment in infants or pre-adolescents, although most authors agree that, except in patients with very severe limitation of the mouth opening (33, 34), it is best to perform the operation once the growth process has finished, in order to avoid recurrence, deformity or even restricted movement.

Hyperplasia of the coronoid process is usually treated by intraoral coronoidectomy. Some authors suggest a submandibular approach, but that has not been accepted widely. A coronal incision is considered when the coronoid process hyperplasia is accompanied by TMD; the intraoral incision is not used, because serious opening restriction usually exists in this situation.

Obtaining a satisfactory outcome will depend largely on post-operative rehabilitation. Cases of limitation of mouth opening caused by fibrosis, secondary to incorrect reorganization if there is a hematoma in the operated area (1) and even recurrence in the growth of the coronoid process have been described.

In a review of 31 cases, McLoughlin et al. (1) describes how only half of patients treated can open their mouths more than 30 mm, and suggests inadequate rehabilitation as a possible cause of this failure. Accordingly, the importance of early post-operative physiotherapy is emphasized by many authors (11, 20, 35), although most of them do not describes the protocols used. Many instruments, such as spatulas, wedges or tweezers, are habitually

used in mouth opening exercises.

It is recommended that physiotherapy begin between 3 days and 1 week after surgery. We recommend the use of the TeraBite®, a simple manual physiotherapy device

CONCLUSIONS

Successful treatment of bilateral hyperplasia of the coronoid process depends on the right surgical technique on the one hand, for which the correct approach needs to be established in each case, and on the other hand, on early post-operative physiotherapy.

The only successful treatment to restore the mouth opening caused by coronoid process hyperplasia is surgical correction of coronoid-malar interference by coronoidectomy or coronoidotomy.

Usually the intraoral approach provides sufficient access for the resection of the coronoid process and the cutting of the temporal tendon. For long-term results of surgical intervention, McLoughlin et al. (1) reported that they were disappointing for the organization of postoperative hematoma with consequent fibrosis and regeneration of the coronoid apophysis. But Gerbino et al. (20) suggested that intraoral coronoidotomy combined with prolonged postoperative physiotherapy could give satisfactory and stable results in the correction of coronoid-malar interference. Nevertheless, the sectioned part of the coronoid process united with the mandibular ascending ramus again. Regrowth of the coronoid process was observed in only 3 cases with youths (36). Except for one case in which a false joint was found between the coronoid process and the zygomatic bone, recurrence of mouth opening limitation was not observed.

For the selection of surgical treatment, if an intraoral approach is insufficient for coronoidectomy in preoperative imaging or under operation, it was considered that another approach could be selected immediately to avoid the risk of hematoma formation and consequent scarring. In addition, in our opinion, in accordance with Yoshida sagittal application of the Titanium mini screw, it is a very efficient method for removal of the coronoid process with less invasiveness.

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ORAL MELANOMA WITH OSTEOCARTILAGINEOUS DIFFERENTIATION: A CASE REPORT AND LITERATURE REVIEW

M. L. PALLOTTA^{1,2}, G. A. BELTRAMINI^{1,2}, L. MONEGHINI³,
R. MONTEVERDI⁴, A. B. GIANNI^{1,2}, F. J. SILVESTRE⁵ and A. BAJ^{1,2}

¹Maxillofacial and Dental Unit, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico di Milano, Milan, Italy; ²Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy; ³Department of Pathology "Azienda Ospedaliera Santi Paolo e Carlo", University of Milan, Milan, Italy; ⁴Department of Maxillofacial Surgery, IRCCS, Istituto ortopedico Galeazzi Milan, Italy; ⁵Stomatology & Oral Surgery, Dr Peset University Hospital, Department of Stomatology, University of Valencia, Spain

Osteocartilaginous differentiation within malignant melanoma is a rare occurrence with several implications for diagnosis. Most of the reported cases have occurred in acral lentiginous malignant melanomas. In this paper, the authors describe the clinical, morphological, immunohistochemical features and surgical treatment of a case of primary oral mucosal melanoma with osteocartilaginous differentiation and they review the existing literature. The clinical history of a 67-year-old man affected of oral malignant melanoma was described from the first presentation to the second recurrence. FISH analysis on primary lesion and on relapses showed positive results both in epithelioid and in osteocondroblastic areas. Because of the scarcity of literature in osteogenic melanoma, histological identification may be problematic and prognostic factors and therapeutic protocols are not well established. Immunohistochemical and molecular techniques can help to diagnosis this rare lesion.

Oral malignant melanoma (1) (OMM) is an extremely rare and aggressive tumour arising from the mucosal epithelium of the oral cavity. It accounts for only 0.5% of all oral malignancies, and oral melanoma represents 0.2-8.0% of all melanomas (2-4). OMM usually occurs in the fourth to sixth decades of life and is extremely rare before 30 years-of-age. OMM is known to occur more frequently in Japanese, African and North American Indian populations rather than European populations (5). Some studies have shown a male predilection (6). Most commonly involved intraoral site is palate and maxillary alveolar gingival; the labial and buccal mucosa, tongue and floor of mouth are less frequently affected (7).

Etiology of OMM is essentially unknown. Unlike cutaneous melanoma, which are mainly associated with long-term sun exposure, no definite risk factors for OMM have been defined: tobacco use as well as chronic irritation from ill-fitting dentures, have been mentioned as possible risk factors (1, 6, 7). Oral mucosal melanoma tends to be diagnosed on an average age of 55-65, in an invasive vertical growth phase, with frequent metastasis and a 5-year survival ranging from 15-40% in the literature (5).

Osteogenic melanoma is an extremely rare variant of malignant melanoma displaying a sarcomatous heterologous features with osseous and/or cartilaginous differentiation. Osteocartilaginous

Key words: melanoma, oral cancer, head and neck neoplasms

Mailing address:

Dr Giada Anna Beltrami,
Maxillofacial and Dental Unit, Fondazione IRCCS Ca' Granda,
Ospedale Maggiore Policlinico di Milano,
Via Francesco Sforza 35, 20122 Milan, Italy
Tel.: +39 0255032532 - Fax: +39 0255032558
e-mail: giada.beltrami@policlinico.mi.it

0393-974X (2016)

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sarcomatous changes in malignant melanoma are exceedingly rare findings, with still debated histogenesis, mainly occurring in acral lentiginous malignant melanoma. Since the first description of melanoma with heterologous differentiation by Wahlstrom in 1976 (8) followed by a report of bone formation in a melanoma metastasis by Urmacher in 1984 (9), only 23 cases of osteogenic melanomas have been reported in literature (10-21), 5 of which in human mucosa, 4 in the head and neck region and only one arising in human oral cavity (Table I).

The histogenesis and clinical significance of this divergent differentiation are still debated. The exact histological identification of unusual variants of malignant melanoma based on an accurate differential diagnosis with soft tissue tumours exhibiting osseous differentiation is crucial for therapeutic patient management (22).

We report the second case in literature of primary

oral melanoma with osteocartilaginous differentiation in two local recurrences arising in the hard palate.

MATERIALS AND METHODS

A 67-year-old man with no history of alcohol or nicotine abuse reported to our department with a 3 month history of an asymptomatic painful, firm, non-pulsatile, non-fluctuant, whitish mucosal nodule of the right hard palate, increasing in size over this time. A biopsy of the lesion was performed and diagnosis of amelanotic malignant melanoma was made. After surgical treatment, the tumour recurred locally twice, in 5 and in 7 years. No other primary sites and/or metastatic lesions were found at a full body computed tomography scan (CT). The histopathology report in both relapses showed the diagnosis of malignant melanoma with osteocartilaginous differentiation, which is actually, to our knowledge, the second case in literature of this kind of lesion occurring in human oral cavity (Table II).

Table I. *Patient oncological data.*

Timing	Site	Treatment	Histological pattern
T0 - Initial presentation	Right hard palate	• Biopsy • Right subtotal maxillectomy, temporalis muscle flap • No adjuvant therapies	Melanoma
T1 - 5 years	Right hard palate/reconstructive flap	• Biopsy • Excision, forearm flap, elective supraomohyoid neck dissection • No adjuvant therapies	Amelanotic malignant melanoma
T2 - 7 years	Right retromolar trigone	• Biopsy • Excision and marginal mandibulectomy	Amelanotic malignant melanoma – osteosarcomatous metaplasia
T3 - 8 years	Right upper gingiva	• Excision	Atypical melanotic elements
T4 - 9 years	Posterior oropharyngeal wall	• Excision	Malignant Melanoma

Table II. Synopsis of reports reviewed.

Author	Patient	Histological pattern	Site	Treatment	Recurrence
<i>Arising in oral cavity</i>					
Banarjee ¹³	67 yr old man	Osseous (bone and osteoid) and chondroid.	Mucosal (oral cavity, oropharynx, nasopharynx and nasal cavity)	Surgery – Radiotherapy at third relapse	Multiple local recurrences over 22 years
<i>Arising in mucosa</i>					
Hoorweg ¹⁰	75 yy old man	Bone and osteoid formation features	Nasal cavity mucosa	Surgery	Cervical lymph node metastasis in 1 y time
Takeshita ¹¹	75 yy old woman	Osteocartilaginous differentiation	Sinonasal cavity	Surgery	/
Rinaggio ⁵	76 yy old man	High grade malignant epithelioid and spindle cell neoplasm with irregular areas of cartilage formation	Nasal cavity mucosa	Radiation treatment followed by surgery	/
<i>Arising on the skin</i>					
Urmacher ²	57 yy old woman	Osseous (bone and osteoid)	Retroauricular	Surgery	/
Grunwald ³	50 yy old woman	Desmoplastic cartilaginous formation in malignant melanoma	Calf	Surgery	Further metastases in leg in 2 months
Weidner ³¹	54 yy old man	Osseous (focal osteoid-like material) and neurosarcomatous	Buttock	Surgery	Satellites and metastases over following 24 months
Moreno ⁴	77 yy old man	Malignant melanoma with heterotopic bone formation	Subungual	Surgery	Pulmonary metastases
Brisigotti ⁶	83 yy old woman	Osseous (bone and osteoid) and chondroid	Subungual	Surgery	Local recurrence in 12 months; subcutaneous metastasis in 24 months
Nakagawa ⁷	43 yy old man	Osseous (osteoid), chondroid and 'fibrosarcomatous'	Acral (sole of foot)	Surgery	/
Pellegrini ⁸	73 yy old man	Osseous (osteoid) associated with multinucleated giant cells	Back	Surgery	/
Lucas ⁹	Average age 56 yy	Amelanotic sarcomatoid malignancies with abundant osteoid matrix	Subungual /sole of foot	Surgery	Regional lymph node metastases
Toda ¹⁴	71 yy old man	Osteogenic melanoma	Subungual	Surgery	/
Cachia ¹²	57 yy old woman	Malignant melanoma with cartilaginous differentiation	Subungual	Surgery	/
Giele ³²	50 yy old man	Osseous (osteoid) and chondroid	Subungual	Surgery	/

The studies are not comparable for therapeutic approaches; in some of these, the characteristics of the patients (age, sex) are not furnished.

Surgical specimens were formalin-fixed and paraffin-embedded for histopathological examination. Standard 5 µm tissue sections were stained with hematoxylin and eosin for light microscopy examination. Immunohistochemistry was carried out on 5 µm tissue sections using the standard avidin-biotin-peroxidase complex method with antibodies for protein s-100, melanocytic antigen HMB-45, melanocytic antigen MART-1, osteonectin and vimentin. Fluorescence *in situ* hybridization (FISH) on the primary lesion and recurrences was added for the first time to immunohistochemistry in order to improve accuracy in diagnosis and knowledge in histogenesis. FISH analyse was carried out with 4 probes targeting RREB1 (RAS Responsive Element-Binding Protein 1) on 6p25, MYB (Myeloblastosis gene) on 6q23, CCND1 (Cyclin D1 gene) on 11q13, and CEP6 (Centrometric Probe of Chromosome 6) (23).

The patient had been followed with clinical examination every 2 months and Total Body CT scan to rule out metastasis. We conducted a literature review about osteocartilaginous change in oral melanoma using the database PubMed.

RESULTS

The primitive lesion was a nodular, ulcerated lesion of the right hard palate measuring 1.4 cm in its greatest dimension. The patient underwent right hemimaxillectomy with omolateral laterocervical lymphnode excision (I-II-III levels) and reconstruction with temporal flap.

Histopathologic exam showed malignant epithelioid cells characterized by nuclear round to oval pleomorphism and hyperchromasia, high nucleocytoplasmatic ratio and absent/low content of melanin, infiltrating the epithelial mucosal lining and the corion beneath. Neoplastic cells were S-100+, HMB-45+, MART-1+ and were surrounded by an intense acute inflammatory infiltrate, squamous metaplasia of ducts of minor salivary glands and focal osseous erosion. Excision was radical. No lymph node metastasis was found and no adjuvant therapies followed the surgical, radical, excision of the mass.

The lesion recurred locally 5 years later: a brownish ulcerated nodule of the hard palate of about 2 cm in diameter and a second nodular lesion of 0.8 cm in greatest dimension posteriorly, sited in

the superior alveolar process. The lesion was treated with excision and reconstruction with microvascular forearm flap.

The nodules were composed of amelanotic melanoma cells similar to those of primary lesion with small groups of circumscribed malignant cells, surrounded by clear spaces resembling chondroid lacune and with hyaline deposit of condroid matrix; foci of osteoid with osseous deposition were also present. The lesion infiltrated the chorion beneath.

Diffuse intraepithelial atypical melanocytic hyperplasia surrounded the lesion and was present in the anterior and posterior surgical margins. The lesion was immunoreactive to S-100 protein, vimentin and osteonectin; a plurifocal immunoreactivity to HMB-45 and MART-1 was observed both in epithelioid and in osteocartilaginous neoplastic components.

Seven years after the first tumour was treated, a second recurrence occurred: it was a melanoma with sarcomatoid osteocartilaginous features in vertical growth fase, invading the chorion, located in the right superior vestibular fornice. Surgery was again the treatment of choice. Neoplastic cells infiltrated the stroma beneath for 1.06 mm; atypical menocytic hyperplasia was diffusely present at the anterior and posterior margins of excision.

The patient denied adjuvant radiotherapy. At follow up, 16 months after the last surgery, the patient is free of disease, with no macroscopical radiological signs of local recurrent tumour nor distant metastasis.

FISH analysis on primary lesion and on relapses showed positive results both in epithelioid and in osteocondroblastic areas.

DISCUSSION

Diagnosis of OMM can be made based on clinical presentation of the pigmented lesion with the so-called ABCD checklist (asymmetry, border irregularities, colour variegation, and diameter) that is commonly used for cutaneous melanomas (1, 7). The most common presenting symptom is a pigmented swelling. During the initial radial growth phase, melanomas do not tend to invade the underlining tissue; haemorrhage and ulceration are

usually late findings when the lesion has entered vertical growth. OMM can be uniformly brown or black, or variable pigmentation may be present; 10% of cases are amelanotic. The lesions are asymmetric, irregular in outline and occasionally multiple. Umeda et al. (24) noted that on close examination typical OMM usually presents with 3 distinct components: a nodular component usually affecting the centre, a flat or slightly elevated, deep brownish-black pigmented plaque component and a non-elevated light brown macular component.

Induration is usually absent in cases with prolonged radial growth phase or with minimal invasion. Other signs and symptoms include bleeding, ill-fitting dentures, pain, increased mobility of teeth, and delayed healing of extraction sockets. Tanaka et al. (25) identified 5 types of OMM based on clinical appearance: pigmented nodular type, non-pigmented nodular type, pigmented macular type, pigmented mixed type and non-pigmented mixed type. Because most of the melanomas are painless in their early stages, the diagnosis is often unfortunately delayed until symptoms resulting from ulceration, growth or bleeding are noted (26). Regional lymphadenopathy may be present and connotes a poor prognosis (7).

Mucosal melanomas with osteocartilaginous differentiation are extremely rare events. Osteocartilaginous differentiation may represent a form of host response, alteration of benign stromal fibroblastic cells to differentiate along cartilaginous or osseous lines. However, the malignant appearance of the osteogenic and/or chondrogenic cells in many reported cases argues against a reactive process. Alternatively, melanoma cells may themselves undergo heterologous mesenchymal differentiation (27).

Melanomas exhibiting cartilaginous differentiation occur in older patients with an approximate equal sex distribution. Many are associated with a history of antecedent trauma, especially acral tumours. Most of the cases in literature occurred on the skin, mainly in subungual sites. Only 4 cases have been described since now arising from mucosal surfaces (12). Three cases of melanoma with osseous or osteocartilaginous differentiation have been described in the nasal cavity (12, 17, 18).

Banerjee et al. (20, 28) described the first case

of mucosal melanoma with osteocartilaginous differentiation developing in the oral cavity. The patient was a 67-year-old man who developed a multiple pigmented and non-pigmented ulcerated or nodular lesion in the oral cavity, oropharynx and nasal cavity. The biopsy revealed melanotic malignant melanoma consisting of pleomorphic spindle and epithelioid cells. The patient had 17 biopsies and excisions of lesions over 22 years, one of which showed *in-situ* and invasive melanoma with transition to an osteogenic tumour.

Melanoma is notable for an impressive degree of morphologic variability that often presents enough diagnostic challenge to make additional studies such as immunohistochemical analysis a necessity. Approximately one third of specimens lack melanin pigmentation (12). Differential diagnosis includes melanotic macule, oral pigmented nevus, smoker's melanosis, amalgam tattoo, and Kaposi's sarcoma (29). Mucosal malignant melanoma with osteocartilaginous differentiation further extend the differential diagnosis associated with melanoma in unusual sites.

In the case of the oral/nasal cavity malignant melanoma with osteocartilaginous differentiation includes sarcomatoid squamous cell carcinoma, osteogenic sarcoma, mesenchymal chondrosarcoma and malignant change in a pleomorphic adenoma (20). If an associated junctional melanocytic component or areas of conventional melanoma are identified, the diagnosis of melanoma with cartilaginous differentiation is relatively straightforward. If characteristic features of melanoma are lacking, careful histological evaluation and correlation of the microscopic findings with the clinical features is critical to arriving at the correct diagnosis. Absence of primary bony involvement and presence of regional lymph node metastases are clinical features in favour of melanoma. Furthermore, despite the presence of cytological atypia in sarcomas, location of the tumour in distal extremities (particularly subungual) favours a diagnosis of melanoma exhibiting osteocartilaginous differentiation over osteosarcoma or chondrosarcoma, which are exceedingly rare in such locations in the absence of underlying skeletal diseases (27). Formation

of osteoid, bone and cartilage may occasionally occur in melanomas osteocartilaginous differentiation with complication for diagnosis, because they could be misdiagnosed as osteosarcomas or chondrosarcomas. More specifically mucosal melanoma with osteocartilaginous differentiation can be misdiagnosed as pleomorphic adenoma, pleomorphic adenoma with osteosarcomatous change and pure osteosarcoma and mesenchymal chondrosarcoma, while subungual melanomas resemble bone/cartilage-forming pseudosarcomatous lesions of digits, such as florid reactive periostitis (also known as fibro-osseous pseudotumour), bizarre parosteal osteochondromatous proliferation and myositis ossificans.

Immunohistochemistry may also assist in the diagnosis of melanoma through the presence of an *in-situ* melanoma component and diffuse S100 protein positivity helps in making the correct diagnosis (20).

The main treatment modality for melanoma is complete excision with wide margins, the extent of which is largely determined by tumour thickness (30). Umeda et al. (24) suggested a protocol for management of OMM which refers to the extent of margins: 1) excision of the primary lesion, preferably using an intraoral approach and involving at least 1.5 cm of healthy tissue; 2) excision of any lymph node metastasis; 3) consider chemotherapy.

Local control is not a strong predictor for survival. Many patients with a presumptive radical excision of tumours develop a recurrence, which may be explained by the presence of atypical melanocytes in the surgical margins and/or the presence of satellites (7).

Achieving satisfactory tumour-free margins may be difficult in some anatomic sites, where extensive surgery is likely to result in significant cosmetic and functional defects and quality of life impairment (12).

According to Tanaka et al. (25), neck dissection should be reserved for cases with preoperatively confirmed lymph node metastases and the extent and the level of the nodes should guide the choice of the neck dissection modality. There is no proof that elective nodal dissection improves survival. Besides, it is difficult to improve the prognosis of this disease with surgery alone and sometimes, the site and size of the tumour limits radical surgery therefore adjuvant

therapy is then important (31-35).

In advanced disease, radiotherapy is a primary treatment for palliation and it is most effective for unresectable disease (36). Adjuvant chemotherapy decreased the relapse rates of both local and distant metastasis disease (37).

In contrast to cutaneous melanoma, a standardized correlation of histopathologic features and prognosis for mucosal melanomas of the head and neck has not been completed (30). Clinical stage at presentation is probably the most important prognostic factor. Thickness of the tumour, cervical lymph node metastasis, ulceration, and the anatomic sites are all independent risk factors.

Mucosal melanomas invade and spread more quickly and metastasize locally more frequently and are therefore associated with much poorer prognosis than cutaneous melanomas.

The reasons for poor prognosis are not known with certainty, but various probable reasons are put forward. The most important of them being late diagnosis of OMM due to nonspecific symptoms, high vascularity of oral mucous membrane, which along with earlier bone invasion can contribute to the high incidence of distant metastases and anatomic location, sometimes it is difficult to achieve clear margins during resection (1, 38).

CONCLUSIONS

The paucity of literature of osteogenic melanoma does not allow to attribute prognostic implication to the osteocartilaginous component. Because of its rareness, the pathogenesis and risk factor are elusive, histological identification may be problematic and prognostic factors and therapeutic protocols are not well established yet. As malignant melanomas and other human cancers with osteo- and/or condroblastic sarcomatous features have different therapies and prognosis, recognition and accurate diagnosis of this unusual variant of malignant melanoma is crucial in patient management.

The best likelihood for favourable outcome is early detection and excision of melanocytic mucosal lesions of the head and neck. The anatomy of the head and neck region will always be an obstacle during

treatment, but success can be achieved in its early diagnosis when the lesion is at an innocuous stage. Immunohistochemical and molecular techniques can help to diagnosis this rare lesion.

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BIDIRECTIONAL FLUX OF FLUIDS AND MICROBIOTA AT IMPLANT-ABUTMENT CONNECTION OF FMD STORM IMPLANT SYSTEM: AN *IN VITRO* STUDY USING RT-PCR

A. BAJ^{1,2}, M. ROMANO^{1,2}, E. SEGNA^{1,2}, A. PALMIERI³, F. CURA³, A. SCARANO⁴,
L. OTTRIA⁵ and A. B. GIANNI^{1,2}

¹Maxillofacial and Dental Unit, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico di Milano, Milan, Italy; ²Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy; ³Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna, Bologna, Italy; ⁴Department of Medical, Oral and Biotechnological Sciences and CeSi-MeT, University of Chieti-Pescara, Chieti, Italy; ⁵Dental School, Tor Vergata University, Rome, Italy

The purpose of the present microbiological study was to evaluate bacterial leakage at implant-abutment connection level of a new type of implant (Storm implant (FMD, Falappa Medical Devices®, Rome, Italy) using Real-Time Polymerase Chain Reaction (RT-PCR). This implant presents a polygonal external implant-abutment connection with a geometry that provides a hex on which engage complementary abutments. To identify the capability of the implant to protect the internal space from the external environment, the passage of genetically modified *Escherichia coli* across implant-abutment interface was evaluated. Four Storm implants (FMD, Falappa Medical Devices®, Rome, Italy) were immersed in a bacterial culture for 24 h and bacteria amount was measured inside implant-abutment interface with Real-time PCR. Bacteria were detected inside all studied implants, with a median percentage of 15% for *P. gingivalis* and 14% for *T. forsythia*. Our results are similar to those reported in the English literature. Additional studies are needed to explore the relationship in terms of microbiota between the internal implant and implant-prosthetic connection. In addition, the dynamics of internal colonization needs to be thoroughly documented in longitudinal *in vivo* studies. As a result, microbial leakage along the implant abutment interface was acceptable and considered the most probable explanation for peri-implantitis.

Every two-piece implant systems (TPISs) are composed of two or three parts. The TPIS includes the endosseous fixture, an abutment and a denture or fixed prosthesis, which ultimately restores function and aesthetics. Even though the TPIS two parts are intimately attached, a micro-gap seems inevitable at the interface (1, 2). A second and deeper micro-gap is typically formed at abutment-prosthetic level. In fact, TPISs include an abutment connected to the implant on one hand and to the prosthesis on the other hand. So that, in a TPIS, an implant-abutment and an

abutment-prosthesis interface are created. The mean size of the TPISs micro gaps is more or less 50 µm for commonly used TPIS. Larger gaps may be expected at the TPIS abutment-prosthesis interface, and, in addition, both micro-gaps may be further widened under loading conditions. Because most of the oral bacteria are about 10 µm large, bacterial leakage is unavoidable along the interfaces between implant-abutment-prosthetic connections (3, 4).

A number of *in vitro* studies and some *in vivo* studies have confirmed this bacterial leakage, which

Key words: Implant-abutment connection, implant dentistry, bacterial leakage, perimplantitis, bone resorption.

Mailing address:

Prof. Aldo Bruno Gianni,
Maxillo-Facial and Dental Unit, Fondazione Ca' Granda IRCCS,
Ospedale Maggiore Policlinico Milan,
Via Francesco Sforza 35, 20122 Milano, MI, Italy
Tel.: +39 0255032532 - Fax: +390255032558
e-mail: aldo.gianni@unimi.it

is considered the most plausible cause of peri-implantitis (5-13). These bacterial flows may also explain more initial bone loss around TPIS, so that TPISs implant-abutment interface is located much closer to the alveolar crest than the one-piece implant types. In this regard, more inflammation and bone loss may be anticipated as more implants become submerged. TPIS components has been thoroughly described, including data on many implants systems with no clinical signs of peri-implantitis supporting screw-retained full-arch bridges for an average of 9.6 years. The purpose of the present microbiological study was to evaluate bacterial leakage at implant-abutment connection level of a new type of implant: Storm implant (FMD, Falappa Medical Devices®, Rome, Italy) using Real-Time Polymerase Chain Reaction (RT-PCR).

FMD Storm Implant System

Cylindrical implants of Storm line are used in most surgical situations including the GBR (guided bone regeneration) with the simultaneous insertion of the implant, cases in which the macro-morphology allows high primary stability. In fact, the reduced pitch of loop allows the perfect adaptation of the implant in the alveolar bone, favors the fixture insertion without difficulty, even in conditions of reduced residual ridge. Born as submerged fixture, it is also used with the transmucosal immediate loading techniques. The polygonal external implant-abutment connection has a geometry that provides a hex on which complementary abutments engage. They can be used both in fixed prosthesis, available in different diameters and for the over-denture prosthetic rehabilitation. The external hexagon was developed to facilitate the insertion of components such as abutments and impression copings, rather than to provide an antirotational ability and therefore, external systems exhibit weakness due to micro movements of the abutments under a high occlusal load due to their limited hexagonal height.

The external hexagon of TPISs was initially designed to transmit the rotational torque, which was applied on the external surface (external torque), for dental implant placement. Although TPIS Storm implants have been the most frequently applied,

deformation of the hexagon may occur due to fatigue or overload failures. In a TPIS, a higher value insertion torque reduces the risk of implant micro movements at the bone-implant interface, thereby obtaining higher rates of success of immediately loaded implants. On the other hand, TPIS implants can undergo morphological changes during torsion when inserted into bone. These changes, associated with the masticatory load, may affect the implant/abutment stability. Therefore, an accurate adaptation between the external hexagon of a TPIS and the prosthetic component results in good biomechanical and aesthetic conditions for rehabilitation by osseointegrated implants.

Application of rotational force to the internal surface of the external hexagon may reduce the possibility of geometrical deformation of the external hexagon, due to the greater resistance of the internal surface as compared to the external surface. These TPISs use the internal contact with the implant walls instead of mounting devices to apply the force, which simplifies the procedure and diminishes the cost of materials. However, TPIS mechanisms will always reach limited resistance, and excessive torque may cause damage to the upper part of the implant, and to the connection to which prosthetic components are attached (14, 15).

Storm implant is specifically designed for the high primary stability in soft bone and is ideal for immediate loading. Its rapid protocol reduces the time of prosthetic rehabilitation. Storm implant is the system preferred by many professionals for full-arch fixed prostheses, is the original facility and widely documented for the concept of All-on-4®.

MATERIALS AND METHODS

Implant preparation

In order to size up the ability of the implant to isolate the heart of the device from the external environment, we evaluated the passage of modified *E. coli* across the joint of the implant. The peculiarity of these bacteria is that they contain synthetic DNA target sequences in their plasmid. In detail, the plasmid contains two-sequence specific for two bacterial species (*P. gingivalis* and *T. forsythia*) and two genes for antibiotic selection (Kanamycin and Ampicillin).

Bacteria were cultured in lysogeny broth (LB) containing both Kanamycin and Ampicillin (at a final concentration of 50 µg/ml) at 37°C for 12-18 h in a shaking incubator. Four Storm implants (FMD, Falappa Medical Devices®, Rome, Italy) were used in this study. Few microliters of LB with antibiotics were put inside the implants. The implants and the abutment were screwed with a force of 35 Newton.

Few microliters of this culture was used to “contaminate” fresh LB with antibiotics contained in a microcentrifuge tube together with the implant. Tubes were then left at 37°C for 48 h in a heater, in order to allow bacterial growth and their hypothetical passage within the implant. Inside the implant, we just put LB and antibiotics without bacteria instead. To ascertain that there were no contaminations, a negative control containing only LB and antibiotics was prepared.

Forty-eight hours later, implants were opened and samples were collected by dipping a paper probe in both the sites containing LB (external and internal to the implant) for each implant and in the negative control.

DNA extraction

Once collected, paper probe were put on a new micro centrifuge tube and processed for bacterial DNA extraction by using the GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, St., St. Louis, MO, USA), following the manufacturing procedures. Briefly, samples were incubated with lysozyme and subsequently with proteinase K to isolate DNA. Once extracted, DNA was purified by spin-column method.

Real-time polymerase chain reaction

Bacterial quantification was performed by Real-Time Polymerase Chain Reaction using the absolute quantification with the standard curve method.

Primers and probes oligonucleotides for *P. gingivalis* and *T. forsythia* were designed based on 16S rRNA gene sequences of the Human Oral Microbiome Database (HOMD 16S rRNA RefSeq Version 10.1).

For the quantitative analysis, plasmid (Eurofin MWG Operon, Ebersberg Germany) containing the specific DNA target sequence was employed as standard.

All reactions were performed in duplex, in 20 µl final volumes, with 2X TaqMan Universal PCR master mix (Applied Biosystems, Foster City, CA, USA) and 50 nM concentration of each primers and 200 nM of the probes.

Amplifications were carried out by using the ABI PRISM 7500 (Applied Bio systems, Foster City, CA, USA).

Statistical analysis

To evaluate if the difference in viability between outside and inside the implant was statistically significant, we applied Student's *t*-test on average bacteria quantification at each time point.

RESULTS

Bacteria quantification is reported in Table I. In all the tested implants, bacteria were found in the inner side, with a median percentage of 15% for *P. gingivalis* and 14% for *T. forsythia*.

The analysis revealed that in both cases (internally and externally), bacteria grew for the first 48 h but subsequently they started to die, probably as a consequence of nutrient consumption. Moreover, the difference between outer and inner bacteria concentration was statistically significant at each time point.

DISCUSSION

Malò recently presented the “All-on-4” concept for rehabilitation of edentulous mandible and maxilla with TPIS. The “All-on- 4” technique has been well established in the clinical practice. Favorable survival rates and clinical results of immediate rehabilitation of fully edentulous jaws by two posterior tilted and two axial implants have been reported in the literature. Immediate loading rehabilitation of edentulous jaws and its effectiveness has been extensively discussed in the literature. Primary stability is suggested to be one of the main prerequisites for successful immediate loading techniques and is influenced by bone quality, implant macro-design and surgical techniques. In standard “All-on-4” technique after insertion of TPIS with external hexagon, patients receive an immediate provisional acrylic prosthesis a few days after surgery; but there are clinical situations, in which due to low insertion torque or the necessity of bone augmentation, simultaneous with implantation, immediate loading is not feasible.

Table I: Absolute quantification of *P. gingivalis* and *T. forsythia*, outside and inside the implant.

PJ-16-005 N12						
IMPLANT	BACTERIA	BACTERIA QUANTITY	IMPLANT	BACTERIA	BACTERIA QUANTITY	Passage of bacteria from outside to inside the implant (%)
1 OUTSIDE	<i>P. gingivalis</i>	1103555	1 INSIDE	<i>P. gingivalis</i>	214175	19%
	<i>T. forsythia</i>	1483326		<i>T. forsythia</i>	105536	7%
2 OUTSIDE	<i>P. gingivalis</i>	6743770	2 INSIDE	<i>P. gingivalis</i>	1101295	16%
	<i>T. forsythia</i>	588492		<i>T. forsythia</i>	44553	8%
3 OUTSIDE	<i>P. gingivalis</i>	2197054	3 INSIDE	<i>P. gingivalis</i>	500291	23%
	<i>T. forsythia</i>	2002942		<i>T. forsythia</i>	231635	12%
4 OUTSIDE	<i>P. gingivalis</i>	1128726	4 INSIDE	<i>P. gingivalis</i>	212933	19%
	<i>T. forsythia</i>	1224427		<i>T. forsythia</i>	225993	18%
5 OUTSIDE	<i>P. gingivalis</i>	5441674	5 INSIDE	<i>P. gingivalis</i>	495853	9%
	<i>T. forsythia</i>	2081744		<i>T. forsythia</i>	470422	23%
6 OUTSIDE	<i>P. gingivalis</i>	4620030	6 INSIDE	<i>P. gingivalis</i>	616082	13%
	<i>T. forsythia</i>	299719		<i>T. forsythia</i>	24262	8%
Negative Control OUTSIDE	<i>P. gingivalis</i>	0	Negative Control INSIDE	<i>P. gingivalis</i>	0	0
	<i>T. forsythia</i>	0		<i>T. forsythia</i>	0	0
Media Outside			Media Inside			
PorG			PorG			15%
TanF			TanF			14%

Implant permeability is expressed as a percent rate of the internal vs external bacteria quantity.

This method provides the insertion of TPIS distal and longer implants in a tilted position, so that bone-implant-connection is increased improving prosthetic support. The “All-on-4®” treatment allow edentulous patients to an immediately loaded full arch restoration with only four TPIS implants.

The “All-on-4®” provided in the mandible, makes it possible to achieve good bone anchorage without interfering with mental foramina in severely resorbed jaw. Additionally, in the maxilla, TPIS tilted implants are an alternative to sinus floor augmentation.

The role of the external hex in survival rate of “All-on-4” oral rehabilitation is fundamental; in fact, preventing bacterial leakage at level of implant-abutment connection is a main aim of a good interface (16, 17).

TPIS implant-abutment interface have been modified over the years in order to fit a better connection. Considering the importance of the TPIS abutment stability and the bacterial penetration through the micro gap, this study was performed to evaluate bacterial leakage of a new type of implant with an external hex. In fact, that external hex may favors bacterial leakage in the implant-abutment connection.

In a TPIS, the connection is related to the spinning of a screw with a predetermined amount of torque during the installation. A number of *in vitro* studies have been published documenting bacterial leakage along the TPIS interface at implant-abutment connections. These studies indicated a bidirectional flux of fluids and bacteria via the micro-gap, which occurred within hours or days following inoculation



Fig. 1. Storm implant (FMD, Falappa Medical Devices®, Rome, Italy), with a hexagonal external implant-abutment connection.

on all TPIS implant types (18). The phenomenon was reduced when the closing torque of the abutment screw was increased. These findings are interesting, but should be interpreted with caution; in fact, bacterial leakage may be underestimated, especially when relatively large species such as *P. gingivalis* and *T. forsythia* are selected for this purpose. In addition, bacterial leakages were not examined under loading and may underestimate this phenomena. These findings indicate that *in vivo* studies are mandatory to evaluate bacterial leakage.

The presence and quantity of a large number of species at the internal implant have been previously described. Bacterial leakage in vivo data are lacking: Van Aelst et al. (19) showed that a high proportion of the TPIS implants was contaminated by the species included in the panel in frequently high numbers, as well as on the abutment screw in the intra-coronal compartment. In addition, the bacterial leakage in the internal compartments showed high variation: organisms such as *Helicobacter pylori* and *Staphylococcus aureus* were identified in at least one-third of the samples. Anaerobic species associated with peri-implantitis were even more

frequently found. The RT-PCR method has shown a high diagnostic sensitivity for the detection of microbiota around dental implants.

The purpose of the present study was to document bacterial leakage of a new type of implant. Our results indicated that the microbiota under investigation would result in death of all microorganisms. As a result, microbial leakage along the implant abutment interface was acceptable and considered the most probable explanation for peri-implantitis.

Under the assumption that bacterial leakage is truly related to peri-implantitis, the following implications should be taken into account. It could be speculated that it may only be a matter of time until this bacterial burden starts to initiate pathology around these TIPS implants that have been in a clinically healthy state for years. If peri-implantitis has developed, the presence of these species at the internal implant and the abutment-prosthetic connection form a microbial reservoir explaining why nonsurgical treatment strategies for peri-implantitis are ineffective.

Additional studies are needed to explore the relationship in terms of microbiota between the

internal implant and implant-prosthetic connection. In addition, the dynamics of internal colonization needs to be thoroughly documented in longitudinal *in vivo* studies.

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BACTERIAL COLONIZATION OF THE IMPLANT-ABUTMENT INTERFACE OF CONICAL CONNECTION WITH AN INTERNAL OCTAGON: AN *IN VITRO* STUDY USING REAL-TIME PCR

A. BAJ^{1,2}, G. A. BELTRAMINI^{1,2}, A. BOLZONI^{1,2}, F. CURA³, A. PALMIERI³,
A. SCARANO⁴, L. OTTRIA⁵, and A. B. GIANNI^{1,2}

¹Maxillofacial and Dental Unit, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico di Milano, Milan, Italy; ²Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy; ³Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna, Bologna, Italy; ⁴Department of Medical, Oral and Biotechnological Sciences and CeSi-MeT, University of Chieti-Pescara, Chieti, Italy; ⁵Dental School, University of Tor Vergata, Rome, Italy

Bacterial leakage at the implant-abutment connection of a two-piece implant system is considered the main cause of peri-implantitis. Prevention of bacterial leakage at the implant-abutment connection is mandatory for reducing inflammation process around implant neck and achieving bone stability. Micro-cavities at implant-abutment connection level can favour bacterial leakage, even in modern two-piece implant systems. The conical connection with an internal octagon (CCIO) is considered to be more stable mechanically, and allows a more tight link between implant and abutment. As *P. gingivalis* and *T. forsythia* penetration might have clinical relevance, it was the purpose of this investigation to evaluate molecular leakage of these two bacteria in a new two-implant system with an internal conical implant-abutment connection with internal octagon (Shiner XT, FMD Falappa Medical Devices S.p.A. Rome, Italy). To verify the ability of the implant in protecting the internal space from the external environment, the passage of genetically modified *Escherichia coli* across implant-abutment interface was evaluated. Four Shiner XT implants (FMD, Falappa Medical Devices®, Rome, Italy) were immersed in a bacterial culture for 24 h and bacteria amount was measured inside implant-abutment interface with Real-time PCR. Bacteria were detected inside all studied implants, with a median percentage of 6% for *P. gingivalis* and 5% for *T. forsythia*. Other comparable studies about the tightness of the tested implant system reported similar results. The gap size at the implant-abutment connection of CCIOs was measured by other authors discovering a gap size of 1–2µm of the AstraTech system and of 4µm for the Ankylos system. Bacterial leakage along implant-abutment connection of cylindrical and tapered implants, Shiner XT, (FMD Falappa Medical Devices S.p.A. Rome, Italy) showed better results compared to other implants. Additional studies are needed to explore the relationship in terms of microbiota of the CCIO. In addition, the dynamics of internal colonization needs to be thoroughly documented in longitudinal *in vivo* studies.

Bacterial leakage at the implant-abutment connection of a two-piece implant system is considered the main cause of peri-implantitis (1-

9). Prevention of bacterial leakage at the implant-abutment connection is mandatory for reducing inflammation process around implant neck and

Key words: Implant-abutment connection, conical connection, internal octagon, bacterial leakage, perimplantitis

Mailing address:

Prof. Aldo Bruno Gianni,
Maxillo-Facial and Dental Unit, Fondazione Ca' Granda IRCCS,
Ospedale Maggiore Policlinico Milan,
Via Francesco Sforza 35, 20122 Milano, MI, Italy
Tel.: +39 0255032532 - Fax: +390255032558
e-mail: aldo.gianni@unimi.it

achieving bone stability. Micro-cavities at implant-abutment connection level can favour bacterial leakage, even in modern two-piece implant systems. The conical connection with an internal octagon (CCIO) is considered to be more stable mechanically and allows a tighter link between implant and abutment.

The constant flux of microbiota between implant and abutment is an important factor for chronic inflammatory infiltration and marginal bone resorption (10, 11). CCIO aims to reduce the leakage by increasing the stability of the implant-abutment connection. In fact, a CCIO is manufactured to reduce the mobility of the screw by constructing tight physical connections with a high level of precision. Few investigators aimed to quantify microbial leakage of CCIOs (12, 13). These studies investigated bacterial loading (size 1-10µm, living bacteria). However, bacteria like *P. gingivalis* and *T. forsythia* are responsible for inflammatory reactions. These bacteria can penetrate in smaller gaps. It is well known that *P. gingivalis* and *T. forsythia* are the most important bacteria of gram-negative group and play a major role in bone destruction processes.

To the authors' knowledge, only few data exist concerning micro-leakage phenomena of CCIOs. The sealing capability of different CCIOs was tested in few studies (12). All the tested CCIOs presented micro-leakage. This is in agreement with other studies showing micro-movement between the abutment and its implant (14, 15).

As *P. gingivalis* and *T. forsythia* penetration might have clinical relevance, it was the purpose of this investigation to evaluate molecular leakage of these two bacteria in a new two-implant system with a conical implant-abutment connection with internal octagon (Shiner XT, FMD Falappa Medical Devices S.p.A. Rome, Italy) (Fig. 1). Therefore, the hypothesis of the present study was that this CCIO is tight enough to prevent bacterial leakage.

Shiner XT implants

Bacterial leakage along implant-abutment connection of Shiner XT, A cylindrical and tapered implant, (FMD Falappa Medical Devices S.p.A. Rome, Italy) was studied. Shiner XT implant system

is characterized by cylindrical and tapered implants, being equipped with a CCIO, and having a 1.8 mm smooth neck.

Shiner XT implant system is suitable for the replacement of single-rooted teeth in edentulous crests or for post-extractive surgery. The threaded form makes it easy to achieve optimal primary stability in all bone conditions and a considerable long-term stability in osseointegration.

MATERIALS AND METHODS

Implant preparation

In order to size up the ability of the implant to isolate the heart of the device from the external environment, we evaluated the passage of modified *E. coli* across the joint of the implant. The peculiarity of these bacteria is that they contain synthetic DNA target sequences in their plasmid. In detail, the plasmid contains two-sequence specific for two bacterial species (*P. gingivalis* and *T. forsythia*) and two genes for antibiotic selection (Kanamycin and Ampicillin).

Bacteria were cultured in lysogeny broth (LB) containing both Kanamycin and Ampicillin (at a final concentration of 50ug/ml) at 37°C for 12-18 h in a shaking incubator. Four Shiner XT implants (FMD, Falappa Medical Devices®, Rome, Italy) were used in this study. a few microliters of LB with antibiotics were put inside the implants. The implants and the abutment were screwed with a 35 Newton force.

A few microliters of this culture was used to "contaminate" fresh LB with antibiotics contained in a micro-centrifuge tube together with the implant. Tubes were then left at 37°C for 48 h in a heater, in order to allow bacterial growth and their hypothetical passage within the implant. Inside the implant, we placed LB and antibiotics without bacteria instead.

To assure that there were no contaminations, a negative control containing only LB and antibiotics was prepared.

Forty-eight hours later, implants were opened and samples were collected by dipping a paper probe in both the sites containing LB (external and internal to the implant) for each implant as well as in the negative control.

DNA extraction

Once collected, paper probe were put on a new micro centrifuge tube and processed for bacterial DNA extraction, by using the GenElute™ Bacterial Genomic

DNA Kit (Sigma-Aldrich, St., St. Louis, MO, USA), following the manufacturing procedures. Briefly, samples were incubated with lysozyme and subsequently with proteinase K to isolate DNA. Once extracted, DNA was purified by spin-column method.

Real-time polymerase chain reaction

Bacterial quantification was performed by Real-Time Polymerase Chain Reaction using the absolute quantification with the standard curve method.

Primers and probes oligonucleotides for *P. gingivalis* and *T. forsythia* were designed based on 16S rRNA gene sequences of the Human Oral Microbiome Database

(HOMD 16S rRNA RefSeq Version 10.1).

For the quantitative analysis, plasmid (Eurofin MWG Operon, Ebersberg Germany) containing the specific DNA target sequence was employed as standard.

All reactions were performed in duplex, in 20 µl final volumes; with 2X TaqMan Universal PCR master mix (Applied Biosystems, Foster City, CA, USA) and 50nM concentration of each primers and 200nM of the probes. Amplifications were carried out by using the ABI PRISM 7500 (Applied Bio systems, Foster City, CA, USA).

Statistical analysis

To evaluate if the difference in viability between the

Table I: Absolute quantification of *P. gingivalis* and *T. forsythia*, outside and inside the implant. Implant permeability is expressed as percent rate of the internal vs. external bacteria quantity.

XT-29-002 N12						
IMPLANT	BACTERIA	BACTERIA QUANTITY	IMPLANT	BACTERIA	BACTERIA QUANTITY	Passage of bacteria from outside to inside the implant (%)
1 OUTSIDE	<i>P. gingivalis</i>	5114182	1 INSIDE	<i>P. gingivalis</i>	117481	2%
	<i>T. forsythia</i>	1700640		<i>T. forsythia</i>	102421	6%
2 OUTSIDE	<i>P. gingivalis</i>	1094558	2 INSIDE	<i>P. gingivalis</i>	12403	1%
	<i>T. forsythia</i>	7014977		<i>T. forsythia</i>	134075	2%
3 OUTSIDE	<i>P. gingivalis</i>	2157567	3 INSIDE	<i>P. gingivalis</i>	22289	1%
	<i>T. forsythia</i>	1616843		<i>T. forsythia</i>	22761	1%
4 OUTSIDE	<i>P. gingivalis</i>	7540425	4 INSIDE	<i>P. gingivalis</i>	254716	3%
	<i>T. forsythia</i>	5329711		<i>T. forsythia</i>	284677	5%
5 OUTSIDE	<i>P. gingivalis</i>	21431324	5 INSIDE	<i>P. gingivalis</i>	696785	3%
	<i>T. forsythia</i>	17769637		<i>T. forsythia</i>	154882	1%
6 OUTSIDE	<i>P. gingivalis</i>	55702475	6 INSIDE	<i>P. gingivalis</i>	4242865	8%
	<i>T. forsythia</i>	70541849		<i>T. forsythia</i>	4320393	6%
Negative Control OUTSIDE	<i>P. gingivalis</i>	0	Negative Control INSIDE	<i>P. gingivalis</i>	0	0
	<i>T. forsythia</i>	0		<i>T. forsythia</i>	0	0
Media Outside			Media Inside			
PorG			PorG			6%
TanF			TanF			5%



Fig. 1. FMD Shiner XT Implants with conical connection with internal hexagon.

outside and the inside of the implant was statistically significant, we applied Student's *t*-test on average bacteria quantification at each time point.

RESULTS

Bacteria quantification is reported in Table I. In all the tested implants, bacteria were found in the inner side, with a median percentage of 6% for *P. gingivalis* and 5% for *T. forsythia*.

The analysis revealed that in both cases (internally and externally), bacteria grew for the first 48 h but subsequently started to die, probably as a consequence of nutrient consumption. Moreover, the difference between outer and inner bacteria concentration was statistically significant at each time point.

DISCUSSION

The present investigation has demonstrated that even CCIOs were not tight on the molecular level.

Our results reveal that bacterial leakage occurs in two-stage implants. Our findings are similar to other authors who found that different types of two-stage implants showed a significant increase in flux of fluids after inoculating the internal implant parts with vital colour markers.

Since the discovery of microbial colonization of the internal parts of two-stage implants, there is very little data regarding the analysis of the micro leakage at the CCIO. Other comparable studies about the tightness of the tested implant system reported similar results (15, 16). The gap size at the implant-abutment connection of CCIOs was measured by other authors finding a gap size of 1–2 µm of the AstraTech system and of 4 µm for the Ankylos system (14). It appears that *P. gingivalis* and *T. forsythia* are able to pass through gaps in this dimension more quickly than bacteria with a diameter of 1.1 to 1.5 µm. These findings might explain why a higher rate of leakage and a faster increase in bacterial loading for this CCIO implant system was found in our study. Nevertheless, the use of *P. gingivalis* and *T. forsythia* as markers to verify molecular micro-leakage makes high demands on laboratory processes and hygiene standards during testing.

Based on the findings it was assumed that the micro-gaps in CCIO leads to accumulation of inflammatory cells, which, when combined with osteoclast formation/growth, results in alveolar bone loss (17, 18). The role of *P. gingivalis* and *T. forsythia* in bone destruction has been well described in recent years (19). This underlines the importance of evaluating the tightness of CCIO regarding bacteria.

CONCLUSIONS

The experimental setup was considered valid and produced consistent data. The hypothesis that CCIOs are tight enough to prevent bacteria penetration is accepted.

Prevention of microbial leakage at the level of CCIO is the main aim for the construction of two-piece implant systems to avoid inflammation in peri-implant tissues.

The design of CCIO can limit the microbial penetration into the internal part of a dental implant. Some microbiological studies confirmed the passage of bacteria around CCIO at level of peri-implant tissues. Other studies have investigated microorganism penetration through CCIO in order to find an efficient bacterial seal system. With two-stage implant system, the abutment is retained in the fixture with mechanical methods. This results in gaps and cavities between the CCIO becoming a bacteriological reservoir. These gaps favour an inflammatory process in peri-implant tissues. Microbial colonization of the CCIO may have consequences, such as bone resorption.

Bacterial leakage along implant-abutment connection of cylindrical and tapered implants, Shiner XT, (FMD Falappa Medical Devices S.p.A. Rome, Italy) showed better results compared to others implants. Additional studies are needed to explore the relationship in terms of microbiota of the CCIO. In addition, the dynamics of internal colonization needs to be thoroughly documented in longitudinal *in vivo* studies.

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NASAL RECONSTRUCTION: OUR EXPERIENCE

A. BAJ^{1,2}, A. RUSSILLO^{1,2}, E. SEGNA^{1,2}, M. ROMANO^{1,2},
A. BOLZONI^{1,2}, F. J. SILVESTRE³, A.B. GIANNI^{1,2} and G.A. BELTRAMINI^{1,2}

¹Maxillofacial and Dental Unit, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico di Milano, Milan, Italy; ²Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy; ³Stomatology & Oral Surgery, Dr Peset University Hospital, Department of Stomatology, University of Valencia, Spain

The nose is a critically important aesthetic and functional portion of the face. We can explore nasal defects following trauma or tumor resections. Reconstructive techniques based on primary closure, skin graft, local flap, regional flap and microvascular free tissue transfer provides an algorithmic framework applicable to any reconstructive problem. From 2010 to 2016, 33 patients were treated with nasal reconstruction. Malignant tumor resection was the main cause of nasal defect. The most common tumor resected was BCC (basal cell carcinoma) followed by SCC (squamous cell carcinoma). Recurrence of malignant tumor were also treated. As described in the literature, we used the most common reconstructive techniques to handle post-resection defects. Most of the defects involved the dorsum and the ala of the nose, only rarely had the skeleton been affected by the resection. When the cartilage had been sacrificed, a graft from the septum or from the ear flap was harvested. All patients resulted in optimal reconstruction. In any case, a second surgery was needed. All the surgical procedures were performed in one single stage except for the paramedian forehead flap that required a second stage for division and inset of the flap. All donor sites did not show functional and aesthetic damage. All patients resulted in a morpho-functional restoration of the area affected by the resection with a high success rate. Following the algorithm when restoring the anatomical subunit is the base purpose has determined satisfactory results.

The nose is a critically important aesthetic and functional portion of the face. We can explore nasal defects following trauma or tumor resections (1). Reconstruction of the nose with cheek advancement flaps was first described in the Sushruta Samhita in the Hindu literature around 600 B.C. The origins of forehead rhinoplasty were described as early as 1440 A.D. in the Indian literature, although these operations were probably performed well before that (2). The first English translation of the Indian

midline forehead flap appeared in 1794. The classic description of the paramedian forehead flap appeared in 1946, where authors described a vertical midline forehead flap, elevated from nasofrontal angle to hairline, receiving its blood supply from paired forehead arteries and rotated 180° at its pivot point above the eyebrow (3).

Many variations of the forehead flap have surfaced since the mid-twentieth century, although none has achieved the widespread acceptance and

Key words: nose, flap, aesthetic, subunit, reconstruction

Mailing address:

Dr Antonio Russillo,
Maxillo-Facial and Dental Unit, Fondazione Ca' Granda IRCCS,
Ospedale Maggiore Policlinico Milan,
Via Francesco Sforza 35, 20122 Milano, MI, Italy
Tel.: +39 0255032532 - Fax: +39 0255032558
e-mail: anto.russillo@gmail.com

0393-974X (2016)

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outstanding results of the paramedian forehead flap. A thorough knowledge of anatomy provides the basis for understanding the defect, described according to the anatomic site and the acquired absence of tissue. The missing tissue is defined by the lining, support and cover referring to the mucosa, cartilaginous and bony skeleton and the overlying skin. Currently, algorithms for medium to large nasal soft tissue defects have been determined and published in the medical literature (4-6).

Anatomy

An important, relatively recent concept with respect to these reconstructions is the definition and description of the aesthetic nasal subunits. The concept of aesthetic subunits of the face was first described by Gonzales-Ulloa (1957) (7). He characterized facial units such as the nose to describe the location of a defect in order to design the reconstruction. Burget and Menick (8) refined this principle by describing nasal subunits with specific reference to nasal reconstruction. These subunits remain consistent across patient populations, though different ethnic features should be considered when planning any reconstruction. The nose is divided into the following subunits:

1. The dorsal subunit
2. The nasal sidewall
3. The soft triangle
4. The ala
5. The columella
6. The nasal tip

The nasal skeleton

The bony and cartilaginous framework of the nose provides the support for the nasal cover and its arrangement is reflected in the contour of the skin. The bony structure of the nose begins with the radix or root, at the naso-frontal suture and extends caudally as the nasal bones. Proceeding inferiorly, the bony framework gives way to the cartilaginous framework, beginning with the upper lateral cartilages. The midline of the nose is formed by the bony and cartilaginous septum, the perpendicular plate of the ethmoid, the vomer and quadrangular cartilage. Moving caudally, the septum

and upper lateral cartilages submit to the lower lateral or alar cartilages. Many important anatomic landmarks exist with respect to these structures. The most lateral portion is known as the lateral crus. Medially, we find the lateral genu of the alar cartilage, which provides the projection of each hemi-tip. The columellar portion of the alar cartilage is composed of the middle and medial crus. The junction of these components is known as the medial genu.

Blood supply

Knowledge of the arterial anatomy is crucial in planning any nasal reconstruction, as the rotation of local/regional flaps are centered on the vessels that supply these tissues. The arterial supply to the external portion of the nose is comprised of the alar and septal branches of the maxillary artery, which provide blood for the nasal alae and septum. The dorsum and sidewalls of the nose are supplied by the dorsal, nasal and angular branches of the ophthalmic artery and the infraorbital branch of the maxillary artery. The anterior facial and ophthalmic veins provide venous drainage.

Nerve supply

The nervous supply to the integument of the nose is derived from branches of the infratrochlear and nasociliary branches of the ophthalmic nerve and from the infraorbital branch of the maxillary nerve. The nerves that innervate the lining of the nose include the nasociliary branch of the ophthalmic nerve, fascicles of the alveolar branch of the maxillary nerve, the nerve of the pterygoid canal, the nasopalatine, the anterior palatine and the nasal branches of the sphenopalatine ganglion.

Reconstructive techniques

Reconstructive techniques can be based from primary closure, to skin graft, to local flap, to regional flap, to microvascular free tissue transfer provides an algorithmic framework applicable to any reconstructive problem. Skin grafts can be used to reconstruct small defects with excellent results; the Banner flap, a local tissue rearrangement first described by Elliot in 1969, can be used to reconstruct defects up to 2.5 cm anywhere on the

nose. The bilobed flap, whose original design was attributed to Esser around World War I (9), can be used to reconstruct small defects of the lower two-thirds of the nose. Defects involving the caudal extent of the dorsum, and/or including a portion of the nasal tip totaling less than 2 cm, are well served by the Rintala flap (10). In the Nasolabial Flap the redundant skin of the cheek, adjacent to the nasolabial crease, should be considered in the reconstruction of partial or complete alar defects. Defects of the nasal sidewall can be effectively covered using a cheek advancement flap or a rhomboid flap from the adjacent cheek skin. The Paramedian forehead flap can provide an excellent functional and aesthetic outcome in the reconstruction of large nasal defects particularly involving the nasal tip and/or ala; it is done in two surgical steps, step 1 elevation of the flap and coverage of the defect and step 2, thinning or defatting and modification of the framework with division and inseting.

MATERIAL AND METHODS

From 2010 to 2016, 33 patients were treated with nasal reconstruction. Malignant tumor resection was the main cause of nasal defect. The most common tumor resected was BCC (basal cell carcinoma) followed by SCC (squamous cell carcinoma). Recurrence of malignant tumor were treated to. As described in the literature, we used the most common reconstructive techniques to cope with post-resection defects. Most of the defects involved the dorsum and the ala of the nose, only rarely had the skeleton been affected by the resection. When the cartilage had been sacrificed, a graft from the septum or from the earflap was harvested.

In our paper, we only considered the cases where resection was followed by a nasal reconstruction (Table I).

RESULTS

All patients resulted in a morpho-functional restoration of the area affected by the resection with a high success rate (Fig. 1, 2).

Most patients, as described in Table I, underwent BCC resection, only 5 patients were suffering of SCC and an extended resection was necessary; in 3 cases,

a forehead flap was harvested and in 2 cases a cheek advancement flap was used; in 2 cases, cartilage graft was required to restore the ala contour. All the surgical procedures were performed in a single stage except for the paramedian forehead flap that required a second stage for division and inset of the flap.

The most common reconstruction applied was the bilobed flap. No patient required a secondary reconstruction, in one case a dehiscence of the surgical wound had been detected and healed by secondary intention. In 2 cases after reconstruction with paramedian forehead flap, lipofilling was performed to refine the paramedian scar.

Only in one case was a skin graft required, since the poor clinical condition of the patient and the extension of the defect had not allowed the use of other reconstructive techniques. The donor site did not show any functional and aesthetic damage.

DISCUSSION

The knowledge of the surgical algorithm, which perceives the extent of the fault and reconstructive options, is the basis of an optimal result and restoration of the facial symmetry (11-18). A good knowledge of the anatomy and of the subunits of which the nose is constituted is required for a successful recovery. In accordance with literature, we believe that when the surgical defect is wide, it is necessary to reconstruct the entire anatomical subunit to avoid inadequate aesthetical and functional reconstruction.

As described in literature, when the defect contains more subunits or a subunit with an extension greater than 2.5 cm, a forehead flap is necessary. Bilobed flap was the most used, because in most cases the disease had been treated in an early stage. Defect of the ala with extension less than 2.5 cm can be managed with Banner flap or bilobed flap. Rintala flap has proved an excellent solution in lesions involving the nasal dorsum with an extension of less than 2 cm. When the defect involves the bony skeleton or the cartilage as well, grafts are to be considered.

Whenever a nasal reconstruction is necessary, we suggest the principle of reconstruction on the anatomical subunits, this will get more satisfactory results from a morphological and functional point of

Table I. *Patient treated with nasal reconstruction.*

Patient	Region (subunit)	Cause (histology)	Type of reconstruction
1	Ala	Recurrent (BCC)	Bilobed
2	Tip	BCC	Banner
3	Nasal side wall	BCC	Banner
4	Ala	BCC	Nasolabial Flap
5	Tip	BCC	Rintala
6	Tip/Dorsal	BCC	Paramedian Forehead flap
7	Tip/dorsal	BCC	Skin graft
8	Dorsal/side wall	BCC	cheek advancement flap
9	Ala	BCC	Bilobed
10	Tip	BCC	Bilobed
11	Tip	BCC	Bilobed
12	Tip	BCC	Rintala
13	Tip	BCC	Banner
14	Ala/ Dorsal	SCC	Paramedian Forehead flap, cartilage graft
15	Ala	SCC	cheek advancement flap, cartilage graft
16	Ala	BCC	Bilobed
17	Dorsal/Ala/Tip	Recurrent (BCC)	Paramedian Forehead flap
18	Ala	BCC	Banner
19	Tip	BCC	Paramedian Forehead flap
20	Ala	BCC	Bilobed
21	Ala	BCC	Bilobed
22	Sidewall	BCC	Bilobed
23	Tip	SCC	Paramedian Forehead flap
24	Ala	BCC	Cheek advancement flap, cartilage graft
25	Tip	Recurrent (BCC)	Skin graft
26	Ala	BCC	Nasolabial Flap
27	Dorsal	BCC	Banner
28	Ala	BCC	Bilobed
29	Tip/dorsal	SCC	Paramedian Forehead flap
30	Ala	SCC	Nasolabial Flap
31	Ala	BCC	Bilobed
32	Sidewall	BCC	Nasolabial Flap
33	Tip/Dorsal	BCC	Paramedian Forehead flap

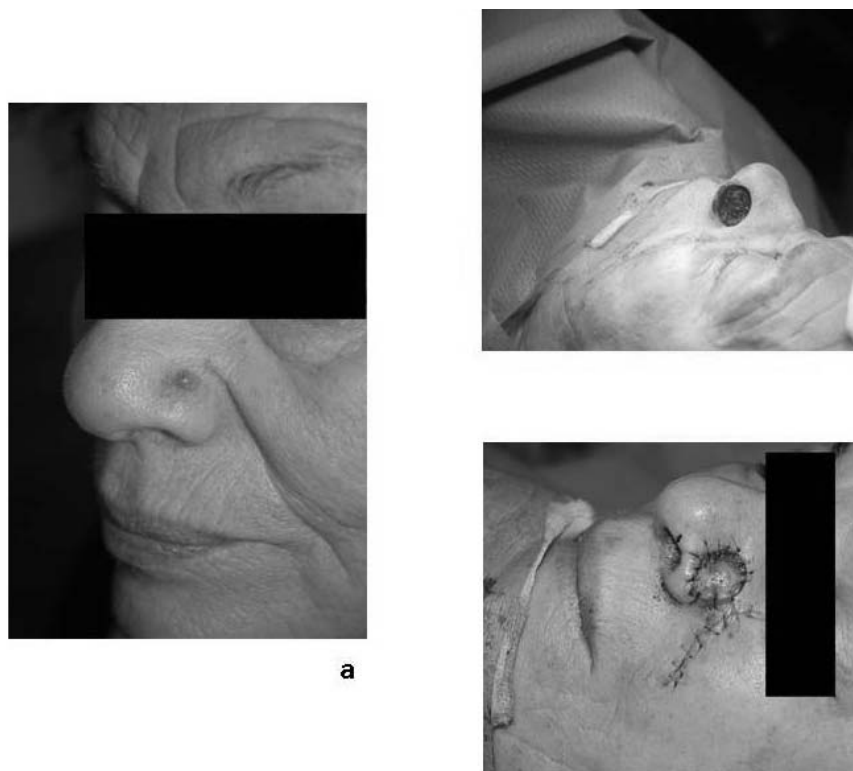


Fig. 1. Nasolabial flap. *a)* Pre operative lateral view (BCC); *b)* Surgical defect; *c)* Flap inseting.

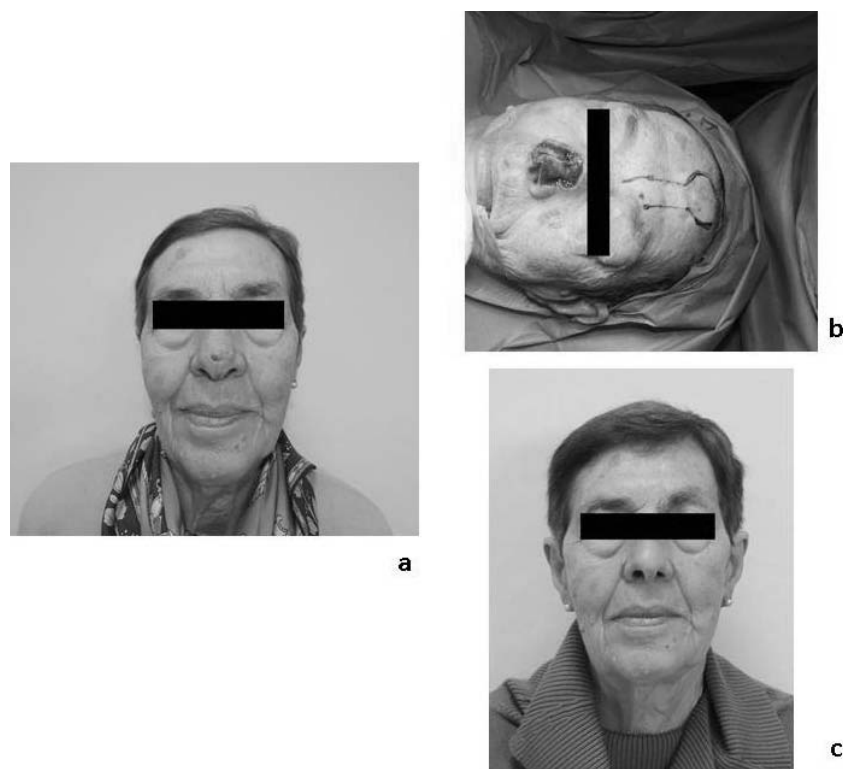


Fig. 2. Paramedian Forehead flap. *a)* Pre operative frontal view (BCC); *b)* Tumor resection; *c)* Six months' postoperative step 2 frontal view.

view, even in composite defects where experience and careful treatment plan will determine the difference between an unsatisfactory and optimal outcome. In any case, it is basic to choose a surgical solution that best suits the defect area allowing to minimize the sequelae and to restore the area widely known as a crucial part of the face, from a functional and morphologic point of view.

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REPLICA-GUIDED TRAUMA SURGERY

F. LAGANÀ¹, E. SEGNA², A. BAJ^{2,3}, G. BELTRAMINI^{2,3},
A. RUSSILLO², V. CANDOTTO⁴, F. J. SILVESTRE⁵ and A. B. GIANNI²

¹UO di Chirurgia Maxillo-facciale e Odontostomatologia, I.R.C.C.S. Azienda Ospedaliera Universitaria San Martino, Genoa, Italy; ²UOC di Chirurgia Maxillo-facciale e Odontostomatologia, Fondazione Cà Granda I.R.C.C.S. Ospedale Maggiore Policlinico di Milano, Milan, Italy; ³Dipartimento di Scienze Biomediche, Chirurgiche e Odontoiatriche Università degli Studi di Milano, Milan, Italy; ⁴Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, Italy; ⁵Stomatology & Oral Surgery, Dr Peset University Hospital, Department of Stomatology, University of Valencia, Spain

The aim of this study is to present a new approach to the surgical treatment of fractures of the middle third of the face, based on the use of resorbable materials and stereolithographic models. From 2009 to 2014, we treated 10 cases of orbitozygomatic trauma mostly as secondary surgery. For each case, an anatomical replica had been produced simulating the expected result and was then used to contour the resorbable plates on its surface, even before the beginning of the surgical procedure itself. The plates and meshes already bended act as a guide to bony reduction procedure. No complications occurred: all patients healed well, without inflammatory reactions. The results obtained corresponded to the expected results. This technique has been employed for primary or secondary treatment of fractures of the middle third facial, where it is not always easy to understand the displacement of bony segments and where the use of resorbables plates could be particularly beneficial, as they do not interfere with the maxillofacial skeleton physiology and avoid the necessity to remove plates and screws. Combined use of stereolithographic model and resorbable materials allow a new surgical approach based on the realisation of the surgical procedure on the model, then transferred from the patient to the model, thanks to plates and meshes. This technique helps to overcome many of the limits of the conventional technique: the need of minimal surgical access, in order to minimize facial scarring and the vasculo-nervous structures that cross the head and neck district and the obligation to restore not only the morphology but also the biological functions.

We present the results and the potential of a new approach to the treatment of fractures of the middle third of the face, a complex anatomical region with multiple functions (1) such as, the containment and the protection of the orbital, sino-nasal and oral structures and the dissipation of traumatic energy which ensures the

preservations of these organs as much as possible.

The standard fractures treatment requires the reduction and the fixation of the bony segments, passing through multiple surgical accesses and using plates and screws made of titanium (2-9). The surgical result is evaluated clinically, immediately after the procedure, then post-

Key words: fracture, bone, face, jaw, skull

Mailing address:

Prof. Aldo Bruno Gianni,
Maxillofacial and Dental Unit,
Fondazione IRCCS Cà Granda, Ospedale Maggiore Policlinico,
Via Francesco Sforza 35, 20122 Milan, MI, Italy
Tel.: +39.02.55032510 - Fax: +39.02.55032815
e-mail: aldo.gianni@unimi.it

175(S)

0393-974X (2016)

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operatively confirmed by a radiological assessment.

The method presented suggests the use of resorbable material bended on a stereolithographic model, printed according to the expected surgical result. Resorbable plates and meshes are conformed on the resin model, and consequently the shape obtained could guide the reduction *in vivo*. This approach could limit significantly surgical access to the fracture areas, with the obvious advantage of creating fewer wounds and reducing the risk of vasculonervous damage (10).

We describe the advantages derived from the simultaneous use of two well-known technologies, resorbable fixation system (11) and stereolithographic modelling (12), formulating a new approach to trauma surgery on the middle third of the face. We successfully applied this technique to 10 cases of primary or secondary treatment of orbitozygomatic fractures.

MATERIALS AND METHODS

From 2009 to 2014, we treated 10 cases of orbitozygomatic trauma (Table I), mostly as secondary surgery; the cases vary greatly in terms of type, age of patient and time of the injury and/or primary surgery. For each surgical procedure, an anatomical replica has been printed using a SOMOS family resin, sterilisable through a low temperature procedure (STERRAD®) (FIG. 1). The average print time is 40 h. Once sterilised, the model is carried to the operating theatre and the surgeon contours the resorbable plates on its surface, even before the beginning of the surgical procedure itself. Modelling is performed after soaking in warm water. While they dry, the plates return to their solid state so more water is needed if they need to be re-modeled. The skull replica was printed thanks to the laser stereolithography process, by BIOSOLUTIONS®. Anatomical replica represents the expected surgical result based on analogue and digital measurements taken during the production process.

The resorbable fixation system chosen was SonicWeld® line, manufactured by KLS Martin, which uses ultrasound-welded pins instead of conventional ones to fasten plates and membranes. Plates and mesh are made of 100% amorphous poly-DL-lactide (PDLLA) that degrades due to hydrolysis, resulting in CO₂ and H₂O with no foreign body reaction, no cellular reaction and no tissue necrosis or heat damage (13). The system is slowly

degraded (14), permitting bone regeneration. However, the stiffness of the material lasts until the healing process has been completed (15), with the material completely resorbed in 12 months.

All surgical procedures were conducted by the same surgeon, in accordance with current guidelines, therefore there was no need to prepare an informed consent form different from the one already used. Every patient underwent a CT scan evaluation, pre- and post-operatively.

RESULTS

The 10 cases presented show the efficacy of the method proposed. We treated 4 males and 6 women; the average age was 35.7 years. In 5 cases we performed primary surgery while the others were cases of secondary long-lasting deformities consequent to untreated fractures, not only with aesthetic complaints but also with function impairment. We reported 2 cases of diplopia (patient #4 and #5), one case of dacryocystitis (patient #7) and one case of coronoid-mandibular interference (patient #8). The suggested procedure had been applied to all cases and the results had been well evaluated from both the surgeon and the patient. The functionality had been successfully restored in all patients (Fig. 2); all primary fractures healed well without the necessity of further surgery. No post-operative complications either medical or surgical occurred.

DISCUSSION

The ultimate objective of any surgical trauma procedure is the restoration of anatomy and function of the fractured area. The technological evolution of the past 20 years has projected maxillofacial surgeons into a new era. These innovations improve their ability to interpret and examine the clinical situation. Digital data enhance the capacity of analysis giving more accurate information. In addition, rapid prototyping technology, specifically laser stereolithography, produces anatomical models extremely faithful to the real clinical situation (16).

Fundamental fields of technological evolution are osteosynthesis and resorbable materials, among the

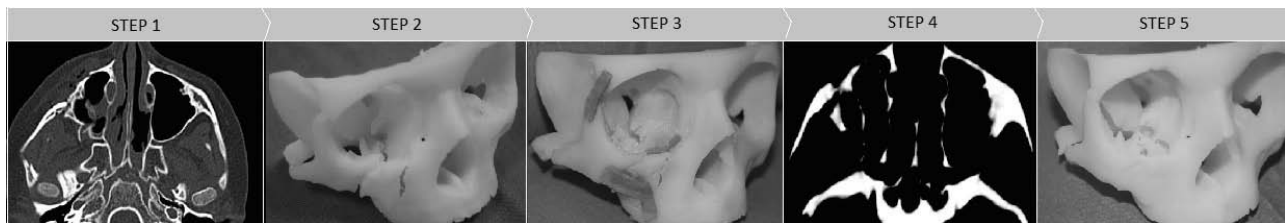


Fig. 1. *The steps of the procedure.*



Fig. 2. *a): the pre-operative frontal view; b): the post-operative frontal view, at 2 months after surgery.*

most innovative. Biomechanical capacities are high in the blends [P(L/DL)LA 70/30] and the “foreign body” reactions required for polymer breakdown are almost always subclinical cytological phenomena, due to the reduced presence of crystals in the highly purified products. The difficulties in the three-dimensional modelling of absorbable synthesis devices persist; they can be manipulated exclusively in a sterile water bath at a temperature of about 60°C and they regain solidity in a few seconds as they cool down. These characteristics make it difficult to obtain the quick and accurate three-dimensional conformation necessary to reconstruct complex anatomical areas such as the orbit and the maxillary-zygomatic region. Stereolithographic model permits to overcome this annoying aspect, that to date has prevented the exploitation of resorbable materials in complex maxilla–facial traumatology. The model offers an

open surface on which it is possible to contour plates and meshes, without soft tissues limitation.

The easy conformation of resorbable materials facilitate their use in orbital walls reconstruction, especially the inferior one. We strongly advise the use of resorbable meshes in orbital floor reconstruction because they have great advantages, such as the possibility to restore the proper orbital volume, providing sufficient stability during bony structure restoration. It maintains the biology of the orbital floor with its capacity to collapse in case of further facial trauma and eliminates the morbidity associated with the use of autogenous bone graft. Furthermore, considering that the peak of incidence for facial injuries occurs between the second and fourth decade of life, the possibility of a second injury in the same site cannot be excluded. Titanium meshes could represent a great risk to the eyeball in

such a situation (17, 18) while resorbable meshes are suitable because they restore the pre-traumatic condition perfectly, with the orbital walls acting like a safety valve (19). These benefits are even more evident if we consider the pediatric population (20).

Pre-surgical study on stereolithographic model

offers other advantages; surgical access could be reduced, limiting scars and risks of complications. In addition, as there is no need to separate the fractured bone fragments from the periosteum, fracture reduction is easier and bone fragment vascularisation is better preserved.

Table 1 Patients treated according to the proposed technique. PS: primary surgery; SS: secondary surgery; R: right; L: left

Patient #	Age at treatment	Sex	Site	Surgical access
#1 (m.g.)	53y	M	zygomatic fracture + orbital floor involvement (R) PS	supraorbital + lower lid + intraoral
#2 (s.s.)	7y	F	zygomatic fracture + orbital floor involvement (R) PS	transconjunctival + intraoral (vestibular)
#3 (c.f.)	30y	M	zygomatic fracture (R) PS	supraorbital + lower lid + intraoral
#4 (d.e)	38y	F	post-traumatic orbitozygomatic deformity (R) SS	transcutaneous to the zygomatic arch + transconjunctival with cantotomy
#5 (f.a.)	57y	F	post-traumatic orbitozygomatic deformity (R) SS	supraorbital + lower lid + intraoral
#6 (e.m.)	35y	M	post-traumatic orbitozygomatic deformity (L) SS	hemicoronal + intraoral + supraorbital + lower lid
#7 (d.cristian)	32y	M	post-traumatic orbitozygomatic deformity (R) SS	supraorbital + lower lid + intraoral + Lynch
#8 (montuschi)	40y	F	post-traumatic orbitozygomatic deformity (L) SS	intraoral + lower lid
#9 (p.p.?)	30y	F	zygomatic fracture + orbital floor involvement (R) PS	transconjunctival with cantotomy + supraorbital
#10 (d.i.)	20y	F	zygomatic fracture (R) PS	supraorbital + lower lid + intraoral

Our technique expresses a new surgical philosophy, transferring the surgical procedure from the patient to the model, the surgery “planned” on the model is then re-transferred to the patient thanks to the resorbable devices that, already shaped, embody the surgical project and guides the repositioning of bony segments. Surgery itself on the patient will therefore be limited to mobilisation of the fractured fragments and to obtaining synthesis-guided fracture reduction.

CONCLUSIONS

Our experience illustrates the possibility of modifying the approach to the surgical treatment of head injuries. In maxillofacial traumatology it is difficult and sometimes impossible to obtain large operator access fields to the fracture sites. The vasculonervous anatomy, the presence of highly specialised structures and the importance of minimising facial scarring impose limited access. Similarly, in the case of extensive damage, the reconstruction of the orbit walls, floor and medial wall is a complex challenge and the need to restore both anatomy and function is essential. Combined use of stereolithographic model technologies and resorbable plates and screws allows a new surgical approach based on transferring the action from the patient to the model, thus overcoming many of the limits of the conventional technique. Furthermore, the accuracy of the reconstruction has been evaluated on the anatomical model in advance.

For the patient there is the undeniable advantage of avoiding the need to remove titanium plates (21). At the same time, this aspect constitutes an economic advantage for the health service. Another economic advantage is related to the optimisation of the number and type of plates.

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SALIVARY GLUCOSE AS A METABOLIC CONTROL MARKER IN PATIENTS WITH TYPE 2 DIABETES

E. CARRAMOLINO-CUÉLLAR¹, D. LAURITANO², F. CARINCI³,
J. SILVESTRE-RANGIL¹, C. BAÑULS-MORANT⁴,
F. J. SILVESTRE⁵ and A. HERNÁNDEZ-MIJARES⁴

¹*Stomatology & Oral Surgery, Dr Peset University Hospital, Department of Stomatology, University of Valencia, Spain;* ²*Department of Medicine and Surgery, University of Milan-Bicocca, Monza, Italy;* ³*Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, Italy;* ⁴*Department of Endocrinology and Nutrition, Dr Peset University Hospital, Department of Medicine, University of Valencia, Spain;* ⁵*Sección Pacientes Especiales, Clínica Odontológica Universitaria, Valencia, Spain*

A study was made of the correlation between the serum and salivary glucose levels in healthy subjects and in patients with type 2 diabetes, in order to establish the validity of salivary glucose determination in monitoring glycemia. Ninety-seven subjects were included in the study: 47 diabetic patients and 46 controls, aged between 40- and 80-years-of-age. Venous blood and saliva samples were collected in both groups under fasting conditions and after administering a test meal (15% proteins, 55% carbohydrates and 30% lipids). The glucose levels were measured using the glucose oxidase technique. The salivary glucose levels were seen to be greater in the diabetic group vs the controls both under fasting conditions (baseline) and after the meal (postprandial) ($p=0.023$ and $p=0.008$, respectively). A significant positive correlation was found between the serum and salivary glucose levels at baseline and under resting conditions, particularly in the diabetic group ($r=0.389$, $p=0.002$). The coefficient of determination of the simple linear regression model was $R^2=0.042$, showing salivary glucose to be related to the blood glucose levels. In conclusion, salivary glucose concentration is correlated to serum glucose, particularly in type 2 diabetics.

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by hyperglycemia. Its etiopathogenesis involves a number of mechanisms, ranging from autoimmune destruction of the pancreatic β cells to anomalies resulting in resistance to insulin action. It has been estimated that the worldwide prevalence of type 2 diabetes will reach 366 million people by the year 2030, making it one of the most important public health problems of our time. In Spain, approximately 13.8% of the population suffers the disease (1). Although it is

well known that chronic hyperglycemia can produce micro- and macrovascular complications, it may also cause oral disorders such as salivary gland alterations that can result in changes in saliva production and composition, periodontal disease (2-8), dental caries, increased vulnerability to infections, and slowed healing of oral lesions (9).

Saliva offers an alternative to serum as a biological fluid, since it contains markers that are derived from serum. The collection of saliva samples has the advantage of being easy to perform, noninvasive

Key words: Salivary glucose, glycemia, saliva, diabetes mellitus

Mailing address:

Prof. F. Javier Silvestre Donat,
Sección Pacientes Especiales,
Clínica Odontológica Universitaria,
C/ Gascó Oliag 1,
46010-Valencia, SPAIN
e-mail: francisco.silvestre@uv.es

0393-974X (2016)

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and with a good cost/benefit ratio for use in large studies. In this regard, saliva sampling may be an alternative in cases where blood sampling involves risk or proves difficult (10).

The increased basal membrane permeability observed in the salivary gland acini of diabetic patients can lead to an increased presence in saliva of certain components found in serum, such as glucose, thanks to the small size of the molecule (11). As a result, some studies have suggested that saliva could be used as a substitute for blood in determining glycemia in diabetic patients, though the results to date have been very controversial (12-16).

Saliva thus might be useful as a tool for the diagnosis and even the monitoring or control of certain disease processes. The present study was therefore carried out to analyze the relationship between the concentrations of glucose in serum and saliva, in both healthy individuals and in diabetic patients, with the aim of assessing the usefulness of salivary glucose concentration in clinically monitoring patient glycemia.

MATERIAL AND METHODS

Study design and patient selection

A cross-sectional prospective study was carried out in the Departments of Stomatology and Endocrinology (Dr. Peset University Hospital, Valencia, Spain). The study group consisted of 47 patients with type 2 diabetes randomly recruited from among the diabetic patients seen in the hospital, while the control group consisted of 46 non-diabetic individuals accompanying the mentioned patients, though with no kinship between them.

The subjects in both groups were required to be between 40- and 80-years-of-age, with no smoking at least during the last 12 months, and with at least 10 teeth in the mouth. Pregnant women were excluded, as were individuals with decompensated systemic conditions such as cardiac, liver, thyroid gland or renal diseases. The diabetic individuals were required to have a glycosylated hemoglobin (HbA1c) concentration of $\leq 8\%$, with treatment limited to oral antidiabetic drugs. The control group consisted of healthy individuals without diabetes or glucose intolerance, matched by age and gender to the diabetic group. Written informed consent to participation

in the study was obtained from all the participants.

The study was approved by the local Clinical Research Ethics Committee (reference 09/093), in accordance with the principles of the Declaration of Helsinki referred to experimental studies in humans.

Blood and saliva sample collection and analysis

On the day of the test, the patients were required to come to the center early in the morning, after a fasting period of at least 10 hours and without having performed oral hygiene in the last two hours. After compiling the case history, blood and whole saliva samples were collected. The saliva samples were obtained after rinsing the oral cavity with distilled water, under both resting conditions and following stimulation by chewing paraffin tablets (Ivoclar Vivadent®, Liechtenstein). The subjects then received a test meal in the form of Nestlé Resource® Energy (nutritional supplement: 15% de proteins, 55% carbohydrates and 30% lipids), and the samples were analyzed immediately before deterioration could occur. The drainage technique was used for saliva collection, discarding the two min of secretion prior to the start of the test. The accumulated saliva was decanted each min for a total of 5 min, into a funnel connected to a millimetered glass tube. The blood and saliva samples were centrifuged (2000 g at 4°C during 10 min) and analyzed using the glucose oxidase technique (Trinder GOD-POD Spinreact®, Gerona, Spain). Two hours after the test meal, we repeated the process with the collection and analysis of blood and saliva samples.

Statistical analysis

In order to ensure a statistical power of 80%, with the detection of a correlation of 0.4 using a two-tailed test ($c=2$) and a significance level of 5% ($\alpha=0.05$), we required the inclusion of at least 44 subjects in each group (total=88). The final sample consisted of 47 patients with type 2 diabetes and 46 controls.

Quantitative variables were reported as the mean of each group and the standard deviation (SD) as a measure of sample dispersion. The Mann-Whitney U-test was used to evaluate differences between the groups. The Spearman correlation coefficient was used to establish associations between the saliva and serum glucose concentrations, and a simple linear regression model was developed to predict serum glucose from salivary glucose.

RESULTS

We analyzed 47 patients with type 2 diabetes and 46 controls, with a mean age of 61 ± 6 and 59.4 ± 5.2 years, respectively. The diabetic group consisted of 19 males (40.4%) and 28 females (59.6%), while the control group consisted of 18 males (39.1%) and 28 females (60.9%). The anthropometric data are shown in Table I.

At baseline the salivary glucose levels were found to be higher in the diabetic patients than in the control group under both resting conditions (GLUSALRB) and in response to stimulation (GLUSALEB) - though statistical significance was only reached for resting saliva, with a mean concentration of 5.57 ± 5.08 mg/dl and 3.73 ± 2.53 mg/dl, respectively ($p=0.023$). Following the test meal, the stimulated salivary glucose concentration (GLUSALE120) was

significantly higher in the diabetic group ($p=0.008$) (Table II).

A significant positive correlation was found between the serum and salivary glucose levels at baseline and under resting conditions, particularly in the diabetic group ($r=0.389$, $p=0.002$). In addition, under postprandial conditions, we recorded a correlation between the stimulated salivary glucose and serum glucose levels in the overall sample ($r=0.309$, $p=0.003$) (Fig. 1).

A simple linear regression model was developed to predict serum glucose from salivary glucose. The estimated coefficient of the slope proved significant ($b=1.63$; $p=0.048$), thus confirming the existence of a correlation. Specifically, for every 1 mg/dl increase in salivary glucose, a 1.63 mg/dl increase can be expected in serum glucose concentration (serum glucose = $107.20 + 1.63$ salivary glucose). The coefficient of determination was $R^2=0.042$.

Table I. Anthropometric data of the study groups.

	DM (n=47)	CONTROLS (n=46)	P-value (MW)
Age (years)	61 ± 6	59.4 ± 5.2	>0.05
Gender	M 40.4% / F 59.6%	M 39.1% / F 60.9%	>0.05
Height (m)	1.62 ± 0.1	1.63 ± 0.1	>0.05
Weight (kg)	82 ± 15.3	72.1 ± 12.2	<0.01
BMI (kg/m ²)	31.2 ± 4.7	27.2 ± 3.8	<0.001
Waist (cm)	105.8 ± 10.7	91.9 ± 12.7	<0.001
Hip (cm)	109.6 ± 9.5	104.8 ± 7.4	<0.01
WHR	0.97 ± 0.1	0.88 ± 0.1	<0.001
SBP (mmHg)	143.9 ± 22.5	131.8 ± 19.6	<0.01
DBP (mmHg)	84.4 ± 16.9	76.7 ± 8.8	<0.01

Data expressed as the mean $\pm$ standard deviation (SD); M: males; F: females; BMI: body mass index; WHR: waist-hip ratio; SBP: systolic blood pressure; DBP: diastolic blood pressure; MW: Mann-Whitney U-test.

Table II. Salivary glucose concentrations according to the presence or absence of type 2 diabetes.

GLUCOSE (mg/dl)	DM (n=47)	CONTROLS (n=46)	P-value (MW)
GLUSALRB	5.57±5.08	3.73±2.53	0.023
GLUSALEB	4.31±3.08	3.46±2.73	0.150
GLUSALR120	5.47±4.77	4.27±2.74	0.593
GLUSALE120	5.09±4.25	3.16±2.48	0.008

Data expressed as the mean $\pm$ standard deviation (SD); **GLUSALRB**: baseline resting salivary glucose; **GLUSALEB**: baseline stimulated salivary glucose; **GLUSALR120**: postprandial resting salivary glucose; **GLUSALE120**: postprandial stimulated salivary glucose; **MW**: Mann-Whitney U-test.

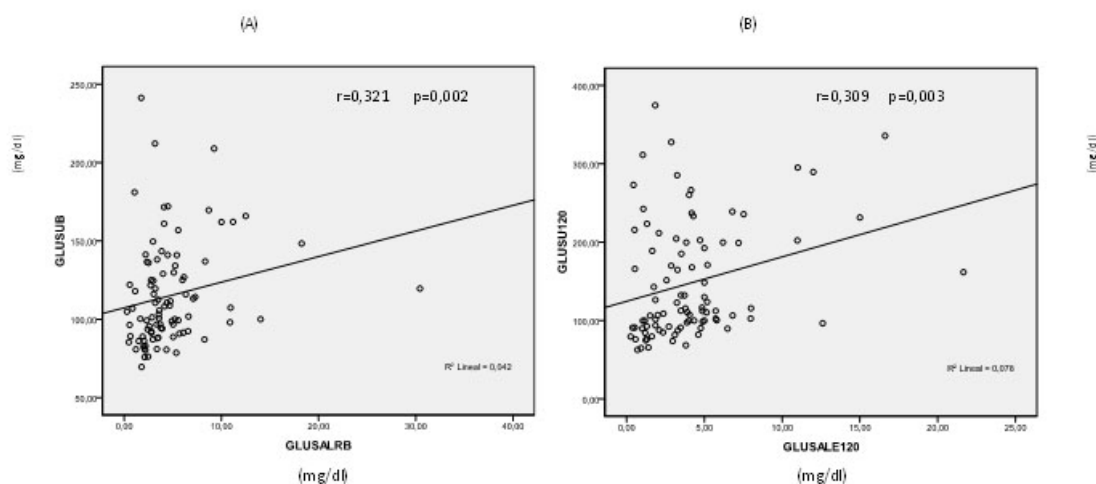


Fig. 1. Correlation between serum and salivary glucose in the overall study sample at baseline (A) and after the test meal (B). **GLUSALRB**: baseline resting salivary glucose; **GLUSALE120**: postprandial stimulated salivary glucose; **GLUSUB**: baseline serum glucose; **GLUSU120**: postprandial serum glucose.

DISCUSSION

In accord with the observations of most authors (12, 15, 17-30), we recorded a significant increase in salivary glucose levels in the type 2 diabetic patients versus the controls, both at baseline and following the test meal. This elevation could be a result of the increase in basal membrane permeability found in diabetic individuals (31), which in turn would favor glucose penetration from blood into saliva. Some investigators have only observed this increase among diabetic patients with poor metabolic control (13, 32), thus suggesting that the phenomenon only occurs when the blood

glucose levels are particularly high. These results must be interpreted with caution, however, since the mentioned studies involved small sample sizes.

Likewise, as with other authors (16-19, 25, 28-30, 33), we observed a positive correlation between the serum and salivary glucose levels in both groups, the relationship being more evident among the type 2 diabetics (18, 34). This suggests the possibility of estimating serum glucose levels from the salivary glucose concentrations. Other investigators have also reported this association, though with comparatively greater correlation values (19, 25, 26, 29, 35, 36). Balan et al. (35) found that 80.28% of the patients with controlled diabetes and 74.13%

of those with poor diabetes control exhibited an association between both parameters, i.e. the correlation being lower among the non-diabetics. Likewise, Amer et al. (19) recorded a strong correlation ($r=0.78$) in type 2 diabetic individuals, suggesting that saliva could be a good marker for monitoring blood glucose conditions in diabetic patients, in line with the findings of Borgnakke (26), who obtained similar results in the case of diabetic individuals with poor metabolic control. A recent meta-analysis has explored this possible association, including a total of 10 clinical trials with rigorous screening criteria. A stronger overall significant correlation was observed ($r=0.49$), with greater correlation in the diabetic group versus the control group ($r=0.67$ and 0.30 , respectively) (36).

Although many authors have observed a relationship between glucose levels in blood and saliva, a number of factors prevent the firm definition of salivary glucose as a marker for monitoring glycemia. The fact that glucose can undergo rapid degradation mediated by bacteria and enzymes in the oral cavity or on penetrating from blood into saliva (37), together with the great heterogeneity in the designs of the studies found in literature (with important differences in the saliva collecting methods, glucose tests, mean age or metabolic control of the patients), all complicate the comparison of results among different studies and the possibility of drawing firm conclusions. In our case we analyzed the samples immediately after collection in order to reduce the risk of glucose degradation in saliva, and we only included diabetic patients with acceptable metabolic control, in order to control for this variable.

In conclusion, glucose appears to penetrate saliva from blood, associated to increments in the blood glucose levels with a closer correlation between the two parameters being observed in the diabetic population. However, the methodological heterogeneity of the studies found in the literature makes it necessary to conduct further controlled studies in order to correlate serum glucose to salivary glucose levels and thus determine the possibility of using salivary glucose as a noninvasive diagnostic test.

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NEUROINFLAMMATION, ALZHEIMER'S DISEASE AND PERIODONTAL DISEASE: IS THERE AN ASSOCIATION BETWEEN THE TWO PROCESSES?

F.J. SILVESTRE¹, D. LAURITANO², F. CARINCI³, J. SILVESTRE-RANGIL¹,
M. MARTINEZ-HERRERA¹, and A. DEL OLMO⁴

¹*Stomatology & Oral Surgery, Dr Peset University Hospital, Department of Stomatology, University of Valencia, Spain;* ²*Department of Medicine and Surgery, University of Milan-Bicocca, Monza, Italy;* ³*Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, Italy;* ⁴*Neurology, Dr Peset University Hospital, Department of Medicine University of Valencia, Spain*

Alzheimer's disease (AD) is a very common cause of dementia in developed countries and increases its prevalence progressively with age. AD etiopathogenesis is not yet understood. However, it is recognized that neuroinflammation plays a key role in its pathogenesis with the activation of microglia and the secretion of proinflammatory cytokines triggering irreversible neurodegenerative deterioration. This paper is a short review of the relationship between AD and periodontal disease (PD). Both processes may have common causes: both are inflammatory diseases and the prevalence and progression increases with ageing. However, we must consider that AD begins to develop many years before its clinical diagnosis. It is thought that in this prodromal period a connection could be established between both processes, both sustained by low intensity inflammation. There are several studies that relate both processes such as the possible systemic exposure to certain periodontopathogenic bacteria or the proinflammatory cytokines and other elements. It is argued that there are no modifiable factors such as age, or genetic factors, but that there are other factors that could be avoided, modified or controlled such as periodontal peripheral inflammation.

Alzheimer's disease (AD) is a degenerative and progressive process that is clinically characterized by cognitive and functional impairment and by the presence of amyloid plaques and neurofibrillary tangles in the brains of these patients (1,2). It represents about 70% of cases of dementia. It is estimated that in Spain, the prevalence of AD among people over 65 years of age exceeds 5% (1) and prevalence values of 7% are reached in Western Europe (3). AD has a significant impact due to its irreversible nature, lack of effective curative treatment and is

considered a burden for families. It is an important cause of morbidity and mortality in developed countries. In addition AD has a significant impact on public and private health systems (4).

For the last few years, thanks to the introduction of biomarkers (cephalo-spinal fluid measurements), liquid cephalo-rachidian (LCR)- of beta-amyloid, Tau proteins, and the amyloid image by positron emission tomography (PET), (amyloid PET), diagnosis of AD can be performed in the initial clinical phases (before dementia - prodromal phase), or even

Key words: Alzheimer's disease, dementia, neuroinflammation, periodontal disease, periodontitis, proinflammatory cytokines

Mailing address:

Dorina Lauritano DDS,
Department of Medicine and Surgery,
University of Milano Bicocca,
Via Cadore 48, 20052 Monza, Italy
Tel.: +39.0392332301 - Fax: +39. 03923329892
e-mail: dorina.lauritano@unimib.it

0393-974X (2016)

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detected before the appearance of the first clinical symptoms (preclinical phase). In addition, better knowledge of the clinical phenotype of the disease also facilitates early diagnosis (5-7) and, therefore, facilitates the development of research on this disease. It has been shown that the first biological alterations of AD begin decades before the first clinical symptoms, which is known as the preclinical phase. This phase is biologically active and there are anatomical and physiological changes in the patient's brain that may even modify cognitive performance. In this phase patients are "cognitively normal" (8). Initially, when the patient also shows good functionality, the symptoms can be detected by anamnesis and neuropsychological tests. This phase is considered the prodromal phase of the disease (before dementia) (9). The dementia phase implies the appearance of symptoms with an evident social and occupational deterioration of the patient. At present, it is believed that to be effective, treatment should initiate in the very early stages of the disease, so early detection is essential (8).

From the neuropathological point of view, AD is characterized by amyloid beta ($A\beta$) deposits derived

from the degradation of the amyloid precursor protein (APP); neurofibrillary tangles proteins (Tau protein), as well as neuronal and synaptic loss. Both pathological proteins present a temporal and spatial dissociation between them. Amyloid initially accumulates in the cerebral neocortex, before the onset of symptoms. Over time, it extends to other areas. The Tau proteins begin to accumulate and initially appear in the hippocampus and in the entorhinal cortex, subsequently in cortical areas. In addition, there are cellular losses in the Meynert basal nucleus and the Locus Coeruleus (10). Nowadays, the accumulation of $A\beta$ (products of oxidative stress), the synaptic dysfunction and the neurodegeneration depend on the state of inflammation of the nervous system (neuroinflammation). In turn, neuroinflammation can deregulate glial activation. Therefore, it could be hypothesized that neuroinflammation could act as a triggering factor for accumulation of $A\beta$ deposits, known as one of the causes of neurodegeneration in AD (11, 12). In addition, the amyloidogenic process may cause an increase in the oxidative capacity of $A\beta$ and generate an inflammatory response with an increase of cytokines, nitrous oxide (NO), products

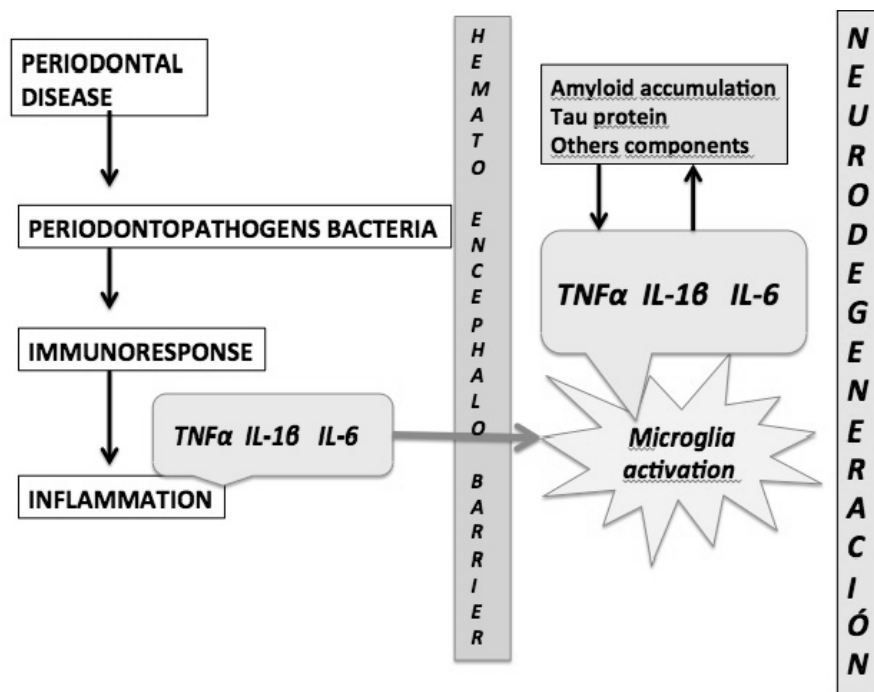


Fig. 1. Mechanism of action of periodontal disease on Alzheimer's disease.

derived from oxidative stress and microglial reactivity. Microglial inflammation and activation anticipate neuronal damage. Proinflammatory conditions appear to favour the development of AD whereas sustained anti-inflammatory treatment modifies its incidence (13-15).

The objective of the present review is to describe the relationship between AD and periodontal disease (16-22), through a common inflammatory pathogenesis. In fact, periodontal inflammation may contribute to the maintenance and aggravation of neurodegeneration.

Neuroinflammation and Alzheimer's disease

Abnormal protein is associated with numerous forms of brain damage and when aggregated, leads to overproduction of reactive oxygen species (ROS) and oxidative stress. This may trigger the activation of neuroprotective mechanisms such as inflammation, but may be neurotoxic (23, 24).

When microglia is activated, it triggers the release of proinflammatory cytokines such as tumour necrosis factor α (TNF α), interleukin 1 β (IL-1 β), interleukin 6 (IL-6) and proteins such as C-reactive protein (CRP). In turn, this accumulation of proinflammatory products act in a paracrine or autocrine way on the glial cells, favouring a greater formation of A β -42 due to the activation of the amyloidogenic pathway of amyloid precursor proteins (APP), Tau protein and other inflammatory molecules. Thus, this cycle is self-supported with glial stimulation and activation of proinflammatory molecules producing cytoskeletal alterations by Tau aggregation and ultimately, neurodegeneration (25). In turn, the aggregates of altered Tau proteins are released into the extracellular medium acting again as neurotoxic products. The microglial stimulation influences the type of stimulus, the intensity, and the activation time. In response to A β accumulation, neuroinflammation in its early stages may be protective. Prolongation over time without removing the triggering stimulus, would result in chronic low-grade inflammation, microglial over activation with cytokine release, neuronal sensitivity to free radicals and neurodegeneration (23).

The senile plaques are associated with the reactivity of astrocytes and previous activation of the

microglia and immunoreaction antibodies against TNF α , IL-1 β , IL-6 (26, 27). C-reactive protein has also been studied in patients with cognitive impairment and risk of AD. In these patients CRP showed elevated mean values many years before the on-set of AD (28). Other studies have observed the effective slowing-down of the onset of AD in patients taking non-steroid antiinflammatory drugs (NSAIDs) (29), although the results depend on the type of anti-inflammatory used. Cyclooxygenase-1 (COX-1) inhibitors, administered in effective concentration and adequate duration, have an agonist effect on gamma receptor activator of peroxisome proliferation (PPAR- γ), inhibiting the proinflammatory mediators, with effective results, if used in very early stages of the disease (30).

Periodontal disease as generator of inflammation

Periodontal disease (PD) is one of the most common pathologies in humans. It is a chronic, infectious and inflammatory process of the supporting structures of the tooth that if untreated, ends with tooth loss. PD is associated with a number of host-related factors such as the type of immune response and genetic determinants, but may also be influenced by environmental factors such as oral hygiene, smoking, stress, and systemic health status (31, 32).

The presence of a biofilm initially on the surface of the teeth and gingiva will facilitate successive microbial communities inducing a host immune response. Initially this host response is mediated by the activity of phagocytes such as neutrophils and macrophages, subsequently activates an adaptive response by lymphocytes (33).

Fundamentally, the initial inflammatory state has a protective mission against pathogenic bacteria but if it persists, it will damage the periodontal tissues of the host. A migration of the epithelium towards the apical will occur, enlarging the periodontal pocket along with the progressive destruction of the alveolar bone and the periodontal ligament. This in turn favours the growth and development of bacterial plaque of periodontopathogenic bacteria that will extend and perpetuate inflammation (34).

Currently, it is believed that the dysbiotic

community that forms the evolved plaque will favour the destruction of these tissues and increase their protection against the host. The products derived from this tissue degradation will serve as a source of nutrients, favouring the maintenance of an inflammatory medium. Hence, they have been defined as inflamophilic communities (35). In turn, chronic inflammation may favour selection and enrichment of these communities creating a vicious circle that increases the progression of the disease. More precisely, the periodontal treatment is based on the removal of the periodontal calculus and the affected tissues, in order to break this vicious circle and decrease inflammation (36).

Association of periodontitis with Alzheimer's disease

Aging has been associated with a significant deterioration of oral health with an increase in edentulism, dental caries and especially periodontitis (37, 38).

The evidence of the relationship between periodontitis and AD has been established in several hypotheses. Firstly, the patient with AD has poor oral hygiene due to the abandonment of hygiene habits and a lack of manual ability to perform such care. If major tooth loss occurs, they will lead to poorer feeding due to reduction of masticatory efficiency and the stimulation of chewing will be lost. This alteration in the masticatory reflex may lead to a decrease in the pyramidal cells and in the level of acetylcholine in the hippocampus, which may contribute to memory loss. On the other hand, prolonged poor oral hygiene can cause chronic periodontitis and this may favour the risk in the development of AD by providing proinflammatory cytokines at the systemic level from chronic periodontal inflammation. This situation could contribute to modify the low-grade inflammation at the cerebral level. These small contributions of cytokines from peripheral periodontal inflammation may increase the degree of neuroinflammation (39-41). Studies have been carried out to demonstrate the possible systemic exposure of periodontopathogenic bacteria. A model of relationship between chronic systemic exposure to certain periodontal bacteria such as *Porphyromonas gingivalis* and alteration of the cognitive level has been proposed (Fig. 1). The systemic presence of these

microorganisms has been demonstrated in association with atherosclerotic lesions or with rheumatic diseases such as rheumatoid arthritis (42).

We observed the level of cognitive ability through the test and the level of antibodies against *Porphyromonas gingivalis* in patients over 60-years-of-age. When the level of antibodies against this bacteria increased, the level of verbal agility and ability to calculate in a dose-dependent manner decreased, proving an evident degree of correlation between both (43). Steir et al. (44) also performed a cohort study on serum antibodies in PD. At the beginning, none of the participants had cognitive alterations, but throughout the group, individuals with higher levels of serum antibodies against periodontopathogenic bacteria were associated with the occurrence of AD. However, although this association has been described, the possible mechanisms involved have not been clarified. Multiple factors such as age, environmental factors, genetic factors, and degeneration in neurotransmission, involvement of the blood-brain barrier, immune system alterations and infectious agents directly or indirectly have been implicated in association with AD.

Elderly patients have shown a certain degree of bacteraemia following routine oral hygiene measures such as dental brushing, especially if gingivitis is present. This bacteraemia with presence of periodontopathogens (*Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola*) can produce a low-grade infection by disseminating these bacteria and their toxic products, inducing in the host an inflammatory response with proinflammatory cytokine release. Prolonged low brain exposure to these pathogens and related proinflammatory factors would initially act by activating the microglia and increasing the degree of inflammation.

Experimental studies with mice have demonstrated the presence of pathogens such as *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia* in the brain tissue several weeks after inoculating them at the oral level (45). DNA from *Porphyromonas gingivalis* was found in the brains of mono-infected in 9 out of 12 mice at 24 weeks. Activation of complement was demonstrated with involvement of pyramidal neurons of the hippocampus.

Lipopolysaccharides (LPS), which form part of

the germ wall, may promote increased permeability of the blood-brain barrier facilitating the entry of proinflammatory molecules into the brain and accelerating neuroinflammation (44).

These same pathogens have also been identified in post-mortem studies of patients with AD. Years ago, a study by Miklossy et al. (46) was conducted to find out possible post-mortem and central nervous system spirochete infection using dark field microscopy. We selected 14 patients with a history of AD and 13 with different brain pathologies without dementia. Spirochetes were observed in central nervous system of the 14 cases with AD and not observed in any of the 13 controls.

In another study, we attempted to determine if any species of oral *Treponema* could be detected in the brains of persons with AD (16 AD and 18 controls). *Treponemas* were detected in 14/16 of the AD group and only 4/18 of controls (47). In a more recent post-mortem study, periodontal pathogens such as *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia* were identified in 10 brains of AD cases. The presence of *Porphyromonas gingivalis* LPS in brain tissue was confirmed in 4 cases (48).

Inflammation treatment

Most risk factors in AD are not modifiable as age and genetic factors are involved. However, there may be factors associated with inflammation such as PD that could be controlled by taking preventive measures and performing periodontal treatment.

PD is produced as a host reaction to the anaerobic and Gram-negative bacterial plaque of periodontopathogenic bacteria that causes and favours inflammation, which is perpetuated if the plaque is not removed at the sub gingival and supragingival levels (49,50). Dental brushing can remove the biofilm and the initial bacterial plaque but at the sub gingival level a mature, well-established plaque is impossible to remove without attending a specialized treatment with ultrasound and root scaling. With this periodontal treatment, the affected periodontal tissues and the sub gingival calculus are debrided and can be supplemented with the use of mouthwashes or effective antiseptic gels such as 0.12% chlorhexidine gluconate.

It is also necessary to avoid those factors that may favour the accumulation of sub gingival plaque, which can be treated in a preventive and corrected way, such as performing a correct oral hygiene or the removal of possible irritating periodontal factors such as dental caries, small dental fractures or incorrect dental restorations (39).

Other types of treatments have also been proposed to decrease proinflammatory products derived from host responses such as prostaglandins (PG2) and cytokines (IL-1, IL-6, TNF). No steroidal anti-inflammatory drugs (NSAIDs) and anti-cytokine drugs were proposed (51).

Among NSAIDs, cyclo-oxygenase (COX) inhibitors such as rofecoxib and non-selective inhibitors such as indomethacin have been proposed (52-54). With the administration of indomethacin, IL-6 and TNF α levels were reduced. However, no response was obtained regarding the decrease of A β . In addition, side effects such as gastric irritation and cardiovascular adverse effects were observed, so these drugs had to be discontinued.

Recent studies using drugs active on prostaglandin receptors have shown, in experimental animal studies, improvements in cognitive function, and reduction of free radicals (51).

CONCLUSIONS

The correlation between AD and periodontal disease is still a controversial topic, though numerous studies support this hypothesis.

Although the intended therapeutic action in the future will be to decrease and control neuroinflammation at a central level early, peripheral inflammation should also be controlled, which may in some way negatively influence neuroinflammation and AD.

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ORAL PROBIOTICS IN THE MANAGEMENT OF GINGIVITIS IN DIABETIC PATIENTS: A DOUBLE BLINDED RANDOMIZED CONTROLLED STUDY

S. SABATINI¹, D. LAURITANO², V. CANDOTTO³,
F. J. SILVESTRE⁴ and G. M. NARDI¹

¹Department of Oral and Maxillo-Facial Sciences, Sapienza University of Rome; ²Department of Medicine and Surgery, University of Milan-Bicocca, Monza, Italy; ³Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, Italy; ⁴Stomatology & Oral Surgery, Dr Peset University Hospital, Department of Stomatology, University of Valencia, Spain

Diabetic patients are more prone to develop infections such as periodontal diseases. Bacteriotherapy with the concept of using good bacterial strains to replace the pathogenic ones emerged as a fascinating field due to the increasing incidence of antibiotic resistance. The aim of this study was to evaluate if a particular combination of probiotics (Lactobacillus Reuteri DSM 17938 and Lactobacillus Reuteri ATCC PTA 5289) is useful in treating gingivitis in diabetic patients. Eighty adult patients with diagnosis of controlled diabetes type II and gingivitis were enrolled for this study and randomized in two groups. Only oral hygiene instructions were given. Bacteriotherapy (Lactobacillus Reuteri) was administered to test group. Outcome measures were presence of Plaque (P.I.) and Bleeding on Probing (B.O.P.). Data were collected at baseline and after 30 days. At 30 days, both groups showed a statistically significant clinical indexes reduction from baseline. More reduction was present in the test group for P.I. (14%±6) and for B.O.P. (18%±4) and was statistically significant (p<0.05). Within the limitations of this study, bacteriotherapy can be considered effective in reducing plaque and BOP in patients with controlled diabetes type II and gingivitis.

Diabetes mellitus is a metabolic syndrome considered to be caused by multiple factors resulting from a deficiency of insulin, which may be absolute due to pancreatic β -cell destruction (type 1) or relative due to an increased resistance of the tissues to insulin (type 2) (1). In Europe it is estimated that about over one million people have diagnosis of diabetes, though the actual number is certainly larger, in view of the individuals with the syndrome but without the diagnosis.

Gingivitis is a non-destructive periodontal disease (2-8). The most common form of gingivitis is in response to bacterial biofilms adherent to

tooth surfaces, termed *plaque-induced gingivitis*. Gingivitis is reversible with good oral hygiene. Gingivitis and periodontitis often affect diabetic patients.

Through extensive research, dental plaque has been proved to be paramount in the initiation and progression of gingival and periodontal diseases (9,10).

Probiotics are living microbial feed, which provide benefits to the host. They are a heterogeneous group of non-pathologic bacteria that are functionally defined by their ability to allay inflammation when introduced into the inflamed intestine (10, 11).

Key words: oral hygiene, gingivitis, probiotics, diabetes mellitus type 2

Mailing address:
Dr Silvia Sabatini,
Via Caserta 6,
00161 Rome, Italy
Tel.: +393495061580
e-mail: silviasabatini01@gmail.com

197(S)

0393-974X (2016)
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Bacteriotherapy, with the concept of using good bacterial strain to replace the pathogenic ones, emerged as a fascinating field due to the increasing incidence of antibiotic resistance (11, 12). Probiotics traditionally used in medicine are now being used in an attempt to control and treat periodontal disease (13-16).

The aim of this study was to evaluate if a particular combination of probiotics (Lactobacillus Reuteri DSM 17938 and Lactobacillus Reuteri ATCC PTA 5289) is useful in treating gingivitis in diabetic patients. The hypothesis was that the use of probiotics is effective in lowering the indicators of the presence of gingivitis against the lack of effect.

The Consort statement (Consolidated Standards of Reporting Trials) in the present study (<http://www.consort-statement.org>) was followed.

MATERIALS AND METHODS

The study was designed as a pilot randomized controlled study with 30 days follow-up. This study was conducted according to the Declaration of Helsinki. Any patient having diagnosis of diabetes type II reporting gingivitis between October 2015 and January 2016 was considered eligible for the study. Patients were enrolled in one dental office in Sarzana. It was decided to include 40 patients per group, since no previous study was available to calculate a sample size.

Inclusion criteria:

- age >18 years
- diagnosis of diabetes type II
- diagnosis of generalized gingivitis (bleeding on probing in 30% of sites at least, absence of PPD >4mm; absence of PAL > 4mm)

Exclusion criteria:

- additional systemic diseases other than diabetes
- uncontrolled diabetes
- smoker
- pregnancy
- antibiotic therapy during the previous 6 months
- presence of dental implants
- previous orthodontic therapy
- impossibility to come to the follow-ups

Main outcome measures

- BOP: defined as percentage of bleeding sites considering six sites per each tooth.
- P.I.: defined as percentage of sites with plaque considering six sites per each tooth.
- Side effects or/and adverse events

Subjects were required to be available for the duration of the study and to sign an informed written consent form.

Clinical protocol

Bleeding on probing (BoP) and Plaque Index (PII) was collected from all patients enrolled in the study using a periodontal probe CP15 and a fluorescein plaque detector (Fig. 1a, b) by an experienced dental hygienist (SS).

No professional cleaning was performed. All patients were asked to brush twice per day for at least 2 min with a compact size medium bristle toothbrush (Gum® Technique Pro Compact Medium) and to clean interproximal areas with medium bristles interproximal brushes of different sizes (Gum® Trav-ler) (Fig. 1c, d). No attention was paid to the movement but to the instrument, according the Tailor-made protocol (17). The toothbrush was chosen by the operator after evaluating the oral condition of gingivitis and the decision was shared with the patient, underlying the design characteristics of the instrument that allows bristles to enter the gingival sulcus however it is used. The interproximal brushes were chosen as they fit well in each area that needed to be cleaned. The decision was shared with the patient.

After brushing instructions, an envelope with a code was opened and the patient was enrolled in group 1 or group 2. Dedicated randomization software (<https://www.randomizer.org>) generated the codes. The patient was not blinded to the treatment. The operator who took the measurements before and after the treatment and the statisticians did not know to which group the patients belonged to. Another operator administered the probiotic according to the group (G.N.) (Fig. 2). Patients of group 1 were asked to take a tablet of probiotics (Reuterinos®) twice per day after brushing. After 30 days BoP and PII were collected again (Fig. 1e).

Statistical analysis

The patient was the unit of analysis. Differences within groups from baseline to 30 days should be evaluated with *T*-test for paired data. Difference between groups

should be evaluated with *T*-test for paired data. A different operator evaluated the results and carried out statistical analysis. *T*-test for paired data was applied ($p < 0.05$).

RESULTS

One hundred and fifty diabetic type II patients were examined between October 2015 and January 2016 and 80 patients were included in the study.

Reasons for exclusion of the 70 patients were:

- 20 declined to participate as unable to come to follow ups;
- 18 had periodontitis;
- 19 had implants;
- 8 received antibiotic therapy during the previous six months;
- 2 were pregnant women;
- 3 had no controlled diabetes.

All the 80 subjects completed the study. The mean age was 47 ± 10.5 years with a female/male ratio of 0.7/1. In group 1 the mean age was 49.5 ± 12.2 years with a female/male ratio of 0.8/1. In group 2 the mean age was 54.4 ± 9.07 years with a female/male ratio of 0.8/1. No statistical differences in age and sex were recorded between the two groups.

Group 1 (test group) had a mean PII of $35\% \pm 8$ at baseline. After 30 days it was $12\% \pm 3$, showing a statistically significant reduction ($p < 0.005$) from baseline. The mean BoP was $52\% \pm 7$ at baseline and $15\% \pm 2$ after 30 days. The result was statistically significant ($p < 0.001$).

Group 2 had a mean PII of $34\% \pm 6$ at baseline and after 30 days it was $26\% \pm 4$, showing a statistically significant reduction ($p < 0.005$). The mean BoP was $51\% \pm 5$ at baseline and $33\% \pm 7$ after 30 days, showing a statistically significant reduction ($p < 0.005$).

The difference of Mean PII and BoP at baseline were not statistically significant between Group I and Group II, while the difference was statistically significant at 30 days ($p < 0.005$). Tables I and II summarize the results. No adverse or side effects were noticed or recorded.

DISCUSSION

The study showed that taking a probiotic with *Lactobacillus Reuteri* produced a statistically

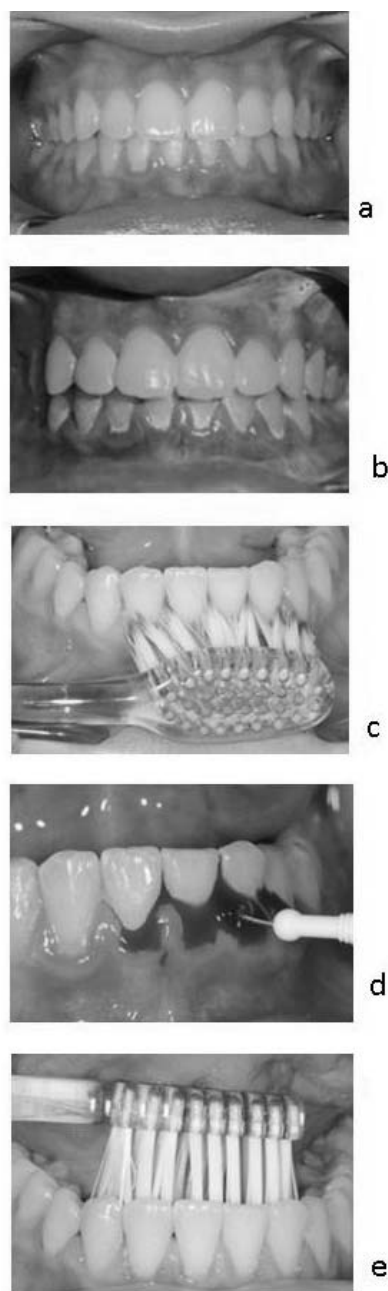


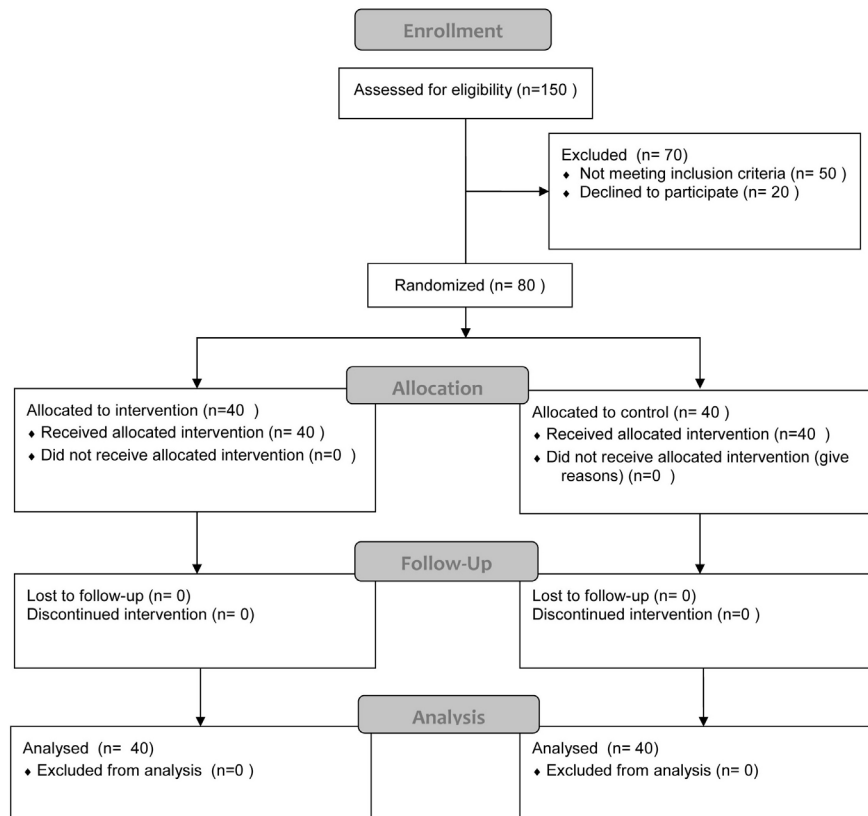
Fig. 1. *a): At baseline, patients showed gingivitis due to plaque accumulation. Gingival inflammation was easily noticeable; b): A plaque detector was used to facilitate the collection of Plaque Index; c): Patients were asked to brush twice a day with a compact size medium bristle toothbrush; d): Interproximal brushes were chosen to complete the home maintenance protocol; e): After a month, clinical indexes improved. Patients were indicated to continue the home maintenance protocol, as it was effective in reducing clinical indexes.*

Table I. *Plaque Index variation in both groups*

	Baseline	T (30)	P value
Group I	35% $\pm$ 8	12% $\pm$ 3	<0.05
Group II	34% $\pm$ 6	26% $\pm$ 4	<0.05
Differences between groups	1% $\pm$ 3; no significative, p <0.05	14% $\pm$ 6 significative, p <0.05	<0.05

Table II. *Bleeding on Probing variation in both groups.*

	Baseline	T (30)	P value
Group I	52% $\pm$ 7	15% $\pm$ 2	<0.005
Group II	51% $\pm$ 5	33% $\pm$ 7	<0.005
Differences between groups	1% $\pm$ 4 no significative, p <0.05	18% $\pm$ 4 significative, p <0.05	<0.05

**Fig. 2.** *Experimental design.*

significant decrease of P.I and G.I. in controlled diabetic type II patient suffering from gingivitis.

The rationale for the study was that diabetic patients are more prone to develop infections, such as gingivitis and periodontitis and that probiotics used as bacteriotherapy can play a key-role in managing oral biofilm alteration such as gingivitis. No study up to now tested the efficacy of probiotics in the management of gingivitis in diabetic patients.

No adverse event or complications were present during the study. This is in accordance with the lack of adverse effect found in literature.

The probiotic and the placebo groups showed similar scores of PII and BoP at baseline. A month later PII and BoP improved in both groups. This can be related to the efficacy of the customized home maintenance protocol and the short follow-up period. However, the test group had better results. The combination of probiotics used for this study could have been able to inhibit the pathogenic bacteria growth and to reduce inflammation.

Lactobacillus Reuteri DSM 17938 produces a natural antimicrobial, denominated reuterin. This has been shown to reduce parodontal pathogens *in vivo* (18). Besides, *Lactobacillus Reuteri* ATCC PTA 5289 showed to have an anti-inflammatory effect *in vitro* inhibiting the production of α TNF (19).

The addition of bacteriotherapy to a good home maintenance protocol seems to be effective in the management of gingivitis even in diabetic patients. This is in accordance with a RCT conducted on 45 pregnant women using the same probiotic. The protocol proved to be effective in managing gingivitis without any other intervention (20). However, these results are difficult to compare with the one of the present study due to the differences in the population examined.

A reduction in G.I. without scaling and root planning was noted also in a study on periodontitis patients (18).

A sample size of 80 patients was chosen as no other study was available for a sample size calculation and it seemed useful for gathering information. A greater cohort of patients will be examined in the future. The follow up was short, but the aim was to evaluate the change that could be obtained using only oral

hygiene instruction and the probiotics. So this short term follow-up was considered acceptable before giving the patient a professional treatment.

This study included patients with similar gingivitis, following a tailored made program of home care, so the generalizability of data should take into account the population examined.

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CONE-MORSE IMPLANT CONNECTION SYSTEM SIGNIFICANTLY REDUCES BACTERIAL LEAKAGE BETWEEN IMPLANT AND ABUTMENT: AN *IN VITRO* STUDY

A. BAJ^{1,2}, A. BOLZONI^{1,2}, A. RUSSILLO^{1,2}, D. LAURITANO³, A. PALMIERI⁴,
F. CURA⁴, F. J. SILVESTRE⁵, and A. B. GIANNI^{1,2}

¹Maxillofacial and Dental Unit, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico di Milano, Milan, Italy; ²Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy; ³Department of Medicine and Surgery, University of Milano Bicocca, Monza, Italy; ⁴Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna, Bologna, Italy; ⁵Stomatology & Oral Surgery, Dr Peset University Hospital, Department of Stomatology, University of Valencia, Spain

Osseointegrated implants are very popular dental treatments today in the world. In osseointegrated implants, the occlusal forces are transmitted from prosthesis through an abutment to a dental implant. The abutment is connected to the implant by mean of a screw. A screw is the most used mean for connecting an implant to an abutment. Frequently the screws break and are lost. There is an alternative to screw retained abutment systems: the cone-morse connection (CMC). The CMC, thanks to the absence of the abutment screw, guarantees no micro-gaps, no micro-movements, and a reduction of bacterial leakage between implant and abutment. As *P. gingivalis* and *T. forsythia* penetration might have clinical relevance, it was the purpose of this investigation to evaluate molecular leakage of these two bacteria in a new CMC implants systems (Leone Spa®, Florence, Italy). To identify the capability of the implant to protect the internal space from the external environment, the passage of genetically modified *Escherichia coli* across implant-abutment interface was evaluated. Four cone-morse Leone implants (Leone® Spa, Florence, Italy) were immersed in a bacterial culture for 24 h and bacteria amount was then measured inside implant-abutment interface with Real-time PCR. Bacteria were detected inside all studied implants, with a median percentage of 3% for *P. gingivalis* and 4% for *T. forsythia*. Cone-morse connection implant system has very low bacterial leakage percentage and is similar to one-piece implants.

Osseointegrated implants are very popular dental treatments in the world today. In osseointegrated implants, the occlusal forces are transmitted from prosthesis through an abutment to a dental implant. The abutment is connected to the implant by mean of a screw (1-15). A screw is the most used mean for connecting an implant to an abutment. Frequently, the screws break and are lost. There is an alternative to screw retained abutment systems: the cone-morse connection (CMC).

The cone-morse connection (Leone Spa®, Florence, Italy), thanks to the absence of the abutment screw and the presence of an internal hexagon, guarantees the outstanding resistance to masticatory forces (Ø3, 3mm abutment fatigue strength: 240 N, Ø4, 1mm abutment fatigue strength: 392 N – tests performed according to the ISO 14801 international standard). This connection system allows a dramatic reduction of prosthetic complications due to the absence of the abutment screw, eases abutment

Key words: Implant-abutment connection, implant dentistry, bacterial leakage, perimplantitis, bone resorption

Mailing address:

Prof. Aldo Bruno Gianni,
Maxillo-Facial and Dental Unit, Fondazione Ca' Granda IRCCS,
Ospedale Maggiore Policlinico Milan,
Via Francesco Sforza, 35 - 20122 Milano, MI, Italy
Tel.: +39 0255032532 - Fax +390255032558
e-mail: aldo.gianni@unimi.it

0393-974X (2016)

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preparation, due to abutments without a screw access hole. In addition, CMC favours a precise transfer of implant position between the dental office and the laboratory.

The CMC, thanks to the absence of the abutment screw, guarantees no micro-gaps, thus perfect bacterial seal, no micro-movements, thus absolute stability, allowing the option of sub-crestal placement of the implants.

Furthermore, the presence of an internal hexagon allows an easy and precise implant placement with reduced number of components and easy and quick insertion of straight and angled abutments in any clinical situation.

Implant restorations have been in use for over 30 years, but recent studies have stated that peri-implantitis and implant loss concern about 30-50% of all implant restorations. Bacterial leakage at the implant-abutment connection is considered the main cause of peri-implantitis (16-24). Prevention of bacterial leakage at the implant-abutment connection is mandatory for reducing inflammation process around implant necks and achieving bone stability, whereby CMC should prevent bacterial leakage and peri-implantitis on-set. In this paper, we want to study bacterial leakage of locking tapered connection of a new implant system. CMC is an important factor to prevent the passage of bacteria and the occurrence of

peri-implantitis. In fact, the bacteria of periodontal disease are the same that cause peri-implantitis. Even systemic diseases such as lesions of the oral mucosa (25-29) and the precancerous or systemic deficiency (30-35) can affect implant success; therefore, it is important to diagnose the presence of these diseases prior to implant therapy.

MATERIALS AND METHODS

Implant preparation

In order to establish the ability of the implant to isolate the heart of the device from the external environment, we evaluated the passage of modified *E. coli* across the cone morse connection. The peculiarity of these bacteria is that they contain synthetic DNA target sequences in their plasmid. In detail, the plasmid contains two sequences specific for two bacterial species (*P. gingivalis* and *T. forsythia*) and two genes for antibiotic selection (Kanamycin and Ampicillin).

Bacteria were cultured in lysogeny broth (LB) containing both Kanamycin and Ampicillin (at a final concentration of 50ug/ml) at 37°C for 12-18 h in a shaking incubator. Four cone-morse connection implants (Leone®, Florence, Italy) were used in this study. Few microliters of LB with antibiotics were put inside the implants. The implants and the abutment are screwed with a force of 35 Newton.

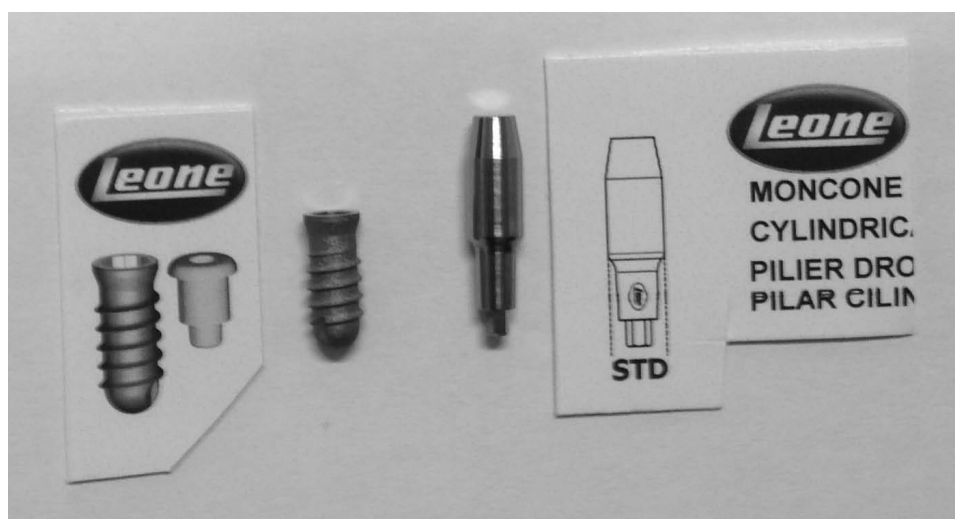


Fig. 1: Implants with cone morse connection.

A few microliters of this culture were used to “contaminate” fresh LB with antibiotics contained in a micro-centrifuge tube together with the implant. Tubes were then left at 37°C for 48 h in a heater, in order to allow bacterial growth and their hypothetical passage within the implant. Inside the implant, we just put LB and antibiotics without bacteria instead.

To ensure that there were no contaminations, a negative control containing only LB and antibiotics was prepared.

Forty-eight h later, implants were opened and samples were collected by dipping a paper probe in both the sites containing LB (external and internal to the implant) for each implant and in the negative control too.

DNA extraction

Once collected, paper probes were put on a new microcentrifuge tube and processed for bacterial DNA extraction using the GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, St., St. Louis, MO, USA), following the manufacturing procedures. Briefly, samples were incubated with lysozyme and subsequently with proteinase K to isolate DNA. Once extracted, DNA was purified by spin-column method.

Real-time polymerase chain reaction

Real-Time Polymerase Chain Reaction using the absolute quantification with the standard curve method performed bacterial quantification.

Primers and probes oligonucleotides for *P. gingivalis* and *T. forsythia* were designed basing on 16S rRNA gene sequences of the Human Oral Microbiome Database (HOMD 16S rRNA RefSeq Version 10.1).

For the quantitative analysis, plasmid (Eurofin MWG Operon, Ebersberg Germany) containing the specific DNA target sequence was employed as standard.

All reactions were performed in duplex, in 20ul final volumes with 2X TaqMan Universal PCR master mix (Applied Biosystems, Foster City, CA, USA) and 50nM concentration of each primers and 200nM of the probes. Amplifications were carried out by using the ABI PRISM 7500 (Applied Bio systems, Foster City, CA, USA).

Statistical analysis

To evaluate if the difference in viability between outside

and inside the implant was statistically significant, we applied Student's *t*-test on average bacteria quantification at each time point.

RESULTS

Bacteria quantification is reported in Table I. Bacteria were detected inside all studied implants, with a median percentage of 3% for *P. gingivalis* and 4% for *T. forsythia*.

The analysis revealed that in both cases (internally and externally), bacteria grew for the first 48 h but subsequently they started to dye, probably as a consequence of nutrient consumption. Moreover, the difference between outer and inner bacteria concentration was statistically significant at each time point.

DISCUSSION

Bacterial leakage at implant-abutment connection is the main cause of peri-implantitis. The current CMC can completely prevent micro leakage and consequent bacterial colonization of the inner part of the implants. Although several investigations have shown that bacterial oral leakage along the implant-abutment interface may constitute a potential risk of inflammation of the supporting tissues, compromising the long-term success of the treatment with implants, CMC have demonstrated to reduce bacterial leakage at significant percentage. The CMC has a friction similar to a screw-retained abutment. The tight link of CMC is achieved by driving the 1.5-degree morse taper into the matching socket in the implant. A high clamping force of CMC between abutment and implant is created by this mechanism. The difference is that this clamping force of CMC is almost lateral to the axis of the implant and therefore almost normal or perpendicular to the friction plane. This means that lateral forces of CMC and moments about the abutment do not tend to wear down the surface asperity. The high friction force of CMC is the result of a relative slip between the two friction surfaces occurring at high contact pressure. This results in the surface oxide layers of CMC breaking down and the asperity fusing, sometimes referred to

Table I. Absolute quantification of *P. gingivalis* and *T. forsythia*, outside and inside the implant.

LEONE						
IMPLANT	BACTERIA	BACTERIA QUANTITY	IMPLANT	BACTERIA	BACTERIA QUANTITY	Passage of bacteria from outside to inside the implant (%)
1 OUTSIDE	P. gingivalis	252784	1 INSIDE	P. gingivalis	8356	3%
	T. forsythia	280893		T. forsythia	6998	2%
2 OUTSIDE	P. gingivalis	285467	2 INSIDE	P. gingivalis	14093	5%
	T. forsythia	259494		T. forsythia	16127	6%
3 OUTSIDE	P. gingivalis	227998	3 INSIDE	P. gingivalis	11318	5%
	T. forsythia	203656		T. forsythia	11963	6%
4 OUTSIDE	P. gingivalis	454227	4 INSIDE	P. gingivalis	6060	1%
	T. forsythia	405013		T. forsythia	7109	2%
Negative Control OUTSIDE	P. gingivalis	0	Negative Control INSIDE	P. gingivalis	0	0
	T. forsythia	0		T. forsythia	0	0
	Media Outside			Media Inside		
	PorG	305119		PorG	9957	3%
	TanF	287264		TanF	10549	4%

Implant permeability is expressed as percent rate of the internal vs external bacteria quantity.

as cold welding. Therefore, there are few gaps CMC, unlike screw assemblies. Locking taper assemblies of CMC are rigid and can withstand high compressive axial forces and lateral forces without loosening. Because of the high friction force of CMC, they will resist pullout forces unless accompanied with torque to break the fused asperity.

CONCLUSION

Blocking the passage of bacteria, CMC is essential to prevent peri-implantitis, in fact the presence of a cavity near the bone may influence the development of peri-implant inflammation and bone resorption. An intense inflammatory cell infiltrate may be the cause

of a significant bone resorption, on the contrary, one-piece implants showed a minimal inflammation and bone loss around peri-implant tissues. In conclusion, CMC have a very low bacterial leakage percentage and are similar to one-piece implants.

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GENETIC EFFECTS OF BIOPAD® ON FIBROBLAST PRIMARY CULTURE

A. BAJ^{1,2}, G. A. BELTRAMINI^{1,2}, M. ROMANO^{1,2}, D. LAURITANO³, R.M. GAUDIO⁴,
A. PALMIERI⁵, F. CURA⁵ and A.B. GIANNI^{1,2}

¹Maxillofacial and Dental Unit, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico di Milano, Milan, Italy; ²Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy; ³Department of Medicine and Surgery, University of Milano Bicocca, Monza, Italy; ⁴Department of Medical Sciences, University of Ferrara, Ferrara, Italy; ⁵Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna, Bologna, Italy

BIOPAD® is an ivory-white soft sponge, made exclusively of lyophilized type I native heterologous collagen extracted from horse flexor tendon, gelatine free, that keeps its native structure specific to the body's skin tissue. **BIOPAD®** is an active dressing, playing an active role in all stages of wound healing process, stimulating granulation tissue growth and enhancing regeneration tissues. It ensures balance between absorption and humidity at wound surface, gaseous exchange of soft tissues during healing process, barrier to prevent bacterial infections and it is completely non-adherent. The use of **BIOPAD®** is painless for the patient and does not require removal or change of dressing. In this work, we evaluated the effect of **BIOPAD®** on fibroblasts behavior in term of cell viability, survival and growth and collagen production. The expression levels of some adhesion and traction-resistance related genes (ELN, DSP, FN1, FBN1, ITGB1, ITGA1, ITGA5, ITGA2, COL1A1, COL3A1) were analyzed using real time Reverse Transcription-Polymerase Chain Reaction (real time RT-PCR). All genes, except for ELN, DSP, ITGB1 and ITGA1 are up-regulated after 48 h of treatment. Altogether, our results point out the good potential of **BIOPAD®** as a biocompatible and regenerative tool in medicine.

Bone grafts are a very common surgical technique for jaws bone augmentation (1-10). One of the most common problems in bone grafting is to keep bone grafts stable in the native bone during healing phase. This problem can be overcome by positioning collagen membranes that prevent them from dislocating in post-surgical period. Collagen membranes (CMs) are frequently used in implant dentistry for stabilizing bone grafts in bone augmentation techniques. CMs are widely used throughout oral surgery and implant dentistry (11-18) and in extractive sites to preserve the volume of cortical bone and induce the clot.

The most common CMs are freeze-dried and sterile equine heterologous type 1 collagen in the

form of membranes that are easily adaptable to application areas. The protection time of the CMs membrane is 2 weeks, after which the membrane begins to degrade. The CMs do not need stabilization by screws as they adhere perfectly to the graft site due to the presence of collagen, which acts as a hemostatic. CMs actions are manifold: they induce a hemostatic action favoring platelet activation and growth factors, they act as support for fibroblast proliferation and granulation tissue, they promote biological stimulus with activation of fibronectin, fibroblasts and monocytes and finally, they excite an angiogenetic action, stimulating neoformation of capillaries.

Key words: bone, regeneration, gene, expression, mucosa

Mailing address:

Prof. Aldo Bruno Gianni,
Maxillo-Facial and Dental Unit, Fondazione Ca' Granda IRCCS,
Ospedale Maggiore Policlinico Milan,
Via Francesco Sforza 35, 20122 Milano, MI, Italy
Tel.: +39 0255032532 - Fax: +390255032558
e-mail: aldo.gianni@unimi.it

0393-974X (2016)

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BIOPAD®

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BIOPAD® is an active dressing, playing an active role in all stages of wound healing process, stimulating granulation tissue growth and enhancing regeneration tissues. It ensures balance between absorption and humidity at wound surface, gaseous exchange of soft tissues during healing process, barrier to prevent bacterial infections, and it is completely non-adherent. BIOPAD® use is painless for the patient and does not require removal or change of dressing.

Previous studies (19) tested the efficacy of BIOPAD® on wounds using an animal model where a surface wound was induced to obtain a "pocket" where BIOPAD® and reference product (polyurethane sponge) were packed in. The results shown demonstrate that BIOPAD® exerts a substantial and beneficial chemotactic effect on the macrophages and neutrophils, which are important in wound healing. The increase in the number of macrophages and neutrophils were shown to be statistically significant ($p < 0.01$) compared to the control group. Cell migration, towards and in the induced wound, was faster in the control group.

Studies with electron microscopy and electrophoresis showed that the preservation of the native structure of the collagen protein of BIOPAD® has been verified. The electrophoresis of the protein confirms that BIOPAD® is structurally unaltered. Purity and nativity of BIOPAD® was also confirmed by the determination of water-soluble peptides, hydrolysed collagen products originated by extraction, lyophilisation and sterilization processes. Such tests were conducted in comparison with Purity Standard Collagens (SIGMA 129F8130, SIGMA 128F81451 and CALBIOCHEM 014491). The statistical evaluation performed with Student's *t*-test confirmed that groups of tested values were statistically significant. Denatured collagen protein may also be "damaged" by manufacturing processes. Such "damage" may be measured by means of

enzymatic hydrolysis of bacterial collagenases.

Long-term evaluation (24 h) of the BIOPAD® integrity proves comparable to the Purity Standard Collagen. The BIOPAD® dressing has a high collagen content, in fact its structure provides more real native collagen to "feed" elastase and active MMP's, speeding-up the fibroblast depletion of autologous collagen. The most important and appropriate application of collagen is in wound healing, but it is used successfully as a membrane for protecting bone grafts of the jaw.

In this work, we evaluated the effect of BIOPAD® on fibroblasts behavior in term of cell viability, survival, growth and collagen production.

Fibroblasts are the main cells of connective tissue that synthesize extracellular matrix, an important body component for support in cell anchorage and to give individuality to each tissue. To this aim, the expression levels of some adhesion and traction-resistance related genes (ELN, DSP, FN1, FBN1, ITGB1, ITGA1, ITGA5, ITGA2, COL1A1, COL3A1) were analyzed using real time Reverse Transcription-Polymerase Chain Reaction (real time RT-PCR).

MATERIALS AND METHODS

Primary Human Fibroblast cells culture

Gingival fragments of 3 healthy volunteers (mean age 35 years) were collected during tooth extraction. The pieces were transferred in 75 cm² culture flasks containing DMEM medium (Sigma Aldrich, Inc., St Louis, Mo, USA) supplemented with 20% fetal calf serum, antibiotics (Penicillin 100U/ml and Streptomycin 100 micrograms/ml-Sigma Aldrich, Inc., St Louis, Mo, USA).

Cells were incubated in a humified atmosphere of 5% CO₂ at 37°C. The medium was changed the next day and twice a week. After 15 days, the pieces of dermal tissue were removed from the culture flask. Cells were harvested after 24 days of incubation.

Cells Culture

For the investigation, Human Fibroblasts (HFb) were seeded on a layer of BIOPAD® at the second passage. A set of untreated cells were used as controls. The cells were maintained in a humified atmosphere of 5% CO₂

at 37°C for 48 h. At the end of exposure time, cells were trypsinized and lysed for RNA extraction.

RNA processing and Real Time PCR

Total RNA was isolated from cells using GenElute mammalian total RNA purification miniprep kit (Sigma-Aldrich, Inc., St Louis, Mo, USA) according to manufacturer's instructions. Total RNA concentration and quality were measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Complementary cDNA was obtained using PrimeScript RT Master Mix (Takara Bio Inc., Shiga, Japan) starting from 500 ng of total RNA. Finally, the cDNA was amplified by real-time PCR. For all but two genes, (COL1A1 and COL3A1), amplification was performed by using Power SYBR® Green PCR Master

Mix (Applied Biosystems, Foster City, CA, USA) and the specific assay designed for the investigated genes. For COL1A1 and COL3A1, we performed amplification by using the TaqMan universal PCR master mix (Applied Biosystem, Foster City, CA, USA) instead.

SYBER assays reactions were performed in a 20 µl volume using the ABI PRISM 7500 (Applied Biosystems, Foster City, CA, USA). Each reaction contained 10 µl 2X Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 400 nM concentration of each primer, and cDNA, while TaqMan assays amplification were performed in a 20 µl volume using the ABI PRISM 7500 (Applied Biosystems, Foster City, CA, USA). Each reaction contained 10 µl 2X Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 400

Table I. Primers sequences for SYBR® Green assay.

Gene symbol	Gene name	Primer sequence (5'>3')
DSP	Desmoplakin	F- ATGACCTGAGGAGAGGACGAA R- AGGCTCTCTCTTCCTGTACCAC
ELN	Elastin	F- CTAATAACGGTGCTGCTGGC R- CATGGGATGGGGTTACAAAG
FN1	Fibronectin 1	F – CCATAGCTGAGAAGTGTTTTG R- CAAGTACAATCTACCATCATCC
FBN1	Fibrillin-1	F- AAAGATCTTGATGAGAGTGTGC R- GTATGGTGTGGGTAAATCC
ITGA1	Integrin Subunit Alpha 1	F- CAGTTGGAATTGTACAGTATG R- TGTCTATTCCAAGAGCTGTC
ITGA2	Integrin Subunit Alpha 2	F- GGTGGGGTTAATTCAGTATG R- ATATTGGGATGTCTGGGATG
ITGA5	Integrin Subunit Alpha 5	F- AAGCTTGGATTCTCAAACG R- TCCTTTCAGTAGAATGAGGG
ITGB1	Integrin Subunit Beta 1	F- ATTCCTTTCCTCAGAAGTC R- TTTTCTTCCATTTCCCTG
RPL13	Ribosomal protein 13	F - ATTCACAAGAAGGGAGACAG R - GAAATCTTCTCTCCTCAGTG

Table II. Primers and probes sequences for TaqMan® Gene Expression Assay.

Gene symbol	Gene name	Primer and Probe sequence (5'>3')
COL1A1	Collagen type I alpha1	F- TAGGGTCTAGACATGTTCACTTTGT R- GTGATTGGTGGGATGTCTTCGT P- CCTCTTAGCGCCACCGCCCT
COL3A1	Collagen type III alpha1	F- CCCACTATTATTTGGCACAACAG R- AACGGATCCTGAGTCACAGACA P- ATGTTCCCATCTTGGTCAGTCCTATGCG
RPL13	Ribosomal protein 13	F- AAAGCGGATGGTGGTTCCT R- GCCCCAGATAGGCAAACTTC P- CTGCCCTCAAGGTCGTGCGTCTG

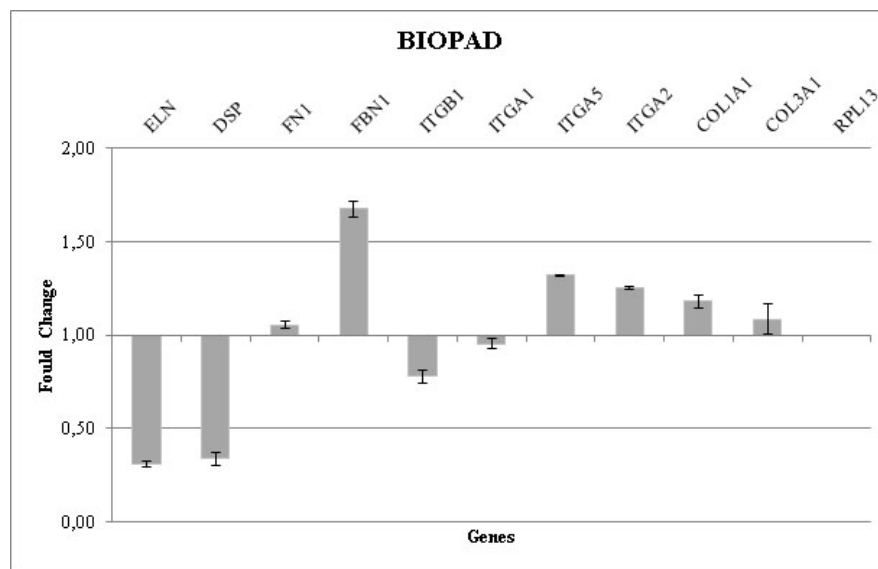


Fig. 1: Human gingival fibroblast gene expression profile after BIOPAD® Collagen Scaffold treated. *ELN* = 0.31; *DSP* = 0.34; *FN1* = 1.05; *FBN1* = 1.67; *ITGB1* = 0.78; *ITGA1* = 0.95; *ITGA5* = 1.32; *ITGA2* = 1.25; *COL1A1* = 1.18; *COL3A1* = 1.08; *RPL13* = 1.00.

nM concentration of each primer, 200nM of the specific probe and cDNA.

All experiments were performed with non-template controls to exclude reagents contamination. PCRs were performed with two biological replicates.

Expression was quantified using real time RT-PCR. The gene expression levels were normalized to the expression of the housekeeping gene *RPL13*. In both cases, we evaluated the expression as fold changes relative to the expression of the untreated HFb. Quantification was carried out with the delta/delta calculation method.

Forward and reverse primers for the selected genes were designed using primer express software (Applied Biosystems, Foster City, CA, USA) (Table I, II).

RESULTS

Fibroblast behavior was evaluated by measuring the gene expression levels of several growth, wealth and elasticity related genes after treatment with BIOPAD®.

All but 4 genes resulted up-regulated after 48 h of treatment. In fact, *ELN*, *DSP*, *ITGB1* and *ITGA1* were the only down-expressed genes (Fig. 1).

DISCUSSION

Previous studies (19) tested the efficacy of BIOPAD® on wounds using an animal model where a surface wound was induced to obtain a “pocket” where BIOPAD® and reference product (polyurethane sponge) were packed in. The results shown demonstrate that BIOPAD® exerts a substantial and beneficial chemotactic effect on the macrophages and neutrophils, which are important in wound healing.

In this work, we evaluated the *in vitro* effect of BIOPAD® on fibroblasts behavior in term of cell viability, survival, growth and collagen production.

Human gingival fibroblasts were exposed to BIOPAD for 48 h and later expression profile of *ELN*, *DSP*, *FN1*, *FBN1*, *ITGB1*, *ITGA1*, *ITGA5*, *ITGA2*, *COL1A1*, *COL3A1* were analyzed by real time RT-PCR. BIOPAD® induces the up-regulation of *COL1A1* and *COL3A1* in treated cells.

Collagen I and collagen III are the primary extracellular matrix (ECM) proteins expressed by myofibroblasts, during wound repair and fibrosis (21). Myofibroblasts activation is a key event in physiological and pathological tissue repair. These

cells exhibit a developed endoplasmic reticulum and bundles of contractile microfilaments (22).

Another up-regulated gene in treated cell was fibrillin 1 (FBN1). Members of the fibrillin family are involved in many processes related to the structural integrity of tissues, binding tropoelastin during elastic fiber formation and targeting growth factors such as TGF- β and bone morphogenic protein (BMP) to the ECM (23,24).

Fibronectin 1 (FN1) was moderately up-regulated in treated cells. FN1 plays a key role in cell adhesive and migratory behavior (27).

Integrin Subunit Alpha 5 (ITGA5) is a receptor for fibronectin and fibrinogen. ITGA5 were found to be up-regulated in treated cells. This gene has been suggested as an important factor in the healing cascade (28, 29). Integrin family members participate in a variety of processes including cell adhesion, immune response, metastasis of tumor cells and as embryogenesis, hemostasis and tissue repair (28).

Altogether, our results point out the positive potential of BIOPAD[®] as a biocompatible and regenerative tool in medicine.

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GENETIC EFFECTS OF VICRYL® ON FIBROBLAST PRIMARY CULTURE

A. BAJ^{1,2}, G. A. BELTRAMINI^{1,2}, M. ROMANO^{1,2}, D. LAURITANO³, R.M. GAUDIO⁴,
A. PALMIERI⁵, F. CURA⁵ and A.B. GIANNI^{1,2}

¹Maxillofacial and Dental Unit, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico di Milano, Milan, Italy; ²Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy; ³Department of Medicine and Surgery, University of Milano Bicocca, Monza, Italy; ⁴Department of Medical Sciences, University of Ferrara, Ferrara, Italy; ⁵Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna, Bologna, Italy

Vicryl® (polyglactin 910) is an absorbable, synthetic, usually braided suture, indicated for soft tissue approximation and ligation. Vicryl® has a special coating for minimizing friction, easing passage through tissue and easy knot tie down. It is synthetic for minimal tissue reaction. Fibroblasts are the main cells of connective tissue that synthesize extracellular matrix. In this work, we tried to judge the action of Vicryl® on fibroblasts behaviour. We evaluated the expression levels of some adhesion and traction-resistance related genes (ELN, DSP, FN1, FBN1, ITGB1, ITGA1, ITGA5, ITGA2, COL1A1, COL3A1) by using real time Reverse Transcription-Polymerase Chain Reaction (real time RT-PCR). All but 2 genes resulted up-regulated after 48 h of treatment. Our preliminary results point out the potential of Vicryl® as a biocompatible and regenerative tool in medicine.

Vicryl® sutures have recently become popular among dentists and oral surgeons because of their fast absorption rate. Vicryl® (polyglactin 910) is an absorbable, synthetic, usually braided suture, indicated for soft tissue approximation and ligation. The suture holds its tensile strength for approximately two to three weeks in tissue, and is completely absorbed by hydrolysis within 56-70 days. In practice, Vicryl® comes as braided, dyed or undyed with the following decay schedule: 75% at 2 weeks, 50% at 3 weeks, and 25% at 4 weeks. The sutures retain that proportion of tensile strength at those dates. In addition, Vicryl® has a special coating for minimizing friction, facilitating passage through tissue and easy knot tie down. It is synthetic for minimal tissue reaction.

There are wide varieties of factors that affect the hydrolytic rate of hydrolytically degradable sutures

such as Vicryl®. These include temperature, pH, salt concentrations, tension, external stress and body fluids such as saliva (1). The healing mucosa or gingiva undergoes many changes during the first days of healing: primarily inflammation and activation of fibroblasts and myofibroblasts and secondly, the production of free radicals and various enzymes. These enzymes are known to accelerate the hydrolysis of some hydrolytically degradable suture in the course of wound healing. Additionally, the physical movement of cells within the mucosa during this phase may further damage the suture strand. So Vicryl® is the ideal suture to induce hydrolysis.

Vicryl Rapide® Vicryl Plus Antibacterial®

Vicryl® and other polyglycolic-acid sutures may also be treated for more rapid breakdown ("Vicryl Rapide") in rapidly healing tissues such as mucous

Key words: bone, regeneration, gene, expression, mucosa

Mailing address:

Prof. Aldo Bruno Gianni,
Maxillo-Facial and Dental Unit, Fondazione Ca' Granda IRCCS,
Ospedale Maggiore Policlinico Milan,
Via Francesco Sforza 35, 20122 Milano, MI, Italy
Tel.: +39 0255032532 - Fax: +390255032558
e-mail: aldo.gianni@unimi.it

0393-974X (2016)

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membrane, or impregnated with triclosan ("Vicryl Plus Antibacterial") to provide antimicrobial protection of the suture line. The Coated Vicryl® Plus Antibacterial (polyglactin 910) suture is indicated for use in oral surgery.

Coated Vicryl Plus® sutures provide multiple benefits; support for 7-10 days while tissues heal, inhibition of bacterial colonization of the suture (*in vitro*), minimization of drag force and only a slight tissue reaction during absorption. It tends to induce lesser foreign body reaction and consequently, less adhesion formation. The anti-bacteria property is of paramount importance since wound colonization can support implant infection (2-19). However, it is far more brittle than nylon sutures and will therefore snap if tugged on suddenly, frustrating the novice use.

Finally, even if the properties of Vicryl® make it ideal for a wide range of applications when an absorbable suture is needed, especially in implant dentistry and oral surgery, future studies should evaluate the potential benefits of using polyglactin 910 sutures, and also the potential benefits of other materials and modalities in improving wound healing.

Fibroblasts are the main cells of connective tissue that synthesize extracellular matrix, a body component important in giving support to cell anchorage, individuality to each tissue and in promoting a good skin response.

In order to judge the action of Vicryl® on fibroblasts behaviour we evaluated the expression levels of some adhesion and traction-resistance related genes (ELN, DSP, FN1, FBN1, ITGB1, ITGA1, ITGA5, ITGA2, COL1A1, COL3A1) by using real time Reverse Transcription-Polymerase Chain Reaction (real time RT-PCR).

MATERIAL AND METHODS

Primary Human Fibroblast cells culture

Fragments of gingiva of healthy volunteers (mean age 35 years) were collected during tooth extraction. The pieces were transferred in 75 cm² culture flasks containing DMEM medium (Sigma Aldrich, Inc., St Louis, Mo, USA) supplemented with 20% fetal calf serum, antibiotics (Penicillin 100U/ml and Streptomycin 100 micrograms/ml-Sigma Aldrich,

Inc., St Louis, Mo, USA).

Cells were incubated in a humidified atmosphere of 5% CO₂ at 37°C. The medium was changed the next day and twice a week. After 15 days, the pieces of dermal tissue were removed from the culture flask. Cells were harvested after 24 days of incubation.

Cells Culture

For the investigation, Human Fibroblast cells (HFb) at the second passage were seeded on Vicryl®. A set of untreated cells were used as controls. The cells were maintained in a humidified atmosphere of 5% CO₂ at 37°C for 48 h. At the end of the exposure time, cells were trypsinized and lysed for RNA extraction.

RNA processing and Real Time PCR

Total RNA was isolated from cells using GenElute mammalian total RNA purification miniprep kit (Sigma-Aldrich, Inc., St Louis, Mo, USA) according to manufacturer's instructions. Total RNA concentration and quality were measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Complementary cDNA was obtained using PrimeScript RT Master Mix (Takara Bio Inc., Shiga, Japan) starting from 500 ng of total RNA.

Finally the cDNA was amplified by real-time PCR. For all but two genes, (COL1A1 and COL3A1) amplification was performed by using Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and the specific assay designed for the investigated genes. For COL1A1 and COL3A1, we performed amplification by using the TaqMan universal PCR master mix (Applied Biosystem, Foster City, CA, USA) instead.

SYBER assay reactions were performed in a 20 µl volume using the ABI PRISM 7500 (Applied Biosystems, Foster City, CA, USA). Each reaction contained 10 µl 2X Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 400 nM concentration of each primer, and cDNA.

TaqMan assays amplification were performed in a 20 µl volume using the ABI PRISM 7500 (Applied Biosystems, Foster City, CA, USA) instead. Each reaction contained 10 µl 2X Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 400 nM concentration of each primer, 200nM of the specific probe and cDNA.

All experiments were performed including non-template controls to exclude reagents contamination. PCRs were performed with two biological replicates.

Expression was quantified using real time RT-PCR. The gene expression levels were normalized to the expression of the housekeeping gene RPL13. In both cases, we evaluated the expression as fold changes relative to the expression of the untreated HFb. Quantification was done with the delta/delta calculation method.

Forward and reverse primers for the selected genes were designed using primer express software (Applied Biosystems, Foster City, CA, USA) (Table I, II).

RESULTS

Fibroblasts behaviour in term of cell viability,

survival and growth and collagen production was analyzed by measuring the gene expression levels of several growth, wealth and elasticity related genes.

All but 2 genes resulted up-regulated after 48 h of treatment. In fact, ELN and COL3A1 were the only down-expressed genes (Fig. 1).

DISCUSSION

The Coated Vicryl® Plus Antibacterial (polyglactin 910) suture is indicated for use in oral surgery. Coated Vicryl Plus® sutures inhibits bacterial colonization of the suture (*in vitro*), it minimizes drag force and elicits only a slight tissue reaction during absorption. It tends to induce lesser foreign body reaction and consequently, less adhesion formation. It is ideal

Table I. Primers sequences for SYBR® Green assay

Gene symbol	Gene name	Primer sequence (5'>3')
DSP	Desmoplakin	F- ATGACCTGAGGAGAGGACGAA R- AGGCTCTCTCTTCTGTACCAC
ELN	Elastin	F- CTAATACGGTGCTGCTGGC R- CATGGGATGGGGTTACAAAG
FN1	Fibronectin 1	F – CCATAGCTGAGAAGTGTTTTG R- CAAGTACAATCTACCATCATCC
FBN1	Fibrillin-1	F- AAAGATCTTGATGAGAGTGTGC R- GTATGGTGTGGGTAAATCC
ITGA1	Integrin Subunit Alpha 1	F- CAGTTGGAATTGTACAGTATG R- TGTCTATTCCAAGAGCTGTC
ITGA2	Integrin Subunit Alpha 2	F- GGTGGGGTTAATTCAAGTATG R- ATATTGGGATGTCTGGGATG
ITGA5	Integrin Subunit Alpha 5	F- AAGCTTGGATCTCTCAAACG R- TCCTTTCAGTAGAATGAGGG
ITGB1	Integrin Subunit Beta 1	F- ATTCCTTTCTCCTCAGAAATC R- TTTTCTTCCATTTTCCCCTG
RPL13	Ribosomal protein 13	F - ATTCACAAGAAGGGAGACAG R - GAAATTCTTCTTCTCCTCAGTG

Table II. Primers and probes sequences for TaqMan® Gene Expression Assay.

Gene symbol	Gene name	Primer and Probe sequence (5'>3')
COL1A1	Collagen type I alpha1	F- TAGGGTCTAGACATGTTCTTGT R- GTGATTGGTGGGATGCTTCGT P- CCTCTTAGCGGCCACCGCCCT
COL3A1	Collagen type III alpha1	F- CCCACTATTATTTGGCACAACAG R- AACGGATCCTGAGTCACAGACA P- ATGTTCCCATCTTGGTCAGTCCTATGCG
RPL13	Ribosomal protein 13	F- AAAGCGGATGGTGGTTCCT R- GCCCCAGATAGGCAAACTTTC P- CTGCCCTCAAGGTCGTGCGTCTG

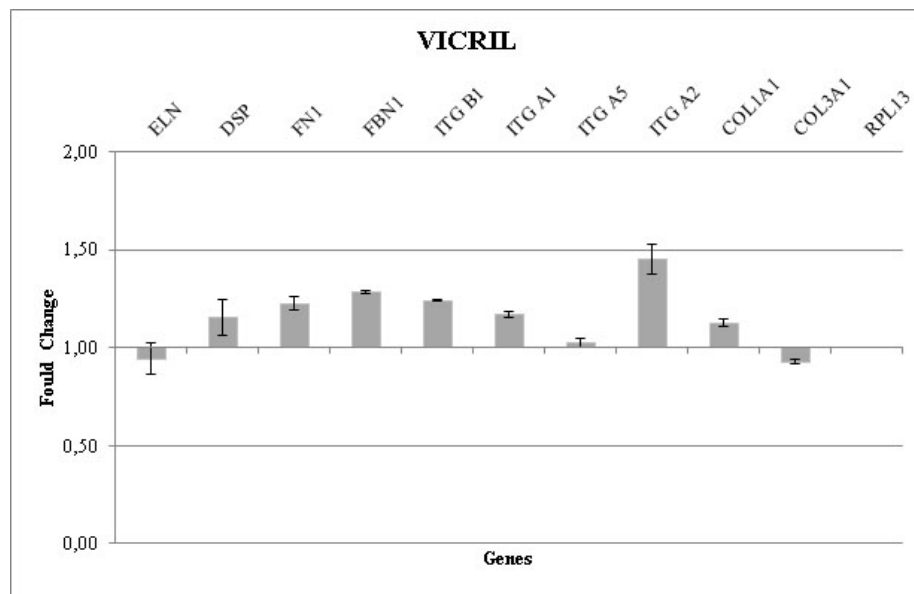


Fig. 1. Human gingival fibroblast gene expression profile after Ethicon Vicryl Sutures treated. SYBR Green assay. *ELN* = 0.94; *DSP* = 1.16; *FN1* = 1.23; *FBN1* = 1.28; *ITGB1* = 1.25; *ITGA1* = 1.17; *ITGA5* = 1.03; *ITGA2* = 1.45; *COL1A1* = 1.13; *COL3A1* = 0.93; *RPL13* = 1.00.

for a wide range of applications when an absorbable suture is needed, especially in implant dentistry and oral surgery.

In this work, we investigated the expression profile of some of the genes indirectly involved in regeneration and repair processes. All but 2 of the analyzed genes were upregulated in treated cells, in comparison with untreated cells.

Among the up-regulated genes, *DSP* encodes desmoplakine, an essential component of desmosomes that anchor intermediate filaments to desmosomal plaques. Its up-regulation suggests the involvement of this protein in cell-cell and cell-matrix adhesion. Vicryl® induces an increase in *FN1* expression by favoring tissue regeneration processes.

Fibrillin is of great importance in cellular migration and adhesion. The main function of *FN1* is to serve as a scaffold regulating cell proliferation and differentiation (20, 21). In healthy adult tissue, *FN1* is expressed at low levels; its expression is reactivated transiently because of tissue injuries, ranging from skin wounds to tissue inflammation (22). Fibronectin favors the degradation of tissue residues, migration, proliferation and contraction of

the cells involved in the healing process as well as angiogenesis.

Vicryl® up-regulates the expression of three genes, *ITGB1*, *FN1* and *COL1A1*, all involved in tissue repair and interacting between them. Integrin $\beta 1$ is a common mediator of fibroblast attachment to collagen type I (*COL1A1*) and its expression has a key role in tissue repair (23). Fibroblasts migrate into the wound to produce and remodel extracellular matrix (ECM).

Integrin- $\beta 1$ -deficient fibroblasts showed reduced expression of type I collagen and connective tissue growth factor and failed to differentiate the wound reparation into myofibroblasts (24).

ITGB1 gene encodes the beta 1 subunit of integrin receptors. This protein heterodimerizes with the alpha 1 subunit encoded by the *ITGA1* gene to form a cell-surface receptor for collagen and laminin (25).

This receptor is involved in cell adhesion, inflammation and fibrosis. Both *ITGB1* and *ITGA1* are up-regulated in fibroblast exposed to Vicryl®. These results suggest a positive role of Vicryl® in the processes of tissue regeneration.

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SURFACE MODIFICATION OF TITANIUM ALLOYS FOR BIOMEDICAL APPLICATION: FROM MACRO TO NANO SCALE

D. PEDREIRA DE OLIVEIRA¹, L. OTTRIA², M. GARGARI³, V. CANDOTTO⁴,
F.J. SILVESTRE⁵, and D. LAURITANO⁶

¹Department of Materials Engineering, Federal University of São Carlos, Brazil; ²Dental School, Tor Vergata University, Rome, Italy; ³Department of Clinical Sciences and Translational Medicine, University of Tor Vergata, Rome, Italy; ⁴Stomatology & Oral Surgery, Dr Peset University Hospital, Department of Stomatology, University of Valencia, Spain; ⁵Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, Italy; ⁶Department of Medicine and Surgery, University of Milan-Bicocca, Monza, Italy

Bone is a complexly structured tissue and hierarchically organized from macro to nano levels of dimensions. Biomaterial devices developed to replace and interact with bone tissues have been designed from the macro to nano perspective to provide suitable performance in acquiring primary and biological stability. Macro level designed features provide mechanical interlock and primary stability to implants inside the bone. Surface micro and nano structures influence cells behavior to promote the secondary or biological stability, reaching successful osseointegration.

Bone is an important tissue for our body, suitable to perform mechanical, biological and chemical functions. It provides structural support, cell healing storage and ions reservoir for biochemical reactions, of vital importance to body homeostasis (1). From a materials science perspective, the bone is interestingly composed and investigated from a macro to nano hierarchical structure (1, 2). Bone has a proper macro design depending on its function and the region where it is allocated. Intrinsically it is composed of distinguishable and organized structures such as compact and spongy bones. At micro scale level, structural unities denominated osteon, lamellae and collagen fibers can be observed and resolved; furthermore at nanometric level, structures known as collagen fibrils, collagen molecules and hydroxyapatite nanocrystals comprise the hierarchical structure of bone (3-5).

This complex bone tissue is preserved in homeostasis by mean of a remodeling process, performed by four cells types: osteoblast, osteoclast, bone-lining cells and osteocytes. With the exception of osteoclast cells which are derived from hemopoietic tissues, osteoblasts, bone-lining and osteocytes have the same origin, i.e. the osteoprogenitor cells (5).

When healthy, osteoblastic cells are responsible for producing bone matrix during mineralization processes. Osteoclast, inversely, stimulates bone resorption, also a renewal mechanism to tissue homeostasis. Bone-lining cells are found at especially undifferentiated or unspecialized stages of osteoprogenitor cells (6, 7). Osteocyte was previously an osteoblastic cell and is now an immobilized cell in bone matrix, functioning as structural component for bone, providing gap-junction and communications into matrix regions (5,

Key words: titanium, surface, modification, cell, alloy

Mailing address:

Dorina Lauritano, DDS,
Department of Medicine and Surgery,
University of Milano Bicocca,
Via Cadore 48, 20052 Monza, Italy
Tel.: +39.0392332301 - Fax: +39. 03923329892
e-mail: dorina.lauritano@unimib.it

0393-974X (2016)

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8). Architecture from macro to nano design support ions storage, blood, molecules and host diverse cells performing in symphony for the homeostasis maintenance of the bone tissue.

During our routine activities through the years we noted that some tissues can present dysfunctions caused by trauma, losses, ageing, congenital or degenerative origin. The consequences sometimes lead to diagnosis that suggest the replacement or substitution of compromised tissue, aiming to restore functionality and improve quality of life for patients.

Medical procedures and protocols have been developed or researched to improve bone and joint replacements and biomaterials are in constant development to enhance the biological activity to stimulate interface reconstitution (9). Synthetic and natural, exogenous or endogenous biomaterials have been used for humans for many years, to reestablish functionality to the damaged region of the tissue. Nowadays, medical implants are used worldwide to replace joints, bones, teeth and ears with an elevated success rate (10).

Implants are biomedical devices used in invasive surgery to restore functionality to a tissue and can be classified according to the interaction with a given biological tissue such as bioinert, biotolerant, bioactive or bioabsorbable, depending on the level of acceptance between tissue and implant biomaterial (10).

Titanium-based materials have demonstrated a combination of properties making them important raw materials for orthopedic joints devices and dental implants (11). Corrosion resistance, specific strength, toughness, resilience and low stiffness properties demonstrated by titanium alloys have led to advantageous application for permanent intraosseous or endosseous implants devices (12).

Titanium alloys are inherently bioinert, especially due to passive layer, very thin film spontaneously formed for oxygen binding to outermost metallic atoms of bulk titanium alloys, providing intrinsic physical-chemical stability, resulting in high corrosion resistance encountered in many titanium alloys. Additionally, surface modification processing can enhance the surface features and produce bioactive interaction with synthetic or natural biofluids (13-16). According

to bone tissue interaction, intraosseous implants with a modified surface can induce specific cellular responses and osteoinduction, osteoconduction and optimized osseointegration (17-21); circumventing the bioinertness therefore, surface can develop bioactive performance attracting biofluid ions, such as phosphates, calcium, magnesium, potassium, zinc and strontium after periods of immersion and modulate the cells' fate (13, 22-24). Osseointegration can be affected by oral bacteria (25-33) and new nano-surfaces can potentially help to reduce or prevent these infections.

Furthermore, surface modification processes are especially concerned in creating bio-inspired microenvironments from nature to promote cell interaction from the nano levels (34, 35). According to these interactions, surface features are able to stimulate or promote bone apposition and mineralization events. In such conditions, titanium surface demonstrates osteoinductive or osteoconductive behavior, depending on stimulated and differentiation stage of the lineage cells (18).

Osteoinduction relates to phenomenon in which the stem cells are induced to differentiate in osteoblasts cells, while osteoconduction relates to substrate able to conduct osteoblast cells and expression of bone mineralization events. Briefly, the sequence of events after an implantation are related to the absorption of water and of biofluid components, after interaction of proteins take place and cell attachments are established (10). Stimulating, accelerating or modulating these events is important for a suitable performance of implants. Interestingly, implants and bone interactions can be analyzed hierarchically from macro to nano scale or *vv* aiming to a more comprehensive and realistic evaluation for the design of intraosseous biomaterials (36).

Since nano interactions with ions and molecules until the geometric design of implants, at macro scale they have regulated osseointegration related events (4).

TITANIUM ALLOYS

In general, titanium alloys present good mechanical strength, toughness, low density, excellent corrosion

resistance, low Young's moduli or low stiffness compared to others biomedical metallic alloys. These properties enable these materials as candidates for a wide range of applications in dental and orthopedic implants. Usually, for low load bearing conditions, commercially pure titanium (CP-Ti) can provide these requirements. When high loading conditions are necessary, some titanium alloys classified as alpha plus beta or beta alloys, have demonstrated to support these service loads (37).

Titanium alpha is a crystalline phase of titanium, represented as a compact hexagonal crystalline (HCP) cell unity. In particular, commercially pure titanium is composed entirely of alpha phase and used for dental implants, due to high biocompatibility performance. Beta phase is described as a body centered cubic (BCC) cell unity, developed mainly to acquire low rigidity in order to provide the mechanical compatibility necessary to avoid stress shielding and osteoporotic-related events (37). Combining portions of both alpha (HCP) and beta (BCC) stabilizing elements, alpha plus beta titanium alloys were developed firstly for aerospace industry to reach high mechanical performance, excellent corrosion resistance with low density and was adopted and adapted for biomedical applications.

In the biomedical intraosseous field of application, mechanical compatibility is required for better performance of assembly implant-interface-bone. Therefore, beta titanium alloys have been developed to provide high mechanical performance associated to low rigidity, important to prevent stress shielding related factors (9, 38-40). Additionally, these materials have demonstrated excellent corrosion resistance and biological test evidence have shown advantageous responses compared to conventional alloys (41,42).

For orthopedic implants, additions of atomic elements to the bulk alloy such as niobium, tantalum, molybdenum and iron have been reported and special thermo-mechanical routes have been developed to optimize and enhance the microstructure for titanium alloys, in order to perform static and dynamic mechanical requirements properly, with no cytotoxic potential (40, 42-46).

Metallurgical processes have been researched

and developed, aiming to obtain metallic alloys with outstanding mechanical responses. Alterations on the metal bulk can induce extraordinary grain refinement resulting in very high mechanical strength. The ultrafine grain metals can be obtained by severe plastic deformations techniques which deform the material internally, aiming to produce micro and nano grains material (47, 48).

SURFACE MODIFICATION

This topic covers the surface modifications and the consequent properties to biological responses activities. Topography, composition, wettability and surface effective area are especially important surface characteristics, which directly influence osteoblasts-related cell activities. Surface sensitive mechanical property such as fatigue resistance, crack propagation or notch sensitivity have to be taken into consideration during surface design of implants, therefore cyclic loading tests are carried out aiming to evaluate surface influence on cyclic fatigue properties.

Topography: from macro to nano scale

Topography includes the morphological aspects of the surface of a 3D space. According to the manner in which roughness parameters and surface features are organized, surface topography can be defined. Roughness parameters related to *Amplitude* (S_a : Arithmetic mean height, S_z : Maximum height, S_{sk} : Skewness, S_{ku} : Kurtosis), *Spatial* (S_{tr} : Texture-aspect ratio, S_{al} : Autocorrelation Length) and *Hybrid* (S_{dr} : developed interfacial area) can provide quantitative characterization of morphological aspects, enabling comparison between processing and surfaces. Roughness parameters associated to electron microscopy images can then provide a useful tool for topographic biomaterial surface characterization.

Not surprisingly, observing the bone hierarchically, the act of designing a surface for intraosseous implants can be conceived in macro to nano scales. Usually, macro scale alterations provide primary or mechanical stability while micro and nano features can guide biological events. Primary stability has an important function in osseointegration

providing the mechanical interlock between implant and tissues, avoiding micro movements, deleterious to osseointegration evolution (49). Micro and nano scale surface modification has been designed to create a topography and increased interfacial area to guide cell behavior overall, stimulating bone mineralization events for osseointegration of implants (15, 50).

Increased implant area after geometric design of implants can improve bone-to-implant contact (BIC) and the torque removal. Macro scale parameters can be designed for: length of implant, outer and inner diameters, pitch-height of threads for dental implants while for femoral stems, length, angle, thickness and macro surface roughness are relevant for increased primary stability, BIC, torque removal and also for a homogeneous stress distribution on interface implant/bone, preventing stress shielding problems.

The macro design can be evaluated by finite elements modeling (FEM) simulations. Increased implant external area aiming to improve mechanical or primary stability in the bone can be developed. Additionally, stress distribution around the 3D implant virtual implant model can also be simulated by FEM analysis and stress and strain distribution maps can be produced (51). Adopting geometry design to generate homogeneous and not punctually concentrated stress for both bone or implants regions is important for osseointegration and survival of intraosseous implants. These macro modification processes can be obtained through a physical modification process: machining, grit blasting, plasma spraying deposition, thermal deposition, physical vapor deposition and electro-erosion (52-54).

Micro and nano environments can be obtained by physical or chemical modification methods. These levels of surface alteration aim to control the cellular cascade events prior to osseointegration and catalyze the bone formation on the implant/bone interface. Structures such as porous, spikes, columns, ripples, fibers and sponges can be developed using chemical and physical manipulation of matter (55-57). In particular, nano features have a more sensitive cell control behavior, where nanotopography promotes mechanotransduction by stretching the cells resulting

in gene expression and differentiation fate for cells (58). Adhesion, spreading, cells morphologies events are very important to influence and express gene markers that regulate cells behavior. For osteoblastic-related cells the spreaded shape can be induce by nanotopography and recognized as important morphology for osteoinduction and osteoconduction capability (34,59-61).

There are various ways to obtain micro and nano surfaces, using physical and chemical processing in order to organize the surface architecture by extractive or additive process. Oxide, calcium phosphate rich phases (CaP) and mineral growth can be developed so that the incorporation of biomolecules and growth factors can be special tools to build a nanotopographic surface (62, 63).

There is a wide variety of morphologies for surface modification processing: Conditions and parameters can be modified to develop interesting surfaces topologies (34, 64-66). Nanotubes have been developed in titanium alloys (α , $\alpha+\beta$ and β) aiming to improve biological responses. *In vitro* tests and more recently, *in vivo* tests reports have demonstrated the importance in regulating nano scale topography to suitable cell responses on biomaterial substrates (34, 67, 68).

Since the rising interest in osseointegration or tissue engineering, preservation of chemistry environment associated to nanotopographic formation is aimed at various applications in the biomaterial field. Interestingly, diameter of nanotubes has been controlled to investigate the geometry conditions, to obtain for example, optimized effect of cells adhesion and differentiation outcomes, conserving very similar chemistry. Park et al. have considered an average diameter of around 15 nanometers as ideal for biological responses with osteoblasts and osteoclasts and co-culture experiments were executed to corroborate these findings (60). Nano dimensions are also important for integrin cluster formation on the surface substrate, responsible for focal adhesion of cytoskeleton observed after staining, where deformation or stretching are reported, influencing nucleus deformation, gene expression and differentiation results (67, 69).

These findings are important for the development

process of surface modification to obtain and enhance micro- to nano- structured surfaces. Chemically, films deposition, electrochemically, laser irradiated are reported as tools to regulate and optimize bio responses. Additionally, the immobilization of biomolecules have attracted the attention of researchers aiming to assess the influence of integrins, fibronectins molecules and implications on the adhesion until differentiation paths followed by cells (70).

Composition

Bulk metallic composition of alloys are important to establish a well-designed sequence of metallurgical processing that assure mechanical strength, elasticity, corrosion resistance, low density and biocompatibility.

A special tendency to mimic the bone environment also in the environmental chemistry can provide affinity to osteoconductive behavior, exposing bio active elements or compounds have been object of researchers developing surface modification of implants. Generally, a surface reactivity is investigated providing a substrate as deposit to adsorb specific ions and proteins from biological surroundings.

Bioactive responses have been reported based on the enrichment of physiological related elements such as calcium, phosphorous, magnesium, siliceous, zinc, magnesium, strontium and potassium (71, 72).

Cells have demonstrated to respond in a distinct manner to materials that have the same stoichiometry but different crystalline structure parameters. Rutile and anatase, both TiO_2 and tetragonal crystalline structure, however with different parameters have induced distinct osteogenesis potentials (73, 74).

Additionally, absorption of atoms, ions and molecules have been researched and developed as modification tools to functionalize biomaterials surfaces. Growth factor components have been reported as successful tools for tissue regeneration as substances having the potential to act directly on cells (75, 76).

Wettability and effective area

Events related to protein and cell adhesion have

been reported as strongly influenced by wettability and surface energy or charge (77-79).

Wettability can be measured analyzing the angle formation of the drop due to surface force actuating. When using water, the surface tension of the drop will be more or less attracted to the surface according to the zeta potential, influence also by roughness of surface (80,81). Interestingly, hydrophilic surface tends to attract determined type of protein while hydrophobic ones another type. On literature, there is not a consensus; however an increased surface energy tends to be more reactive. Some researchers have shown that fibronectin are preferentially attracted on hydrophilic surfaces, while albumin on hydrophobic ones (82,83). Fibronectin is a protein actuating on cells adhesion so important mechanism to osteoinduction or osteoconduction activities (70,78,84,85).

Mechanical concern on cyclic loading

Titanium and its alloys for biomaterials applications are developed to exert a long term function after implantation, and the failure susceptibility of titanium alloys has a fundamental importance to avoid premature revision surgeries and the damage occasioned to the patient.

Fatigue can be examined as a surface-dependent mechanical property of a given material. So, the surface finishing can exert important role for implant performance. Sand blasting and thermal oxidation can provide a reduction on fatigue behavior of implant, forming crack nucleation sites that induce premature failure (86). Anodic oxidation, chemical treatments have demonstrated some favorable results during fatigue testing (87-89).

The tendency of alloys for orthopedic implant application includes beta titanium alloy design; low rigidity material, suitable to an efficient stress/strain distribution around the implant, avoiding stress shielding able to prevent osteoporosis related events around the implant environment. Severe Plastic deformation (SPD) has been pointed as candidate for thermo mechanical processing to produce biomedical raw-materials for implants fabrication. Prominent mechanical strengthening is frequently reported for SPD processed materials, owing to the grain size

reduction after deformation of bulk material in a die mold (44,90).

Carrying out equal channel angular pressing and heat treatments protocols, outstanding mechanical properties can be realized [84]. Additionally, *in vitro* tests have demonstrated biological activity compared to conventional grains materials (91).

BIO – RESPONSES

Cells can present distinct behavior according to surface substrates and environment where the contact is established. Cytoskeletons can assume a determined configuration by mean of actins filaments deformations and integrin unities have important influence on cells morphology. Surface can provide anchoring since nano level where filopodia can be stimulated, these protrusions are important to sense the environment before cells motilities (92). Lamellopodia and actin bundles can be formed also in smooth and micro structured surfaces. These prolongations have important influence on cellular differentiation fates and cells motilities of cytoskeleton (93). Adhesions, cell viability, morphology, gene expression and others have been recognized tests to evaluate biological responses on biomaterials surfaces for many applications include for osseointegration.

Cellular test

There are several tests assessing biological behavior of bone replacement materials. Very often reported during investigations can be cited cellular viability tests, cellular attachment and adhesion experiments, cellular morphology and orientation, gene expression and apoptosis (94).

Cell types

There a variety of cells aiming to perform or mimic the osteoblastic behavior, since stem cells until osteoblastic-like cells. Very often reported experiments are carried out with pre-osteoblastic cells (MC3T3-E1), human mesenchimal stem cells, adipose stem cells, human osteoblastic cells, or even with human osteoblast-like cells (MG63), osteosarcoma-derived cells line (SaOS-2) (22,95-

103). According to reports few occurrences a wide study about different cells line on biological responses could be performed separately or even co-cultured to comparative and more realistic understanding of cells types implication on bio responses and markers (104). There is no conclusive investigating delimiting the suitable culture for biocompatibility essays.

Adhesion

Cell adhesions are intimately related to surface hydrophilicity, surface energy or charge. According to the wettability and increased surface area a improve adhesion performance is reported for researchers. Specially bridged by fibronectin proteins, that bind to hydrophilic preferentially surface, cell adhesion is amplified and increased cells number are adhered into hydrophilic substrates (55,105,106).

Cells shape and spreading

Interestingly, a strong influence of micro and nanotopography are attributing to cell shape after culture on surface modified titanium samples. Nanotopography presents a direct relation to influence the formation of filopodia and micro topography which determines the lamellopodia directions. Filopodia presents an important correspondence to surface nanotopography, imposing cytoskeleton mechano-transduction determining differentiation fates. The cytoskeleton senses the surface nanotopography by mean of small levels actins protusions called filopodia while in higher dimension, micro levels a lamellopodia determines cells at micro level (107-109).

Gene expression

Gene expression has fundamental importance in cells fate. Osteoblastic cells are stem cells differentiate by some inductive signals and investigators have shown the importance of Runx2 transcription factor to maintain osteoblasts as immature condition, blocking the potential to differentiate in osteocytes (110, 111). According to the micro and nanotopographic features the osteoblast lineage cells can express Runx2 and a cascade of osteogenic events. There are correspondences of

nanotopographic and lamelopodia, filopodia and adhesion points of cell membrane on the phenotypic expression of osteoblasts. For this assumption the surface modification of titanium surfaces aims to control cells behavior and accelerate and improve interface modeling and construction. Finally, a biological stability can be consolidated.

Interestingly investigate ultimately, correspondence among cytoskeleton morphology and substrate micro and nano environment have attracted attention for many researchers. Actin fibers can induce cell adhesion, spreading, motility and even gene expression. Alfa and beta unities of integrin proteins exert the adhesive function regulating this phenomenon during the interaction between cell membrane and materials surface. Schonichen et al. assess molecular model to describe fifteen formins actin molecules filaments, stating the importance for stability and motility of eukaryotic cells (108). Cells membrane forming lamelopodia and filopodia morphologies are important behavior for differentiation fate and motility of osteoblastic lineage cells.

Interesting results were found demonstrating the importance of nano scale control for optimized biological responses (67,68). Focal adhesions are established in nano scale of dimension supported by integrin having clusters in nano scale, the focal adhesions and filopodia have demonstrated importance for a cascade of events related to biological and osteogenic activities (112).

In vivo tests

Additional to cellular responses examined *in vitro*, the *in vivo* tests are carried out usually for investigate on final stage especially to validate cells experiments and beneficial effects to biomaterials application of a given material or surface. *In vivo* responses provide information on tissue level, concerning about tissue growth around surface materials (113). Parameters related to osseointegration phenomena can be extracted from biomechanical, morphological and histological evaluations; bone-to-implant contact, bone mineralization, removal torque, histomorphometric images and quantifications, all them can illustrate the osseointegration efficacy of

a given implant material. Macro associated to micro and nano features have demonstrated the main tendency to development of implantable materials for orthopedic, dental and tissue engineering (65,68,114,115).

ACKNOWLEDGEMENTS

FAPESP (2013/06258-0) and CAPES – PDSE, (0111/12-0), University of Ferrara.

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MONDANI INTRAORAL WELDING: HISTORICAL PROCESS AND MAIN PRACTICAL APPLICATIONS

L. DAL CARLO¹, M. E. PASQUALINI², P. M. MONDANI³,
F. ROSSI⁴, E. MOGLIONI⁵ and M. SHULMAN⁶

¹Private practice in Venice, Italy; ²Practice in Milan, Italy; ³Private practice in Genoa, Italy

⁴Private practice in Busto Arsizio, Italy; ⁵Private practice in Rome, Italy; ⁶Private practice in
Cliffside Park, NJ USA

The intraoral welder was invented by Dr. Pierluigi Mondani during the early 70's to weld titanium needle implants to a titanium bar in patient's mouth and to load them immediately by means of resin prosthesis. The clinical use documented dates back to 1972. Over the years, many practical applications have been added to the initial one, which have expanded the use of this device. In this scientific work, main applications are described. The aim of the work was to trace the historical process of intra-oral welding according to Mondani and describe the main practical applications. Intra-oral welding is a process introduced by dr. Pier Luigi Mondani of Genova (Italy) which allows to firmly conjoin titanium implants of any shape by means of a titanium bar or also directly between them in the mouth during surgery. The immediate stabilization achieved by intraoral welding increases implants success rate, allows immediate loading even in situations of bone atrophy, saves implants that are running into failure, re-evaluates fractured implants, allows to stabilize submerged implants postponing prosthesis management, allows to achieve efficient rehabilitation protocols to deal with difficult cases. The 40-years' experience with intra-oral welding described in this article, confirms the ease of use and efficiency in providing immediate stabilization of titanium implants of all types.

The fact that immediate solidarization increases the success rate of immediately loaded implants is accepted in the international literature (1-3).

The first insights proven by clinical series, were described by Ugo Pasqualini in 1972. He published the following, describing his experience: "In our experience, even in light of quite a few puzzling exceptions, we can prudently recommend to block implants on each other or to other natural elements as early as possible, noting that immediate prosthesis (performed according to the rules of gnathology) helps to decrease the number of failures" (4).

The most important innovation in the field of implants solidarization occurred during the 70's,

when Dr. Pier Luigi Mondani, a dentist of Genoa (Italy), presented his intraoral welder, created in collaboration with the University of Modena (Fig. 1). It was finally possible to firmly conjoin implants together in a single structure, without any play between the parts put in solidarization. Mondani brought his invention to the GISI congress, the main event in Italy, held in Bologna twice a year by Prof. Giordano Muratori. He published the technical description only later, in 1982 (5).

Mondani strove to create this device to put titanium needle implants into immediate contention, enabling the needle technique to become a repeatable technique and therefore, scientifically reliable (6-9).

Key words: intra-oral welding, titanium implants, retention, Mondani

Mailing address:

Dott. Luca Dal Carlo,
Studio Dentistico Dott. Luca Dal Carlo,
San Marco 924,
30124 Venezia, Italy
Tel.: 0039 041 5227465
e-mail: lucadalcarlo@yahoo.it

The needle implant solution is particularly suited to thin bone crests and the exploitation of very small recesses in the context of the alveolar process, utilizing the principle of bicorticalism. This proves particularly suitable to ridges containing rarefied cancellous bone (10-17).

Numerous colleagues soon began to think about how to apply this technique of intra-oral welding to other forms of implants. Several applications were identified.

MATERIALS AND METHODS

The welding procedure according to Mondani is performed immediately after the placement of the titanium implants.

The “intraoral welder” unit comes with an energy accumulator, a potentiometer and a clamp. This device emits a very intense electric charge, but for such a short period (4 sec) that with proper material, the heat released does not propagate over the areas adjacent to the point at which the gripper is applied. As a safety precaution, the welding should be carried out under continuous cooling with cold water.

A fundamental requirement is that the two pieces of titanium to be welded are in contact with each other and contact the spouts of the clamp, so that there is a fluid passage of energy. It is possible to weld two or more implants between themselves directly or by means of a titanium bar.

By welding the bar, a single structure is created, formed by implants and bar, without solution of continuity. This produces a single body, known as implant structure. The solidarization can also be achieved by means of several titanium wires.

To try to reduce the internal volume of the bar to a minimum, you can mold it before performing the welding, in order to ensure that it overhangs the ridge occlusal surface (Fig. 2).

In general, the intraoral welder can be used to join any titanium implant type. Specifically, we list below its most common uses, dividing them in two categories: main and additional.

Main uses

1. Join endosseous implants during intervention

2. Join new implants to already osseointegrated implants
3. Stabilize other implants

Additional uses

4. Connect already osseointegrated implants
5. Join endosseous and iuxtaosseous implants
6. Join teeth and implants with appropriate connectors
7. Rebuild abutments of inadequate length
8. Rebuild fractured implants
9. Connect new implants to fractured implants
10. Join implants between the submucosa

1. Join endosseous implants during intervention

This is the main use of intra-oral welding proceedings. The immediate solidarization of the implants positively affects their stability, promoting bony inclusion without interposed connective tissue. Immediate loading of the implant structure causes bone apposition that follows trajectories lead by force.

Immediate solidarization by welding can be made between two or more implants, to allow immediate load and to protect implants from the expansive action of the tongue during swallowing (18, 19). A surgical protocol for the superior arch was published in 2005 and 2015, with exhaustive indications about prosthesis management (20, 21). In Fig. 3, you can see a full-arch inferior jaw implant structure with prosthesis including the bar. Recent studies have demonstrated that the presence of the bar does not create inflammatory problems (22, 23).

One of the advantages of this method of solidarization is that emerging and submerged implants of different shape can be welded together, allowing to freely choose the suitable implant. This aspect frees the dentist from a single implant factory, facilitating treatment of difficult cases in which a single type of implant does not fit anatomical ridge variations.

Normally, when the plan is to maintain the welded bar also in the final phase, one-piece implants are used. When the plan is to eliminate the welded bar before building the final prosthesis, you can either use one-piece implant planning to mill them as if they were natural teeth, or use submerged implants to postpone and manage the prosthetic stages at will.

In general, the choice of keeping the bar depends on the difficulty of the clinical case. The bar gives strength to the structure not only when there is poor bone depth,

but also when the implants are inserted in a tilting manner so as not to damage deep anatomical structures, such as the mental foramen. A study on the rules of keeping or eliminating the bar has been recently published (24). The presence of welded bar allows you to mitigate the effect of negative factors, extending the possibility of treatment even in difficult cases (25).

The welding of a titanium bar can be used to join implants at the end of surgery, which is then removed before performing the definitive prosthesis.

After osseointegration is reached under load, the bar is removed. The subsequent prosthetic steps are facilitated by the fact that soft tissues are already healed and stabilized around the circumference of the stumps (Fig. 4) (26).

Connecting submerged implants by intra-oral welding is described in literature since 1998 (27-29).

2. Connect new endosseous implants to already osseointegrated endosseous implants

This type of management of the clinical case can be programmed in the therapeutic plan [Auriga technique, (30)] or may arise from the fact that included implants placed years before are already exploited.

3. Stabilize other implants to prevent or correct stability problems

In situations where, due to the need for immediate loading and scarceness of bone offered by the “receptor” site, the inserted implant does not guarantee stability *per se*. A needle implant can be soldered to the main implant to provide immediate stability, making this implant structure

suitable to immediate loading (Fig. 4) (31). In particular cases, the welding is also used to solder an implant to an adjacent tooth (32).

RESULTS

The analysis of the statistical studies performed by our research group lead us to suggest the use of intraoral welding as a standard aid when you want to load implants immediately. The choice of whether to keep it in the final prosthesis or remove it before completing it depends on the evaluation of the biomechanical requirements of the implant structure in the context in which it is inserted. Some rehabilitation protocols long tested by our research group have given very encouraging statistical results. In particular, as noted above, a study of 193 screw and blade implants used in cases of higher atrophy with Auriga technique gave results of complete success (30) and a study of 351 thin cylindrical titanium implants inserted in the posterior atrophic mandible gave results of 99% at 5 years and 95.8% at 10 years (8). It must be underlined that the correct use of the intra-oral welding implies an adequate learning curve.

DISCUSSION

The solidarization of the implants is a solution whose effectiveness is now universally accepted by the world literature (1-3). In Italy and in Latin

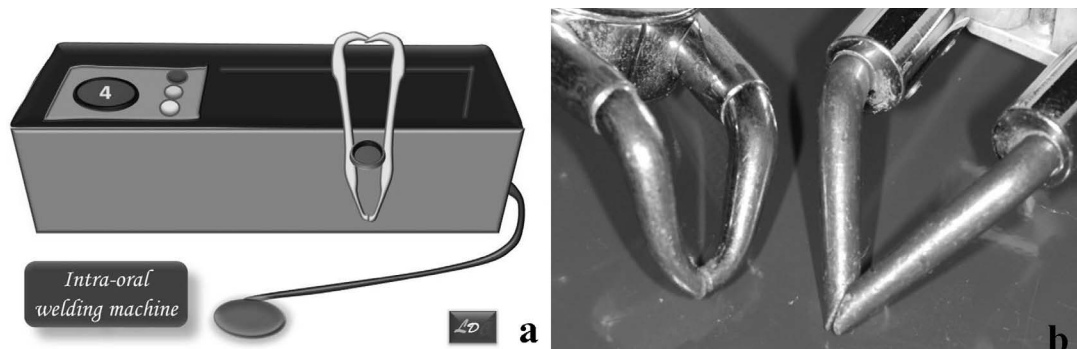


Fig. 1. a): drawing of the intra-oral welding machine; **b):** different types of clamps fit to different situations. Flat edges are suitable to buccal-palatal welding, chamfer edges to mesio-distal welding between teeth.

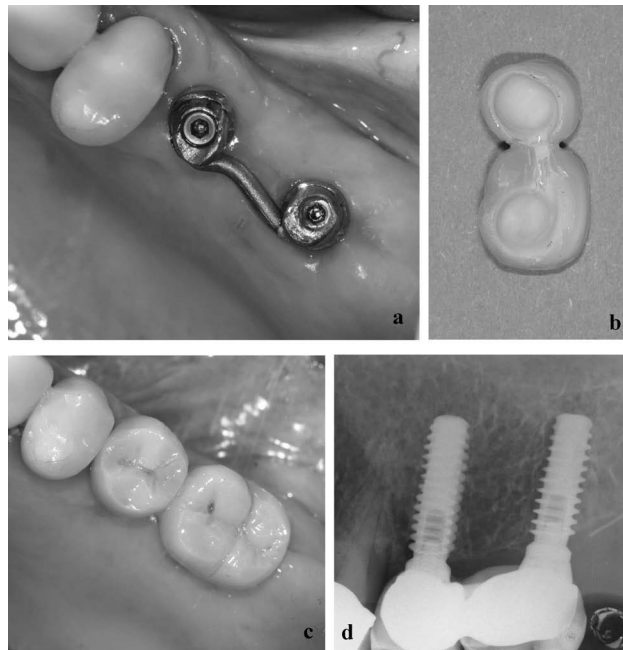


Fig. 2. *a): a titanium bar has been curved and then welded to the abutments of two titanium screw implants; b): the zirconia fix prosthesis including the bar; c): due to bar shaping, no volume augmentation has been necessary; d): X-ray. The bar is inside the prosthesis.*

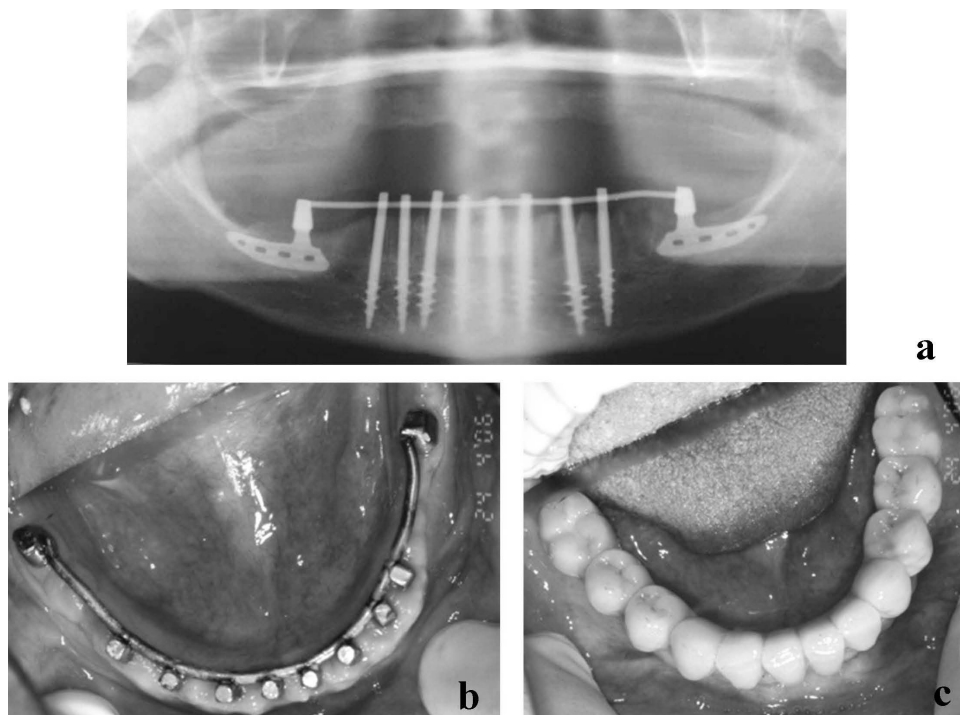


Fig. 3. *a): panoramic X-ray taken at end of intervention in which 10 one-piece implants were intraorally welded after insertion in the inferior jaw. Immediate load by means of provisional prosthesis was applied immediately after; b): view of the gums after healing; c): final prosthesis.*

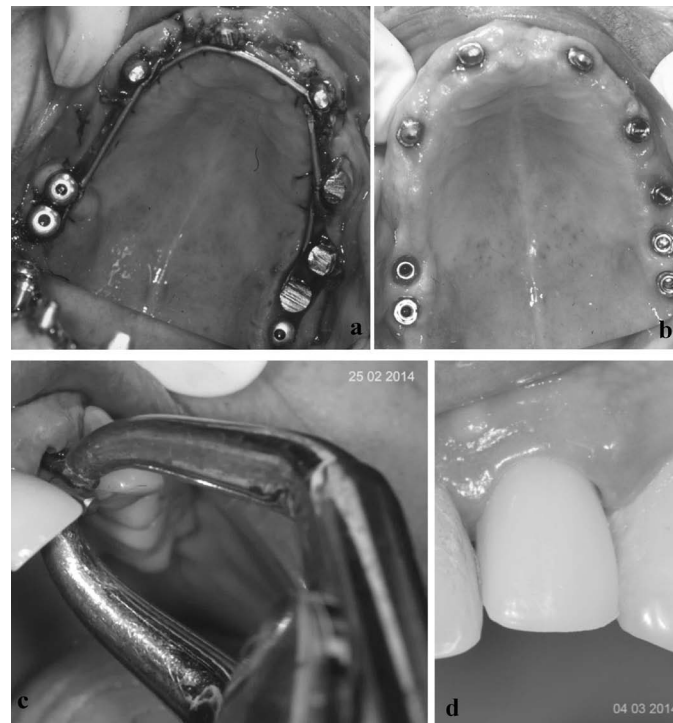


Fig. 4. *a): upper atrophic arch treated with 9 implants of different shapes (3 one-piece blades in the area in which ridge was thin and 6 submerged screws) joined together with welded bar and immediately loaded; b): after 4 months, provisional prosthesis was removed, bar was cut and implants were ready for impressions; c): intra-oral welding of screw and needle inserted in 2.2.socket immediately after extraction; d): clinical view one week after intervention.*

American countries, it has been continuously used since the 70's. It is the quickest and safest way to join the implants between them. To get CE recognition, welders today must pass a series of safety tests. This device allows welding with any titanium implant of any shape or brand. We leave readers to imagine the resistance there is in the industry and in its "opinion-leaders", engaged in the promotion of complex and expensive screwed solidarization methods.

Even with submerged implants, welding is a much more simple and efficient procedure, and has documented the support of over 20 years of clinical experience. The welding of emerging and submerged implants for immediate loading follows coded rules where possibility of failure is minimum.

The intraoral welder is a simple and versatile device to use, allowing you to improve and simplify the procedure of solidarization of titanium implants. Its applications are numerous. This makes it a safe investment in the dental profession, changing for the better the quality of work.

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EFFECT OF PLATELET REACH PLASMA ON FIBROBLAST PRIMARY CULTURE: GENE EXPRESSION EVALUATION

V. CANDOTTO¹, A. PALMIERI², F. CURA², F.J. SILVESTRE³, D. LAURITANO⁴,
R.M. GAUDIO⁵, A. BAJ⁶ and A. B. GIANNI⁶

¹*Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, Italy;* ²*Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna, Bologna, Italy;* ³*Stomatology & Oral Surgery, Dr Peset University Hospital, Department of Stomatology, University of Valencia, Spain;* ⁴*Department of Medicine and Surgery, University of Milan-Bicocca, Monza, Italy;* ⁵*Department of Medical Sciences, University of Ferrara, Ferrara, Italy;* ⁶*Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy*

To correct skin imperfections, aesthetic medicine today makes use of injectable dermal fillers that allow filling skin by increasing the volume of soft tissue. Currently, there are many fillers usable for their excellent biocompatibility and low allergic reactions that ensure good results and good compliance. Among these, the most widely used filler is Human Plasma, obtained by centrifugation from patient blood and processed through cycles of centrifugation and heating, until it becomes a gel. In this work, we evaluated the effect of the Plasma on fibroblasts behavior in terms of cell viability, survival, growth and collagen production. To this aim, the expression levels of some adhesion and traction-resistance related genes (COL11A1, ELN, GDF6, IGF1, DSP, ELANE, HAS1, HYAL1, COL1A1 and COL3A1) were analyzed using real time Reverse Transcription-Polymerase Chain Reaction (real time RT-PCR). All genes, except for COL1A1, COL3A1 and COL11A1, were up-regulated after 24 h of treatment. Altogether, our results point out the good potential of Plasma. In particular, we appreciated an up-regulation in some of the most important genes involved in bio-revitalization, such as elastin and hyaluronic acid.

To correct skin imperfections, aesthetic-medicine today makes use of injectable dermal fillers that allow filling the skin by increasing the volume of soft tissue. Such fillers restore skin volume and rejuvenate appearance (1-9).

Among these substances, the most widely used is Human Plasma. It represents the liquid component of blood and is obtained through centrifugation of the biological fluid. Once it is physically separated from blood cells, patient plasma is processed through several cycles of centrifugation and heating, until it

becomes a gel (Platelet Reach Plasma, i.e. PRP). At this point, plasma has a semi-solid consistency and is injectable into the tissue in order to create volume and correct wrinkles and depressions.

PRP perfectly complies with vital requirements of a good injectable filler. In fact, it is easily and rapidly reabsorbed from the body (4-6 months) and has no allergic reactions so it is usable even in people with autoimmune problems. Moreover, it is possible to use large amounts of gel at a reduced cost. Finally, given the regenerative capacity of the proteins present in

Key words: plasma, gene expression, fibroblasts, RT-PCR

Mailing address:

Prof. Aldo Bruno Gianni,
Maxillofacial and Dental Unit, Fondazione IRCCS Cà Granda,
Ospedale Maggiore Policlinico,
Via Francesco Sforza 35, 20122 Milan, MI, Italy
Tel: +39.02.55032510 - Fax: +39.02.55032815
e-mail: aldo.gianni@unimi.it

0393-974X (2016)

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plasma, its employment leads not only to an effective filling of the skin but also to an improvement of the quality of the treated tissues. The infiltration of the tissue is no different from that obtained with others fillers. In fact, the use of micro needles or cannulas, causes the same side effects related to the trauma of injection (10).

In this work, we evaluated the effect of Plasma on fibroblasts behavior in terms of cell viability, survival, growth and collagen production.

Fibroblasts are the main cells of connective tissue that synthesize extracellular matrix, an important body component for giving support to cell anchorage and individuality to each tissue. Due to some of its components such as hyaluronic acid, elastin and collagen, it ensures elasticity, turgor and stress-resistance to tissues. The importance of fibroblasts in cosmetic surgery is highlighted by their use as autogenous filler (11).

To this aim, the expression levels of some adhesion and traction-resistant related genes (COL11A1, ELN, GDF6, IGF1, DSP, ELANE, HAS1, HYAL1, COL1A1 and COL3A1) were analyzed using real time Reverse Transcription-Polymerase Chain Reaction (real time RT-PCR).

MATERIAL AND METHODS

Primary Human Dermal Fibroblast cells culture

Fragments of dermal tissue from healthy volunteers were collected during operation. The pieces were transferred in 75 cm² culture flasks containing DMEM medium (Sigma Aldrich, Inc., St Louis, Mo, USA) supplemented with 20% fetal calf serum and antibiotics (Penicillin 100U/ml and Streptomycin 100 micrograms/ml-Sigma Aldrich, Inc., St Louis, Mo, USA).

Cells were incubated in a humified atmosphere of 5% CO₂ at 37°C. The medium was changed the next day and twice a week. After 15 days, the pieces of dermal tissue were removed from the culture flask. Cells were harvested after 24 days of incubation.

Cells Culture

For the investigation, HFb at the second passage were seeded on a layer of Plasma. A set of untreated

cells were used as controls. The cells were maintained in a humified atmosphere of 5% CO₂ at 37°C for 24h. After the end of the exposure time, cells were trypsinized and lysed for RNA extraction.

RNA processing and Real Time PCR

Reverse transcription to cDNA was performed directly from cultured cell lysate using the TaqMan Gene Expression Cells-to-Ct Kit (Ambion Inc., Austin, TX, USA) following manufacturer's instructions. Briefly, cultured cells were lysed with lysis buffer and RNA released in this solution. Cell lysate were reverse transcribed to cDNA using the RT Enzyme Mix and appropriate RT buffer (Ambion Inc., Austin, TX, USA).

Finally, the cDNA was amplified by real-time PCR. For all but two genes, (COL1A1 and COL3A1), amplification was performed by using Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and the specific assay designed for the investigated genes. For COL1A1 and COL3A1, we performed amplification by using the TaqMan universal PCR master mix (Applied Biosystem, Foster City, CA, USA) instead.

SYBER assays reactions were performed in a 20 µl volume using the ABI PRISM 7500 (Applied Biosystems, Foster City, CA, USA). Each reaction contained 10 µl 2X Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 400 nM concentration of each primer, and cDNA.

TaqMan assays amplification were performed in a 20 µl volume using the ABI PRISM 7500 (Applied Biosystems, Foster City, CA, USA) instead. Each reaction contained 10 µl 2X Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 400 nM concentration of each primer, 200nM of the specific probe and cDNA.

All experiments were performed including non-template controls to exclude reagents contamination. PCRs were performed with two biological replicates.

Expression was quantified using real time RT-PCR. The gene expression levels were normalized to the expression of the housekeeping gene TFRC in the former case, and RPL13 in the latter. In both cases, we evaluated the expression as fold

changes relative to the expression of the untreated HFb. Quantification was done with the delta/delta calculation method (12).

Forward and reverse primers for the selected genes were designed using primer express software (Applied Biosystems, Foster City, CA, USA) and are listed in Table I.

RESULTS

Fibroblast behaviour was evaluated by measuring the gene expression levels of several genes growth, wealth and elasticity related genes after treatment with Plasma.

All but 3 genes resulted up-regulated after 24 h of

treatment. In fact, COL1A1, COL3A1 and COL11A1 were the only down-expressed genes (Fig. 1a, b).

DISCUSSION

Human Dermal Fibroblasts were exposed to Plasma for 24 h and later, expression profile of COL11A1, ELN, GDF6, IGF1, DSP, ELANE, HAS1, HYAL1, COL1A1 and COL3A1 were analyzed by real time RT-PCR.

ELN encodes elastin, the major component of connective tissue. This protein is one of the main ones responsible for conferring dermal elasticity and flexibility. Moreover it has a known role in damaged skin repair and regeneration (12).

Table I. Primers sequences for SYBR® Green assay.

Gene symbol	Gene name	Primer sequence (5'>3')
COL11A1	Homo sapiens collagen, type XI, alpha 1	F- AGATGAGGCAAACATCGTTGA R- ATCAGAATCCCTGCCGTCTA
DSP	Homo sapiens desmoplakin	F- ATGACCTGAGGAGAGGACGAA R- AGGCTCTCTCTTTCCTGTACCAC
ELN	Homo sapiens elastin	F- CTAAATACGGTGCTGCTGGC R- CATGGGATGGGGTTACAAAG
HAS1	Homo sapiens hyaluronan synthase 1	F- CTCGGAGATTTCGGTGGACTA R- CGCTGATGCAGGATACACAG
GDF6	Homo sapiens growth differentiation factor 6	F- CCCCACGAGTACATGCTGTC R- GAGCATGGACACATCAAACAA
IGF1	Homo sapiens insulin-like growth factor 1	F- GCGCAATGGAATAAAGTCCT R- ACAGCGCCAGGTAGAAGAGA
ELANE	Homo sapiens elastase, neutrophil expressed	F- CTACGACCCCGTAAACTTGCT R- CCTCACGAGAGTGCAGACGTT
HYAL1	Homo sapiens hyaluronoglucosaminidase 1	F- ACAGATGTATGTGCAACACCG R- AAGGGCCCCAGTGTAGTGTC
TFRC	Homo sapiens transferrin receptor protein 1	F- CGCTGGTCAGTTCGTGATTA R- GCATTCCCGAAATCTGTTGT

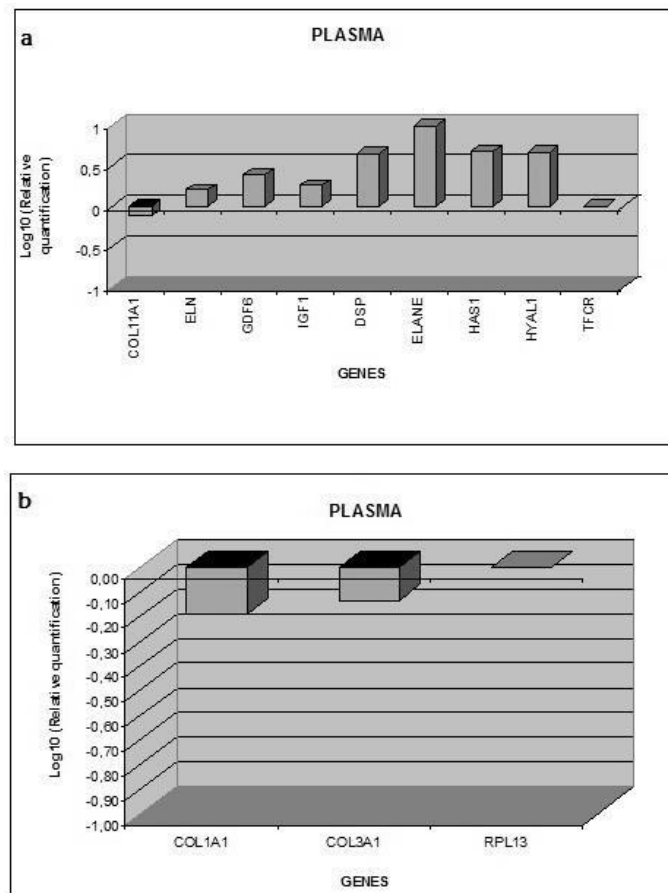


Fig. 1. a): Human Dermal Fibroblasts gene expression profile after 24h of treatment with Plasma. SYBR® Green assay; **b):** Human Dermal Fibroblasts gene expression profile after 24h of treatment with Plasma. TaqMan® Gene Expression Assay

Table II. Primers and probes sequences for TaqMan® Gene Expression Assay.

Gene symbol	Gene name	Primer and Probe sequence (5'>3')
COL1A1	Collagen type I alpha1	F- TAGGGTCTAGACATGTTTCAGCTTTGT R- GTGATTGGTGGGATGTCTTCGT P- CCTCTTAGCGGCCACCGCCCT
COL3A1	Collagen type III alpha1	F- CCCACTATTATTTTGGCACAACAG R- AACGGATCCTGAGTCACAGACA P- ATGTTCCCATCTTGGTCAGTCCTATGCG
RPL13	Ribosomal protein 13	F- AAAGCGGATGGTGGTTCCT R- GCCCCAGATAGGCAAACCTTC P- CTGCCCTCAAGGTCGTGCGTCTG

DSP gene encodes desmoplakin, an important protein associated with desmosomes (13). We can easily note that an undamaged tissue prevents cells from further environmental damages (e.g. UV) and bacterial infections (14, 15). All these features lead to a valuable skin protection from aging.

GDF6 gene encodes growth differentiation factor of 6 proteins. This is involved in the patterning of the epidermidis. GDF6 induces genes related to the development of the epidermis and can bind directly to noggin, a gene that controls neural development, therefore blocking its effect (16).

Insulin-like growth factor 1 (IGF-1), also called somatomedin C, is a protein that in humans is encoded by the IGF1 gene. This protein plays an important role in embryos development and growth as well as dermal youth maintenance (17). Their upregulation indicates that Plasma enhances cellular survival and growth, suggesting that its use could lead to filling depressed areas.

Hyaluronic acid (HA) is a component of the extracellular matrix. HA is synthesized from a gene named HAS1 (18), which resulted over expressed in this work. HAS1 up-regulation leads to a higher HA production and consequently, is associated with toning up, moisturizing and volume restoration effects.

Altogether, our results highlight the great potential of plasma. However, to evaluate the long-term effects, we will set other experiments prolonging the exposure time.

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COMPUTER-GUIDED IMPLANT INSERTION IN A PATIENT WITH IMPACTED MAXILLARY CANINES: CASE REPORT

S. LICO¹, C. ANDRISANI², M. ANDREASI BASSI³, V. CANDOTTO⁴,
F.J. SILVESTRE⁵, and D. LAURITANO⁶

¹Private practitioner in Olevano Romano, Rome, Italy; ²Private practitioner in Matera, Italy

³Private practitioner in Rome, Italy; ⁴Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, Italy; ⁵Stomatology & Oral Surgery, Dr Peset University Hospital, Department of Stomatology, University of Valencia, Spain; ⁶Department of Medicine and Surgery, University of Milan-Bicocca, Monza, Italy

The use of minimally invasive surgical techniques undoubtedly represents a huge advantage for both the clinician and the patient. This case report shows the possibility of making an implant-prosthetic rehabilitation in an upper jaw in a patient who presented dental elements 1.3 and 2.3 in bone inclusion. The use of computer-guided surgery offers us the possibility to carry out this type of implant rehabilitation without performing the extraction of the above-mentioned dental elements combined with the bone regeneration of the extraction sockets. Consequently, surgery invasiveness and post-surgery complications are reduced to a minimum. Additionally, the predictability of the methodology is evaluated with specific software that enables a comparison between what is virtually planned and what occurs in clinical practice.

The replacement of missing teeth through the use of implants is now considered a predictable procedure in modern dentistry, with high rates of success with regards to both the recovery of the function and aesthetic (1).

Planning plays a fundamental role in the implantology clinical success, there is therefore a strong need to faithfully transfer the therapeutic plan to clinical practice (2, 3).

The development of new three-dimensional diagnostic techniques, along with the commercial availability of specific software that allows the clinician to make a virtual planning of surgery, makes it feasible today to have an accurate and predictable implant placement based on an accurate computer planning (3, 4).

The virtual design also allows us to have a prosthetic driven approach, resulting in the possibility of having a better prosthetic design, better aesthetics and the ability to optimize the occlusion and implant-prosthetic load (3).

The information obtained by means of X-ray examination and processed by a dedicated software are converted to allow us to create a surgical guide for implant placement, with or without lifting a mucoperiosteal flap. In this way, it is possible to reduce chair time and surgery stress, as it greatly reduces the risk of intra and post-surgery complications (2-6). However, it must take into account some controversial issues that may arise in the various steps leading to the creation of a surgical guide:

Key words: Implant, fixture, computer, guided, insertion

Mailing address:

Dott. Carlo Andrisani,
Via 4 Novembre 22,
75100 Matera, Italy
e-mail: carlo.andrisani@gmail.com

1. Creation of an accurate radiological template.
2. Radiographic examination TC ConeBeam with the same template in the correct position.
3. Planning of the implant placement with the dedicated software.
4. Manufacturing of the surgical guide.
5. Positioning adequate fixing of the surgical guide on the day of surgery.

Of course, during all these steps errors can be made but by paying close attention to every step we can reach a level of adequacy that enables us to work in maximum safety, making the most of all the advantages that the computer guided surgery offers us (7).

The following case report shows an implant-supported prosthetic rehabilitation aided by a surgical template created with a 3D printer, in a patient with maxillary canines in bone inclusion. This procedure has allowed us to place implants without colliding with the impacted canines, which avoids the patient having to be subjected to guided bone regeneration techniques, with all the complications that this could imply.

Case report

A 67 year-old woman has come to our attention for a prosthetic rehabilitation of the upper jaw. The medical history examination did not reveal any systemic disease and the patient claimed to take no medication.

A Cone Beam CT (Promax 3D, Planmeca, Helsinki, Finland) showed the presence of two impacted dental elements (1.3, 2.3) with no radiographic signs of degeneration of the

follicular sac (Fig. 1).

The patient refused the extraction of the aforementioned dental elements associated with guided bone regeneration techniques to allow the implant placement. Therefore, we proposed the patient to place titanium implants bypassing the impacted dental elements. This procedure can be achieved thanks to the possibility of planning the intervention on a specific 3D software and the subsequent creation of a surgical guide (Fig. 2) with a 3D printer. The surgical guide allows us to transfer the surgical project from computer planning to clinical practice.

Surgical procedures

The pre-surgery planning was divided into the following steps:

1. A radiological template made of radiopaque



Fig. 2. *Surgical guide.*

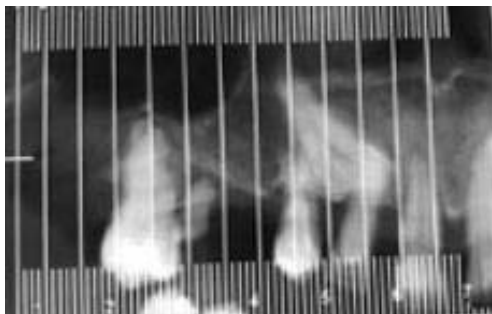
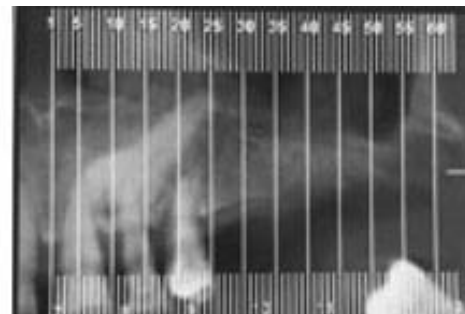


Fig. 1. *Initial Cone Beam CT of the patient.*



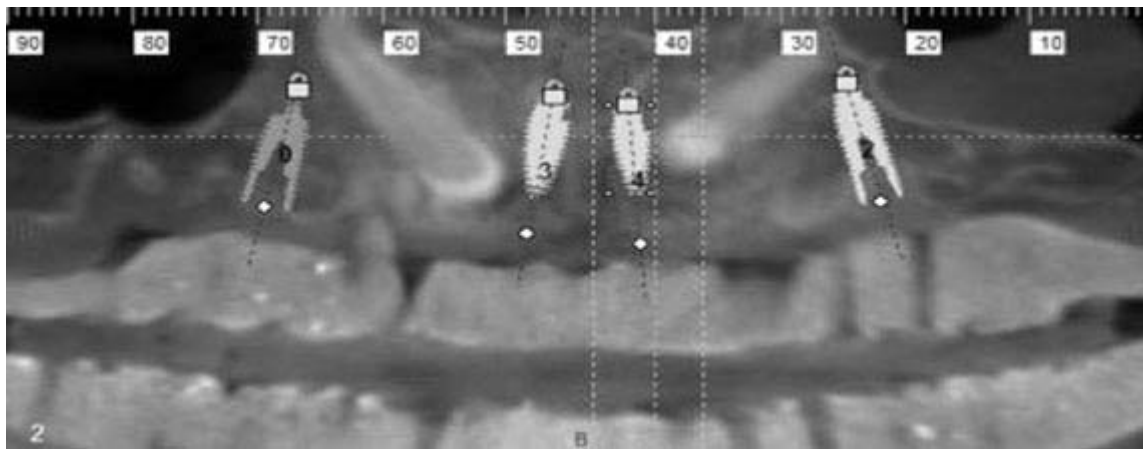


Fig. 3. Initial project, from which it is possible to notice the placement of the implants without any interference with the impacted canines.

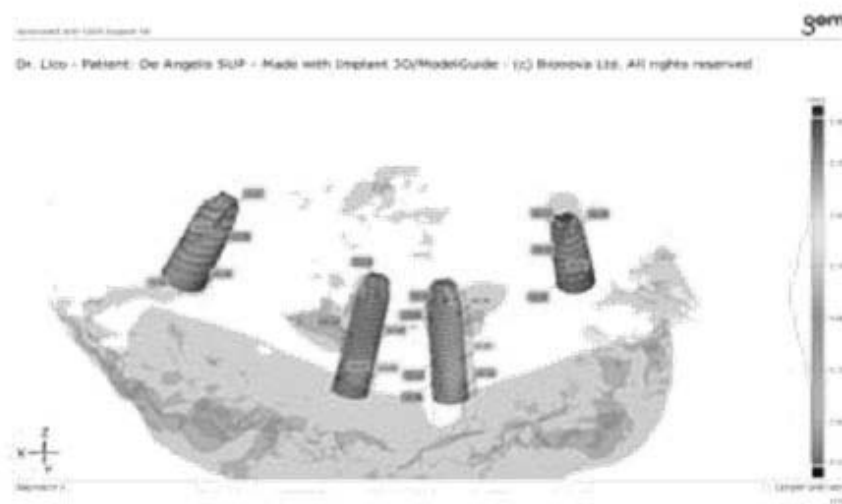


Fig. 4. Comparison using the dedicated software Gom Inspect V8.

resin (VivoTac, Ivoclar, Casalecchio di Reno, Italy) was fixed to a Universal Stent in the mouth of the patient (Bionova, Follo-Sp, Italy) by means of a polyether impression material (Impregum, 3M Espe Italy, Pioltello-Mi, Italy). This template has some extraoral radiopaque markers, which must be worn by the patient during the X-ray examination in order to highlight any artefact.

2. The Dicom 3.0 files obtained were processed through a specific software (3D Implant, Medialab,

Follo-Sp, Italy) in order to virtually simulate the placement of the implants in a position that was prosthetically guided and at the same time, to not interfere with the adjacent teeth, including the impacted canines (Fig. 3).

3. The surgical template in acrylic resin was created using a 3D printer (Form 2, 3DZ, Castelfranco Veneto-TV, Italy).

The patient was prescribed an antibiotic therapy with amoxicillin clavulanate (Augmentin,

GlaxoSmithKline, Verona, Italy), in the dose of 1 g every 12 h for 6 days, from the day before surgery. The area of the surgery was anesthetised using Articaine with Epinephrine 1:100000 (Septanest, Septodont). The surgical template was then placed and blocked by means of lateral pins. Subsequently, four implants were placed (I-Fix, FMD, Ladispoli-Rm, Italy).

At the end of the surgery, the gingival punches were repositioned and sutured in place. Immediate loading protocol was not performed, because as agreed with the patient, the decision was to proceed with the creation of the final prosthesis after implant osseointegration period. The prosthetic finalization was carried out after 4 months with a Toronto Bridge full arch rehabilitation.

After surgery, the patient was asked to perform a Cone Beam CT radiographical checkup exam, using the radiological template paired with the Universal Stent again, in order to make a comparison between what was our virtual project and what happened in clinical practice, thus confirming the methodological accuracy.

This comparison was carried out with a specific software (Gom InspectV8, GOM GmbH, Braunschweig, Germany) (Fig. 4).

The following variables were taken into account:

- Discrepancy of the depth of insertion of the implant (DPI), measured at the apical level;
- Radial discrepancy, measured at the level of the implant neck (MRD = mesial radial discrepancy, DRD = distal radial discrepancy).

RESULTS

DPI had an average value of 0.27 mm with a maximum value of 0.67 mm, measured at implant apical level.

MDR had an average value of 0.40 mm with a maximum value of 0.87, measured at implant neck level, while DRD always measured at implant neck level, had a value of 0.35 mm, with a maximum value of 0.69 mm.

DISCUSSION

Undoubtedly, computer-guided surgery represents

a valid support in everyday clinical practice, both for the possibility to build an accurate prosthetic device before surgery and consequently, to predict the placement of implants in prosthetically-guided position. This is an important result for a number of reasons, primarily for the reduction of chair time (with resulting reduction of stress for both the surgeon and the patient) and for the significant reduction of post-surgery discomfort of the patient in terms of pain and edema (8).

There are studies showing that even inexperienced surgeons are capable of placing implants with extreme accuracy using the computer guided insertion protocol, if all steps required by the methodology are respected and under the supervision of an experienced surgeon (9, 10).

Literature related to the accuracy of computer-guided surgery has been revised by several authors with the aim of assessing the validity of the different methodologies. The meta-analysis carried out have taken into account both *in vivo* and *in vitro* studies. The latter, on corpses and on models, were found to be more accurate for both the best visual control of the implant insertion axis and the absence of movements by the patient (11-14). Accuracy is an essential parameter to evaluate the reliability and security of a procedure performed by computer-guided surgery in our daily practice, which allows us to work within the anatomical limits and the prosthetic needs without the risk of intra and post-surgery complications (15).

The aim of this work was to suggest a valid alternative to the extraction of impacted dental elements with the support of computer-guided surgery for placement of implants adjacent to the impacted canines without coming into direct contact with them. This treatment allowed us to have final maxillary rehabilitation, with a full-arch Toronto Bridge after 4 months from surgery. This greatly reduced time compared to post extractive regenerative technique and allowed us to treat the patient with a significantly less invasive technique and reducing the risk of complications (compared to the extraction of the dental elements) with consequent guided bone regeneration to allow the implant placement.

In addition, the following comparative control allowed us to demonstrate that, by means of a dedicated

software, although literature shows greater accuracy in studies that uses surgical guide with dental support compared to surgical guide with bone or mucosa support (11), we can get an optimal accuracy even in cases of totally edentulous patients by respecting the provided protocols.

CONCLUSIONS

Previous considerations leads us to deem computer-guided surgery a suitably and safe method, which respects the anatomical limits and helps the clinician with complex surgical cases that would otherwise require a more invasive approach, not always tolerated by the patient. Thus, it represents a valuable aid in simplifying our work.

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