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COVERAGE OF MULTIPLE RECESSIONS USING THE TUNNEL TECHNIQUE AND A COLLAGEN MATRIX IN THE MAXILLA OR MANDIBLE: A 6 MONTH STUDY

M. CIEŚLIK-WEGEMUND^{1*}, V. CANDOTTO^{2*}, B. WIERUCKA-MŁYNARCZYK¹,
M. TANASIEWICZ³, Ł. GIŁOWSKI⁴, M. DUDA⁵,
D. LAURITANO⁶ and Z. ORMIANER⁷

¹*Department of Periodontal and Oral Mucosa Diseases, Department of Conservative Dentistry with Endodontics, School of Medicine with the Division of Dentistry in Zabrze, Medical University of Silesia in Katowice, Poland;* ²*Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy;* ³*Department of Conservative Dentistry with Endodontics, School of Medicine with the Division of Dentistry in Bytom, Medical University of Silesia in Katowice, Poland;* ⁴*Department of Pharmacology, School of Medicine with the Division of Dentistry in Zabrze, Medical University of Silesia, Poland;* ⁵*Duda Clinic College of Dental Medicine, Katowice, Poland;* ⁶*Department of Medicine and Surgery, University of Milan-Bicocca, Milan, Italy;* ⁷*Department of Oral Rehabilitation, The Maurice and Gabriela Goldschleger School of Dental Medicine, Tel Aviv University, Tel Aviv, Israel*

*These authors contributed equally to this work.

Multiple recession defects in the dentition of the patients are routinely encountered in clinical practice and as such present a challenge for clinicians. Periodontal plastic surgical procedures aim to restore both esthetics as function in periodontal tissues. The objective of this study was to evaluate and compare the clinical efficacy of using a tunnel technique with a collagen matrix to cover multiple recessions in the maxilla or mandible. Fourteen patients were enrolled in the study. Patients in the maxilla-group and mandible-group were treated with xenogeneic collagen matrix using the tunnel technique. Clinical recordings were obtained at baseline and after 3 and 6 months. The percentage of average recession coverage (ARC), the percentage rate of patients with complete coverage of all recessions (CRC-1) and the percentage rate of complete coverage of recession defects (CRC-2) were evaluated after 3 and 6 months after the surgery. Statistically significant differences were observed in every parameter except probing depth between the baseline values compared to the values 3 and 6 months after the procedure in both groups. The mean ARC 6 months after the procedure was 96.8% in the maxilla and 81.3% in the mandible. At 6 months after the procedure, a complete root coverage was obtained in 2 out of 9 patients and 31 out of 39 recessions (79%) in the maxilla and 0 out of 5 patients and 10 out of 20 recessions (50%) in the mandible. A collagen matrix combined with the tunnel technique led to a satisfactory ARC, CRC-2 and resulted in an unsatisfactory CRC-1.

A recession defect is the pathological, apical displacement of the gingival margin from its normal position on the crown of the tooth to a position on

the root surface that is beyond the cemento-enamel junction (CEJ) (1). Hypersensitivity that is associated with exposed roots, problems with eliminating

Key words: tunnel technique, collagen matrix, multiple recessions

Mailing address:

Dr Dorina Lauritano,
Department of Medicine and Surgery,
University of Milan-Bicocca, Via Cadore 48, 20052 Monza, Italy
Tel.: +390392332301 - Fax: +3903923329892
e-mail: dorina.lauritano@unimib.it

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dental plaque, and a patient's desire to improve aesthetics are among the most common reasons for choosing periodontal plastic surgery (2). The etiopathogenesis of recession defects is multifactorial and commonly related to alveolar bone dehiscence's and fenestrations, malposition of the dental arch and overcontoured tooth shapes, thin biotypes, high muscle and frenum attachments, traumatic brushing, localized and generalized periodontal diseases and iatrogenic factors (3-18).

Recently, the correction of exposed roots for esthetic and functional reasons has become one of the primary goals of plastic surgery. A variety of surgical techniques has been proposed to cover exposed tooth surfaces. Using a connective tissue graft with a coronally advanced flap has been suggested as the most predictable technique to cover exposed roots that are Miller's Class I and II (19). However, this procedure has been associated with the release of incisions on the buccal flap that may delay early esthetic results.

To avoid releasing incisions in the receptor site, Blanes and Allen, Zabalegui et al., and Santarelli et al. proposed using the tunnel technique, along with modifications of this method, to cover multiple recessions; this technique has the advantages of minimal surgical trauma, early esthetic results and satisfactory healing (20-23). Procedures using connective tissue grafts and the tunnel technique result in good predictability of root coverage, but they require a second surgical site, can cause discomfort for patients, and increase the risk of postoperative complications, such as pain, bleeding and numbness in the donor area (24).

A disadvantage of techniques that use connective tissue grafts to cover multiple recessions is that there are limited amounts of donor tissue available. A xenogeneic collagen matrix could be useful as a substitute for a connective tissue graft to treat generalized recessions because the amount of material available is not limited by the patient's palatal anatomy.

Mucoderm is an acellular sterilized collagen matrix derived from porcine dermis that consists of collagen types I and III and elastin. A natural collagen matrix has a positive impact on wound

healing and tissue integration because of its hemostatic properties and chemotactic effects on fibroblasts by promoting proliferation and promoting the migration and adhesion of cells to the matrix (25). To our knowledge, has been published limited clinical trials that has differentiated between the maxillary and mandibular sites, and no studies have specifically addressed the predictability of using a tunnel technique with a collagen matrix. The objective of the current report was to study the influence of the location (maxilla versus mandible) of multiple recessions on the total root coverage and on the average percentage of root coverage achieved by using the tunnel technique with a collagen matrix.

The purpose of this study was to compare the short-term (3-6 months) root coverage that was achieved using the tunnel technique with a xenogeneic collagen matrix to treat multiple Miller Classes I and II recessions in the maxilla or mandible.

MATERIAL AND METHODS

Study design

The study was conducted with the approval of the local ethical committee (the Institutional Review Board associated with the Medical University of Silesia, Katowice, Poland; protocol resolution no. KNW/0022/KB1/108/12). Patients who were eligible for the study were informed of the potential risks and benefits of participating in the study and they signed informed consent statements prior to participating. The primary outcome variable was the assessment of the CAL (clinical attachment level). The secondary outcome variables included the assessment of the recession depth and width, keratinized tissue width, complete recession coverage and average percentage of root coverage.

Patient population

Fourteen systemically healthy patients with 59 Miller's Class I and II recessions who were between 20- and 50-years-old were selected from the Department of Periodontal and Oral Mucosa Diseases in Zabrze, Poland. The patients were treated with a xenogeneic collagen matrix (Mucoderm, Botiss Dental, Zossen, Germany) using the tunnel technique. The maxilla-group consisted of 9 patients with 39 recessions and the mandible-group

consisted of 5 patients with 20 Miller Class I and II recessions.

The following inclusion criteria were used:

- 1) 18 to 60 years old;
- 2) Systemically healthy with no contraindications for periodontal surgery;
- 3) No clinical signs of active periodontal disease;
- 4) A minimum of two adjacent periodontal Miller Class I or II recessions on both sides of the maxillary or mandibular arch with recession depths of at least 2 mm;
- 5) Presence of identifiable CEJ (a step ≤ 1 mm at the CEJ level and/or the presence of a root abrasion with identifiable CEJ were both accepted) (26);
- 6) An Approximal Hygiene Index (API) of $\leq 5\%$ and a Sulcus Bleeding Index (SBI) of $\leq 10\%$.

Patients were excluded from the study based on the following criteria:

- 1) Pregnant or lactating;
- 2) Presence of systemic diseases that compromise healing potential or infectious diseases;
- 3) Usage of medications known to affect gingival conditions (hyperplasia) or interfere with healing (e.g., steroids);
- 4) Non-vital teeth, caries, prosthetic crown or restoration involving the cemento-enamel junction area;
- 5) Tobacco smoking;
- 6) Drug or alcohol abuse.

Before surgery, each patient received periodontal treatment and oral hygiene instructions to reduce improper habits that may have contributed to the recession defects, and occlusal adjustments were performed when necessary. All of the participants were enrolled by one investigator (MCW). The Sulcus Bleeding Index and API were evaluated (by LG) one month and also one week before patients underwent surgery. A final examination was conducted to confirm the patient's compliance with the study enrollment criteria.

Clinical measurements

The following clinical parameters were measured using a standard CP15 (Carl Martin GmbH, Solingen, Germany)

periodontal probe with markings every millimeter. The measurements were rounded to the nearest 0.5 mm.

At baseline and 3 and 6 months post-surgery, the following measurements were recorded: recession depth (RD) was measured from the mid-facial point of the CEJ to the free gingival margin; recession width (RW) was measured horizontally at the CEJ; KTW (width of keratinized tissue) was measured from the mucogingival junction to the free gingival margin; clinical attachment level (CAL) was measured from the CEJ to the deepest point of the gingival sulcus; the distance from the CEJ to the mucogingival junction (CEJ-MGJ) was recorded; depth of the gingival sulcus (PD) was measured from the free gingival margin to the deepest point in the gingival sulcus; RA (exposed root surface area) was measured as the area of a triangle that had the recession width as the base and the recession depth as the height of the triangle).

The recession defects coverage ratings were calculated at 3 and 6 months after the surgery and these ratings were referred to as the average percentage of root coverage (ARC), the percentage rate of patients with complete coverage of all recessions (CRC-1) and the percentage rate of complete coverage of recession defects (CRC-2).

All pre- and postoperative measurements were obtained by the same previously calibrated examiner (LG), who was blinded to the treatment assignments.

Surgical protocol

After local anesthesia, the exposed roots were prepared using ultrasonic devices, curettes and finishing burs (fig.1, 2, 3). Then, using 15 C blades, sulcular incisions were made around each tooth to enable access for the suprapariosteal preparation of the buccal flap. The incision was extended to the adjacent tooth on both sides of each recession. The dissection of the entire buccal aspect was performed as a partial-thickness flap using tunneling instruments according to the method described by Blanes and Allen (21). At this point, all the buccal tissues were undermined and connected. The papillae were fully detached, and the periosteum was performed as a full-thickness flap, which was the authors' modification of the original method. This was performed with a small elevator to minimize trauma. The xenogeneic collagen matrix had been rehydrated for 15 min in saline and was then placed into the tunnel. Then, the matrix was stabilized using 6-0 absorbable suspensory sutures.

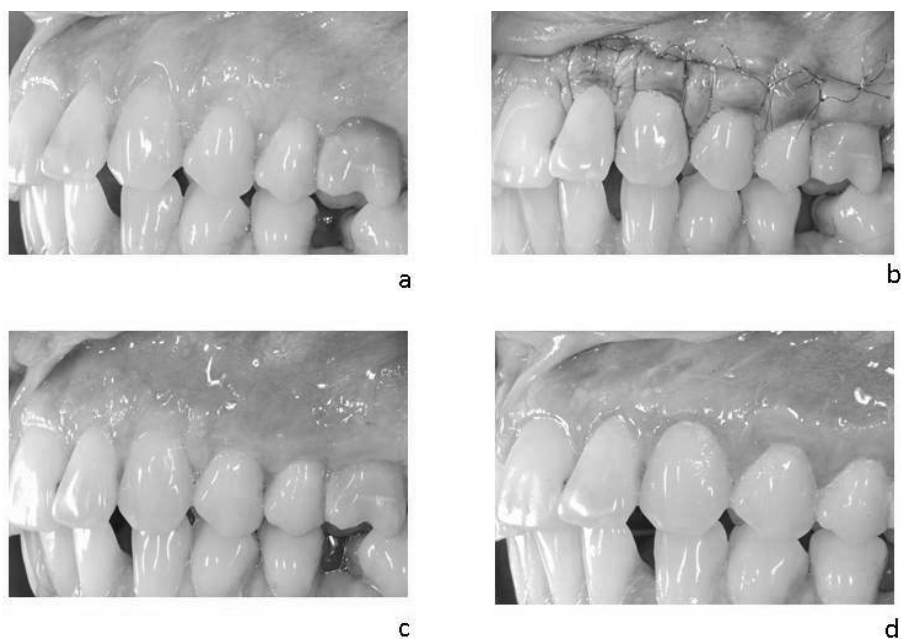


Fig. 1. *a):* recessions in maxilla before treatment; *b):* day of surgery procedure (suturing technique); *c):* 14 days post surgery (after removal of the sutures); *d):* 6 months post surgery.

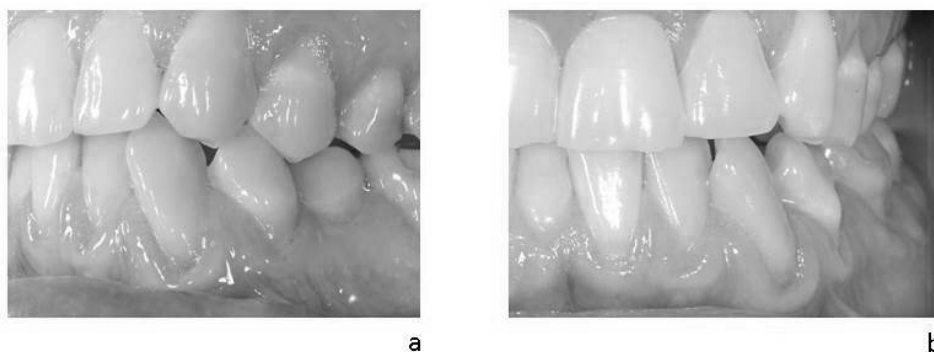


Fig. 2. *a):* recessions in mandible before treatment (front view); *b):* recessions in mandible before treatment (side view).

The mobilized mucous flap was advanced coronally, and the 6-0 non-absorbable suspensory sutures were secured. Healing progression was inspected on days 1, 7, and 14 after surgical procedure. Sutures were removed 2 weeks after the procedure. All patients received instructions for proper home care. The patients did not brush the surgical site for 2 weeks and rinsed twice daily with a 0.12% solution of chlorhexidine gluconate.

They were prescribed antibiotics (Amoxicillin with clavulanic acid, 625 mg every 12 hours for 7 days) because of the implanted xenogeneic material. At all of the postoperative appointments, the surgical areas were

deplaqued and polished. When the sutures were removed, all patients received instructions to clean the teeth in the surgical area using a soft toothbrush and the roll technique. All of the surgical procedures were performed by the same clinician (MCW). At the 3 and 6 month postoperative visits, clinical measurements were recorded again (LG).

Statistical analysis

Shapiro-Wilk tests were used to determine whether the studied parameters had normal distributions. Intragroup differences between measurements of the clinical parameters at the three time points (the initial examination

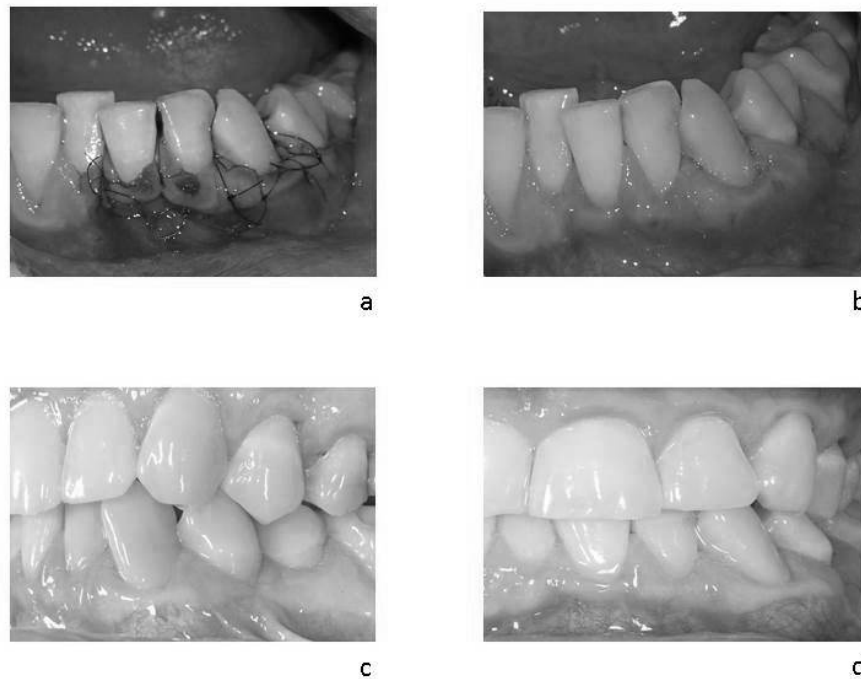


Fig. 3. *a): day of surgery procedure (suturing technique); b): 14 days post surgery (after removal of the sutures); c): 6 months post surgery (front view); d): 6 months post surgery (side view).*

and 3 and 6 months post-surgery) were analyzed by Friedman's Differences and differences in the ARC results between the maxilla-group and the mandible-group at the different time points were tested with Mann-Whitney U tests. The Fisher's exact test was applied to categorical data as a frequency of the percentage rate of patients with complete coverage of all recessions (CRC-1) and the percentage rate of complete coverage of recession defects (CRC-2). A P-value of less than 0.05 was considered to be statistically significant. All findings were analyzed using Statistica software, version 10.

RESULTS

All patients completed the study and attended all follow-up visits. A total of 59 Miller's Class I and II recessions (20 recessions in the mandible-group, 39 in the maxilla-group) were treated in 14 patients using the tunnel technique and a collagen matrix. At 3 and 6 months after the treatment, the following clinical measurements were recorded. The mean depth of the sulcus (PD) was not changed

after surgery in either the mandible or the maxilla group, and there were no statistically significant differences between the treatment groups ($p > 0.05$) at each time point that was observed. Significant decreases were observed in both groups in CAL, RD, RW, RA and CEJ-MGJ at the 3 and 6 month follow-up visits compared to the baseline measurements, and there were no statistically significant differences between the treatment groups ($p > 0.05$) observed in RD, PD, CAL and RA at baseline. Significant ($p < 0.001$) increases in the width of keratinized tissue (KTW) were observed in both groups 3 and 6 months after surgery in comparison to the baseline measurements and there were statistically significant differences between the treatment groups ($p < 0.001$) observed at baseline and 3 and 6 months after surgery. There were no significant differences in CAL between the groups 3 months after surgery and no significant differences in RW or PD 3 and 6 months after surgery.

Statistically significant differences between the treatment groups ($p < 0.001$) were observed in RD,

KTW, RA, and CEJ-MGJ 3 months after surgery and in RD, KTW, RA, CEJ-MGJ, and CAL 6 months post-surgery (Table I). The complete coverage of all recessions in individual patients (CRC-1) was 22.2% (2/9) in the maxilla-group and 0% (0/5) in the mandible-group and the percentage rate of complete coverage of all recession defects (CRC-2) was 79% (n=31/39) in the maxilla-group and 50% (n=10/20) in the mandible-group 3 and 6 months post-surgery. There were no statistically significant differences in CRC-1 and CRC-2 between the groups 3 and 6 months post-surgery. The mean ARC value 3 and 6 months post-surgery was 96.8% in the maxilla-group and 81.3% in the mandible-group. There were statistically significant differences in ARC between the groups during the period of observation (Table II).

DISCUSSION

The use of soft tissue grafts is an accepted and routine procedure in recession coverage surgery because of functional and esthetic factors. However, if a

connective tissue graft is used, palatal-site morbidity can often occur. Many clinicians have reported complications associated with soft tissue grafts from the palate such as excessive hemorrhaging from the donor site, bone exposure, permanent anesthesia and paresthesia, infection, prolonged pain and discomfort, the occurrence of a surgical cyst following CTG and increased chair side for the harvesting graft (27-29). The use of a xenogeneic collagen matrix completely eliminates the issue of donor area morbidity. The use of Mucoderm with the tunnel technique is a new approach that allows for the coverage of multiple recessions.

Mucoderm is a collagen tissue monolayered matrix with a rough and open-porous structure that guides blood vessels and soft-tissue cells to promote rapid vascularization and soft tissue integration (30). The tunnel technique is a bilaminar method that has predictable results in the achievement of multiple recession coverage and has early initial healing, an enhanced blood supply and good esthetic results. An advantage of the coronal positioning of the tissue margin in the tunneling procedure is that the risk of collagen matrix exposure is decreased, and the possibility of complete coverage of the recessions is increased. However, this method requires a specific suturing technique (31, 32). Pini Prato et al. (33) and Nieri et al. (34) concluded that a complete coverage of the recessions can only be achieved if the margin of the gums is displaced coronally in relation to the cement-enamel junction.

It should be noted that the current study included multiple recessions located on the mandibular teeth and molars, which may increase surgical difficulty and the risk of failure due to anatomical conditions. Therefore, the comparison with recessions localized only in the upper jaw without molars is inadequate. To our knowledge, no study has yet been published comparing the outcomes of the coverage of multiple recessions in the maxilla and mandible using the tunnel technique and CM. Therefore, comparing our study with other studies is difficult.

The results of the current study confirm that this method was effective in reducing the following clinical parameters: RD, RW, CAL, RA, and CEJ-MGJ by 3 and 6 months after surgery in comparison to

Parameters	Maxilla M	Maxilla SD	Mandible M	Mandible SD	*p-value
RD 0	3,13	0,74	2,88	0,84	p>0.05
RW 0	3,92	0,65	2,98	0,9	p<0.001
PD 0	1	0	1	0	p>0.05
KWT 0	3,78	0,62	0,36	0,37	p<0.001
CAL 0	4,13	0,74	3,88	0,84	p>0.05
RA 0	6,61	2,47	4,98	3,54	p>0.05
CEJ-MGJ 0	6,92	0,82	3,25	1,15	p<0.001
RD 3	0,21	0,16	0,73	0,31	p<0.001
RW 3	0,5	0,45	1,12	0,63	p>0.05
PD 3	1	0	0,94	0,13	p>0.05
KWT 3	4,44	0,54	1,59	0,5	p<0.001
CAL 3	1,21	0,16	1,51	0,35	p>0.05
RA 3	0,29	0,28	0,93	0,62	p<0.001
CEJ-MGJ 3	4,71	0,63	2,41	0,53	p<0.001
RD 6	0,21	0,16	0,73	0,31	p<0.001
RW 6	0,5	0,45	1,12	0,63	p>0.05
PD 6	1	0	1	0	p>0.05
KWT 6	4,44	0,54	1,54	0,5	p<0.001
CAL 6	1,21	0,16	1,73	0,31	p<0.001
RA 6	0,29	0,28	0,93	0,62	p<0.001
CEJ-MGJ 6	4,71	0,63	2,36	0,51	p<0.001

Table I. Comparison of clinical parameters at 3 and 6 months post-surgically in maxilla an mandible. *: Test U-Mann-Whitney'a.

Parameters	Maxilla	Mandible	p-value
ARC 3 months post surgery	96.8%	81.3%	*p<0.001
ARC 6 months post surgery	96.8%	81.3%	*p<0.001
CRC-1 3 months post surgery	22.2%	0%	**p>0,05
CRC-1 6 months post surgery	22.2%	0%	**p>0,05
CRC-2 3 months post surgery	79%	50%	**p>0,05
CRC-2 6 months post surgery	79%	50%	**p>0,05

Table II. Comparison of parameters ARC and CRC in maxilla and mandible at 3 and 6 months post-surgery. *:Test U-Mann-Whitney'a; **:Test U Fisher.

the baseline measurements in both groups. Chaparro et al. (35) obtained similar clinical outcomes in RD after 12 months of observation after using the tunnel technique with AMD.

The coverage of maxilla recessions was higher in our study for the ARC, CRC-1 and CRC-2 parameters than that of mandible recessions. Three and six months after the surgical procedures, ARC was 96.8% in the maxilla group and 81.3% in the mandible group, while CRC-2 was 79% (n=31/39) in the maxilla group and 50% (n=10/20) in the mandible group. In comparison to our study, Chaparro et al. had a lower percentage rate of complete coverage of recession defects (CRC-2) obtained for maxilla recessions 67.9% (n=36/53) but a similar percentage rate for mandible recessions 52.5% (n=21/40).

Although the mean area of root coverage (96.8% in the maxilla group and 81.3% in the mandible group) are satisfactory for both groups in the current study, the number of patients with complete coverage of all recessions (CRC-1) was low and unsatisfactorily (maxilla n=2/9 and mandible n=0/5). There were no statistically significant differences in CRC-1 and CRC-2 between the groups and statistically significant differences in ARC between the maxilla and mandible groups in the observed period. De Sanctis et al. (36) reported significant recession reductions after treating multiple recessions in mandibular teeth with SCTG+CAF. The mean

coverage 12 months post-surgery in that study was higher (91.2%) and the percentage rate of complete coverage of recession defects was similar (50%) to the values in the current study. The number of patients with complete coverage of all recessions (CRC-1) was low and similar to those in the current study (n=1/10). Carvalho et al. (37) achieved higher coverage of maxillary recessions than we observed in the current study. Papageorgakopoulos et al. (38) had similar results to those in the current study, while Shepherd et al. (39) achieved greater root coverage in the maxilla than in the mandible, which is similar to the current study and the study by Chaparro et al. (35).

Complete root coverage is the ultimate goal when treating recession defects in patients with esthetic and functional demands. Frequently, the most coronal millimeter(s) of root exposure after the surgery procedure is still visible when the patient smiles; therefore, the persistence of shallow recessions after surgery may represent an unsatisfactory outcome for esthetic reasons (40). Complete root coverage is also associated with a greater reduction of hypersensitivity than partial root coverage. Differences between the maxilla and mandible in the percentage of root coverage using the tunnel technique and CM may be due to several factors, including depth and amount of keratinized tissue, complete coverage of the collagen matrix with a tension-free flap, the postoperative level of the flap and adequate blood supply (41, 42). The coverage of the molars is still a challenge because of the difficulty in accessing those teeth, wider mesiodistal cervical contours, and flat interproximal bone morphology with larger buccolingual dimensions (43). The lack of predictability for mandibular recessions may be due to the release of the flap with passive coronal positioning because this area has a larger number of muscles with strong tension in comparison to those in the maxilla (35). In the current study, in treating recession defects of the mandible, it was difficult to achieve a flap without tension.

According to some researchers, factors such as total coverage of the collagen matrix and the degree of flap tension after the surgery are factors for the predictability of the total coverage of the recession.

The advantage of using a collagen matrix in the coverage of recessions is the impact on increasing the width of keratinized gingiva, which is consistent with the observations of Rotundo and Pini-Prato (44). In their 2014 study involving animal material, Pabst et al. showed that the Mucoderm supports metabolic activity and proliferation of fibroblasts (25). In studies investigating the recession defects in the maxilla and the mandible, it has been reported that significantly greater improvements in RD and KWT were observed for maxillary defects treated with CTG and a coronal advanced flap compared with the improvement of recessions in the mandible (45). In the current study, the mean of the width of keratinized tissue (KTW) increased in the maxilla (from $3,78 \pm 0,62$ mm to $4,44 \pm 0,54$) and the mandible (from $0,36 \pm 0,37$ mm to $1,54 \pm 0,50$) at 3 and 6 months post-surgery compared to the baseline measurements and there were statistically significant greater improvements in the maxilla group. It is preferable to achieve a certain amount of attached gingiva for the maintenance of the integrity of the mucogingival junction, but the amount of attached gingiva that is required to minimize the recurrence or progression of recession defects has never been established (46,47). The tunnel technique does have disadvantages. In our study, there were multiple recessions with different recession depths that increased the difficulty of the procedure and the risk of failure compared to recessions that have a similar depth. The limitation of flap mobilization, the coronal advancement and direct access to the operating field especially in the lower arch could have contributed to the lack of complete root coverage in the current study.

CONCLUSION

Surgical treatments using a xenogeneic collagen matrix in combination with the tunnel technique resulted in satisfactory average-recession coverage-rates of multiple Miller's Class I and II recessions in the maxilla and mandible but yielded lower complete recession coverage-rates. Therefore, choosing an appropriate surgical technique for each specific clinical case is important to achieve predictability of root coverage. In the future, studies on coverage

of recessions should be conducted separately in the lower and upper jaw using different treatment techniques and different substitute tissues because the results in the mandible and around the molars often cause decreases in the final success rate because of anatomical conditions. In addition, longer-term studies and histological and gingival thickness examinations would be beneficial.

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THE EFFECT OF GINGIVAL WALL LOCATION ON THE MARGINAL SEAL OF CLASS II RESTORATIONS PREPARED WITH A FLOWABLE BULK-FILL RESIN-BASED COMPOSITE

P. SEGAL^{1*}, V. CANDOTTO^{2*}, A. BEN-AMAR¹, M. EGER¹, S. MATALON¹,
D. LAURITANO³ and Z. ORMIANER¹

¹*Department of Oral Rehabilitation, the Maurice and Gabriela Goldschleger School of Dental Medicine, Tel Aviv University, Tel Aviv, Israel;* ²*Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy;* ³*Department of Medicine and Surgery, University of Milan-Bicocca, Milan, Italy*

*These authors contributed equally to this paper.

SureFil SDR is a flowable resin-based composite that allows a single incremental bulk placement. The marginal seal of SureFil SDR at the gingival margins of class II restorations located apical to the cemento-enamel-junction (CEJ) has not been adequately evaluated compared to those located occlusal to the CEJ. Forty class II cavities were prepared in human molars. The gingival margins of 20 preparations were located 0.5 mm occlusal to the CEJ, and the other 20 preparations were located 0.5 mm apical to the CEJ. The cavities surfaces were bonded with XenoV dental adhesive and filled with SDR in one bulk increment up to 4 mm, after which they were covered with CeramX. The teeth were subjected to thermo- and load-cycling, and their gingival margins were exposed to 0.5% basic-fuchsin solution. The specimens were sectioned mesio-distally and scored for microleakage. A Wilcoxon test for pairwise comparison was performed to determine significance. Dye penetration was observed in 30% of the 20 restorations with cavo-surface margins located occlusal to the CEJ and in 55% of the 20 restorations with cavo-surface margins located apical to the CEJ. The bulk-fill flowable resin base SureFil SDR with XenoV dental adhesive provided a better marginal seal in class II restorations with gingival margins above the CEJ compared to restorations with gingival margins below the CEJ. SDR should not be recommended for class II cavity preparations with gingival margins located below the CEJ.

Tooth-colored restorative materials are increasingly requested by patients and clinicians. Resin composite materials and techniques are constantly being improved with the aim of producing ideal restorative material. Efforts have been undertaken to develop new products with simplified application techniques, reduced polymerization shrinkage, and increased wear resistance (1, 2). The physical properties of esthetic restorative materials, such as polymerization

shrinkage, water sorption, solubility, elastic modulus and shear bond strength, all affect marginal behavior and influence the integrity of the restoration (3-5). Poor adaptation of the restoration to the prepared cavity walls may lead to the formation of gaps (6) and consequently to microleakage, postoperative sensitivity, recurrent caries and, finally, failure of the adhesive resin composite restoration (3).

One of the most crucial physical features of resin

Key words: Bulk-fill, flowable resin-based composite, SureFil SDR, marginal seal

Mailing address:

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

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composite materials is the fact that they undergo different degrees of volumetric shrinkage during polymerization. The amount of volumetric change depends, among other aspects, on the elastic moduli that influences the accumulation of stress build-up in the material during polymerization (4). In order to decrease volumetric shrinkage and to reduce the amount of stress buildup, a meticulous incremental layering technique is mandatory during the application of resin composite restorative materials (5). The need for several applications, however, jeopardizes restoration integrity, and individual light-curing of each layering increases treatment duration, a characteristic that is considered inconvenient for the clinician and patient alike. Another important characteristic of resin composite materials that requires multiple application steps is their resistance to flow, i.e., the Bingham body behavior that displays a time-dependent response known as “viscoelastic and viscoplastic strain” (6, 7). During condensation, the material undergoes viscoelastic recovery, at the end of which some deformations are reversed. Therefore, multiple packing/pushing steps are required to achieve final adaptation of the filling material to the cavity walls (7).

The continuing search for improved restorative materials that would overcome the above features led to the development of flowable resin composites in the 1990s (8). The “flowables”, as they are called, are low viscosity resin composites with 20%-25% less filler loading than the conventional resin composites (9). They possess good wetting ability, which favors their adaptation to the cavity walls with decreased risk for air entrapment and void inclusion (10, 11) and makes it possible to place them by injection with syringes. The first-generation of flowable resin composites was used only as liners and fissure sealant due to their inferior mechanical properties, low elastic modulus and increased volumetric shrinkage (9, 11, 12). The latest generations of flowable resin composites, however, have higher filler content and are claimed to have increased mechanical properties (13), and so they have been proposed for use in one increment bulk up to a 4-mm thickness (14-17). One of these new flowable resin composites is SureFil SDR® (Smart Dentin Replacement) (DENTSPLY, Konstanz,

Germany). SureFil SDR was introduced into the market as a flowable resin composite with the claim that it would allow a 4-mm bulk placement in one layer. This new product is a one-component, fluoride-containing, visible blue light-cured, radiopaque resin composite restorative material. It was designed to be used as a base in class I and class II restorations, and it must be overlaid with a methacrylate-based universal/posterior composite. The information on the chemical composition of flowable resin, provided by the manufacturer, indicates that the polymerization kinetics are better controlled than they are with other resins due to an organic matrix that consists of a patented urethane dimethacrylate with incorporated photoactive groups and to an enhanced translucency that allows light passage to the inner layers of the applied material.

Studies that analyzed the micromechanical properties and the shrinkage behavior of this new material described a high modulus of elasticity and a low shrinkage stress buildup after polymerization. It has minimal internal polymerization stresses due to a longer pre-gel phase, which is accomplished by the use of a “polymerization modulator” that interacts with camphoroquinone to reduce the contraction modulus and increase the number of linear bonds (14-18). These attractive properties are expected to provide good marginal adaptation, with reduced gap formation at the cavo-surface margin, thereby leading to an excellent marginal seal when using SDR restorative material.

The objective of the present study was to assess the marginal sealing of class II restorations done with SureFil SDR and covered with high filler-containing resin composite, and to compare the marginal seal at the gingival wall located above the cemento-enamel-junction (CEJ) to that located below the CEJ.

The tested null hypotheses were: (1) there will be penetration of the dye in class II restorations prepared with SDR flowable material, and (2) penetration of the dye would be greater in class II restorations with gingival walls below the CEJ.

MATERIALS AND METHODS

This study was carried out at the laboratory of Dental

Materials, Faculty of Dentistry, Tel-Aviv University, Israel and with the approval of the Tel Aviv Sourasky Medical Center Institutional Review Board (Helsinki Committee). Twenty caries-free human molars freshly extracted for periodontal reasons were randomly assigned to the study groups (Fig. 1). They were cleaned with an ultrasonic device and stored in tap water at 4°C prior to use. Forty class II cavities were prepared using a no. 330 tungsten-carbide burr (S.S.W. London, UK), with a high-speed hand piece (8000B GENTLEsilence LUX, KaVo Dental GmbH, Biberach, Germany) and an air-water spray. Two class II independent cavities, i.e., occluso-mesial and occluso-distal, were prepared for each tooth. The gingival margin of the preparation on one side was located 0.5 mm above the CEJ (enamel margins), and the gingival margin of the other preparation on the other side of the same tooth was located 0.5 mm below the CEJ (dentin/cement margins). The study was designed so that one side of the tooth served as the control of the other side. There was a total of 20 preparations with gingival margins located above the CEJ and 20 preparations with gingival margins located below the CEJ. The preparations measured 3 mm buccolingually at the occlusal surface and 3.5 mm at the gingival surface. All the preparations and restorations were made by the same operator. The prepared teeth were surrounded with Toffelmire steel matrix bands to prevent cavity overfill. They were cleaned with a cavity cleanser, chlorhexidinedigluconate 2% (BISCO Corp. Schaumburg IL, USA), prior to restoration. Excess water was removed

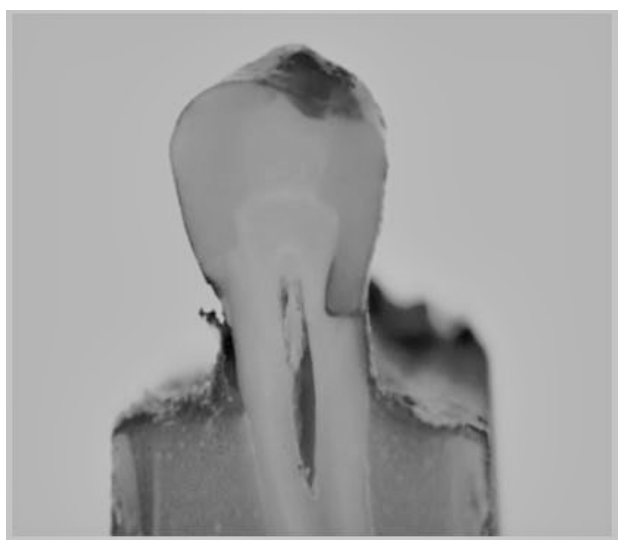


Fig. 1. Section of tooth preparation.

with a triple-action syringe. A one-component, self-etching dental adhesive, XenoV (Dentsplay De Tray, Konstanz, Germany), was applied uniformly with a small applicator and agitated for 20 sec to ensure sufficient wetting of all cavity surfaces. The solvent was evaporated by gentle blowing of air by means of an air-water syringe, until no movement of the adhesive was observed, and then light-cured for 20 sec.

The gingival box of the cavity was filled with the SDR flowable composite in one bulk increment up to 4 mm, using slow and steady pressure. Dispensation began at the deepest portion of the cavity, while the tip was maintained close to the cavity floor and then gradually withdrawn as the cavity became filled. Curing followed after a few sec to allow the material to level itself. The material was then cured for 20 sec using a conventional one-level output method. The residual height of the cavity was filled with 2-mm oblique incremental layers of shade-D3, the Ceram X nano ceramic. Each layer was cured for 40 sec, according to the manufacturer's instructions. The dentin-bonding adhesive and the resin composite materials were polymerized using an ESPE Elipar TriLight unit (ESPE, Seefeld, Germany). To ensure a consistent light output of 650 mW/cm², the light was checked periodically during the experiment using a Demetron light-emitting diode and a halogen radiometer (Kerr Corporation, CA, USA).

Following removal of the matrix band, curing continued for an additional 20 sec for both sides of the restoration, i.e., bucco-proximal and linguo-proximal. Visible overhangs were removed using a no. 15 scalpel blade (Swann-Morton, UK). The proximal margins were immediately finished with flexible disks (SofLex Pop-on, 3M-ESPE, St. Paul, MN, USA), starting with the roughest flexible disk and finishing with the gentlest flexible disk, using a low-speed handpiece with air-water spray. No pressure was applied to the restorations. All the restored teeth were stored in distilled water at 37°C for 7 days and then subjected to thermo-cycling, i.e., thermal stress in water baths of 4°C and 55°C with 1,000 cycles of 10 sec in each bath and a dwelling time of 10 sec between baths using a TC-2000 automatic device (Y. Manes, Tel-Aviv, Israel). The root apices were sealed with Duralay (Reliance Dental Mfg. Co., IL, USA). All of the other surfaces, except for the restoration and 1-2 mm of the cavo-surface margins, were covered with two layers of nail varnish to prevent dye penetration. The teeth were embedded in

auto-polymerizing acrylic material within a metal cylinder up to 2 mm apical to the restoration's margins and subjected to an occlusal load of 100 N for 1,000 cycles using an Ami III Stress Cycling Machine (Y. Manes, Tel-Aviv, Israel). The load was pressed against the occlusal surface of the restoration during 1000 cycles, 0.5 seconds each. The teeth were submerged in 0.5% basic fuchsin solution at 37°C for 7 days and then rinsed under running tap water, carefully dried, and sectioned mesio-distally on their longitudinal axes into three sections using an ISOMET Plus low-speed saw (Buehler Ltd., IL, USA) (19). The sections were examined under a light-reflecting stereomicroscope at x18 magnification and by 2 independent examiners to assess dye penetration at the restoration margins (19). The depth of dye penetration at the gingival margins, if any, was assessed on the monitor attached to the microscope. All of the measurements were categorized according to the following scale:

0 = No dye penetration (no leakage);

1 = Dye penetration up to 1/3 of the gingival wall from the cavity margins

2 = Dye penetration up to 2/3 of the gingival wall from the cavity margins

3 = Dye penetration up to the axial wall of the cavity margins

4 = Dye penetration along the axial wall into the tubules

The section with the highest dye penetration score was chosen for statistical analysis (Fig. 2). Two independent examiners evaluated all of the samples. Discrepancies between the two examiners were rare. In the event of a discrepancy, the section was reexamined by both examiners, and the higher score was always chosen. A non-parametric test (Wilcoxon's matched-pair signed-ranks test) for pairwise comparisons was performed to demonstrate significant differences ($P=0.005$) since the two study groups (enamel or dentin margins) did not exhibit a normal data distribution (Kolmogorov-Smirnov test).

RESULTS

Over one-half (56.5%) of all 40 restorations had no dye penetration (score 0). Of them, 65.5% were above the CEJ, and 25% were below the CEJ. Out of all of the restorations, 12.5% were scored 4, and they were all below the CEJ (Table I). Seventy percent of all the restorations above the CEJ had no dye penetration whatsoever and 30% had a dye penetration score between 1 and 3. No restorations had a dye penetration score of 4 (Fig. 2). Out of all the restorations below the CEJ, 45% had no dye penetration, 25% had a dye penetration score of 4 and 30% had a dye penetration score between 1 and 3 (Fig. 2).

There was a significant difference in the dye penetration for the two restorations with gingival margins located above and below the CEJ for the same tooth ($P=0.005$). All the teeth but one showed the same or more dye penetration in the restorations below the CEJ compared to the restorations above CEJ for the same tooth (Tables II).

DISCUSSION

The present study evaluated the marginal seal of

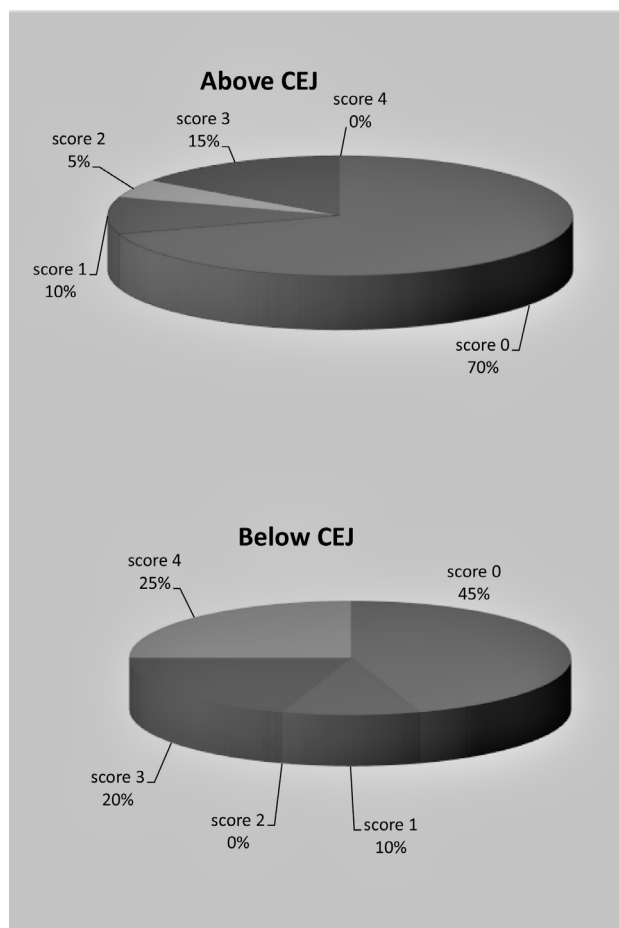


Fig. 2. Dye penetration. Below the cemento-enamel-junction (CEJ): score = 4 (right); Above the CEJ: score = 1 (left).

Table I. *Distribution of dye penetration.*

Dye penetration scale along the gingival wall	0	1	2	3	4
Restorations with gingival wall above the CEJ (No.)	14	2	1	3	0
Restorations with gingival wall below the CEJ (No.)	9	2	0	4	5

CEJ: cemento-enamel-junction. **0:** No dye penetration (no leakage); **1:** Dye penetration up to 1/3 of the gingival wall from the cavity margins; **2:** Dye penetration up to 2/3 of the gingival wall from the cavity margins; **3:** Dye penetration up to the axial wall of the cavity margins; **4:** Dye penetration along the axial wall into the tubules.

Table II. *Comparison of dye penetration for both sides of the same tooth.*

Tooth No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Above CEJ	2	0	0	3	0	1	0	0	0	0	0	0	3	0	0	1	0	0	3	0
Below CEJ*	4	0	3	4	0	0	0	0	0	3	0	1	4	0	1	3	3	0	4	4

CEJ: cemento-enamel-junction. *: The same or more dye penetration was found in the restorations below the CEJ in all but one tooth.

SureFil SDR by dye penetration in class II restorations with a gingival wall placement above or below the CEJ. The cavities were restored with a bulk-fill flowable composite, SureFil SDR and covered with a more wear-resistant resin composite, Ceram X. The restored teeth were subjected to thermo-cycling and occlusal loading cycling, which simulate months of daily use.

Marginal seal is one of the key factors for long-lasting restorations. Previous studies had evaluated the marginal integrity of standardized class II cavities, which were incrementally filled with resin-bonded composites with or without bulk-fill flowable resin base and after thermo-mechanical loading. Those investigations found no difference in the cervical microleakage scores between the groups when the gingival walls were located above the CEJ (14-17). Roggendorf et al. (17) also found that SDR had no advantages over conventional posterior resin composites in reducing marginal microleakage in class II restorations with gingival walls below the CEJ. The degradation of the marginal seal quality was further exacerbated after thermo-mechanical loading (17).

Another study (20) that examined the microleakage of Class-V restorations, carried out with three restorative materials on both dentin and enamel substrates, also reported that all groups showed dye penetration but, in opposition to the finding of Roggendorf et al. (17) study, those authors found that the SDR group were advantaged as they had obtained a better seal, especially in the dentin margins. They also reported that there was less dye penetration on the enamel for all the restorative material (20).

In the present study, overall poor margin sealing performance was documented in 30% and 55% of the enamel and dentin margins, respectively, which were leaking to some degree at the gingival floor interface. A comparison of the performance of SDR below the CEJ and above the CEJ of the same tooth revealed dye penetration of different depths, regardless of gingival wall placement, in 17 of 40 cases (representing 42.5% of the studied restorations). There had been no dye penetration in 70% of the restorations above the CEJ, and in only 45% of the restorations below the CEJ.

With regard to the marginal seal on both sides of each tooth, all of the restorations above the CEJ consistently showed less or the same extent of dye penetration relative to those below the CEJ, with only one exception. The marginal seal quality of the enamel/SDR interface was much better than that of the dentin/cement/SDR interface ($P=0.005$), but still not good enough.

The finding that inadequate marginal seals were obtained with SDR restorations even when the cavo-surface had been located on the enamel may be explained by the Roggendorf et al. (17) report of para-marginal enamel fractures occurring slightly more frequently with SDR restorations. Other studies that analyzed the shrinkage behavior of SDR concluded that the experimental flowable material showed the lowest shrinkage-stress-rate but was also more rigid (higher modulus of elasticity) and more plastic than the regular flowable materials, thus making its effect on interfacial stress build-up difficult to predict (14, 16).

On the other hand, Ilie and Hickel's study on the bulk-fill SDR flowable material showed different behavior when compared to other flowable composites and to nano- and micro-hybrid composites. Those authors demonstrated a low shrinkage-stress rate after polymerization, a high modulus of elasticity and high creep values (16). Van Ende et al. (15) evaluated SDR's bond strength to dentin in different C-factor cavities. They found that bulk-fill flowable base SDR can be cured in bulk without harming the bond to cavity-bottom dentin, in addition to having good adaptation to cavity walls (15). Those features are regarded as being optimal for cavity adaptation and sealing. However, the marginal seal was less than optimal in both of the present study groups and there was greater dye penetration in the group with marginal walls below the CEJ. The difference between the two groups can be explained by the importance of the remaining enamel in class II preparations above the CEJ in providing improved and more reliable micromechanical adhesion between the tooth and the flowable composite SDR (21).

The bonding of resin to enamel is largely micromechanical and relies on the preparation of a high-energy etched surface that can be wetted easily by low-viscosity bonding resins to produce micromechanical resin tags (21). The bonding of resin to dentin involves a similar procedure, although dentine

substrate parameters vary greatly from the enamel in the organic composition and water content. Dentin surface preparation is critical for creating a "hybrid zone" of demineralized dentin into which a hydrophilic monomer can penetrate and interlock with exposed collagen to provide another form of micromechanical retention (22). The hybrid layer is the resulting resin-infiltrated surface layer in dentin.

A good adhesive should prevent leakage along the restoration margins and provide a long-lasting marginal seal. Inadequate surface preparation and inadequate surface wetting are major factors associated with bonding failure. In the present study, the self-etching dental adhesive material, XenoV, was used according to the manufacturer's recommendations. An earlier study that compared the adhesive performance of several adhesive materials used with SDR did not observe any incompatibility between other adhesives and the SDR flowable resin composite (18). It was also confirmed that the use of a total etch technique provided a better marginal seal (23-25). On the other hand, Roggendorf et al. (17) reported that para-marginal fractures occurred slightly more frequently in SDR restorations when an etch-and-rinse bonding technique was used in comparison to a self-etching technique. Mazzoni et al. (26) found that there are no differences in the activities of MMP-2 and MMP-9 after treatment with different etch-and-rinse or self-etch adhesives, including XenoV. They also noted that MMPs have been considered as having a major role in the degradation of poorly resin-infiltrated hybrid layers. We chose XenoV, a self-etch adhesive for the present study, based on those findings and on the facts that self-etch bonding is less sensitive to the technique applied, that it is easy to work with, and that it may be more compatible with dentin.

Cavity preparation is another important parameter. The current study was designed to evaluate the marginal seals of non-beveled class II preparations. A non-beveled protocol is quite common for studies that primarily investigate clinical bond strengths to dentin, under the controversial premise that beveling the enamel margins increases the surface area and may increase the marginal seal (26, 27) and fracture resistance (28), while reducing the occurrence of micro-cracks (29). Therefore, restorations placed on beveled enamel in class II preparations have the potential to exhibit even

higher marginal seals than that those shown in the present study.

The main advantages of SDR bulk-fill flowable resin are its low shrinkage on polymerization (14, 30), its self-leveling characteristic and its stress-relieving properties during polymerization, that would allow the placement of 4-mm bulk increments and the use of light-curing without requiring multiple packing or pushing steps. These physical properties have clinical value. They increase the efficiency, simplify the restorative technique and decrease operating time. Nonetheless, SDR bulk-fill flowable resin bonded with XenoV did not yield a perfect gingival seal in the current study.

CONCLUSION

Within the limitations of the present *in vitro* study, the following conclusion can be drawn about bulk-fill flowable resin composite Surefil SDR. It provides poor sealing of the gingival margins located below the CEJ in class II restorations compared to gingival margins above the CEJ. Further studies are needed in order to clarify the high rate of dye penetration associated with its use.

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REHABILITATIONS WITH IMMEDIATE LOADING OF ONE-PIECE IMPLANTS STABILIZED WITH INTRAORAL WELDING

M. E. PASQUALINI^{1*}, D. LAURITANO^{2*}, F. ROSSI³, L. DAL CARLO⁴, M. SHULMAN⁵, F. MEYNARDI⁶, D. COLOMBO⁷, P. MANENTI⁸, G. COMOLA⁹ and P. ZAMPETTI¹⁰

¹MD DDS Private practice Milano, Italy; ²Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy; ³MD DDS Private Practice Busto Arsizio, Italy ⁴DDS Private Practice Venezia Italy; ⁵Private practice, Clifton, NJ. USA; ⁶Private Practice Mondovì Italy; ⁷Private Practice Como, Italy; ⁸Private Practice Bergamo, Italy; ⁹Università Alfonso X El Sabio Madrid, Spagna; ¹⁰Università di Pavia, Italy

*These authors equally contributed to this paper.

The authors present an implant prosthesis procedure that uses screws on one-piece implants connected with a titanium pin at their abutment level and one supporter titanium bar in order to guarantee immediate stabilization. These can be implanted and fitted with customized temporary crowns in a single surgical procedure, restoring function and aesthetics and consenting recovery of the bone deficit with reduced healing times and limited patient discomfort. One-piece wide-diameter titanium screw implants with thread measurements of 2.1 and 2.6 mm (smaller diameter) up to diameter of 4.5 mm with one abutment of 2.0 and 2.5 mm respectively, were positioned and splinted by intraoral welding. One-piece titanium implants were used together with a pin (needle) titanium implant as supporting structure to achieve deep stabilization. The Scialom-like pin has a diameter of 1.2 mm and it is long enough to reach deep cortical bone that is “bicorticalism”. The One-piece implant is tightly connected to the needle implant by means of Mondani intra-oral welding technique. In severely atrophic anterior maxilla, the use of this method allows the immediate loading of a fixed resin prosthesis soon after surgery. These implants yielded satisfactory functional and aesthetic outcome in bone-deficient upper anterior sectors, without invasive regenerative procedures. The low invasiveness of this approach also consents rapid healing, reduced biological burden and greater patient benefit.

International conferences have stated procedures and techniques for immediate loading based on scientific and clinical evidence and considering various edentulous anatomic forms, type of bone, length and structure of the implants used. Clinicians from the study group of Italian Immediate Loading School have a long and successful experience with immediate loading in implants connected together by intraoral welding. Intraoral welding can join and

stabilize implants by the use of a titanium wire that is permanently connected to the implants or can be connected in a single one-piece implant stabilized with one titanium pin (needle).

Welding procedure is performed intraorally before immediate loading. Stabilization and fixation of the implants allows immediate loading by means of provisional prosthesis occurring the same day of surgery. By inserting a prosthesis with adequate

Key words: one-piece implant, pin implant, narrow-diameter screw, intraoral welding, immediate loading

Mailing address:

Dr Marco Pasqualini,
Galleria Strasburgo 3,
20121 Milano, Italy
Tel.: +39. 02799651
e-mail: dott.marcopasqualini@tiscali.it

retention and stability on the same day as surgery, complaints and discomfort can be avoided or substantially reduced. The instantaneous stability that results from the splinting can reduce the risk of failure during the healing period.

The intraoral welding was presented in 1978 at the Congress of Implantology and Maxillofacial Surgery held in Ortisei (Bolzano, Italy), this “machine”, invented by Pier Luigi Mondani sparked enormous interest. Initially, however, no one except the inventor dared to use an electric device that had to reach a melting point of 1678° C in order to welder the ends of metallic artifacts placed in the bone directly in the patient’s mouth (1-7).

Mondani’s technique was later employed at the Specialization School of Dentistry of the University of Modena, where it was tested and certified as safe, as it does not harm the tissues with which it comes in contact.

Mondani’s intraoral welding is still an essential tool for professionals who exploit all implant techniques to rehabilitate most cases of edentulous ridges (1, 8-11).

MATERIALS AND METHODS

The “biocompatibility” of the welding, which occurs at 1678°C on the protruding and closely set portion of the needles placed in living tissue, is because the electric current needs a working time of just 2-3 milliseconds. This micro-time, combined with the calculated pressure of the electrodes on the structures to be soldered, prevents the diffusion of the tremendous heat gradient beyond the welding point. Apart from being a bad conductor, titanium also has low thermal conductivity very similar to that of enamel.

While industrial welders melt titanium only in the presence of argon and in the absence of atmospheric oxygen, Mondani’s small solder welds the titanium in the presence of air, blood, mouth saliva or even underwater. To summarize, spot welding involves three stages.

1) Current passes through the welding circuit at a voltage proportional to required energy, according to the formula $E=V^2 \times C/2$, where E is energy, V is the

voltage of the loaded capacitor, and C is the electrical value of the capacitor. The current flows through the needles, heating and welding them together;

2) The welding is aided and facilitated by the constant pressure exerted by the pliers on the surfaces to be welded;

3) The welding time (2-3 milliseconds) and the timing distribution of the two foregoing parameters create welding cycles that are repeated at each spot.

Mondani’s intraoral solder also makes it possible to:

1) connect and splint the various tripodial (or quadripodial) abutments or the single needles of a whole arch using bars, forming a single block that is very resistant to the occlusal stress of temporary or definitive prostheses, which can be placed on it immediately;

2) splint other types of implants for both permanent and temporary stabilization during integrating osteogenesis;

3) remedy possible stress fractures of the needles or any other type of titanium implant (screws, blades, etc.) by welding a new abutment directly in the mouth;

4) increase the stability of different types of implants by soldering one or two balancing needles, thus distributing the load to the cortical bone (12).

Regarding the technical knowledge required to correctly perform intraoral soldering, the physical difference between fusion, soldering and syncrystallization has no practical consequence.

We believe that this brief overview of Mondani’s intraoral welding can suffice to present it as a remarkable step forward in the evolution of implantology (13-16).

“Deep balancing” of the implant. Surgical protocol

To improve resistance to static and dynamic occlusal stress, one-step implants can also exploit the supporting structures that, joined together, are able to distribute stress across a broader surface.

A natural example can be found in the morphology of molar roots, which are designed to bear most of the static stress of mastication and deglutition. In addition to being in a region of compact bone, these teeth also distribute the load across areas that are

broader than their occlusal surface (17).

Deep balancing of the implant is achieved by infixing a needle that diverges with respect to the axis of the main implant, which must reach and penetrate the cortical bone deeply and be soldered to the screw at the point where it emerges from the bone.

Titanium pin (Scialom) with diameters of 1.1, 1.2 and 1.3 mm are used as the supporting structures of screw implants, quick screws and especially MUM mini-implants, which are progenitor of all the small-diameter screws (Fig. 1). They must be placed so as to diverge in respect to the screw and must be pushed deeply until they touch and penetrate the cortical bone. They must then be soldered to the screw at the point where they emerge from the bone, using Mondani's intraoral welding: this is the only way that they can exert their stabilizing effect. In addition to increasing resistance to static and dynamic stress, deep balancing also exerts a counter clockwise action and provides great and immediate primary stability due to the cortical support. This prevents dangerous micromovements of more than 150 microns, as they are particularly harmful and can potentially inhibit osseointegration of the implants (18). Deep balancing is particularly useful for all single-tooth implants (including post-extractive ones and those placed in poorly mineralized bone) that will be loaded immediately. For example, we can cite post-extractive implants placed in shallow

sockets, in which it is virtually impossible to achieve immediate primary stability. Thus, they cannot be loaded immediately without deep balancing (19-22).

Even when dealing with particularly thin ridges, when augment surgery is impossible or undesirable, MUM mini-implants (with a smaller diameter of 2.1 and 2.6 mm) are feasible. These implants are particularly effective because they are placed between the two close cortical walls (buccal and palatal or lingual) and as a rule, the deep cortical bone supports them. This represents the ideal bone for implants with very small core diameters, which can withstand static and dynamic masticatory stress for years without undergoing stress fractures (12, 23, 24), as long as an adequate number is inserted and protocol is followed (25).

In all of these cases, deep balancing (i.e. stabilization by means of titanium needles) ensures increased resistance that can withstand the test of time. The front sector of the mandible is composed of very compact bone. The screws placed into this type of bone may sometimes be expelled due to necrosis, as the tissue is overly compressed by the screwing action and suffers reduced venous and lymphatic flow. In these cases, it is advisable to prepare osteotomies larger than the implant cores in order to avoid compression.

Deep balancing of screws inserted in tunnels wider than their core (to avoid ischemia of the walls)

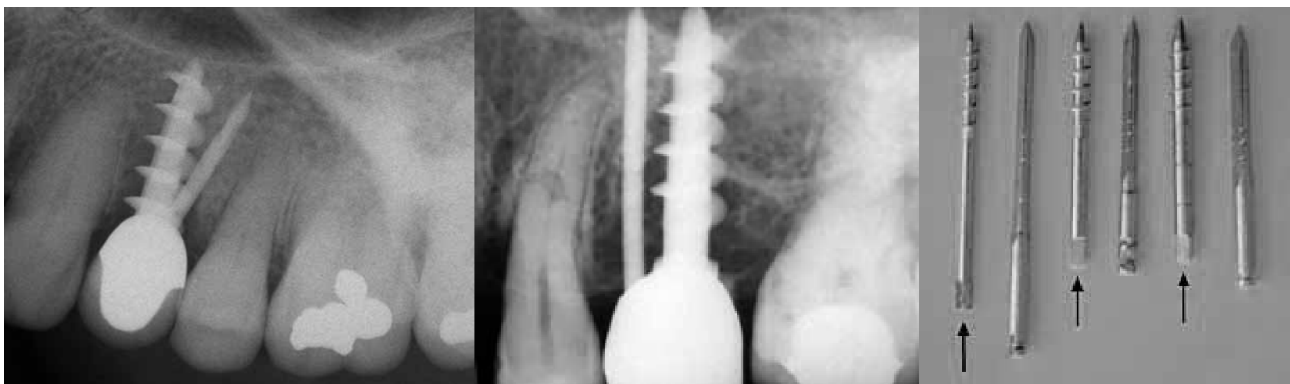


Fig. 1. *Left: Example of deep balancing of a screw with a diverging needle replacing uppers premolars; Right: Three MUM implants with different diameters (arrows) and the Pasqualini drills used for their placement.*

ensures the implant stability required to achieve osseointegration even with immediate loading. This assures:

- an antirotational function
- immediate loading
- primary stability
- greater resistance to static and dynamic stress.

The screw exploits deep balancing when it is used as a double substitute implant for multi-rooted teeth. Deep balancing can be obtained by soldering one or two diverging needles to the abutment, or by joining the abutments of two adjacent screws to form a single block. The figures of the case report exhaustively illustrate the surgical technique as well as the long-term outcomes of this implant method, which is specifically designed for immediate loading (26-28).

Clinical case report

Rehabilitation with immediately loaded implants in the completely edentulous upper maxilla of a 46-year-old male patient (1996). Bone loss in the front

area is evident in both height and width. Implants were inserted according to the protocol of Italian Immediate Loading Implant School, with immediate placement of a temporary resin prosthesis that had the correct vertical height and was occlusally balanced. Due to the minimal bone thickness, implants with a smaller diameter were placed (MUM mini-implants) in the front sector; the thread diameter ranged from 2.1 to 2.6 mm. A bicorticalized needle was inserted besides the mini-implants for reinforcing purposes. It was placed in the bone with a slightly divergent axis with respect to the main implant, but parallel to the abutment emerging into the oral cavity (for prosthetic needs); it was then welded with Mondani's intraoral solder, thus achieving deep balancing. In addition, two implants placed in the right and left maxillary tubers, respectively, made it possible to bypass the large maxillary sinuses (29).

The exceptional stability of the implant permitted good healing of the soft tissues; the definitive gold-ceramic prosthesis was placed after 3 months. The panoramic X-ray (dated 1996) demonstrates the good



Fig. 2. The number of implants placed in a single session should correspond as closely as possible to the number of missing teeth (Step 1 of the protocol). All implants must reach and penetrate the deep cortical bone to achieve the bicorticalization that determines primary stability (Step 2 of the protocol). Immediate splinting is performed with a titanium supporting bar with a diameter of 1.2 mm, set on the mucosa without any compression and welded to each implant by means of the intraoral solder (Step 3 of the protocol). The supporting bar must be placed so that the emerging pre-prosthetic abutments of the implants is free in the oral cavity. A resin provisional restoration is immediately applied during the same session. It has a correct height and is occlusally balanced, without interfering with static and dynamic equilibrium (Step 4 of the protocol).



Fig. 3. *Left:* Correct positioning of the supporting bar and abutments before cementation of the definitive prosthesis and definitive gold-porcelain prosthesis that respects oral physiology, without compressing the soft tissues (1996); *Right:* X-ray of the finished case. Reparative osteogenesis is complete (90 days). These data are particularly interesting because they demonstrate that immediate loading on very stable implants (as per protocol) not only fails to cause bone resorption. Note the spaces between the crowns. Good oral hygiene can be maintained even when there is a titanium supporting bar.

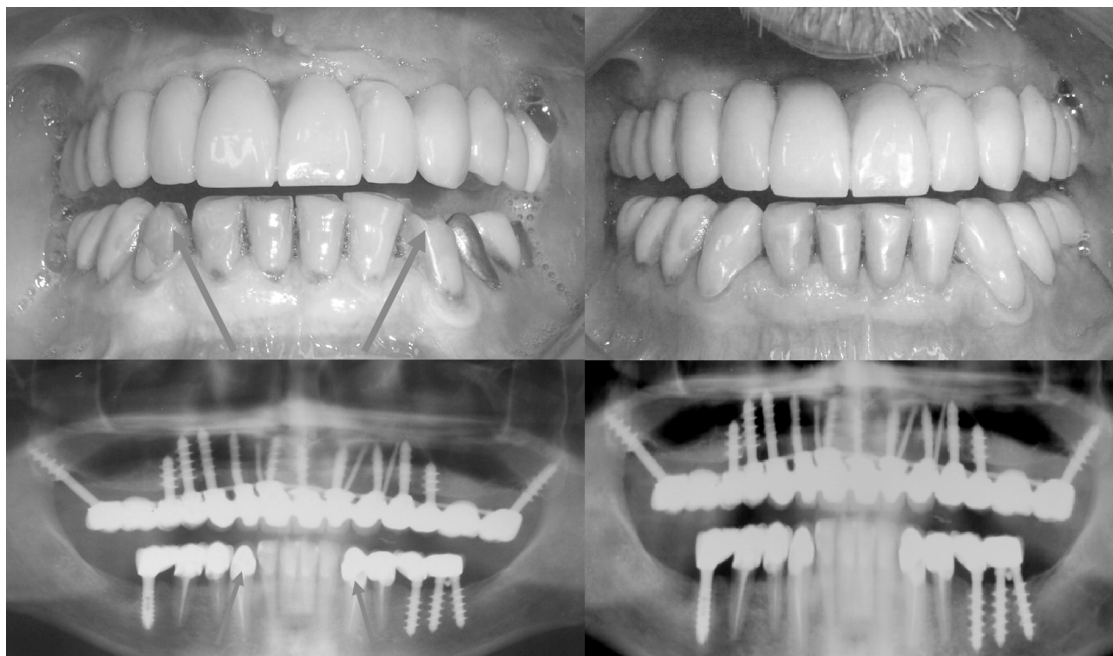


Fig. 4. *Left:* Follow up to 11 years (2007) note the partial destruction of the lower canines. Orthopantomography (2007) shows the prosthetic reconstruction to allow proper canine disclusion; *Right:* Follow-up (2014) also shows two new crowns on the lower canines and the OPT taken 18 years later (2014) shows the good condition of the bone around all of the implants.

status of the bone tissue, with no signs of resorption cones around the implant (Fig. 2). Fig. 3 shows the palatal image of the supporting bar. The prosthetic crowns rest on the bar without covering it. These conditions clearly facilitate correct oral hygiene. The picture taken at the eighteen-year follow-up (2014) also shows two new crowns on the lower canines (arrows). The OPG X-ray, also taken 18 years later, confirms healthy bone conditions with no signs of lesions (Fig. 4).

Scrupulous periodic checks of the occlusion and of oral hygiene conditions are imperative in such cases (30-38).

CONCLUSIONS

Colleagues that employ the intraoral solder should apply deep balancing to screw implants in cases similar to those presented here and in many other clinical situations. This makes it feasible to treat cases that would be possible to solve only with invasive surgery (39, 40). The definitive prosthesis can be placed in furcation areas, with optimal and enduring aesthetic results.

This contradicts the mistaken conviction that in implantology bi- and/or tri-furcations are harmful for the periodontium (see the cases described here) (23, 41-43).

Deep balancing immediately stabilizes the implants by means of strong mechanical osseointegration that allows immediate cementation of the temporary prosthesis, also favoring enduring biological osseointegration, with all the benefits that ensue.

Among the advantages of intraoral welding are immediate stabilization of the implants, immediate provisionalization, reduced risk of failure during the healing period, elimination of errors caused by unsatisfactory impression-making and a potential reduction in patient complaints and discomfort (12, 24, 44, 45).

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THE IMPORTANCE OF OCCLUSAL TRAUMA IN THE PRIMARY ETIOLOGY OF PERIODONTAL DISEASE

F. MEYNARDI^{1*}, D. LAURITANO^{2*}, M.E. PASQUALINI³, F. ROSSI⁴,
L. GRIVET-BRANCOT⁵, G. COMOLA⁶, L. DAL CARLO⁷,
E. MOGLIONI⁸ and P. ZAMPETTI⁹

¹Private Practice, Mondovì (CN), Italy; ²Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy; ³Private Practice, Milan, Italy; ⁴Private Practice, Busto Arsizio, Italy; ⁵Visiting Professor, Turin University, Italy; ⁶Universidad Alfonso X El Sabio, Madrid, Spain; ⁷Private Practice, Venice, Italy; ⁸Private Practice, Rome, Italy; ⁹Pavia University

*These authors contributed equally to this paper.

The presence of periodontal disease can be ascertained by clinical examination (redness, edema and probe depth, bleeding-on-probing). Occlusal adjustment can lead to a marked, stable improvement in periodontal health in terms of bacterial profile and clinical appearance, presumably by obviating tissue distress caused by occlusal dysfunction, thereby providing unfavorable conditions for bacterial growth. Therefore, occlusal trauma can be an essential indicator of the periodontal disease and, if not corrected, could lead to a relapse.

In periodontology, periodontal disease is regarded as an *ab initio* infective condition. According to this angle, the saprophytic bacterial component works on healthy tissues to cause the disease. Therefore, the pathologic event is thought to be the result of an accumulation of a bacterial film due to an inadequate mechanical deletion and bacteria could spread through the deeper tissues, bypassing defense barriers. In this scenario, the salivary turn-over does not prove to be as effective as it should be in order to prevent the disease.

Evolution of periodontal condition is hard to predict and forces the patient to undergo several exams. The bacteriologic scenario takes into account different hypothesis in the field of infectiology, according to which the bacterial component within the dental and gingival plaque might prove to be the combination of numerous species and responsible

for the infective process.

Another hypothesis regards the rise of a bacterial imbalance, which is able to induce microbiotic conditions that fit an infective process (1). According to Listgarten, it might be about specific bacterial strains such as the *Treponema Denticola* (2). Nevertheless, these theories do not take into account the significance of the conditions that the host organism shows in its dynamic-adaptive relation to the microbiotic component. The host organism represents the actual counterpart of the bacterial elements, whose invasive capacity is independent to defensive factors.

Approaching the periodontal disease from a bacteriological angle means that the responsibility of the pathologic event is given to bacteria only, while any other cause is not taken into account.

Realistically, the theory according to which an

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Mailing address:

Dr Marco Pasqualini,
Galleria Strasburgo, 3,
20121, Milano, Italy
Tel.: +3902799651
e-mail: dott.marcopasqualini@tiscali.it

organism with healthy and fully working structural, functional, metabolic and immune components could be damaged *ab initio* by every kind of microbes in the mouth, without considering the factors that allow and help the switch from saprophytic to pathogenic, does not conform to biology principles (3-7). The idea that oral hygiene is the only factor preventing periodontal disease is ambiguous.

The analysis highlights some questions about the patterns that are supposed to produce *ab initio* a periodontal disease in a totally healthy soil and exclusively because of the accumulation of dento-gingival plaque due to poor oral hygiene (8-15).

In this scenario, it is necessary to know what the circumstances are determining the beginning of the periodontal disease *ab initio* in a healthy soil. More specifically:

- when does the infection begin precisely?
- which conditions of the tissue help the bacteria to proliferate?
- which microbiotic conditions are required to begin the process and which turn out to be just supportive?
- what actually breaks the balance between host organism and the saprophytic bacterial flora?
- which bacterial patterns make it possible to bypass the epithelial and immune barriers, both localized and systemic, while they are fully working (16)?

Those patterns result in the penetration of the corion directory to the alveolar bone, and consequently in the switch of the bacterial flora from saprophytic to pathogenic. The bibliographic analysis begins from significant authors, such as Karoly (17) the first who supported a causal connection between bruxism and periodontal disease, also known as “Kolyo effect”, in 1901. In 1922, Stillman and McCall recognized that traumatic occlusion is an enormous occlusive stress, which is able to damage the periodontium (18). Glickmann, in the 60s, stated that occlusal trauma is a predisposing factor to the subsequent bacterial infection, according to the theory of the irritation area and chronic obstructive trauma (19).

Soon after those who believed in the theory of bacterial pathogenesis, other authors thought a new idea out (20-24). That new angle was not aimed to

be substitutive. It was, indeed, an addition to the existing hypothesis, regarding a biomechanical trauma as one of the conditions required to let the periodontal disease appear (25, 26).

In 1993, Professor Ugo Pasqualini issued a considerable book (*Le Patologie Occlusali*) written in Italian, which provided all the scientific notions about the complex biomechanics of occlusion. He showed a clinical correlation between the continuous action of a dysfunctional loading vector, malocclusion, and the resulting medium-long term effects on the periodontal support system. According to his studies, this results in dystrophy with micro-ulcerations of the basal membrane, a wound in the connection of the epithelial attachment and, simultaneously, in alveolar-bone atrophy. An inflammatory-aseptic-chronic damage event takes place in the tissue. It is defined as “periodontosis”, a predisposing condition to the septic-bacterial inflammatory process known as periodontitis.

There are many studies showing that the periodontal disease features this kind of pathogenesis and evolution (27-32). As far as the infectivologic *ab initio* angle is concerned, the clinical method of diagnosis, treatment, prognosis and prevention, deals with protocols that may be about oral hygiene only. Some others require the use of ultrasounds and radicular smoothing, while every now and then it is necessary to approach the situation surgically by employing the technique of guided bone regeneration (33-38).

In the case report which is about to be shown, the treatment follows a protocol requiring a verification and a possible occlusal rebalancing, according to Pasqualini's principles, and, besides, to check on the correlation between the periodontal lesion evolution and the site-specific bacteriological profile, according to the morphology (39, 40).

On the other hand, there is clinical evidence that the evolution is changeable and unpredictable, whenever the treatment is limited to the professional hygienic approach. It looks like other variables interact in the relation between the bacterial component and the host reactivity, which results in all the evolutionary conditions, that are totally site-specific (41-44).

A screening of the periodontal disease is then performed, starting from the comparison between the evolution of the bacterial profile and the static and dynamic occlusal load conditions.

The functionalist approach can define a functional pathogenic diagnosis through morphologic-bacterial analysis *in vivo* (by employing phase contrast microscopy) and simultaneously, the detection of the occlusal profile. The most frequent and significant detections are possible because of the evolutionary behavior of the bacterial profile. In fact, it is directly related to the functional occlusal loading conditions of the site.

Once the biomechanical congruity of occlusion is restored, the bacterial morphological profile is highlighted by a significant increase in cocci, which is a periodontal health index (2, 45).

MATERIAL AND METHODS

This case report is about a 60-year-old female patient showing signs of a stomatognathic morpho-functional scenario, featured by severe inflammation of the frontal incisor gum. She also showed a functional overload in habitual occlusion (not in a centric relationship), causing a chronic occlusive trauma.

The periodontal setting showed in the antero-superior sector, from 1.1 to 2.1, septic-bacterial inflammation with edema and congestion due to stasis, more specifically on papillas (Fig. 1). The use of a probe highlighted lesions in the epithelial attachment and bleedings, atrophy of the bone-alveolar crest and tooth mobility (grade 1-2) (Fig. 2).

Plaque samples showed a significant bacterial profile for the diagnosis of septic-bacterial periodontitis, according to Listgarten criteria. Then, the diagnosis was dento-prosthetic malocclusion with functional anterior overload due to distal displacement pre-contact (Fig. 3-4). Moreover, a bilateral latero-posterior occlusion defect was diagnosed, in addition to periodontal gingivitis, chronic inflammation and congestive edema, which was the result of both biomechanical dysfunction and bacterial infection.

The treatment was performed basically with a functional-biomechanical approach based on the principles of the Ugo Pasqualini school. It consisted in rationalizing prosthetic/conservative rehabilitation by making a stable occlusal support with the maximum intercuspitation

(Pim).

In the very first phase of the procedure, the occlusal table was structurally and functionally restored, in order to set an occlusal balance. Later, the antero-superior gingival and periodontal scaling was performed, as well as the analysis of the evolution of the microbiological scenario. This was possible by monitoring the bacterial profile of the dental-gingival plaque, with monthly follow-up, lasted two years.

It was to be noticed the spontaneous healing of the gingival profile of groups 1.1, 2.1 was achieved in only one month after occlusion balance (Fig.5). This was been possible due to fact that the frontal teeth were no longer stressed by the traumatic pressure of the displacement due to the posterior pre-contact.

The check on the bacterial profile was performed by using the technique of *in vivo* sample and bacterial count, employing phase contrast microscopy. The clinical connotation of these profiles was considered in relation to Listgarten's indications about the clinical and prognostic significance. Furthermore, throughout the observation-time, professional hygiene sessions were performed in order to remove the dento-gingival plaque (46,47).

RESULTS

The evolution of the case, after the removal of the distal pre-contact, improved both the periodontal purview and the clinical condition. The periodontal parameters have progressively fallen within the normal range (probe use and bleeding) (Table I). The bacterial component in the sites affected by periodontal disease gradually turned into a standard profile (Table II).

CONCLUSIONS

In light of the clinical outcomes and most of all, of the current knowledge about the nature of the pathogenic bases, the periodontal disease is not to be considered as an infective *ab initio* disease that depends on the bacterial component in the dento-gingival plaque only. It is, rather, an actual atrophic-dystrophic involutional chronic syndrome, based on a biomechanical dysfunction.

The periodontal damage is indeed the result of



Fig. 1. The 2 yellow upper arrows indicate the severe gingival disease around the prosthetic border of the superior crowns. The inferior arrow indicates the same problem in the inferior opposite tooth due to occlusal overload. The biological width was not violated as will be proved in the following figures. General hygiene is quite good.



Fig. 2. The radiological situation of the subject indicating the extrusion of the two lower molars due to absence of the opposite teeth, please notice the correct contour of the frontal crowns.

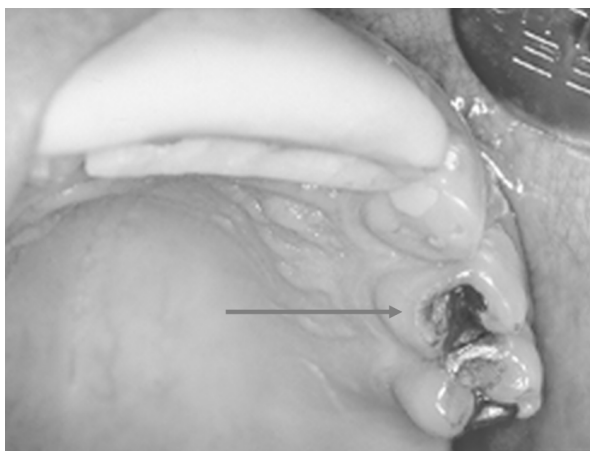


Fig. 3. The yellow arrow indicates the posterior pre-contact, identified thanks to the Pasqualini Stopper, a modified version of the Lucia Jig. The pre-contact on the 2.4 has been marked with an ultrafine silk strip (8u), the red sign must be removed.



Fig. 4. *The spontaneous healing of both hard and soft tissue around the two upper frontal crowns, without any surgery, but only with a re-balance of the occlusion and the prosthetic rehabilitation of the distal zone.*



Fig. 5. *The radiological outcome that shows the healing of the hard tissue.*

the influence that the environment exerts on the periodontium. More specifically, the damage comes from the chronic dysfunctional occlusal overload, which can induce the support system to have a reaction of adaptive aseptic inflammation (47-53).

The bone tissue, when continuously targeted by dysfunctional actions, responds, according to genetics and epigenetics, with an inflammatory evolution, far from physiological remodeling. Besides, another response consists in the increase of osteoclastic events, instead of osteoblastic ones, which results in a progressive atrophy (54-57).

The authors suggest a critical analysis of interpretative attitudes about the clinical approach to the periodontal disease, which is to be considered as the response to complex and multi-factorial environmental pressures (bio-chemical, bio-

mechanical, metabolic, immune, micro-vascular and psychological). According to this principle, an inflammatory septic bacterial evolution, known as periodontitis, might consequently occur.

To sum up, it is clear that the prevention of the periodontal disease and any recurrence must not take into account the hygienic approach only. Instead, it needs to be the result of a multi-factorial analysis focused on the causes of every kind, applying an interdisciplinary therapeutic method (3, 54, 58).

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CLINICAL APPLICATIONS OF NATURAL BONE MORPHOPROTEINS IN DENTISTRY: A NARRATIVE REVIEW

L. OTTRIA^{1*}, A. PALMIERI^{2*}, M. ANDREASI BASSI³, D. LAURITANO⁴,
V. CANDOTTO⁵, A. TAGLIABUE^{6**} and L. TETTAMANTI^{6**}

¹Dental School, Tor Vergata University, Rome, Italy; ²Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna, Bologna, Italy; ³Private Practitioner in Rome, Italy; ⁴Department of Medicine and Surgery, University of Milano Bicocca, Monza, Italy; ⁵Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy; ⁶Department of Medicine and Surgery, University of Insubria, Varese, Italy

*these authors contributed equally to this work and they are co-first authors.

**these authors contributed equally to this work and they are co-last authors

The need to restore bone loss in maxilla and mandible has led to find natural bone substitutes, such as fresh autogenous bone grafts. Fresh autogenous bone grafts (FABGs) have a remarkable capacity to induce new bone formation, a phenomenon called ‘osteinduction.’ FABGs are useful in craniomaxillofacial and oral applications to restore bone deficiencies. The isolation of those proteins believed to be responsible for the osteoinductive activity of FABGs, namely Natural Bone Morphogenetic Proteins (NBMPs), led to a new era in bone regeneration. NBMPs have been approved for use in specific oral and maxillofacial applications. Clinical trials and studies of oral and craniofacial surgery have indicated that NBMPs can promote bone repair. Information about the biology, chemistry, and actions of NBMPs has called into question whether NBMPs would result in clinically useful bone induction and morphogenesis. Preclinical and specific clinical trials have indicated the efficacy of NBMPs either combined with autograft or compared with an autograft alone. In light of questions about potency and safety of NBMPs, however, additional high-level evidence is needed for specific clinical indications and appropriate patient populations that would benefit from their use.

Fresh autogenous bone grafts (FABGs) is considered the reference standard for skeletal reconstruction because of the safety in using the patient’s own cells and cellular viability upon transfer to the new site. The limitations of FABGs include the need for harvesting and the often-inadequate amount of available graft, especially in infants and frail elders. Synthetic materials are a topic in research of bone substitutes that includes bone-derived products, metallic devices, and biologic agents. Also, interest

has been generated in combining FABGs with synthetic materials, but sometimes the results led to loss of osteoconductivity (1-19).

The present brief review concerns the clinical use of FABGs and natural bone morphogenetic proteins (NBMPs) and the clinical applications and limitations in dentistry and oral surgery.

Applications of FABGs

When properly prepared and used, FABGs have a

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Mailing address:

Prof. Angelo Tagliabue,
Department of Medicine and Surgery,
University of Insubria, Via Piatti 10,
21100 Varese, Italy
Tel.: +39.0332-825625 - Fax: +39.0332-825655
e-mail: angelo.tagliabue@uninsubria.it

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record of safety and efficacy. The history of FABGs for reconstructive surgery can be traced back to when they were used to repair mandible and maxilla defects. FABGs were used in conjunction with demineralized bone in oral and maxillo-facial applications. The use of FABGs alone for regeneration of bone loss and for periodontal defects was reported until 1980 (7, 20-26). The major advantages of FABGs alone include a shortened operative time and reduced blood loss, the potentially unlimited osteogenetic properties and the rapidity of the osteoinductive process. The limitations are related to the lack of rigidity of FABGs, but the addition of rigid additives demonstrated to limit the osteoinductivity of FABGs (eg, resorbable tricalcium phosphate).

Allogeneic bone is the most common type of FABGs used clinically, although autogenous and xenogeneic FABGs can also be successful (27). Donor screening, aseptic procurement, processing and distribution for specific clinical indications are accomplished according to frequently updated guidelines advocated by the American Association of Tissue Banks and regulated by the Food and Drug Administration (FDA) as human cell, tissue or cellular and tissue- based products. The different applications of FABGs can be attributed to their safety and adaptability with many uses in oral and maxillofacial surgery (14, 16, 18, 28-33).

NBMPs and their clinical use

Urist (34) proposed that the osteoinductive activity in FABGs resided in a component they named 'natural bone morphogenetic proteins' (NBMPs). Academic and industrial laboratories sought to clone the responsible genes and express the proteins using recombinant technology. Fifteen NBMPs have been identified on the basis of the amino acid sequence, with NBMP-2, NBMP- 4, and NBMP-7 [also known as osteogenic protein (OP- 1)] displaying osteoinductive activity (35). FDA indication-specific approval of NBMPs resulted in rapid acceptance for use in spinal fusion (36, 37) and fracture repair (36, 38). Favourable reports provided the basis for oral and craniofacial uses of both NBMP-2 and NBMP-7/OP-1 (39). According to the Centres for Medicare and Medicaid Service, however, up to 85%

of NBMPs use has been for off-label indications (available at: <http://www.cms.gov/medicare-coverage-database/details/technology-assessments-details.aspx?TAId=75&b c=BAAGAAAAAAAAA>).

Activity of NBMPs

Bone is a human tissue primarily formed of calcium phosphate in the chemical arrangement named calcium hydroxyapatite. Human bone is a rigid structure, but during a life of a person, is continuously remodelled by bone cells. Bone cells enable the turnover and replacement of the matrix in the interior of the bone. Three major cell types are found throughout the extracellular bone matrix: osteoblasts, osteocytes, and osteoclasts. NBMPs are active proteins in chemotaxis, mitogenesis and differentiation and neo-angiogenesis of bone cells. They promote bone regeneration through many events: chemotaxis of osteoblasts; proliferation and differentiation of cartilage; vascular invasion and finally mineralization, and remodelling of bone.

When a bone is fractured, a local population of pluripotent progenitor cells is activated by different growth factors. This pluripotent progenitor cells are osteoprogenitors that live in the cambial layers of the periosteum, endosteum, and dura. These progenitor pluripotent cells may differentiate in cartilage- chondrocytes or bone-forming osteoblasts, depending on the presence of NBMPs concentrations. More than 15 known NBMPs are structurally related and can be further categorized into subgroups based on amino acid or nucleotide similarity (40), of which NBMP2/4, NBMP5/6/7/8, NBMP9/NBMP10, and NBMP12/13/14 are subgroups (40).

The activity of natural bone morphogenetic proteins (NBMPs) was first observed in the mid-1960s when it was discovered that they could induce bone regeneration (34). The first NBMP were identified and studied biochemically more or less 20 years later. Further studies have demonstrated that NBMPs have the capacity to induce mesenchymal stem cells to differentiate in bone and cartilage. NBMPs are members of the Transforming Growth Factor- β (TGF- β) superfamily of proteins, including TGF- β s, activins, inhibins,

Growth Differentiation Factors (GDFs), Glial

Derived Neurotrophic Factors (GDNFs), Nodal, Lefty, and anti-Müllerian hormone. NBMPs have an effect on a great amount of cell types and processes beyond bone and osteogenesis. NBMPs play a key role in embryogenesis and cells and tissues homeostasis (41); in addition, they regulate signalling for cell growth, apoptosis, and differentiation. It's also been established that NBMPs are important in maintaining tissue homeostasis, (maintenance of cells contiguity, repair of bone fractures, and new vascularization). Because of these diverse functions in all organ systems, it has been suggested that NBMPs may change their definition into body morphogenetic proteins.

Due to their ubiquitous expression and importance as regulators throughout the body, deficiency in NBMPs production or functionality usually leads to marked defects or severe pathologies (41). Our short review would elucidate the role of NBMPs in dentistry and oral surgery.

NBMPs in dentistry

NBMPs have been studied in dentistry for periodontal regeneration; bone healing after implant osteointegration, oral surgery with orthodontic purposes, distraction osteogenesis and endodontic reparative surgery (42-50). Regeneration of periodontal structures constitutes a complex process regulated by interaction among cells, growth factors, and extracellular matrix. NBMPs have not yet been FDA- approved for dentistry, but they have been indicated for alveolar ridge augmentation and maxillary sinus lift, combined with dental extractions. In the field of periodontal regeneration/ wound healing, several growth factors have been clinically evaluated, namely, PDGF-B, bFGF, IGF-I/-II, NBMP2, NBMP3, NBMP7 (OP-1), and NBMP14/GDF15.

The first successful use of NBMPs for periodontal regeneration applied NBMP3 in combination with demineralized freeze-dried bone allograft and purified bovine collagen. Other studies in humans and animals demonstrated enhanced regeneration with NBMP3 combined with a variety of biomaterials.

Currently, PDGF and enamel matrix derivative (EMD) are recommended for periodontal

regeneration, whereas recombinant NBMP2 contained within an absorbable collagen sponge is indicated to assist with ridge and sinus augmentation (51). PDGF-B is a growth factor involved in wound healing, which stimulates the regenerative potential of periodontal tissues, including bone, cementum, and periodontal ligament, having been approved by the FDA in 2005 for the treatment of localized periodontal defects, as part of a dental bone-filling device only. Several investigations have been conducted, indicating the effectiveness of NBMP2 for correcting intra-bone, supra-alveolar, furcation, and fenestration defects, and in the supra-alveolar periodontal defect dog model, resulting in alveolar bone and cementum formation and fibrovascular tissue matured into bone and fatty marrow. NBMP7 stimulates periodontal wound healing in class III furcation defects (52).

NBMPs represent the future biotechnology for preparation of the implant site for osteointegration and wound healing. NBMP7 has been demonstrated to enlarge the bone ridge in areas of alveolar bone defects associated with dental implant fixtures. Targeted gene transfer of NBMP7 at dental implant osteotomy sites improves regeneration of bone defects. Another approach with NBMP2 and VEGF protein-combined scaffolds showed a synergistic effect in promoting bone healing around the dental implant (53).

Several studies have shown that, NBMPs are effective in treating diseases in oral surgery and dentistry, decreasing surgical morbidity and hospitalization time, and eliminating the need for surgical reinterventions for anatomical corrections, improving the patient's quality of life. Thanks to advances resulting from studies of the clinical use of NBMPs, in the future they will be the standard for treatment of implant and oral surgery. Nevertheless, the technologies must be significantly improved to address the questions remaining on their clinical use. Therefore, it is mandatory to better understand the biological processes of bone regeneration and overall changes occurring in osteoblast. In addition controlled, randomized, double blind clinical trials for NBMPs must be performed to improve survival rate of implants instead of using bone grafts.

Human clinical trials in oral surgery have recently investigated NBMPs (INFUSE® Bone Graft and OP-1) efficacy (ClinicalTrials.gov accessed March 11th, 2013). In addition, NBMPs may be used for restoring craniofacial defects of genetic syndrome (Cruzon, Apert syndrome, etc.) or oral tumors (54).

Further investigations are needed to define secure protocols for different purposes, in oral and maxillofacial surgery and with severe complications with the use of NBMPs.

Future perspective

Several oral and maxillo-facial surgeries require different protocols to regenerate alveolar and basal bone of maxilla and mandible. NBMPs have been demonstrated to be growth factors for bone augmentation. Systematic review and meta-analysis support therapeutic applications of NBMPs in implant and oral surgery, despite adverse effects of their clinical use have been reported. In conclusion, the use of NBMPs in dentistry have been documented and their availability through tissue banks has resulted in continued, increasing use. Many important basic and translational research questions remain concerning the mechanisms of action of these molecules, how to improve new bone regeneration with the delivery systems, the use of combinations with other signalling factors, how to eliminate the risks of complications. However, additional high-level evidence is needed for specific clinical indications and appropriate patient populations that would benefit from their use. Development of other dental biomaterials will be an attractive alternative for NBMPs and other growth factors.

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PERI-IMPLANT TEST IS A PROPOSAL OF A NEW PROCEDURE TO PREVENT PERI-IMPLANTITIS AND FORENSIC CLAIMS

R.M. GAUDIO^{1*}, L. OTTRIA^{2*}, D. LAURITANO³, A. PALMIERI⁴,
F. CURA¹, A. TAGLIABUE^{5**}, and L. TETTAMANTI^{5**}

¹Department of Medical Sciences, University of Ferrara, Ferrara, Italy; ²Dental School, Tor Vergata University, Rome, Italy; ³Department of Medicine and Surgery, University of Milan-Bicocca, Milan, Italy; ⁴Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna, Bologna, Italy; ⁵Department of Medicine and Surgery, University of Insubria, Varese, Italy

*these authors contributed equally to this work and they are co-first authors

**these authors contributed equally to this work and they are co-last authors

Implant dentistry has become one of the most successful techniques for oral rehabilitation over the last 20 years. The success rate of implant oral rehabilitation is above 80% while peri-implant disease (PID) is the most important complication of implant dentistry. The main cause of PID is considered bacterial leakage at the implant-abutment connection of a two-piece implant system. Prevention and control of bacterial leakage at the implant-abutment connection is mandatory for reducing inflammation process around implants neck and achieving bone stability. Since bacteria leakage at implant-abutment connection level is the main cause of PID, a microbiological test should be important to identify bacteria that cause PID. According with the conclusion of workshop of the European Federation on Periodontology, a test that detects the most frequent bacterial species involved in the onset of PID (*Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*) should be used in clinical practice. In fact, PID progression depends on the typology, quantity and composition of bacterial flora in peri-implant pockets, so controlling PID onset and progression, is a keystone for preventing implant failures and consequently forensic conflicts. The effort to prevent PID and consequently assurance or forensic conflicts have become one of the main focal points of all dental professionals. Behind these efforts lie, above all, ethical but also economic reasons, as well as a desire to prevent PID, improving implant care quality and increasing the legal security of health care professionals themselves. Since the legal decisions in our society influence how we practice dentistry, especially in the fast-evolving field of implant dentistry, using diagnostic tools that will allow dentists to demonstrate that they have acted correctly in accordance with the knowledge of modern medicine, it is of great importance to defend themselves in the case of legal-legal disputes.

Implant dentistry has become one of the most successful techniques for oral rehabilitation over the last 20 years (1). The success rate of implant,

oral rehabilitation is above 80% while peri-implant disease (PID) is the most important complication of implant dentistry (2). PID springs from bacterial

Key words: peri-implant disease, microbia, bacterial test, genetic test, forensic dentistry

Mailing address:

Prof. Angelo Tagliabue,
Department of Medicine and Surgery,
University of Insubria,
Via Piatti, 10, 21100 Varese, Italy
Tel: +39.0332-825625 - Fax: +39.0332-825655
e-mail: angelo.tagliabue@uninsubria.it

infection that activates a cytokines cascade leading to inflammation and bone loss (3-11). In addition, the patient-related susceptibility is a critical factor for disease onset (12). Therefore, every factor favouring oral biofilm formation (poor oral hygiene), host defence capability (smoking habit, excessive alcohol consumption, genetic traits, history of periodontitis, use of bisphosphonates), might favour developing of PID (13).

The Consensus of the Seventh European Workshop on Periodontology (14), stated that biofilm causes oral infection and its by-products, but numerous etiological factors are recognized to be specifically associated with PID also, such as biomechanical stress, cement remnants and incorrect finishing line, abutment unscrewing, implant crack or fracture (15).

According to these observations, the most frequent clinical subtypes of PID were recently classified as follows: purely plaque-induced, prosthetically and surgically triggered peri-implantitis (15).

Multiple risk factors act, making the identification of the triggering factors more difficult. In addition, a recent systematic review reported that PID shows an increasing prevalence in general population and that the lack of a standard therapeutic modality aggravates this condition (16). In fact, a positive success rate after PID treatment is observed only in early follow-ups, however, in the long run, absence of resolution as well as progression or recurrence of disease (until implant loss) is normality. The lack of efficacy of PID treatment may be due to insufficient bacterial control.

In fact, misunderstanding that bacterial control is fundamental for positive outcome, the role of other factors such as soft and hard tissue conditions, tridimensional implant positioning and triggering factor of disease might be irrelevant in PID treatment.

The hypothesis of this paper is that different subtypes of peri-implant diseases (purely plaque-induced, prosthetically and surgically triggered peri-implantitis), are directly or indirectly related to bacterial leakage at implant-abutment connection, regardless of specific predictive profiles for patient-based risk assessment and diagnostics. So bacterial control and testing is a keystone in preventing PID.

To evaluate the potential bacterial leaking from implant-abutment junction a meta-analysis of previous published articles by our group was conducted and data reported.

MATERIALS AND METHODS

Implant preparation and DNA extraction

In this study were analysed 11 implant systems with different types of implant-abutment connections; internal hexagon screwed connection: DITRON (MPI, Ditrion Dental, Israel); NOBEL (NobelBiocare®, Vimercate (MB), Italy); SHINER (Shiner XT, FMD Falappa Medical Devices S.p.A. Rome, Italy); NORIS (Noris Medical Dental Implants System, Israel); NQ (uNiQo, FMD Falappa Medical Devices S.p.A. Rome, Italy); FN (nano-fix, FMD Falappa Medical Devices S.p.A. Rome, Italy); ELISIR (FMD Falappa Medical Devices S.p.A. Rome, Italy); PALTOP (Paltop Advanced Dental Solutions Ltd, Caesarea, Israel); LEONE (Leone Spa®, Florence, Italy). External hexagon screwed connection: STORM (FMD, Falappa Medical Devices®, Rome, Italy); KONUS (Konus Implant System®, Industrie biomediche e farmaceutiche, Italy).

In order to evaluate the isolate capacity of the internal lumen of each implant from the external environment, we evaluated the passage of modified *Escherichia coli* across the joint of the implant.

E. coli is a Gram-negative, facultative anaerobe, motile bacteria measuring 1.1 to 1.5µm in diameter and 2 to 6µm in length. The peculiarity is that they contain synthetic DNA target sequences in their plasmid. In this particular study, the plasmid contained two sequences specific for two bacterial species (*Porphyromonas gingivalis* and *Tannerella forsythia*) and two genes for antibiotic resistance (Kanamycin and Ampicillin).

Bacteria were cultured in lysogeny broth (LB) (Sigma-Aldrich) containing both Kanamycin and Ampicillin (50µg/ml both) at 37°C for 24h in a shaking incubator. Sterile culture medium with antibiotics (Kanamycin and Ampicillin, each at 50µg/ml), without bacteria, was carefully pipetted in the internal lumen of each implant. The implants and the abutment were screwed according to the manufacturer's protocols.

Ten microliters of this culture were used to "contaminate" fresh LB with antibiotics contained in a

microcentrifuge tube together with the implant. The tubes were then left at 37°C for 24h in a heater, in order to allow bacterial growth and their hypothetical passage within the implant. To ensure that there were no contaminations, a negative control containing only LB and antibiotics, was prepared.

Twenty-four hours later, implants were opened and bacteria sampling was performed dipping two paper probe in both the sites containing LB (inside and outside to the implant) for each implant, and in the negative control too.

Once collected, the paper probe were put on a new microcentrifuge tube and processed for bacterial DNA extraction using a QIAamp DNA Mini QIAcube Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

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 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 Biosystems, Foster City, CA, USA).

Statistical analysis

To evaluate if the difference in viability between outside and inside the implant was statistically significant, mean values of the bacteria (*P. gingivalis* and *T. forsythia*) present in the external and internal connection implants and percentage of bacterial entry into the implant were calculated.

RESULTS

In Table I, bacterial quantification and their entry percentage are shown. In all the tested implants,

Table I. Bacterial quantification and calculation of their entry's percentage.

Implant name	Implant connection type	Number of investigated implants	BACTERIA	Mean Outside	Mean Inside	Passage of bacteria from outside to inside the implant (%)
DITRON	Internal connection	4	P. gingivalis	354499	5987	2
			T. forsythia	416829	6357	2
NOBEL	Internal connection	3	P. gingivalis	291770	5192	2
			T. forsythia	289571	5991	2
FMD-SHINER	Internal connection	6	P. gingivalis	15506755	891090	6
			T. forsythia	17328943	836535	5
NORIS	Internal connection	12	P. gingivalis	3229381	279502	9
			T. forsythia	3040039	287426	9
FMD-NQ	Internal connection	5	P. gingivalis	37116	230	1
			T. forsythia	36271	277	1
FMD-FN	Internal connection	4	P. gingivalis	34912	713	2
			T. forsythia	40122	725	2
FMD-ELISIR	Internal connection	5	P. gingivalis	138148	463	0
			T. forsythia	120103	538	0
PALTOP	Internal connection	4	P. gingivalis	3774	703	19
			T. forsythia	3763	761	20
LEONE	Internal connection	4	P. gingivalis	305119	9957	3
			T. forsythia	287264	10549	4
FMD-STORM	External connection	6	P. gingivalis	3539135	523438	15
			T. forsythia	1280108	183733	14
KONUS	External connection	12	P. gingivalis	3273565	599419	18
			T. forsythia	3409120	639808	19

bacteria were found in the inner side with a mean percentage of 10% for *P. gingivalis* and 9% for *T. forsythia*. The results show a lower permeability to bacteria for internal hexagon screwed connection implant (DITRON, NOBEL, SHINER, NORIS, NQ, FN, ELISIR, PALTOP, LEONE) than external hexagon screwed connection implant (STORM, KONUS).

DISCUSSION

Bacterial leakage at implant-abutment connection

Bacterial leakage at the implant-abutment connection of a two-piece implant system is considered the main cause of PID. Prevention of bacterial leakage at the implant-abutment connection is mandatory for reducing inflammatory process around implants neck and achieving bone stability (13). Micro-cavities at implant-abutment connection level can favour bacterial leakage, even in modern two-piece implant systems. The constant flux of microbiota between implant and abutment is an important factor for chronic inflammatory infiltration and marginal bone resorption (17). Implant-abutment connection aims to reduce the leakage by increasing the stability. In fact, a good implant-abutment connection should be manufactured for reducing the mobility of the screw by constructing physically tight connections with a high level of precision (17). Few investigators aimed to quantify microbial leakage of implant-abutment connection (18, 19). 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.

Micro-leakage phenomena of implant-abutment connection was well documented. The sealing capability of different implant-abutment connections was tested in some studies (20). All the tested implant-abutment connections presented micro-leakage. This is in agreement with other studies showing micro-movement between the abutment and its implant. One of the possible solutions to prevent

bacteria leakage is to coat the implant-abutment connection with a new coating with bactericidal activity (21).

Bacterial control

Bacteria are the main cause of PID, so microbiological testing of PID is important to identify these bacteria (22). In a workshop of the European Federation on Periodontology (23) about PID, a consensus was reached concerning oral peri-implant infections on the basis of the state of the art in the relevant sciences. Important conclusions were that peri-implant mucositis occurs in 80% of subjects with oral implants, and that PID occurs in 28% - 56% of subjects studied. Important risk factors for developing PID seem to be insufficient oral hygiene, a history of periodontitis and cigarette smoking (24-27). Mechanical treatment in combination with antimicrobial oral mouth rinses may be effective in the treatment of PID. No evidence has been found that mechanical treatment alone is effective. To control the infection of PID, surgical treatment, often in combination with the use of local or systemic antibiotics, is necessary (28-31). It is therefore clear that the parodonto-pathogen bacteria are the main causes of PID, and a test that allows identifying the main bacteria and bacterial load should be a valuable tool in clinical practice.

In accordance with the conclusion of the European Federation on Periodontology workshop (32), a microbiological test that detects the most frequent bacterial species involved in the onset of PID (*Actinobacillus actinomycetecomitans*, *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*) should be used in dental practice.

PID progression depends on the quantity and composition of bacterial flora in peri-implant pockets, so controlling PID onset and progression is a keystone for preventing implant failures and consequently forensic conflicts.

However, many techniques to diagnose PID have not been fully accepted due to low sensitivity or specificity, moreover sometimes they are slow, expensive and laborious. Polymerase chain reaction (PCR) test is rapid and sensitive, especially if few

but high sensitive bacteria are investigated such as *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola* (i.e. red complex bacteria).

PCR-RT allows to identify and quantify the main pathogens of PID and inform the patient about his/her personal microbiological profile, to make the patient knowledgeable about treatment and his/her compliance in home care, and to verify the effectiveness of therapeutic protocols (33-35). In addition, performing periodontal tests prior to implant dentistry, will allow dentists to demonstrate that surgery has been performed under conditions of bacterial control (13, 18, 25, 26, 28-31, 36-50). Periodontal tests during follow-ups also allows to demonstrate that implant failure is not due to surgical or prosthetic errors, but to the lack of oral hygiene by the patient.

Forensic conflict and legal defence

The effort to prevent PID and consequently forensic claims, has become one of the main focal points of all dental professionals. Behind these efforts lie, above all, ethical but also economic reasons, as well as a desire to prevent PID, improving implant care quality and increasing the legal security of health care professionals themselves.

The key concept in implant dentistry is preventing adverse events such as PID. PID is an unfavourable, undesired consequence caused by bacterial leakage, which has negative consequences on clinical outcome. These negative consequences are not caused by the patient's underlying disease or pathology, but by bacterial leakage at implant-abutment connection level. The main objective of patient safety is to avoid PID to the greatest extent possible and to limit the negative consequences of those that are unpreventable.

Therefore, it is essential to ascertain how PID occurs in implant dentistry, in order to study it in-depth and propose measures for prevention. In the field of implant dentistry, most of the available studies about forensic conflicts are limited to descriptions of small series. At this time, we have no information, which contains a reliable reflection of the frequency and importance of the forensic

conflicts caused by PID, which take place in dental practice. Any proposal of measures to prevent PID must necessarily be based on knowledge of the real situation (regarding frequency and severity).

Legal claims and court sentences tend to contain a great deal of information on the implant failures, which have occurred in implant dentistry, as well as their causes, the circumstances surrounding each event and their consequences. On the other hand, they are significantly biased. Most implant failures with minor consequences lead to no legal claims and are therefore unknown (50).

PID is one of the main causes of forensic conflict in dentistry. PID generate medico-legal issues related to difficulties inherent to surgical and prosthetic procedures and objectives (both functional and aesthetic), as well as full patient collaboration as an essential part of successful treatment. An accurate assessment of each case by the clinician is fundamental to prevent forensic conflicts. The patient should therefore receive complete information and be made fully aware of the risk of PID, as well as possible complications, limits to the procedures, and the fact that successful out-come will also depend on his/her scrupulous observance of the practitioner's instructions and oral care. The aim is to make the patient an active partner in order to prevent PID onset. To this aim, an extremely detailed information leaflet is strongly advised in order to inform the patient about the risk of PID; after careful clarification of any doubts the patient may have, written informed consent should be obtained. In this light, monitoring bacterial status is of paramount importance to prevent PID and ensure the success of implant outcome since the main cause of implant failure is bacterial infection.

CONCLUSION

Since the legal decisions in our society influence how we practice dentistry, especially in the fast-evolving field of implant dentistry, using diagnostic tools that will allow us to demonstrate that we have acted correctly in accordance with the knowledge of modern medicine, it is of great importance to defend us in the case of legal-legal disputes.

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EDITORIAL

PREVALENCE OF HIV-RELATED ORAL MANIFESTATIONS AND THEIR ASSOCIATION WITH HAART AND CD4+ T CELL COUNT: A REVIEW

L. OTTRIA^{1*}, D. LAURITANO^{2*}, L. OBERTI², V. CANDOTTO³,
F. CURA⁴, A. TAGLIABUE^{5**} and L. TETTAMANTI^{5**}

¹Dental School, Tor Vergata University, Rome, Italy; ²Department of Medicine and Surgery, University of Milan-Bicocca, Milan, Italy; ³Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy; ⁴Department of Medical Sciences, University of Ferrara, Ferrara, Italy; ⁵Department of Medicine and Surgery, University of Insubria, Varese, Italy

*these authors contributed equally to this work and they are co-first authors

**these authors contributed equally to this work and they are co-last authors

HIV infection is one of the major health problem of the last decades. This disease causes a chronic infection that can lead to acquired immunodeficiency syndrome (AIDS). According to the Global AIDS update, released in 2016 by HIV department of World Health Organization (WHO) and by the Joint United Nations Program on HIV/AIDS (UNAIDS), at the end of 2015, 36.7 million people were infected by HIV: 34.9 million of these were adults and 1.8 million were children under 15 years of age. The same report shows that during 2015, 2.1 million of new infection cases have occurred all over the world and about 1.1 million people have died for HIV. The aim of this short review is to up-date of the main HIV-related oral manifestations and their correlation with HAART (Highly Active Antiretroviral Therapy) and CD4+ T-cell count. Despite that more than 20 years have elapsed, this classification still remains valid: even today, group 1 lesions are found in the majority of HIV-positive patients with oral manifestations. Group 1 includes the following conditions: oral candidiasis (*pseudomembranous candidiasis*, *erythematous candidiasis*, *angle cheilitis*), oral hairy leukoplakia, periodontal diseases (necrotizing gingivitis, necrotizing periodontitis, linear gingival erythema), Kaposi's sarcoma, and non-Hodgkin's lymphoma. Melanotic hyperpigmentation, HSV infection and HPV infection, which are included in group 2, are also common. Oral candidiasis, oral hairy leukoplakia, Kaposi's sarcoma and HSV infection are the lesions that have seen the major drop in their incidence after the HAART introduction. The increase in CD4+ T-cell count is not significantly correlated to the decrease of every type of oral lesions, but it is statistically significant only in relation to oral *candidiasis* (p-value <0.001). Oral lesions are an important sign of immunodepression and with the introduction of HAART their incidence has strongly decreased, particularly in urban areas. Nevertheless, developing countries still have a high prevalence of these manifestations because of the persistence of many risk factors, like the difficulty to access treatment, poor oral hygiene, low socioeconomic status and late diagnosis.

HIV infection is one of the major health problem of the last decades. This disease causes a chronic infection that can lead to acquired immunodeficiency

syndrome (AIDS). According to the Global AIDS update, released in 2016 by HIV department of World Health Organization (WHO) and by the Joint United

Key words: HIV, AIDS, HIV-related oral manifestations, HAART, CD4

Mailing address:

Prof. Angelo Tagliabue,
Department of Medicine and Surgery,
University of Insubria,
Via Piatti 10, 21100 Varese, Italy
Tel.: +39.0332-825625 - Fax: +39.0332-825655
e.mail: angelo.tagliabue@uninsubria.it

Nations Programme on HIV/AIDS (UNAIDS), at the end of 2015, 36.7 million people were infected by HIV; 34.9 million of these were adults and 1.8 million were children under 15 years-of-age. The same report shows that during 2015, 2.1 million of new cases have occurred all over the world and about 1.1 million of people have died from HIV.

In the initial phases, the infection is asymptomatic and it can stay unchanged for years until the first symptoms, due to immunosuppression, show up. This condition is the direct consequence of the destructive effect of HIV on T-helper lymphocytes, which the virus has a strong tropism for and where it completes its replication cycle. The first symptoms, which can be local or systemic, are considered manifestations of opportunistic infections. Among these, oral lesions are one of the most common HIV-related diseases.

In 1981, Gottlieb identified AIDS in a group of young homosexual men and he published a description of the disease, revealing the association between AIDS and oral candidiasis, which represents an important sign of immunodepression (Fig.1). Nowadays it is known that oral candidiasis, as well as other oral manifestations, is not only related to AIDS but it is also a common sign of immunodepression in HIV-positive patients, who have not developed AIDS yet (1).

HIV-related oral manifestations include a large group of lesions, like bacterial, viral or fungal infections, idiopathic lesions, salivary gland diseases

and neoplastic diseases (1). These manifestations are often the first symptom of HIV infection (2).

The majority of studies on this issue concern a population of HIV-positive patients, living in developing countries with a large diffusion of HIV, in particular Sub-Saharan Africa, where about the 65% of the global HIV-infected people live and where 5.5% of inhabitants are HIV-positive; The Caribbean, where 1.1% of population is HIV-infected and South-East Asia/India, where 15% of global seropositive population live (3). On the contrary, the prevalence of these lesions in the urban areas is less, probably because of rapid diagnosis and easier access to treatment (3).

Despite the amount of information that can be collected, it is difficult to find the exact prevalence of oral manifestations in the HIV-infected population, due to many variable factors that can affect this datum. Studies in heterogeneous seropositive populations show discordant prevalences of HIV-related oral lesions: according to Gaurav et al., the prevalence is 80.6% in South India (4), whereas the study conducted by Lourenço et al. reports a prevalence of 29.9% in a HIV-positive Brazilian population (5). In any case, we can presume that HIV-related oral manifestations prevalence is between 30% and 80% (2). This value increases in presence of adverse factors such as diagnosis of AIDS, low CD4+ T-cell count (<200 cell/mm³) and high viral load ($>20,000$ copies/mL); while it decreases in patients undergoing HAART (Highly Active Antiretroviral



Fig. 1. *Pseudomembranous oral candidiasis of tongue in young patients with AIDS.*

Therapy), with high CD4⁺ T-cell count and low viral load. These are the main factors influencing the appearance of oral manifestation in HIV-positive people.

Major HIV-related oral manifestations

In 1993 the EC-Clearinghouse on oral problems related to HIV infection and WHO collaborating center on oral manifestations of the immunodeficiency virus produced a classification of HIV-related oral manifestations (6).

No oral lesion is unique to HIV; nevertheless, three groups were established, sorted by the frequency of association with HIV infection: strongly associated lesions, less commonly associated lesions, possible associated lesions.

Despite more than 20 years have elapsed, this classification still remains adequate: even today, group 1 lesions are found in the majority of HIV-positive patients with oral manifestations (7).

Group 1 includes the following conditions: oral candidiasis (*pseudomembranous candidiasis*, *erythematous candidiasis*, *angle cheilitis*), oral hairy leukoplakia, periodontal diseases (necrotizing gingivitis, necrotizing periodontitis, linear gingival erythema), Kaposi's sarcoma (8-11), and non-Hodgkin's lymphoma (7). Melanotic hyperpigmentation, HSV infection and HPV infection (12-19), which are included in group 2, are also common.



Fig. 2: *Pseudomembranes of candida on soft palate and pharynx in a patient with AIDS.*

The majority of studies conducted around the world agree that candidiasis (OC) is the most common HIV-related oral lesion, both in patients undergoing HAART and in untreated ones (1). In studies by Bodhade et al. and Sontakke et al., oral candidiasis is the main HIV-related oral manifestations, with a 39.3% and 32.3% prevalence respectively (20, 21). These findings are confirmed in the study by Kumar et al. where 36.5% of subjects suffer from OC (22), and in that of Nanteza et al. where this value is 24.9% (23). Gaurav et al. and Da Silva et al. also come to the same conclusion, indicating HIV-related candidiasis as the most common lesion with a 38.8% and 9.3% prevalence respectively (4, 24) (Fig. 2).

Two forms of OC are mainly observed: *pseudomembranous candidiasis* (PC) and *erythematous candidiasis* (EC). A third form, the angular *cheilitis*, is also common in HIV-positive patients.

Khatibi et al. have found that pseudomembranous candidiasis is the most common variant (25). The same conclusion comes from studies by Sontakke et al. and Nanteza et al. that find a prevalence of PC of 20.1% and 12.1% respectively, instead of an 8.1% and a 8.4% prevalence of EC (21, 23). Even Moodley and Wood affirm that this form is the most observed (3%) (26).

However, there are several studies showing the opposite: Gaurav et al. (2011) report a prevalence of erythematous candidiasis of 38.8%, significantly higher than the 8.7% prevalence of pseudomembranous candidiasis (4). According to Bodhade et al. (2011) EC is the most common form (30.6% compared to 12.3% prevalence of PC) (20).

This difference is due to 3 main factors that may influence the onset of one form instead of another: smoking habits of patients, different geographical area of origin and the use of different antibiotic therapies, which are common in HIV-positive patients for eradicating any opportunistic infection (20). It is not clearly defined which is the most common form of candidiasis, however the pseudomembranous form is considered to be the most frequent.

Another common HIV-related oral manifestation is the oral hairy leukoplakia, a lesion caused by EBV. It is frequently observed in many studies (4, 20, 21,

23, 25-27) and it represents on average 6-7% of all HIV-related oral lesions, with a higher prevalence in Western countries (26) and in the homosexual male population (28). According to Nittayananta et al. OHL might also be due to the increased susceptibility to EBV by the epithelium of oral mucosa in HIV-positive patients (29).

Periodontal disease is another relevant HIV-related oral manifestation. This disease, caused by the alteration of the oral microbiota in seropositive patients, includes linear gingival erythema (LGE), necrotizing gingivitis (NUG) and necrotizing periodontitis (NUP).

The periodontal manifestation with the higher prevalence is LGE. This lesion seems to have fungal aetiology: recent studies have shown the role of *Candida albicans* in its pathogenesis (30). The prevalence emerged in many studies is significantly high: Khatibi et al. have found that 22% of patients were affected by LGE (14), while according to Gaurav et al. and Bodhade et al. this percentage decreases to 13.6% and to 10.3% respectively (4, 20).

Sometimes LGE could evolve in NUG and NUG could predispose to the development of NUP (30). The analyzed studies report variable prevalence of these lesions, due to the difficulty in making a diagnosis with universally valid evaluation criteria and due also to the interference of many other factors related to the development of periodontal disease (smoking habits, oral hygiene, systemic diseases etc.) (30). However the average prevalence is between 3% and 8%.

The role of HIV is still unclear in the onset of chronic periodontitis: nevertheless it has been reported that the risk of periodontal attachment loss is 6 times higher in seropositive patients with a CD4+ T-cell count less than 200 cell/mm³ and over 35 years of age (30).

Kaposi's sarcoma is the HIV-related neoplasia with the highest prevalence (28). It predominantly affects palate, oropharynx and gum (31), and its average prevalence varies between 0.5% and 3.5%. Homosexuality represents an important risk factor (20). Non-Hodgkin's lymphoma is also an important HIV-related neoplasia with an average prevalence of 0.5-1.5%.

In addition to all the lesions of group 1 (EC-Clearinghouse classification) there are many other common manifestations, like melanotic hyperpigmentations. According to the study by Denny et al. their prevalence is 42.6% (31), while Satyakiran et al. report a 32.8% prevalence (32). This disease is caused by the increased release of α -MSH (melanocyte-stimulating hormone), due to immunological changes in HIV-positive patient and due to the effects of some antiviral and antifungal drugs (4, 20).

Ulcers, HSV infection and xerostomia could occur in HIV-positive patients. The prevalence of ulcerative lesions in the examined studies varies between 1.5% (Nanteza et al.) and 12% (20). The majority of herpetic lesions are caused by HSV, while those caused by VZV are less common. HSV-related lesions are found in 1-3% of seropositive patients. In the study of Mwangosi and Tyllia, HSV lesions are even the HIV-related oral manifestations with the highest prevalence (11.5%) (27). Finally, xerostomia has a prevalence of 1-4%, even though Gaurav et al. reported a 32% prevalence (4).

While the use of HAART has reduced the prevalence of the most common HIV-related oral lesions, the prevalence of HPV infections has not decreased (8). Many studies have shown a significant increase in the incidence of these manifestations between 1990 and 2000. This phenomenon is caused by the introduction of HAART, which happened exactly in the same years (7). HPV could be considered a manifestation of immune reconstitution inflammatory syndrome (IRIS), an atypical inflammatory response due to a viral load decrease and to the improvement of immune system activity (33). HPV infections may also evolve into malignancy (7). The risk of developing HPV-related oropharyngeal cancer is 2-6 times higher in HIV-positive individuals compared with the general population.

Oral manifestations of HIV and their correlation with HAART

There are many factors that can influence the appearance of these lesions. The majority of the studies published in the last years affirm that use of

HAART, CD4⁺ T-cell count and viral load are the main factors affecting the number and the type of lesions. Other factors include poor oral hygiene (25), low socioeconomic status (3, 22, 34) and alcohol abuse (25).

The introduction of HAART has led to a significant decrease in the prevalence of HIV-related oral lesions (7, 25, 28, 30) (26, 29). In a prospective study by Ortega et al. a cohort of patients were followed from 1989 to 2006, and an important reduction in the incidence of HIV-related oral manifestations was observed (35). This reduction was noted in two specific periods: 2000, right after the introduction of protease inhibitors (HAART-PIs), and 2006, when the first non-nucleoside reverse transcriptase inhibitors (HAART-NNRTIs) were diffused (35).

It is estimated that at the end of 2015, about 17 million people living with HIV/AIDS were receiving HAART: this number is still growing compared to 2010 when subjects undergoing HAART were only 7.5 million.

The main goal of this therapy is to reduce viral load and increase CD4⁺ T-cell count (31). Moreover, HAART seems to alter oral innate immunity, causing an increase in the number of Langerhans cells, neutrophils and monocytes, but also determining a reduction of salivary flow rates in the long term; as a result the risk of chronic opportunistic infections and of malignant transformation increases. Because of that, it is possible to affirm that short-term HAART is the only therapy strengthening oral innate immunity, while long-term adversely affects this immunity (29).

Oral candidiasis, oral hairy leukoplakia, Kaposi's sarcoma and HSV infection are the lesions which have seen the major drop in their incidence after the HAART introduction (26).

Lourenço et al. conducted a study on patients receiving antiretroviral drugs (except protease inhibitors) in 1997 and on subjects undergoing HAART in 2004-2008. This study shows that the prevalence of oral manifestations is of 60.1% in the first group and 29.9% in the second one (p-value <0.0001) (5). An analysis of scientific literature reveals that the majority of studies agree that only two kinds of lesions have seen a statistically significant decrease of their incidence after the introduction of

HAART: oral candidiasis and periodontitis. Patil et al. found that 32% of seropositive individuals with oral lesions receive HAART, while 56% of them are not in therapy: this difference is statistically significant (p-value <0.05) (36). The same study also shows that the use of HAART induces a decrease in the prevalence of oral candidiasis and periodontitis from 8% to 2% and from 14% to 2% respectively (36). The study by Rao et al. had the same result, reporting a 24.4% prevalence of oral candidiasis and a 68.8% prevalence of periodontitis before initiating treatment and a 10.3% and a 46.6% prevalence respectively after therapy initiation (p-value: 0.01) (37). Finally, Satyakiran et al. report a direct correlation between the reduction of oral lesions prevalence and the duration of HAART (32).

However, there are some HIV-related oral manifestations, which have seen an increase in their incidence after the introduction of HAART, like hyperpigmentation, xerostomia and salivary gland hypertrophy. This is due to the effects of antiretroviral drugs on melanocyte-stimulating hormone (MSH) and on salivary flow rate (4, 26). The study by Rao et al. show a statistically significant increase in melanotic hyperpigmentations prevalence from 5.3% to 23.8% after initiation of HAART (p-value <0.001) (38). Even Eweka et al. report a similar increase in melanotic hyperpigmentations prevalence from 10.8% before HAART initiation to 66.7% during treatment (33). The same conclusion is drawn by Satyakiran, which indicates a prevalence of hyperpigmentation and xerostomia of 17.6% and 0% respectively in patients with HAART under 4 years duration; a prevalence of 42.3% and 7.6% in patients with HAART between 4 and 8 years duration; a prevalence of 26.3% and 10.5% in patient with HAART for more than 8 years (32).

A notable study conducted by Batavia et al. examined a sample of HAART-naïve HIV-positive people with a CD4⁺ T-cell count between 200 and 350 cell/mm³ and randomized them into two groups. The first group was immediately subjected to HAART and the second to the same treatment only when the CD4 count was less than 200 cell/mm³ or with the development of an AIDS-defining condition. The study shows that patients undergoing early treatment have a lower risk in developing HIV-related oral

lesions compared to the other participants (p-value <0.01): there is a statistically significant reduction in the incidence of candidiasis (p-value <0.01), oral hairy leukoplakia (p-value: 0.02) and HSV lesions (p-value: 0.05). The effect of HAART on reducing oral lesions prevalence is also confirmed by comparing oral candidiasis before and after treatment initiation in the delayed group. Finally, the increase of HPV-related oral warts during HAART is also statistically significant. It is possible to conclude that the presence of oral lesions in HIV-positive patient is an index of immunosuppression and it represents an important reason for initiating treatment; on the contrary, the presence of HPV-related lesions during HAART is a sign of immune reconstitution (39).

Oral manifestations of HIV and their correlation with CD4+ T-cell count

The effect of HAART in reducing the incidence of HIV-related oral lesions is to be found not only in increasing the CD4 count, but also in the direct effect that therapy has on every kind of lesion. The increase in CD4+ T-cell count is not significantly correlated to

the incidence decrease of every kind of oral lesions, but it is statistically significant only in relation to oral candidiasis (p-value <0.001) (23).

Sontakke et al. reported that pseudomembranous candidiasis was found in 27.4% of patients with a CD4 count <200 cell/mm³, in 8.1% of those with a CD4 count between 200 and 500 cell/mm³, and in 1.6% of those with CD4 count >500 cell/mm³: this correlation is statistically significant (p-value <0.05) (21). A similar result was obtained in the study by Bodhade et al. which analyzed the CD4 count of patients with and without oral candidiasis: the difference indicates a strong association between the lesion and the presence of a CD4+ T-cell count <200 cell/mm³ (p-value <0.0001) (20). Finally, this significant correlation has also been shown by Saini (2), Da Silva et al. (24), Satyakiran et al. (32), Moodley and Wood (26) and Gaurav et al. (4).

For this reason, it is possible to affirm that oral candidiasis could be a valuable index of a low CD4 T-cell count and therefore also of immunosuppression (PPV: 68%) (23).

Finally, some studies analyzed the correlation

Fig. 2: *Pseudomembranes of candida on soft palate and pharynx in a patient with AIDS.*

Reference/N. of subjects	HAART (%)	Any OL (%)	OC (%)	OHL (%)	NUG (%)	NUP (%)	LGE (%)	Neop lasm (%)	Others (%)	Comments	
Lourenço <i>et al.</i> (2011), Brazil	388	79.9	29.9	EC 6.4 PC 9.3	10.3	ND	ND	ND	KS 0.5 NHL 0.3	AC 11.9 Ulcers 1.8 HSV 0.8	Cross-sectional study comparing 2 periods (1997, 2004-2008). These results correspond to 2004-2008. Diagnosis followed established criteria (EC-Clearinghouse, 1993). HAART duration was described.
Khatibi <i>et al.</i> (2011), Iran	200	68.0	74.5	16.5	3.0	4.0	1.5	22.0	NHL 1.5 KS 1.0	PIG 5.5 Ulcers 2.5	Cross-sectional study. Diagnosis based on clinical appearance (except for KS e NHL whose diagnosis was histopathologically confirmed). The study evaluated other possible oral lesions-related factors (drugs and alcohol abuse, smoking habits, oral hygiene, educational status, stage of infection, transmission path).
Gaurav <i>et al.</i> (2011), India	103	40.8	80.6	EC 38.8 PC 8.7	17.5	9.7	3.9	13.6	ND	PIG 35.9 Xerost. 32.0 AC 5.8 Ulcers 4.9 VZV 1.9	Cross-sectional study. Diagnosis followed established criteria (EC-Clearinghouse, 1993). The study evaluated CD4/CD8 ratio, types and PPV of the most common lesions, correlation between HAART, viral load and HIV-related oral lesions.
Bodhade <i>et al.</i> (2011), India	399	0.0	76.7	39.3 EC 30.6 PC 12.3	11.5	8.5	5.3	10.3	NHL 0.8	PIG 19.5 Ulcers 11.8 AC 4.3 HSV 2.0 VZV 0.3 Xerost. 0.3	Cross-sectional study. Diagnosis followed established criteria (EC-Clearinghouse, 1993). Cytology for candidiasis and biopsy for unknown lesions are included. The study analyzed the prevalence of other opportunistic infections (tuberculosis, zoster, molluscum contagiosum...) and the correlation between CD4+ T-cell count and oral lesions.
Sontakke <i>et al.</i> (2011), India	124	ND	71.0	32.3 EC 8.1 PC 20.1	4.1	1.6	5.6	2.4	ND	PIG 30.6 Xerost. 16.1 Ulcers 3.2 HSV 1.6	Cross-sectional study. Diagnosis based on clinical appearance. It was not described the number of patients undergoing HAART. Evaluation of correlation between CD4 T-cell count and oral lesions was included.
Mwangosi e Tyllia (2012), Tanzania	200	100	29.0	7.5	4.0	0.5	ND	ND	KS 3.5	HSV 11.5 Xerost. 1.5 AC 0.5	Cross-sectional study. Diagnosis based on clinical appearance.
Kumar <i>et al.</i> (2014), India	126	80.0	74.6	36.5	ND	ND	ND	ND	4.8	Ulcers 11.9 Leuk. 9.8 LP 5.5	Cross-sectional study, comparing HIV-positive patients with a control group (532 subjects). Diagnosis followed WHO 1997 criteria. The study included DMFT score of patients.
Nanteza <i>et al.</i> (2014), Uganda	346	0.0	48.6	24.9 EC 8.4 PC 12.1	5.5	ND	ND	2.0	KS 9.3	PIG 17.3 SGS 3.8 HSV 1.7 Ulcers 1.5	Cross-sectional study. Diagnosis followed established criteria (EC-Clearinghouse, 1993). The study included an evaluation of the correlation between CD4+ T-cell count and prevalence of oral candidiasis.

between the incidence of HIV-related oral lesions and viral load (HIV-RNA). In this case, there was also a statistically significant correlation: a high viral load (>20.000 copies/ml) increases the risk of developing HIV-related oral manifestations (4, 26). Different studies about HIV and oral manifestation are reported in Table I.

Another topic is implant dentistry in HIV patients. In fact, since many HIV positive patients have a very long life expectancy, they require implant prosthetic rehabilitation. Problems related to survival rate of implants in positive HIV patients are a topic that requires further insights (40-46).

CONCLUSION

Oral lesions are an important sign of immunodepression and with the introduction of HAART their incidence has strongly decreased, particularly in urban areas. Nevertheless, developing countries still have a high prevalence of these manifestations because of the persistence of many risk factors, such as difficulty in accessing treatment, poor oral hygiene, low socioeconomic status and late diagnosis.

Dental surgeons may have a potential role, not only in the treatment of HIV-related oral manifestations, but also in monitoring the evolution of HIV infection, because of the strong correlation between the presence of oral lesions and a low CD4+ T-cell count (<200 cell/mm³) (7, 47).

For these reasons, an oral examination might help in the early diagnosis and in the prognosis of HIV infection, as already happens with oral cancer (2, 48-53).

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HUMAN PAPILLOMA VIRUS ASSOCIATED WITH ORAL CANCER AND PREVENTIVE STRATEGIES: THE ROLE OF VACCINES

L. OTTRIA^{1*}, V. CANDOTTO^{2*}, F. CURA³, L. BAGGI⁴, C. ARCURI⁵, M. NARDONE⁶,
R. M. GAUDIO³, R. GATTO⁷, F. SPADARI⁸ and F. CARINCI⁹

¹Dental School, Tor Vergata University, Rome, Italy; ²Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy; ³Department of Medical Sciences, University of Ferrara, Ferrara, Italy; ⁴Department of Clinical Sciences And Translational Medicine, University of Tor Vergata, Rome, Italy; ⁵Department of Clinical Sciences and Translational Medicine, University of Tor Vergata, Rome, Italy; ⁶Ministry of Health, Rome, Italy; ⁷Department of Life, Health and Environmental Sciences, School of Dentistry, University of L'Aquila, L'Aquila, Italy; ⁸Department of Medicine and Surgery, University of Milano Bicocca, Monza, Italy; ⁹Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, Italy

*these authors contributed equally to this work and they are co-first authors

The aim of this paper is to describe the efficacy of Human Papilloma Virus (HPV) vaccines for preventing oral cancer. A systematic review of the literature was conducted to describe the state of the art about HPV vaccines for preventing oral cancer. The aspects of prevention and control of infection by administering vaccines and the diffusion of sexual education campaigns are discussed also. In recent years there has been a growing interest in HPV in dentistry, suggesting a role of such a family of viruses in the development of oral cancers as well as of the uterine cervix. Even if the mass media have increasingly faced the problem, causing frequent alarming among patients, the dentist therefore needs a complete and up-to-date knowledge of this infectious condition that is one of the most common causes of sexually transmitted mucous membrane infections (eg genital, anal and oral). Recent studies about HPV infection are a basic requirement in order to promote the HPV vaccinations and patient's health.

HPV and oral cancers

Oral Cancers (OCs) are known to have a diverse etiology that includes infectious agents. More than 20% of the global cancers have an associated infectious etiology (1-7). A diverse range of viruses is implicated in human cancers, which originate in different regions of the body, including the liver, genital regions, and the oral cavity, to name a few. A significant proportion of the viral human cancers arises from the oral cavity (8, 9). The viral agents associated with OCs include: (1) human papillomavirus (HPV)-

associated with OCs; (2): Epstein Barr virus (EBV)- associated with nasopharyngeal carcinoma (NPC); (3): Kaposi's sarcoma- associated herpesvirus (KSHV) and associated with oral Kaposi's sarcoma (KS) (10, 11). HPV has been found to be associated with several types of human cancer, inclusive of cervical, vulvar, vaginal, penile, anal, and OCs (12, 13). The incidence OCsHPV related has increased in fact within the last ten years, the percentage of OCs that was HPV- positive went from less than 20% to more than 70% in the United States and

Key words: *human papillomavirus, oropharynx carcinoma, sexual transmission, oral infections, anti-HPV vaccines, HPV test*

Mailing address:

Francesco Carinci, MD,
Department of Morphology, Surgery and Experimental
Medicine, University of Ferrara,
Via Luigi Borsari 46, 44100 Ferrara, Italy
Tel.: +39.0532.455874 - Fax: +39.0532.455876
E-mail: crc@unife.it - Web: www.carinci.org

some European countries. To reduce the incidence of HPV-OCs, it is necessary to encourage research for better understanding of the pathogenesis of HPV-OCs, and to develop research for the treatment of the disease with HPV vaccines.

Squamous cells carcinoma constitutes the sixth leading malignancy globally and arises primarily in the oral cavity, oropharynx, nasopharynx, larynx, and hypopharynx (14, 15). Known etiological risk factors for OCs include tobacco use and alcohol consumption (16, 17). In the United States and some European countries, the overall incidence of OCs associated with the above etiological risk factors has declined in the past two decades, consistent with the decrease in tobacco usage in these regions. By contrast, a rapid rise in the incidence of OCs, especially oropharyngeal squamous cell cancer involving the tonsil and the base of the tongue, has been noticed in men younger than 50 years of age with no prior history of tobacco usage and alcohol consumption. These cancers were found to be specifically associated with HPV infection.

In the United States, HPV prevalence in OCs increased during the last years; similarly, increases in the incidence of HPV-associated OCs have also been reported in many European countries, including Finland, Sweden, the Netherlands, and the United Kingdom (18, 19). There are more than one hundred HPV types that can be divided into two categories, high-risk oncogenic types and low-risk non-oncogenic types. The high-risk type 16 constitutes the most prevalent HPV type globally, being detected in almost 60% to 80% of OCs. However, high-risk type 18 was found in 34% of oral cavity squamous cell cancers and 17% of laryngeal squamous cell cancers (20, 21). The HPV-associated OCs manifests different clinical and biological characteristics in comparison with the HPV-negative OCs. In addition, patients with HPV-positive OCs have a favourable prognosis in comparison with those with HPV-negative OCs, and mutations associated with tumour suppressor genes like p53 are relatively infrequent in the former (22, 23).

Although it has been established that HPV infection has increased the incidence of OCs in the United States and some European countries,

a great many ambiguities exist pertaining to the epidemiological characteristics of these cancers. The increased incidence of HPV-associated OCs could be attributable to changes in sexual behaviours, such as increased oral sex practices and more oral sex partners. However, HPV-positive OCs are also documented in patients reporting very few oral sexual partners, with almost 8-40% of the patients reporting never having had oral sex. Traditional risk factors, such as alcohol consumption and tobacco use, may still have a significant impact on HPV-associated OCs, since around 10-30% of these cancers occur in patients who smoke or consume alcohol. Thus, the contributions of these risk factors in the etiology of HPV-associated OCs cannot be undermined, and a possible synergy of HPV infection and traditional etiological factors should be investigated. In light of the above considerations, the role of vaccines is crucial to preventing infection and the onset of OCs.

Prevention of infection

There are currently three prophylactic vaccines already evaluated by randomized clinical trials, but only the last two are available: monovalent vaccine against HPV 16; direct quadrivalent equivalent vaccine against HPV 6, 11, 16 and 18; bivalent vaccine against HPV 16 and 18.

The quadrivalent vaccine has been approved by the Food and Drug Administration (FDA) in 2006 for the prevention of precancerous lesions and anogenital squamous cells carcinoma (SCC) (cervical, vaginal and vulvar) in girls ages 9 to 26 (24); it was later extended to males of the same age group for the prevention of penile and anal SCC (25). The indication of this vaccine, which also protects against genotypes LR 6 and 11, also includes the prevention of condyloma and anogenital warts. In clinical trials so far, quadrivalent vaccine has proven to be very effective in preventing SCC and premalignant lesions both in native patients (efficacy >98%) and in previously infected individuals (efficacy between 50 and 78 %).

The bivalent vaccine was only tested for cervical SCC and not for other HPV-related neoplasms and was recommended for girls aged 10 to 26 years. The percentages of efficacy in the prevention

of premalignant and malignant lesions of this mucosal region are also extremely high (>97% for native subjects) (26). However, it is important to note that the bivalent vaccine, structurally and functionally similar to quadrivalent, is also effective in preventing HPV 16 and 18-related lesions in other mucosal areas. In both cases, vaccination is tridose and currently most European countries (e.g. Sweden, Italy, France, the Netherlands, Germany, Switzerland, Denmark, Spain and Belgium) and over 70 countries around the world have free vaccination for girls aged 9-17, with a rate of about 50%. These vaccination campaigns, intended for young teenagers before sexual activity, are aimed at obtaining not only the individual immunization of the vaccinated subjects, but also of the so-called “flock immunity”, which occurs when at least 80% of the population is vaccinated against a specific infection and involves a reduced circulation of the virus and consequently less likely that the unvaccinated become ill. In order to reach this result, it is essential to extend vaccination to adolescent males, although the high cost of vaccinations is a limit, at least in developing or underdeveloped countries.

Other crucial and still debated issues concern the ideal age of vaccination. In light of both the ever-early sexual intercourse among adolescents and the need to vaccinate native individuals to obtain higher efficacy rates and extending vaccination to homosexual males who are currently not protected either individually or indirectly as group immunity and are a vulnerable category for anogenital SCC. Although there are still no data in the literature on the efficacy of HPV vaccines in reducing the incidence of SCC of oropharynx and oral cavity, it is reasonable to assume that in the long term the benefits will also be found for this category of tumours. Only in the future will it be possible to evaluate the exact impact of vaccine programs on the epidemiology of positive HPV tumours. The oral cavity is populated by a large number of bacteria species that form polymicrobial communities called biofilm. The biofilm formed by the oral microbiota includes both symbiotic and potentially pathogenic species and viruses such as HPV. In addition HPV may interact with periodontal pathogens in the onset of periodontal

diseases and peri-implantitis, that appear associated with a microbial shift, more commonly known as dysbiosis, that could be considered either a decrease in the number of beneficial symbionts and/or an increase in the number of pathogens(27-32).

In recent decades, the amount of information about HPV infection and its oncogenic potential has increased considerably, in line with advances in molecular biology techniques applied to diagnostic, preventive and therapeutic management of HPV-induced lesions. Understanding interactions between the viral agent and the host provided new targets for infection control and allowed the formulation of new-targeted therapeutic protocols. It is important to emphasize that the general population knows very little about the transmission and clinical presentation of HPV infection, largely due to the lack of informative campaigns and implementation, which is desirable in order to gain better control of the infection

In the expectation that such information and prevention programs are implemented, the role of the dentist is of great importance. Whatever the prevailing discipline to which he/she is referring, the knowledge of the notions relating to this frequent infectious condition, its natural history, clinical manifestations in the oropharyngeal district and other target sites, preventive and therapeutic measures are an important requirement in order to promote patient health and provide them with the most comprehensive indications.

Next-generation preventive HPV vaccines

There have been several approaches for the next generation of preventive HPV vaccines that have broader protection against more high-risk types of HPV at possibly reduced costs. These strategies include targeting multivalent particles like virus vaccines (PLVVs), L1 capsomers, and L2 vaccines.

Existing PLVVs strategies generate a neutralizing antibody response specific for HPV L1 capsid protein (Gardasil and Cervarix). It is now clear that each PLVVs is type-restricted, in fact to generate broader protection, additional types of HPV must be included in vaccines.

Currently, there are ongoing trials to compare

multivalent PLVVs to Gardasil, however, several parameters remain unclear: the optimal dose needed for each type of PLV in a multivalent vaccine is unclear. In addition, it is not clear whether the presence of PLVVs for one type of HPV will influence the immune response generated against another type; furthermore, it is uncertain if the duration of protection is similar for each HPV type included within the vaccine. May be that the inclusion of more types will make vaccines more costly than the current Gardasil and Cervarix; nevertheless, existing multivalent PLVVs are currently being explored, and they warrant further investigation to provide broader protection against the majority of high-risk types of HPV.

CONCLUSION

Significant steps toward the ideal prophylactic and therapeutic vaccines have been made. The effectiveness of commercially available PLVVs in generating a short-term antibody response to HPV-16 and HPV-18 is evident. HPV vaccines must decrease their cost to make them more accessible to susceptible populations in developing countries, and this will make the largest impact on the incidence of persistent HPV infection and cervical cancer. Even if a new preventive nonavalent HPV vaccine may give more protection, cost is the most pertinent issue facing future preventive vaccines.

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HYPNOTIC APPROACH DURING DENTAL TREATMENT: ANALYSIS OF DESCRIPTIVE DATA OF A CASE SERIES

R. FERDEGHINI^{1*}, D. LAURITANO^{2*}, A. TAGLIABUE^{3**} and L. TETTAMANTI^{3**}

¹Private practise, Monza, Italy; ²Department of Medicine and Surgery, University of Milan-Bicocca, Monza, Italy; ³Department of Medicine and Surgery, University of Insubria, Varese, Italy

*these authors contributed equally to this work and they are co-first authors

**these authors contributed equally to this work and they are co-last authors

Anxiety and worry are important components that affect the patient's behaviour during dental sessions and influence the effectiveness of dental treatment. Psychological approach and hypnotic approach (HA) have in recent years assumed increasing prominence as effective treatment regimes. Dentists have used a number of methods in the management of dental phobic disorders through HA. However the efficacy of HA in controlling anxiety and worry is controversial. The aim of the present study is to describe the response of patient to HA during dental treatment. The study of the population consisted of 42 patients, these being 50% random sample of consecutive patients presenting to a private practise over an 8-year period. In 38 patients HA was induced. 4 patients were not susceptible to HA. There were 20 women (52.6%) and 18 men (47.3%) with a mean age of 47.2 years (range 30-69 years). At the end of dental sessions with HA all the patients were asked to answer the following questions: 1) Have you been hypnotized in the past? 2) Were you aware of dental applications of HA? 3) How do you evaluate the use of this technique in the field of dental care? 4) Do you think that you can use this procedure in the future too? The answers were as follows: question 1: 34 patients answered yes, 4 no; question 2: 34 answered yes, 4 no; question 3: the choice was between "very useful", "useful", "Indifferent", "to avoid". Twenty-nine patients indicated "very useful", and 9 "useful"; question 4: the choice was between yes and no. All patients answered yes. This study adds further support to a growing body of evidence relating HA to a better compliance to dental treatment. The dental situation in particular lends itself to carefully controlled investigation providing further evidence to support a robust theory.

Anxiety and worry are important components that affect the patient's behaviour during dental sessions and influence the effectiveness of dental treatment. Despite the development of many new procedures these factors are still issues of major concern to many dental patients.

Although psychological suffering is not directly valuable, we do not have adequate tools to control anxiety and fear related to dental care. The need for further research into these areas is pertinent, in fact

fear causing dental avoidance in these studies ranged from 8% to 16%, which is considerably higher than the often-quoted estimates of 5-6% of some years ago (1). Dentistry has achieved great goals in recent years, but the anxiety related to the environment in a dentist's office is a global problem and, thus, poses a significant challenge for the provision of dental care. Thus, the anxiety related to dental treatment is some of the most difficult aspects for the management of dental patients.

Key words: Hypnosis, dental anxiety, dental fear, dental phobic disorders

Mailing address:

Prof. Angelo Tagliabue,
Department of Medicine and Surgery,
University of Insubria,
Via Piatti, 10, 21100 Varese, Italy
Tel.: +39.0332-825625 - Fax: +39.0332-825655
angelo.tagliabue@uninsubria.it

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Psychological approach and hypnotic approach (HA) have in recent years assumed increasing prominence as effective treatment regimes. Dentists have used a number of methods in the management of dental phobic disorders through HA. They have treated the anxiety directly and symptomatically, relying largely on relaxation and behavioural techniques. HA has also been found to be useful in more sophisticated uncovering techniques of age-regression and hypnoanalysis as part of more analytical insight-oriented psychotherapies.

HA has been found to be a relatively stable characteristic of the individual. Approximately 20% of the population is highly susceptible to HA, 20% is unresponsive and the remaining 60% is moderately susceptible to varying degrees (1). HA has been related positively to the degree of improvement in chronic pain problems, asthma, and various dermatological conditions. Recent studies provide that some specific clinical populations are characterized as more responsive to HA than the general population (1, 2).

While HA has always been a controversial subject, acceptance and understanding of this valuable tool is becoming more widespread. It is being used in many phases of medicine and dentistry and should rightly be applied in many instances where it is not. Its infrequent use is due to an inability of therapists to gain access to training programs. HA is not new. It is as old as mankind. One of the chief misconceptions of today is that HA is sleep. It is not. Other modern erroneous beliefs about HA stem from the thought that the subject becomes unconscious, loses his will power completely to the operator or may never wake up. Others feel that if a symptom is removed, another one of a negative nature will replace it. Such operators are definitely responsible for the overwhelming public opinion that HA is to be taken lightly or is something to "have fun" with. This type of application on a conscientious subject causes many people to avoid HA. Recent HA has been defined as "changed state of consciousness during which the patient's attention is focused on ideas and images that can evoke the desired effects" (3, 4).

Pain is not objectively valuable, and dentistry involves numerous painful procedures causing

psychological distress. HA is widely and often successfully used in a variety of clinical situations to modify patients' thinking, behaviour, and is one of the options being used in dentistry as a method to help the anxious subject relax and could be used to improve the level of patient cooperation by increasing confidence itself. Recently, dentistry is one of the medical fields more accepting of HA, as it has been very effective in controlling "toothache"; furthermore it is being used in oral surgery as a complement to anaesthesia, with its main tools being suggestion and speech. For this reason, HA is an option for reducing the anxiety or pain associated with dental treatment, in particular in children (5).

The aim of the present study is to describe sensations referred by the patient responsive to HA after dental treatment.

Descriptive data

The study of the population consisted of 42 patients, these being 50% random sample of consecutive patients presenting to the clinic over an 8-year period. 4 patients were not susceptible to HA. In 38 patients HA was induced.

There were 20 women (52.6%) and 18 men (47.3%) with a mean age of 47.2 years (range 30-69 years). Most patients presented with a combination of dental fears. The most common fears were fear of not being able to express or cope with feelings of helplessness and defencelessness (93.7%), fear of pain (85.3%), fear of injections (87.8%), generalized dental anxiety (73.4%), fear of drilling (75.6%) and fear of extractions (56.3%). The majority of patients, 32 (84.2%), had other phobic symptoms besides their dental phobia, i.e., multiple phobias.

HA was assessed for 38 out of the 42 patients. The others were selected out because their psychiatric problems, including schizophrenia and severe depressive illness, were contraindications for the use of HA. In 6 patients surgical therapies were performed, in 10 implant dentistry and in 20 oral hygiene.

MATERIALS AND METHODS

At the end of the dental sessions with HA all the patients

were asked to answer the following questions:

- 1) Have you been hypnotized in the past?
- 2) Were you aware of dental applications of HA?
- 3) How do you evaluate the use of this technique in the field of dental care?
- 4) Do you think that you can use this procedure in the future too?

RESULTS

The answers were as follow:

- 1) Have you been hypnotized in the past?
Excluding 4 patients who responded positively, all other patients answered no.
- 2) Were you aware of dental applications of HA?
Except for the 4 above-mentioned patients, all other patients answered no.
- 3) How do you evaluate the use of this technique in the field of dental care?
The choice was between: “very useful”, “useful”, “Indifferent” and “to avoid”.
Twenty-nine patients indicated “very useful” and 9 “useful”.
- 4) Do you think that you can use this procedure in the future too?
The choice was between yes and no. All patients answered yes.

What emerges from this preliminary study on the use of dental HA is that the potentials of the procedure are practically unknown among dental patients, particularly in regards to the benefits that HA can provide.

There is an incorrect perception of HA. Many patients fear that HA may control their will, or may be an intrusion into their private life, or they have no possibility of leaving the state of hypnosis. Television, pointing to the spectacularization of HA, is likely to have some responsibility in consolidating such fears. It would therefore be useful to alter this erroneous perception of the phenomenon in order to allow for wider diffusion of HA among dentists. In this regard, it is useful to evaluate the results of question 4, which shows that all patients, who successfully undergo HA, were ready to perform it again.

Obviously, the most satisfying result emerges from question 3. The overwhelming majority of patients considered the procedure very useful. The usefulness of HA consists of a global state of relaxation and sedation that accompanies the entire dental session, of a modified perception of pain that simulate analgesia, and can alter temporal perception of dental session, generally felt less longer than it really was.

DISCUSSION

The present study is a descriptive analysis of HA in dental patients. The aim of this research was to determine the dental anxiety and pain related to dental session, in order to ascertain whether HA combined with conventional behaviour management techniques reduces anxiety and pain.

In addition, we may assert that HA combined with conventional behaviour management techniques, is a tool more able to help patients to relax than conventional behaviour management techniques alone. HA is beneficial for the operator, as it enables optimal and more efficient clinical work, providing greater comfort and avoiding disruptive behaviours. The scientific evidence regarding the use of HA as a complement to another therapy for reducing anxiety during dental treatments is limited, thus, further research is required to objectively support the reduction of anxiety through the use of HA as a complement to other anxiety reduction techniques (3).

Similarly, as there is no evidence that HA, on its own, is capable of producing an aesthetic effect for dental procedures, it should always be combined with local anesthetic techniques or as a complement to sedation or general anaesthetic techniques (2). HA may be used for implant dentistry and perimplantitis treatment. In fact peri-implantitis is the most important delayed complication of implant dentistry (6-12). Peri-implantitis and periodontal disease spring from bacterial infection that activates a cytokines cascade leading to inflammation and bone loss (13-16). In addition, the patient-related susceptibility is a critical factor for disease onset. So, every factor favouring oral biofilm formation (poor oral hygiene), host defence capability (smoking habit, excessive alcohol

consumption, genetic traits, history of periodontitis, use of bisphosphonates), might favour developing of peri-implantitis and periodontal disease, which diagnosis and treatment require dentist's engagement (17-23).

In conclusion HA would be eminently suitable in the dental treatment. The literature is replete with accounts of techniques and a multitude of applications of HA techniques to dentistry. HA had varied periods of popularity followed by its rejection and appears to warrant far wider use than it receives. This may be related in the past to the lack of undergraduate training, misinformation, superstition, exaggerated claims, and the re-introduction of conscious sedation techniques.

CONCLUSION

Recently, the attempts to relate HA to the production of dental analgesia have ranged a moderate relationship between HA and pain reduction. This study adds further support to a growing body of evidence relating HA to a better compliance to dental treatment. The dental situation in particular lends itself to carefully controlled investigation providing further evidence to support a robust theory.

However HA must be used under strict supervision of the doctor. He chooses to work in this manner just as a physiotherapist or other technician does.

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HPV ACTING ON E-CADHERIN, P53 AND P16: LITERATURE REVIEW

L. OTTRIA^{1*}, V. CANDOTTO^{2*}, F. CURA³, L. BAGGI⁴, C. ARCURI⁵, M. NARDONE⁶,
R. M. GAUDIO³, R. GATTO⁷, F. SPADARI⁸ and F. CARINCI⁹

¹Dental School, Tor Vergata University, Rome, Italy; ²Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy; ³Department of Medical Sciences, University of Ferrara, Ferrara, Italy; ⁴Department of Clinical Sciences and Translational Medicine, University of Tor Vergata, Rome, Italy; ⁵Department of Clinical Sciences and Translational Medicine, University of Tor Vergata, Rome, Italy; ⁶Ministry of Health, Rome, Italy; ⁷Department of Life, Health and Environmental Sciences, School of Dentistry, University of L'Aquila, L'Aquila, Italy; ⁸Department of Medicine and Surgery, University of Milano Bicocca, Monza, Italy; ⁹Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, Italy

*these authors contributed equally to this work and they are co-first authors

In addition to tobacco and alcohol consumption, the two main risk factors for oral squamous cell carcinoma (OSCC), recent studies have revealed infections with human papilloma virus (HPV) as an additional risk factor for OSCC development. In the field of head and neck malignancies, the prevalence of HPV infections in oropharyngeal cancer (OC) ranges in different studies up to 84%. While HPV infection is discussed as an independent risk factor in this region, its distinguished role in carcinogenesis of tumours localized to the oral cavity remains uncertain. A systematic literature search was performed using PubMed, Cochrane library, Science Direct, and the Internet search, with language restricted to English. The search included published studies which dealt with detection methods of HPV-related oral and oropharyngeal cancers and biomolecular studies, particularly regarding the compromising of p53 p16 and e-cadherin's. P53 Tumour suppressor protein p53 has several functions that are related to maintaining genomic stability and inhibiting cell proliferation in response to DNA damage. For preventing neoplasia to occur, the most important of these functions are cessation of cell growth and induction of either apoptosis or senescence. P16 is a cellular protein involved in cell cycle regulation. It is a cyclin-dependent kinase 4 (CDK4) inhibitor, and is integral to Rb mediated regulation of G1-S phase of the cell cycle. P16 is expressed at a very low level in normal cell as Rb inhibits transcription of p16. Various detection methods ranging from immunohistochemistry (IHC) to molecular techniques have been used to determine the HPV status of HNSCC. E-cadherin, a 120 kDa Type I classical cadherin, is expressed primarily on epithelial cells. It is found on the surface of keratinocytes and Langerhans cells (LC) and E-cadherin mediated adhesion between these cell types is required for LC retention in the epidermis. It is also an important tumour suppressor protein: its loss or inactivation is associated with epithelial-to-mesenchymal transition (EMT), a process involving dedifferentiation, infiltration and metastasis of tumours. Oral carcinogenesis is a multi-factorial process involving socioeconomic, environmental and microbial factors leading to multistep changes. Smoking and tobacco exposure seems to modify the survival and recurrence of HPV positive tumours and should be considered in future trials for risk stratification of HPV positive patients. HPV associated oropharynx cancer represents a distinct clinical and biologic entity with many unresolved

Mailing address:

Francesco Carinci, MD,
Department of Morphology, Surgery and Experimental Medicine,
University of Ferrara, Via Luigi Borsari 46, 44100 Ferrara, Italy
Tel: +39.0532.455874 - Fax: +39.0532.455876
e-mail: crc@unife.it - Web: www.carinci.org

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issues that will be investigated in future translational, clinical research. We need to further explore and understand why the disease occurs predominantly in males, and whether the natural history of oral HPV infection differences in men and women.

Key-words: e-cadherin, HPV, oral and head squamous cell carcinoma, P16, P53

Cancers occurring in the oral cavity account for 2-3% of all malignancies. Incidence of oral cancer has been increasing in most regions of the world. Tobacco smoking and alcohol consumption are the major causative factors for head and neck squamous cell carcinoma (HNSCC) (1-6). The exact role of human papilloma viruses, (HPVs) in the case of oral squamous cell carcinoma (OSCC) and premalignant, continues to be a debated topic despite the well-established fact that the vast majority of cervical squamous cell carcinoma of uteri was attributable to HPV infection (7). Across the world, HPV infections are the most common, sexually transmitted viral infections (8).

The HPV is a double-stranded DNA virus and its genome contains eight open-reading frames, which are potential coding sites of six early (E) proteins and two late (L) proteins (9). Over 120 types of HPVs have been identified to date (8). Most of the HPVs that infect human mucosae belong to alpha papilloma viruses, which include 15 species (9).

HPV infections have been postulated as a possible risk factor for at least some varieties of oral cancer. Quite a few studies have detected the DNA of HPV in a considerable proportion of malignant tumours of the head and neck cancers, with rates of detection ranging from 0% to 100 % (10).

Various epidemiological studies have evaluated the relation between HPV infection and OSCC using techniques which detect only viral DNA using polymerase chain reaction (PCR), etc., only serological assays using enzyme-linked immunosorbent assay (ELISA), etc., or using both techniques. There were only a few studies which focused on evaluating the reliable diagnostic method in the detection of HPV-related oral and oropharyngeal cancers (10). For almost 2 decades, papillomaviruses have constituted their own virus family of papillomaviridae. Originally, they were

lumped together with the polyomaviruses in one family, the papovaviridae. After recognition of the complete differences in their genome, they were officially recognized by the International Committee on the Taxonomy of Viruses (ICTV) as two separate families. The distinct mechanism of HPV-induced malignancy is degradation of the tumour suppressor p53 by the viral protein E6 and binding of pRb by E7 with consecutive degradation through the ubiquitin-proteasome pathway. Recent studies have noticed an increased frequency of HPV positive oropharyngeal cancers (OCs) (11) even describing HPV-associated OCs as its own tumour entity.

A systematic literature search was performed using PubMed, Cochrane library, Science Direct and Internet search, with language restricted to English. The search included published studies which dealt with detection methods of HPV-related oral and oropharyngeal cancers and biomolecular studies, particularly regarding the compromising of p53 p16 and e-cadherin's. The dates taken into account went from January 2012 to today. Literature search was done using keywords such as oral cancer and HPV, oropharyngeal cancer and HPV and detection method of HPV-related oral cancers.

Articles that were not published in English or where the full text could not be acquired were excluded from the search. Case reports, reviews, non-RCTs, non case-control studies, and articles that included only cases that tested positive for HPV, studies based on pediatric population, animals and *in vitro* studies were not included in the study.

P53

Tumor-suppressor protein p53 has several functions that are related to maintaining genomic stability and inhibiting cell proliferation in response to DNA damage. To preventing neoplasia occurrence, the most important of these functions is cessation of

cell growth (12, 13) and induction of either apoptosis (14, 15) or senescence (16, 17). P53 performs these functions both by acting as a transcription factor (inhibiting or stimulating the transcription of specific genes) and by acting directly on various elements of the regulatory machinery of the cell cycle (18). Under normal conditions, the protein is expressed only in small quantities and with a very short half-life (only a few minutes) and is normally undetectable by methods such as immunofluorescence. When mutated or inactivated, it becomes unable to impede the progression of the carcinogenic process and begins to accumulate in cells thus, can be easily identifiable.

In contrast to HPV-negative HNSCC, p53 is predominantly wild type in HPV-positive HNSCC (19, 20). However, high-risk HPV E6 inactivates p53 through two distinct mechanisms. E6 associates with E6AP to degrade p53 through the proteasome pathway and associates with p300 to block p300-mediated p53 acetylation (21). Acetylation of p53 enhances p53 stability, and transcriptional activity (22-26). Inactivation of p53 by E6 is indispensable for HPV-mediated tumorigenesis, suggesting that reactivation of p53 may be a strategy to ablate HPV-positive carcinoma cells. Several genetic and chemical strategies to reactivate p53 have been demonstrated in HPV-positive cervical carcinomas. Most of these approaches focused on targeting E6 levels, E6AP levels, or E6-E6AP association to increase p53 stability and accumulation (27). Crystal structural analyses of the effect of p53 mutations on DNA binding classifies p53 mutations into two distinct types, disruptive mutations and non-disruptive mutations, according to their ability to knock out p53 function (14, 15).

Disruptive mutations are non-conservative mutations occurring within the core DNA binding domain L2/L3, stop mutations, or frameshift mutations and thus entirely abrogate p53 function. All other mutations defined as non-disruptive mutations affect p53 function in a limited fashion. It has been previously shown that HPV-positive oropharyngeal carcinoma is less likely to harbor p53 mutations as compared with HPV-negative oropharyngeal carcinoma (16). Recently, an inverse relationship between HPV infection and

disruptive mutations in oropharyngeal carcinoma has been reported, though in a small number. Of note is an increasing HPV prevalence in oropharyngeal carcinoma over time, which currently exceeds 70% in the USA and 90% in Europe (19, 20). Human papillomavirus-positive tumors are significantly less frequent in black patients than in white patients. Despite these findings, the prevalence and pattern of HPV infection and p53 mutations in HNSCC among Japanese patients remain less well established.

P16

Human papillomavirus (HPV), most frequently HPV type 16, has been identified in 40-80% of oropharyngeal cancers in the United States (28). HPV-positive squamous cell carcinomas of the head and neck (HNSCC) most commonly arise in the lingual and palatine tonsils (29). Patients are more likely to be non-smokers and non-drinkers than their HPV-negative counterparts are, although HPV-positive HNSCC may also be seen in heavy smokers and drinkers, and are younger than patients with tobacco-associated lesions. Oral sex and increased numbers of sexual partners are also considered major risk factors. P16 is a cellular protein involved in cell cycle regulation. It is a cyclin-dependent kinase 4 (CDK4) inhibitor, and is integral to Rb mediated regulation of G1-S phase of the cell cycle. P16 is expressed at a very low level in normal cell as Rb inhibits transcription of p16. Various detection methods ranging from immunohistochemistry (IHC) to molecular techniques have been used to determine the HPV status of HNSCC. P16 IHC has been regarded by some authors as a cost effective and practical surrogate marker of HPV status in HNSCC (26-29). However, there is no clear guidance on the definition of a "positive" p16 IHC result. Some studies have used a cut-off of 70% of tumour cells expressing diffuse, nuclear and cytoplasmic p16 (29) while others have developed a 3-tier scoring system akin to Her2/ neu IHC in the breast (30-33).

The correlation between p16 and HPV in oral cavity and oropharyngeal cancers has been so close that p16 is considered to be not only a surrogate marker for HPV infection, but also a prognostic factor in SCCHN (34).

Retrospective studies have signalled improved outcomes in HPV associated SCCHN especially in oropharyngeal cancer (35).

Wilson et al. (36) investigated the association between hypo-pharyngeal carcinoma and p16 INK4A status. Twenty-seven patients with hypo pharyngeal carcinoma treated with radio-chemotherapy (22 of 27 patients) or radiotherapy alone (5 of 27 patients) included in this study. They found p16 INK4A-positivity in 9 patients. There was no significant difference in OS, DFS and loco-regional control (LRC) between p16 INK4A- positive or p16 INK4A-negative groups. They also demonstrated that p16 INK4A was not a useful prognostic biomarker in hypopharyngeal cancer (36). Also, there is lack of information regarding the prognostic significance of p16 INK4A and HPV in nasopharynx carcinomas other than oral cavity and oropharynx cancers too.

Only two studies conducted in India exhibited correlation between HPV and p16 status in oral cancer (28, 37). Vokes et al. (38) detected p16 in 85.71% cases of OSCC and suggested p16 as a surrogate marker for HPV presence in OSCC and OED; however, the sample size was much less (n=20) for establishment of p16 as surrogate marker. The other study by Prakash et al. (39) also identified a high positivity of p16 in OSCC (71%), but they did not detect HPV in these cases. So overexpression of p16 cannot be suggested due to HPV infection.

E-cadherin

As previous said, the majority of cases of oral cancer are a consequence of infection with high-risk, oncogenic human papillomaviruses (HPV). These small, non-lytic, non-enveloped, dsDNA viruses are tropic for squamous epidermis (40). The two viral proteins E6 and E7 from high-risk HPV types are the major oncogenes and are necessary for the induction and maintenance of the transformed phenotype (41). The E6 open reading frame (ORF) encodes an 18 kDa protein containing four Cys-X-X-Cys motifs, which form two zinc finger structures. E6 manipulates a range of cellular functions important in viral genome amplification, replication and persistence in the host, including inhibition of apoptosis as a result of degradation of p53 (42) and increased genomic

instability mediated by activation of hTERT (43). There is increasing evidence that E6 also affects cell adhesion and polarity, via targets such as hDlg, MAGI, hScrib and E-cadherin (41). E-cadherin, a 120 kDa Type I classical cadherin, is expressed primarily on epithelial cells (42). It's found on the surface of keratinocytes (43) and Langerhans cells (LC) and E-cadherin mediated adhesion between these cell types is required for LC retention in the epidermis. It is also an important tumour suppressor protein: its loss or inactivation is associated with epithelial-to-mesenchymal transition (EMT), a process involving dedifferentiation, infiltration and metastasis of tumours. Furthermore, in in vitro studies, surface E-cadherin expression is reduced on cells expressing E6 or E7, implicating these proteins in its regulation (40). Although E7 is reported to repress E-cadherin by augmenting DNA methyltransferase 1 (DNMT1) activity (41), no pathway for E6 regulation of E-cadherin has yet been described. Our objective is to elucidate the mechanism by which E6 regulates E-cadherin, in order to gain an understanding of how HPV16 controls this important cell adhesion and tumour suppressor protein.

Transcriptional repression of E-cadherin has been widely reported in cancer cells, through repression of the E-cadherin promoter, and this might be the case in cells expressing HPV16 E6. On measuring the levels of E-cadherin transcript in E6 expressing cells, they were found to be reduced by around half compared with the parental HCT116 cell line.

In addition to its tumour suppressor function, E-cadherin is an important immune regulatory protein in the epidermis, where it regulates adhesion between LC and keratinocytes. We have previously reported that E-cadherin expression is reduced or lost in HPV infected tissues and that E6 has a role in its reduced expression. In this study, we provide the first evidence for reduced levels of transcripts in E6 cells resulting from E-cadherin promoter repression and go some way towards establishing how this regulation might occur.

CONCLUSIONS

Oral carcinogenesis is a multi-factorial process

involving socioeconomic, environmental and microbial factors leading to multistep changes. We observed that HPV infection was associated with significant number of cases and was found to be an independent risk factor as well as prognostic factor for OSCC. This might be useful for individualization of the therapeutic strategies in HPV related tumours. p16 is a surrogate marker for the virus and is technically less challenging to perform and hence can be utilised for determining the viral status. While our current knowledge of epidemiology, natural history and prevention of oral HPV infection remains limited, studies show that oral HPV infection is sexually transmitted and that male gender, current tobacco use, and HIV infection are associated with increased prevalence.

In summary, tumor HPV status is a prognostic factor for overall survival and progression free survival and may also be a predictive marker of response to treatment. The method of *in situ* hybridization provides a feasible tool for implementation in most diagnostic pathology laboratories; and immunohistochemically staining for p16 may be useful as a surrogate marker for HPV status. It appears that loco regional recurrence but not the rate of distant disease is lower in the HPV positive patients. Smoking and tobacco exposure seems to modify the survival and recurrence of HPV positive tumours and should be considered in future trials for risk stratification of HPV positive patients. HPV associated oropharynx cancer represents a distinct clinical and biologic entity with many unresolved issues that will be investigated in future translational, clinical research. We need to further explore and understand why the disease occurs predominantly in males, and whether the natural history of oral HPV infection differences in men and women (44).

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MECHANICAL, CHEMICAL AND BIOLOGICAL ASPECTS OF TITANIUM AND TITANIUM ALLOYS IN IMPLANT DENTISTRY

L. OTTRIA^{1*}, D. LAURITANO^{2*}, M. ANDREASI BASSI³, A. PALMIERI⁴,
V. CANDOTTO⁵, A. TAGLIABUE^{6**} and L. TETTAMANTI^{6**}

¹Dental School, Tor Vergata University, Rome, Italy; ²Department of Medicine and Surgery, University of Milano Bicocca, Monza, Italy; ³Private Practitioner in Rome, Italy; ⁴Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna, Bologna, Italy; ⁵Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy; ⁶Department of Medicine and Surgery, University of Insubria, Varese, Italy

* these authors contributed equally to this work and they are co-first authors

**these authors contributed equally to this work and they are co-last authors

Implant dentistry has become a popular restorative option in clinical practice. Titanium and titanium alloys are the gold standard for endo-osseous dental implants production, thanks to their biocompatibility, resistance to corrosion and mechanical properties. The characteristics of the titanium implant surface seem to be particularly relevant in the early phase of osseointegration. Furthermore, the microstructure of implant surface can largely influence the bone remodelling at the level of the bone-implant surface. Recently, research has stated on the long-term of both survival and success rates of osseointegrated implants and mainly on biomechanical aspects, such as load distribution and biochemical and histological processes at the bone-implant interface. This short review reports recent knowledge on chemical and mechanical properties, biological aspects, innovations in preventing peri-implantitis, describing clinical applications and recent improvements of titanium dental implants. In addition, it highlights current knowledge about a new implant coating that has been demonstrated to reduce the number of initially adhering bacteria and peri-implantitis.

Titanium characteristics

The characteristics of the titanium implant surface (TIS) seem to be particularly relevant in the early phase of osseointegration, furthermore implant microstructure influences the bone remodelling at level of the bone-TIS (1-10).

Recent studies have reported higher bone-to-implant contact (BIC) percentages in rougher TIS than in machined surfaces (11). Irregular morphologies of TIS allow significantly higher levels of cellular attachment in osteoblast-like cells and may play a crucial role in biomolecular

adsorption and cell adhesion to the TIS as well as in osteoblast cells maturation (12). In fact, the success of TIS is highly related to the surface properties of the implant materials that influence molecular interactions, cellular response and thereby, bone regeneration. Mesenchymal stem cell involvement, cell-cell communication at the bone-TIS and in particular interactions between the surface oxide and the biological host, are the underlying mechanisms of osseointegration (13-16).

In particular, improved biological host response (e.g. increased cell proliferation and cell activities,

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Mailing address:

Prof. Angelo Tagliabue,
Department of Medicine and Surgery,
University of Insubria, Via Piatti, 10, 21100 Varese, Italy
Tel.: +39.0332-825625 - Fax: +39.0332-825655
e-mail: angelo.tagliabue@uninsubria.it

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higher mRNA expression for osteoblast markers and enhancement in matrix mineralization) has been reported as an effect of interaction with TIS (17-19). Moreover, it was reported that different microtopography of TIS may induce mesenchymal stem cell (MSCs) differentiation at a more accelerated rate, compared to similar material with a smooth surface (19). To achieve osseointegration of TIS, rougher surfaces seemed to improve the *de novo* bone formation due to the early surface adhesion of non-collagenous proteins such as osteopontin and bone sialoprotein (20). Subsequently, calcium phosphate nucleation at the calcium binding sites on these proteins continues the process of osseointegration of TIS, which is completed by crystal growth and collagen production with mineralization (21,22).

Different studies on the TIS micromorphology are difficult to compare due to the variability of surface treatment methods, sources of cells and the experimental methods. Perrotti et al. described fractal dimension of TIS with rough surfaces and concluded that roughness organization rather than roughness amplitude could be crucial in differentiating rough surfaces with different potentials of osseointegration (23).

TIS of oral implants have been modified to play an important role for cellular reactions, tissue healing and implant stability (24,25). Different methods such as machining, air-abrasion, acid etching, and electrochemical oxidation and laser treatment were applied to model TIS at various dimensions.

TIS corrosion can occur if titanium is put in contact with anoxic acid media, with a complete dissolution of anodic oxide (26,27). To overcome these problems, typically the TIS are air-abraded with aluminium trioxide particles (27). Blasted TIS demonstrated better bone integration than turned/machined implants. In addition, blasted TIS are also etched in some types of implants. In contrast to animal studies, clinical studies didn't find any advantages with blasted TIS when compared with turned implants (28).

Biological aspects

Bone regeneration around oral TIS is a process that comprises inflammation, regeneration and

remodelling with possible overlap of all these phases. TIS influence bone healing at cellular and molecular level. In fact, the implant itself acts as an osteoconductive substrate decreasing the size of the defect bridged by new tissues. The TIS influences the initial sequences of protein adsorption, platelet adhesion, haemostasis, inflammation and osteogenic cell response (29). Further studies should define physic-chemical properties of the TIS interacting with cellular pheno- and genotypes, so as the molecular mechanism with which the cells are recruited and become adherent to the surface. Moreover, the cell-cell communication during the early phase of osseointegration of TIS requires better understanding (29).

Immunohistochemistry and SEM analysis show gene expression of cells adherent to TIS during the first hours and days after implantation (30,31) with higher expression of monocyte chemo attractant protein-1 (MCP-1) coupled with higher expression of pro-inflammatory cytokines TNF- α (3 h and 1 day after implantation) and IL-1 β (1 day and 6 days after implantation) at machined TIS. On the other hand, the expression of the chemokine receptor CXCR4, a receptor for stromal derived factor-1 α (SDF-1 α), was highly expressed at oxidized surfaces of TIS as early as 12 h after implantation. Coexistence of monocytes/macrophages and mesenchymal stem cells (MSCs) at the interfacial region with predominance of MSCs at the oxidized TIS has been identified. It has been also shown that early peak expression of SDF-1 α during the first day after surgery was associated with the highest MSCs at the TIS level. The role of SDF-1 α /CXCR4 chemotactic axis in mediating the recruitment of progenitor cells is of current research interest to reveal the mesenchymal cell recruitment to different sites of osseointegration (31).

Cell attachment to the TIS is one of the critical first steps in the cell response to a biomaterial. The cellular attachment to TIS is mediated through a protein-rich layer through adhesion receptors including the integrins. Osteoblasts-like cells near TIS revealed up regulation of integrin β 1 during the 24 h of osseointegration (30).

Inflammation at the bone-TIS has not received much attention as that given to the soft tissue-

TIS. Histological studies in bone revealed that macrophages and multinucleated cells are present in machined TIS (32) as well as HA-coated TIS (33) during the early stage after implantation. These cells are known to express a wide range of pro-inflammatory and anti-inflammatory cytokines, growth and differentiation factors and chemotactic mediators. Major pro-inflammatory cytokine, TNF- α , was unregulated after 3 hours at the machined TIS compared with oxidized TIS (30). Higher expression was also observed after 1 day. Down regulatory effect was observed on the expression of IL-6 at TIS blasted with TiO₂ particles and subsequently treated with hydrofluoric acid (34).

Many cell types, including osteoblasts, can express these cytokines and growth factors, so it is important to define which cell type is responsible for these changes in gene expression. For instance, the expression of IGF-1 was also up regulated at TIS level during the eight-week evaluation period. *In vitro* studies have demonstrated that monocyte cell line expressed BMP-2 (35) that contributed principally to the osteogenic differentiation. However, *in vivo* data are not available showing the expression of BMP-2 from monocytes at TIS level. Research based on antibody-labelling strategies such as immunohistochemistry and fluorescence assisted cell sorting are suggested to verify these findings (36).

The regulation of gene expression at TIS clinically is a complex phenomenon. The TIS properties possibly influence the gene expression by affecting transcription factor, such as RUNX2, in the differentiation of mesenchymal cells towards the osteoblastic lineage. This factor has also been shown to contribute to the osteoclastic differentiation (37). The higher expression of osteoblast markers alkaline phosphatase (ALP) and osteocalcin (OC) and osteoclast marker cathepsin K (CATK) was similar to a higher expression of RUNX2 at the oxidized TIS compared with machined ones after 3 days of osseointegration (31). Similar results were demonstrated for acid etching TIS in comparison to surfaces without acid etching. Although all of these studies suggest influence of the different TIS properties on the expression of critical switching

factors, it is still not clear in which way and which specific surface properties contribute to such effects.

Recent studies on early osseointegration of TIS (hours-days) have demonstrated that the upregulation of genes responsible for bone formation ALP and OC was coupled with up regulation of genes expressed by osteoclasts, indicating that the bone remodelling phase is triggered much earlier than what has previously been assumed (31). Osteogenic cells and osteoclasts show a strictly cross talk between each other. For instance, the surface receptor RANK on osteoclasts recognizes and binds to osteoblast membrane-associated factor (RANKL) during the osteoclastic differentiation from the monocytic lineage (37). In fact, bone remodeling begins during the first days after osseointegration and continues over time. Further studies on gene expression of osteoblasts in relation to properties of the TIS are desirable to establish possible changes at implant-bone interface.

Others studies showed that different titanium surfaces led to osteoblasts recruitment, maturation, and differentiation, thus promoting osseointegration at the tissue-implant interface (1). In addition these studies demonstrated that titanium nanotubes could lead to osteoblast differentiation and extracellular matrix deposition and mineralization in dental pulp stem cells by the activation of osteoblast related genes SPP1, FOSL1 and RUNX2 (9,38-44).

Peri-implantitis

Peri-implantitis can happen with high frequencies in patients affected by periodontal diseases (8,11,45-63) (64-67) (68), after cancer resection (69) and in some syndromic diseases (70).

Peri-implantitis is caused by the formation of a biofilm at TIS level and by compromised immune ability at the bone-TIS. The biocompatibility of TIS can be attributed to a surface protein layer formed under physiological conditions (71).

This protein layer actually makes the TIS suitable for bacterial colonization and biofilm formation. Biofilms are defined as a microbial derived sessile community characterized by cells irreversibly attached to a substratum, interface or to each other, embedded in a matrix of extracellular

polymeric substances that they have produced, and exhibiting an altered phenotype with respect to growth rate and gene transcription (72). The role that biofilm plays at TIS level in developing peri-implantitis is well documented (73, 74). The biofilm protects adherent bacteria from the host defence system and bactericidal agents via several proposed mechanisms (75). The host immunity ability on the TIS is consequently impaired. The time immediately after surgery is the most favourable for developing infection at TIS level, in fact the local defence system is severely disturbed by the surgical trauma. Even after osseointegration the small number of blood vessels at TIS level compromises local immunity (76). The reduced defence mechanism facilitates biofilm formation and infection may occur. Even if different aseptic protocols have been proposed to reduce bacterial leakage at TIS level, there is still evidence that bacterial invasion usually occurs after surgery (77). Bacterial contamination can also arise from haematogenous sources later on (78). The nanostructured surface of biocompatible materials strongly influences the adhesion and proliferation of biofilm at TIS level. The observation of this phenomenon has led to an increased effort to develop new strategies to prevent bacterial leakage and biofilm formation, primarily through nano engineering of TIS. Bacterial species *Staphylococcus aureus* and *Escherichia coli* interaction with nanostructured TIS showed an increase in adhesion and biofilm formation with increasing nano scale morphological properties (72,73,78).

As previously stated, bacterial adherence to implants is considered to be an important event in the pathogenesis of bacterial infections. In fact, this infection process is a first stage of peri-implant mucositis and peri-implantitis, and a positive correlation has been found between oral hygiene and marginal bone loss around implants in the edentulous mandible. Surface properties of trans gingival implant components are important determinants in bacterial adhesion. In addition, coating characteristics of dental implants such as composition and topography regulate cell response during implant healing. In fact, the characteristics of

the implant surface seem to be particularly relevant in the early phase of peri-implant bone healing, and the bone tissue microstructure is mainly related to the remodeling processes at level of the bone-TIS. The modification of the surfaces or the use of different materials has been shown to play a relevant role in the bacterial adhesion to implant surfaces (74,79). Bacterial adherence to implants is considered an important event in the pathogenesis of bacterial infections and the infectious process can be viewed as a stepwise process in which the bacteria must first adhere to an implant surface. The failure of adhesion would result in their being swept away in the fluids, which constantly bathe the tissue surface. Surface properties of trans gingival implant components are important determinants in bacterial adhesion. The study of correlation between TIS and periodontal disease could improve also the battle against peri-implantitis (55, 57, 75, 80-84). In fact, dental implants had a great success in the last decades for replacing missing teeth in partially or totally edentulous patients. Even if the main factor for implant dentistry success is the quality of bone of receiving sites, TS could be an important co-factor in developing peri-implantitis.

Recently a new implant coating has been produced called Anatase-Bactercline®. Bactercline® is essentially an aqueous suspension of titanium dioxide nanoparticles functionalized with organic and inorganic ions with antibacterial, virucidal and fungicidal properties. Silver ions, stabilized in their one electron oxidized form, are covalently bound to high surface area titanium dioxide nanoparticles, acting in synergy with cationic species, which improve the bactericidal, fungicidal and virucidal effect of the product. Bactercline® is a new disinfecting formulation that has passed the examination of the Italian National Institute of Health and is now classified as Presidio Medico Chirurgico N. 19258. This product was applied on the healing screws by dip coating after the deposition of an anatase layer. This new screw coating has been demonstrated to reduce the number of initially adhering bacteria, formed mainly of microorganisms gram positive, while, on the contrary, the micro flora covering the titanium

alloy healing screws was, for the most part, gram negative (55). In conclusion, the patient's local and systemic conditions represent a relevant factor for the success of the implant therapy (85-99).

CONCLUSIONS

Titanium, based on its physical, chemical and biological properties, appears to be especially suitable for dental implants and prostheses. For the production of dental implants, titanium and its alloys have become well accepted and can be considered the golden standard. Surface activation or tuning of titanium surfaces certainly will improve biological integrity in compromised situations, increasing clinical service of implant therapies even further. Titanium chemical and mechanical characteristics, however, have limited usefulness in fixed and removable prostheses in dentistry. For crown and bridge prostheses, dentists can consider titanium and its alloys as a viable options to more traditional noble and base metal alloys, but careful selection of processing methods and laboratory skill is necessary to ensure the therapy success.

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DEPRESSION AND DISTRESS IN BURNING MOUTH SYNDROME: A CASE CONTROL STUDY

D. DI STASIO^{1*}, V. CANDOTTO^{2*}, R. SERPICO¹, R. MIGLIOZZI³, M. PETRUZZI⁴,
M. TAMMARO¹, C. MAIO¹, P. GRITTI³, D. LAURITANO^{5**} and A. LUCCHESI^{1**}

¹Multidisciplinary Department of Medical-Surgical and Dental Specialties, University of Campania Luigi Vanvitelli, Naples, Italy; ²Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy; ³Department of Mental and Physical Health and Preventive Medicine, Second University of Naples, Naples, Italy; ⁴Interdisciplinary Department of Medicine, University of Bari, Bari Italy; ⁵Department of Medicine and Surgery, University of Milano Bicocca, Monza, Italy

*these authors contributed equally to this work and they are co-first authors

**these authors contributed equally to this work and they are co-last authors

To compare the results of psychometric tests in patients with BMS and controls, participants were tested for depression, anxiety, fatigue and distress. Patients with BMS had noticeably higher scores for depression, fatigue and distress, compared to controls. Depression and distress were significantly correlated with the burning symptom. Moreover, distress and burning symptoms proved to be interdependent. Depression seems to play a specific role in BMS. Burning symptoms affect quality of life of patients and they could be a predictor of distress.

Burning mouth syndrome (BMS) is a condition characterized by a chronic sensation of burning and stinging in the oral mucosa without accompanying abnormal clinical or laboratory finding (1-4). The pathogenesis of BMS is poorly understood, and several theories exist (5, 6). Discomfort reported by the patient can significantly influence the quality of life (7). In recent years, much of the work on the BMS have placed emphasis on the possible comorbidity with anxiety disorders (8), mood disorders or personality disorders (6, 9).

Patients with BMS significantly exhibit symptoms of somatization (10). The psychological analysis of the subject should never be underestimated; several studies have shown an association with distress (11, 12), cancer-phobia, anxiety and depression (13-19) and sleep disorders (20, 21), nevertheless it is difficult

to determine whether the psychiatric manifestations are primary or secondary to the disorder or whether they are part of the syndrome itself (15, 22-25). The aim of this study is to compare the results of psychometric tests in patients with BMS and controls. In particular, the participants were tested for depression, anxiety, fatigue and distress.

MATERIALS AND METHODS

This study evaluated 77 patients attended at the Oral Pathology clinic of the University of Campania "Luigi Vanvitelli". The BMS group consisted of 53 patients, 44 women and 9 men. The control group consisted of 20 women and 4 men. This study was approved by the local Human Research Ethics Committee. All participants provided signed informed consent forms.

Key words: burning mouth syndrome, depression, distress, anxiety, fatigue

Mailing address:

Prof. Rosario Serpico,
Multidisciplinary Department of Medical and Dental Specialties,
Second University of Naples,
Via L. De Crecchio 6, 80138 Naples, Italy
Tel.: +39 081566 7674 - Fax: +39 081566 7674
e-mail: rosario.serpico@unicampania.it

Inclusion and exclusion criteria

To be included in the study, patients had to have a diagnosis of BMS. Patients were excluded if they had local or systemic problems, including use of drugs like angiotensin-converting enzyme inhibitors, tricyclic antidepressants and anticholinergics.

The control group consisted of patients seen in the Dental Clinic of University of Campania “Luigi Vanvitelli” in the Department of Oral Pathology. They were not receiving any analgesic treatment at the time of study and the patients in the group had no history of chronic pain syndrome or concomitant oro-facial pain.

Assessment

The study group and the control group were evaluated by collecting demographic data (age, gender, employment) and medical history (disease history, clinical features and previous psychiatric diagnosis), as shown in Table I. Visual analogue scale (VAS) was used to assess the intensity of the “burning” symptom (26). For the prevalence and intensity of depressive symptoms, Hamilton Depression Rating Scale (HAM-D) was administered (27) and the assessment of trait and state anxiety was performed using the STAI (State-Trait Anxiety Inventory) Form Y (28) (cut-off for this scale is 40).

For the first time, in this study the Distress Thermometer (DT) was administered to BMS patients (29), to measure the emotional distress related to the presence of a chronic disease and problems related to it and for the evaluation of Fatigue, the Brief Fatigue Inventory (BFI) was used (30). Spearman’s coefficients were calculated for the variables of psychometric scales and VAS in the BMS group where there was a correlation and a linear regression analysis was performed.

RESULTS

Out of 53 patients selected for this study with diagnosis of BMS, only 25 subjects, 23 women (92%) and 2 men (8%) agreed to perform psychometric tests.

The control group consisted of 24 patients, 20 women (83.4%) and 4 men (16.6%). The mean (SD) duration of symptoms in BMS group was 59.12 (49.41) months. The mean (SD) VAS score was 6.22 (2.13). Patients with BMS and controls did not differ

in gender distribution or age, occupational data and in previous psychiatric diagnosis as shown in Table I. The severity of depressive symptoms was higher in patients with BMS compared to controls ($p=0.020$) as well as the mean score of the BFI ($p<0.001$) and DT values ($p<0.001$).

Patients with BMS did not show higher scores in both scales of the State-Trait Anxiety Inventory, in comparison with control group (Table I). Severity of burning sensation (VAS) was successfully correlated only with depressive symptoms (HAM_D) ($r=0.437$; $p=0.029$) and Distress (DT) ($r=0.697$; $p<0.001$) as well as these last between them ($r=0.605$; $p=0.001$); however when linear regression model is performed, only Distress and VAS scores were interdependent ($\beta=0.723$; $p<0.001^{**}$) (Table II).

DISCUSSION

The results presented in this work are in line with those reported in other literary studies. The subjects of our sample in fact, have an important association of psychiatric symptoms (14, 15, 31, 32) and oral burning condition, with higher prevalence for depressive disorders and anxiety. These observations therefore seem to suggest the importance of assessing the psychological profile of patients with BMS as a diagnostic therapeutic act essential to the setting. For the first time, we had a significant correlation between “burning” symptom intensity, depression and distress. Furthermore, a greater intensity of the “burning” symptom has proven to be a predictor for the development of distress.

CONCLUSIONS

These results corroborate clinical observations found in previous studies on the role of psychiatric disorders in the pathophysiology of BMS. However, disputes may persist regarding the significance of the presence of these disorders in the clinical course of the syndrome. It is important, moreover, to emphasize the great impact that these disorders have on the quality of life of patients and it would therefore be desirable that dentists suggested a psychiatric consultation for these patients. In the presence of

Table I. Demographic, clinical data and scores from BMS patients and controls in different psychometric scales.

	BMS (n=25)	Controls (n=24)	p value
Age, mean (SD), years	61 (10.9)	64.9 (10.2)	0.195*
Gender (M/F)	02:23	04:20	0.312**
Duration symptoms, mean (SD), months	59,12 (49.41)		
VAS, mean (SD)	6,22 (2.13)		
Homeworkers (%)	16 (64%)	6 (25%)	0,309***
Previous psychiatric diagnosis	12 (48%)	11 (46%)	0,682**
Hamilton Rating Scale for Depression, mean (SD)	9,68 (7.0)	5.46 (5.2)	0.020*
STAI-State, mean (SD)	53 (9.63)	51.20 (3.76)	0.422*
STAI-Trait, mean (SD)	52.20 (11.10)	51.33 (4.01)	0.718*
Brief Fatigue Inventory, mean (SD)	6.83 (1.95)	1.24 (2.33)	<0.001*
Distress Thermometer, mean (SD)	7.32 (2.15)	3.33 (2.80)	<0.001*

*: T-Test; **: Fisher exact test; ***: Chi-square test.

Table II. Spearman's correlation analysis of psychometric scales that held statistically significant differences in patients with BMS and linear regression model.

Variables	Spearman's coefficient	p value	linear regression (p value)
HAM_D X BFI	(-)0.300	0.26	
HAM_D X VAS	0.437	0.029*	0.208 (0.329)
HAM_D X DISTRESS	0.605	0.001**	
VAS X DISTRESS	0.697	<0.001**	0.723(<0.001**)
VAS X BFI	0.263	0.325	
DISTRESS X BFI	0.144	0.596	
duration of syptoms X HAM_D	(-) 0.60	0.775	
duration of syptoms X VAS	0.011	0.960	
duration of syptoms X DISTRESS	(-)0.91	0.664	
duration of syptoms X BFI	(-)0-90	0.740	

*: Correlation is significant at 0.05 (2-tailed); **: Correlation is significant at 0.01 (2-tailed).

clinical, significant psychiatric symptoms, which could interfere in the treatment of BMS, patients should be properly addressed and treated.

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PSYCHIATRIC DISORDERS IN ORAL LICHEN PLANUS: A PRELIMINARY CASE CONTROL STUDY

D. DI STASIO^{1*}, D. LAURITANO^{2*}, P. GRITTI³, R. MIGLIOZZI³, C. MAIO¹, G. MINERVINI¹,
M. PETRUZZI⁴, R. SERPICO¹, V. CANDOTTO^{5**} and A. LUCCHESI^{1**}

¹*Multidisciplinary Department of Medical-Surgical and Dental Specialties, University of Campania Luigi Vanvitelli, Naples, Italy;* ²*Department of Medicine and Surgery, University of Milano Bicocca, Monza, Italy;* ³*Department of Mental and Physical Health and Preventive Medicine, Second University of Naples, Naples, Italy;* ⁴*Interdisciplinary Department of Medicine, University of Bari, Bari, Italy;* ⁵*Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy*

*these authors contributed equally to this work and they are co-first authors

**these authors contributed equally to this work and they are co-last authors

The aim of this study was to evaluate the prevalence of psychiatric symptoms in patients with oral lichen planus (OLP) compared to a control group. 11 patients (mean age 65.2 years-old), with diagnosis of OLP and 13 controls (mean age 64.8 years-old) underwent a psychiatric evaluation with five psychometric scales: Visual Analogue Scale (VAS), Hamilton Rating Scale for Depression (HAM-D), State-Trait Anxiety Inventory (STAI 1-2), Distress Thermometer (DT) and Brief Fatigue Inventory (BFI). Seventy-three % of OLP group presented a VAS score of mild type; 9% had depressive symptoms; 100% of the sample had a score above the cut-off for state and trait anxiety; 45% presented Distress (36% moderate type). However, no statistical difference was found in comparison with the control group. In this study, there was no difference in these parameters between the OLP group and the control group. Perhaps, a larger cohort of patients could give different results.

Oral Lichen planus (OLP) is an idiopathic inflammatory disease of mucous membranes, characterized by an autoimmune epithelial attack by infiltrating T cells. The disease has a predominance in the general population of 1-2%, 50% of which has both skin and oral lesions, while 25% of patients shows only oral lesions (1). The oral sites generally involved are buccal mucosa, tongue and gingiva (2). Extra-oral sites (i.e. scalp, skin, nails, conjunctiva, esophagus, larynx urethra, vulva and vagina, and perianal area) are commonly involved (3). It results in severe morbidity; thus, a multidisciplinary approach is needed (4). It should be noted that the mechanisms underlying

this pathology are still discussed (5). For example, psychological disturbances could play a role in the onset of OLP (6-12) as in other oral mucosa diseases (13,14) but the results are conflicting (15). In particular, the psychiatric comorbidities most frequently discussed in the literature were stress or stressful events (16,17), anger suppression (18) anxiety (6,19), depression (4,8). In addition, the quality of life of patients with OLP was analyzed and in this context, an analysis of the burning and pain symptoms was made through the VAS scale. The purpose of this preliminary case control study is to verify the prevalence of psychiatric disorders in a sample of patients with OLP through

Key words: oral lichen planus, distress, anxiety, fatigue, depression

Mailing address:

Prof. Rosario Serpico,
Multidisciplinary Department of Medical and Dental Specialties,
Second University of Naples, Via L. De Crecchio 6,
80138 Naples, Italy
Tel.: +39 081566 7674 - Fax: +39 081566 7674
e-mail: rosario.serpico@unicampania.it

the administration of five psychometric scales. Furthermore, the symptom pain in patients with OLP was assessed with the VAS scale.

MATERIALS AND METHODS

This study evaluated 24 patients attended at the Oral Pathology Clinic of the University of Campania “Luigi Vanvitelli”. The OLP group consisted of 11 patients, 10 women and 1 man. The control group consisted of 10 women and 3 men. The study was approved by the local Human Research Ethics Committee. All participants provided signed informed consent forms.

Inclusion and exclusion criteria

To be included in the study, patients had to have a diagnosis of OLP (all forms). Patients were excluded if they had Burning Mouth Syndrome, systemic problems, including use of drugs like tricyclic antidepressants and anticholinergics. The control group consisted of patients seen in the Dental Clinic of Second University of Naples in the Department of Oral Pathology.

Assessment

The study group and the control group were evaluated by collecting demographic data, and medical history; the administration of psychiatric scales was preceded by an examination of the oral cavity.

Visual analogue scale (VAS) was used to assess the intensity of the pain (20, 21). For the prevalence and intensity of depressive symptoms Hamilton Depression Rating Scale (HAM-D) was used (22). The assessment of anxiety symptoms was performed using the STAI (State-Trait Anxiety Inventory) Form Y (23). A Distress Thermometer (DT) (24) was administered for the emotional distress in a chronic disease. Brief Fatigue Inventory (BFI) was used to evaluate the fatigue (25).

A database was assembled using Microsoft Excel and elaborated with R[®]. Demographic and clinical features of the two groups as well as the results of psychometric variables were recorded.

RESULTS

Both groups did not differ in gender distribution or age (mean age 66.6 years-old OLP; 63.5 years-

old controls). There are no significant differences between the two groups regarding depression ($p > 0.05$). In fact, 9% of patients with OLP have mild depressive symptoms, compared to 13% of the control group that also has a 4% of severe symptoms. Furthermore, if it is true that 100% of the group with OLP exceeds the cut-off (40) for state and trait anxiety, this also occurs in the control group ($p > 0.05$). Only 1 patient (9%) with OLP has severe fatigue, while in the control group 15% have mild fatigue and 23% moderate fatigue. Forty-five % of patients with OLP have Distress, of which 36% are moderate and 9% are severe; in the control group, only 30% have distress values for 15% moderate and for 15% severe. However, comparing the groups with an unpaired *t*-test there is no significant difference ($p > 0.05$). Only VAS score is significantly higher in patients with OLP than in the control group ($p < 0.05$), and in particular 73% have mild pain, while 61.5% of the control group report no pain (Fig. 1).

DISCUSSION

In this preliminary study, there do not seem to be any differences in psychometric test results between patients with OLP and control group. In fact, even if 100% of the study group has high values of state and trait anxiety, there seems to be no difference compared to the control group. The data reported in this study appear to be in line with some previous work (12, 15). For example, the results provided by Allen and coworkers (15) indicate that no difference exists among OLP group and controls respect the tendency toward anxious feelings; even Alshahrani et al. in a cross-sectional study admits that despite reports of positive association, evidence on psychological factors and OLP remains inconclusive (12). In contrast, Gavic et al. (19) as well as Chaudhary et al. (6) have shown a prevalence of anxiety and depressive disorders in patients with OLP. As for the other parameters, such as fatigue and depression measured in the two groups, we have higher values in the control group even if there is no significant difference. These data are in contrast with previous work (8, 11, 26). As far as distress

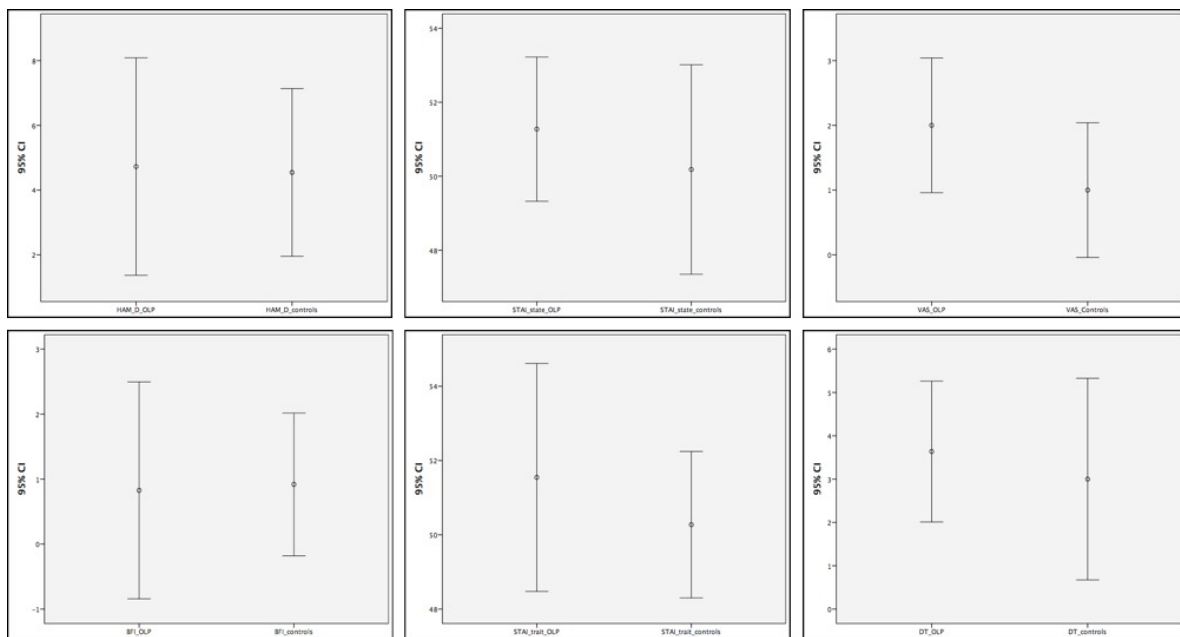


Fig. 1. Error Bars. When OLP group was compared to controls, only VAS results significantly higher in OLP group.

is concerned, there seems to be greater prevalence in the OLP group, although not significant. Other studies have analyzed the “stress” or even better the “stressful events” (16, 17, 27) but not the “distress”. In any case, a study with a larger sample could be useful in determining the prevalence of these psychometric values in the OLP.

CONCLUSION

In this study, patients with OLP have elevated levels of anxiety and distress, although not significant compared to controls. Only VAS score is significantly higher in patients with OLP. Studies with a larger cohort of patients can better clarify the role of psychiatric comorbidities in oral lichen planus.

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HOW SOCIAL MEDIA MEET PATIENTS' QUESTIONS: YOUTUBE™ REVIEW FOR CHILDREN ORAL THRUSH

D. DI STASIO^{1*}, AN. ROMANO^{1*}, R. S. PAPARELLA¹, C. GENTILE¹, G. MINERVINI¹,
R. SERPICO¹, V. CANDOTTO^{2**} AND L. LAINO^{1**}

¹Multidisciplinary Department of Medical-Surgical and Dental Specialties, University of Campania Luigi Vanvitelli, Naples, Italy; ²Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy

*these authors contributed equally to this work and they are co-first authors

**these authors contributed equally to this work and they are co-last authors

YouTube™ is increasingly being used by patients to obtain health-related information. No studies have evaluated the content of YouTube™ videos on children oral thrush. The aim of this work is to examine the quality of information offered by this platform about oral thrush in children. Searching term “oral thrush in children” (OTC) displayed a total of 2.790 results. Of the top 60 videos analyzed, 27 were excluded. The main source of upload was from generalist information YouTube® channels (GC) followed by healthcare professionals (HP), individual users (IU), and healthcare information channels (HC); usefulness of videos is successfully correlated with the number of visualization, number of likes and viewing rate and was interdependent with the number of visualizations, number of likes and VR. However, videos on the oral thrush do not have satisfactory quality information. HP themselves, along with HC, do not seem to provide more appropriate information on COT, than GC or IU.

Nowadays, patients look up the Internet to gather medical information (1). YouTube™ is being increasingly used as a platform for dissemination of health information (2), but the probability for a lay user to find this kind of content is relatively high (3). Recent studies have shown that YouTube™ is very effective in improving patients' attitude toward cancer risk reduction (4-8).

Surprisingly, though, the majority of the most popular health related YouTube™ videos were not created by public health professionals (9). Not being subject to peer reviewing or any other form or professional control, this platform has the potential to change the beliefs of patients concerning controversial topics (10-13). Nevertheless, it could also be used as a priceless tool for healthcare

professionals to bridge the gap between health care and patients (14-16).

Diagnosis and management of Pediatric stomatological diseases are an important challenge; they may affect both hard tissues (17, 18) and soft tissues of the oral cavity. Pseudomembranous candidiasis is the most commonly recognized type of candidiasis and is also known as thrush (19-22).

The prevalence of oral colonization with *Candida* varies from 11 to 15% during the first year of life and newborns may acquire *Candida* through the vagina, and later from the environment (23-25). The main local factors are ventilator status (26), interferences with a normal salivary flow (27, 28) and poor oral hygiene (19) due to bottle and pacifiers (25).

Systemic factors include: immunodeficiency (23, 29),

Key words: YouTube™, child, dental, oral, videos, healthcare

Mailing address:

Prof. Rosario Serpico,
Multidisciplinary Department of Medical and Dental Specialties,
Second University of Naples, Via L. De Crecchio 6,
80138 Naples, Italy
Tel.: +39 081566 7674 - Fax: +39 081566 7674
e-mail: rosario.serpico@unicampania.it

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antibiotic (22, 30), steroid exposure (21, 31) and low birth weight (32). To prevent oral thrush in infants, attention has to be paid to bottle and pacifier hygiene (33). Therapies include antifungal molecules such as azoles (28, 34-36) and other alternative solutions (37-39). This study systematically examined the available information about children oral thrush on this platform, comparing the obtained data with evidences in scientific literature and international guidelines.

MATERIALS AND METHODS

This work followed a previously described systematic method for searching on Youtube™ (1, 4, 11). The string “oral thrush in children” was searched on YouTube™. The search was conducted on the 24th October 2017 using the default settings of YouTube™, without any filters; the first 60 videos were examined; the flow chart (Fig. 1) explains the standardized method used in this work. Remaining videos were reviewed to extract video demographics such as video’s length (VL), date and source of upload (SOU), and numbers of views (NV), likes (NL), dislikes (NDL) and number of comments (NC). In addition, each video was given a score from 0 to 11 to indicate its usefulness as specified in Table I. *Viewers’ interaction (VI)*, *viewing rate (VR)* (1,4) and *community interaction rate* [(NC/total NV)*100] (*CR*) were calculated. Descriptive statistics were generated with R®; Pearson’s analysis was used to examine correlations between variables; analysis of univariate variance and linear regression analysis were made to confirm the correlation hypothesis.

RESULTS

The term search “oral thrush in children” (OTC) displayed a total of 2.790 results. The first 60 videos were screened of which 27 were excluded following the exclusion criteria (Fig. 1). The main SOU was from generalist information YouTube® channels (GC-42.4%). The rest of the videos were uploaded by healthcare professionals (HP-24.2%), individual users (IU-18.2%) and healthcare information YouTube® channels (HC-15.2%). The mean VL was 03:50±04:58 min (230±298 sec),

with a range from 00:42 min up to 28:40 min (1720 sec) and a median of 02:34 min (154 sec). The mean of NV was 17.496±3.6971, 27views (from 5 to 203.166 views – median: 6.589); each video was viewed with a mean of 30.9±70.81 views/day (from 0.1 to 338.04 views/day). The VI mean was 0.39%±0.45, and the CR was 0.69%±3.47. The average NL for each video was 72.45±225.66, with a peak of 1265, and a median of 9, while the NDL was 4.48±13.67, with a peak of 41; 69.7% of selected videos treated therapy themes but of these the majority are more focused on natural remedies (24.2%) and home remedies (33.3%), rather than on some true therapeutic strategies (33.3%) or probiotics (9.09%). Only 27.3% of videos deal with prevention issues, and of which only 33.3% are related to a healthy lifestyle (excessive use of antibiotics, healthy diet, and general behavior), and 66.6% discuss the topic of the management of baby bottles and pacifiers. Moreover, 30.3% discussed about risk factors and of these the 80%

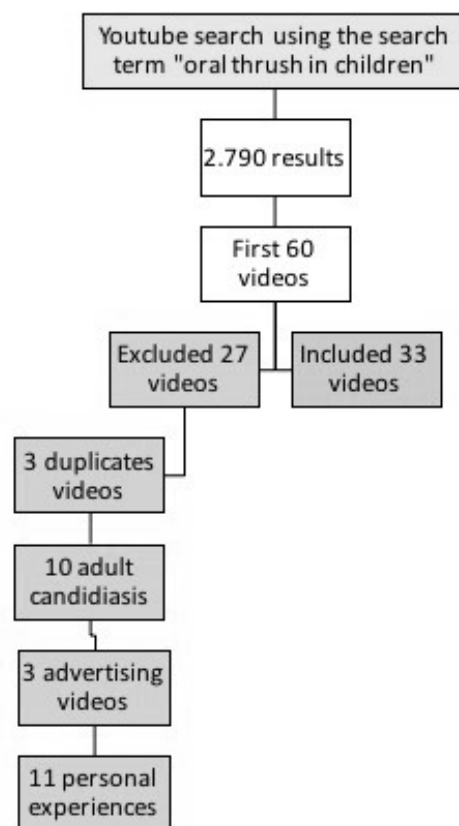


Fig. 1. YouTube™ flow chart.

discussed about a possible immune depression of mother or child, 50% about diabetes, 20% about the use of steroid drugs, poor oral hygiene, dry mouth condition, and eventually a stress or a trauma. None of the videos mentioned any bibliography or source of information regarding the presented content.

Usefulness score and Pearson's analysis

The mean US of videos analyzed was 3.18 ± 2.28 with a maximum score of 8 and a minimum score of 1; 69.7% of them resulted to be slightly useful, the 24.2% moderately useful and only the 6.1% of them resulted to be very useful for the patient; anyway, no videos has been catalogued as not useful. The *Pearson's analysis* revealed that the US is successfully correlated with NV ($R=0.389$; $p \leq 0.05$), NL ($R=0.371$; $p \leq 0.05$) and VR ($R=0.429$; $p \leq 0.05$); when linear regression model is performed, US was interdependent with NV ($\beta=0.389$; $p=0.025$), NL ($\beta=0.371$; $p=0.033$) and VR ($\beta=0.429$; $p=0.013$). US, VR and NL were divided into groups based on the different sources of upload: univariate ANOVA and Turkey tests were carried out and no mean difference between groups was significant ($p > 0.05$).

DISCUSSION

The world-wide-web and in particular, social media platforms, play a key role in informing patients on health related issues (3, 10, 12, 15). This work analyses the data available on YouTube™ both quantitatively and qualitatively. The content of YouTube™ on oral thrush is highly heterogeneous and the information provided is almost never complete. Apparently, this might be due to the fact that only the 39.4% of the videos analyzed were uploaded by health care professionals or health care channels, while the majority (60.6%) were uploaded by general information YouTube™ channels, or individual users. In fact, as shown by the results, not only is there no significant difference in US based on the different sources of upload ($p > 0.05$), but also the maximum score was made by IU and the other one by GP. This data underline the need for higher quality of information provided to patients by HC and HP through social platforms, even more so, given the potential that these tools have in educating patients (14-16). Moreover, prevention strategies and risk factors have been presented in an inadequate percentage of videos (27.7% and 30% of analyzed videos).

Table I. *Usefulness score.*

Usefulness score (US)		
Scoring Item	Description	score
the presence of representative images	Images demonstrating oral candidiasis, thrush, white pseudomembranous lesion, red erythematous lesions	1
local health effects	pain and clinical features associated with thrush	1
general health effects	malnutrition, loss of school days, diminished oral health-related quality of life	1
etiology	diabetes	1
	Immune deficiencies	1
	topical and systemic corticosteroid therapies	1
	antibiotic therapies	1
	oral hygiene	1
	(sub tot)	5
prevention	preventions strategies	1
therapy	anti-mycotic agents	1
	Home remedies	1
Total score		11

0=not useful; 1-3=slightly useful; 4-7=moderately useful; 8-11=very useful.

Most videos suggest home remedies or therapy based on natural extract (57.54%). Drug therapy (33.3%) or probiotics (9.09%), even when presented, are not connected to any scientific reference (1). Probably because of the low degree of appropriateness of the data presented in the videos, the average usefulness score is very low (3.18 ± 2.28), in line with other studies evaluating YouTube™ videos regarding various healthcare issues (1, 4, 13, 40). However, this work presents, for the first time, a positive correlation and an interdependence between US, VR, NV, and NL ($p \leq 0.05$) compared to previously published articles (1, 4, 13, 40); this may mean that the final user begins to recognize the usefulness of the content that is provided to him.

CONCLUSIONS

YouTube™ is a growing platform in informing patients about health issues. Videos on the oral thrush do not have satisfactory quality information. HP themselves, along with HC, do not seem to provide more appropriate information on COT, than GC or IU. Patients may be receiving biased information from videos and, therefore, physicians and dentists should be aware of the type of information patients may be accessing on YouTube™. These works on analyzing health content on social platforms are a starting point for providing greater quality of information, as the main point that emerges from this paper is that patients require more quality content.

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ANALYSIS OF SALIVA SAMPLES IN PATIENTS WITH PRADER-WILLI SYNDROME

M. BOCCCELLINO^{1*}, D. DI STASIO^{2*}, R. SERPICO², A. LUCCHESI², A. GUIDA³,
G. SETTEMBRE⁴, M. DI DOMENICO^{1,5} and A. RIZZO⁶

¹Department of Biochemistry, Biophysics and General Pathology, University of Campania “Luigi Vanvitelli”, Naples, Italy; ²Multidisciplinary Department of Medical-Surgical and Dental Specialties, University of Campania Luigi Vanvitelli, Naples, Italy; ³INT – IRCCS “Fondazione G. Pascal” – U.O.C. Maxillo-facial surgery and otolaryngology; ⁴Clinical Pathology Unit, University of Campania “Luigi Vanvitelli”, Naples, Italy; ⁵Sbarro Institute for Cancer Research and Molecular Medicine, Center for Biotechnology, Temple University, Philadelphia, PA, USA; ⁶ Department of Experimental Medicine, Section of Microbiology and Clinical Microbiology, University of Campania “Luigi Vanvitelli”, Naples, Italy

*these authors contributed equally to this work and they are co-first authors

Patients affected by Prader-Willi Syndrome (PWS) usually show orofacial dysfunction, poor oral hygiene, severe tooth wear, generalized caries and thick sticky saliva. The aim of this study was to evaluate molecular/ionic changings in PWS patients compared to controls, as well as unstimulated salivary flow rate (SFR); 7 patients with a mean age of 20.0 ± 5.45 years were enrolled in the study group (PWS group) and 5 patients with a mean age of 22.6 ± 3.05 years, in the control group. Results showed a greater Na^+ ($p=0.003$), Cl^- ($p=0.004$) and P ($p=0.001$) concentration in saliva of PWS group as well as a greater concentration of secretory IgA ($p=0.003$) with a reduction of SFR ($p=0.004$) compared to controls. A Spearman’s analysis (based on the SFR of both groups) revealed an inverse correlation with Na ($\rho=-0.747$), Cl ($\rho=-0.723$), P ($\rho=-0.637$) and sIgA ($\rho=-0.707$) concentration and SFR, when linear regression model was performed only P and SFR were interdependent ($\beta=-0.748$; $p=0.005$).

Prader-Willi syndrome (PWS) is a complex multisystem genetic disorder caused by an abnormality of chromosome 15. The disease develops if the paternal alleles are silenced, missing or defective in any way. Epidemiological studies estimate the population prevalence to be up to 1:52000 and the gender ratio about 1/1 (1-3). Symptoms of PWS are linked to high pain threshold, compulsive appetite, sleep disorders, body temperature instability, altered growth hormone (GH) secretion and hypogonadism (4). Other clinical features are small hands and feet, scoliosis, developmental delay with significant neurological, cognitive and behavioral

abnormalities, learning problems and characteristic facial appearance (5-8).

A hypothalamic dysfunction is believed to be responsible for this multifaceted phenotype, including a dysregulation of GH/insulin-like growth factor I axis, hypogonadism, adrenal insufficiency, and altered pituitary-thyroid axis (9, 10). Initially, PWS usually begins with severe neonatal hypotonia; during the first year of life, these individuals have feeding problems and difficult growth. Thriving issues change into hyperphagia and weight gain between the ages of 1 and 6. At this stage, most PWS patients develop morbid obesity (11). Furthermore,

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Mailing address:

Prof. Rosario Serpico,
Multidisciplinary Department of Medical and Dental Specialties,
Second University of Naples, Via L. De Crecchio 6,
80138 Naples, Italy
Tel.: +39 081566 7674 - Fax: +39 081566 7674
e-mail: rosario.serpico@unicampania.it

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hypogonadism and osteoporosis are present in the vast majority of adult patients, together with psychosis and self-aggressive behavior (12-14).

The Challenging Behavior Interview provides to establish the degree of severity of pathology. The effectiveness of the interventions is related to the guidance of four-point Likert scale (15). Treatment of manifestations consists of intensive rehabilitation, psychological support, speech therapy and if the patient meets the appropriate criteria, growth hormone replacement therapy to normalize height, increase lean mass and mobility and reduce the fat mass. An extremely important element of therapy is implemented nutritional management adequately planned. In particular, during childhood, strict supervision of daily food intake according to height, weight and body mass index (BMI) is required to provide the right energy requirements by limiting excessive weight gain and encouraging physical activity (16, 17).

It has been reported that PW subjects show an orofacial dysfunction, poor oral hygiene and severe tooth wear (both erosive wear and attrition) (18). Moreover, they show generalized caries that lead to an almost complete destruction of the teeth and thick sticky saliva (19-24).

In neonates with PWS, viscous saliva has been reported to be a diagnostic indicator. In addition, in individuals with the syndrome, decreased salivary flow rate and increased amounts of salivary ions

and proteins has been reported (25-27). Saliva plays a key role in maintaining dental health, it is involved in lubrication, protection, buffering action, maintenance of teeth integrity, antibacterial activity, taste and digestion. It consists of various types of electrolytes, immunoglobulins, proteins, cytokines, enzymes and large quantity of mucins. A large part of these substances plays a crucial role against oral microorganisms and could serve as biomarkers, to reflect the status of oral health and hygiene (28-32). The aim of this study was to evaluate molecular/ionic changings in PWS patients, as well as unstimulated salivary flow rate, to understand if poor oral health in this subject is related to organic causes rather than low compliance in oral hygiene only. In particular, secretory immunoglobulin A (sIgA), with a well-known role in the immune response level was investigated and furthermore, Nordic Orofacial Test-Screening (NOT-S) test was administered in order to evaluate any orofacial dysfunction.

MATERIALS AND METHODS

This study was approved by the ethical committee of the University of Campania "Luigi Vanvitelli". For the purposes of the study, a group of 15 patients with a genetic diagnosis of PWS was enrolled (6F and 9M), with an average age of 22 years, compared with a control group of 22 healthy subjects (10F and 12M with a mean of age 21).

Nordic Orofacial Test-Screening (NOT-S) was used to

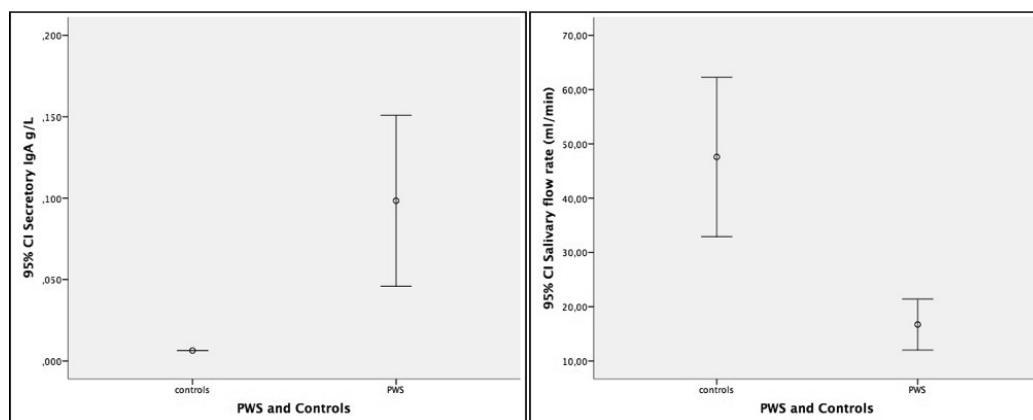


Fig 1.

support anamnestic and clinical data. Informed consent was signed by parents of all patients and by all subjects in the control group. Subjects were asked to collect unstimulated whole saliva (UWS) produced in 5 minutes in 5 cl sterile tubes; in the two hours before saliva collection, patients of the two groups had to refrain from eating and drinking (with the exception of water) and did not smoke. Salivary flow rate (milliliters of saliva secreted per minute) was calculated; saliva samples were subjected to biochemical analysis of inorganic components such as sodium (Na^+), potassium (K^+), chlorine (Cl^-), calcium (Ca^{++}) and phosphorous (P); salivary secretory immunoglobulin A levels (sIgA-g/L),

cystatine C (CysC-mg/L), salivary amylase, salivary transaminase (ALT/AST) and total protein concentration were analyzed.

A two-sided independent sample *t*-test was used to compare UWS, total protein/ionic concentrations between the PWS and control groups. A two-sided Mann-Whitney test was used to compare salivary flow rate (SFR), CysC, sIgA and ALT/AST concentration between the two groups. Spearman correlation coefficient (ρ) was used to analyze the association between variables and in case of that linear regression analyses was performed. The statistical analysis was carried out using the statistical software program R®.

Table I. Demographic data, Anamnesis, ionic, IgA, CysC, salivary amylase, salivary transaminase, total protein and SF rate of PWS group and controls.

	PWS	controls	p-value
mean age	20.0 (± 5.45)	22.6 (± 3.05)	
gender	4F; 3M	3F; 2M	
genetic diagnosis	2UPD; 5 Del15		
diabetescomorb.	2		
epilepsycomorb.	1		
cardiovascular diseases	1		
psychiatric drugs	3		
antihistamines drugs	2		
antiepileptic drugs	1		
Ca^{++} (sd)	1.84 (± 0.61)	1.82 (± 0.15)	0.988*
Na^+ (sd)	108.71 (± 8.75)	92.6 (± 2.19)	0.331*
K^+ (sd)	9.07 (± 3.08)	9.87 (± 0.65)	0.586*
Cl^- (sd)	92.81 (± 7.39)	79.94 (± 1.95)	0.004*
P (sd)	25.52 (± 1.55)	22.02 (± 0.72)	0.001*
sIgA g/L (sd)	0.10 (± 0.06)	0.006 (± 0.0001)	0.003**
CysC mg/L (sd)	0.54 (± 0.32)	0.46 (± 0.24)	0.808**
Salivary amylase (sd)	5491.7 (± 5413.8)	12536 (± 10547)	0.380**
sAST (sd)	40.97 (± 49.59)	41.34 (± 24.45)	0.570**
sALT (sd)	19.50 (± 27.3)	32.60 (± 24.19)	0.167**
totprotein (sd)	0.09 (± 0.05)	0.06 (± 0.04)	0.331*
SF rate (ml/min) (sd)	16.71 (± 5.09)	47.60 (± 11.82)	0.004**

*: *t*-test for independent variables; **: Man-Whitney U-test.

RESULTS

Only 7 (4F and 3M with a mean age of 20.0 ± 5.45 years) of the 15 PWS patients underwent the study; the control sample also passed from 22 to 5 patients (3F and 2M with a mean age of 22.6 ± 3.05 years). Demographic and mean of SFR, sIgA levels, ALT/AST and ionic/total protein concentrations of PWS group and controls are shown in Table I. *T*-test revealed a significant greater Na^+ ($p=0.003$), Cl^- ($p=0.004$), and P ($p=0.001$) concentration in saliva of PWS group compared to controls.

Mann-Whitney U test (2-tailed) revealed a greater concentration of secretory IgA ($p=0.003$) in PW patients with a reduction of SFR ($p=0.004$), compared to controls (Fig. 1).

To verify the “concentration effect”, Spearman correlation was performed without splitting the two groups and revealed an inverse correlation with Na ($\rho=-0.747$), Cl ($\rho=-0.723$), P ($\rho=-0.637$) and sIgA ($\rho=-0.707$) concentration and SFR. Unfortunately when linear regression model was performed only P and SFR were interdependent ($\beta=-0.748$; $p=0.005$) as shown in Fig. 2.

DISCUSSION

The data presented in this study, even if the experiments were performed on a small sample, confirm the data present in the literature. Several studies have reported reduced salivary flow in PWS patients (18, 23, 26, 28). In particular, Hart et al. (26) in a 1998 study found a 20% reduction in

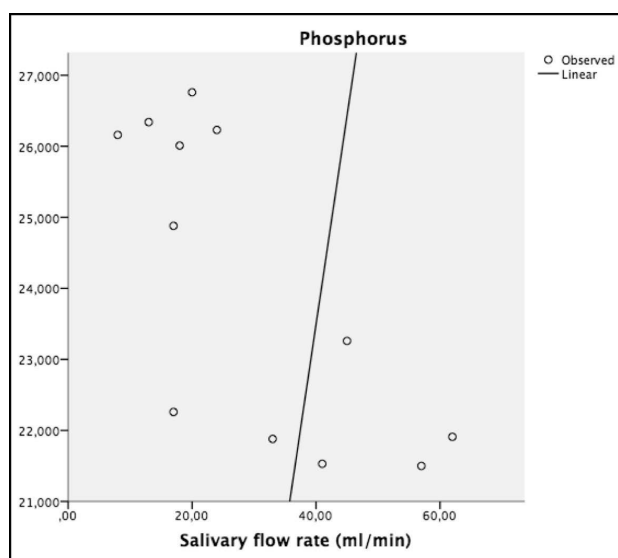


Fig 2.

salivary flow and an increased ionic concentration in patients with PWS, hypothesizing that this could be due to a “concentration effect”, due to the decrease of the aqueous component in saliva. In the present study, correlation investigations demonstrated an inverse proportionality between salivary flow and ionic and IgA levels in the PWS group saliva. It should be emphasized that linear regression was demonstrated only an interdependence for P and SFR; however, further studies and a wider group of patients could verify the correlation hypotheses. Furthermore, higher levels of secretory IgA, which normally have a protective function of oral tissues, in the PWS group may be due to the presence of infections (e.g. from *Streptococcus mutans*), as reported by Gonzalez (28) and in any case from an oral mucosa continually traumatized with the chronicization of candidiasis, as reported by Olczak-Kowalczyk (18).

CONCLUSION

This study confirms the presence of salivary alterations in patients with PWS. Saliva could be an easy aid for screening oral diseases of these difficult-to-manage patients. However further studies are needed.

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MANAGEMENT OF DENTURE STOMATITIS: A NARRATIVE REVIEW

D. DI STASIO^{1*}, D. LAURITANO^{2*}, G. MINERVINI¹, R. S. PAPARELLA¹, M. PETRUZZI³, A. ROMANO¹, V. CANDOTTO^{4**} and A. LUCCHESI^{1**}

¹Multidisciplinary Department of Medical-Surgical and Dental Specialties, University of Campania Luigi Vanvitelli, Naples, Italy; ²Department of Medicine and Surgery, University of Milano Bicocca, Monza, Italy; ³Interdisciplinary Department of Medicine, University of Bari, Bari, Italy; ⁴Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy

*these authors contributed equally to this work and they are co-first authors

**these authors contributed equally to this work and they are co-last authors

***Candida albicans* is a typical commensal microorganism of the oral cavity that can become virulent in certain conditions and cause denture stomatitis (DS). Many conditions can predispose for DS, including local and systemic alterations. The therapeutical approach to DS is various and both topical and systemic alternatives can be considered. Azoles molecules are the most common used, both topically and systemically, but in recent years various alternatives have been proposed. This review aims to examine the scientific literature to evaluate the effectiveness of conventional therapies and the potential of the new ones.**

Denture stomatitis (DS) is the most frequent, opportunistic, infectious disease in the oral cavity among denture wearers, with a multifactorial etiology and caused by yeast of the genus *Candida* (1-3). *Candida albicans* has been shown to be the principal *Candida* species responsible for the pathology, but also other species such as *C. dubliniensis*, *C. parapsilosis*, *C. krusei*, *C. tropicalis* and *C. glabrata* are involved in the pathogenesis of oral infections. *Candida albicans* can be found in the normal oral microflora with percentages ranging from 25% to 50% of the total flora (1) and the presence of this fungus in the oral cavity is considered normal commensalism.

In a healthy individual, the inherent microflora usually suppresses the overgrowth of pathogenic fungal organisms and the integrity of mucosa prevents the spreading of these organisms (4). To allow the

infection, both local and systemic factors have to be altered (5). Different conditions can predispose to the development of *candidiasis*. These include the use of broad-spectrum antibiotics, immunosuppression, xerostomia, physical disabilities that make oral hygiene less effective or improper nutrition, as well as use of removable oral prostheses (4).

When *Candida* turns into the pathogenic form, immunological reaction can cause inflammation and edema of tissues under the prosthesis known as DS; there are several clinical forms in which DS can occur, involving one or more oral sites and can affect the whole oral cavity and disseminate into invasive forms (2).

The importance of an accurate diagnosis is because of the similarity of symptoms with other pathological conditions of the oral cavity (6). This can be achieved by a combination of clinical features and

Key words: Candida, inflammation, mucosa, oral, cavity

Mailing address:

Prof. Rosario Serpico,
Multidisciplinary Department of Medical and Dental Specialties,
Second University of Naples, Via L. De Crecchio 6,
80138 Naples, Italy
Tel.: +39 081566 7674 - Fax: +39 081566 7674
e-mail: rosario.serpico@unicampania.it

cultures and is frequently confirmed by a response to antifungal therapy (4). The high prevalence of complete denture wearers, changes related to aging, poor oral hygiene and motor limitations, predispose elderly patients to the development of DS (1), indeed DS has been reported in 11-67% of denture wearers (3) and in 11-36-7% of partial removable dental prostheses wearers (1).

Clinical classification

There are many classifications of DS, but the most common used is the Newton's classification proposed in 1962 that identifies three different degrees of clinical manifestation of DS: a localized simple inflammation (type I), generalized erythema (type II) and papillary hyperplasia of the palate (type III).

Treatment

In literature, there are many references to the treatment of denture stomatitis (6). There are both topical and systemic antifungal therapies (7, 8). The standard antifungal regime for this condition embraces local treatment with nystatin or amphotericin B tablets (q.i.d.) and/or frequent applications (3-4 times/day) of nystatin ointment or miconazole gel on the fitting surface of the denture for 2-4 weeks. However, the diluent effect of saliva and the cleansing action of the oral musculature tend to reduce the concentration of these agents to sub-therapeutic levels (9). In addition, a problem with topical antifungal therapy is patient compliance (8). To overcome these problems, efforts have been directed to develop methods and devices for sustained delivery of the medication in therapeutic concentration to focal points of infection. Systemic antifungal agents such as amphotericin B and fluconazole are also effective, but they do not eradicate the microorganisms that colonize the denture. This makes it necessary to seek new therapeutic approaches (8, 9). DS is a problem of frequent presentation in the clinical practice of oral medicine and needs a correct approach to obtain a clinical and microbiological resolution. The therapeutic strategies concern the oral mucosa and the correct denture care (1). This work identifies three topics: Prevention strategies, Topic therapy and Systemic therapy.

Prevention strategies

Prevention concerns the management of denture wearing and denture and oral hygiene (10, 11). A therapeutic plan, based on palatal brushing with a soft-bristle manual toothbrush after each meal and before sleeping, shows an improvement of the inflammation of the palate (12), but this approach is not enough to eradicate the fungus. It is important to remove the prosthesis during the night before going to sleep, carefully brush it and store it in a dry place (5). Some authors suggest soaking the prosthesis in 0.5% chlorhexidine and alcohol solution (13) or sodium hypochlorite 0.02% overnight (6). Also microwaving dentures seems to be an effective procedure on *Candida* colonies on the prosthesis surface (7, 14). However, no author proposes a real strategy of primary prevention: enrolled subjects are already affected by *Candida*, so the prevention measures may be defined in the field of tertiary prevention.

Topic therapy

The orientation of most authors in regards to topic therapy is to use molecules with yeast-killing action in formulations such as gels, oral rinses and mouthwashes, which seems to be the simplest way to deliver the active components through the mucosa of the oral cavity (15). Products based on Azoles: Ketoconazole (16), Miconazole (8) and Nystatin (17), seem to show a good therapeutic efficacy against the fungus. Some authors propose alternative products based on molecules of natural extraction (3, 15, 18-20). Despite the encouraging results, further studies with a wider population are needed to evaluate their effectiveness. Studies with a longer follow-up are necessary to confirm the efficacy of other molecules such as chlorine dioxide (4), histidine-rich polypeptides (21), Hexetidine (22), Lawsone Methyl Ether (23) and Amolfine (10). For some authors, the lack of topic therapies seems to be due to the drug warding off because of the constant flow of saliva and mobility of involved tissues, before it has explicated its therapeutic effect (15). To overcome this limit, some studies evaluate the possible use of laser technology for the treatment of DS (5, 9, 24). These protocols provide the irradiation of both the

denture and the mucosa and each protocol is different depending on the laser device used. The parameters to be taken in consideration are, the type of laser (LED; diode), the wavelength of the light emitted, the possible use of a photosensitizer and the irradiation time. These techniques show a potential therapeutic role, but further investigations should be made.

Systemic therapy

Few authors treat systemic therapy exclusively and again, azoles drugs as Fluconazole (11, 25), Itraconazole (11), and Nystatin, both in pastilles and suspension (13) are effective in the treatment of DS. Some authors suggests that a topical therapy may be combined with a systemic one together with a correct denture hygiene, especially in medium-lasting therapies. The topical action in fact, is determined by the salivary concentration of the drug, but if the worn denture is in close contact with oral tissues, it can exclude saliva from the two surfaces, losing the topical effect of the drug (26). This data is supported by the results of some studies. In fact, the association of systemic fluconazole with hexetidine mouth-rinses seems to be more effective in terms of *Candida* presence on the mucosa rather than the use of the two molecules alone (22). Moreover, topic Chlorhexidine solution 2% in conjunction with Fluconazole pointed out a similar result (26).

CONCLUSIONS

DS is an important challenge in everyday clinical practice for the dentist. The analysis of this works shows that the correct therapeutic approach seems to be a combined one: the association of systemic and topic therapy can give better clinical and microbiological results than the single therapy alone. Oral hygiene and daily denture care are of great importance to maintain good results and a low level of infection.

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HOW SOCIAL MEDIA MEET PATIENTS' QUESTIONS: YOUTUBE™ REVIEW FOR MOUTH SORES IN CHILDREN

D. DI STASIO^{1*}, A. ROMANO^{1*}, R. S. PAPARELLA¹, C. GENTILE¹, R. SERPICO¹,
G. MINERVINI¹, V. CANDOTTO^{2**} AND L. LAINO^{1**}

¹*Multidisciplinary Department of Medical-Surgical and Dental Specialties, University of Campania Luigi Vanvitelli, Naples, Italy;* ²*Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy*

*these authors contributed equally to this work and they are co-first authors

**these authors contributed equally to this work and they are co-last authors

Recurrent aphthous stomatitis (RAS) is one of the most common causes of mouth sores in children so the management of this condition is a matter of great importance. YouTube™ is increasingly being used by patients to obtain health-related information. The aim of this work is to examine the quality of information offered by YouTube™ about mouth sores in children (MSC). Searching the term ‘mouth sores in children’, (MSC) displayed 12.300 results. Of the top 60 videos analyzed, 31 were excluded following exclusion criteria. The major source of upload was from healthcare information channels (HC-41,38%), followed by individual users (HP-25.59%), healthcare professionals (IU-17.24%) and generalist information channels (HC-13.78%); 20.69% of them deal with predisposing factors, and related pathologies, the majority of these propose home remedies (60.72%) rather than topical analgesic drugs (21.43%), antimicrobials (7.14%) and topical steroids (3.57). Most of the videos analyzed were slightly useful (68.97%). Information about mouth sores in children on YouTube™ was poor regardless of the upload source. Analyzing health content on social platforms is a starting point for providing greater quality of health-related information.

Pediatric odontostomatologic diseases may affect both hard tissues (1, 2) and soft tissues of the oral cavity. Recurrent aphthous stomatitis (RAS) is the most common type of oral ulceration and painful condition seen in children (3, 4) and it is characterized by multiple recurrent small, ovoid ulcers on the less keratinized oral mucosal tissues, with circumscribed margins, erythematous haloes and yellow or gray floor and usually regress spontaneously within 14 days (5-7).

Aphthous ulcers can cause functional complications, including difficulties in speaking, tooth brushing and eating (8). The precise aetiology

of aphthous lesions is still not clear (6, 9). Treatment is symptomatic. The goal is to decrease symptoms, reduce ulcer number and size, increase disease-free periods (10, 11). Topical and systemic corticosteroid can reduce the duration and severity of RAS ulcers, but this therapy is not always easy to manage and can cause some side effects (5, 11-14). In modern times, patients consult the Internet not only to find medical information but also to find a diagnosis or treatments for their illnesses (15). YouTube™ is increasingly being used as a platform for health-related information (16). Since YouTube™ videos are not subjected to peer reviewing, they could

Key words: YouTube™, stomatitis, inflammation, videos, pain

Mailing address:

Prof. Rosario Serpico,
Multidisciplinary Department of Medical and Dental Specialties,
Second University of Naples, Via L. De Crecchio 6,
80138 Naples, Italy
Tel.: +39 081566 7674 - Fax: +39 081566 7674
e-mail: rosario.serpico@unicampania.it

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contain misleading information (17-21); moreover, the most popular health related YouTube™ videos were not created by public health professionals (22). However, YouTube™ could be a useful tool for healthcare professionals to bridge the gap with patients, with a great educational potential (23-25).

This study systematically examined the available information on YouTube™ about mouth sores in children on this platform comparing the obtained data with evidences in scientific literature and international guidelines.

MATERIALS AND METHODS

This work followed a previously described systematic method for searching on YouTube™ (10, 14, 16). The term ‘mouth sores in children’ (MSC) was searched on YouTube™ because this term was the most frequently used search term for RAS on the web according to ‘Google™ Trends’, with a maximum search rate in the United States (100). The search was conducted on the 23th of November

2017 using the default settings of YouTube™ without any filters and the first 60 videos were examined. The flow chart (Fig. 1) explains the standardized method used in this work.

Remaining videos were reviewed to extract video demographics such as length (VL), date and source of upload (SOU), number of views (NV), likes (NL), dislikes (NDL) and number of comments (NC). In addition, each video was given a score from 0 to 11 to indicate its usefulness as specified in Table 1. Viewers’ interaction (VI), viewing rate (VR) (1, 4, 26) and community interaction rate $[(NC/total\ NV)*100]$ (CR) were calculated. Descriptive statistics were generated with R®, Pearson’s analysis was used to examine correlations between variables and analysis of univariate variance and linear regression analysis were made to confirm the correlation hypothesis.

RESULTS

The searching term ‘mouth sores in children’ (MSC) displayed 12.300 results. The first 60 videos were screened but 31 videos (52%) were excluded following the exclusion criteria (Fig. 1). The major source of upload was from healthcare information YouTube® channels (HC-41.38%). The rest of the videos were uploaded by individual users (HP-25.59%), healthcare professionals (IU-17.24%) and generalist information YouTube® channels (HC-13.78%) as shown in Figure 2a. The mean VL was $03:09 \pm 01:48$ min (189 ± 108 sec) with a range from 00:35 min up to 08:41 min (521 sec) and a median of 02:45 min (165 sec).

The mean of NV was 108.790 ± 173.550 views (from 49 to 574.916 views – median: 38.048). Each video was viewed with a mean of 178 ± 463 views/day (from 0.2 to 2467.5 views/day). VI mean was $0.95\% \pm 3.67$ and CR was $0.09\% \pm 0.13$. The average NL for each video was 726 ± 2.535 with a peak of 13.734 likes and a median of 69, while the NDL was 49 ± 87 with a peak of 373 dislikes. Only 20.69% of videos deal with predisposing factors (stress, local trauma, nutritional deficiencies), while the same percentage (but different videos) presents information on related pathologies. Otherwise, almost all videos deal with the topic of therapy (96.55%). The majority

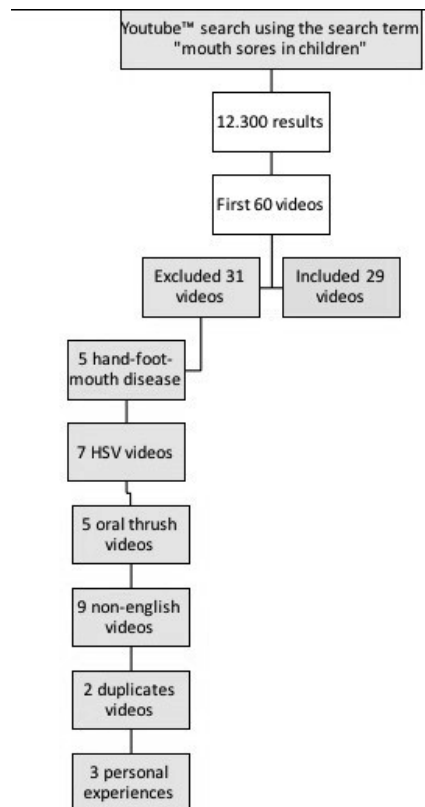


Fig. 1. Youtube™ flow chart.

Table I. *Usefulness score (US) has been attributed based on the knowledge available in scientific literature.*

Usefulness score		
Scoring Item	Description	score
Clinical features	Images demonstrating oral lesions as one or multiple rounded, shallow, painful ulcers; minor/major aphthous ulceration	1
local health effects	pain and clinical features associated with ulcers	1
general health effects	malnutrition, hospitalizations, loss of school days, diminished oral health-related quality of life	1
etiology	local trauma	1
	deficiencies of hematinics	1
	physical and psychological stress	1
	nutritional deficiency	1
	gastro-intestinal disorders	1
	(sub tot)	6
medical disorders associated	Behcet's syndrome	1
	MAGIC / Sweet / PFAPA syndrome	1
	HIV	1
	Crohn's disease	1
	celiac disease	1
	sub tot	5
therapy	antimicrobials	1
	topical corticosteroids	1
	topical analgesis	1
tot		16

of these propose home remedies (60.72%) rather than topical analgesic drugs (21.43%), antimicrobials (7.14%) and topical steroids (3.57). finally, only two videos (7.14%) propose the association of the aforementioned therapies.

No video mentioned any bibliography or source of information regarding the content presented. The mean US of all the videos analyzed was 4.34 ± 4.81 with a maximum score of 11 and a minimum score of 1; 68.97% of them resulted to be slightly useful, the 27.58% moderately useful and only the 3.45% of them resulted to be very useful for the patient. In any case, no video was cataloged as not useful. The US, VR, VI, CR, likes and dislikes values were grouped based on the different sources of upload. ANOVA univariate and Turkey test showed no significant difference in SOU (Fig. 2b). Pearson's analysis revealed that the US is not correlated with any other score analyzed.

DISCUSSION

Recurrent aphthous stomatitis (RAS) is one of the most common causes of mouth sores in children so the management of this condition is a matter of great importance for parents and little patients. The world wide web and in particular, social media platforms play a very important role in informing patients about health related problems (17, 18, 20, 24). This work analyzes data on YouTube™ both quantitatively and qualitatively. This work achieved results that are in agreement with previous studies evaluating YouTube™ videos on healthcare issues (15, 21, 27).

The content of Youtube™ on mouth sores is very heterogeneous and the information provided are for the most part incomplete. Although 58.62% of videos analyzed have been uploaded by HC (41.38%) and HP (17.24%), this does not seem to correlate,

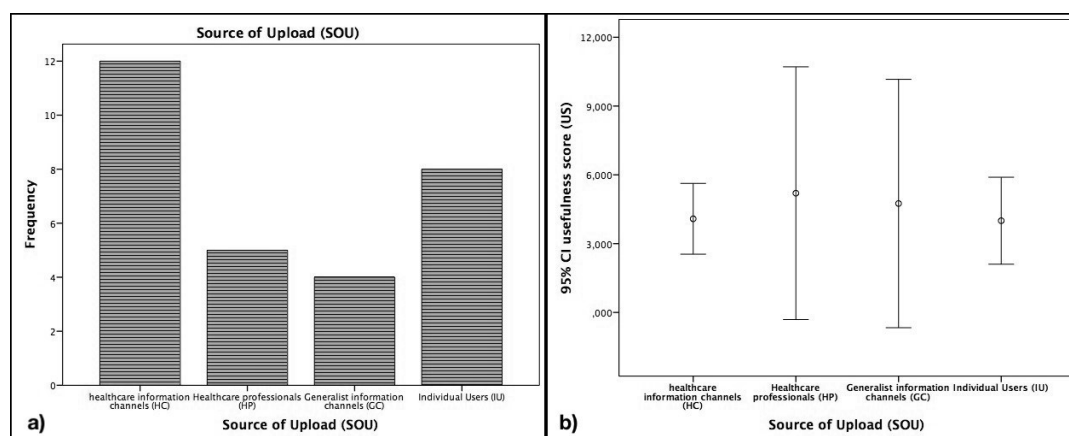


Fig. 2. (a): Frequencies of Source of Upload in analyzed video; (b): There were no differences in Usefulness Score (US) between Different Source of Upload (SOU).

however, with the higher quality of the information provided. The 68.97% of videos presented a US slightly useful and there is no significant difference among usefulness score values between different sources of upload. This is the most worrying data, as clinicians and healthcare professionals should be responsible for a higher quality of information expressed in YouTube™ videos. These works on analyzing health content on social platforms are a starting point for providing greater quality of health-related information.

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SYSTEMIC AND TOPICAL PHOTODYNAMIC THERAPY (PDT) ON ORAL MUCOSA LESIONS: AN OVERVIEW

D. DI STASIO^{1*}, A. ROMANO^{1*}, C. GENTILE¹, C. MAIO¹, A. LUCCHESI¹,
R. SERPICO¹, R. PAPARELLA¹, G. MINERVINI¹, V. CANDOTTO^{2**} and L. LAINO^{1**}

¹*Multidisciplinary Department of Medical-Surgical and Dental Specialties, University of Campania Luigi Vanvitelli, Naples, Italy;* ²*Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy*

*these authors contributed equally to this work and they are co-first authors

**these authors contributed equally to this work and they are co-last authors

Photodynamic Therapy (PDT) is a minimally invasive treatment that has shown promising results in treating preneoplastic lesions. PDT reckons on the administration of a drug (photosensitizer), which can be used in a topical or systemic form. Photodynamic therapy has been successfully used in the management of a variety of pathologies from different anatomical sites, including the head, neck, brain and lungs, hepatobiliary tree and other gastrointestinal and urological pathologies, skin, gynaecological conditions and in vascular anomalies. Starting from these same considerations, PDT has also been adopted for oral malignant and premalignant lesions' treatment, and should be kept under investigation as a promising therapeutic management of leukoplakia and preneoplastic lesions of the oral cavity.

Photodynamic therapy (PDT) is considered as a minimally invasive approach to the treatment of malignant and premalignant lesions (1), which results in resolution through selective and localized destruction of dysplastic cells. Tissue death, due to direct cytotoxic damage and microcirculation damage, as well as possible immune response, occur with swelling and formation of necrotic tissue (2). This tissue, finally, separates itself from the surrounding healthy tissue and results in a normal healing and restoration of the treated site (full repopulation). The therapeutic effects of PDT are obtained through the production of free radicals of oxygen.

In clinical practice, a photosensitizing molecule (also called "fluorophore"), which tends to accumulate in mitochondria, lysosomes and cytoplasmic membranes, is administered and subsequently

activated by a light source direct on the area to be treated. The action of the light beam damages the subcellular structures in which the fluorophores have accumulated, through mechanisms of apoptosis, necrosis and microcirculation-induced collapse, triggered by the production of radical oxygen (3).

LED lamps (Light Emitting Diode) are therapeutically advantageous light sources; their fibers, in fact, are able to easily reach every corner of the oral cavity. LEDs allow a much more delicate irradiation and a considerably lower energy production compared to lasers of equal wavelengths. These aspects are strategic because LEDs do not provide enough energy to damage tissues and there is no risk of accidental damage to the eye compared to lasers. Other advantages compared to the laser include the possibility of combining various wavelengths. The LED light disperses over a larger

Key words: Diode, mucosa, oral, cavity, lesion

Mailing address:

Prof. Rosario Serpico,
Multidisciplinary Department of Medical and Dental Specialties,
Second University of Naples,
Via L. De Crecchio 6, 80138 Naples, Italy
Tel.: +39 081566 7674
e-mail: rosario.serpico@unicampania.it

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surface than the laser and can be applied on larger target areas, allowing faster treatment (4).

Systemic Photodynamic therapy

Many systemic photosensitizers are composed of interconnected pentacyclic hydrocarbons, similar to the tetrapyrrolic group, i.e. the basic structure of porphyrin (5). Porphyrins are photosensitizers normally present in healthy tissue. While the porphyrin skeleton is not a prerequisite for photodynamic activity, the advantages offered by this molecule are related to its components, since it seems that, once activated by light, they are efficient generators of radical oxygen, needful for oxygen-mediated cytotoxicity mechanisms. In 1995 Photofrin® was adopted as the first photosensitizer approved by the FDA for PDT. Photofrin®'s biological activity results quite modest, with a very low molar extinction coefficient (ϵ) in the single red band at 630 nm; it was necessary to give the patient high doses to get a good activity, with low selectivity towards the target tissues. It showed the appearance of numerous side effects, sometimes even serious. Firstly, an elevated photosensitization of the skin, with limitations of exposure to light sources for many weeks (6). Foscan® was introduced in the second generation of photosensitizer drugs, based on temoporfin or m-tetrahydroxyphenylchlorine (mTHPC). Foscan® is the most effective generator of radical oxygen, with a quantum yield of about 0.87, which makes it superior to other photosensitizers (Photofrin® has a quantum yield of about 0.5). For this reason, it is the most used drug for the execution of PDT on preneoplastic lesions. Despite the improvement in physico-chemical and pharmacological characteristics, systemic photosensitizers still present numerous clinical disadvantages. In fact, although systemic photosensitizers currently in use or under development are characterized by very low systemic toxicity, in the absence of light, however, administration of these drugs involves a period of unwanted residual photosensitization, which must be managed as long as the drug is not deleted. Another risk is related to incorrect administration of the drug: due to the poor water solubility, high concentrations of photosensitizer can accumulate in extra-vascular sites, close to the inoculation's point. Moreover, it

the theoretical risk of an allergic reaction must be considered for intravenous administration of these drugs.

Topical Photodynamic therapy

In order to overcome the disadvantages associated with systemic PDT, 5-aminolevulinic-acid (ALA) was the first topical photosensitizer drug introduced for photodynamic application. The rationale for using ALA in PDT is based on the ability of this drug to induce the production of endogenous protoporphyrins. Photosensitization lasts no more than a few hours; ALA, in fact, has an optimal drug-light interlude, ranging from 2 to 6 h. Depending on the size of the lesion, 5-10 g of cream containing 20% ALA is applied topically on the lesions (7).

After 2 h, the cream is removed, and the lesion and the adjacent healthy tissue are illuminated for 1 h with red monochromatic light obtained with an argon laser and dyes (argon-dye) or LED lamp at 630 nm. The benefits of ALA therapy are related to the selective accumulation of the drug, which translates into a necrotizing action towards the cells that need treatment and complete harmlessness to healthy tissue (8). This way, a larger area around the lesion can be irradiated with the activating light, inducing necrosis to the lesional margins, but saving the surrounding healthy tissue. However, according to some authors, the use of ALA would be limited to superficial lesions, due to its low effect's depth (between 0.2 and 0.5 mm) (9); it makes the drug suitable for the treatment of lesions that do not cross the epithelial layer. Being performed at a superficial level, PDT with ALA does not require sterile conditions; therefore, it can be performed on outpatient procedure under local anesthesia (10).

At the moment, the use of ALA is authorized for treatment of actinic keratosis only and has shown good results in the treatment of basal cell carcinomas (11). In 2006, a case series by Aghahosseini et al. showed how topical Methylene Blue-Mediated PDT had a quick and significant beneficial effect in the control of the main symptoms and signs of Oral Lichen Planus (OLP) lesions with minimal adverse effects (12). In their study, they selected 26 OLP lesions from 13 patients, dividing, sorting by the type and the size, all the lesions in 5 groups. OLP sign score decreased in

almost all scoring groups, but complete remission was achieved only in four reticular lesions. Toluidine blue, highlighting as a nuclear marker areas corresponding to more marked mitotic activity of the cell, typically attributable to dysplastic and potentially malignant lesions, can be used as a photosensitizer too (13).

The potential of toluidine blue in PDT of cancer has been confirmed by its ability to induce an *in vitro* phototoxic effect against different varieties of tumor cell lines, including human cells of submandibular gland epidermoid carcinoma (14), HeLa cells (15) and human cells of laryngeal carcinoma (HEP-2) (16).

DISCUSSION

Photodynamic Therapy (PDT) has shown promising results as a minimally invasive treatment of oral, head and neck lesions, and can be used alone or in combination with other conventional treatments (surgery, chemotherapy and radiotherapy) (17-21). Systemically administered drugs have the advantage of reaching the deeper tissue, but cause residual photosensitivity in the patient (22). Topically administered drugs have the advantage of being more manageable, as it minimizes the uptake by non-neoplastic cells and ensures high comfort to patients, as there is neither residual photosensitivity nor theoretically, risk of an allergic reaction (which is present with intravenous administration) (8). However, the reduced absorption of the drug at the tissue level makes the topical method suitable especially for non-invasive pathologies (precancerous lesions).

Since the most used photosensitizers for PDT do not accumulate in cell nuclei, PDT does not seem to cause DNA damage, mutations or carcinogenesis, as opposed to cytotoxic chemotherapy, specifically designed to interfere with cell replication.

In recent years, there has been an increase in the interest of the scientific community in identifying a non-invasive, repeatable therapeutic pathway that is capable of determining the complete healing of premalignant oral lesions. Minimal invasive techniques are really needed because surgery may not be applicable to all cases. Despite the need for further studies, PDT fits on these new non-invasiveness horizons, also being part of a non-invasive diagnostic iter (23-27), as a promising

and hopeful minimally invasive approach for the premalignant and malignant lesion.

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ORAL-FACIAL-DIGITAL SYNDROME (OFD): 31-YEAR FOLLOW-UP MANAGEMENT AND MONITORING

G. MINERVINI^{1*}, A. ROMANO^{1*}, M. PETRUZZI², C. MAIO¹, R. SERPICO¹,
D. DI STASIO^{1**} and A. LUCCHESI^{1**}

¹*Multidisciplinary Department of Medical-Surgical and Dental Specialties, University of Campania Luigi Vanvitelli, Naples, Italy;* ²*Interdisciplinary Department of Medicine, University of Bari, Bari, Italy*

*these authors contributed equally to this work and they are co-first authors

**these authors contributed equally to this work and they are co-last authors

Orofaciodigital syndrome (OFD) is a group of hereditary disorders identified by malformations of the mouth (oris), face (facies), hands and feet (digitus=finger and toe). Although there are several different types reported in the literature, there is a great overlap in their clinical presentation. The full spectrum of all disorders due to OFD is not yet fully understood, since each patient shows variations in the expression of the syndrome. In the oral cavity, teeth are often affected by various alterations, such as dental caries, abnormal teeth, enamel hypoplasia, supernumerary teeth and dental agenesis. Treatment of a syndromic patient with a complex picture showing conditions such as palatine fissures and a severe hypodontia involves a multidisciplinary approach and a careful periodical follow-up.

Oral-facial-digital syndrome (OFD) was first described as hereditary malformation of the buccal mucous membrane and abnormal frenula in 1954 (1). The orofacioidigital (OFD) syndromes are a group of disorders that consist of facial anomalies, abnormalities of the oral cavity and malformations of fingers and toes (2). Nine different subtypes have subsequently been identified (2-4), based on various clinical findings (4). However, the spectrum of the OFD syndrome has not yet been fully revealed, since each patient shows variations in the expression of the syndrome, with a great overlap in the clinical presentation (1, 4).

Various oral features have been described in the literature. These include hyperplastic oral frenuli, buccal frenuli, median cleft lip (in 45% of patients), pseudocleft of the upper lip, lobulated (30% to

45%), bifid tongue (30% to 45%), tongue nodule, cleft palate, tongue hamartoma (70%), and high-arched palate. Hard tissue features include dental caries, anomalous anterior teeth, supernumerary teeth, missing teeth and enamel hypoplasia that are responsible for higher caries receptivity (5-7). OFD was classified into two different types; OFD I, inherited as an X-linked or sex-linked autosomal dominant and OFD II (Mohr syndrome), probably inherited as an autosomal recessive (8). OFD type I was described as a dominant trait with variable penetrance, manifested only in females and lethal in males (1, 2). Oral-facial-digital syndrome type I (OFD1) is the most common type (3). Half of the patients with OFD type I show manifestations in all areas (oral, facial and digital) (1). Papillon-Le'age and Psaume syndrome (OFD 1) have a reported

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Mailing address:

Prof. Rosario Serpico,
Multidisciplinary Department of Medical and Dental Specialties,
Second University of Naples, Via L. De Crecchio 6,
80138 Naples, Italy
Tel.: +39 081566 7674 - Fax: +39 081566 7674
e-mail: rosario.serpico@unicampania.it

frequency of 1/50,000 births (2, 4). It is characterized by the presence of multiple oral abnormalities, such as aberrant frenula, tongue abnormalities, clefts of the alveolar ridge, cleft/high arched palate, and teeth abnormalities (4). Anomalies of tooth number are common manifestations of this syndrome (5). Facial deformities include frontal bossing, hypoplastic mandible ramus and zygoma, broad nasal root and pseudocleft of mid-upper lip (2).

Other manifestations that have been described are microglossia, ankyloglossia or tongue-tie, T-shaped canine crown form, tooth fusion, infraocclusion of teeth, high caries susceptibility, small or hypoplastic mandible with a short ramus and an extended bony ridge from the alveolar crest medially to the midline in the canine premolar area (1). The cephalometric characteristics of OFD1 show micrognathia, with a large gonial angle, high mandibular plane (3) and a tendency to increase in the skull-base angle (8). Facial anomalies show in 69.1% of cases, evanescent milia (29.4%) of the face and ears (which usually disappear before the third year of life), dryness, brittleness and/or alopecia of the scalp hair (21.5%) (4, 9). Mental retardation and/or selective cognitive impairment has also been described (2, 4, 8). Digital malformations affect the hands (50%-70%) more frequently than the feet (25%) and include polydactyly, clinodactyly, brachydactyly, syndactyly (2, 3), camptodactyly and extra digits (1) and, more rarely, preaxial or postaxial polydactyly (3). Short tubular hand and foot bones, hypoplasia of the second to fifth toes and the hallux often bent in a tubular direction (2).

Cilia dysfunction give rise to a wide spectrum of diseases ranging from organ specific such as polycystic kidney disease to more pleiotropic disorders, such as Bardet-Biedl, Alstrom, Jeune short rib polydactyly, and OFD1 syndromes (5).

Mohr syndrome or OFD type II (OFD II) has a frequency of 1/330,000 births and is characterized by oral/maxiofacial deformities such as low nasal bridge, broad nasal tip sometimes slightly bifid, hypertelorism, nodules on tongue, midline cleft of lip, high arched or cleft palate, hypertrophy of usual frenula, hypoplasia of zygomatic arch and maxilla and micrognathia (2). Malposition of teeth is a consistent finding, as well as agenesis,

supernumerary teeth, delayed eruption, microdontia, talonism, supernumerary cusps and abnormal root morphology. The canines are occasionally separated from the incisors or premolars by a gap resulting from a hypertrophied frenulum. Other abnormalities include partial reduplication of hallux, brachydactyly, syndactyly, polydactyly and conductive hearing loss (2, 9).

The therapeutic approach involves a multidisciplinary course of action involving surgeons, pediatricians, genetic experts, orthodontists, orthophonists and psychologists. The management of the various orofacial abnormalities appears to be based on the characteristics of the individual cases (5).

Clinical Report

Patient reported was a Caucasian girl who first presented to the dental clinic at the age of 9.2 years, referred by a general practitioner for observation of the development of her dentition.

The patient had already been diagnosed with OFD syndrome. Pregnancy was uneventful and the patient was born at full term; she presented the icterus neonatorum. On first clinical examination, the patient did not show many typical manifestations of the OFD syndrome. In fact, there was no cleft of either of the lips and neither the hair nor the skin appeared to be affected. Mental deficiency had not been reported. Her face showed asymmetry: the right orbit was lower than the left; the patient has oval facies with underdeveloped left half face. There was a hypertrophied ethmoid, the sella turcica was small, but regular on the bottom, the aditus was restricted and there was a hypertrophy of clinoid and an Olympic skull. She also had small demineralized hands, brachydactyly, clinodactyly and camptodactyly of the second and third finger of right hand, the phalange of third finger deflect, neither presented polydactyly and there was a Metacarpus hypoplasia and osteoporosis carpal bones (Fig. 1). Right foot shows brachydactyly and camptodactyly; second and third metatarsus of left foot are short and large. Abnormal features common to both were essential severe valgus, flat feet and a bilateral varus tibia (Fig. 1).

According to her medical history, the girl had an operation to correct her limb deformity and brachydactyly. Radiograph of both feet showed short metatarsals and phalanges. The form and the structure of the kidneys is normal, but the right one is rotated. Oral examination showed anterior and lateral open bite, with basal and alveolar hypoplasia of the maxillary and mandibular bones. The malocclusion consisted of a dental skeletal class III. The cephalometric analysis showed a mild Class III pattern (ANB, -1° ; mean, 0° -4°) (Fig. 2).

Oral hygiene was reasonable, the patient was caries free. On her initial oral examination, the patient was in the primary dentition stage of development, and showed a severe enamel hypoplasia. Primary teeth eruption was tardive at the age of 1 y as well as the exchange that started at 7 y.o. In the upper arch, there were impacted and ectopic rudimentary teeth, confirmed by the OPG. 13, 15 and 24 were ectopic, the traction fouls because the teeth were ankylosed and so were extracted with remaining tooth formula 17;16;14;22;23;25;26;27 (Fig. 2).

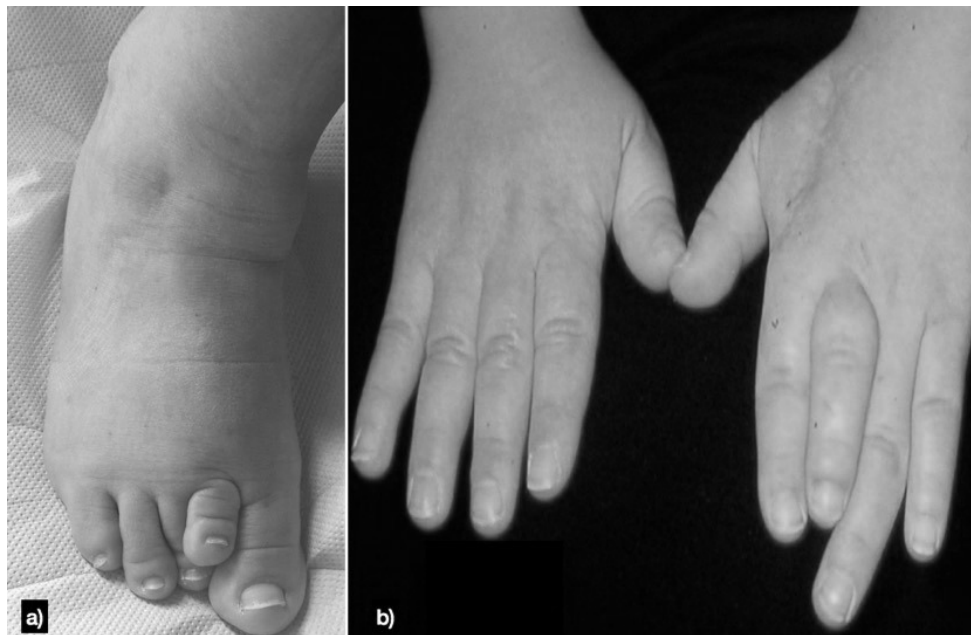


Fig. 1. *Morphology of hand and foot.*



Fig. 2. *Radiography of skull and jaws.*

Marked hypertrophy of the buccal and lingual frenula was observed. The patient has always refused any orthodontic and surgical treatment. She was rehabilitated with a removable prosthesis. The use of a full acrylic flange extension was used for the replacement of lost soft and hard tissues. The patient has been monitored with several short-term follow-ups until today (40 years-of-age), and is kept in a non-surgical periodontal supportive therapy and manages to preserve all dental elements.

DISCUSSION

The occurrence of various oral conditions such as a cleft palate, hypodontia and supernumerary teeth in a single patient requires multidisciplinary approach, treatment planning, dental care and a long-term monitoring (5). Dental caries can be a problem for patients with OFD syndrome type 1. The presence of crowding caused by the eruption of supplemental teeth and a constricted dental arch can result in areas where plaque can easily accumulate, and tooth brushing is likely to be further compromised by hyperplastic frena. A preventive program consisting of oral hygiene instructions and dietary advice is needed (5).

Definitely, for the clinician, the key to success of OFD syndromic patient management is to intervene following the right course of action. First of all, it should have to deal with supernumerary and other teeth as needed to surgically remove; then, malocclusion should be treated with orthodontic approach and dental rehabilitation, including restorations and prosthetic treatment should be needed (9).

Finally, careful and adequate periodical follow-ups are needed to better manage patient's

compliance, his domestic oral care and his responsiveness over the time.

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TELESCOPIC OVERDENTURE ON NATURAL TEETH: PROSTHETIC REHABILITATION ON OFD SYNDROMIC PATIENT AND A REVIEW ON AVAILABLE LITERATURE

G. MINERVINI^{1*}, A. ROMANO^{1*}, M. PETRUZZI³, C. MAIO¹, R. SERPICO¹,
A. LUCCHESI¹, V. CANDOTTO^{2**} and D. DI STASIO^{1**}

¹Multidisciplinary Department of Medical-Surgical and Dental Specialties, University of Campania Luigi Vanvitelli, Naples, Italy; ²Department of Biomedical, Surgical and Dental Sciences, University of Milan, Italy; ³Interdisciplinary Department of Medicine, University of Bari, Bari, Italy

*these authors contributed equally to this work and they are co-first authors

**these authors contributed equally to this work and they are co-last authors

Abstract Orofaciodigital syndromes (OFD) are rare genetical disorders characterized by malformations of the mouth (oris), face (facies), hands and feet (digitus = finger, toe). It is still impossible to fully understand the whole spectrum of all dysfunctions due to OFD, since the expressions of the syndrome vary in each patient. There are various alterations, thoroughly described in literature that can affect the oral cavity such as dental caries, abnormal teeth, enamel hypoplasia, supernumerary teeth and dental agenesis. A syndromic patient with a complex picture showing conditions such as palatine fissures and a severe hypodontia has to be treated using a multidisciplinary approach, and in particular, needs a prosthetic rehabilitation for the restoration of missing dental elements in the arch.

The orofacioidigital (OFD) are a group of syndromes that consist of facial anomalies, abnormalities of the oral cavity and malformations of fingers and toes (1, 2). There are nine different subtypes that have been identified (1, 3, 4), based on several clinical findings (4). Hard tissue features include dental caries, anomalous anterior teeth, supernumerary teeth (1, 4-6), missing teeth (2, 5, 7) and enamel hypoplasia (8, 9). Only a multidisciplinary course of action involving surgeons, endodontist, orthodontist and prosthodontist (1, 10) can constitute an effective therapeutic approach. The goal of a choice involving prosthetic rehabilitation should not only aim to restore dental structures, but also to give an adequate prosthetic support to the defective osteo-mucous tissues that both compensate aesthetically the mandibular hypoplasia, and the condition of the

III skeletal class. For this reason, the opportunity to rehabilitate the patient with an overdenture solution on natural teeth was taken into consideration.

Clinical Report

The patient considered in this paper was a Caucasian girl who was presented to the dental hospital clinic for the first time when she was 9 years- and 2-months-old. The patient had already received a diagnosis for orofacioidigital syndrome. In the upper arch 13, 15 and 24 were ectopic. These rudimental teeth were ankylosed causing traction issues. Therefore, these teeth were extracted with remaining tooth formula 17;16;14;22;23;25;26;27. There was a basal and alveolar hypoplasia of the maxillary with a malocclusion consisted of a dental skeletal class III (ANB, -1°). Patients affected by orofacioidigital

Key words: orofacioidigital syndromes, prosthetic rehabilitation, telescopic prosthesis

Mailing address:

Prof. Rosario Serpico,
Multidisciplinary Department of Medical and Dental Specialties,
Second University of Naples, Via L. De Crecchio 6,
80138 Naples, Italy
Tel.: +39 081566 7674
e-mail: rosario.serpico@unicampania.it

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syndrome present some peculiar anatomical features in their oral cavity, such as particularly deep grooves and vertically reduced alveolar ridges. These features constitute major impediments to the construction of traditional removable prosthetic artifact for restoring the missing dental elements. Since the patient had always refused any orthodontic or surgical treatment, we could only proceed with a prosthetic approach.

Firstly, the rehabilitation was achieved with removable prosthesis; the lower arch was arranged in centrically to improve inter-arch relationship (ANB +1). Alginate impressions were then made and using facial arch and articulator, a diagnostics waxing was obtained. The parallelometer was used to evaluate frameworks; showing that only the 2.5 needed an elective root treatment in order to better obtain the necessary parallelism with the other elements. Abutments were prepared on finish line using a dime. Subsequently all the elements were imprinted by single step, double elastomeric impression for the realization of the type III-IV gold alloy mesostructures (male). Since in the lower arch there were still natural teeth, in order to avoid usury and vertical dimension loss, it was decided that it was best to develop ceramic inlays in the occlusal surfaces. When the prosthetic rehabilitation was completed (25th January 2005), the patient had been observed and treated with several short-term follow-ups for 13 years.

During this time, due to periodontal disease, the 2.5 had been extracted. The lost abutment was dealt with by simply filling the overcrown with acrylic resin to form a pontic (11). To date, the patient is still being treated with a non-surgical periodontal supportive therapy and she still manages to preserve all the abutments.

Available therapeutic strategies

There are three prosthetic options available to the practitioner. They depend on the strategic decision that can either be to maintain a certain number of teeth with a good prognosis; to extract all remaining teeth in a jaw; or to place implants at prosthetically favorable positions (12).

However, also other issues can influence the priorities of the patient or restrict the range of choices;

for instance, financial constraints may prevent the patient from choosing the most desirable treatment (13). Zitzmann and Marinello (14) introduced a new notion of treatment based on a method that enables the practitioner to choose the appropriate type of restoration. The presented systematical analysis and the following classifications give the dentists the possibility to evaluate in detail the relationship between anatomical structures, and implant and teeth position. These data allows the practitioner to explain and discuss specific aspects with the patient (12).

In the first case, the implant rehabilitation was unsuitable because the goal that had to be achieved with prosthetic rehabilitation was not only to restore the dental structures, but also to rehabilitate the osteo-mucous tissues, with adequate prosthetic support flange. Literature clearly shows that fixed prosthetic therapy is extremely more complex when facing either moderate or severe loss of hard and soft tissues (12). In literature, acrylic materials, composites, and pink porcelain are described as suitable replacements for lost soft and hard tissues, as the most aesthetic advantage for the support of facial tissues (12).

An alternative treatment can be found in endosseous implants, but considering the age and potential growth of our patient, postponing the osseointegrated implants seemed the best option (15). It is easier to have right prosthodontic outcomes than making predictions for patients with removable overdentures supported and retained either by tooth roots or implants (16-18). Combined tooth-implant support in removable prosthetics is not often discussed in literature (10). The presence of healthy periodontal ligaments help maintain alveolar ridge morphology and dimensions (18). Overdentures realized on natural teeth enhance proprioception, improve prosthesis stability and retention (19-22) and may have positive effect on the psyche of patients (13). The masseter muscles' electromyographic activity in tooth-supported overdenture group is lower than others (22); this seems to suggest that the role of periodontal ligament is very important during chewing, for the efficiency of the muscular activity in patients wearing overdenture prosthesis

(17, 23). The overdenture has many advantages and many options are available for retention of the prosthesis, including magnets, clips, bars and balls (17) (21); however, every case has a best option and there is no universal fit. Therefore, it is critical that the appropriate attachment is selected for each individual situation (18). It has been established that the telescopic dentures, supported by the roots of natural teeth, tend to have more predictable prosthodontics outcomes, thanks to the increased support, stability, retention and decrease in rate of the residual ridge resorption (6).

CONCLUSION

The selection of strategic roots or teeth for retention plays a key role in the results of an overdenture (24). It has been largely discussed that the risk of loss of vitality of the tooth constitutes the main disadvantage that can be encountered in the housing of the telescopic device. Thus, the tooth must be treated endodontically to ensure adequate preparation of the tooth for prosthesis. In our case, on 8 total elements in the upper arch, it was necessary to treat only 1 for the preparation of the abutment on which to dwell the telescopic device. The main cause of denture failures nowadays, seems to be the denture fractures and loss of abutment teeth (12). These problems can be overcome by simply filling the overcrown with acrylic resin to form a pontic (11). A major disadvantage is the high demand on precision and special skills required of both the dental technician and the clinician. The total cost of the prosthesis may end up being very expensive. This issue can be solved thanks to the introduction of cheaper and less dense casting alloys (12). Currently, the recommended alloy for fabrication of telescopic and conical crowns is the one that has elevated quantities of gold and elastic modulus together with being very hard (ADA Type III-IV) (25). Follow-up studies on telescopic and conical crown-retained dentures report survival rates between 92% and 100% (26) (27), 3.8% abutment teeth were extracted and 4.7% dentures were reported as failures. Subjects with few remaining abutment teeth have significantly higher failure rates (25). Telescopic

prosthesis can be designed to be removed daily, enabling the patient to effectively remove plaque at the gingival crest, while taking better care of oral hygiene around the abutments (13, 17). Important factors in the abutments longevity are also the regular maintenance care, topical fluoride use and oral hygiene (17) (22). When all these factors are well managed and taken care of, even teeth with a relatively poor prognosis can be successfully utilized as overdenture abutments (26). Our rehabilitation outcomes seem to be as accurately predictable as the literature reported; the removable prosthesis by using telescopic overdenture on natural teeth has guaranteed both the expected aesthetic result and the prosthetic success after 13 years of follow-ups.

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LICHEN PLANUS: MOLECULAR PATHWAY AND CLINICAL IMPLICATIONS IN ORAL DISORDERS

M. BOCCELLINO^{1*}, D. DI STASIO^{2*}, A. ROMANO², M. PETRUZZI³, A. LUCCHESI²,
R. SERPICO², L. FRATI⁴ and M. DI DOMENICO^{1,5}

¹*Department of Biochemistry, Biophysics and General Pathology, University of Campania “Luigi Vanvitelli”, Naples, Italy;* ²*Multidisciplinary Department of Medical-Surgical and Dental Specialties, University of Campania Luigi Vanvitelli, Naples, Italy;* ³*Interdisciplinary Department of Medicine, University of Bari, Bari, Italy;* ⁴*Department of Molecular Medicine, Policlinico Umberto I, Sapienza University of Rome, Rome, Italy;* ⁵*Sbarro Institute for Cancer Research and Molecular Medicine, Center for Biotechnology, Temple University, Philadelphia, PA, USA*

*these authors contributed equally to this work and they are co-first authors

Stem cells play a role in many mucosal disorders characterised by abnormal proliferation and differentiation of keratinocytes, such as oral lichen planus (OLP). In OLP there were changes in stem cell markers as component of integrin complexes $\alpha 6$ and $\beta 1$ integrin increased along with increase of melanoma-associated chondroitin sulphate proteoglycan (MCSP) and decreased of notch1 (N1) and keratin 15 (K15). Stem cell marker expression may be altered by pathological signalling in these lesions. Cadherins are transmembrane receptors that provide cell-cell contact and communication function through calcium-dependent homophilic and heterophilic interactions. In actively diseased areas of OLP lesions, basal keratinocytes downregulate CD40 and were focally E-cadherin-negative, in contrast to non-diseased areas and normal oral mucosa. This loss of E-cadherin expression may contribute to epithelial basal cell destruction and T-cell migration into the epithelial compartment in OLP. In addition, Growth factor pathways as a role in OLP and has been analyzed in this review.

Oral lichen planus (OLP) is a chronic inflammatory mucous-cutaneous disease (1) and is classified as a potentially malignant condition by the World Health Organization (WHO) (2). Clinical presentation of OLP ranges from asymptomatic reticular white lichen to symptomatic atrophic-erosive red lichen with symptoms of burning, irritation and pain (3, 4). For the erosive forms, a differential diagnosis must be made with pemphigus and pemphigoid, both through careful clinical examination, through instruments that allow an *in vivo*, non-invasive diagnosis, even if the gold standard remains biopsy (5). OLP has a protracted

clinical course despite various available treatments, with a substantial negative impact on the patients' quality of life (6). Evidence indicates that OLP is an autoimmune disease mediated by cytotoxic T cells attack against epithelial cells, causing disruption of basement membrane, liquefaction degeneration of the basal cells and sub-epithelial band-like infiltration of predominantly T lymphocytes as well as a keratinocyte dysregulation. The precise aetiology, however, remains unclear (7). Stem cells play a role in many mucosal disorders characterised by abnormal proliferation and differentiation of keratinocytes,

Key words: Stem cells, keratinocytes, lichen planus, cadherins, integrins, growth factors pathways

Mailing address:

Prof. Rosario Serpico,
Multidisciplinary Department of Medical and Dental Specialties,
Second University of Naples, Via L. De Crecchio 6,
80138 Naples, Italy
Tel.: +39 081566 7674
e-mail: rosario.serpico@unicampania.it

such as OLP (8-10); despite the degree of malignant transformation reported in the literature is low, an analysis of the molecular and cellular pathway of this pathology could be an important diagnostic and prognostic factor (9, 11-14).

Nuclear Targets

Notch

Notch signaling is an evolutionarily conserved pathway that regulates numerous cellular processes. Altered notch signaling affects the function of a variety of mammalian stem cells including those from marrow, skin and intestine (15). In mammals, there are four notch receptors. Notch signaling is triggered by activation of cell-surface receptors which lead to proteolytic cleavage and translocation of the Notch cytoplasmic domain to the nucleus, where it binds to the transcriptional repressor CSL (RBP-J) to activate target gene expression (16, 17).

Another direct target of Notch/RBP-J transcription is p21, which mediates cell cycle arrest in primary keratinocytes (18). Notch-mediated RBP-J signaling is important for terminal differentiation and maintenance of hair follicles and sebaceous glands (19). Notch signaling pathway has pleiotropic effect in different self-renewing organs such as skin, intestine, lymphoid system (20). Oral lichen planus is histologically characterized by T lymphocyte-mediated degeneration of basal layer keratinocytes. This basal cell damage was accompanied by changes in basal cell phenotype such as reduced expression of keratin 5 (K5) and keratin 14 (K14). In OLP there were other changes in stem cell markers as increased of component of integrin complexes $\alpha 6$ and $\beta 1$ integrin; increased of melanoma-associated chondroitin sulphate proteoglycan (MCSP), decreased of notch1 (N1) and keratin 15 (K15). Then, pathological signaling in these lesions (8) may alter stem cell marker expression.

Cadherins

Cadherins are transmembrane receptors that provide cell-cell contact and communication function through calcium-dependent homophilic and heterophilic interactions. These interactions are crucial for enhancing the cell's ability both to interact

with neighboring cells, but also for cell signaling (21-23). According to their structural features, cadherins are classified into type I, type II, and type III or atypical cadherins. Type I cadherins possess a unique histidine-alanine-valine (HAV) motif in their amino acid sequence and has a single tryptophan; type II cadherins contain the HAV motif, but this has two tryptophan residues, type III or atypical cadherins possess calcium binding sites, but do not typically have HAV or tryptophan residues in their amino acid sequence (24).

E-cadherin, a type-I cadherin, is mainly expressed in the epithelial cells of humans and animals and is the first cadherin expressed during mammalian development. E-cadherin is essential for the maintenance of cell polarity, cohesion of tissues, morphology of epithelial cell and tissue integrity. E-cadherin-mediated junctions play an important role in tumorigenesis; in fact, impairment of the junction increases cellular invasion and migration, facilitating the growth and proliferation of tumor cells at metastatic positions (22, 25, 26). Recent studies shown that E-cadherin expression is abnormal in a variety of malignant tumors of epithelial origin, such as colorectal/gastric carcinoma, hepatoma, lung neoplasms, breast carcinoma, prostate cancer and endometrial malignant tumors (27, 28). Since an abnormal expression of E-cadherin has been observed in oral leukoplakia and cervical intraepithelial neoplasia, its involvement in precancerous lesions has been suggested.

Therefore, it is possible that E-cadherin may be a suitable indicator for early diagnosis and prevention of tumors. DU Y. and Li H. in their immunohistochemical study reported the E-cadherin expression both in OLP and in normal oral mucosa samples. They found that 25 of 52 OLP samples exhibited normal E-cadherin expression and 27 had abnormal expression corresponding to 51.92% abnormal rate. The abnormal positive rate in the normal oral mucosa was significantly lower than in the OLP group (4.88%). This suggests the involvement of E-cadherin in the malignant transformation of OLP and supports the malignant potential of OLP. Ebrahimi M et al. (12) showed decreased expression of beta-catenin, E-cadherin and EGFR in samples

from patients with OLP compared with the normal controls.

In actively diseased areas of OLP lesions, basal keratinocytes downregulate CD40 and were focally E-cadherin-negative, in contrast to non-diseased areas and normal oral mucosa. This loss of E-cadherin expression may contribute to epithelial basal cell destruction and T-cell migration into the epithelial compartment in OLP (29).

Growth factors pathways

The protein Epidermal Growth Factor (EGF) was explained by Stanley Cohen (30). EGF is capable to generate epidermis proliferation and to bring out precocious teeth eruption. Keratinocyte growth factor (KGF) could cause the evolution and expansion of many tumor entities (31).

EGF and Notch signaling also promote epithelial Mesenchymal Transition (EMT). In EMT processes physiological and pathological Notch signaling pathway is implicated (32). A premalignant condition in oral mucosa is named lichen planus (OLP) that is an oral's mucosa chronic inflammatory disorder. Epithelial mesenchymal transition (EMT) is a step in the hostile progression, where epithelial cells obtain mesenchymal characteristics. Growth factor- β (TGF- β) utilizes Smad proteins (those proteins are an integral part of the TGF β signaling pathway), like mediators for the signaling to get EMT (33). Epithelial-Mesenchymal Transition (EMT) modifies in oral lichen planus because of malignant behavior of this disorder and its tendency to change to head and neck squamous cell carcinoma (HNSCC). Smad3 is a protein that has function in EMT. Why? Because it is required in TGF- β pathway and is eminently expressed in various cancers (33,34). The SMAD arrests the cell cycle: losing these genes may ensure the inhibited cell growth. The tumoral advancement is operated by Smad3 together with co-factor SNAIL in suppression of E-cadherin and Occludin (34).

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COLONIZATION OF LEGIONELLA SPP. IN DENTAL UNIT WATERLINES

F. CARINCI^{1*}, L. SCAPOLI^{2*}, M. CONTALDO³, R. SANTORO³, A. PALMIERI²,
F. PEZZETTI², D. LAURITANO⁴, V. CANDOTTO⁵, D. MUCCHI⁶, L. BAGGI⁷,
A. TAGLIABUE^{8**} and L. TETTAMANTI^{8**}

¹Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, Italy; ²Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna, Bologna, Italy; ³Multidisciplinary Department of Medical-Surgical and Dental Specialties, University of Campania Luigi Vanvitelli, Naples, Italy; ⁴Department of Medicine and Surgery, University of Milano Bicocca, Monza, Italy; ⁵Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy; ⁶LAB S.r.l., Codigoro, Ferrara, Italy; ⁷Department of Clinical Sciences and Translational Medicine, University of Tor Vergata, Rome, Italy; ⁸Department of Medicine and Surgery, University of Insubria, Varese, Italy

*these authors contributed equally to this work and they are co-first authors

**these authors contributed equally to this work and they are co-last authors

Legionella spp. are ubiquitous in aquatic habitats and water distribution systems, including dental unit waterlines. Surveys have shown that the percentage of samples taken at different dental sites that were positive for Legionella spp. was highly variable and ranged from 0% to 100%. Cultivation is the principal approach to evaluating bacterial contamination employed in the past, but applying this approach to testing for Legionella spp. may result in false-negative data or underestimated bacterial counts. PCR and direct fluorescent counts can detect viable non-cultivable bacteria, which are not counted by plating procedures. Legionella spp., commonly form such viable non-culturable cells and it is likely that they contribute to the difference between plate count results and those of PCR and fluorescent-antibody detection. However, studies have shown that Legionella is present in the municipal water source in spite of the current filtration and chlorination procedures. Once Legionella reaches the building water system, it settles down into a biofilm layer of stagnant water. By means of this layer, Legionella can protect itself from antimicrobial agents and then multiply. Dental unit waterlines may be contaminated with opportunistic bacteria. The water quality in the dental units should be controlled to eliminate opportunistic pathogens and to provide water for dental treatment that meets public health standards for potable water.

Legionella spp. are ubiquitous in aquatic habitats and water distribution systems, including dental unit waterlines. Surveys have shown that the percentage of samples taken at different dental sites that were positive for Legionella spp. was highly variable and ranged from 0% to 100% (1-6).

The concentration of Legionella spp. in the water of dental units may reach 1000 organisms per mL. The primary route of Legionella spp. transmission is inhalation or aspiration of environmentally contaminated aerosol. Bio-aerosols generated during dental procedures are the main source of legionella,

Key words: legionella, oral pathogens, dental chair, dental infection control, biofilm

Mailing address:

Prof. Angelo Tagliabue,
Department of Medicine and Surgery,
University of Insubria, Via Piatti, 10,
21100 Varese, Italy
Tel.: +39.0332-825625 - Fax: +39.0332-825655
e-mail: angelo.tagliabue@uninsubria.it

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the agents that cause both Legionnaires' disease and Pontiac fever. Raised levels of antibodies to legionella confirm that dental surgeons, nurses, hygienists and patients are exposed to contaminated aerosols. This may reflect continuous exposure to small numbers of these microorganisms that may result in mild Pontiac fever or subclinical infections (1, 7-9).

Biofilms formed within the small bore tubing of dental units create an environment that favours the attachment and growth of *Legionella* species. Once colonized, other microorganisms as well as protozoa may attach to this biofilm (8). Under adverse conditions, amoebae will increase their uptake of bacteria and provide a continuous supply of bacteria that are released into the environment (9). This may be problematic because legionella-laden amoebae may contain infective numbers of organisms. The recognition of amoebae as reservoirs and vehicles for bacterial spread leads to public health concerns (10).

The oral cavity is a unique environment, which provides an ideal medium for bacterial growth. Microorganisms present in the oral cavity may be transmitted from person to person through aerosol, water contamination or surface contact. Aerosol produced during use of scaler or airtor of dental chair contains droplet nuclei particles, which remain in the environment for long periods, and is a source of infection for the patient as well as the health care provider (11). The contaminated water in dental chair waterlines is yet another source for transmission of infection. The second argument is that dental instruments, such as air-water syringe, dental turbine and ultrasonic scaler handpieces, produce aerosols. Thus, if dental unit water is contaminated with legionella, these microorganisms could be spread in the environment during dental therapy and inhaled by patients and staff (12).

Detachment of Legionella

Cultivation is the principal approach to evaluating bacterial contamination employed in the past, but applying this approach to testing for *Legionella* spp. may result in false-negative data or underestimated bacterial counts. The rates of recovery of *Legionella* spp. using culture methods are generally markedly lower than 100% (3), due to the fastidious growth

requirements of these bacteria, the overgrowth by other bacteria, and the legionella damage/loss when samples are concentrated. In contrast, PCR methods can be used to detect non-culturable legionella, those living within amoebae (4, 5) and doublets or chains of *Legionella* cells, which are counted as only 1 CFU when using the culture method but can be counted as individual cells using qPCR.

PCR and direct fluorescent counts can detect viable non-culturable bacteria, which are not counted by plating procedures. *Legionella* spp. commonly form such viable non-culturable cells (13), and it is likely that they contributed to the difference between plate count results and those of PCR and fluorescent-antibody detection. In addition, plate counts of *Legionella* spp. often underestimate the presence of *Legionella* spp. in water samples because of interference by other heterotrophic bacteria or because the *Legionella* spp. are within protozoa. Many of the *Legionella* spp. observed by fluorescence microscopy appeared to be within or on the surfaces of protozoa in the biofilm lining the flexible thin lines used in the dental-unit water systems (14).

The 2015 Italian guidelines for the prevention of Legionnaires' disease (15) included safety recommendations for dental surgeries for the first time. All dentists are required to conduct a statutory risk assessment of their practices. To comply with their legal duties, employers must identify and assess the sources of risk and prepare a scheme for preventing and controlling risks. Moreover, they must monitor the quality of their DUWLs at least annually to ensure that the waterlines are "legionella free".

DISCUSSION

Several studies have indicated that dentists and dental staff have higher rates of respiratory infections than the public (16). Contaminated handpieces are believed to be at least partially responsible for these higher rates of respiratory disease (17). Appropriate procedures to decontaminate handpieces, including autoclaving and handpiece replacement between patients, have been developed and implemented

in many dental practices (18). These procedures are aimed at reducing the likelihood of aerosol dissemination of pathogens within dental operatories and the resulting infections. However, decontamination of handpieces such as high-speed drills and syringes does not remove the potential for exposure to pathogens that originate within the water lines of dental units.

Challacombe and Fernandes (11) indicated that units contaminated with *Legionella pneumophila* also could contain high numbers of other bacteria (mostly *Pseudomonas*). Furthermore, dental units are suspected as an important source of *Legionella* spp. In one case, a dentist in Northern California died due to *Legionella* infection acquired from the dental unit (1). According to a study by Costerton et al. (19), biofilms, once established, are notoriously difficult to remove because of their complex nature. Additionally, Walker et al. (20) claimed that chemicals, such as hydrogen peroxide and iodine could not totally remove the biofilm in DUWL. By contrast, Meiller et al. (21) assert that chlorhexidine and glutaraldehyde agents have reduced the volume of biofilm.

However, studies have shown that *Legionella* is present in the municipal water source in spite of the current filtration and chlorination procedures (22). Once *Legionella* reaches the buildings' water system, it settles down into a biofilm layer of stagnant water. By means of this layer, *Legionella* can protect itself from antimicrobial agents, and then multiply.

The American Dental Association (ADA) (23), has set a heterotrophic bacteria load of ≤ 200 cfu/mL for water delivered from dental unit waterlines, while the CDC (24) guidelines for infection Control in Dental Health-Care Settings suggest that the numbers of microorganisms should meet nationally recognized standards for safe drinking water, considering that the standard set for the USA by the Environmental Protection Agency (EPA) is ≤ 500 cfu/mL.

In Italy (25) standards for drinking water include ≤ 100 cfu/mL of heterotrophic bacteria (HPC at 22°C) and ≤ 20 cfu/mL of mesophilic bacteria (HPC at 37°C), in accordance with the European and national directives.

CONCLUSIONS

Dental unit waterlines can be contaminated with opportunistic bacteria. The water quality in the dental units should be controlled to eliminate opportunistic pathogens and to provide water for dental treatment that meets public health standards for potable water (12). Clinicians can apply some preventive methods, as follows: flushing through the chair for 3 min at the start of each day; supplying the dental unit reservoir with sterile and good quality water; autoclaving dental hand pieces after each use; regular maintenance of the dental unit system and regular chlorination of the water system of the dental clinic.

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FOCUS ON PERIODONTAL DISEASE AND DEVELOPMENT OF ENDOCARDITIS

F. CARINCI^{1*}, M. MARTINELLI^{2*}, M. CONTALDO³, R. SANTORO³, F. PEZZETTI²,
D. LAURITANO⁴, V. CANDOTTO⁵, D. MUCCHI⁶, A. PALMIERI²,
A. TAGLIABUE^{7**} and L. TETTAMANTI^{7**}

¹Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, Italy; ²Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna, Bologna, Italy; ³Multidisciplinary Department of Medical-Surgical and Dental Specialties, University of Campania Luigi Vanvitelli, Naples, Italy; ⁴Department of Medicine and Surgery, University of Milano Bicocca, Monza, Italy; ⁵Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy; ⁶LAB S.r.l., Codigoro, Ferrara, Italy; ⁷Department of Medicine and Surgery, University of Insubria, Varese, Italy

*these authors contributed equally to this work and they are co-first authors

**these authors contributed equally to this work and they are co-last authors

Abstract Infective endocarditis is a devastating disease with high morbidity and mortality. The link to oral bacteria has been known for many decades and has caused ongoing concern for dentists, patients and cardiologists. The microbiota of the mouth is extremely diverse and more than 700 bacterial species have been detected. Half of them are uncultivable so far. Oral microbiota is not uniform, specific sites exist in the mouth such as tongue, palate, cheek, teeth and periodontal pockets that have their own microbiota. Factors involved in the development of a bacterial endocarditis are difficult to define but a vulnerable surface (i.e. a damaged endocardium) and a high bacterial load in the blood seems to be decisive. The cause of microorganisms, in 90% of cases, are staphylococcus, streptococcus and enterococcus. Oral streptococci belong to viridans group (streptococcus mutans and streptococcus sanguis). As they are part of dental plaque, they could enter the bloodstream causing bacteraemia through daily habits like chewing or tooth brushing. Effective treatment of periodontal infections is important to reduce local inflammation and bacteraemia. In addition, poor periodontal health appears to increase the risk of cardiovascular disease, pulmonary disease, and preterm and low birth weight. **Conclusions:** Long-standing oral disease prevention protocols reduce the risk of developing periodontal disease. Data suggest that methods used to prevent cases of IE that originate from oral bacteria should focus on improving oral hygiene and reducing or eliminating gingivitis, which should reduce the incidence of bacteraemia after tooth-brushing and the need to extract teeth owing to periodontal disease and caries.

Infective endocarditis is a devastating disease with high morbidity and mortality. The link to oral bacteria has been known for many decades and has caused ongoing concern for dentists, patients and cardiologists.

Infective endocarditis (IE) is a disease caused by a bacteraemia that affects different organs or tissues, including the oral cavity. Although it has a low incidence, it might imply a potential threat to the life of the affected individual. Predominantly it tends

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Mailing address:

Prof. Angelo Tagliabue,
Department of Medicine and Surgery,
University of Insubria, Via Piatti, 10,
21100 Varese, Italy
Tel.: +39.0332-825625 - Fax: +39.0332-825655
e-mail: angelo.tagliabue@uninsubria.it

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to develop on cardiac valves previously damaged, the mitral valve being its most frequent location, followed by the aortic and in rare occasions the pulmonary valve.

The incidence is estimated to be 1 to 5 cases per 100.000 inhabitants (1). It is rare amongst younger population with the exception of intravenous drug users. Heart valves, sometimes damaged by diseases (i.e. a rheumatic heart disease), can frequently be affected by bacterial endocarditis (BE), although this disease might equally affect those people suffering from a congenital heart disease or after undergoing a valve surgery. Conversely, those people affected by a non-complex myocardial infarction or having faced non-complex angioplasties, coronary bypass or cardiac pacemakers are not likely to risk an infected endocarditis (IE) (2).

Microbiota of the mouth

The microbiota of the mouth is extremely diverse. More than 700 bacterial species have been detected here (3). Half of them are uncultivable so far. The oral microbiota is not uniform. Specific sites exist in the mouth such as tongue, palate, cheek, teeth and periodontal pockets that have its own microbiota (4). Most of it is located in biofilm on the teeth and sub-gingivally. In periodontitis bacteria of the periodontal pocket have easy access to the blood circulation; this affects about 75% of the population in the USA, with 20% to 30% having severe forms (5, 6). It has been suggested that bacteraemia released daily from periodontitis due to chewing and tooth brushing can contribute to the cumulative deleterious inflammatory effects on cardiovascular tissue causing atherosclerosis and at the end endocarditis.

Oral bacteria and development of endocarditis

Factors involved in the development of a bacterial endocarditis are difficult to define but a vulnerable surface (i.e. a damaged endocardium) and a high bacterial load in the blood seem to be decisive. Causing microorganisms, in 90% of the cases, are staphylococcus, streptococcus and enterococcus. Oral streptococci belongs to viridans group (streptococcus mutans and streptococcus sanguis) (7). As they are part of the dental plaque, they can

enter the bloodstream causing bacteraemia through daily habits like chewing or tooth brushing. Dental extraction or other dental procedures might cause bacteraemia as well. Because of dental treatment, only a small amount of patients contract bacterial endocarditis. Developing IE in valve disease patients is statistically 1 every 3000 cases (8).

The role of dental hygiene is also confusing. Tooth brushing or flossing may increase the risk of oral streptococcal bacteraemia on a short-term basis, but may also decrease this risk of IE on a long-term basis. The development of IE because of everyday-life bacteraemia may be determined by bacteraemia characteristics, themselves related to oral hygiene habits and/or orodental status (9).

Furthermore, a trend toward an increased incidence of IE in the United Kingdom, after the 2008 National Institute for Health and Care Excellence guidelines were implemented, was recently reported, bringing back to the forefront the possible role of dental procedures in the development of IE (10).

Diagnosis and prognosis

In the presence of infection, tooth-supporting tissues become highly vascularized and enter an intimate relationship with microbial bio-film, increasing the risk of bacteraemia (7, 11, 12).

Surmounting evidence has indicated that dental treatment in patients at risk of developing IE could be beneficial because the elimination and/or control of acute or chronic oral infections can reduce the source of microorganisms and consequently the likelihood of bacteraemia. However, the costs and benefits of dental intervention prior to cardiac valve surgery (CVS) have not been well-defined (13).

Additional risk factors include genetic susceptibility, tobacco smoke, alcohol use and systemic conditions such as diabetes, osteoporosis, malnutrition and stress. Effective treatment of periodontal infections is important to reduce local inflammation and bacteraemia (14-21). In addition, poor periodontal health appears to increase the risk of cardiovascular disease, pulmonary disease, and preterm and low birth weight.

The diagnosis of bacterial endocarditis is based on four factors: changing murmurs, ECG diagnosing

abnormal rhythms of the heart, echocardiography identifying adenoids and evaluating valve and heart functions and blood culture. It is essential to perform blood culture before antibiotic treatment begins, at half hourly intervals in order to increase the prospects of positive blood cultures. Without treatment, bacterial endocarditis is a fatal disease in 30% of the cases. Patient should be sent to a hospital for intravenous antibiotic therapy (benzylpenicillin and gentamicin are normally used) (22). Generally, an extended treatment is needed. For this reason, programs of home hospitalization service have been progressively adopted administering antibiotics intravenously. If staphylococcal endocarditis is suspected, penicillin could be replaced for vancomycin. In severe cases like prosthetic valve endocarditis patients, it might be necessary to replace the infected valve with a new valve (23).

DISCUSSION

In many countries, there are national guidelines in order to use antimicrobial prophylaxis having a bacterial endocarditis condition, in case dental surgery is needed. The reduction of bacteraemia, preventing the adherence of bacteria to the endocardium (24), is the main benefit of the use of prophylaxis. Nonetheless, the effectiveness of these guidelines have been questioned by several authors, publications and societies, such as; the recommendations published in France in 2002, the British Society for Antimicrobial Chemotherapy (BSAC) in England (2006), the American Heart Association (AHA) in 2007 and the National Institute for Health and Clinical Excellence (NICE) in the UK in 2008. Finally, these guidelines have been questioned also by Australian recommendations in 2008 and the European Society of Cardiology (ESC) in 2009 (25).

The literature indicates that most cases of infective endocarditis are not related to procedures (26). Eykyn (27) stated that it is becoming increasingly clear that poor dental hygiene rather than dental procedures are responsible for most, if not all, cases of viridans streptococcal endocarditis.

In the study by Pogrel and Welsby (28), oral

sepsis was associated with infective endocarditis in 12% of cases. Guntheroth (29) pointed out that bacteraemia was found in 11% of patients with oral sepsis and no intervention.

Minimizing the occurrence of postoperative bacteraemia has been considered the most important factor in the prevention of IE, and the results of several clinical studies using conventional blood culture systems have demonstrated a marked reduction in bacteraemia following dental extraction with the use of antibiotic prophylaxis. In recent studies using lysing and filtration of blood, prophylactic administration of penicillin V, amoxicillin, erythromycin, clindamycin, or cefaclor did not reduce the incidence or the magnitude of bacteraemia after dental extraction, as compared to placebo (30). The antimicrobial mechanism of protection for IE is apparently different from a mere killing in blood. The implications of this for prophylaxis for IE in humans is still not fully understood, but studies of animals suggests that the protective effect may be exerted by inhibiting bacterial growth on the vegetation, thus allowing host defence mechanisms to gradually eliminate the bacteria from the valves (31).

Long-standing oral disease prevention protocols reduce the risk of developing periodontal disease (32). Data suggest that methods used to prevent cases of IE that originate from oral bacteria should focus on improving oral hygiene and reducing or eliminating gingivitis, which should reduce the incidence of bacteraemia after tooth-brushing and the need to extract teeth owing to periodontal disease and caries. These findings also may provide measures by which physicians and dentists can monitor the risk of developing IE from oral pathogens by means of both patient histories (assessment of bleeding with tooth-brushing) and clinical examinations (general assessment of plaque and calculus). Although a large clinical study would be necessary to substantiate this claim, our data suggest that maintenance of optimal oral hygiene and the absence of gingival disease should result in fewer cases of IE.

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ANALYZE MY FACE

V. QUINZI¹, E.T. SCIBETTA², E. MARCHETTI¹, S. MUMMOLO¹,
A. BRUNO GIANNI³, M. ROMANO³, G. BELTRAMINI³ and G. MARZO¹

¹*Department of Life, Health, Environmental Sciences, University of L'Aquila, L'Aquila, Italy;*

²*Freelance, Rome, Italy;* ³*Department of Biomedical, Surgical and Dental Sciences, University of Milan, Italy; UOC Ch Maxillo faciale e odontoiatria, IRCCS Fondazione Ca Granda Ospedale Maggiore Policlinico Milano*

Plastic surgery is gaining more and more popularity, while stigma and popular myths about it are gradually decreasing. Analyze My Face conjunctly deals with the two main problems of facial plastic surgery: the excessive rate of dissatisfaction, which results in high revision requests and negative psychological side-effects and the “diagnosis by procedure” approach, which leads to erroneous measurements. This new and innovative method of Digital Facial Analysis is a direct non-expensive online service that provides professional and documented in-depth consultation to patients before they decide to undergo any type of facial intervention. The paper thoroughly explains the scientific method with which professionals provide customers with a facial assessment based on specific parameters which will be discussed (height, width, proportions, direction of facial growth, the way they assess each facial area in detail (eyes, mouth, cartilage), and the motivations for which they suggest to correct eventual defects through precise measurements, indicators and suggested interventions. Long-term evaluation of stability of surgical results and patient satisfaction achieved with digital facial analysis has not yet been established and needs further research. However, it is important to underline that the AMF approach tends to consider exclusively possible and feasible procedures that do not compromise functionality and that do not put patients in danger of serious damage. Problems or deformities that cannot be treated are always indicated. AMF aims to maximize professionalism by giving practitioners an additional tool to aid their work, give unbiased opinions and look at the overall picture. It also aims to help patients by soothing their way into the complicated world of aesthetic surgery.

Plastic surgery is gaining more and more popularity, while stigma and popular myths about it are gradually decreasing (1). While it was commonly believed that surgery, especially when it concerns the face, is a mere tool for aesthetic amelioration, in recent studies it was confirmed that 88% of patients felt healthier on many levels after their treatments. Furthermore, the decrease in prices of procedures and the presence in the market of less invasive new technologies helped to boost numbers as never before. In the United States

only, in 2017 cosmetic procedures increased by 4%, reaching 1.7 million; cosmetic procedures increased by 3% reaching 17.1 million and minimally invasive procedures have increased by 3% reaching 15.4 million (2). Reconstructive procedures kept a steady rate with almost 6 million patients. Over the last years, it has also been observed that an increasing number of men are turning to treatment for restoration, reconstruction or alteration of body parts. An article published on the 22nd of June on Bloomberg News, explains a new

Key words: plastic surgery, Digital Facial Analysis, deformities, aesthetic

Mailing address:

Dr Vincenzo Quinzi,
Department of Life, Health and Environmental Sciences,
University of L'Aquila,
P. le G. Liberatore, Ed. Delta 6,
67100 L'Aquila, Italy
e-mail: vincenzo.quinzi@univaq.it

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report by the American Academy of Facial Plastic and Reconstructive Surgery that says that 31% of male respondents admit they would seriously contemplate surgery to improve their appearance. Fifty-eight percent of these men are between 24- and 34- years-of-age. This is just the latest data in a long-brewing trend in which more and more men, especially the youngest, are realizing that surgical benefits are not merely a female issue (1).

While the market and its possibilities are as open as never before, there remains a substantial threshold before seeking any actual treatment. Deducing and discovering the correct aesthetic issue and appropriate treatment is a task that should be left to professionals and not to the patients themselves. Despite the advances and increasing interest in the market, not much progress has been made to simplify this step or increase its accessibility.

To detect the presence and source of aesthetic imbalance and how it may be alleviated, the professional is able to perform a facial analysis. It can vary greatly between different techniques, but in its essence, facial analysis evaluates the suitability of patients for aesthetic, maxillofacial and craniofacial intervention by quantifying and interpreting facial proportions, shape, and relationships and the degree of any aesthetic or functional facial deformity (2). Some approaches and their clinical application have been well described in medical literature. The most common method, clinical facial analysis (CFA), generally involves a two-step approach: a live assessment of the patient as well as the assessment of standardized photographs that are normally produced during the consultation or shortly thereafter.

This crucial procedure for selecting the most beneficial applicable aesthetic treatment has so far been limited to being performed exclusively in the office of a practitioner and, as such, been limited to a narrow group of potential patients. Furthermore, it depends on the medical surgical expertise and knowledge of the practitioner performing the clinical facial analysis (3).

A new approach in this field is the Digital Facial Analysis, which relies entirely on photographs that are digitally sent to clinicians by the potential patients that wish to receive a comprehensive facial analysis

without having to book a consultation or having any prior knowledge about their possible or assumed aesthetic deviations that would require specialist consultation. We can already detect a growing popularity of such an approach by the emergence of video conference-based consultations offered by medical practitioners in general and cosmetic plastic surgeons in particular, and web-based services like Analyze MyFace and Realself (4).

The typical, most common plastic surgery patient is female, married, upper middle class and between 30 to 50 years old. The current method of clinical facial analysis needs to be performed locally, within a medical office, thus limiting the target of aesthetic intervention to a narrow group. Using digital facial analysis eliminates the need for physical presence. Furthermore, geographical restrictions for the practitioner and patient are eliminated as well. There is a increasing number of patients willing to travel significant distances to undergo specific aesthetic treatments with specific surgeons. This method can furthermore assist reputable surgeons and experts to increase their reach and educate and diagnose patients that would otherwise not be able to travel to their offices (5).

However, since such method of facial analysis is conducted solely on the basis of digital photographs produced by the patients themselves, limitations as to which cases it can be applied to for satisfactory and reliable results occur. By using images provided by the patients themselves, the photographs and models will naturally exhibit significant deviations in camera distance, camera angle, camera mode, head position and head tilt that must be accounted for. Since the main focus is evaluation from an aesthetic point of view, these deviations force the analyst, while conducting this method, to shift the focus on relative metrics and relationships instead of absolute distances. From the aesthetic point of evaluation however, relative metrics play a far greater role in the perception of facial attractiveness and unattractiveness.

As the scientific elite becomes increasingly aware of benefits linked to aesthetic ameliorations, both in terms of health and in terms of self-confidence and psychological returns, it is important to notice that

in many cases the market share is rendered higher by revision rates (6).

The problem of recurring revision surgery is extremely common, even though surgeons record much lower numbers, it is most probable that disappointed patients look for solutions elsewhere, rendering the self-reported “coming back for repair” statistics biased (7).

One of the main reasons for revision, as stated by David Sarver and Fabio Meneghini in their work, involves the problem of obtaining “diagnosis by procedure” from specialists rather than basing their judgment on patients’ needs on an average assessment of facial proportions. Too many times, absolute and standardized proportions are taken into consideration without considering relative proportions, facial equilibrium and harmonious overall results.

Analyze My Face conjunctly deals with the two main problems of facial plastic surgery: the excessive rate of dissatisfaction, which results in high revision requests and negative psychological side-effects and the “diagnosis by procedure” approach, which leads to erroneous measurements. This new and innovative method of Digital Facial Analysis is a direct non-expensive online service that provides professional and documented in-depth consultation to patients before they decide to undergo any type of facial intervention (8).

The consultation includes a professional assessment of the patient’s face, based on relative ratios and proportions according to the pictures that will be requested by different perspectives; a detailed explanation of the objective defects and eventual surgical or non-surgical solutions when possible; a studied opinion on the extent to which the procedures are recommended or not and an analysis, reinforced by connected ad-hoc created pictures, of possible short term and long-term outcomes.

The service includes different packages according to the extent to which patients want their analysis performed. The elaboration of hard data through objective and precise measurements is only the first step towards a new frontier of facial surgery: the data is interpreted according to a new approach which reflects relative rather than absolute proportions according to a vision based on an in-depth overall

view of the face, that considers every detail to pursue the most aesthetic possible version of customers (9).

The paper thoroughly explains the scientific method with which professionals provide customers with a facial assessment based on specific parameters which will be discussed (height, width, proportions, direction of facial growth, the way they assess each facial area in detail (eyes, mouth, cartilage), and the motivations for which they suggest to correct eventual defects through precise measurements, indicators and suggested interventions (10).

Furthermore, the paper assesses the approach professionals utilize when interpreting the ratios and hard data collected in the first phase of the service. This innovative evaluation method sets a turning point between AMF specialists’ conception of aesthetic surgery and the prevalent approach. While rhinoplasty is nowadays the second most recurrent facial surgical operation, the AMF team considers it greatly overused and should, in most cases, be viewed as ultima ratio if other interventions cannot be applied. The paper will give details on why this happens and what other features of the face confer this illusion. Moreover, it will mark another difference with mainstream approach by analyzing the importance of ancillary procedures that are nowadays much less common though they should be implemented in much higher percentages as their aesthetic importance is underestimated.

Finally, the paper will focus on the reasons why AMF shifts the attention towards results that are possible and convenient to pursue under a medical point of view rather than whatever operation is deemed necessary by the patient, who may at time be discouraged.

MATERIALS AND METHODS

Technical requirements

Before the method can be applied, photographs must be requested from the patient, which exhibit sagittal view and frontal view of their face. If complex and unusual three-dimensional deformities are detected, additional images exhibiting basal and oblique view may be requested and analyzed to verify the findings.

To improve the reliability and efficiency of the

facial analysis, these images need to meet a baseline of photographic requirements in resolution, positioning and compression, in order to minimize the variance. If the deviations in head tilt or camera angle are extreme, new images must be requested and used, as it can lessen or enhance the aesthetic impact of points of interest.

These images are then altered using photo-editing software to standardize the format ratio (any standard format can be used) and leveled, using an approximation of the Frankfort horizontal plane (*tragus – infraorbitale*) as a plane of reference to allow comparability.

These initial alterations serve only to improve the workflow of the practitioner, allow for greater comparability of the images and cases, and lastly identify any deviations too extreme to proceed with the analysis.

Analysis

Before any further alterations are made and before facial landmarks and other hard data is identified and processed, the images should first be observed manually, without any influence of hard data. This forming of a “first impression” is similar to when non-experts observe faces of strangers, even though the impression is supported by medical knowledge and surgical expertise, which is important to interpret data correctly. It includes the observation of general proportions, points and zones of interests, obvious distractions and deviations (11). The next step is extrapolation of relevant data, that includes facial landmarks, angles, lines used using facial recognition or photo editing software.

There are many described facial landmarks, lines, angles in medical literature that are considered to play an important role in the perception of facial attractiveness. Many methods, masks and models have been described, each claiming to be able to quantify facial attractiveness. There is however an ongoing debate and thus far, there is no clear consensus in the field. Using even most of these facial landmarks and lines altogether would not prove to be a valid method because of the considerable increase in workload and the redundant nature of many of these indicators (12).

Instead, it may be said that less is often more. Facial analysis serves to provide initial indicators for underlying aesthetic deficiencies or deformities and the overall relationships of the face. The general method of evaluating the aesthetics of the side profile, using

facial planes such as the Frankfort horizontal or others, and assessing the level of convexity or concavity of the outline, has already been tested and applied with good results. While an aesthetic profile and an aesthetic frontal face are positively correlated, creating an attractive profile projection often does not create a beautiful face. On the other hand, a face that is deemed beautiful may not have a particularly aesthetic profile.

It is the frontal face that is essential to the perception of an attractive face. Medical literature and research however, has often placed its focus on the analysis and enhancement of the profile view and there is much more data on this matter. In the process of developing this method, while we assess the profile using the conventional method, we could not apply a thorough analysis to the frontal face, which we deem more important (13).

For the analysis of the frontal face, we use a simple yet conclusive approach, using 19 base facial landmarks that play a paramount role in overall proportions, facial projection and facial symmetry to assess the attractiveness of a face. They are the basis to form many lines and calculate angles and crucial relationships. These can be supplemented by the use of additional facial landmarks and measurements in complex cases. Except trichion, that marks the beginning of the hairline, all of the base facial landmarks lie in the lower and middle facial third of the face. The landmarks are used to assess general proportion, facial feature relationship and bilateral symmetry (14).

The proportions, the placement of the facial features relative to each other, especially of the lips and the eyes play a pivotal role to facial aesthetics, and it must be assessed thoroughly. While changing the proportions directly can only be achieved with highly invasive reconstructive procedures, and therefore never recommended but in most extreme cases, its perception can be influenced with aesthetic intervention and therefore must be an important part of a facial analysis (15).

One important proportion metric that is considered is the inclination of the intercanthal axis. This angle can be measured between a horizontal line passing through the medial canthus and a line passing through medial and lateral canthus. Most people will have a slightly positive tilt, but it can anatomically be negative, neutral or positive. A negatively inclined intercanthal axis has a strong negative effect on facial aesthetics and perceived emotional or physical state (“tired-looking”, “sad eyes”).

The average angle differs depending on ethnicity and sex, but it is about $2^{\circ} \pm 2^{\circ}$ in men and $4^{\circ} \pm 2^{\circ}$ in women. This shows that it should be noticeably positive in female faces, and at least neutral in male faces. A positively inclined intercanthal axis is correlated with strong skeletal support of the upper maxilla and zygoma.

Similarly, we use a line that passes through the medial canthus and the midpoint of the upper eyelid (vertically above the center of the pupil) to calculate the angle of eye opening. Significant deviations may indicate aesthetic problems such as proptosis or upper eyelid ptosis.

Other measurements we can use to determine the proportions of the face that we can apply using facial landmarks, include: the total upper and lower anterior face height (TAFH, UAFH, LAFH); the relative length of the upper lip and philtrum (subnasale – stomion) compared to the relative length of the chin (stomion-menton); bigonial width; bizygomatic width and bimental width. All these measurements play an important role in determining aesthetic problems or sometimes functional deformities.

Only succeeding this step should the facial analysis focus on individual parts (such as the nose, with a nasal assessment) if the case requires and the general analysis indicate aesthetic problems in that area. It is often not required, and the aesthetic problems can be identified without this additional procedure in most patients.

Interpretation

Interpretation of data that is extracted from the facial analysis to form a conclusive report is obviously critical, for this we can rely on many described correlations and relationships in medical science. For example, an increased LAFH (>0.6) is strongly correlated with the presence of moderate to severe dentofacial deformities such as vertical maxillary excess (long face syndrome). This strong correlation is well described and applied in maxillofacial surgical and orthodontic practice.

Moreover, we need to interpret the relationship and facial shape and lateral contours of the face. By assessing the bimental width, bigonial width and bizygomatic width, we have clear width indicators and can assess the face shape and lateral projection. We find that not only are well defined projecting zygomatic and gonial bones highly important to the aesthetics of the frontal face, they are also easily alterable with aesthetic intervention. They serve as a frame to the frontal face and provide structural

support just like a well-developed upper and lower maxilla does to the eyes, lips and nose. Especially in male faces, the lateral projection and shape of the zygomatic arch, mandibular angles and lateral temporal bone should form a distinct straight contour in a frontal view, while the jaw angles in the female face should project slightly less while a distinct contour is still desired to achieve a beautiful face. There are many treatment options to achieve this distinct contour, improvement of the facial frame is a viable option (16).

It becomes clear that the interpretation of the data must be handled with utmost care, as there is no fully automated “cookie-cutter” approach and it differs widely depending on a set of variables, such as sex, age, and height. In a medical field where most facial aesthetic procedures are targeted for and performed on women, this distinction is often not made. Aesthetic problems must also not be assessed singularly, but always on how they fit into the composite face, in order to avoid “diagnosis by procedure” as David Sarver described this phenomenon. Additionally, the negative impact of aesthetic deviations (and positive impact of aesthetically pleasant facial features) can vary widely between individual faces despite a similar level of severity, so it is important not to rely on such metrics completely, but to use them as a tool that complements the clinician’s practical expertise and experience (15).

In his research, Patzer categorizes such determinants of facial attractiveness in compensatory and non-compensatory relationships - if a favorable feature can compensate other unfavorable features and vice versa. Very strong features do not necessarily compensate aesthetically poor features, and poor features do not necessarily negate aesthetic features.

Ultimately, the facial analysis incorporates, if available, solutions to the aesthetic problems, if present. This requires the clinician to individually assess the case and weigh the level of invasiveness and complication rate of possible procedures against the expected aesthetic (and in some cases, functional) improvement – for example, while orbital decompression surgery for mild proptosis (solely for aesthetic reasons) is possible and has been done, such an invasive procedure cannot be considered feasible since there are other, less invasive ways to improve the appearance of proptosis from an aesthetic point of view.

When presented with mild to moderate structural deformity, camouflage aesthetic surgery and treatments

will routinely be suggested alongside the ideal procedure, when the expected aesthetic outcome is close to the ideal, inform the patients on their treatment options. When presented with severe structural deformity that may or may not yield to functional impairments, such a distinction is not made, since in such case camouflage surgery cannot achieve an aesthetically satisfactory result.

Presentation

Following interpretation, a table of hard data which includes the most important measurements and a brief individual assessment is presented to the patient, describing the findings in a technical, but accessible way. This includes listing the most problematic aesthetic areas (if any), if and how they can be improved aesthetically and listing any present structural deformities. Lastly, it compiles possible treatment options in order to achieve such an aesthetic improvement. They are not complete, but rather a selection of most suitable procedures decided by the analyst.

The patient can be presented with a list of referrals, either in a general way to guide him to practitioners of the relevant medical field, or in a more individualized approach considering his geographical location and the specific expertise of surgeons in a particular procedure etcetera.

To conclude the digital facial analysis, we present altered images of the photographs in sagittal and frontal view, or “virtual simulations” (17). They serve as a tangible visual representation of the treatment options and their expected aesthetic amelioration, providing immediate feedback not only as to how a post-surgical result could look like from an aesthetic point of view, but also to highlight the immediate aesthetic impact of present structural deformities or aesthetic imbalances which educates the patient and assists in making an informed decision. The simulations also assist the operating surgeon or performing practitioner intra-consultation, by providing an approximation of the desired aesthetic result.

DICUSSION

The market is in dire need of an immediate, easily accessible way of providing potential patients and customers important information so they can make a more informed decision whether they want to pursue

their treatment options. Digital facial analysis, in many cases, can be considered an entry-level medium that provides this and can significantly increase the reach of aesthetic analysis and practitioners. Due to its limitations, it never is and should not be viewed as a singular assessment before any aesthetic intervention: As the decision in favor of aesthetic intervention is made, a further live consultation and clinical assessment by the performer is customary before eliciting surgical treatment. AMF’s digital facial analysis is however not performed by the operating surgeon. This consultation and live assessment therefore serves as a second opinion and another assessment that complement each other in a “checks and balances” fashion.

Currently, many people do not get second opinions and solely base their decision on one assessment: This is a poor state that can be significantly improved using this medium. This state is especially concerning because patients that seek to address aesthetic issues

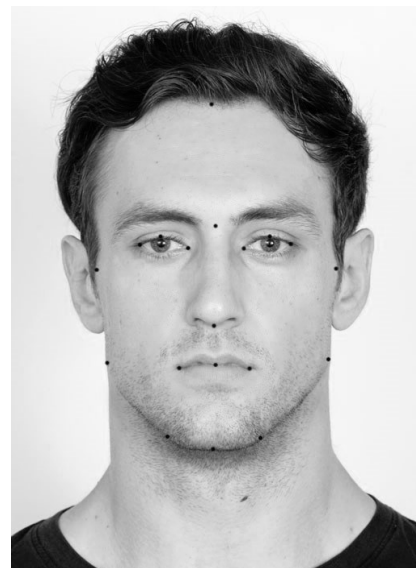


Fig. 1. A simplified model illustrating the 19 base facial landmarks that are used: Trichion, nasion, subnasale, stomion, menton, center of pupil left, center of pupil right, medial canthus left, medial canthus right, lateral canthus left, lateral canthus right, apex of the zygomatic arch left, apex of the zygomatic arch right, lip commissure left, lip commissure right, gonion left, gonion right, most superior point of the chin left, most superior point of the chin right.

usually do not possess a critical unbiased perspective of all aspects of their features, so they tend to look for the “most visible” imperfections, underestimating the reasons that underlie behind such defects. The most central and three-dimensional part of the face is the nose, combined with the ubiquity of rhinoplasty in the beauty market and advertising compared almost to every other facial procedure that is constantly presented to the patient, commonly leads them to identify the nose as the main culprit of aesthetic problems. Such apparent information and power asymmetry places the aesthetic outcome in the hands of a single person, and if that person has been chosen for reasons of proximity and word of mouth, the aesthetic outcome is unpredictable.

The facial analysis, if performed digitally, can either reinforce a patient's suspicions, increasing their likelihood to achieve a good aesthetic result, or more commonly, rebut them which allows them for a more objective self-assessment as many become obsessed

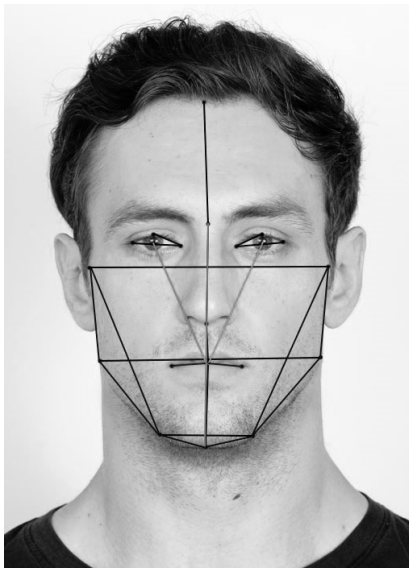


Fig. 2. Overlay of selected measurements that can be obtained from using the base facial landmarks, including TAFH (LAFH+UAFH, indicated purple), eye-mouth-eye angle (indicated gold), relative bizygomatic width, relative bigonial width, relative bimental width, lip turn, philtrum height to chin height, intercanthal axis inclination, eye opening angle and fWHR that can be used to assess general proportion, facial feature relationship and bilateral symmetry.

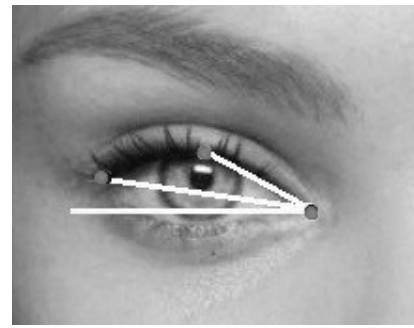


Fig. 3. Facial landmarks medial and lateral canthus and medial canthus and midpoint of upper eyelid and angles illustrated.

with their perceived flaw. In any case, incidence of revision surgery may greatly be reduced.

While rhinoplasty is a powerful and highly effective tool in the right cases and aesthetic deformities, it is currently highly over performed – it is the second most commonly performed facial surgical procedure, accounting for 954,423 or 25.3% of facial aesthetic surgeries in the year 2013 which yields to poor aesthetic outcomes, patient dissatisfaction and unacceptably high revision rates. The revision rate of rhinoplasty is still debated. It is commonly stated to be 5-15% by self-reporting surgeons. However, this method is flawed, as a significant part of patients choose to have a revision rhinoplasty with another practitioner and are therefore not accounted for. Other studies suggest it might be up to 40%. Not included are the poor aesthetic results that are not revised, either because it is not wished for or not identified by the patient. In any case, the revision rate is unacceptably high.

Rhinoplasty must not be performed so carelessly and as such, it must not be considered an entry-level facial plastic surgery as it is now, but rather as a last resort, if other measures cannot achieve adequate proportion without incorporating rhinoplasty.

AMF's method of facial analysis incorporates analysis of all relevant facial features and overall proportions. It may incorporate a nasal assessment if needed. As such, it aims to detect if, and the exact reasons for which a patient's nose is perceived as unaesthetic. The nasal assessment can provide important information about size, width, height

and rotation. But in many cases the defects depend on more complex structural issues that have little or nothing to do with the dimension of the nose. Many professionals however, do not consider essential ratios such as the *subnasale-stomion* and *subnasale-menton* ratio which determines important aesthetic balances: the distance between nose and lips or lips and chin and how a rhinoplasty may affect this important relationship. It is very hard to reverse negative changes to these ratios induced by rhinoplasty, so an overall evaluation is of primary importance before deciding to render the nose “better” as a single feature without considering facial harmony as a whole.

In the case of structural deficiencies, maxillofacial deformities such as maxillary retrognathia or mandibular hypoplasia, which often greatly exaggerate the perceived dimension of the nose, despite normal dimensions, many patients that initially only considered the nose the aesthetic problem and desired rhinoplasty may decide against this treatment due to invasiveness and prolonged convalescence, however it is important to inform them of all viable options, and educate them about the aesthetic limitations and risks of undergoing rhinoplasty to camouflage the problem. This is rarely done, adding to a high patient dissatisfaction and exaggerated patient expectations.

Informing patients about their overall facial aesthetics and presence of aesthetic deformities on the one hand, may yield or enforce negative psychological effects, but on the other hand, it can prevent unnecessary, aesthetically unfavorable surgery and subsequent revision surgery.

Another factor that AMF’s digital facial analysis aims to improve with its holistic approach to improving facial attractiveness is the widespread mistake of “diagnosis by procedure”, singling an aesthetic problem out, analyzing and treating it by itself. This mistake is evident in the prevalence of rhinoplasty and high revision rate, but it doesn’t stop there. According to these considerations the next step is assessing the relevance of many ancillary procedures that have up to now been underestimated in terms of priority when searching for the best possible, feasible aesthetic result.

Previously, orthognathic surgery was mostly reserved for treatment of severe maxillofacial deformities to improve both form and function. This has changed in recent years and nowadays, many patients undergoing orthognathic surgery are mainly motivated by aesthetic reasons and exhibit moderate or even only mild maxillofacial deformities. The commonly used techniques, Le Fort I osteotomy and BSSO only affect a limited area. Despite this limitation, in many cases ancillary surgery to eliminate aesthetic imbalances is not performed, causing dissatisfaction in these patients. This is unjustified, as such intervention does not greatly increase invasiveness or risk, would be relatively easy to perform and would yield immediate aesthetic improvements and an overall greatly improved result. Furthermore, such patients would be responsive to those additional measures.

The assessment of the jaw angles and “ZYGOMO” relationship, i.e. zygoma and gonial projection and shape in relationship to the mouth, should be associated to orthognathic surgery in all cases, which is needed, especially since the surgery itself may alter facial proportions (it causes issues on a skeletal level: movements of the left create malocclusion, upper midface volume deficiency, tissue descent, nasolabial folds).

We already established that with the measurements we use during the analysis, such as UAFH and LAFH, we can reliably identify dentofacial deformities such as long face syndrome. This deformity is characterized by excessive vertical growth, a steep occlusal plane and a hyper divergent profile. In most of these cases, linear advancement may increase the aesthetic imbalances. Instead, a more effective approach would be to perform a counterclockwise rotation. However, this rotation by itself cannot contrast the skeletal limits: contouring is necessary (for example enhancing the lateral projection of the jaw angles with medpor implants or segmented osteotomies on the border of the mandible; fillers may also be considered after post-surgical swelling decreased) and it should be considered much more frequently. It is in fact very rare that patients who present such deformities that require orthognathic surgery, present no other skeletal defects. In most

cases ancillary surgery is mandatory to achieve a good aesthetic outcome.

Since the target of AMF's digital facial analysis is to open up the market and make it more accessible whilst reducing common problems, it serves as an entry for many potential patients and interested clients. To provide a satisfying first contact experience, it is important to provide comprehensible feedback, and mustn't overload it with unnecessary information. In light of this selection we focus exclusively on suggesting aesthetic intervention that are deemed as possible, feasible and beneficial for the patient's overall aesthetic and medical condition. This requires the clinician to individually assess the singular cases and weigh the level of invasiveness and complication rate against the expected improvement – for example, while orbital decompression surgery for mild proptosis is possible, such an invasive procedure cannot be considered feasible for mild proptosis since there are other, less invasive aesthetic treatment options. However, in moderate cases where there are no functional issues, such a step may be considered and presented to the patient. When presented with mild to moderate structural deformity, camouflage aesthetic surgery and treatments will routinely be suggested alongside the ideal procedure, when the expected aesthetic outcome is close to the ideal, the patient has to be educated about their treatment options. When presented with severe structural deformity, such a distinction is not made since in such case camouflage surgery cannot achieve an aesthetically satisfactory result.

In all cases, the report is not meant to satisfy patients regardless of possible harms or unfavorable consequences. If such information is available, mental health disorders such as body dysmorphic disorder, obsessive compulsive disorder or delusions that may cause customers to have wrongful or greatly exaggerated impressions on their necessities, will be considered and no customer will be assessed on biased criteria.

Conclusively, by analyzing a patient's face, we can identify and quantify most relevant facial proportions, imbalances and a considerable amount of deformities in order to directly provide tangible

feedback to the patient (patient education). Due to the limitations of facial analysis performed on non-standardized photographs, it cannot be considered a method that fully replaces established ones, but it should assist and improve existing techniques. The AMF approach is born to contrast the conflict of interest which may bring specialists to direct patients' attention mostly on specific problems that they can take care of, rather than overall problems which determine aesthetic imbalances.

Long-term evaluation of stability of surgical results and patient satisfaction achieved with digital facial analysis has not yet been established and needs further research. However, it is important to underline that the AMF approach tends to consider exclusively possible and feasible procedures that do not compromise functionality and that do not put patients in danger of serious damage. Problems or deformities that cannot be treated are always indicated. AMF aims to maximize professionalism by giving practitioners an additional tool to aid their work, give unbiased opinions and look at the overall picture. It also aims to help patients by soothing their way into the complicated world of aesthetic surgery. Patients are given a chance to have a professional overview of their situation, a scientific method based analysis and a photographic projection of possible results, other than important advice on what procedures they can have access to.

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TRAUMATIC AND SURGICAL SCARS: SUCCESSFUL TREATMENT WITH A 1,565NM ERBIUM-GLASS NAFL COMBINED WITH IPL

D. QUARATO¹, M. PASCALI² and F. CARINCI³

¹Private Practice, Rome Italy; ²Department of Plastic and Reconstructive Surgery, University of "Tor Vergata" Rome, Italy; ³Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Italy

Scars are a very common condition of the general population and can have a profound impact on the psyche of the patient such as low self-esteem and feelings of psychosocial isolation. Various therapeutic approaches have been proposed for improving the clinical appearance of scars. Fractional mode of ablative and non-ablative lasers has become a novel strategy for the treatment of scars. A total of 43 patients (Fitzpatrick skin type II to IV), clinically diagnosed of surgical and post-traumatic scars from January 2015 to December 2016, were treated. Each treatment comprised of several passes over the scars with different devices, using a 1.565nm scanned erbium-doped fiber NAFL and an IPL. All patients noted subjective improvement in cosmesis and functionality after treatment, also with a decreased pain and an increased mobility on the underlying plans. Numerous therapeutic strategies for traumatic and surgical scars have been suggested to date, but no consistent treatment modality has been established yet. In our study, we have shown that there was a significant collagen remodelling with decrease of scar vascularity and significant improvement of pliability of scar after combined treatment with non-ablative fractional resurfacing and IPL resulting in a remarkable improvement in scar vascularity, pigmentation and height.

Scars are a very common condition that affect approximately 4.5-16% of the general population and depending on the degree of disfigurement, can have a profound impact on the psyche of the patient such as low self-esteem and feelings of psychosocial isolation (1). Clinically, scars can impede the patient's range of motion, and can cause pain, dysesthesia, and pruritus.

Various therapeutic approaches have been proposed for improving the clinical appearance of scars, including corticosteroids, hydrant creams, different chemical acid peels, surgical revision, microdermabrasion, silicone gel application, pressure therapy, radiation and skin needling, with different opinions on the results (2-10).

Technologies designed primarily for cosmetic

applications, such as fractional laser resurfacing and laser hair reduction, have been adapted for consistent functional enhancement.

Fractional mode of ablative and non-ablative lasers has become a novel strategy for the treatment of scars, and some authors have suggested that treatment with fractional lasers for various scars, such as postoperative, atrophic and acne scars, has demonstrated to safely improve the appearance of the scars (6).

Fractional treatment is achieved through a pattern of microscopic thermal zones produced by the laser beams at specific depths in the dermis. Fractional photothermolysis stimulates the epidermal turnover and dermal collagen remodeling (11).

Recently, non-ablative fractional laser and

Key words: Scars, lasers, collagen, remodeling

Mailing address:

Prof. Francesco Carinci, MD,
Department of Morphology, Surgery and Experimental Medicine,
University of Ferrara, Via Luigi Borsari 46, 44121 Ferrara, Italy
Tel.: +39.0532.455874 - Fax: +39.0532.455876
e-mail: crc@unife.it - Web: www.carinci.org

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ablative 10.600 nm carbon dioxide fractional laser have been suggested as successful approaches for improving the appearance of scars (12-16).

A 1.565nm near-infrared, erbium-doped fiber NAFL (Lumenis Ltd., Yokneam, Israel) that has been cleared by the FDA for skin resurfacing and coagulation of soft tissue was released in 2013. The device delivers laser energy non-sequentially using a sophisticated patent pending scanning algorithm. Variations in microbeam distribution and clustering of microthermal treatment zones due to multiple passes of laser energy delivery, as seen with rolling pattern handpieces, are thereby prevented (17). An integrated, thermokinetically cooled sapphire crystal treatment tip enhances epidermal protection and reduces intraoperative discomfort. The combination of non-sequential energy delivery and contact cooling also limit the degree of dermal bulk heating, minimizing the risk of post inflammatory hyperpigmentation, bullae formation, and scarring.

As well described in Literature, IPL works on the principles of laser and selective photothermolysis; the light energy is absorbed by particular target chromophore and then converted to heat energy, which causes damage to the specific target area (18).

In the current study, we showed the effectiveness of this novel 1.565nm scanned erbium-doped fiber NAFL combined with IPL, for the treatment of traumatic and surgical scars.

MATERIALS AND METHODS

Forty-three patients (Fitzpatrick skin type II to IV), clinically diagnosed with surgical and post-traumatic scars from January 2015 to December 2016, were treated. Informed consents were obtained from all patients before treatment.

This study included 17 males and 26 females aged between 27- and 63-years-old; 14 patients had Fitzpatrick skin type II, 25 had Fitzpatrick skin type III, 4 had Fitzpatrick skin type IV. Exclusion criteria were photosensitivity, use of photosensitizing drugs and history of keloid formation, active local or systemic infection, use of botulinumtoxin A, chemical peels, dermal fillers in areas to be treated within the previous 3 or 4 months and use of isotretinoin or ablative laser to target areas within

the past 12 months.

The clinical evaluation was objectively based on clinical photography before treatment and one month after last laser treatment session by means of clinical improvement and patient satisfaction. Side effects and complications were also recorded at each session.

The cosmetic improvement of the scars was evaluated by the physician and the patients independently, using a quartile grading scale; grade 0: no improvement, grade 1: minimal improvement, grade 2: moderate improvement, grade 3: marked improvement, grade 4: near-total improvement. The patients were also asked to scale their subjective satisfaction with the treatment on a quartile grading scale; grade 0: no satisfaction, grade 1: little satisfied, grade 2: somewhat satisfied, grade 3: satisfied, grade 4: very satisfied.

Devices

The patients were treated with a non-ablative fractional resurfacing (NAFR) module (ResurFX) and Intense Pulsed Light (IPL) for the M22 multi-application platform (Lumenis Ltd., Yokneam, Israel).

The ResurFX system delivers pulsed near-infrared laser light with a nominal wavelength of 1.565 nm via a sapphire contact-cooled tip. At each impact, the laser generates an array of focused microspots with a density adjustable by the operator and varying from 50 to 500 μ beams/cm. The practitioner can choose any of the six different shapes (hexagon, circle, doughnut, square, rectangle, or line), and scan sizes ranging from 5 to 18 mm, with energy of up to 70 mJ/ μ spot.

The light spectrum, for IPL, ranges from 515 nm to 755 nm. The light is absorbed by the chromophores within the skin, principally the melanin in the pigmented lesions, and hemoglobin with its derivatives (oxy- and deoxyhemoglobin) within the blood vessels. The chromophores absorb different light wavelengths, and the absorbed light is transformed into thermal energy, causing damage to the chromophores and the lesions where they are located.

Procedure

Half an hour before each treatment, a very thin layer of an anesthetic cream containing 7% lidocaine and 7% tetracaine was applied to the right hemi-face. After removal of the anesthetic cream, a careful disinfection

of the area was performed. Each treatment comprised several passes over the scars, each with a different setting as follows.

At first, using the 1.565nm scanned erbium-doped fiber NAFL, each scar was first treated using a small spot size covering the whole extension of the scarring areas (settings: 300 microspots/cm² with 40mJ/cm²). Then, it runs a second pass on the entire area, including the healthy surrounding areas, using a large spot size (settings: 200 microspots/cm² and 50 mJ/cm²). These treatment settings were repeated at each of the first two/three treatment sessions, in order to remodel the collagen, and to guide the healing of the wounds. Subsequently, when the appearance of the scar is improved, further two therapeutic sessions will be performed to improve the redness resulting from residual inflammation.

In the second step, two passes of IPL (590 nm /560 nm – 560 nm/515nm) are performed covering the whole extension of the scarring areas, and then proceed with the 1.565nm scanned erbium-doped fiber NAFL, with the same modes as previous treatments.

Immediately after each treatment, a water-based emulsion containing triethanolamine was applied to the

treated area, and the patients were requested to apply at home the cream two times a day for a week to maintain hydration of the skin and to apply a protective sunscreen 50+ three times a day throughout the treatment course. The patients received a total of four/five treatments, with a four weeks interval between each treatment. The start of the treatment was 1 week after suture was removed for patients with surgical scars, and as early as possible, after the healing of the wounds, for post-traumatic scars.

Case 1

A 29-year-old male with Fitzpatrick skin phototype III presented with extensive traumatic scars of the right half of his face after a car accident six months prior. He reported tarmac abrasions on his right cheek and forehead and a wound on the right eyelid, which was sutured at the emergency room. A hypertrophic scar remained along with impaired eyelid functionality. Over the course of the months, he suffered from the aesthetics dissatisfaction and the limited mobility in his right eyelid (Fig. 1).

The patient received 5 treatments, the first 3 sessions of only 1.565nm scanned erbium-doped fiber NAFL, followed by a further 2 sessions of 1.565nm scanned erbium-doped



Fig 1. Traumatic scar of the face. A 29-years-old male with Fitzpatrick skin phototype III six months after car accident.

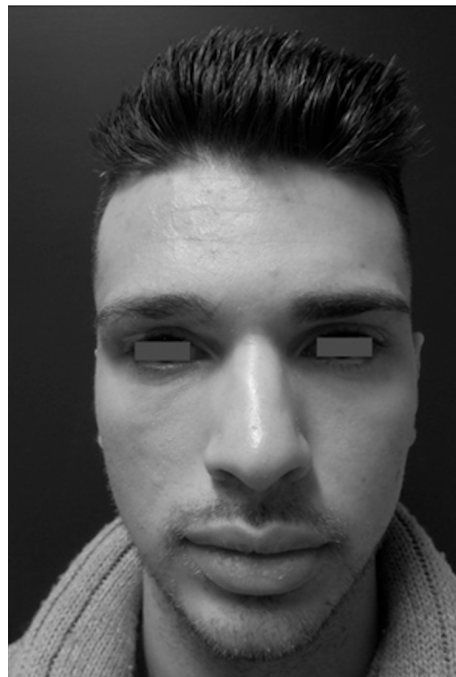


Fig. 2. Patient after 5 treatments. The patient was completely satisfied with significant improvement in both texture and pigmentation.

fiber NAFL combined with IPL (590nm - 560nm and 560nm – 515nm).

The treatments were well tolerated with mild, transient erythema and edema lasting 5/7 days after each treatment. The patient noted subjective improvement in cosmesis and functionality after each treatment. He also reported decreased pain and an increased mobility in his right eyelid throughout the treatment.

After the first treatment, the patient was able to resume working and a normal social life with minimal discomfort. From an aesthetic point of view, the patient noted significant subjective improvement in both texture and pigmentation with each treatment. After two treatments, the patient noted a 50% improvement in cosmesis and was very pleased with his results. After the last treatment, the patient was completely satisfied and agreed to perform two maintenance sessions of resurfacing every year in order to improve the appearance of his face further (Fig. 2).

Case 2

A 46-year-old female with Fitzpatrick skin phototype II presented with scars of the upper arms bilaterally after surgical brachioplasty one month prior. The patient began

laser scar treatment, to improve the aesthetic appearance, after approximately 1 week from the removal of the suture (Fig. 3).

The patient received 4 treatments, the first 2 sessions of only 1.565nm scanned erbium-doped fiber NAFL, followed by a further 2 sessions of 1.565nm scanned erbium-doped fiber NAFL combined with IPL (590nm - 560nm and 560nm – 515nm).

The treatments were well tolerated with mild, transient erythema and edema lasting 5/7 days after each treatment. The patient noted subjective improvement in cosmesis after each treatment; in fact, the patient noted significant subjective improvement in both texture and pigmentation with each treatment. After two treatments, the patient noted a 60% improvement in cosmesis and was very pleased with her results. After the last treatment, the patient was completely satisfied and agreed to perform two maintenance sessions of resurfacing every year in order to improve the appearance of her arms further (Fig. 4).

RESULTS

This study included 43 patients having surgical and post-traumatic scars with a broad range of atrophic and hypertrophic scars. The age of patients varied from 27-63 years with a mean of 46.7 years. The study included 17 males (39.5%) and 26 females (60.5%).

There was remarkable improvement in scar vascularity, pigmentation and height. All patients noted subjective improvement in cosmesis and functionality after treatment, also with a decreased pain and an increased mobility on the underlying plans. Clinical improvement assessment showed a mean grade of 3.6 for physician evaluation and a mean grade of 3.4 for patient evaluation. Patient's subjective satisfaction score was 3.3. No major complications were reported.

DISCUSSION

Numerous therapeutic strategies for traumatic and surgical scars have been suggested to date, but no consistent treatment modality has been established yet. From the numerous modalities used to improve



Fig. 3. Surgical scars. A 46-years-old female with Fitzpatrick skin phototype II one month after brachioplasty and one week after the removal of the sutures.

scars, more effective laser treatments continue to emerge, becoming a popular therapeutic alternative.

Lasers such as the vasculature-targeting 595-nm pulsed dye laser and the full-field ablative 10 600-nm CO₂ laser continue to be effectively integrated into the treatment of various scar types. These modalities are, however, somewhat limited in large traumatic scars because of modest efficacy and excessive thermal damage, respectively (19-22).

Treatment with pulsed dye laser is based on selective photothermolysis, with hemoglobin serving as the target chromophore. Moderate damage to local blood vessels results in a remodeling response that can help reduce scar erythema, pain, itch, and prominence (23-28).

Although not technically a laser, IPL works on the principles of laser, that is, selective photothermolysis, light energy is absorbed by particular target chromophore and then converted to heat energy, which causes damage to the specific target area (18).

Though the exact mechanism is unknown, (29) such laser-induced remodelling may be associated with evidence of new dermal collagen formation.



Fig. 4. Surgical scars after 4 treatments. It highlighted a significant improvement in both texture and pigmentation with the resolution of the inflammatory state. The early treatment with non-ablative fractional laser allowed the collagen remodeling during the healing phase of the scars.

A study by Goldberg showed histologic evidence of new upper papillary dermal collagen formation, associated to clinical improvement (30). Also Feng et al., found increased activity of fibroblasts; increased type-I and type-III collagens; and decreased elastin content, though the elastin fibres gets neatly rearranged, thus, there was a morphological evidence of clinical improvement(31).

The IPL today is also the most employed in treatment of variety vascular lesion (29). Here the IPL targeted on the chromophoreoxyhaemoglobin, which is abundant in the blood vessels. When activated, by IPL it causes photocoagulation of vascular endothelium, leading to fibrosis and obliteration of the blood vessels (32), thus ensuring improvement of the clinical condition.

Some of the most exciting recent advances in scar treatment are associated with the emergence of fractional photothermolysis in 2004 (7). Fractional photothermolysis is a recently developed laser technology that creates thousands of microscopic columns of thermal damage in the epidermis and dermis surrounded by islands of normal tissue, limiting the amount of injury and resulting in rapid epidermal repair. This involves the generation of a pixelated pattern of narrow columns of thermal injury (vaporization or coagulation of tissue) in the treatment area, based on the heating of tissue water. An early inflammatory response is followed by cell proliferation, matrix metalloproteinases guided turnover of extracellular proteins, and long-term neocollagenesis and dermal remodeling (33-35). As noted, fractional laser therapy is associated with a relatively low rate of complications, especially compared with full-field ablative devices.

In a study comparing the effect of ablative CO₂ fractional laser with that of non-ablative 1.550 nm Erbium (ER), both modalities showed similar improvements of scar; however, treatment with the ablative CO₂ fractional laser was considered more painful than the treatment with the non-ablative fractional laser, and resulted in more post-inflammatory hyperpigmentation and longer post-treatment erythema (36, 37).

Non-ablative fractional photothermolysis at wavelengths of 1.565 nm has been found to be

effective for the treatment of scars, but fractional ablative laser has gained popularity in the last years due to its potentially greater efficacy in the treatment of both the dermal and epidermal components of scar tissue.

In our study, we have shown that there was a significant collagen remodelling with decrease of scar vascularity and significant improvement of pliability of scar after combiner treatment with non-ablative fractional resurfacing and IPL. These were because NAFL has its most effect in dermal remodeling while the IPL reduces residual inflammation coagulating the blood vessel and, furthermore, it may hasten the collagen metabolism as described above.

CONCLUSION

This work demonstrates the efficacy of treating traumatic and surgical scars using two different laser technologies. The low invasiveness of the two individual treatments allows us to associate them, benefiting from their specific targets. The NAFL's main target is water; its application will result in collagen remodeling. The IPL, with the wavelengths used, has the primary target for melanin and hemoglobin, its application will mainly result in the reduction of inflammation and, secondly, in collagen stimulation.

The combination of the two treatments in the same session is well tolerated. Procedure pain was reported as mild to moderate in most subjects and adverse events were generally limited to immediate post-treatment trace-to-moderate erythema and edema. The results achieved were remarkable improvement in scar vascularity, pigmentation and height.

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PRE-TREATMENT WITH BERBERINE ENHANCES EFFECT OF 5-FLUOROURACIL AND CISPLATIN IN HEP2 LARYNGEAL CANCER CELL LINE

A. PALMIERI¹, A. IAPICHINO¹, F. CURA², L. SCAPOLI¹, F. CARINCI³,
M. MANDRONE⁴ and M. MARTINELLI¹

¹*Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna, Bologna, Italy;* ²*Department of Medical Sciences, University of Ferrara, Ferrara, Italy;* ³*Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, Italy;*

⁴*Department of Pharmacy and Biotechnology, University of Bologna, Bologna, Italy*

Larynx squamous cell carcinoma represents one of the most common head and neck cancers in the world. Herbal drugs are popularly emerging as complementary and alternative therapies in cancer because of their cost effectiveness and minimal side effects. The present study was undertaken to explore the anti-tumor potential of berberine, an isoquinolone present in the extract of *Tinospora cordifolia* in HEP2 human laryngeal cancer cell line. Besides, it was aimed to investigate whether berberine could enhance the anti-cancer effect of 5-fluorouracil and cisplatin in HEP2. Our data seem to support a role for berberine in decreasing the expression of genes usually seen overexpressed in larynx squamous cell carcinoma and involved in pathways such as those of cell cycle and regulation, differentiation, and epithelial-mesenchymal transition. Moreover, a downregulation of these genes caused by cisplatin or 5-fluorouracil, treatment of election in laryngeal cancers was enhanced by a 4h pre-treatment with berberine.

Among head and neck squamous cell carcinomas (HNSCC), laryngeal cancer accounts for more than 20% of all cases and it represents the 20th most common cancer in Europe (1). It occurs with increasing age, showing the highest incidence in the 7th decade. The early development of symptoms favors diagnosis of laryngeal tumor at an earlier stage compared to other tumors of the head and neck and a higher survival rate. Recognized risk factors are tobacco use, alcohol consumption and human papilloma virus infection. Other potential risk factors are: a diet low in leafy green vegetables, laryngopharyngeal reflux, exposure to paint, asbestos, radiation, diesel and gasoline fumes. Moreover, a potential genetic predisposition induced researchers to investigate genomic profiles in order to identify novel prognostic factors at the

molecular level (2-9).

The final aim of treatment strategies is to provide the maximal cure whilst preserving a functioning larynx without compromising survival. A combination of surgery, radio and chemotherapy is preferred to treat patients presenting local or regionally advanced larynx cancer. The most successful cure appears to be radiation therapy with concurrent chemotherapy based on 5-fluorouracil (5-FU) and cisplatin administration (10). Despite the effectiveness of this kind of strategy, the role of induced chemotherapy in the management of locally advanced laryngeal squamous cell carcinoma (LSCC) remains a matter of discussion.

Several studies have been focused on alternative antiproliferative compounds, such as berberine (BBR), an alkaloid of natural origin, obtained from

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Mailing address:

Luca Scapoli, Ph.D.,
Dept. of Experimental, Diagnostic and Specialty Medicine,
University of Bologna, Via Belmeloro 8,
40126 Bologna, Italy
Tel.: +39.051.2094106 - Fax: +39.051.2094110
e-mail: luca.scapoli2@unibo.it

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plants widely used in Ayurvedic medicine. The anti-inflammatory, anti-angiogenic, anti-proliferative and apoptosis inducing properties of BBR have been evidenced in hamsters with induced oral carcinogenesis (11), while its anti-cancer activity has been tested *in vitro* with different cell lines (12), as well as with squamous carcinoma cells (13-15). BBR could be able to induce apoptosis of cancer cells, selectively (16). A limited number of studies investigated genes expression modulation induced by BBR of genes involved in proliferation, apoptosis and invasion process (17, 18).

In this study, the expression levels of a set of gene involved in proliferation, differentiation and epithelial-mesenchymal transition (EMT) were investigated in a larynx cancer cell line. The effects of BBR and *Tinospora cordifolia* were evaluated. Finally, we aimed to verify if a pre-treatment with BBR could improve the anticancer effect of 5-FU and cisplatin treatment. A validation of such hypothesis could suggest the use of a possibly reduced concentration of 5-FU and cisplatin and consequently, with lower side effects.

MATERIALS AND METHODS

Plant material, preparation and analysis of extracts

Tinospora cordifolia (Willd.) stem was supplied by Maharishi Ayurveda Product Italy (Verona, Italy). Dried and powdered plant material was collected from Ram Bagh (Rajasthan, India) and authenticated by Dr. MR Uniyal, Maharishi Ayurveda Product Ltd., Noida, India. *T. cordifolia* extract was obtained in MeOH/H₂O (1:1) and 1 h sonication, then filtered. The extract was finally analysed by HPLC-UV and LC-MS and subjected to standardization using BBR pure powder as a reference standard (Sigma-Aldrich, Inc., Milan, Italy). The title of BBR in the extract of *T. cordifolia* was 120 mg/g dry extract.

Cell culture

Laryngeal carcinoma cell line HEP2, kindly provided by Prof. L. Lo Muzio (University of Foggia, Italy), was cultured in DMEM (Sigma-Aldrich, Inc., St. Louis, Mo) supplemented with 10% heat-inactivated fetal bovine serum, 0.6 mg/mL, glutamine and 200 U/mL

penicillin/streptomycin (Sigma-Aldrich, Milan, Italy). Cell cultures were replicated for subsequent experiments and maintained in a water-saturated atmosphere at 37°C, under 5% CO₂.

Cell viability test

PrestoBlue™ Reagent Protocol (Invitrogen, Carlsbad, CA) was used to evaluate viability of cells treated with *T. cordifolia*, BBR, cisplatin, or 5-FU.

HEP2 cell line was seeded into 96-well plates at a density of 10⁴ cells per well containing 100 µl of cell culture medium. After 24 h of incubation, adherent cells were treated with serial dilution of the mentioned solutions.

After 48 h of incubation, cell viability was measured using PrestoBlue™ Reagent Protocol (Invitrogen, Carlsbad, CA). The percentage of viable cells was determined by comparing the average absorbance in drug treated wells with average absorbance in control wells exposed to vehicle alone. The results were presented as the mean ± standard deviation of three measures.

A. T. cordifolia and BBR solutions:

The stock solution of *T. cordifolia* was prepared dissolving 0.0167 g of dry extract in 1 ml of DMSO. The pure berberine purchased from Sigma-Aldrich (Milan, Italy) was resuspended in a stock solution 5.4 mM. In these two stock solutions, the absolute concentration of BBR was the same. Both solutions were prepared freshly.

Corresponding dilutions of the stock solutions, i.e. *T. cordifolia* solution (30.9 µg/ml, 92.7 µg/ml, 154.5 µg/ml, 309.0 µg/ml) and BBR solution (10 µM, 30 µM, 50 µM, 100 µM) were made with the culture medium. Three wells for each concentration were made. The vehicle DMSO dissolved in cell culture medium was used as negative control.

B. Cisplatin and 5-fluorouracil solutions:

The stock solution of 5-FU (380 mM) and cisplatin (3.3 mM) have been diluted 1:10 in sterile water to reach a final concentration of 380 µM and 330 µM, respectively. Afterwards, serial dilutions of 5-FU (15 µM, 20 µM, 25 µM, 30 µM, 50 µM) and cisplatin (5 µM, 8 µM, 10 µM, 15 µM, 25 µM, 40 µM) were made with the culture medium. Two wells for each concentration of both solutions were made. The cell culture medium alone was used as negative control.

Cell treatment

HEP2 cell line was seeded at a density of 1.0×10^5 cells/ml into 9 cm² (3 ml) wells and subjected to serum starvation for 18 h at 37°C.

Two biological replicates were performed for each treatment. Cells were maintained in a humidified atmosphere of 5% CO₂ at 37°C in DMEM 2% FBS. After the end of the exposure time (48 h), cells were trypsinized and processed for RNA extraction.

A. *T. cordifolia* and BBR solutions treatment:

Treatment was performed using 92.7 µg/ml of *T. cordifolia* methanol extract, 30 µM of BBR solution and DMSO solution at the same concentration used to dissolve the extracts.

B. Cisplatin and 5-fluorouracil treatment:

HEP2 was treated with 15 µM of cisplatin and 30 µM of 5-FU for 48h. Cell medium alone was used as negative control.

C. Berberine pre-treatment:

HEP2 was pre-treated with 30 µM of BBR solution for 4 h and then 15 µM of cisplatin was added with 30 µM of 5-fluorouracil for 48 h. Cell medium alone was used as negative control.

RNA isolation, reverse transcription and quantitative real-time RT-PCR

Total RNA was isolated from cell lines using GenElute mammalian total RNA purification miniprep kit (Sigma-Aldrich, Milan, Italy) according to manufacturer's instructions. Pure RNA was quantified at NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). cDNA synthesis was performed starting from 500 ng of total RNA, using PrimeScript RT Master Mix (Takara Bio Inc., Shiga, Japan). The reaction was incubated at 37°C for 15 min and inactivated by heating at 70°C for 10 sec. cDNA was amplified by Real Time Quantitative PCR using the ABI PRISM 7500 (Applied Biosystems, Foster City, CA, USA).

Expression profile of a panel of 12 genes was performed in HEP2 cell line treated with *T. cordifolia* and BBR (Table I). These genes were selected because our group found them differentially expressed in a HCA7 colon cancer cell line (that shares the same epithelial origin as HEP2) treated in the same way. Ten additional genes were analyzed because specifically involved in oral tumorigenesis (Table II).

All PCR reactions were performed in a 20 µl volume.

Table I. Effects on gene expression levels caused by berberine, or *T. cordifolia* treatment, in HEP2 cell line; the genes selection includes genes de-regulated in the colon cancer cell line HCA7 by the same treatments.

Gene	Berberine 30 µM		<i>T. cordifolia</i> solution 92.7 µg/ml		Pathway
	Fold change	p value	Fold change	p value	
ANAPC2	0.40	0.298741	0.67	0.695338	Cell cycle
CCNA2	1.07	0.798156	0.55	0.028626	Cell cycle
BMP2	0.51	0.368157	1.12	0.918405	Differentiation
PTP4A1	0.10	0.000226	0.60	0.125879	Differentiation
TGFB1	0.79	0.126576	0.86	0.323770	Differentiation
TSPAN13	0.58	0.138404	0.63	0.166395	Differentiation
TIMP1	0.26	0.034118	0.34	0.168617	Differentiation
CALD1	0.51	0.004025	1.20	0.204722	EMT
CaMKII	0.52	0.059237	0.66	0.128259	EMT
CDH2	0.42	0.001403	0.82	0.215958	EMT
ITGA5	0.16	0.000228	1.12	0.660160	EMT
SNAI2	0.44	0.005989	0.63	0.004880	EMT

In bold significant gene expression level. **Significantly up-regulated gene:** fold change ≥ 2 (p value ≤ 0.05); **Significantly down-regulated gene:** fold change ≤ 0.5 (p value ≤ 0.05).

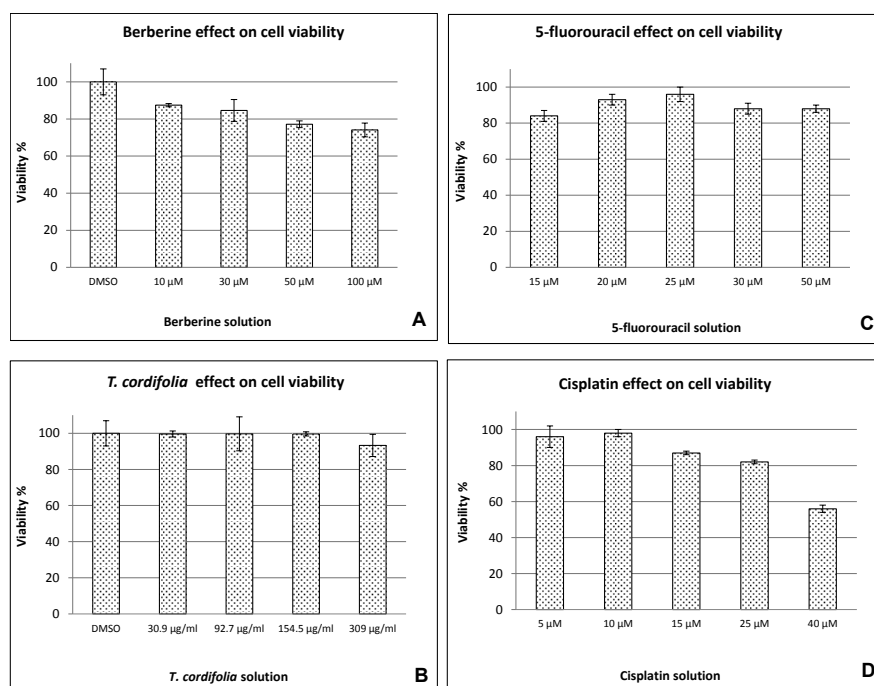


Fig. 1. Cell viability of HEP2 cell line after 48 h treatments with drugs at different concentrations. (A): berberine solution (10 μ M, 30 μ M, 50 μ M, 100 μ M); (B): *T. cordifolia* solution (30.9 μ g/ml, 92.7 μ g/ml, 154.5 μ g/ml, 309 μ g/ml); (C): 5-fluorouracil (15 μ M, 20 μ M, 25 μ M, 30 μ M, 50 μ M); (D): Cisplatin (5 μ M, 10 μ M, 15 μ M, 25 μ M, 40 μ M).

Each reaction contained 10 μ l 2X Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 400 nM concentration of each primer, and cDNA. All experiments were performed including non-template controls to exclude reagents contamination. PCR was performed including two analytical replicates.

The amplification profile was initiated with a 10 min incubation at 95°C, followed by two-step amplification of 15 sec at 95°C and 60 sec at 60°C for 40 cycles. As final step, a melt curve dissociation analysis was performed.

Statistical analysis

A preliminary test was performed to select the reference gene among three housekeeping genes. The TFRC expression appeared most consistent with the amount of RNA input.

The gene expression levels were normalized to the expression of the reference gene and were expressed as fold changes relative to the expression of the untreated cells. Quantification was carried out with the delta/delta Ct calculation method (19). After normalization, quantification cycles of treated cells and control were compared by *t*-test.

RESULTS

Cell cultures of HEP2 were treated with increasing concentration of each substance in order to evaluate the optimal concentration levels that did not significantly affect cell viability (Fig. 1). Based on these data, the following concentration were adopted for the next experiments: 30 μ M of BBR, 92.7 μ g/ml of *T. cordifolia* extract, 30 μ M of 5-FU, and 15 μ M of cisplatin.

In the first set of experiments, we tested the effect of BBR and *T. cordifolia* extract on gene expression of two panels of genes. In order to make these treatments more comparable, the amount of *T. cordifolia* extract administered was calibrated to provide the same dosage of BBR.

Gene expression profile was carried out for 12 genes belong to different pathways involved in tumor progression and cell differentiation that were previously seen significantly modulated by BBR in HCA7 colon cancer cell line. Both treatments induced the down-regulation of most of the analyzed genes

Table II. Effects on gene expression levels caused by berberine, or *T. cordifolia* treatment, in HEP2 cell line; the genes selection includes genes that are usually de-regulated in oral cancers.

Gene	Berberine 30 μ M		<i>T. cordifolia</i> solution 92.7 μ g/ml		Pathway
	Fold change	<i>p</i> value	Fold change	<i>p</i> value	
CCND1	0.70	0.319683	0.28	0.018853	Cell cycle
CCNE1	0.21	0.009910	0.20	0.004208	Cell cycle
CCNT1	0.0003	0.000953	0.42	0.031853	Cell cycle
ABCB5	2.87	0.009541	0.06	0.053010	Cell regulation
TP63	1.88	0.375011	0.57	0.109616	Cell regulation
AKT1	0.41	0.060346	0.32	0.041042	Differentiation
EGFR	0.10	0.001485	0.35	0.060696	Differentiation
NOTCH1	0.80	0.756635	0.25	0.033096	Differentiation
CDH1	1.82	0.609265	0.05	0.060399	EMT
KRT19	0.22	0.041368	0.32	0.103706	EMT

In bold significant gene expression level. **Significantly up-regulated gene:** fold change ≥ 2 (*p* value ≤ 0.05); **Significantly down-regulated gene:** fold change ≤ 0.5 (*p* value ≤ 0.05)

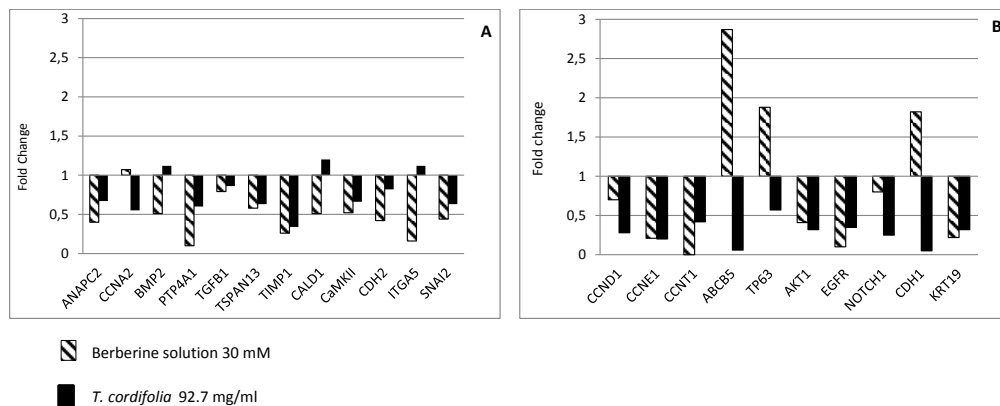


Fig. 2. Gene expression profile of HEP2 cell line treated with *T. cordifolia* 92.7 μ g/ml or berberine 30 μ M. (A): Panel of genes selected because were significantly de-regulated in HCA7 colon cancer cell line by the same treatments; (B): Panel of genes that are usually de-regulated in oral cancers.

(Fig. 2A). However, statistical analysis revealed that significant deviation were obtained only with the BBR solution that determines the down-regulation of five genes: CDH2, ITGA5 and SNAI2, PTP4A1 and TIMP1 (Table I).

Additional 10 genes were investigated because properly associated with oral cancer. The substances determine the down-regulation of most of the investigated genes (Fig. 2B). In particular, the

following genes were significantly down-regulated: the catenin genes CCND1, CCNE1, and CCNT1; the genes AKT1, EGFR and NOTCH1 belonging to the differentiation pathway and KRT19 involved in EMT (Table II).

In the second group of experiments, we intended to investigate whether BBR could modulate the effect of the commonest drugs for chemotherapy: CP and cisplatin. The expression profile of three

Table IV. Gene expression levels after 4h pre-treatment with berberine and 48h treatment with 5-fluorouracil or cisplatin of HEP2 cell line, as compared with untreated cells.

Gene	5-fluorouracil 30 μ M		Cisplatin 15 μ M		Pathway
	Fold change	p value	Fold change	p value	
AKT1	2.12	0.009297	3.55	0.000831	Differentiation
EGFR	0.76	0.522518	0.93	0.841696	Differentiation
NOTCH1	0.32	0.018115	0.64	0.217822	Differentiation
PTP4A1	0.41	0.052773	0.32	0.029666	Differentiation
TIMP1	0.98	0.960372	0.70	0.284505	Differentiation
CCND1	1.31	0.430785	2.48	0.045731	Cell cycle
CCNE1	0.22	0.009614	0.25	0.007049	Cell cycle
CCNT1	1.29	0.320594	1.40	0.174576	Cell cycle
ABCB5	0.12	0.000003	0.12	0.000001	Cell regulation
CDH2	0.76	0.486504	0.76	0.432325	EMT
ITGA5	0.50	0.057443	1.04	0.889447	EMT
SNAI2	0.67	0.130948	1.03	0.894964	EMT
KRT19	0.90	0.617203	1.81	0.037377	EMT

In bold significant gene expression changes. **Significantly up-regulated gene:** fold change ≥ 2 (p value ≤ 0.05); **Significantly down-regulated gene:** fold change ≤ 0.5 (p value ≤ 0.05).

group of cells was compared, namely untreated cells, chemotherapies treated cells, and cells pre-treated with BBR for 4h before the chemotherapies. The effects of 5-FU and cisplatin treatments limited to the expression of a selection of genes, those impacted by BBR or *T. cordifolia*, were summarized in Table III and Figure 3A. Interestingly, the two chemotherapies induced significant fold change variation in expression of CCNT1 (up-regulated) and ABCB5 (down-expressed) genes. Cisplatin had the most significant impact, down-regulating the expression of AKT1, CCND1 and CCNE1 and up-regulating NOTCH1 and KRT19 (Table III, Fig. 3A).

The effect of a 4h pre-treatment with pure BBR followed by a 48h treatment with the chemotherapy drugs was investigated in order to evaluate the potential benefit of a pre-treatment with natural compound before the standard therapies. In this case, compared to the untreated cells, a significant up-regulation of AKT1 gene and a significant down-regulation of PTP41, CCNE1 and ABCB5 genes were observed (Table IV, Fig. 3B).

The effects of the 4^h pre-treatment by BBR could

be better understood by comparing cells treated with BBR plus 5-FU or cisplatin versus cells that underwent chemotherapy treatments only (Table V and Fig. 3C).

DISCUSSION

For a very long time, traditional and Ayurvedic medicine have inspired drug discovery research. Combination between surgical resection and treatment with anti-cancer activity compounds could help the successful resolution of different type of tumors. Among active principles that have been investigated for their anti-proliferative properties, berberine plays an important role because of its apoptosis-inducing activity. In fact, such activity is considered an essential criterion for antitumor agents by many researchers. Nevertheless, the abolished proliferation and invasiveness could also keep tumor cell population under control and possibly avoid relapse.

A chemical/active principle can be considered a valuable anti-tumor agent when it is able to limit

Table V. Gene expression levels after 4h pre-treatment with berberine and 48h treatment with 5-fluorouracil or cisplatin of HEP2 cell line, as compared with cells treated only with 5-fluorouracil or cisplatin.

Gene	5-fluorouracil 30 μ M		Cisplatin 15 μ M		Pathway
	Fold change	p value	Fold change	p value	
AKT1	1.26	0.340325	10.28	0.000006	Differentiation
EGFR	0.57	0.084563	0.73	0.077854	Differentiation
NOTCH1	0.22	0.000405	0.26	0.000463	Differentiation
PTP4A1	0.42	0.045105	0.29	0.000109	Differentiation
TIMP1	0.98	0.961853	0.47	0.002342	Differentiation
CCND1	0.86	0.520667	8.16	0.000010	Cell cycle
CCNE1	0.45	0.086413	0.72	0.175363	Cell cycle
CCNT1	0.63	0.086498	0.45	0.002043	Cell cycle
ABCB5	0.26	0.002430	0.25	0.000003	Cell regulation
CDH2	1.06	0.898998	0.77	0.032792	EMT
ITGA5	0.35	0.005791	2.24	0.003501	EMT
SNAI2	0.37	0.003721	2.24	0.001871	EMT
KRT19	0.74	0.170711	0.42	0.000364	EMT

In bold significant gene expression level. **Significantly up-regulated gene:** fold change ≥ 2 (p value ≤ 0.05); **Significantly down-regulated gene:** fold change ≤ 0.5 (p value ≤ 0.05).

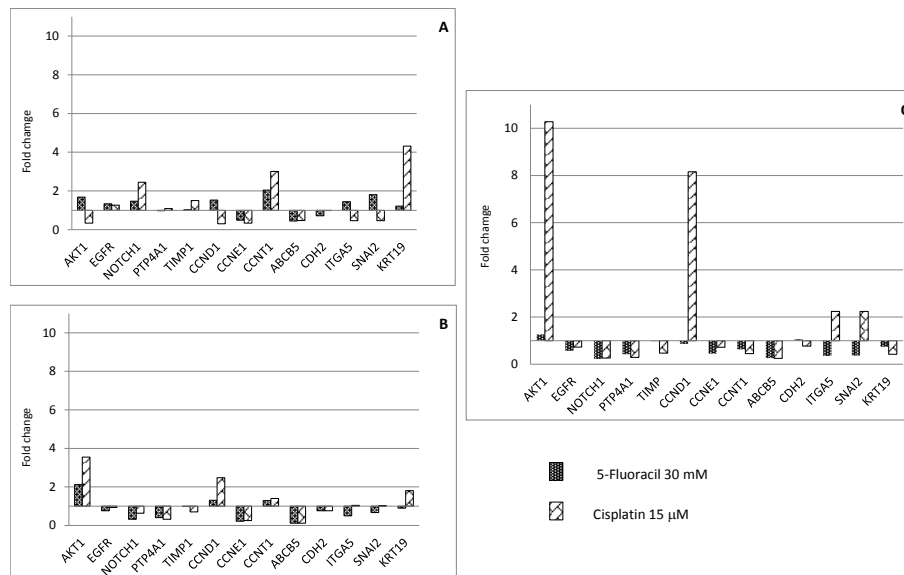


Fig. 3. Gene expression profile of genes de-regulated by treatment with berberine or *T. cordifolia*. (A): treatment with 5-fluorouracil or cisplatin versus untreated cells; (B): pre-treatment with berberine and treatment with 5-fluorouracil and cisplatin, versus untreated cells; (C): effect of the berberine pre-treatment in HEP2 cell line treated with 5-fluorouracil or cisplatin, i.e. berberine pre-treatment plus chemotherapies vs chemotherapies only.

or impair the cell cycle progression of tumor cells. It has been demonstrated that the combination of different chemicals can have synergistically apoptotic effects on tumors. For example, a soy-derived isoflavone cooperates with trichostatin A in promoting apoptosis in cultured HEP2 cells (20). Similarly, Chan and colleagues (21) demonstrated, in a human HNSCC well differentiated cell line (SCC25), a dose-dependent growth inhibition when treated with a combination of apigenin, a natural plant flavone and 5-FU or cisplatin.

The antifolate 5-FU, used as chemotherapeutic in laryngeal cancer (22), exerts its cytotoxic effect through inhibition of the nucleotide synthetic enzyme thymidylate synthase (TYMS), as well as by incorporating fluoronucleotides into RNA and DNA. Likewise, cisplatin, a commonly used chemotherapeutic drug for treatment of cancer of the larynx (22, 23), is able to crosslink with the purine bases on the DNA and it induces apoptosis in tumor cells by interfering with DNA repair mechanisms. Unfortunately, both compounds cause numerous undesirable side effects that vary from severe kidney problems to allergic reactions, decreased immunity against infections, gastrointestinal disorders, hemorrhage and ototoxicity. From this, the need of effective drugs arose, with lower deleterious impact on patients' general health. In this context, phytotherapy could represent a help, aimed to enhance the effect of the adjuvant therapy, thus allowing lower dosages that limit the side effects.

Our study intended to identify specific genes or pathways significantly deregulated by treatment with berberine, or extract of *T. cordifolia*, in the HEP2 laryngeal cancer cell line. Moreover, we wanted to verify if a pre-treatment as short as 4 h with BBR could amplify the action of 5-FU and cisplatin treatments.

Among the five genes chosen to evaluate the modulation of the differentiation pathway in HEP2 cell line, four were downregulated by 4h pre-treatment with BBR: EGFR, NOTCH1, PTP4A1 and TIMP-1. Among these, epidermal growth factor receptor (EGFR) was investigated in many human cancers and its high expression has often been associated to a poorer diagnosis (24). Dai

and colleagues found NOTCH1 upregulated in laryngeal squamous cell carcinoma tissues, while the knockdown of the gene induced apoptosis in the HEP2 cells (25). Protein tyrosine phosphorylation is crucial in regulating signaling events essential for processes that fundamental for cellular life, such as proliferation, metabolism, differentiation, survival/apoptosis, as well as adhesion and motility (26). Increased PTP4A1 (also named PRL-1) expression in some tumor cell lines (27) results in activation of Src and ERK1/2, which stimulates MMP2 and MMP9 production, leading to increased cell migration and invasion (28). Besides, human PRL-1 or PRL-2-transfected epithelial cells form malignant tumors in athymic mice (29). TIMP-1 is an important regulator of MMP-2 and MMP-9 (two subtypes of gelatinases) expression and Cao et al. (30) reported an increased TIMP-1 protein expression LSCC compared to normal tissues. Notably, the 4h pre-treatment with BBR was able to downregulate PTP4A1 expression while 5-FU and cisplatin treatments alone did not affect its level. Besides, TIMP-1 expression seems not affected by any kind of treatment involving 5-FU while it is downregulated by cisplatin preceded by 4h BBR pre-treatment.

Aberrant expression of cell cycle-related cyclins, proteins responsible of the normal cell division has been reported for different oral cancers (31). For example, cyclin D1 (CCND1) is a crucial regulator that governs the entry of cells to S phase from G0/G1 phase and its downregulation attenuates the cell cycle (32). CCND1 gene amplification and/or protein overexpression has frequently been reported in HNSCC, not only as negative prognostic marker, but also as probable predictor of resistance to chemotherapy (33). In addition, low CCNE1 expression in larynx carcinoma was associated with a more favorable outcome (34). CCND1, CCNE1 and CCNT1, another member of the highly conserved cyclin C subfamily were downregulated by our 4h BBR pre-treatment followed by 5-FU or cisplatin, except that CCND1 with cisplatin.

High ABCB5 expression has been significantly associated with OSCC progression and recurrence of the tumor (35). In our HEP2 cell line, ABCB5

is upregulated and both treatments (5-FU and cisplatin) lowered its expression. More, 4h pre-treatment with BBR further downregulates ABCB5 expression.

Epithelial-mesenchymal transition (EMT) allows neoplastic cells to gain the plasticity and motility required for tumor progression and metastasis. As indicators for this process, we studied the expression profile of CDH2, ITGA5, SNAI2, and KRT19 genes. It is reported that the increased presence of integrins within tumor microenvironments seems aimed at contributing to malignant phenotype of the tumor, promote angiogenesis and developmental neovascularization of the neoplasia. Besides, as reported by Janouskova et al. (36), inhibition of $\alpha 5$ integrin subunit expression by siRNA affects the survival of HCT116 highly invasive colon cancer cell-line. In our model, the 4h pre-treatment with BBR does not significantly affect CDH2, while it downregulates ITGA5, SNAI2 and KRT19 when associated to 5-FU treatment. SNAI2 is an E-cadherin repressor EMT inducer, which was found significantly overexpressed in HNSCC compared to normal mucosa (37). Keratins are epithelial-specific intermediate filament protein. KRT19 is expressed by the basal cells in the non-cornified squamous epithelium of oral mucosa and its expression in OSCC has been documented in several studies, as either downregulated (38,39) or upregulated (40). Zhong et al. correlated KRT19 overexpression in OSCC distant tissue with a higher tumor recurrence rate and a lower survival rate (41). Our 4h BBR pre-treatment decreased significantly KRT19 expression in HEP2.

Taken together, our data seem to support a potential role for BBR in decreasing the expression of genes usually upregulated in oral cancer cells or, specifically, in laryngeal cancer cells and possibly considered markers of tumorigenicity. Moreover, we found that BBR pre-treatment affects cisplatin/5-FU treatment at least on HEP2 laryngeal carcinoma cell line. Specifically, BBR seems able to enhance the sensitivity to cisplatin/5-FU. This could be considered an initial step in order to design a cancer therapy with improved efficacy and reduced side effects.

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DETECTION OF ENDOCARDITIS BACTERIA IN TONSILLAR MUCOSA OF AFGHAN POPULATION

F. RUGGIERO¹, D. CARBONE², R. MUGAVERO^{3,4,5}, A. PALMIERI⁶, D. LAURITANO⁷, L. BAGGI⁸, M. NARDONE⁹, F. CARINCI^{5,10**} and M. MARTINELLI^{6**}

¹Military Policlinic of Rome, Medical Department, Cardiological Unit, Rome, Italy; ²Military Policlinic of Rome, Department of Urgency and Surgery, Operative Unit of Odontostomatology, Rome, Italy; ³Department of Electronic Engineering, University of Tor Vergata, Rome, Italy; ⁴OSDIFE, University of Tor Vergata, Rome, Italy; ⁵CUFS, University of San Marino; ⁶Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna, Bologna, Italy; ⁷Department of Medicine and Surgery, University of Milano Bicocca, Monza, Italy; ⁸Department of Clinical Sciences and Translational Medicine, University of Tor Vergata, Rome, Italy; ⁹Ministry of Public Health, Rome, Italy; ¹⁰Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, Italy

**these authors contributed equally to this work and they are co-last authors

Endocarditis is a cardiovascular disease caused by the inflammation of the inner tissues of the heart, the endocardium, usually of the valves. Bacteraemia is essential in the development of endocarditis, and there are some findings that the main pathogens of endocarditis are viridans group streptococci: *Streptococcus oralis*, *Streptococcus sanguinis*, and *Enterococcus faecalis*. There is strong evidence that endocarditis bacteria are present in the tonsillar microbiota, so that tonsillar infection is associated with an increased risk of endocarditis. The aim of this manuscript is to investigate the presence of the main pathogens of endocarditis in tonsillar microbiota of an Afghan population group. A sample of 80 tonsil swabs was analyzed by quantitative real time PCR to detect endocarditis pathogens and an estimation of the total bacterial load. The median bacterial load in PCR reaction was 1.4×10^6 (interquartile range 4.7×10^5 - 2.9×10^6). Three species, *S. oralis*, *S. sanguinis*, and *E. faecalis* were found in large amounts in all specimens. On the other hand, *S. mitis* was never detected. The *S. aureus* was found in 3 samples with a prevalence of 0.04 (C.I. 0.01-0.10). The *S. mutans* was found in 33 samples with a prevalence of 0.41 (C.I. 0.31-0.52). Endocarditis bacteria has been found into the tonsillar microbiota, so there is sufficient evidence to justify that the oral cavity is a reservoir of endocarditis bacteria that can have a significant impact on the cardiovascular function.

The term tonsillitis is generally used to describe infection of tonsillae (1). Specific bacteria can cause tonsillitis. These bacteria leak into the tonsillar microbiota creating a cascade of events, which end with the production of inflammatory mediators

and bacterial metabolites. At least two potential metabolic pathways could develop from the tonsillar microbiota and the resulting from this condition could lead to a localized infectious-inflammatory disease involving other organs. The first step is the passage

Key words: endocarditis, tonsillar microbioma, cardiovascular disease, inflammation, infection.

Mailing address:

Prof. Francesco Carinci, MD,
Department of Morphology, Surgery and Experimental Medicine,
University of Ferrara, Via Luigi Borsari 46, 44121 Ferrara, Italy
Tel.: +39.0532.455874 - Fax: +39.0532.455876
e-mail: crc@unife.it - Web: www.carinci.org

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of tonsillar microbiota and their products through the ulcerated epithelium into the bloodstream, leading to bacteraemia and/or causing a systemic immune response. The second step is the passage of inflammatory mediators from the epithelium into bloodstream (2).

The pathological mechanisms by which microbiota may contribute to the pathogenesis of systemic inflammatory diseases are currently topic of intensive research. Thousands of different kinds of bacteria and various viruses are associated to the composition of the microbiota and are involved in the etiopathogenesis of tonsillitis.

Endocarditis is a cardiovascular disease caused by the inflammation of the inner tissues of the heart, the endocardium, usually of the valves. The main pathogens that cause bacteraemia and endocarditis are Viridans Group Streptococci and *Enterococcus faecalis*. Bacteraemia is essential for development of endocarditis. Throat infection may provoke bacteraemia and consequently endocarditis (3). It is well known that tonsillar microbiota were found in cardiovascular valves (4, 5). Maintaining oral health may be important to prevent endocarditis, but there are no studies to confirm this (6). In fact, some studies reported no impact of throat infection on bacteraemia (7, 8). Other studies support an association between

bacteraemia and routine daily activities (9, 10). Virtually, tonsillar microbiota have the potential to create a transient bacteraemia. A recent study found very low quality evidence that was insufficient to support or refute whether tonsillar microbiota can influence the recurrence of endocarditis (11).

The aim of this investigation was to investigate the presence of the main pathogens that cause endocarditis in tonsillar microbiota in an Afghan population group.

MATERIALS AND METHODS

Eighty healthy volunteers were recruited for this investigation. The study was approved by the local committee. All subjects were male employers in the Italian military campus in Herat (Afghanistan). Median age was 30.2 ± 9.7 years (Fig. 1). A sample of the tonsillar microbiota was taken from a single site using a sterile oral swab paper stick. Specimens were processed to extract and purify DNA using a method that includes 2 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.

Quantification of 16S rRNA genes Quantitative PCR of 16S rRNA genes was performed with the hydrolysis probes method to identify and evaluate the amount of 6 bacterial species including *Enterococcus faecalis*, and members of the Viridans Group Streptococci: *S. oralis*, *S. sanguinis*, *S. mitis*, *S. aureus*, and *S. mutans*. PCR oligonucleotide sequences were designed by dedicated softwares. Absolute quantification assays were performed using a Real Time PCR thermal cycler. 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 nontemplate controls to exclude reagents contamination and serial dilutions of the specific synthetic template. 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 (12-15).

Statistical analysis data from quantitative PCR included the amount of each of the 6 investigated bacterial

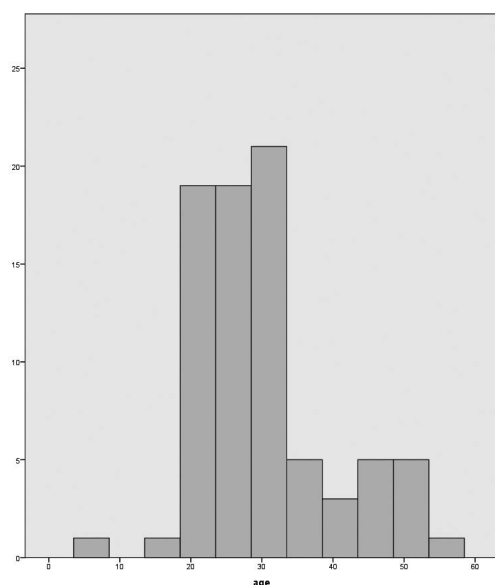


Fig. 1. Age distribution of volunteers in the study.

species and a measurement of the bacterial load. These amounts matched the number of DNA molecules detected in the real time PCR tubes that was directly related with the number of bacteria in the specimens. Ratios between the amount of each species and the total bacterial load were calculated. This reduced variability due to random factors such as specimen conservation, DNA extraction efficiency, and purification yield. The Wilson's method was used to calculate the confidence limits of prevalence.

RESULTS

A sample of 80 tonsil swabs were analyzed by quantitative real time PCR to detect endocarditis pathogens. Each specimen was previously analyzed with an aspecific PCR assay in order to obtain an estimation of the total bacterial load. All 80 samples provided enough DNA for the analysis. The median bacterial load in PCR reaction was 1.4×10^6 (interquartile range 4.7×10^5 - 2.9×10^6). Specific assays were performed to detect 5 members of Viridans Group Streptococci and *E. faecalis*. The three species *S. oralis*, *S. sanguinis*, and *E. faecalis* were found in large amounts in all the specimens. These species appeared to be normal commensals in the throat; indeed the detected amount was statistically correlated to the total bacterial load (Pearson correlation >0.75 ; P value <0.001). On the other hand, *S. mitis* was never detected. The *S. aureus* was found in 3 samples, with an estimated amount of up to 6% of the total bacterial load. The calculated prevalence of *S. aureus* was 0.04 (C.I. 0.01-0.10). The *S. mutans* was found in 33 samples with a mean relative amount of the total bacterial load of 0.15%. Its prevalence was 0.41 (C.I. 0.31-0.52).

DISCUSSION

Endocarditis can be lethal so the prevention of this disease and its various complications is mandatory (16). The most important aspects to consider are appropriate prevention for high-risk patients, appropriate diagnosis, effective antibacterial drug choice, and early detection of complications. Tonsillar infections could cause transient bacteraemia and consequently endocarditis.

The present investigation revealed that relevant bacterial species for endocarditis were widely spread in tonsillar microbiota of Afghan population.

There is lack of knowledge on the relationship between tonsillar microbiota and endocarditis. It is known that tonsillar microbiota mainly invade blood vessels from the oral cavity, thus oral surgery is one of the high-risk procedures for developing endocarditis (17-25). In the United Kingdom, guidelines from the National Institute for Health and Clinical Excellence in 2008 for the prevention of endocarditis before surgery, did not recommend administration of antibiotics in patients at risk of endocarditis, but the incidence of infective endocarditis has increased significantly after 2008 (7).

It is reported that the main pathogens that cause endocarditis are members of Viridans Group Streptococci, and *Enterococcus faecalis*, however, fungi and HACEK organisms such as *Haemophilus* species, *Aggregatibacter* species, *Cardiobacterium hominis*, *Eikenella corrodens*, and *Kingella* species are low-pathogenic gram-negative bacteria that are also detected by blood culture examination (26).

The microbiota causing endocarditis has been studied significantly over the last century. The tonsillar mucosa is populated by a variety of pathogens relevant for endocarditis. Tonsillar microbiota can cause oral inflammation thus increasing the chances of bacteraemia. The vast majority of oral surgery result in some degree of risk for bacteraemia, and likely endocarditis is provoked by bacteraemia. The role of duration of bacteraemia as a risk factor for endocarditis is uncertain. The degree to which antibiotics reduce the risk of endocarditis associated with inflammation is controversial. Some studies suggest that amoxicillin has a highly statistically significant impact on reducing the incidence of endocarditis during infective diseases.

In conclusion, since a growing numbers of patients at risk of endocarditis need oral surgery, the recommendation for endocarditis prevention will be a part of their healthcare maintenance program.

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HUMAN POLYOMAVIRUS IN TONSILLAR MICROBIOTA OF AN AFGHAN POPULATION GROUP

F. RUGGIERO¹, D. CARBONE², R. MUGAVERO^{3,4,5}, A. PALMIERI⁶, D. LAURITANO⁷,
L. BAGGI⁸, M. NARDONE⁹, M. MARTINELLI^{6**} and F. CARINCI^{5,10**}

¹Military Policlinic of Rome, Medical Department, Cardiological Unit, Rome, Italy; ²Military Policlinic of Rome, Department of Urgency and Surgery, Operative Unit of Odontostomatology, Rome, Italy; ³Department of Electronic Engineering, University of Tor Vergata, Rome, Italy; ⁴OSDIFE, University of Tor Vergata, Rome, Italy; ⁵CUFS, University of San Marino; ⁶Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna, Bologna, Italy; ⁷Department of Medicine and Surgery, University of Milano Bicocca, Monza, Italy; ⁸Department of Clinical Sciences and Translational Medicine, University of Tor Vergata, Rome, Italy; ⁹Ministry of Public Health, Rome, Italy; ¹⁰Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, Italy

**these authors contributed equally to this work and they are co-last authors

Some studies have evidenced the role of human polyomaviruses in head and neck squamous cell carcinoma. BK, JC and SV40 human polyoma viruses are widely recognized as etiological agents associated with malignancies. The aim of this study was to analyse the prevalence of BK, IC and SV40 in tonsillar microbiota in a group of Afghan volunteers. A sample of the tonsillar microbiota was taken from a single site using a sterile oral swab paper stick. A fixed volume of purified DNA from each sample was tested by quantitative real-time polymerase chain reactions to evaluate the number of human cells and the number of viral genomes in each sample. The cell number was evaluated via the quantification of a single copy genomic sequence, which is located in the HMBS locus. The median analyzed cell number in each reaction was 4343 (interquartile range 2074-8470). SV40 was never detected, while prevalence rate was 0.11 (C.I. 0.06-0.20) for BK and 0.10 (C.I. 0.05-0.19) for JC. Further studies are necessary to clarify whether polyomaviruses can be considered a risk factor of oral, oropharyngeal and laryngeal malignancies.

Head and neck cancer affects more than 550,000 patients and 380,000 deaths every year worldwide (1-6). The etiopathogenesis of squamous cell carcinoma involves many risk factors. Molecular and epidemiological research has provided evidence of the role of oncogenic viruses in squamous cell carcinoma development (7-11).

The word polyomavirus (PyV) derives from

Greek words poly- (multiple) and -oma (tumors) and was coined when this virus was discovered in neonatal mice with multiple tumors in 1953 (1). Human polyomaviruses (HPOVs) are a viral cluster diffuse worldwide, in fact currently, there are 13 subtypes of viruses infecting humans. BK (2), JC (7), SV40 HPOVs and the more recently discovered Merkel cell polyomavirus (MCPyV) (8)

Key words: human polyomavirus, BK virus, JC virus, SV40 virus, malignancies

Mailing address:

Prof. Francesco Carinci, MD,
Department of Morphology, Surgery and Experimental Medicine,
University of Ferrara, Via Luigi Borsari 46, 44121 Ferrara, Italy
Tel.: +39.0532.455874 - Fax: +39.0532.455876
e-mail: crc@unife.it

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are widely recognized etiological agents of BKPyV-associated nephropathy, progressive multifocal leukoencephalopathy and Merkel cell carcinoma, respectively (9, 10).

Based on epidemiological and phylogenetic findings, it has been established that HPOVS co-evolved with their hosts, showing high prevalence, low pathogenicity and symptomless latency in immune competent people (12). HPOVs become latent post-primary infection and are reactivated among immune-compromised patients, including HIV positive individuals, organ transplant patients, and patients with autoimmune diseases (13-16).

Clinical debilitating conditions including chronic alcohol abuse are sufficient to lead to HPOVs reactivation (10), in fact, the specific factors leading to reactivation are yet to be defined and it has not been determined whether the viruses undergo true latency, expressing only a subset of specific viral genes. However, it is unknown if HPOVs DNA is integrated into the host genome or whether integration is a rare event specific to some virus's subtypes. The identification of factors associated with reactivation, latency and integration of HPOVs is of increasing importance as the prevalence of these viruses and their associated diseases are increasing rapidly.

The best-studied member of the polyoma viruses is the simian vacuolating virus 40 (SV40) (17, 18). Discovered in 1960, SV40 was one of the first DNA tumor viruses discovered linked to the antipolio vaccine development in the 1950s. Antipolio vaccines were prepared in primary cell cultures of rhesus monkey kidney cells, furthermore SV40 was found to provoke salivary gland carcinomas in mice (19) and mesotheliomas, lymphomas, brain tumors, bone tumors and sarcomas in hamsters (20).

In humans, SV40 was found in mesotheliomas and brain tumors (20). However, the range of SV40 disease associations has varied from 0 to 100% (20). While SV40 is been hypothesized a co-factor in human carcinogenesis, may be that SV40 infection alone is sufficient to cause human cancers, and no cancer epidemics were observed following the administration of antipolio and adenovirus vaccines contaminated with SV40 (17).

SV40 was broadly studied after the potential

public health risk induced by antipolio vaccinations (18) and the subsequent findings became important to both molecular and cancer biology.

BK virus may infect almost 90% of population, moreover, BK DNA has been found in many types of tumors, e.g., human brain tumors, in neuroblastoma, in urinary tract tumors, in uterine cervix vulva, lips and tongue carcinomas, as well as in Kaposi's sarcoma (12). BK has been found in saliva (19), and BK is suspected to have role in oral cancer development (20).

The present research investigated the prevalence of SV40, JC and BK in tonsillar microbiota from an Afghan population group.

MATERIALS AND METHODS

Eighty healthy volunteers were recruited for this investigation. The study was approved by the local committee. All the subjects were male employees in the Italian military campus in Herat (Afghanistan). Median age was 30.2 ± 9.7 years (Fig. 1). A sample of the tonsillar microbiota was taken from a single site using a sterile oral swab paper stick. Specimens were processed to extract and purify DNA using a method that includes two consecutive incubations with lysozyme and proteinase K. Once extracted, DNA was purified through a silica spin column.

Two real-time PCR runs were performed for each sample. Absolute quantification assays were performed with a Real-Time PCR system in a 20 μ L reaction volume as detailed before (21) (22-25). Briefly, the first reaction simultaneously detected and quantified both the amount of human single copy gene (HMBS) and SV40. The second reaction detected and quantified BKV and JCV together. BE3, JE3, and SE3 primer and probe sets were used to detect the different BKV, JCV, and SV40 polyomaviruses, respectively (26), although a different combination of dyes and quenchers were adopted to account for the different real-time PCR apparatus as described before (21). Samples were scored as positive for SV40, BKV, or JCV when the number of viral genome were >5 copies/PCR-reaction. The Wilson's method was used to calculate the confidence limits of prevalence.

RESULTS

A fixed volume of purified DNA from each sample was tested by quantitative real-time polymerase chain reactions to evaluate the number of human cells and the number of viral genomes in each sample. The cell number was evaluated via the quantification of a single copy genomic sequence, which is located in the HMBS locus. All samples provided a cells number higher to the established threshold of 100 cells. The median analyzed cell number in each reaction was 4343 (interquartile range 2074-8470).

The 80 samples were investigated for the presence of polyoma viruses SV40, BK and JC. The SV40 was never detected, while 9 and 8 samples produced some positive endpoint signals for BK and JC, respectively. In all these cases, the multiplicity of infection was as low as 10^{-3} viral genomes per cell. The calculated prevalence rate was 0.11 (C.I. 0.06-0.20) for BK and 0.10 (C.I. 0.05-0.19) for JC.

DISCUSSION

A number of studies available in the literature concern the role of viruses in the development of head and neck squamous cell carcinoma. However, they are mainly focused on one type of HOPV, while only some recent reports analyse the possible correlation between the infection of two HOPVs and carcinogenesis. Our research is the first original observation that implicates BK, JC, and SV40 in tonsillar microbiota of an Afghan population group and results reveal the absence of SV40, while a number of cases were positive for BK and JC, respectively.

Recent data has suggested a correlation between BK and various types of human cancers (27). The presence of BK DNA was confirmed in high-grade squamous intraepithelial cervical lesions (precancerous lesions) (28). Burger-Calderon et al. (29) suggests a connection between BK and the oral cavity. Several studies have suggested BK to be oral-tropic (30). It has not been determined whether the HPOVs undergo true latency, expressing only a subset of specific viral genes. It is unclear whether HOPVs DNA is commonly integrated into the host

genome or whether integration is a rare event specific to some subtypes (31).

Some studies have investigated the role of the mucosae of the oral cavity such as reservoir of these viruses, and the role of chemical devices in prevention therapies, in fact BK DNA was detected in tonsillar microbiota (32-47).

Besides, Moens et al. (47) suggests that HOPVs, including those induced by other oncogenic viruses, may be a co-factor in the development of cancer. Some authors suggest that BK may be a potential co-factor for HPV in the development of cervical neoplasia (48), especially together with the HPV genotype 16 (28). Finally, we cannot exclude the role of BK in squamous cell carcinoma, considering the fact that the genetic material of BK was detected in saliva (30). The 'hit and run' mechanism may validate the co-factorial role of BK in cancer onset and progression (29).

Double or mixed infection with other HOPVs may induce transformation. A limitation of our study is, however, only the epidemiological character of the research carried out. Thus, further studies are needed to clarify whether BK plays a role in oral squamous cell carcinoma or is a co-factor for

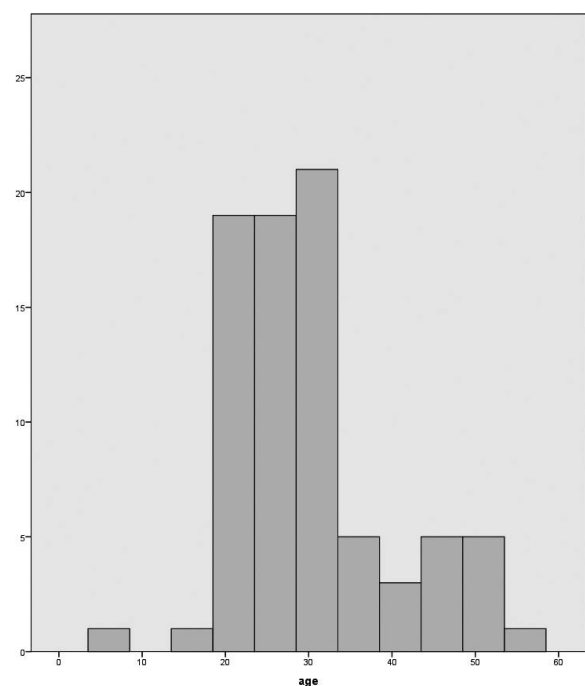


Fig. 1. Age distribution of volunteers in the study.

cancers induced by other HOPVs. Chronic infections influence the immunological system of the host; in fact, primary infection may favour super infection with a HOPVs capable of tumor transformation. Toll-like receptors (TLRs) play a critical role in the early innate immune response to invading pathogens by sensing microorganisms and they are involved in sensing endogenous danger signals. There are few studies examining the association between HOPVs in the progression of oral cancers. A detailed understanding of co-infection will enable the targeting of new methods for the early detection, prevention and treatment of viral-associated cancers.

Future epidemiological studies regarding the relationship between HOPVs and gender, tobacco, alcohol and chronic inflammation in the development of oral cancer as well as studies on the mechanisms of co-infection and/or super infection and their role in oral squamous cell carcinoma are necessary. Knowledge about the pathways of HOPVs may provide targets for therapy and for devising diagnostic methods.

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HUMAN PAPILLOMA VIRUS IN THE TONSILLAR MICROBIOTA OF AN AFGHAN POPULATION GROUP

F. RUGGIERO¹, D. CARBONE², R. MUGAVERO^{3,4,5}, F. CURA⁶, L. BAGGI⁷,
C. ARCURI⁷, M. NARDONE⁸, R.M. GAUDIO⁶, R. GATTO⁹,
L. SCAPOLI^{**10} and F. CARINCI^{5,11**}

¹Military Policlinic of Rome, Medical Department, Cardiological Unit, Rome, Italy; ²Military Policlinic of Rome, Department of Urgency and Surgery, Operative Unit of Odontostomatology, Rome, Italy; ³Department of Electronic Engineering, University of Tor Vergata, Rome, Italy; ⁴OSDIFE, University of Tor Vergata, Rome, Italy; ⁵CUFS, University of San Marino; ⁶Department of Medical Sciences, University of Ferrara, Ferrara, Italy; ⁷Department of Clinical Sciences and Translational Medicine, University of Tor Vergata, Rome, Italy; ⁸Ministry of Public Health, Rome, Italy; ⁹Department of Life, Health and Environmental Sciences, School of Dentistry, University of L'Aquila, L'Aquila, Italy; ¹⁰Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna, Bologna, Italy; ¹¹Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, Italy

****these authors contributed equally to this work and they are co-last authors**

Cancer of the oral cavity is known to have a diverse aetiology that includes infectious agents. Human papilloma virus has been found to be associated with several types of human cancer, inclusive of cervical, vulvar, vaginal, penile, anal, and cancer of tonsil. The aim of this manuscript is to investigate the presence of human papilloma virus in tonsillar microbiota of an Afghan population group. A sample of the tonsillar microbiota was collected by oral swab paper stick from 80 healthy donors. The sample was investigated for the presence of high-risk human papillomavirus types 16, 18, 31 and 45 by real time PCR. Eight samples produced some positive endpoint signals for human papillomaviruses. The human papillomavirus 31 was the unique papillomavirus detected; its calculated prevalence rate was 0.10 (C.I. 0.05-0.19). However, the viral load was always very low, in the order of 10^{-3} viral genomes per cell. The high prevalence of high-risk human papillomavirus in healthy population suggest a need for further investigation on virus spreading and supports the development of vaccination strategies.

Human papilloma virus and tonsillar microbiota

Carcinoma of tonsil (CT) is known to have a diverse aetiology that includes infectious agents. Greater than 20% of the global cancers burden has an associated infectious etiology (1, 2). A diverse range of viruses is implicated in human cancers, which originate in different regions of the body, including

the liver, genital regions, and the oral cavity, to name a few. A significant proportion of the viral human cancers arise from the oral cavity (3, 4).

In fact, the viral agents associated with cancers include: human papillomavirus-associated with CT, Epstein Barr virus-associated with nasopharyngeal carcinoma, and Kaposi's sarcoma-associated herpes

Key words: Human papilloma virus, tonsillar microbiota, oral disorders, oral cancer, squamous cell carcinoma

Mailing address:

Prof. Francesco Carinci, MD,
Department of Morphology, Surgery and Experimental Medicine,
University of Ferrara, Via Luigi Borsari 46, 44121 Ferrara, Italy
Tel.: +39.0532.455874 - Fax: +39.0532.455876
e-mail: crc@unife.it - Web: www.carinci.org

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virus (5, 6). HPV has been found to be associated with several types of human cancer, inclusive of cervical, vulvar, vaginal, penile, anal, and CT (7,8). The incidence CT HPV-related has increased in the last ten years, the percentage of CT that was HPV- positive went from less than 20% to more than 70% in the United States and some European countries. To reduce the incidence of CT HPV-related, it is necessary to encourage research for better understanding of the pathogenesis of this type of cancer, and to develop research for the treatment of the disease with HPV vaccines.

Head and neck cancers constitute the sixth leading malignancy globally (9, 10). Known etiological risk factors include tobacco use and alcohol consumption (11, 12). In the United States and in some European countries, the overall incidence of head and neck cancers associated with the above etiological risk factors has declined in the past two decades, consistent with the decrease in tobacco usage in these regions. By contrast, a rapid rise in the incidence of CT, has been noticed in men younger than 50 years of age with no prior history of tobacco usage and alcohol consumption. These cancers were found to be specifically associated with HPV infection. In the United States, HPV prevalence in CT increased during the last years; similarly, increases in the incidence of CT HPV-related have also been reported in many European countries, including Finland, Sweden, the Netherlands, and the United Kingdom (13, 14). There are more than one hundred HPV types that can be divided into two categories: high-risk oncogenic types and low-risk non- oncogenic types. The high-risk type 16 constitutes the most prevalent HPV type globally, being detected in almost 60% to 80% of CT. However, high-risk type 18 was found in 34% of oral cavity squamous cell cancers and 17% of laryngeal squamous cell cancers (15). The CT HPV-related manifests different clinical and biological characteristics in comparison with the CT not HPV-related. In addition, patients with CT HPV-related have a favourable prognosis in comparison with those with CT not HPV-related, and mutations associated with tumour suppressor genes like p53 are relatively infrequent in the former (16, 17).

The aim of this manuscript is to investigate the

prevalence of high-risk human papilloma virus in tonsillar microbiota of an Afghan population group.

MATERIALS AND METHODS

A sample of the tonsillar microbiota was taken from a single site using a sterile oral swab paper stick. The study was approved by the local committee. Specimens were processed to extract and purify DNA using a method that includes two consecutive incubations with lysozyme and proteinase K. Once extracted, DNA was purified through a silica spin column.

Two real-time polymerase chain reaction runs were performed for each sample. The first reaction detected and quantified the amount of the human single copy gene HMBS and included two primers and a single probe. The second reaction detected and quantified high-risk human papillomavirus type 16, -31 and -18 together with -45. The primers and probe for the amplification of a unique sequence in the first intron of the human gene hydroxymethylbilane synthase isoform 2 (HMBS) were designed using a specific software (HMBSf: 5'-AAGACACGTTCCACTTTTGATTCA-3'; HMBSr: 5'-ACACAAAAGAAGGCGCACTTC-3'; HMBSProbe:

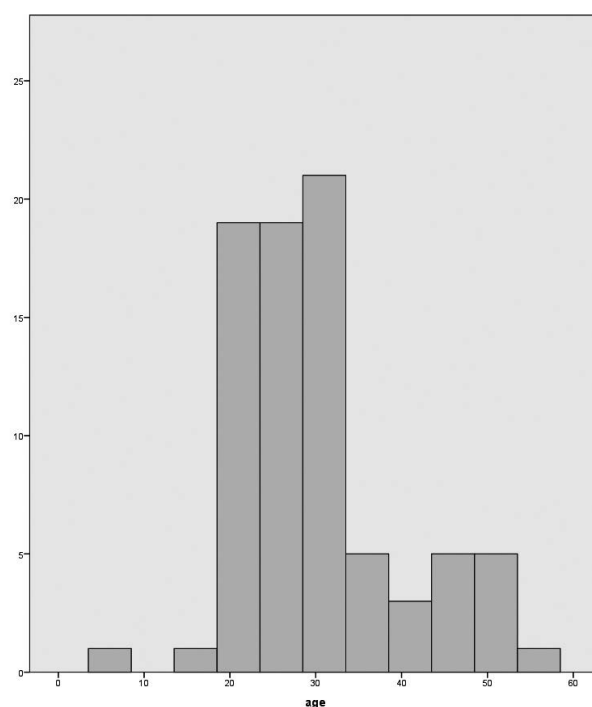


Fig. 1. Age distribution of volunteers in the study.

5'-AAGCCTCCGAACTGCACACAAACGTC-3'). The probe was quenched with BHQ1 at the 3' end and labelled with JOE at the 5' end. The primer and probe sequences used to simultaneously detect human papillomavirus types 16, 18, 31 and 45 in a single tube reaction, were those previously described by Moberg et al. (18) although a different combination of dyes and quenchers were adopted to account for the different real-time polymerase chain reaction apparatus, as described before (19-23).

The absolute quantification of genome copies in samples allowed for the quantification of the input cell number and the calculation of the viral load, which is the number of viral genomes per cell. The Wilson's method was used to calculate the confidence limits of prevalence.

RESULTS

A fixed volume of purified DNA from each sample was tested by quantitative real-time polymerase chain reactions to evaluate the number of cells and the number of viral genomes in each sample. The cell number was evaluated via the quantification of a single copy genomic sequence, which is located in the HMBS locus. All samples provided a cell number higher to the established threshold of 100 cells. The median analysed cell number in each reaction was 4343 (interquartile range 2074-8470).

The sample was investigated for the presence of high-risk human papillomavirus types 16, 18, 31 and 45. Eight samples produced some positive endpoint signals for human papillomaviruses.

The human papillomavirus 31 was the unique papillomavirus detected; its calculated prevalence rate was 0.10% (C.I. 0.05-0.19). However, the viral load was always very low, in the order of 10^{-3} viral genomes per cell.

DISCUSSION

Our research is the first original observation that implicates HPV in tonsillar microbiota of an Afghan population group and results reveal the presence of human papillomavirus 31 as the unique papillomavirus detected and a very low viral load. This data can be explained by the fact that Afghan people are Muslims, and Islamic religion dictates

behaviours that limit the spread of the virus in the population. In fact, in the United States and some European countries HPV infection has increased the incidence of CT, although a great many ambiguities exist pertaining to the epidemiological characteristics of these cancers. Some studies have investigated the role of the mucosae of the oral cavity such as reservoir of these viruses, and the role of chemical devices in prevention therapies (24-31).

The increased incidence of CT HPV-related could be attributable to changes in sexual behaviours, such as increased oral sex practices and more oral sex partners. However, CT HPV-related are also documented in patients reporting very few oral sexual partners, with almost 8-40% of the patients reporting never having had oral sex. Traditional risk factors, such as alcohol consumption and tobacco use, may still have a significant impact on CT HPV-related, since around 10-30% of these cancers occur in patients who smoke or consume alcohol. Thus, the contributions of these risk factors in the etiology of CT HPV-related cannot be undermined, and a possible synergy of HPV infection and traditional etiological factors should be investigated. In light of the above considerations, the role of vaccines is crucial to preventing infection and the onset of CT.

Several groups of researchers screened the hypothesis of HPV role in promoting carcinogenesis in the tonsillar epithelium, considering that oncogene proteins E6 and E7 act at a very early stage of carcinogenesis, significantly increasing the capacity of the epithelial cell of duplicate by blocking the apoptotic pathways. The hypothesis that HPV contributes to the genesis of CT is still on debate. The most common malignant neoplasm of the oropharynx is CT. Recent estimates, both the annual incidence rates and the mortality rates of CT, have not diminished in recent years, so CT is still a serious problem of public health. This disease is characterized by strong local aggression and a poor prognosis, with a five-year survival ranging from 59.4% to 67%. The following evidence subsequently supported initial hypotheses about a possible etiologic role of the virus in oral and oropharyngeal carcinogenesis, dating back to the early 1980s: epithelial tropism of virus; the confirmed etiologic role of HPV in the

almost total squamous cell carcinomas of the uterine cervix and in approximately 40% of the cases of other squamous cell carcinomas.

Despite the histological similarities of the two epithelial, it must be stressed that the environment of the uterine cervix is much more receptive to the presence of the virus than all the other anatomic sites. The role of the virus in carcinogenesis of the CT is very controversial.

The scientific evidence available for oropharynx is therefore differentiated from those available for the oral cavity. Only the CT is most closely related to HPV infection, while squamous cell carcinoma of the mouth have not demonstrated a strong relationship, in fact HPV has a more marginal role in etiopathogenesis.

The most alarming data is that, according to the latest epidemiological studies, due to the success of alcoholism and tobacco awareness campaigns, confirmed by the reduction of new cases of oral squamous cell carcinomas alcohol-related. The annual incidence of HPV verrucous carcinoma of oropharynx has increased exponentially over the last twenty years, so that it is estimated that by 2020 their frequency will be higher than squamous cell carcinoma in the uterine cervix and in the anogenital tract (32).

CONCLUSION

In recent decades, the amount of information about HPV infection and its oncogenic potential has increased considerably, in line with advances in molecular biology techniques applied to diagnostic, preventive and therapeutic management of HPV-induced lesions. Understanding interactions between the viral agent and the host provides new targets for infection control and allow the formulation of new-targeted therapeutic protocols.

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RADIOSURGERY-ASSISTED GINGIVAL DISPLACEMENT: A PROOF OF CONCEPT TECHNIQUE TO ENHANCE THE EFFICIENCY OF INTRAORAL DIGITAL IMPRESSION

L. ARCURI¹, M. CONTALDO², R. SANTORO², A. POZZI³, C. LORENZI¹, C. ARCURI¹,
A. BARLATTANI¹, L. TETTAMANTI^{4**} and L. OTTRIA^{1**}

¹*Department of Clinical Sciences and Translational Medicine, School of Dentistry, University of Tor Vergata, Rome, Italy;* ²*Multidisciplinary Department of Medical-Surgical and Dental Specialties, University of Campania Luigi Vanvitelli, Naples, Italy;* ³*Oral Surgery and Implant Dentistry, Marche Polytechnic University, Ancona, Italy;* ⁴*Department of Medicine and Surgery, University of Insubria, Varese, Italy*

****these authors contributed equally to this work and they are co-last authors**

Nowadays, an increasing number of dentists are using intraoral scanners (IOS) in their daily practice as an alternative to conventional impression taking. One of the main concerns is related to the capability of scanning the subgingival anatomy of the die, usually very challenging due to the limited operative field and the presence of oral fluids. The radiosurgery assisted gingival displacement technique (RAGD) may enhance the intraoral optical scanning of the finish line and neighbor tooth anatomy. The contour of the interim prosthesis is used to drive the tip of radiosurgery electrode along the tooth surface and open selectively the gingival crevice with a prosthetically-driven and minimally invasive approach. The clinical implication of this technique is related to increasing the efficiency and accuracy of the digital impression technique in the critical zone of the prosthetic shoulder.

Lack of margin precision and vertical and horizontal overhangs may cause periodontal tissues inflammation and may increase the risk of recurrent caries, especially for subgingivally-located crown margins (1, 2).

Although a supragingival margin is preferable to maintain a sound periodontal attachment (3), the patient can refuse such approach particularly in the area of the mouth of esthetic concern. Moreover, in certain clinical conditions the finish line needs to be placed subgingivally to increase the retention form, refine the preexisting margins, treat cervical caries or abrasion and cover the root surface to eliminate the sensitivity (2, 4-6).

During the impression, taking the gingival

displacement is necessary to record the finish line regardless of the conventional or digital methods used (6, 7). Besides, gingival fluid seepage and hemorrhage should be controlled to ensure acceptable impressions (8, 9). Several gingival displacement methods were described in the literature, classified as mechanic, mechano-chemical (chemicals embedded in cords or in an injectable matrix form), surgical (RS, electrosurgery (ES), lasers, and rotary curettage), or combinations of these (10, 11).

The precision of the IOS was reported to be clinically acceptable (12, 13) and their use seems easier for taking the impression of the implants than for natural teeth, because of the use of dedicated components (scan-bodies) detectable by the scanner regardless

Key words: Intraoral scanner, digital impression technique, radiosurgery, electrosurgery, gingival displacement

Mailing address:

Prof. Liliana Ottria,
Department of Clinical Sciences and Translational Medicine,
School of Dentistry, University of Rome "Tor Vergata"
Via Montpellier 1, 00133 Rome, Italy
Tel.: +39 06 20900268
e-mail: lilianaottria@gmail.com

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how much deep is the implant platform respect to the gingival margin (13-20).

The use of the IOS for taking the impression of the die and surrounded soft tissue architecture is limited by the operative access to the site. Opening of the gingival crevice is mandatory to ensure accurate recording of the finish line by means of the scanner light beam. The retraction cord may assist the clinician to displace the marginal gingiva, however later on, discerning the finish line from the cord itself in the software can be challenging (21). Moreover in case of retraction cords are associated with bleeding in more than 50 percent of situations (21). The need of a direct vision of the subgingival finish line as well as the absence of bleeding and cord artifacts requires a way for the clinician to clearly detect the emerging anatomy of subgingival dental preparations through IOS.

The RAGD technique is a proof of concept technique to enhance the digital impression of the prosthetic die, discerning the anatomy of the tooth from that of the gingival tissue at the finish line level. The RAGD technique uses the contour of the interim prosthesis to guide the tip of the radiosurgery electrode along the tooth surface to open the gingival crevice selectively with a prosthetically driven and minimally invasive approach.

Technique

Step 1: Fabricate an anatomically-contoured interim dental prosthesis.

Step 2: Prepare the tooth placing the finish line 1 mm subgingivally.

Step 3: Reline the interim prosthesis onto the die with an acrylic resin and refine chairside to deliver a well-polished surface and contour

Step 4: Let the soft tissues heal and mature for 3 weeks around the interim prosthesis contour (Fig. 1).

Step 5: Use a single-wire needle-type electrode with a monopolar cut mode RS [Surgitron® Dual RF; Ellman International, Inc (4.0 MHz frequency, fully rectified, filtered waveform)] with a 45 degrees angle, along the prosthetic contour all around the die except for the interproximal area adjacent to the contact surface, placing the tip of the electrode at the finish line level (Fig. 2). Step 6: Remove the interim

fixed dental prosthesis and gently apply the tip of the electrode in the interproximal areas to open the gingival crevice underneath the contact surface. Step 7: Wait 5 min to control the bleeding and in case, use a hemostatic solution.

Step 8: Dry the interested area with an air syringe.

Step 9: Perform digital impression of the interested area through an IOS (Trios3; 3Shape A/S) (Fig. 3, 4).

Step 10: Wait three weeks for recovery of the soft tissues, before delivering the definitive cement-retained FDP (Fig. 5-8).

DISCUSSION

Detecting the subgingival dental preparations through an intraoral scanner is considered the weak part of the digital impression (13, 21). To the best of our knowledge, no techniques have been published to enhance the optical reading of the finish line of the subgingival preparations. The RAGD technique uses an anatomically-contoured provisional restoration to guide the radiosurgery electrode tip through the gingival crevice and displace the gingival margin horizontally.

Contraindications to its use are the presence of flammable anesthetics, liquids, or skin preparations and its use should be avoided in patients with severe periodontal disease and/or with metal restorations close to the interested area. Besides, when radio wave equipment is used in the presence of cardiac pacemakers or defibrillators, a cardiology consultation should be obtained (22).

Radio wave surgery causes tissue vaporization and potential smoke hazard from particulate inhalation. Precautions include careful and controlled smoke plume evacuation and wearing surgical masks rated for micro particle filtration. Although not a complication, inadequate removal of smoke will cause an unpleasant smell throughout the office (22). The operator's experience and capability in the use of RS are relevant to the success of the technique. The comprehension of the device characteristics is fundamental to handle the high-frequency radiosurgery effectively and to limit the invasion of the periodontal tissues (22, 23). After the application of RAGD, it should wait 3 weeks for the soft tissues



Fig. 1. *Interim prosthesis temporarily cemented.*



Fig. 2. *RAGD Technique applied on the buccal side.*



Fig. 3. *Digital impression buccal view.*

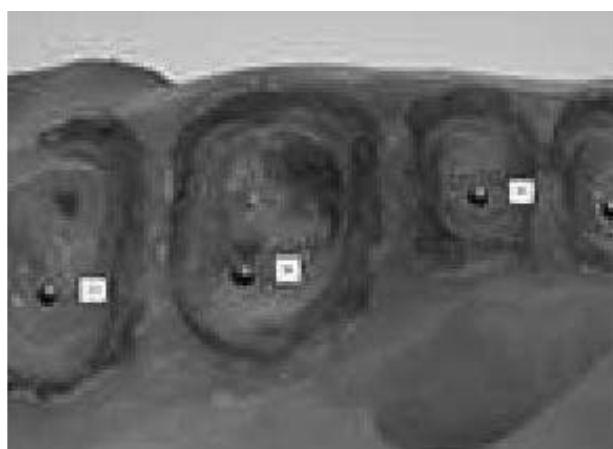


Fig. 4. *Digital impression occlusal view.*



Fig. 5. *3D-printed dental cast buccal view.*



Fig. 6. *3D-Printed dental cast occlusal view.*



Fig. 7. Periodontal tissues after 3 weeks of healing.



Fig. 8. Definitive porcelain fused to zirconia FDP.

healing. However, irreversible changes may occur because of the heat degeneration leading to possible gingival retraction. To the best of our knowledge, no studies were published describing the RS as a gingival displacement technique.

While few and old articles have analyzed the effects of ES as a gingival displacement technique. Azzi et al. (6) analyzed clinically and histologically the effects of ES, retraction cord, and the rotary gingival curettage technique in dogs. Postoperative periods analyzed ranged from 6 hours to 14 days. The results showed that all methods induce some type of damage of a transient nature and that tissues heal after their use without any permanent loss of attachment. Gingival recession was greater and of probable clinical significance with the rotary gingival curettage technique, minimal with ES, and nonexistent with the cord. Another study showed that although there is loss of tissue soon after ES, 70 to 100% of the lost tissue is regained over a period of months (24). It has also to be considered that the histological effect of ES varies depending on the power output (25), frequency (26), the waveform selected (27) and size and shape of active electrode (28). These studies can roughly figure out the effects of RS on the periodontal tissues as a displacement technique, taking into account that Schoinohoriti et al. (29) found no statistically significant differences regarding lateral thermal damage and epithelial

cell proliferation between ES and RS. Long term studies over a greater specimen are necessary to better analyze the RAGD technique. A detailed evaluation of the soft tissues response should be performed, considering different variables such as the periodontal biotype, systemic diseases and smoking habits.

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TEMPOROMANDIBULAR JOINT AND RELATED STRUCTURES: ANATOMICAL AND HISTOLOGICAL ASPECTS

L. OTTRIA^{1*}, V. CANDOTTO^{2*}, F. GUZZO³, M. GARGARI³ and A. BARLATTANI¹

¹*Department of Clinical Science and Translational Medicine, University of Rome “Tor Vergata”, Rome, Italy;* ²*Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy;* ³*Department of Dentistry “Fra G.B. Orsenigo-Ospedale San Pietro F.B.F.”, Rome, Italy*

*these authors contributed equally to this work and they are co-first authors

In literature, there is no unanimous agreement about the anatomical and functional characteristics of the temporomandibular joint (TMJ) and its components. The aim of this work is to increase the knowledge about components of the temporomandibular joint, starting from the revision of classical anatomy, through macroscopic and microscopic study, 20 samples of human dissected temporomandibular joints.

Over the years several researchers have investigated the anatomy and physiology of temporomandibular joint (TMJ) components, in order to increase knowledge about TMJ disorders (TMD). The literature mainly dealt with anatomical, radiological and electromyographic research orientations. A careful analysis of the studies, leads to the consideration that there is still no general agreement about the function of the different components of TMJ. In particular, there is disagreement for what concerns the physiology of the lateral pterygoid muscle and the presence of the capsular lateral ligament (1-9).

The purpose of the following anatomical and histological research is to bring our contribution about the knowledge on the articular capsule and the anatomical structures close to it.

dimensions of about 7x7 cm, were performed in foreign countries. The autopsy specimens were preserved in 10% formalin. On each sample a careful microdissection was carried out from the outside towards the inside until getting the isolation of the articular capsule and condyle-meniscus-lateral pterygoid muscle triad. Afterwards each sample was subjected to a decalcifying process based on formic acid. Hereinafter, each decalcified dissection has been split into sections of about 4 mm in thickness, along the sagittal or frontal planes, which were subsequently embedded in paraffin.

The serial 4 mm samples were themselves sectioned into “slices” of about 5 microns in thickness, by means of which histological slides were set with different staining techniques: hematoxylin-eosin, Van Gieson, Mallory, Von Kossa-Paragon and Orcein stains.

RESULTS

MATERIALS AND METHODS

Anatomical dissections of 20 human left TMJ with

Macroscopic observations

On the lateral surface of the articular capsule, in

Key words: Temporomandibular joint (TMJ), TMJ anatomy and histology, lateral pterygoid muscle

Mailing address:

Liliana Ottria,
Department of Clinical Science and Translational Medicine,
University of Rome “Tor Vergata”,
Via Montpellier 1, 00133 Rome, Italy
Tel.: +39 06 20900268
e-mail: lilianaottria@gmail.com

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all samples, it has been possible to expose a small but robust whitish and translucent structure, made up of compact tissue bundles oriented along their major axis. We have identified that structure in the lateral capsular ligament. This anatomical entity originates from the upper posterolateral portion of the meniscus, and inserts down onto the anterolateral portion of the condylar head. On the anterior and medial surface of the samples, we noticed the close relationship between the lateral pterygoid muscle and the capsular complex and their anatomical continuity. In addition, during the dissection maneuvers, we observed how fiber residues of temporal and masseter muscles, are intertwined with fibers of the lateral pterygoid muscle and constitute, with them, the anterior and anterolateral portion of the articular capsule.

After releasing the capsule in the anterior part, the lateral capsular ligament and medial collateral ligament, much less representative, are clearly shown. Then, by pulling forward the whole meniscus-condyle-lateral pterygoid muscle complex, it was possible to observe the posterior ligament.

In serial sagittal sections (Fig. 1), perpendicular to the major condylar axis, it has not been possible to show any cleavage sulcus between the upper and lower head of the lateral pterygoid muscle. In fact, in correspondence of its insertion, it appears as a single bundle of muscle fibers, with direct attack on the anterior part of the meniscus, and on the condylar fovea. At this level, a prevalence of condylar fibers over the meniscal fibers has been noticed.

Behind the condyle-meniscus complex it was well highlighted the rear attachment on the meniscus by the posterior ligament. The serial frontal sections, parallel to the major condylar axis, have confirmed the presence of the lateral capsular ligament while, in correspondence of the medial pole, in addition to the exiguous collateral ligament, we have noticed a high prevalence of muscle fibers.

Microscopic observations

The analysis of histological slides, prepared with trichrome stain, confirmed the above macroscopic observations. At the sagittal level (Fig. 2), the



Fig. 1. *Sagittal section of TMJ (macroscopic aspect) the slide shows all the TMJ's main structures: temporal bone, articular disc, TMJ bilaminar zone, lateral pterygoid muscle.*

following was observed: anteriorly, the merger of the two heads of the lateral pterygoid muscle, their anatomical continuity with the joint capsule and the direct insertion of the muscle fibers on the meniscus and the condyle. Posteriorly, the attachment of the posterior meniscal ligament and in the bilaminar area, a high prevalence of elastic fibers over the collagen fibers.

The frontal sections have confirmed the presence, at lateral level, of collagen fiber bundles regularly oriented with each other in order to constitute the lateral capsular ligament.

In correspondence of the medial pole, histologic appearance looked different, because of a smaller number of collagen fiber bundles (collateral ligament) and a high prevalence of muscle fibers attack.

DISCUSSION

The conclusions that can be drawn from previous anatomical and histological observations lead to different insights about the physiology of the

different articular components.

In agreement with those reported by other authors (3), for what concerns the lateral pterygoid muscle, the lack of distinction within two classic insertion heads remove the idea of considering these muscle groups as independent or antagonists. Moreover, our study was concordant with others in showing the presence of temporal, masseterine and lateral pterygoid muscle fibers intertwined at the level of the anterior part and the anterior-lateral capsule (9); it suggests as electromyographic needle recordings, carried out in this area, could possibly record action potentials not only of a single muscle, but more continuous muscular structures. This resizes as well the value of EMG recording itself.

Our thought relates in part to those studies in which the lateral pterygoid muscle is described as divided into two different bellies with distinct functions (10, 11), giving this concept a different interpretation: the two heads are distinct only because they are activated according to different mechanical requirements, related to the various vector forces (Fig. 3). Our



Fig. 2. *Sagittal section of TMJ (histological staining Von Kossa-Paragon) the slide shows the TMJ's main structures, furthermore the section clearly shows the maxillary artery and the auriculo-temporal nerve in position lower than condyle, and a part of the parotid gland.*



Fig. 3. *Sagittal section of TMJ (macroscopic aspect) the slide shows the TMJ's complex without the temporal bone, and in agreement with latest literature, it clearly shows the lateral pterygoid muscle as a multipennate muscle.*

interpretations are in agreement with the study of Bernal-Mañas CM et coll. (2016) that describe the lateral pterygoid muscle as a multipennate muscle (12).

Observations on the presence of the lateral capsular ligament provide a glimpse of it as an important role in stabilizing the meniscus-condyle, for TMJ functional efficiency protection (2, 4, 6, 13). Moreover, in correspondence of the lateral pole, no muscle fibers were found, with the possible destabilizing action; the muscle fibers were instead found in the medial pole.

The presence of a large number of elastic fibers at the level of the posterior ligament indicates its low importance in stabilizing the meniscus on the condyle. It is likely that its function could be the role of sweet antagonist to the tone of the lateral pterygoid muscle fibers.

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ANATOMIC RELATIONSHIP BETWEEN TEMPOROMANDIBULAR JOINT AND MIDDLE EAR

L. OTTRIA^{1*}, D. LAURITANO^{2*}, F. GUZZO³, M. GARGARI³ and A. BARLATTANI¹

¹*Department of Clinical Science and Translational Medicine, University of Rome "Tor Vergata", Rome, Italy;* ²*Department of Medicine and Surgery, University of Milan-Bicocca, Monza, Italy;*

³*Department of Dentistry "Fra G.B. Orsenigo-Ospedale San Pietro F.B.F.", Rome, Italy*

*these authors contributed equally to this work and they are co-first authors

The present study was designed to identify a relationship between temporomandibular joint and tympanic cavity and potentially justify the possible clinical correlations between these two anatomical entities. For this reason the authors conducted an anatomic-pathological study about the temporomandibular joint (TMJ) and the neighboring anatomical areas of the middle ear by autopsy finds of human adults.

Malleus (hammer), incus (anvil), mandible and sphenomandibular ligament (SML) all originate from the first branchial arch as shown by embryological studies. The morphogenesis of the discomalleolar ligament and its relationship with the sphenomandibular ligament were studied in human embryos and fetuses, on histological grounds, and shows us that the discomalleolar ligament is an embryological continuation of the sheath of the lateral pterygoid muscle. The sphenomandibular ligament derives from Meckel's cartilage (1).

The sphenomandibular ligament is a relatively wide band; it develops from the lingula on the medial surface of the ramus of the mandible and it is fixed superiorly to the spine of sphenoid bone, lateral to the foramen ovale. Actually, the sphenomandibular ligament seems to contact the malleus through the middle ear.

This last segment can be considered as a part of another ligament, which appears to define the spatial relationship between the temporomandibular joint (TMJ) and the middle ear. This ligament, known as mandibular ligament of the malleus and not considered by the majority of the texts, was discovered by Pinto. He defined it as a connective structure fixed to the neck of the malleus developed lateral to the chorda tympani nerve (2).

Pinto described a "tiny ligament" which passes through the tympano squamosa fissure downwards and sideways to be inserted into the middle and postero-superior portion of the capsule and meniscus of the temporomandibular joint and the sphenomandibular ligament. The discomalleolar ligament (Pinto's ligament or DML) is not described in the anatomy textbooks but was demonstrated by Pinto and others authors.

Burch and Coleman, have dealt with the possible

Key words: discomalleolar ligament, Pinto's ligament, sphenomandibular ligament, TMJ, middle ear

Mailing address:

Liliana Ottria,
Department of Clinical Science and Translational Medicine,
University of Rome "Tor Vergata", Via Montpellier 1,
00133 Rome, Italy
Tel.: +39 06 20900268
e-mail: lilianaottria@gmail.com

oto-menisal relations (3, 4). In disagreement with Pinto, Coleman claims that he has never observed a direct continuity of retro-discal fibres on the sphenomandibular ligament, because “tiny ligament” is composed only of retro-discal fibres of the malleus anterior ligament (4). Therefore, it is very improbable that the shifting of the disc and the condyle during the motion in adults’ mandible can produce a movement of the malleus, as defined by Pinto (5, 6).

In an anatomic and endoscopic study, Rowicki and Zarkrzewska (7) observed that tension applied to the discomalleolar ligament resulted in movement of the malleus in three specimens on fourteen adult human temporomandibular joint. The DML looks like a band of connective tissue of flat shape and triangular lying on the horizontal plane. The basis of this triangle is in continuity with the portion posteromedial of the disc and of the joint capsule. This ligament passes through the Civinini canal laterally to the anterior malleolar ligament (AML) and lateral to the chorda tympani nerve. The histological study of Mérida-Velasco JR and coll. shows that the extratympanic portion of the discomalleolar ligament (DML) is an intrinsic ligament of the TMJ, composed of collagen fibres and abundant elastic fibres (7), and the authors propose that this ligament could act as a tensor of the synovial membrane in movements of the TMJ.

The study results of Loughner BA and coll. (8) indicate that the anterior malleolar ligament (AML) via the sphenomandibular ligament (SML) has the potential to cause middle ear damage and is more likely to do so than the DML.

Jonck LM (9) with his study presented a possible anatomical explanation for the middle ear symptoms related to disturbance of the temporomandibular joint.

In the macroscopical study of Rodriguez-Vazquez JF and coll. (10), the discomalleolar ligament should be considered as an intrinsic ligament of the temporo-mandibular joint and, and its morphology and anatomical arrangement, should be distinguished from the tympanic portion of the sphenomandibular ligament or anterior ligament of the malleus.

According to the studies of Arai and Sato (11) the DML is stretched by the tension of the retro disc tissue during the opening of the mouth, because

the Tenascin c (elastic protein that connects to fibronectin) and Fibronectin were found to a large extent arranged along the collagen fibers of DML without being present elsewhere in this region.

On the bases of these contrasting researches, both anatomic and functional points of view, the authors found it appropriate to re-examine these structures in the adults in order to be able to identify and to evidence the possible relationship between TMJ and the tympanic cavity.

MATERIALS AND METHODS

Micro-dissections on fifteen blocks of human left TMJ (7x7 cm) have been carried out in foreign countries. The anatomic-pathological study aimed to highlight sequentially the components of the TMJ and the neighboring anatomical areas of the middle ear.

RESULTS

In the current study, among the fifteen TMJ examined, only in two cases was the continuity of the sphenomandibular ligament with the “tiny ligament” described by Pinto confirmed (Fig. 1).

In other six autopsy results, the presence of a part of “tiny ligament” was found which did not present continuity with the sphenomandibular ligament, even though both connected in the periosteum of the petrotympanic fissure. This absence of continuity has been highlighted stretching and extending the sphenomandibular ligament in the sagittal sections and noticing the immobility of the malleus and the tympanic complex.

In the remaining cases, no continuity has been revealed between TMJ and tympanic cavity, even if a bone gap was present which converged from the tympanic cavity and tapered towards the TMJ in not constant location.

DISCUSSION

The current study, in agreement with data reported in the literature by several authors, shows that the discomalleolar ligament (DML), which in fetuses extends from the posterior medial part of the



Fig. 1. Block of human TMJ, the section open as a book shows the prior continuity of sphenomandibular ligament (**SML**) with discomalleolar ligament (**Pinto's ligament or DML**), and the proximity between TMJ and the auditory and cochlear system.

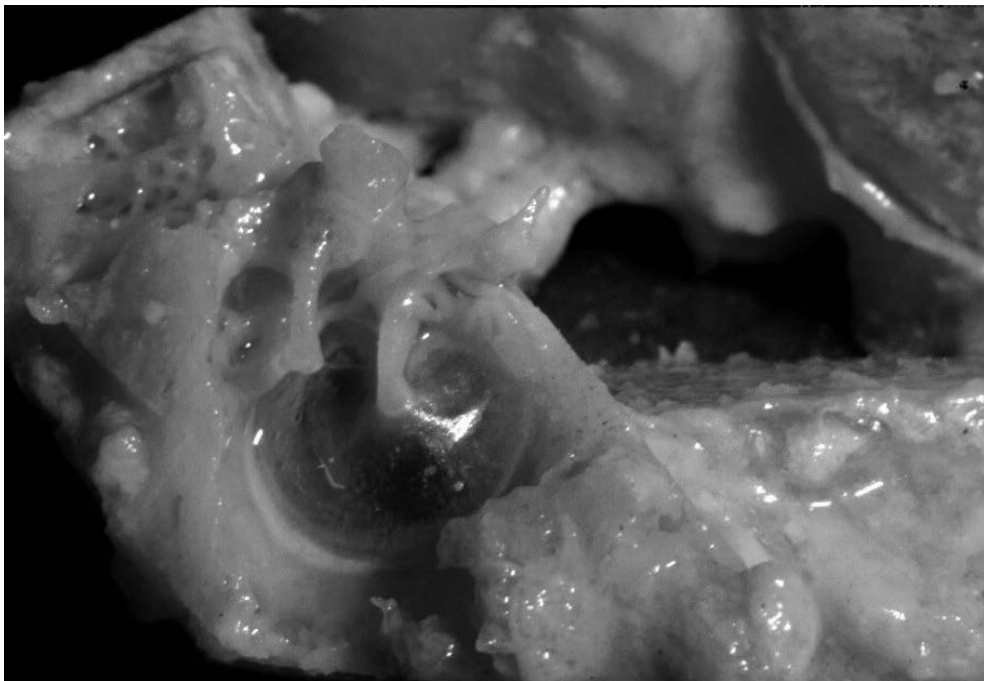


Fig. 2. Block of human TMJ, relationship between TMJ and middle ear (**detail**), sectioned discomalleolar ligament (**Pinto's ligament or DML**).

disc and is attached to the malleus (12, 13), often disappears in adults.

On the bases of the revealed data, we can conclude that Pinto's "tiny ligament" exists, but it is only rarely present as an anatomical and functional entity in some individuals and not as Pinto had supported, in all individuals. Therefore, it is difficult to state that this tympanic-mandibular connection, even when it exists, may be responsible for the onset or continuation of a possible auditory system pathology.

Further studies are necessary to determine any anatomical and physiological correlations that may exist between the TMJ and the auditory and cochlear system (Fig. 2).

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TMJ'S CAPSULE HISTOLOGICAL AND MACROSCOPICAL STUDY: RELATIONSHIP BETWEEN LIGAMENTOUS LAXITY AND TMJ DYSFUNCTIONS

L. OTTRIA^{1*}, V. CANDOTTO^{2*}, F. GUZZO³, M. GARGARI³ and A. BARLATTANI¹

¹*Department of Clinical Science and Translational Medicine, University of Rome "Tor Vergata", Rome, Italy;* ²*Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy;* ³*Department of Dentistry "Fra G.B. Orsenigo-Ospedale San Pietro F.B.F.", Rome, Italy*

*these authors contributed equally to this work and they are co-first authors

In this study, the authors examined the capsular structures of 20 human temporomandibular joints (TMJ) macroscopically and microscopically, in order to improve knowledge of these structures, as part of their possible participation in the genesis of TMJ dysfunctions.

For a better comprehension of what the effects and symptoms are of internal derangements related to the temporomandibular joint (TMJ), we need a deep understanding of the anatomy and of the individual joint components' physiology.

In this regard, the study of the articular capsule structure is of particular interest. Several researchers have already studied these structures, but not all the results agree (1-9). For example, in the anatomical and histological studies of W.P. Savalle, the capsule of 16 post-mortem human specimens was examined both anatomically and histologically. A distinguishable ligamentous formation in the lateral part of the capsule was observed only in three specimens and only in one of these, the distinguishable formation had morphological characteristics of tendinous tissue. This author indicates caution with too generalized opinions about temporomandibular morphology and therefore suggests to consider the existence of a lateral ligament (1).

However, a significant number of different studies, not only confirm the presence of such a

structure, but give it also an important role regarding the stabilization of the meniscus on the condyle and the relative biomechanical restrictions for the TMJ efficiency (2, 3). Regarding the lateral pterygoid muscle (LMP), and its possible physiol-pathological function, several authors reported a distinct separation in superior and inferior heads (10-12); several other studies documented its insertion on condyle and meniscus (13-17). The alteration of the clinical symptoms in the patients with anterior disk displacement with non-reduction (ADDnr) might be associated with the pathological situations and symptoms of the LPM (13).

In this research the authors, as part of a larger anatomical and histological study, wanted to observe the macroscopic and microscopic aspect of 20 human TMJ's capsules, in order to determine if the pathology related to the displacements of the articular meniscus may be in part attributable to a capsular ligamentous laxity or whether such laxity could be not the cause but rather, one of the effects of this pathology.

Key words: TMJ, TMJ capsule, ligamentous laxity, TMJ dysfunction

Mailing address:

Liliana Ottria,
Department of Clinical Science and Translational Medicine,
University of Rome "Tor Vergata", Via Montpellier 1,
00133 Rome, Italy
Tel.: +39 06 20900268
e-mail: lilianaottria@gmail.com

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MATERIAL AND METHODS

Anatomical dissections of 20 human left TMJs (dimensions: 7×7 cm) were performed in foreign countries. The autopsy specimens were stored in 10% formalin. On all samples, an accurate microdissection was performed from the outside towards the inside, up to the isolation of the joint capsule. A decalcification process by formic acid was then run on each sample.

Successively, each decalcified dissection was divided into sections of about 4 mm thickness, along the sagittal or frontal planes and the sections were embedded in paraffin. The 4mm-serial samples were then sectioned into slices of about 5 microns thickness and histological slides were prepared with different staining techniques (hematoxylin-eosin, Van Gieson trichrome, Mallory, Masson, orcein).

RESULTS

The macroscopic analysis of the lateral surface of the joint capsule showed, on all samples, a small whitish translucent structure consisting of small compact parallel bundles of tissue orientated along the long axis of the same structure. We identified such anatomical entity in the lateral capsular ligament, which originates from the upper posterolateral portion of the meniscus and fit down the anterolateral portion of the condylar head.

On the anterior medial surface of the samples, it was possible to note the anatomical continuity of the lateral pterygoid muscle with the capsular complex. On the frontal and lateral surface, temporal and masseterine muscle fibers combine with pterygoid fibers to form the joint capsule. The anterior opening of the capsular compartment has well evidenced the lateral ligamentous structure and on the medial surface, it has been possible to highlight the presence of another ligament, although less representative and numerous muscle fibers too. The endurance offered by this structure to a cranial traction of articular meniscus primarily confirmed their biomechanical relevance, meaning a condyle-meniscal stabilization. Particularly after joint section, the lateral capsular ligament showed us great traction resistance (Fig. 1).

It became possible to observe the posterior ligament after further release of articular capsule in

its anterior segment and after stretching the condyle-meniscus-LMP complex forward. On serial sagittal sections oriented at right angles to condylar long-axis, no separation in superior and inferior heads were noticed in lateral pterygoid muscle, contrary to reports from several authors (10-12). In fact, samples analyzed revealed the fusion of two muscular heads, with condylar insertions probably predominating over meniscal ones. Serial coronal sections, parallel oriented to condylar long-axis, confirmed the presence of capsular lateral ligament and above-mentioned muscular insertions. Histological slides were stained as follows: HE (hematoxylin-eosin), Van Gieson trichrome, Mallory, Masson, orcein.

The histological analysis of the slides prepared by trichrome-staining confirm these previous anatomical observations. On sagittal plane, the following characters have been pointed out: anteriorly and medially, whole fusion of external pterygoid muscle heads with one another and with articular capsule

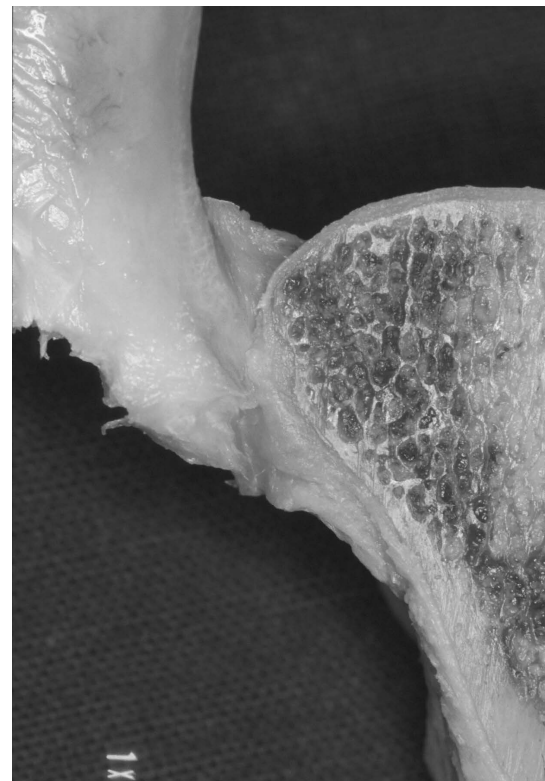


Fig. 1. Block of human TMJ. In the macroscopic analysis, after capsule's preliminary section, the lateral capsular ligament shows us its great traction resistance.

and muscular fibers directly inserting on condyle and meniscus. Posteriorly, in bilaminar zone, high prevalence of elastic fibers (as demonstrated by orcein's stain) over collagen ones (Fig. 2). On lateral histological coronal sections, a significant number of collagen fiber bundles well oriented with one another, to constitute lateral capsular ligament were noticed, while medially collagen fiber bundles in less representative proportion than in lateral anatomy, and significant predominance of muscular fibers were observed (Fig. 3).

DISCUSSION

Interpretations of above-mentioned histo-anatomical observations led us to the following considerations. Communal insertion of lateral pterygoid muscle on condyle and meniscus, with no distinction between two heads (18), refutes the concept that superior and inferior muscular bundles



Fig. 2. Block of human TMJ (histological sagittal section). Orcein's stain in bilaminar zone indicates high prevalence of elastic fibers over collagen ones.



Fig. 3. Block of human TMJ (histological coronal sections). The slide stained Van Giesontrichromic, shows medially collagen fiber bundles in less representative proportion than in lateral anatomy, and significant predominance of muscular fibers.

can be activated not in synergy and independently. Rather, its spatial and anatomical disposition inspires the concept that lateral pterygoid muscle simply operates according to biomechanical requests, with two different strength-vectors.

The low proportion of collagen fibers in posterior ligament, the abundant capillary plexus and the large number of elastic fibers reveal its scarce capacity to bear tractions and destabilize meniscus on condylar neck.

The presence of lateral capsular ligament and the large number of its collagen fiber bundles, well oriented along its major axis, confirm its significance in condylus-meniscal stabilization, ensuring dynamic efficiency of TMJ functionality.

A structural modification in this anatomical region certainly could promote or cause preternatural movements of meniscus on condyle and consequently trigger some feasible articular malfunction. This in agreement with Hirsch et al., in which the authors

analyzed whether the capsular laxity in agreement with generalized joint hypermobility (GJH), could be a risk factor for temporomandibular disorders (TMD). For this purpose, they examined 895 subjects (20-60 yr of age) in a population-based cross-sectional sample, the results were that associations between GJH and reproducible reciprocal clicking or limited mouth opening were statistically significant and in conclusion, GJH was found to be associated with non-painful subtypes of TMD (19).

Unfortunately our anatomic-pathological observations can provide quantitative information (regarding to collagen fibers), but not qualitative information, which could highlight potential structural and metabolic modifications of connective tissue, and so help us to answer our starting question in the first part of our research. Therefore, it is to be hoped that further studies examine articular capsule in its structural, biochemical and metabolic perspectives.

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HYPERBARIC OXYGEN THERAPY (HBOT) AND PERIODONTAL HEALTH

L. OTTRIA^{1*}, L. TETTAMANTI^{2*}, M. GARGARI³, G. VALENTE⁴,
V. PACINI⁴ and C. COSTANZO⁴

¹Department of Clinical Science and Translational Medicine, University of Rome "Tor Vergata", Rome, Italy; ²Department of Medicine and Surgery, University of Insubria, Varese, Italy; ³Department of Dentistry "Fra G.B. Orsenigo-Ospedale San Pietro F.B.F.", Rome, Italy; ⁴Roman Hyperbaric Center, Rome, Italy

*these authors contributed equally to this work and they are co-first authors

This study has tested the effects of hyperbaric oxygen in periodontal structures in agreement with the theories supported by literature research. Eight patients, from 30 to 50 years-of-age, were tested with pure oxygen inhalation, at the 2.5 ATA absolute pressure. Main approved tests of periodontal health were evaluated before and after HBOT's cycles. The results in all patients treated with HBOT, have founded clear improvement of clinical and instrumental parameters.

Hyperbaric oxygen therapy (HBOT) has many uses. The Undersea and Hyperbaric Medical Society sets standards for the use of hyperbaric medicine, currently there are several indications for hyperbaric oxygen therapy in medicine and many more unapproved conditions where HBOT may be useful.

The surrounding ambient pressure at sea level is 1 ATM, each additional ATM of pressure is equal to 33 feet of depth of sea water or 14.7 pounds per square inch (psi). HBOT consists of placing a patient into a pressurized chamber of up to 3 atmospheres (ATM) of pressure, in which the elevated pressure combined with exposure to 100% oxygen has many physiologic effects on the body. The normal oxygen concentration of plasma at sea level is 3 mL/L, instead at the level at a pressure of 3 ATM breathing 100% oxygen, it approaches 60 mL/L.

The literature shows that this is enough to keep swine alive after all of their red blood cells have been removed and is sufficient to supply the resting

oxygen requirement for most tissues. HBOT can potentiate antibiotics (such as aminoglycosides and quinolones), and at higher concentrations it can be bacteriostatic and bactericidal. HBOT also neutralizes alfa exotoxins, produced by bacteria such as Clostridium. Hyperbaric oxygen therapy promotes neutrophil mediated bacterial killing ability in hypoxic tissue, it also prevents the release of proteases and free radicals in certain injuries, thereby decreasing vasoconstriction, edema, and cellular damage (1).

The conditions approved by the Undersea and Hyperbaric Medical Society for hyperbaric oxygen therapy include (2):

1. decompression sickness
2. acute arterial gas emboli
3. necrotizing fasciitis
4. gas gangrene
5. refractory osteomyelitis
6. acute blood loss anemia

Key words: Hyperbaric Oxygen Therapy (HBOT), periodontal health, oxygen, hyperbaric medicine

Mailing address:

Dr Liliana Ottria,
Department of Clinical Science and Translational Medicine,
University of Rome "Tor Vergata", Via Montpellier 1,
00133 Rome, Italy
Tel.: +39 06 20900268
e-mail: lilianaottria@gmail.com

7. failed skin grafts
8. chronic radiation injury
9. carbon monoxide poisoning
10. acute thermal injury
11. compartment syndromes
12. compression injury
13. cranial abscess
14. arterial insufficiency
15. acute sensory hearing loss

There are numerous off label indications such as autism, stroke and attention deficit hyperactivity disorder (ADHD) that did not respond clearly to hyperbaric oxygen therapy. A specific indication for HBOT is in cases of Wagner grade 3 diabetic wounds refractory to at least 30 days of conventional therapy (2).

Most contraindications to hyperbaric oxygen therapy are related to issues of barotrauma. Barotrauma is physical injury to an area where there is a closed air filled space such as the lung or middle ear. Injury results from expansion of trapped gases with the changing pressure from the hyperbaric dive resulting in rupture of the compartment (perforation of a tympanic membrane or pneumothorax) (1).

Cakmak T. et al. think that, additionally to these concerns, barotraumatic lesions can occur in any trapped air e.g. dental abscess after failed filling or in periodontitis and subgingival anaerobes that may cause gaseous lesions. These lesions can cause a very strong sharp pain particularly in the ascent phase of the hyperbaric oxygen therapy when the pressure decreases and volume begins to re-expanse (3).

More relative contraindications are the presence of pulmonary blebs and emphysema with carbon dioxide retention. Administration of certain drugs is a relative contraindication (Disulfiram, Cisplatinum, Sulfamylon, Bleomycin). Sinusitis, seizures, pregnancy, implanted devices such as pacemakers and epidural pumps are relative contraindications. Claustrophobia, especially in cases of the monoplace chamber, can be an issue. The only true absolute contraindication is an untreated pneumothorax. Diving a patient in this situation will lead to fast progression of a tension pneumothorax and certain death.

In reference to theoretical aspects supported by

literature research, the study has tested the effects of hyperbaric oxygen in periodontal structures through a specific experimentation in order to confirm what is explained in literary texts.

Nogueira-Filho et al, evaluate the effect of hyperbaric oxygen therapy (HBO2) as an adjunct to scaling and root planing (SRP) in the treatment of severe cases of chronic periodontitis. Their data suggest that hyperbaric oxygen therapy had a short-term beneficial effect on pocket reduction and bacterial elimination, and may be considered a potential adjunct therapeutic option to improve the clinical outcomes of scaling in severe cases of chronic periodontitis (4).

In our research, the hygienic maintenance of the dental arches was intentionally not improved during the whole research period with other additional treatments, this to test only HBOT's effects by clinical and instrumental data.

MATERIALS AND METHODS

For our research, we selected eight divers in training, non-smokers, with a hygienic maintenance of the dental arches that was intentionally not improved during the whole research period. The informed consent was signed by all the participants prior to the study.

The trial was randomized, controlled and double-blind. Eight subjects without periodontal disease (5 males

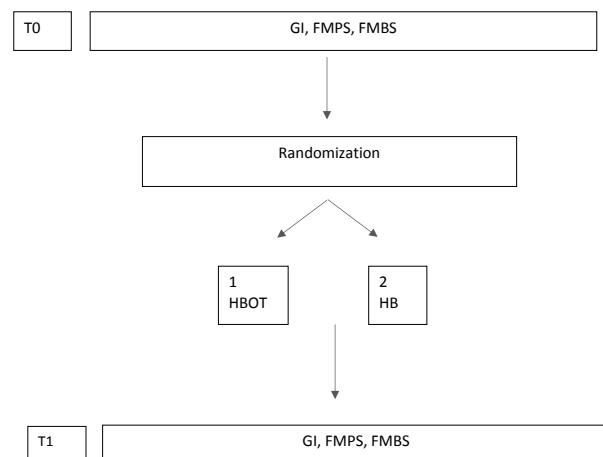


Fig. 1. Flow-chart of the study.

and 3 females) aged 29-50 years, were randomly divided into two groups based on oxygen hyperbaric therapy (HBOT): a groups treated with 100% oxygen, and a group without oxygen (HB placebo).

Inclusion criteria for patients:

Patients with following characteristics:

- at least 20 teeth, to exclusion of 3rd molars
- PSR with values of 1 or 2
- PPD $\leq$ 3 mm

Exclusion criteria:

Patients with following characteristics:

- Last 6 months < periodontal treatment
- Patients with systemic diseases or any contraindications to HBOT
- Presence of orthodontic appliances
- Patients with periodontal disease PPD > 3 mm
- 6 months taking antibiotics <
- Taking 3 months < anti-inflammatory, cortisone
- Pregnant or taking contraceptive hormones
- The absence of consent to the study

In the first group the oxygen hyperbaric therapy was carried out with pure oxygen inhalation, at the 2.5 ATA absolute pressure, through rubber gold-nasal mask, and making the patient breathe with open mouth in order to get advantage from both the indirect oxygen action by endogenous, and the direct action in perfusion contact, on the exposed gum tissues. The treatment provided a 25 +/- 5 cycle of HBOT sessions, according to the scheme:

Table I. Data at T0.

Data at T0	Patient	GI	FMPS %	FMBS %
Group 1 - HBOT	1	1.6	65	59
	2	2	99	76
	3	1.6	73	54
	4	1.9	100	69
Group 2 - HB (Placebo)	1	1.7	88	60
	2	1.4	82	45
	3	1.6	69	65
	4	1.7	55	63

Table I. Data at T1.

Data at T1	Patient	GI	FMPS %	FMBS %
Group 1- HBOT	1	1.3	62	32
	2	1.5	92	47
	3	1.2	74	19
	4	1.5	97	48
Group 2- HB (Placebo)	1	1.5	90	51
	2	1.3	78	32
	3	1.4	67	52
	4	1.5	43	39

30' - 3' break time - 30'. In the second group, the dive was carried out without inhalation of pure oxygen, but always with rubber gold-nasal mask.

Clinical parameters were assessed at baseline (T0) up to 6 months (T1) for the 2 groups (5-10): Gingival Index (GI), Full Mouth Plaque Score (FMPS) (11), Full Mouth Bleeding Score (FMBS) (6). All tests were repeated after a few days at the end of hyperbaric treatment and achieved data. In order to get a simple and comfortable reading, data has been reported on a folder specially created for this research. Each patient was tested by the same operator.

RESULTS

The clinical data collected during the first visit (T0) of the subjects of the various groups, are collected in the table I. The clinical data collected during the last visit (T1) of the subjects of the various groups, are collected in the table II.

As shown in Tables I and II, in all patients treated with HBOT we found a massive improvement of both clinical and instrumental parameters. From a clinical point of view, we found a significant improvement of the gingival health with an important recovery of the mucosa tropism, highlighted especially from the decreasing of bleeding. Indeed instrumental analysis have shown in all patients a significant reduction of

Full Mouth Bleeding Score. No essential increase or decrease of the plaque index has been found, this in agreement hygienic maintenance of the dental arches that was intentionally not improved during the research period.

Because of this whole series of improvements at gingival mucosa's tropism and capillary fragility, in all cases we found an increase of periodontal health.

DISCUSSION

The analysis of the obtained results from this study shows several interesting data. After the application of our protocol of 25 +/- 5 HBOT sessions a clear improvement of the clinical parameters related to gingival tropism has been shown. In the improvement of the gingival mucosa tropism, we can mention the capillary neoangiogenesis due to oxygen "plasma", and anti-inflammatory effects (1).

The clinical efficacy relative to the bactericidal and bacteriostatic action can be explained through the mechanism of oxygen action itself. Furthermore, we supposed that the important improvement of data is also connected to the sequential HOB effect carried on the neocollagen synthesis in the periodontal structures.

In agreement with the neo synthesis action, the effects of daily exposure to HBO on the proliferation and differentiation of human osteoblasts *in vitro* were also evaluated; the data suggest that the exposure of osteoblasts to HBO enhances differentiation toward the osteogenic phenotype, providing cellular evidence of the potential application of HBO in fracture healing and bone regeneration (12).

CONCLUSION

Based on all the considerations previously exposed, we can be optimistic about the possibilities offered by the HBO therapy, as future support for the traditional treatment of periodontal disease.

In fact, with this study we do not want to reach a dogmatic result. Rather, we would like to stimulate further dentistry research into good results achieved by the hyperbaric oxygen therapy in the treatment of other diseases. We are also motivated by the

excellent clinical feedback and by the non-invasive nature of this therapy.

In conclusion, hyperbaric oxygen therapy provides the most tissues with vessels, which have good blood flow, and the anatomical structure of the mouth with its rich vascular beds has the advantage to benefit from this treatment. Therefore, this study supports the idea of using this treatment mode in dentistry. However, even if the data agree in utilizing HBOT in dentistry, the proposed work is to be considered a pilot study given the small sample size. To obtain more statistically significant data, it is important to conduct further studies with a larger sample to determine the HBOT's clinical and instrumental benefits in dentistry.

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FDPS FINISH LINE DESIGN AND TOOTH PREPARATION TECHNIQUE

L. OTTRIA^{1*}, L. TETTAMANTI^{2*}, F. GUZZO³, M. GARGARI³ and A. BARLATTANI¹

¹Department of Clinical Science and Translational Medicine, University of Rome "Tor Vergata", Rome, Italy; ²Department of Medicine and Surgery, University of Insubria, Varese, Italy; ³Department of Dentistry "Fra G.B. Orsenigo-Ospedale San Pietro F.B.F.", Rome, Italy

*these authors contributed equally to this work and they are co-first authors.

The purpose of this research is to perform and verify the "modified 50° preparation" suggested by the authors. This procedure was performed with a scrupulous standardization of the tooth's preparation and then of the laboratory techniques to produce a metal-ceramic crown, and the same for the crown cementation technique of on the preparation of the original sample. After the esthetic evaluations, the obtained sample was included in EPON resin and sectioned in the lingual-vestibular sense. The sections were then observed with an optical microscope in different magnifications for the final evaluations. The geometric design proposed presents the advantages of two great preparation techniques: 50° preparation and circumferential shoulder. The 50° bevel designed in first phase of preparation and the second phase of the circumferential 27° shoulder, together allowed to satisfy the following requirements: aesthetic, marginal accuracy, periodontal compliance, conservation and stability. However, this type of prosthetic preparation is not the only one, but it is one of the marginal designs in prosthetic dentistry.

To ensure the correct housing of the prosthetic restorations in fixed prosthesis, it is necessary to proceed with a procedure known as "preparation" (1-3), meaning a careful removal of the hard tooth tissues first.

This arrangement enables the morphology of the element - after reduction - to provide the following requisites (4, 5): economy of the dental substance (6), respect of the integrity of the adjacent tissues (endodontic and periodontal) (7), adequate restorative material thickness, retention and stability of the prosthetic product, precision of the preparation limits (8, 9). Moreover, further considerations are developed when there are indications for the complete covering of the dental element by a total veneer crown. In fact, in this type of restoration,

due to particular requests concerning the degree of stability, retention and aesthetics, the operator must place the margins of the preparation at the sulcular level (9).

This means that the working tools will inevitably have to follow the delicate and sophisticated biological system, which enables the merging of tooth and gingiva, commonly known as the "dento-gingival junction" procedure (9-11). Especially at this point, it is very important to get enough "space" during the preparation procedure, to provide the necessary thickness for the production of an adequate prosthetic device (9, 12, 13). This involves the execution of a cervical step, which can be provided with different shapes and orientation.

To date, numerous researches have been

Key words: fixed dental prosthesis (FDPs), tooth die, ceramo-metal crown, prosthodontics, geometric principles, finish line design

Mailing address:

Dr Liliana Ottria,

Department of Clinical Science and Translational Medicine,

University of Rome "Tor Vergata", Via Montpellier 1,

00133 Rome, Italy

Tel.: +39 06 20900268

e-mail: lilianaottria@gmail.com

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carried out in relation to the different types of marginal preparation, in an attempt to satisfy basic requirements such as aesthetics, stability, retention and complete bio-mechanical incorporation of the prosthetic restoration (4, 5). The awareness of the advantages and disadvantages of each type of marginal design proposed, which includes the mandatory need to use mixed preparation techniques, motivated us to conduct our experimental research looking for a verification of a new preparation method, supported by the related drawings of marginal geometry (9).

To better understand the following work a good knowledge of the following topics is required, which we will refer to specific texts and lectures: emerging anatomy, over and under-contour, marginal fitting, marginal discrepancy, marginal distortion, knife preparation, shoulder, chamfer, 50 degrees, shoulder with bevel, chamfer with bevel (9, 14-16).

The only one topic we would like to briefly discuss is the "bevelling technique". In prosthetic restoration the advantages due to the presence of a bevelled margin are the reduction of the amount of exposed cement and the increase of stability and retention (17-19).

On the other hand, the presence of a metal edge and the clinical difficulty of a correct bevelling execution constitutes a major disadvantage. In fact, we should remember that there are technological limits in obtaining a bevelled metal corner. In fact, it has been shown that initial bevelling angle must be around 30°, and thickness of the molten edge cannot be less than 24 microns (20). Therefore, it is not possible to reproduce bevels with an angle of less than 30° otherwise, they will remain incomplete causing restoration during the trial or cementation step. The clinical problem of bevelling with an angle equal or greater than 30° has motivated our work (21). We are proposing a new design of marginal geometry, with a bevel in the first step of prosthetic preparation. The design is submitted in a modified 50° preparation.

The modified 50° preparation

The marginal geometry designs previously described, even if always usable, do not offer a total

reliability, as the achievement of certain targets rules out others. The 90° shoulder preparation as an expression of the highest stability values, correspond to the greater space available for the cement and margins. The bevelling, which greatly improves the parameters of marginal closure, but inevitably represents a cause of unlightliness. The Chamfer, which although aesthetic, is noticeably predisposed to a marginal distortion and finally, the aesthetic preparation at 50°, which however leads to problems in the creation of the framework and to distortion at the margin levels (22). The theoretical modified 50° preparation experimented in this study, proposes a marginal geometry design that allows the realization of prosthetic restorations with adequate stability and at the same time good aesthetics parameters.

The assumption that leads to a modification of the 50° preparation have been inspired by the validity of this marginal configuration and from the research carried out in an attempt to improve it. The concreteness of the results obtained is reflected in the chance of using a marginal geometry design, which can simultaneously express the positive qualities of both the circumferential shoulder and the 50° inclined plane. From the operational point of view, the innovation introduced in the execution of the marginal geometry design, includes a first step in which the use of a milling cutter for 50° preparations generates the final marginal 50° bevel, angulated in respect to the longitudinal axis of the tooth (Fig. 1a). The achievement of a geometric configuration of the margins, typical of the preparation devised by Kuwata (22), is very clear at the end of this step.

In the second phase of preparation, in the middle of the inclined plane at 50°, a circumferential shoulder plane is carried out with an angle of 27°, using and taking advantage of the special dental drill conceived for this preparation. In this way, the residual portion of the inclined 50° plane (carried out in the first step) will constitute the bevelling of the preparation. The construction of the bevelling is then performed in the first phase of preparation and the 27° shoulder plane in the second phase (Fig. 1a, b). The orientation of the 27° shoulder - obtained by removal during the second phase of the cutting procedure - is achieved working with the "number two" dental drill at half

thickness, and positioning the instrument parallel to the longitudinal axis of the tooth, with the tip of cutting in contact with the inclined 50° plane (Fig. 1a, b, Fig. 3c). If there is a considerable stability request - thanks to the special dental drill (1mm diameter) studied for this preparation – it's possible to use a circumferential shoulder with 90° angle during the second preparation step (Fig. 2b). The drills used for the execution of the described preparation are multi-blade tungsten carbide burs, which can be found at reference 550 (# 1-2-3) of the “Maillefer” catalog (Fig. 3a). Using these point drills with red ring multiplier (120,000 rpm) we achieve better control, in order to obtain an accurate finishing of the two floors, during both the first and second stages of preparation.

With the introduction of this new method for prosthetic preparation, we hope to achieve an ideal marginal geometry, which can unify the advantages related to both types of preparations (50°-27° or 50°-90°) and therefore matching the following five requirements:

- Esthetic, being able to make metal-ceramic crowns without exposure of metal and in any case positioning the margin at the intrasulcular level (23, 24).
- Marginal precision, since the closure on a sloping surface also offers guarantees of cementation (25, 26).
- Periodontal respect, in preparation (27, 28).
- Control of the quality of cervical reduction (12, 29).
- Stability and retention (12, 30, 31).

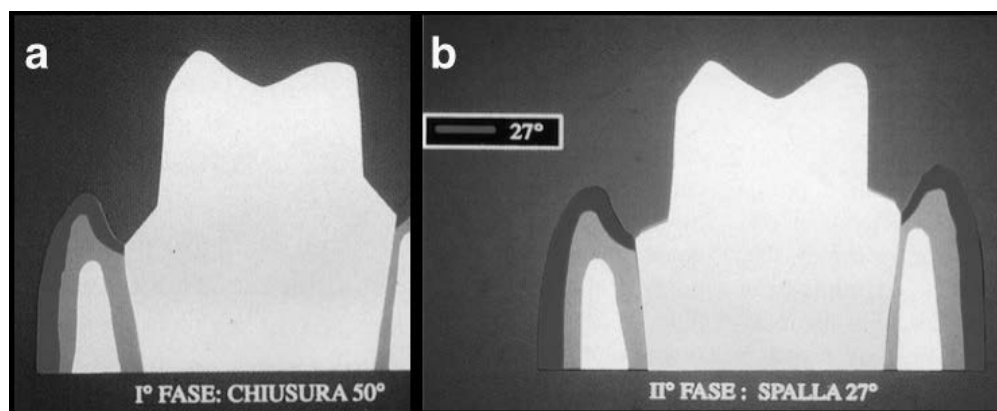
Experimental study

The purpose of this research is to perform and to verify the modified 50° preparation technique's effects, in relation to the reliable characteristics on the relative prosthetic reconstruction. The framework was performed with a scrupulous standardization of laboratory techniques, cemented on the original die. After the aesthetic evaluations, the obtained sample was included in EPON resin and sectioned in the vestibular-lingual sense. The sections were then observed under an optical microscope, at different magnifications for the final evaluations (32).

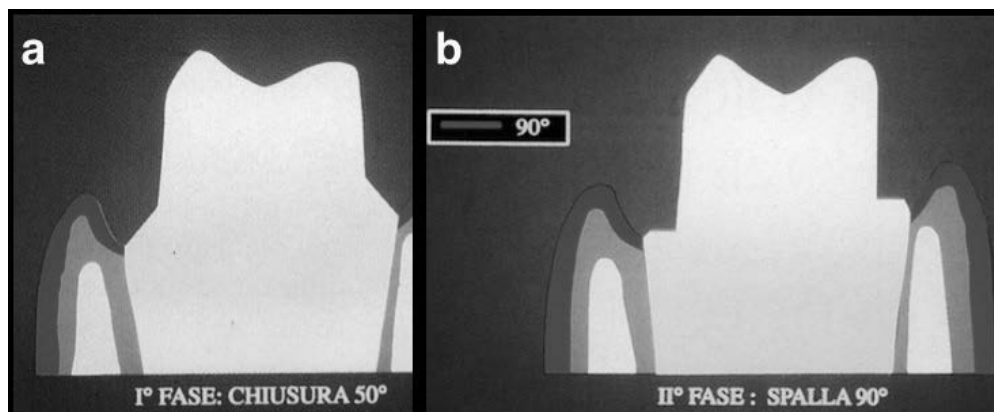
Sample preparation

For the realization of this study, a permanent human premolar has been used, extracted for orthodontic therapy and maintained in a relative 100% humidity. Tartar and organic material were removed from the coronal root by curettage. Subsequently the crown was reduced according to the modified 50° preparation technique described (two-step preparation) (Fig. 1a, b, Fig. 3b, c) using the tungsten carbide multi-tipped cutters (number 550 Maillefer) (Fig. 3a).

The impression of the tooth preparation was detected with additional silicone (Dei Rainbow Putty and Super light). After about 2 hours from setting time, the impression was developed in epoxy resin (Diemet-E ErKodent), scrupulously respecting



Figures from 1a/1b to 5a/5b “legend into the manuscript”

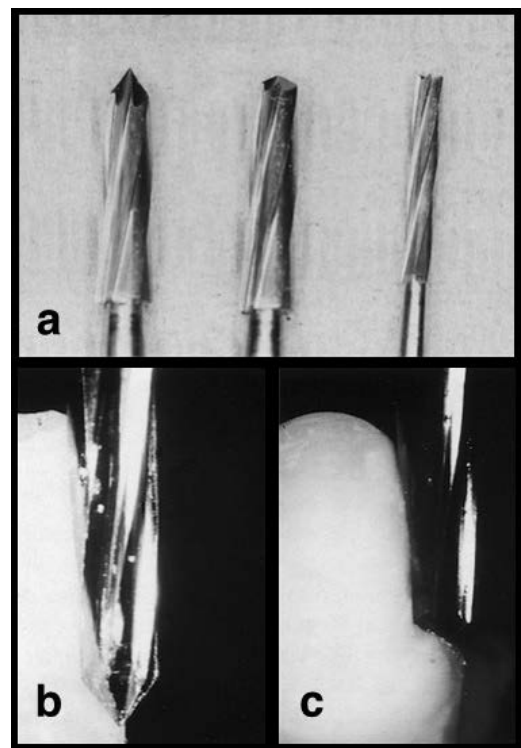


the indications of the manufacturer. After 8 h, the model was analysed under an optical microscope and the marginal finishing line was highlighted with a red wax pencil, always under microscopic control. Prior to waxing procedure, a total thickness of 25 microns was applied with spacers, 0.5 mm from the finishing line. Then, under microscopic vision, the wax modelling was performed, with particular attention to the waxing of the margin. After the wax framework coating (GC vest G), for the gold alloy casting, Shillmen Gold metal-ceramic was used which has excellent hardness and non-deformability characteristics. After pickling, the casting pin was removed, the framework repositioned and checked on the master model and on the tooth preparation.

Details of the finishing line of metal casting (Fig. 4a). After refinement, performed the ceramization (Vita Omega-Vita), the framework has been repositioned and controlled on the master model. Then cementation was carried out on the tooth die with zinc phosphate cement (Harvard cement). The sample was subjected to pressure for about 60 min. Magnification detail of the cementation sample for the evaluation of aesthetic and marginal precision (Fig. 4b). Then the specimen was included in EPON resin. The sections obtained were observed in reflected light, using the Zeiss OPM1 optical microscope at different magnifications (28).

RESULTS

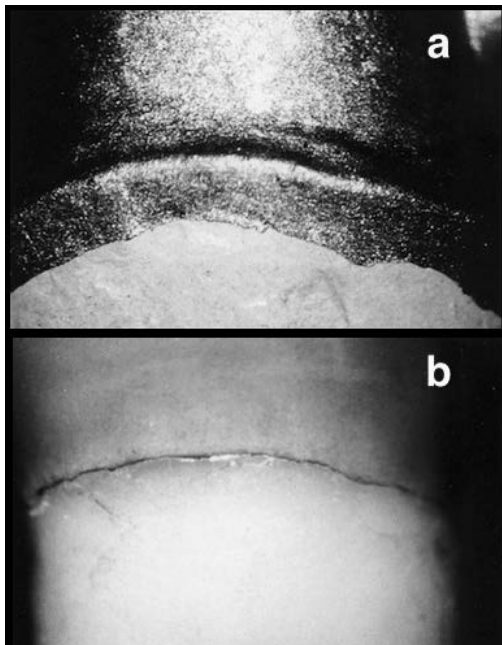
The image of the cemented crown (Fig. 4b)



distinctly shows a positive result on the possibilities of aesthetic expression of the modified 50° preparation. Fig. 4b illustrates the marginal closure, which indeed obtained a reduction to the minimum of the metal collar, thanks to the 50° bevel. Furthermore, Fig. 5a, b clearly reveal the principles of stability in relation to the marginal configuration proposed by authors. Fig. 5b shows a particular of the margin at the vestibular level, with a very good ratio marginal precision between prepared tooth-crown.

DISCUSSION

The analysis of dental and dental technician problems, led us to propose a new type of marginal geometry. The updated geometrical design we made, seems to merge together the advantages of two great techniques of preparation: 50° and circumferential shoulder. The experimental study supported by a bibliographic compilation, gives enough credit to this new intuition which is proved to be achievable. In fact, the preparation we proposed: modified 50°, was used to make a



gold-ceramic crown, cemented and analysed in terms of aesthetics and stability. The 50° bevel, in the first step of preparation, and the 27° shoulder in the second step, allowed to meet the following requirements:

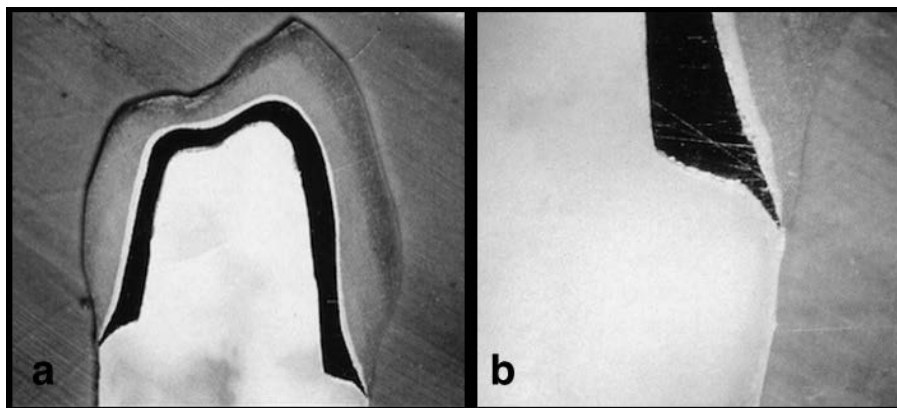
- Aesthetic: in relation to the possibility of making gold-ceramic crowns without metal exposure (Fig. 4b).
- Marginal precision: because the closure on a sloping surface is more precise, and it offers more guarantees after cementation (Fig. 4b, 5a, b).
- Periodontal compliance: in the preparation phase (Fig. 1a, 2a).
- Conservative: on the amount of cervical reduction (Fig. 5a, b).
- Stability: in relation to the possibility of housing on a circumferential support-plane (Fig. 5a).

However, this study does not want to propose or unify a single type of prosthetic preparation, but simply intends to introduce this marginal design into prosthetic dentistry practice.

Further research will be necessary and should include multiple analysis to confirm reliability of this new type of proposed tooth preparation.

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ZINC PLUS OCTENIDINE: A NEW FORMULATION FOR TREATING PERIODONTAL PATHOGENS. A SINGLE BLIND STUDY

D. LAURITANO^{1*}, V. CANDOTTO^{2*}, C.A. BIGNOZZI³, D. PAZZI³, F. CARINCI⁴, F. CURA⁵,
A. TAGLIABUE^{6**} and L. TETTAMANTI^{6**}

¹Department of Medicine and Surgery, University of Milan-Bicocca, Milan, Italy; ²Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy; ³Department of Chemical and Pharmaceutical Sciences, University of Ferrara, Ferrara, Italy; ⁴Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, Italy; ⁵Department of Medical Sciences, University of Ferrara, Ferrara, Italy; ⁶Department of Medicine and Surgery, University of Insubria, Varese, Italy

*these authors contributed equally to this work and they are co-first authors

**these authors contributed equally to this work and they are co-last authors

Periodontal treatment has the aim to reduce oral infection, and prevent the progression of the disease. The potential benefits of new chemical devices for periodontal therapy, include improved patient compliance, an easier access to periodontal pocket and a lower dosage of antimicrobial agent. The objective of this study was to explore the efficacy of a chemical device containing zinc and octenidine in the treatment of chronic periodontitis in adult patients. Ten patients with a diagnosis of chronic periodontitis (20 localized chronic periodontitis sites) in the age group of 35 to 55 were selected. None of these patients received any surgical or non-surgical periodontal therapy and demonstrated radiographic evidence of moderate bone loss. The chemical device zinc plus octenidine was used by each patient after daily oral hygiene. Microbial analysis were analyzed at baseline and on the 15th day. After the treatment, a remarkable decrease in bacteria amount, both for some species and for the total count was observed in the study group. Specifically *T. forsythia* and *T. denticola* were eradicated whereas Total Bacteria Loading and *Fusobacterium Nucleatum* showed a reduction of 38% and 55%, respectively. Our study demonstrated the efficacy of the new chemical device containing zinc and octenidine in a sustained release drug delivery system in the management of moderate to severe chronic periodontitis.

The term periodontal disease (PD) is generally used to describe diseases affecting the gums and tooth support tissues, causing damage to the connective tissue and alveolar bone (1). Specific bacteria of the biofilm formed by plaque cause PD. The bacteria leak into the periodontal ligament space, causing anaerobic

infection creating a cascade of events, which end with the production of inflammatory mediators and bacterial metabolites (1). PD affects about the 50% of adults, or more, while the severe form periodontitis affects around 10% (range: 5-20%) of adults and moderate periodontitis around 30%. In elderly people,

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Mailing address:

Prof. Angelo Tagliabue,
Department of Medicine and Surgery,
University of Insubria, Via Piatti, 10,
21100 Varese, Italy
Tel.: +39.0332-825625 - Fax: +39.0332-825655
e-mail: angelo.tagliabue@uninsubria.it

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the prevalence of PD is estimated about 70-90% of individual's aged 60-74 years (1).

Periodontal therapy is important to reduce local inflammation and bacteremias. Recently it has been stated that periodontal disease appear to increase the risk of cardiovascular disease, pulmonary disease, preterm and low birth weight (2).

The oral microbiota is constituted by a large number of bacteria species that form a biofilm. The biofilm includes both saprophites and potentially pathogenic species. Periodontal treatment has the aim to reduce oral infection, and prevent the progression of the disease³. Periodontal non-surgical therapy with scaling and root planing associated with a good level of domestic oral hygiene can prevent the onset of the disease and allow for proper maintenance of oral health (3, 4).

Good oral hygiene has the aim to control bacterial plaque, but when the patient's attention to oral hygiene decreases, it is possible to experience a recurrence of the disease. In addition, periodontal disease presents a characteristic trend in *pousse* with periods of remission and exacerbation of the disease. Support periodontal therapy is efficacy, but a better microbiological control of oral microbiota can be reached by the administration of topical antimicrobials in association.

The advantages of topical therapy involve the insertion of antimicrobials agents directly into the periodontal pocket, minimizing the adverse effects related to systemic therapy (4). The potential benefits of local drug delivery include improved patient compliance, an easier access to periodontal pocket and a lower dosage of the antimicrobial agent. The most commonly used methods for local drug delivery are topical drugs in gel form (5). The antimicrobial agents in the local drug delivery devices include tetracycline, ofloxacin, clindamycin, chlorhexidine, benzalkonium etc. (4)- and have been used either alone or as adjunct with support periodontal therapy. The local antimicrobials are administered directly into the periodontal pocket and their effectiveness is related to their bactericidal activity and to the subsequent reduction of gingival inflammation (6).

Octenidine has been extensively characterized and poses no risk in terms of acute, subchronic and chronic toxicity, irritation and allergenicity. It exhibits a broad

spectrum of antimicrobial efficacy against Gram-positive and Gram-negative bacteria and fungi and the efficacy is not adversely affected by interfering substances like blood and mucous. On skin, it shows a strong residual effect which can still be observed 24 h after application (7). Octenidine belongs to the bispyridinamines (8), a different class of chemicals with respect to chlorhexidine and benzalkonium and due to its excellent antimicrobial activity and tissue compatibility, has been chosen as a component of the new gel formulation.

Zinc (Zn) has been advocated since stimulates epithelialization of wounds and exhibits a number of beneficial antimicrobial effects. In *in vitro* studies, Zn inhibits a range of microbial enzyme systems, including a range of glycolytic enzymes in streptococci, with concomitant increased sensitivity of the organisms to acid (9). In two typical oral anaerobes, *Fusobacterium nucleatum* and *Prevotella intermedia*, a range of enzymatic processes are inhibited by Zn, including catabolism of amino acids, sugars and peptides, as well as oxidative metabolism (10). Zn can also deliver beneficial effects for bad breath: reducing breath concentrations of volatile sulphur compounds (VSCs) associated with halitosis, (11, 12) as well as have direct and indirect inhibitory effects on anaerobe mediated- VSC production.

The aim of this study was to investigate the efficacy a new hydroxypropyl-cellulose gel, containing zinc in association with octenidine, acting as a drug delivery system in the management of moderate to severe chronic periodontitis.

MATERIALS AND METHODS

Ten patients were randomly selected with a diagnosis of chronic periodontitis. Patients who qualified for the study were in the age group of 35-55 and did not receive any surgical or non-surgical periodontal therapy. The patients were excluded from the study if they meet any of the following criteria: (1) pregnancy; (2) a history of taking antibiotics or using anti bacterial mouth rinses for the past 6 months; (3) has teeth with furcation involvement; (4) patients with a history of smoking, and drug or alcohol abuse.

The subjects that volunteered to participate in the

study, signed consent forms and followed a detailed verbal description of the procedure.

Clinical methods

Twenty sites were selected from the 10 patients. Each selected site was subjected to microbial analysis. At the first visit, after recording the clinical parameters at each site in selected patients at baseline, microbiological samples were collected from pre-selected sites. At 15 days following the first visit, the patients were checked for any adverse reaction. Verbal instructions were given to avoid manipulating the study teeth.

At each visit (Baseline and 15th), the clinical parameters were assessed. A single trained and calibrated examiner who was blinded to the treatment method obtained all readings. Microbiological samples were collected from the sites of the patients at baseline and at the 15th day.

Clinical parameters were assessed sequentially and the pocket depth, the distance between the base of the pocket and gingival margin, measured. For the collection of subgingival samples, the sites were isolated using cotton rolls. Sterile absorbable paper points (size 60) were used for the collection of subgingival samples and were immediately transferred to microbiological laboratory for processing. The microorganisms processed were the three bacterial species more involved in periodontitis that constitute the red complex group: *Porphyromonas gingivalis*, *Tannerella forsythia*, and

Treponema denticola.

Several methods have been used for microbiological testing in periodontitis (13). However, many techniques have not been fully accepted due to low sensitivity or specificity, moreover sometimes they are slow, expensive and laborious. In this study we have used the (LAB-test®) developed by the LAB corporation (Ferrara, Italy), which is rapid and sensitive, allowing to detect and quantify the three bacterial species more involved in periodontitis that constituted the red complex group: *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*. Both *P. gingivalis* and *T. denticola* occur concomitantly with the clinical signs of periodontal destruction (14-22). They appear closely 'linked' topologically in the developing biofilm and are considered the first pathogens involved in the clinical destruction of periodontal tissues (23-28).

Real-Time Polymerase Chain Reaction

Primers and probes oligonucleotides have been designed based on 16S rRNA gene sequences of the Human Oral Microbiome Database (HOMD 16S rRNA RefSeq Version 10.1) counting 845 entries. All the sequences were aligned in order to find either consensus sequences. For each sample, two real-time polymerase chain reaction (PCR) runs were performed. The first reaction quantified the total amount of bacteria using two degenerate primers and a single probe, matching a highly conserved sequence of the 16S ribosomal RNA gene. The second reaction detected and quantified the three red complex bacteria in a multiplex PCR. The reaction included a total of six primers and three probes that were highly specific for each specie. Oligonucleotide concentrations and PCR conditions were optimized to ensure sensitivity, specificity and no inhibitions in case of unbalanced target amounts. Absolute quantification assays were performed by using the Applied Biosystems 7500 Sequence Detection System. The amplification profile was initiated by a 10 min incubation period 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. All the experiments were performed including nontemplate controls to exclude reagents contamination (29-32).

Plasmids containing synthetic DNA target sequences (Eurofin MWG Operon, Ebersberg Germany) were used as a standard for the quantitative analysis. Standard curves for each target were constructed in a triplex reaction, by using a mix of the same amount of plasmids, in serial

Table I. Bacterial count using PCR at T0 (beginning) and T1 (end of treatment).

	Number of cases	Minimum	Maximum
Total Bacterial Loading T0	10	2796	53080
Total Bacterial Loading T1	10	1813	20422
Fusobacterium Nucleatum T0	10	0	1320
Fusobacterium Nucleatum T1	10	0	593
Treponema Denticola T0	10	0	149
Treponema Denticola T1	10	0	0
Tannerella Forsythia T0	10	0	152
Tannerella Forsythia T1	10	0	0

dilutions ranging from 10^1 to 10^7 copies. There was a linear relationship between the threshold cycle values plotted against the log of the copy number over the entire range of dilutions. The copy numbers for individual plasmid preparations was estimated using a Thermo NanoDrop spectrophotometer.

The absolute quantification of total bacterial genome copies in samples allowed for the calculation of the relative amount of red complex species. To prevent samples and polymerase chain reaction contamination, plasmid purification and handling were performed in a separate laboratory with dedicated pipettes.

RESULTS

After the treatment with the zinc-octenidine gel, a remarkable decrease in total bacteria amount and in the individual microbic species was observed in the study group. Specifically *T. forsythia* and *T. denticola* were eradicated whereas the Total Bacteria Loading and *Fusobacterium Nucleatum* showed a percentage reduction of 38% and 55%, respectively (Table I). The zinc-octenidine gel did not show any side effects and was not observed to cause discomfort or to produce adverse reactions in time. No patient reported pain, burning, tingling sensation or numbness. The gel was declared to be tasteless and odorless. Pocket depth at baseline and 15th day was not influenced by the use of the zinc-octenidine gel.

Despite the spread of the data, preventing a correct statistical evaluation, the results obtained clearly demonstrated a reduction of microbial concentration after treatment with the gel.

DISCUSSION

The oral microbiota is constituted by a large number of bacteria species that form a biofilm. The biofilm includes both saprophytes and potentially pathogenic species. It is well understood that most destructive types of periodontal diseases occur due to the presence of pathogenic microorganisms colonizing the subgingival area and the suppression or eradication of these microbes result in improvement in periodontal health. The oral cavity is suitable for invasion of many microorganisms. Mechanical

debridement is effective in both splitting the biofilm and reducing the bacterial load. However, the effects of mechanical instrumentation may be improved with the association of a chemical device, providing an additional benefit in controlling the progress of disease.

Local delivery of chemical devices into the pocket achieves a greater local concentration of the drug, proving bactericidal for most periopathogens, and at the same time, exhibiting negligible impact on the microflora residing in other parts of the body (33).

Our study evaluated the efficacy of a hydroxypropyl-cellulose gel containing zinc in association with octenidine as a sustained release drug delivery system in the management of moderate to severe chronic periodontitis.

The results of this investigation demonstrated an overall improvement in bacterial loading at various time intervals. A new dimension was added to this investigation by microbiological examination of bacterial morphotypes using periodontal tests. It was thought appropriate to evaluate the effect of the zinc-octenidine gel on subgingival microbial population, the primary etiological factor for periodontitis. The results demonstrated a marked reduction in total number of organisms in each patient from baseline to 15 days.

A significant reduction was observed in the number of "red complex" bacteria consistent with the antimicrobial activity of the gel. In a previous *in vitro* study (34) we have demonstrated that zinc plus octenidine inhibits the growth of several bacterial species. Gram-positive organisms appear to be more sensitive to zinc-octenidine than Gram-negative bacteria. In particular, the gel is effective against *Streptococcus* groups A, *Staphylococcus aureus*, *Streptococcus* group B, *Escherichia coli*, *Klebsiella* sp., *Enterobacter* sp., *Proteus* sp., *Pseudomonas aeruginosa*, *Enterococcus* sp. The zinc-octenidine gel also showed antimicrobial activity against aerobic and anaerobic pathogens and inhibition of *S. aureus* growth *in vitro* and *in vivo* (34).

There is currently a trend in the increased use of antimicrobial agents. The effectiveness of antimicrobial therapies depends on host defence mechanisms and virulence factors. Further research,

with relatively larger sample size and longer follow-up period, will be performed to better validate the efficacy of the zinc- octenedine gel as an effective local drug delivery agent in periodontal therapy.

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THE ROLE OF ZINC PLUS OCTENIDINE IN THE REGULATION OF GENE EXPRESSION: AN *IN VITRO* STUDY

D. LAURITANO^{1*}, V. CANDOTTO^{2*}, C.A. BIGNOZZI³, D. PAZZI³, F. CARINCI⁴, F. CURA⁵,
A. TAGLIABUE^{6**} and L. TETTAMANTI^{6**}

¹*Department of Medicine and Surgery, University of Milan-Bicocca, Milan, Italy;* ²*Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy;* ³*Department of Chemical and Pharmaceutical Sciences, University of Ferrara, Ferrara, Italy;* ⁴*Department of Morphology, Surgery and Experimental Medicine, University of Ferrara, Ferrara, Italy;* ⁵*Department of Medical Sciences, University of Ferrara, Ferrara, Italy;* ⁶*Department of Medicine and Surgery, University of Insubria, Varese, Italy*

*these authors contributed equally to this work and they are co-first authors

**these authors contributed equally to this work and they are co-last authors

Zinc was known in ancient times, and is diffused in the environment. The potential benefits offered by zinc supplementary therapy have been demonstrated in numerous clinical trials using oral or topical zinc products. The benefit of zinc can be in principle increased through association with other actives. The aim of this study is to evaluate the effect on primary human gingival fibroblast cell of a new formulation containing zinc and octenidine cations. Human gingival fibroblast cells were obtained from three healthy patients (14-year-old man, 15-year-old woman and 20-year-old man) during extraction of teeth. The gene expression of 14 genes (ELANE, FN1, FBN, ITGA1, HAS1, ELN, DSP, ITGB1, HYAL1, TGFB1, TGFB2, TGFB3, TGFBR1 and TGFBR2) was investigated in HGF cell culture treated with 80µM of Octenidine, 1000µM of Zinc, 80µM Octenidine + Zinc solution and the medium alone at 30 min. PrestoBlue™ data showed that as the active concentration increases (Octenidine, Zinc and Octenidine + Zinc) the percentage of cell vitality compared to that of untreated cells decrease. In this study, no statistically significant gene expression was observed between cells, treated with difference substances, and control cells. Our results points out that zinc plus octenidine shows a positive potential in periodontal disease treatment.

The human mouth is colonised by microbioma, composed by hundreds of species selected from several thousand species found across human populations. The oral microbioma is in relationship with specific oral habitats (1-5). In spite of different genetic patterns, the oral microbioma remains broadly similar across human populations. This stability is the result from the interaction of the entire

oral microbioma and the metabolic interactions between bacteria. Environmental changes often allow the increase of opportunistic microorganism, resulting in conditions such as oral candidiasis (6-12). With these exceptions, the microbioma usually exists in a benign and stable relationship with the host. However, the oral microbioma is also the cause of the most common chronic diseases affecting

Key words: Periodontitis, peri-implantitis, zinc, oral biology, molecular biology

Mailing address:

Prof. Angelo Tagliabue,
Department of Medicine and Surgery,
University of Insubria, Via Piatti 10,
21100 Varese, Italy
Tel.: +39.0332-825625 - Fax: +39.0332-825655
e-mail: angelo.tagliabue@uninsubria.it

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humans, such as dental caries, gingivitis, periodontal disease and peri-implantitis (13-20).

The specific microbioma causing gingivitis remains unclear, however it is known that excessive accumulation of dental plaque produces gingivitis. Periodontitis while started by bacteria also appears to be linked to immune-inflammatory.

Gingivitis is prevented or treated by domestic oral hygiene. The effect of tooth brushing rests on the mechanical removal of dental plaque, with this process assisted by toothpaste formulations containing excipients such as abrasives and surfactants. However, mechanical removal is not sufficient, as a consequence the incorporation of additional antimicrobial actives in toothpastes as an adjunct to mechanical plaque control has been proposed (21).

Zinc (Zn) has been advocated based on a number of beneficial antimicrobial effects. In *in vitro* studies, Zn inhibits a range of microbial enzyme systems, including a range of glycolytic enzymes in streptococci, with concomitant increased sensitivity of the organisms to acid (22). In two typical oral anaerobes, *Fusobacterium nucleatum* and *Prevotella intermedia*, a range of enzymatic processes are inhibited by Zn, including catabolism of amino acids, sugars and peptides, as well as oxidative metabolism (23). Zn can also deliver beneficial effects for bad breath: reducing breath concentrations of volatile sulphur compounds (VSCs) associated with halitosis (24, 25) as well as have direct (26) and indirect inhibitory effects on anaerobe mediated- VSC production.

In this paper we describe the effect on primary human gingival fibroblast cell (HGF) of a new liquid formulation composed by zinc and octenidine (ZO).

MATERIALS AND METHODS

Primary human gingival fibroblast cell (HGF) culture

Human gingival fibroblast cells were obtained from three healthy patients (a 14-year-old man, a 15-year-old woman and a 20-year-old man) during extraction of teeth. The gingival pieces were fragmented with a scalpel and transferred in culture dishes containing Dulbecco's modified Eagle medium (DMEM, Sigma-Aldrich, Inc.,

St. Louis, Mo) supplemented with 20% fetal calf serum (FBS) and antibiotics, i.e. penicillin 100U/ml and streptomycin 100mg/ml (Sigma Aldrich, Inc.).

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 gingival tissue were removed from the culture dishes. Cells were harvested after additional 24h of incubation.

Cell viability test

Stock solutions of Octenidine, Zinc and Octenidine + Zinc 0.2% were prepared. Further dilutions were made with the culture medium to the desired concentrations just before use. HGF cells were seeded into 96-well plates at a density of 10⁴ cells per well, in 100µl of cell culture medium, and incubated for 24h to allow cell adherence.

Serial diluted solutions containing Octenidine, Zinc and Octenidine + Zinc (10µM, 50µM, 150µM, 200µM, 3000µM, 500µM, 1000µM, 1500µM) were added (three wells for each concentration). The cell culture medium alone was used as negative control. After 30 minutes, 3h and 24h of incubation, cell viability was measured using PrestoBlue™ Reagent Protocol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, the PrestoBlue™ solution (10µl) was added into each well containing 90µl of the treatment solution.

Plates were then placed back into the incubator for 1h, after that absorbance was measured at the wavelength of 570 nm by an automated microplate reader (Sunrise™, Tecan Trading AG, Switzerland). The percentage of viable cells was determined by comparing the average absorbance in treated wells with the average absorbance in control wells. The results are presented as the mean ± standard deviation of three measures only for 30 minutes of treatment because at 3h and 24h of treatment the percentage of living cell was less than 60% for all the different tracts.

Cells treatment

Once determined the most appropriate concentration for the treatments, HGF cells were seeded at a density of 1.0 x 10⁵ cells/ml into 9cm² (3ml) wells. Cells were washed two times with PBS and incubated for 18h at 37 °C with serum-free DMEM.

After serum starvation, cells were treated with the following solution: 80µM of Octenidine, 1000µM of

Zinc, 80µM Octenidine + Zinc solution and medium alone for 30 min.

All solutions were obtained in DMEM supplemented with 2% FBS, antibiotics and aminoacids. For each treatment three biological replicates were performed. The cells were maintained in a humidified atmosphere of 5% CO₂ at 37°C. At the end of the exposure time cells were trypsinized and processed for RNA extraction.

RNA Isolation and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The total RNA was isolated using GenElute™ Mammalian Total RNA Miniprep kit (Sigma-Aldrich, Inc., St. Louis, Mo), following the 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. *Real Time Polymerase Chain Reaction (Real Time PCR) and statistical analysis*

The cDNA was amplified by Real Time PCR. The amplification was performed by using Power SYBR[®] Green PCR Master Mix (Applied Biosystems, Foster City, CA), and the specific assay was designed for the investigated genes. SYBR assay reactions were performed in a 20µl volume using the ABI PRISM 7500 (Applied Biosystems). Each reaction contained 10µl 2X Power SYBR[®] Green PCR Master Mix (Applied Biosystems), 400nM concentration of each primer and cDNA.

All experiments performed included non-template controls to exclude contamination of reagents. PCR was performed with two biological replicates.

Expression was quantified using Real Time PCR. The

Table I. Primers sequences for SYBR[®] Green assay.

Gene symbol	Gene name	Primer sequence (5' 3') →
DSP	<i>Homo sapiens</i> desmoplakin	F - ATGACCTGAGGAGAGGACGAA R - AGGCTCTCTCTTCTGTACCAC
ELN	<i>Homo sapiens</i> elastin	F - CTAATACGGTCTGCTGGC R - CATGGGATGGGGTTACAAAG
HAS1	<i>Homo sapiens</i> hyaluronan synthase 1	F - CTCGGAGATTCGGTGGACTA R - CGCTGATGCAGGATACACAG
ELANE	<i>Homo sapiens</i> elastase, neutrophil expressed	F - CTACGACCCCGTAACTTGCT R - CCTCACGAGAGTGCAGACGTT
HYAL1	<i>Homo sapiens</i> hyaluronoglucosaminidase 1	F - ACAGATGTATGTGCAACACCG R - AAGGGCCCCAGTGTAGTGTC
FN1	<i>Homo sapiens</i> fibronectin 1	F - CCATAGCTGGAAGTGTTTTG R - CCAGTAAATCTACCATCATCC
FBN1	<i>Homo sapiens</i> fibrillin 1	F - AAAGATTGATGAGTGTGC R - GTATGGTGTGGGTAAATCC
ITGA1	<i>Homo sapiens</i> integrin subunit alpha 1	F - CAGGTTGGAATGTACAGTATG R - TGCTATTCCAAGAGCTGTC
ITGB1	<i>Homo sapiens</i> integrin subunit beta 1	F - ATTCCCTTCTCAGAAAGTC R - TTTTCTCCATTTCCCTG
TGFB1	<i>Homo sapiens</i> transforming growth factor beta 1	F - AACCCACAACGAAATCTATG R - CTTTAACTTGAGCTCAGC
TGFB2	<i>Homo sapiens</i> transforming growth factor beta 2	F - AGATTTCAGGTATTGATGG R - ATTTCTAAAGCAATAGGCCG
TGFB3	<i>Homo sapiens</i> transforming growth factor beta 3	F - TGTTGAGAAGAGAGTCCAAC R - ATCACCTCGTAATGTTTC
TGFBRI	<i>Homo sapiens</i> transforming growth factor beta receptor 1	F - AGACAATGGTACTTGGACTC R - GTACCAACAATCTCCATGTG
TGFBRI2	<i>Homo sapiens</i> transforming growth factor beta receptor 2	F - GGAGAAAAGATGACGAGAAC R - AGATGATGTTGTCATTGCAC
RPL13	<i>Homo sapiens</i> ribosomal protein L13	F - ATTCACAAGAAGGGAGACAG R - GAAATCTCTCTTCTCCTCAGTG

F: forward; R: reverse.

gene expression levels were normalized to the expression of the housekeeping gene Homo sapiens ribosomal protein L13 (RPL13). Mean expression levels of treated cells were calculated as fold changes relative to the expression of untreated cells with the delta/delta calculation method (27-31). Forward and reverse primers for the selected genes were designed using primer express software (Applied Biosystems), and are listed in Table I.

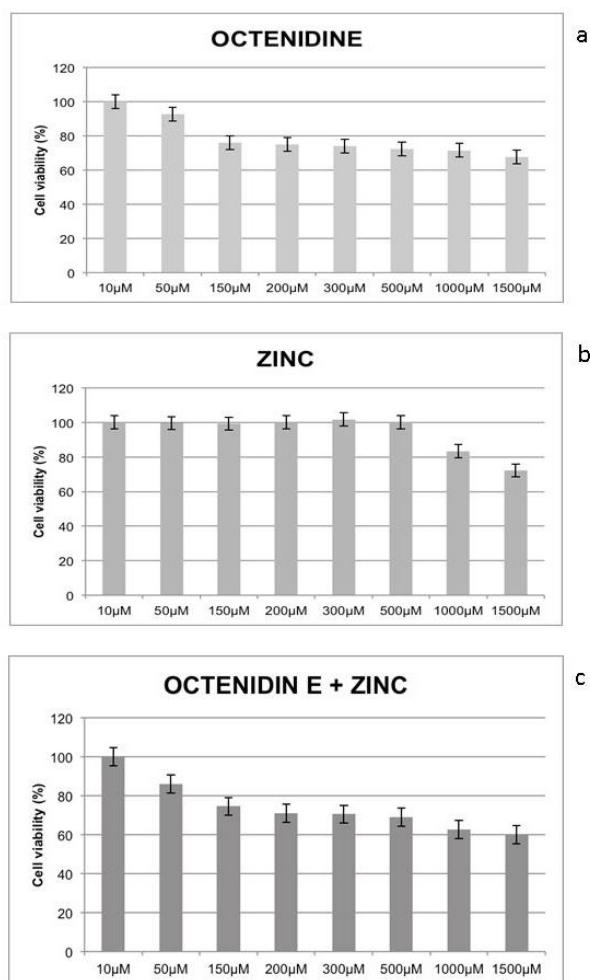


Fig. 1. Cell viability of Octenidine (a); Zinc (b); Octenidine + Zinc (c) solution in HGF cell line assessed by PrestoBlue™ Reagent Protocol.

Cells were treated with different concentrations of Octenidine, Zinc and Octenidine + Zinc solution (10 μM, 50 μM, 150 μM, 200 μM, 300 μM, 500 μM, 1000 μM, 1500 μM) for 30 min.

RESULTS

PrestoBlue™ data showed that as the concentration of actives increases (Octenidine, Zinc and Octenidine + Zinc 0.2%) the percentage of cell vitality compared to that of untreated cells decreases (Fig. 1).

The gene expression of 14 genes (ELANE, FN1, FBN, ITGA1, HAS1, ELN, DSP, ITGB1, HYAL1, TGFB1, TGFB2, TGFB3, TGFBR1 and TGFBR2) was investigated in HGF cell culture treated with 80 μM of Octenidine, 1000 μM of Zinc, 80 μM Octenidine + Zinc solution and medium alone at 30 minutes (Fig. 2-4).

In this study, no statistically significant gene expression was observed between cells treated with different substances, and control cells.

DISCUSSION

Zn is known from ancient times, and is diffused in the environment. Zn is second only to iron in being the most abundant trace element in the human body, and its importance in metabolic processes was evident only in the 1960s, following reports of zinc-responsive growth failure in infants in rural Egypt and Iran (32).

The potential benefits offered by Zn supplementary therapy have been demonstrated in numerous clinical trials using oral or topical zinc therapy. In keeping with the early studies on supplementary Zn therapy, evidence is now available to show that not only Zn is beneficial in the healing profile but that it provides an effective level of anti-infective action.

These roles of Zn are of importance for maintaining biological homeostasis, at both the whole organism and cellular levels. Zn has demonstrated a central role in a number of important disease processes, in fact human genome association studies reveal a strong association of a specific allele of the LC30A8 (solute carrier 30A8) gene with Type 2 diabetes mellitus (33, 34), and results of recent functional studies corroborate this association (35, 36). A role for Zn in the pathology of Alzheimer's disease has been longer-established, supported by many lines of evidence, including the accumulation of Zn in Alzheimer's disease cortex (37) and the

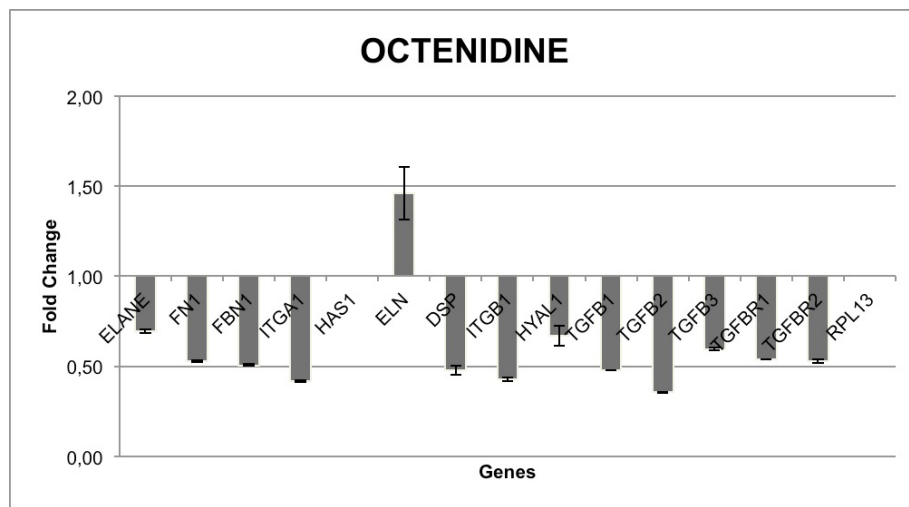


Fig. 2. Gene expression profile of HGF treated cells (80µM of Octenidine) at 30 min. SYBR[®] Green assay. *ELANE* = 0.69; *FN1* = 0.53; *FBN1* = 0.51; *ITGA1* = 0.42; *HAS1* = 1.00; *ELN* = 1.46; *DSP* = 0.48; *ITGB1* = 0.43; *HYAL1* = 0.67; *TGFB1* = 0.48; *TGFB2* = 0.35; *TGFB3* = 0.59; *TGFB1* = 0.54; *TGFB2* = 0.53; *RPL13* = 1.00.

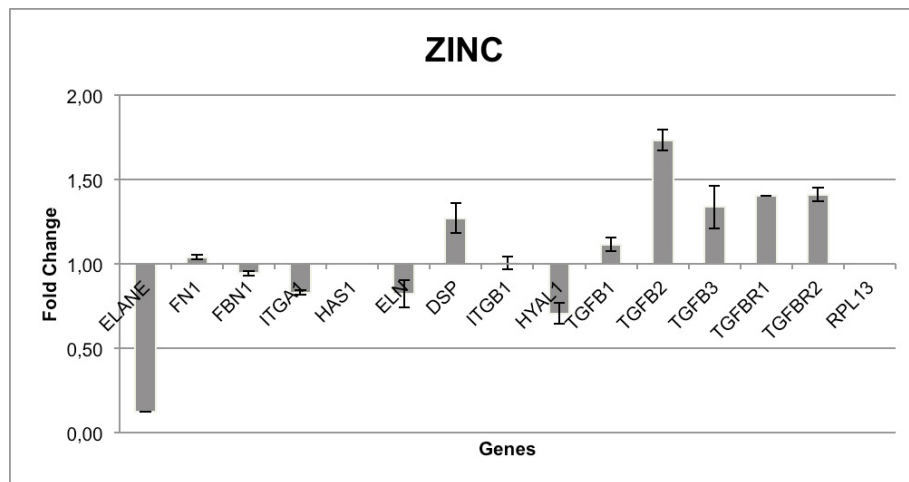


Fig. 3. Gene expression profile of HGF treated cells (1000µM of Zinc) at 30 min. SYBR[®] Green assay. *ELANE* = 0.12; *FN1* = 1.04; *FBN1* = 0.95; *ITGA1* = 0.83; *HAS1* = 1.00; *ELN* = 0.82; *DSP* = 1.27; *ITGB1* = 1.00; *HYAL1* = 0.71; *TGFB1* = 1.12; *TGFB2* = 1.73; *TGFB3* = 1.34; *TGFB1* = 1.41; *TGFB2* = 1.41; *RPL13* = 1.00.

observation that perturbation of Zn homeostasis, either pharmacologically or through diet (38), affects disease symptoms in mouse models. Toothpastes or solutions formulated to contain zinc were tested for antibacterial activity. Previous studies tested the efficacy of ZO as an antimicrobial composition. The results obtained demonstrated that ZO exerts a substantial and beneficial chemotactic effect on the

macrophages and neutrophils (39).

In this work, we evaluated the *in vitro* effect of ZO on fibroblasts behaviour in term of cell viability, survival, growth and collagen production.

Our PrestoBlue[™] data showed that as the concentration of substances, Octenidine, Zinc and Octenidine + Zinc 0.2%, increases the percentage of cell vitality compared to that of untreated cells

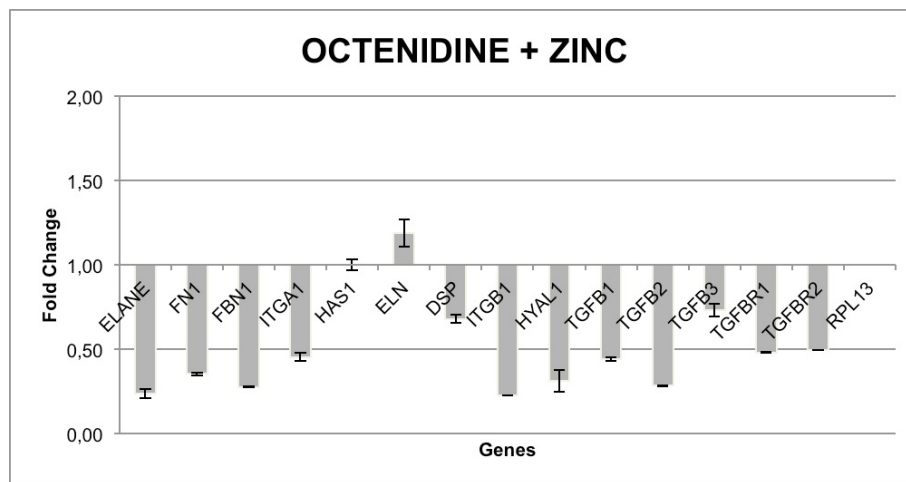


Fig. 4. Gene expression profile of HGF treated cells ($80\mu\text{M}$ of Octenidine + Zinc) at 30 min. SYBR[®] Green assay. *ELANE* = 0.23; *FNI* = 0.35; *FBN1* = 0.28; *ITGA1* = 0.45; *HAS1* = 1.00; *ELN* = 1.19; *DSP* = 0.68; *ITGB1* = 0.23; *HYAL1* = 0.31; *TGFB1* = 0.44; *TGFB2* = 0.28; *TGFB3* = 0.73; *TGFBR1* = 0.48; *TGFBR2* = 0.50; *RPL13* = 1.00.

decreases.

ZO inhibits the growth of several bacterial species. Gram-positive organisms appear to be more sensitive to ZO than Gram-negative bacteria. In particular it is effective against *Streptococcus* groups A, *Staphylococcus aureus*, *Streptococcus* group B, *Escherichia coli*, *Klebsiella sp.*, *Enterobacter sp.*, *Proteus sp.*, *Pseudomonas aeruginosa*, *Enterococcus sp.* ZO shows also antibacterial activity against aerobic and anaerobic pathogens. ZO inhibits attachment and growth of *S. aureus* *in vitro* and *in vivo*.

There is currently a trend in the increased use of antimicrobial agents like silver and iodine. The effectiveness of antimicrobial therapies depends on host defence mechanisms and virulence factors. Altogether, our results point out that the percentage of cell vitality compared to that of untreated cells decreases when in contact with ZO, as a consequence we can foresee an interesting positive potential for the new formula in periodontal disease treatment.

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