THE EFFECT OF TWICE DAILY KIWIFRUIT CONSUMPTION ON PERIODONTAL AND SYSTEMIC CONDITIONS BEFORE AND AFTER TREATMENT: A RANDOMIZED CLINICAL TRIAL

L'EFFETTO DEL CONSUMO DI DUE KIWI AL GIORNO SULLE CONDIZIONI PARODONTALI E SISTEMICHE PRIMA E DOPO IL TRATTAMENTO: STUDIO CLINICO RANDOMIZZATO

Filippo Graziani[1,2,3*], Nicola Discepoli[2*], Stefano Gennai[1,2], Dimitra Karapetsa[1,2], Marco Nisi[2], Lea Bianchi[4], Mario Gabriele[1], Martijn Rosema[5], Ubele Van der Velden[5]

1. Department of Surgical, Medical and Molecular Pathology and Critical Care Medicine, University of Pisa, Pisa, Italy

2. Sub-Unit of Periodontology, Halitosis and Periodontal Medicine, University Hospital of Pisa, Pisa, Italy

3. Interdepartmental Research Center "Nutraceuticals and Food for Health," University of Pisa, Pisa, Italy

4. Chemistry and Endocrinology Laboratory, University Hospital of Pisa, Pisa, Italy

5. Department of Periodontology, Academic Centre for Dentistry of Amsterdam (ACTA), University of Amsterdam and VU University of Amsterdam, The Netherlands

** The first two authors equally contributed to the study*

Running title: Kiwifruit effect on periodontal inflammation

Key words: Periodontal disease, inflammation, kiwifruit, nutraceutical, periodontal treatment

Abstract

Aim: To assess the nutraceutical effects of twice/daily intake of kiwifruit on periodontal parameters and systemic health before and after initial periodontal treatment (IPT).

Matherials and Methods: Included subjects were randomized at baseline (BL) to consumption of 2 kiwifruits or not for 5 months. In the first two months, no periodontal treatment was delivered (2M). Subsequently, a session of full-mouth IPT within 24 hours was performed. Subjects were then re-assessed after 3 months (5M). Blood collection, evaluating lipids profile and C-reactive protein, and vital signs were also collected at BL, 2M and 5M.

Results: Groups were balanced at baseline. At 2M no differences could be detected for any parameter but bleeding score which decreased significantly by 6.67±11.90% ($p<0.01$) in the kiwifruit group. Kiwifruit consumption was also related to lower values of plaque and attachment loss. After treatment both groups experienced significant clinical benefits and no differences were noted but in terms of plaque and gingival inflammation. In the kiwifruit group, a reduction of diastolic blood pressure ($p\leq0.01$) and HDL ($p=0.026$) was noted.

Conclusions: Kiwifruit consumption reduces gingival inflammation despite the lack of any periodontal instrumentation or patient's behavioural changes. No adjunctive effect to periodontal treatment of dietary intake of kiwifruit was noted.

Abstract

Obiettivo: Valutare gli effetti nutraceutici dell'assunzione di 2 kiwi/dì sui parametri parodontali e sistemici prima e dopo trattamento parodontale non-chirurgico (TPNC).

Materiali e Metodi: I soggetti inclusi sono stati randomizzati a baseline (BL) in base al consumo o no di 2 kiwi/dì. Nei primi due mesi, nessun trattamento parodontale è stato effettuato (2M). Successivamente, è stata eseguita una sessione di full-mouth TPNC. I soggetti sono stati rivalutati dopo 3 mesi (5M). I prelievi ematici per la valutazione del profilo lipidico e della proteina C-reattiva e i segni vitali sono stati raccolti a BL, 2M e 5M.

Risultati: I due gruppi erano comparabili a BL. A 2M solo il sanguinamento al sondaggio si è ridotto in modo significativo (6.67 ± 11.90% [$p<0.01$]) nel gruppo kiwi. Il consumo di kiwi è stato associato a valori più bassi di placca e perdita di attacco. Dopo il TPNC entrambi i gruppi hanno tratto significativi benefici clinici e sono emerse differenze solo in termini di placca e infiammazione gengivale. Nel gruppo kiwi, è stata osservata una riduzione della pressione diastolica ($p\leq0.01$) e dell'HDL ($p = 0,026$).

Conclusioni: In assenza di TPNC il consumo di kiwi riduce l'infiammazione gengivale ma non apporta alcun effetto aggiuntivo al TPNC.

Introduction

Periodontitis, a destructive inflammatory disease of the supporting tissues of the teeth, is caused by an imbalance between the host defence and environmental factors like bacteria, smoking and poor nutrition. Nowadays the etiological role of bacteria has been changed from putative periodontal pathogens to periodontal pathobionts (Cugini et al. 2013). A pathobiont is a symbiont that is able to promote pathology only when specific genetic or environmental conditions are altered in the host (Chow & Mazmanian 2010). This insight may revolutionize prevention and treatment of periodontitis. Focus should not only be on plaque control and removal of bacteria but also on improving host resistance through smoking abstention, stress reduction and a healthy diet. The importance of micronutrients has been extensively reviewed and it was concluded that for prevention and treatment of periodontitis daily nutrition should include sufficient antioxidants, vitamin D and calcium (Van der Velden et al. 2011).

Regarding antioxidants, vitamin C has attracted the attention of periodontal researchers. The relationship between necrotizing ulcerative gingivitis and vitamin C deficiency is well-known (Melnick et al. 1988). Furthermore, it has been shown that plasma vitamin C levels are inversely related to the severity of periodontitis (Amarasena et al. 2005, Panjamurthy et al. 2005, Staudte et al. 2005, Amaliya et al. 2007, Chapple et al. 2007). The results of a recent case control study also showed that periodontitis patients have lower plasma vitamin C levels than subjects without periodontal breakdown (Kuzmanova et al. 2012). Moreover, 19% of the periodontitis patients appeared to be depleted of vitamin C (plasma values 2.0-3.9 mg/l), although the estimated dietary intake of vitamin C was comparable. In contrast, Staudte et al. (2012) found that periodontitis patients had lower plasma vitamin C level compared to healthy controls but that they had a reduced intake of vitamin C.

Up to now there is limited available research investigating the effect of vitamin C supplementation on the periodontal condition. Experimental vitamin C depletion/repletion studies in young subjects showed that gingival inflammation was directly related to the vitamin C status (Leggot et al. 1986, Jacob et al. 1987). No effect of vitamin C supplementation was found in relation to the development of gingivitis during experimental gingivitis (Vogel et al. 1986). However, in subjects with gingivitis, vitamin C supplementation resulted in a decrease of gingivitis (Gokhale et al. 2013) while in untreated periodontitis patients vitamin C supplementation had no effect on gingivitis and pocket depth.

Recently, a significant increase of medical literature on the effect of nutraceutical (a term indicating both nutrition and pharmaceutical) dietary aliments on general health has been noted. A nutraceutical "*identifies a food or part of a food, which can be of vegetal or animal origin, that has a beneficial pharmaceutical activity beyond its nutritional value"* (Santini et al. 2016) and, to our knowledge, only one study evaluated in untreated periodontitis patients the nutraceutical effect of vitamin C by means of increasing intake through natural dietary sources i.e. two grapefruits daily for two weeks (Staudte et al. 2005). Results showed that the intake of

grapefruit leads to an increase in plasma vitamin C levels and improved sulcus bleeding scores but no change in pocket depth.

Good sources of vitamin C include peppers, strawberries, broccoli, Brussels sprouts, oranges, grapefruits and kiwifruit. Kiwifruit (Van der Velden et al. 2011) seems most attractive since it contains 93 mg vitamin C/100 g fruit whereas oranges and grapefruits contain 45 and 33 mg/100 g fruit respectively (USDA 2010). Furthermore, it has been reported that consuming two or three kiwifruits per day may reduce blood pressure (Karlsen et al. 2013), platelet aggregation and triglycerides levels (Duttaroy & Jørgensen 2004) and may increase HDL-cholesterol levels (Chang & Liu 2009, Gammon et al. 2013).

Therefore, the aim of the present study was twofold. The first objective was to investigate the effect of twice-daily kiwifruit consumption as sole treatment modality in untreated periodontitis, followed after two months by initial periodontal therapy (IPT) supported by continued kiwifruit consumption. The second objective was to investigate the effect of kiwifruit consumption on periodontal and systemic parameters of these periodontitis patients 3 months after treatment.

Materials e Methods

Experimental patient population

This study was a single-centre randomized, parallel design, clinical trial with a 5-month follow up involving subjects affected by periodontitis. The protocol of the study was approved by the local ethical committee (#3729/2012) and it was conducted according to the principles outlined in the Declaration of Helsinki on experimentation involving human subjects.

Eligible patients were identified from those referring to the Sub-Unit of Periodontology, Halitosis and Periodontal Medicine of the University Hospital of Pisa, Italy. All subjects gave written informed consent, full medical history was recorded and oral examination was completed. Patients presenting with proximal attachment loss of ≥3 mm in ≥2 non-adjacent teeth (Tonetti & Claffey 2005), PPD≥4 mm and BoP on at least 25 % of their total sites, and documented radiographic bone loss were considered eligible to participate in this study. Subjects were excluded from the study if they were (i) younger than 18 years or older than 70 years, (ii) pregnant or lactating females, (iii) females using contraceptive pharmacological medications, (iv) reported diagnosis of any systemic illnesses including cardiovascular, renal, and liver diseases, (v) in need of antibiotic treatment during initial periodontal treatment (IPT), (vi) IPT in the previous 6 months, (vii) allergic to latex, kiwifruit and fruits in general, (viii) suffering of eating/digestive disease or food intolerances and (ix) smoking more than 20 cigarettes per day.

Study design

Subjects who accepted to participate were invited to another clinical session in which a clinical examination was performed and blood collection was taken (Figure 1). Allocation envelopes were then opened and subjects in the test group were prescribed twice daily kiwifruit. They were instructed to eat two kiwifruits per day during the whole study period. The kiwifruits had to be provided by the subjects themselves. No recommendation on the type of kiwifruit was delivered. Kiwifruit was suggested to be taken as a whole and not mixed with sugar or other. No further dietary changes were requested. Subjects were given diaries to annotate the kiwifruit intake and eventual side-effects. In the control group no dietary recommendations were given.

No treatment was delivered in the following 8 weeks which is the approximate waiting list period to receive treatment in our hospital in Pisa. During this period no clinical sessions were performed unless urgency appointment if requested. After 2 months (2M) blood was again withdrawn and another clinical examination was performed. Subsequently, subjects received full mouth IPT within 24h. Subjects were seen once a month to re-enforce oral hygiene. Three months after IPT (5M) another clinical examination was performed and blood samples taken.

Randomization procedures, allocation concealment, masking and sample size calculation

Study participant numbers were assigned in ascending order at the enrolment visit. Subjects were randomly assigned in a 1:1 ratio to either test or control group using a computer-generated table (www.random.org). No stratification on cigarettes/day and years of smoking was made. The randomization table was saved by a research fellow not directly involved in the experimentation. Allocation to treatment was concealed to the clinical examiner and statistician with sealed opaque envelopes which were opened by a clinical staff member on the day of the allocation.

The clinical examiner and the therapists were masked to the allocation. Subjects were asked not to indicate their group allocation. The sample size calculation was based on data of Staudte et al. (2005) showing a drop in bleeding scores from 1.68±0.6 to 1.05±0.6 mg/l after grapefruit consumption. Thus, 24 subjects per treatment arm would be needed to provide 95% power to detect a difference of 0.6 between test and control using the bleeding score after two months of kiwifruit consumption as the primary outcome variable assuming that the standard deviation is 0.6. Thus, a sample of 50 subjects, 25 per arm were recruited to compensate for possible drop-out.

Clinical parameters

Both systemic and periodontal parameters were collected at BL, 2M and 5M.

Periodontal clinical parameters were assessed using a UNC 15-mm periodontal probe by the clinical examiner at six sites/tooth excluding third molars. Calibration of the examiner was performed on a total of 10 non-study subjects affected by periodontitis. The examiner was judged to be reproducible after meeting a percentage of agreement of CAL recording within ≤2 mm between two repeated measurements in separate occasions of at least 98% (Graziani et al. 2010b). During the trial, full-mouth pocket probing depth (PPD) and recession of the gingival margin (REC), positive and negative, were recorded with measurements rounded off to the nearest millimetre. Clinical attachment level (CAL) was calculated as the sum of PPD and REC. The full-mouth plaque score (FMPS) was measured as the percentage of the total surfaces showing plaque assessed dichotomously on six surfaces per tooth (O'Leary et al. 1972). Similarly, a full-mouth percentage bleeding score (FMBS) was calculated after assessing dichotomously the presence of bleeding on probing (Torfason et al. 1979).

During the study, blood samples were also collected and vital signs including systolic (SBP) and diastolic blood pressure (DBP) were measured in triplicates by using an automatic oscillometric device (OMRON- 705IT, Omron, Kyoto, Japan). Average BP was then calculated from the last two measurements. Weight and height were measured and body mass index (BMI) was calculated. Body temperature was measured with tympanic reading by using an ear canal thermometer (Genius TM 2, Covidien LLC, USA). Smoking history was registered dichotomously as current or never/former.

Blood collection and analysis of the serum markers

Serum samples were collected from a venepuncture in the antecubital fossa before 9.00 AM and after an overnight fast for all patients. Blood samples were immediately processed and serum aliquots were stored at -80°C. All laboratory analyses were performed at the laboratory of the University Hospital of Pisa. Glycated haemoglobin, lipid fractions including total cholesterol, HDL, LDL, and triglycerides were measured using standard laboratory procedures. Serum C-Reactive Protein (CRP) was measured by immunoturbidometry (Cobas, Roche Diagnostic, Mannheim, Germany). To assess Vitamin C, within 10 min after collection, sampling tubes were centrifuged with a low-speed centrifuge 3500 rpm for 15 min (Heraeus Megafuge 40R, Thermo Fisher Scientific, Waltham, USA) to separate plasma from blood cells. Immediately thereafter, plasma was stabilized by means of a precipitation reagent in order to minimize the oxidation and subsequently prepared for vitamin C assessment by high-pressure liquid chromatography according to the manufacturer's instructions (Eureka, Chiaravalle. Italy). All samples were analysed at the end of the study by non-staff members masked to the group allocation.

Initial periodontal treatment

Supra- and sub-gingival mechanical instrumentation of the root surface was performed by a single periodontist. Treatment was provided using both hand and ultrasonic instrumentation with periodontal tips (EMS, Nyon, Switzerland). Local anaesthesia was used when needed and no time constraints were enforced. Subjects received treatment within 24 h in two sessions, one side of the mouth for each session.

Oral hygiene instructions (OHI) consisting of electric tooth brushing and interdental brushes were explained and shown to the participants the first day of the IPT. OHI were further re-enforced after one week and once a month during follow-up.

Statistical analysis

Descriptive statistics and data analyses were performed with statistical software from SPSS (version 21.0, SPSS Inc., Chicago, IL, USA). All data are presented as mean and standard deviation unless otherwise specified. Changes of oral and systemic parameters over time within test and control group were analysed with ANOVA for repeated measures at different time points unadjusted for confounding factors. Least significant difference post hoc corrections were adopted. Differences in changes between test and control group over time were analysed with a linear mixed model analysis including regimen variable, session variable and the interaction between regimen and session, adjusting for the particular outcome variable at baseline and the potential confounding factors age, gender, smoking status, BMI, Vitamin C and HbA1c. This model was used because it takes into account the correlation between the repeated measures within the subject.

Results

Subjects accountability, baseline characteristics and dietary compliance

A total of 96 subjects were screened from May 2013 to May 2015 and 50 were finally included. All subjects recruited were Caucasians and they all completed the study providing data for the final database analyses.

Both groups were comparable for age, gender and smoking habits. Included subjects were mainly at their fifties, female subjects and smokers accounted to 60% and 64% and 48% and 40% of the test and control group respectively. No differences in terms of systemic parameters were observed between the two groups. No subjects were undergoing hormone replacement, anti-coagulant or anti-aggregation treatment. Overall subjects self-reported systemically healthy with a tendency for high-end levels of cholesterol, HbA1c and CRP. Low levels of

baseline Vitamin C were noted. Differences were noted for baseline level of HbA1c indicating a lower glycemic control in the test group being at the threshold of prediabetes. Included subjects appeared to be affected by CAL higher than 4 mm on average, FMPS above the 70% and inflammation observed in more than 50% of the sites analysed (Table 1). No differences between the two groups were noted at baseline.

Daily kiwifruit intake was positively perceived by the study population as self-reported at 2M and 5M and adherence to dietary changes was high. The overall percentage of self-reported kiwifruit intake per day on the total of expected assumption (2 kiwis/day over 5 months) was from baseline to month 2 87.62 ± 14.63 % and from month 2 to month 5 78.37 ± 17.35 % (difference BL-2M versus 2M-5M, p= 0.055). No major side-effects were reported. One patient experienced 3 days of diarrhoea and one patient described two days of itching lips 9 days after the beginning of the consumption but kiwifruit intake was not discontinued.

Periodontal parameters

In the first 2M period, in which no treatment was performed, the control group did not show statistically significant changes of the periodontal parameters. In this group FMPS remained above 80%, FMBS above 60% and no changes of PPD and CAL could be assessed (Table 2). Conversely, in the test group, FMBS decreased significantly by 6.67 ± 11.90% ($p \leq 0.01$). The number of pockets also showed a minor reduction in the test group ($p<0.05$). Comparison of test and control group showed for FMPS, FMBS and CAL significant differences in favour of the test group (Table 2). In the second period of the study, IPT appeared to be successful in both test and control group. The only difference in this period was that treatment resulted in more reduction of FMBS, FMPS and CAL in the control group (Table 2).

Systemic biomarkers

In the first 2M no differences were noted among systemic biomarkers and vital signs in both groups compared to baseline. When comparing changes among test and controls, SBP showed a greater reduction in the first 2M ($p=0.034$). After periodontal treatment an increased level of triglycerides ($p \leq 0.01$) and a reduction of HDL ($p \leq 0.05$) were also noted in the test compared to the control group.

Discussion

This clinical trial compared the clinical effect of the consumption of two kiwifruits per day on both untreated and treated periodontal disease. This is the first nutraceutical trial evaluating this nutritional effect in such a particular model. Our data indicate that kiwifruit consumption

establishes a significant decrease of gingival inflammation in the absence of any treatment. This finding is in agreement with a previous observational study indicating reduction of gingival bleeding after two weeks of two grapefruit intake in periodontitis affected subjects, particularly in non-smokers subjects (Staudte et al. 2005). In the present study, comparison of test and control group with regard to the attachment level measurements indicated a significant difference in favour of the test group. The control group experienced a small loss during the two months without treatment whereas the kiwifruit showed a small gain. Although these changes were not statistically significant for each group, the comparison of the two groups by means of linear mixed model analysis adjusted for confounding factors indicated that kiwifruit consumption had a positive influence. Both the reduction in FMBS and the CAL data suggest that due to this supplementation in the diet probably less inflammation is present in the periodontal tissues.

The reason for less inflammation might be due to the high supplementation of vitamin C, provided by kiwifruit, which has a robust modulatory effect of gingival inflammation (Leggott et al. 1991). Indeed, over 5 months a significant increase of Vitamin C level was observed in the test group. Vitamin C consumption through kiwifruit intake may vary among 60 mg to 160 mg per 100 mg of pure fruit flesh according to the type of kiwi with an approximate daily intake of 100-200 Vitamin C per day in this trial (Ma et al. 2017). Vitamin C may actively diminish inflammation through its role in the oxidative stress as potent antioxidant, down regulating inflammation and improving endothelial function (Ashor et al. 2015, Ellulu et al. 2015). Moreover, it has been shown that neutrophil chemotaxis and oxidant generation increased by 20% after kiwifruit supplementation (Bozonet et al. 2015). Recently, it was found in an untreated periodontally diseased population deprived from regular dental care that supplementation with 200 mg vitamin C daily reduced both the subgingival load of all studied periodontal bacteria as well as the serum CRP levels suggesting less inflammation (Amaliya et al. 2015). Furthermore, vitamin C may act as a robust mediator of collagen synthesis as it has been observed in periodontal tissues (Aurer-Kozelj et al. 1982). In that study, vitamin C supplementation in periodontitis-affected subjects was associated with histological repair of the interdental papilla. Furthermore, kiwifruit is also rich of other numerous antioxidants such as lutein, an oxycarotenoid and alpha-linolenic acid, an omega-3 fatty acid (Drummond 2013). Indeed, carotenoids and fatty acids have both anti-inflammatory properties (Helmersson et al 2009).

The reduction of inflammation in the study period without any treatment may also have had an effect on the FMPS. Several studies have reported an increased plaque accumulation in the presence of gingival inflammation (Lang et al. 1973, Goh et al. 1986, Ramberg et al 1994, 1995, Rowshani et al 2004). Also in experimental gingivitis studies it has been shown that subjects develop plaque more rapidly in the presence of gingivitis (Quirynen et al. 1991, Ramberg et al. 1994, Daly & Highfield 1996). Therefore, it may be suggested that a reduction in inflammation may also result in a reduction of plaque. This assumption is supported by the present study

results since in the study period without treatment the FMPS of the test group decreased by 4% whereas the FMPS in the control group increased by 2.3%, a difference that was statistically significant.

No significant effect as adjunctive to periodontal treatment was noted. It is likely that the magnitude of the treatment effect would hide a possible benefit of the fruit consumption. The only difference was that treatment resulted in more reduction of FMBS and FMPS in the control group (Table 2). However, this should be seen in the light of the lower FMBS and FMPS already obtained by the test group after two months of kiwifruit consumption without treatment.

Interestingly, a significant reduction on SBP was also noted in the kiwifruit group when compared to control group. This is in agreement with previous reports indicating significant reduction of both SBP and DBP modulated by kiwifruit consumption (Karlsen et al. 2012, Svendsen et al. 2015). The reason for no variation of the DBP might be due to the different dosage; our trial used two kiwifruits per day versus three in the hypertension studies. Moreover, our population was not constituted by subjects affected by hypertension in which the effects of kiwifruit consumption on blood pressure levels are milder (Gammon et al. 2014). The mechanisms for such effects are hypothesized to rest in the increase of both potassium (Aburto et al. 2013) and the vitamin C (Juraschek et al. 2012) intake provided by the kiwifruit.

Unexpectedly, no beneficial effects on lipid metabolism were seen but a deterioration of the triglycerides in the kiwifruit group. This finding cannot be clearly explained. Kiwifruit consumption usually lessens the plasma values of triglycerides (Rocio-Rodriguez et al. 2015) by approximately 15% (Duttaroy & Jørgensen 2004). However, although significant higher triglyceride values were found compared to month 2 at the start of treatment, they were not different compared to the baseline triglycerides values.

The authors are aware of the strength and the intrinsic limitations of the study. Especially, the sample size was calculated on the effect on the bleeding score. Therefore, the number of subjects in the present study may not be adequate for the other studied parameters. Nevertheless, our findings are of uttermost importance as no other nutraceutical trials are present within the periodontal literature. In particular, the present research model allows measuring the effect of the dietary consumption on both untreated and treated periodontal disease.

In conclusion, daily kiwifruit consumption determines a significant reduction of gingival inflammation in untreated periodontal disease. This may provide support for improved nutritional approaches in the prevention of periodontal diseases.

Acknowledgements

The authors are deeply grateful to the clinical and administrative staff members of the Unit of Dentistry and Oral Surgery of the University Hospital of Pisa for the valuable support throughout the trial and to Dr. Camillo Giacomelli and Dr. Maria Rita Sessa for their precious help in managing blood samples.

References

Aburto, N.J., Hanson, S., Gutierrez, H., Hooper, L., Elliott, P. & Cappuccio, F.P. (2013). Effect of increased potassium intake on cardiovascular risk factors and disease: systematic review and meta-analyses. *British Medical Journal* **346**, f1378.

Amaliya, A., Timmerman, M. F., Abbas, F., Loos, B. G., Van der Weijden, G. A., Van Winkelhoff, A. J., Winkel, E. G. & Van der Velden, U. (2007) Java project on periodontal diseases: the relationship between vitamin C and the severity of periodontitis. *Journal of Clinical Periodontology* **34**, 299-304.

Amaliya, A., Laine, M.L., Loos, B.G., Van der Velden, U. (2015) Java project on periodontal diseases: effect of vitamin C/calcium threonate/citrus flavonoids supplementation on periodontal pathogens, CRP and HbA1c. *Journal of Clinical Periodontology* **42**, 1097-1104.

Amarasena, N., Ogawa, H., Yoshihara, A., Hanada, N. & Miyazaki, H. (2005) Serum vitamin C-periodontal relationship in community-dwelling elderly Japanese. *Journal of Clinical Periodontology* **32**, 93-7.

Ashor, A.W., Siervo, M., Lara, J., Oggioni, C., Afshar, S. & Mathers, J.C. (2015) Effect of vitamin C and vitamin E supplementation on endothelial function: a systematic review and meta-analysis of randomised controlled trials. *The British Journal of Nutrition* **113**, 1182-94.

Aurer-Kozelj, J., Kralj-Klobucar, N., Buzina R & Bacic, M. (1982) The effect of ascorbic acid supplementation on periodontal tissue ultrastructure in subjects with progressive periodontitis. *International Journal of Vitaminal Nutritional Research* **52**, 333-41.

Chang, W. H. & Liu, J. F. (2009) Effects of kiwifruit consumption on serum lipid profiles and antioxidative status in hyperlipidemic subjects. *International Journal of* Food Sciences and *Nutrition* **60**, 709-16.

Chapple, I. L. & Matthews, J. B. (2007) The role of reactive oxygen and antioxidant species in periodontal tissue destruction. *Periodontology 2000* **43**, 160-232.

Chow, J. & Mazmanian, S. K. (2010) A pathobiont of the microbiota balances host colonization and intestinal inflammation. *Cell Host & Microbe* **22**, 265-76.

Cugini, C., Klepac-Ceraj, V., Rackaityte, E., Riggs, J. E. & Davey, M. E. (2013) Porphyromonas gingivalis: keeping the pathos out of the biont. Journal of Oral Microbiology **5**, 19804.

Daly, C.G. & Highfield, J.E. (1996) Effect of localized experimental gingivitis on early supragingival plaque accumulation. *Journal of Clinical Periodontology* **23**, 160-164.

Dietrich, T., Joshipura, K.J., Dawson-Hughes, B. & Bischoff-Ferrari, H. A. (2004) Association between serum concentrations of 25-hydroxyvitamin D3 and periodontal disease in the US population. American Journal of Clinical Nutrition **80,** 108-13.

Dodington, D. W., Fritz, P. C., Sullivan, P. J. & Ward, W. E. (2015) Higher Intakes of Fruits and Vegetables, β-Carotene, Vitamin C, α-Tocopherol, EPA, and DHA Are Positively Associated with Periodontal Healing after Nonsurgical Periodontal Therapy in Nonsmokers but Not in Smokers. *Journal of Nutrition* **145**, 2512-9.

Drummond, L. (2013) The composition and nutritional value of kiwifruit. *Advances in Food and Nutrition Research* **68**, 33-57.

Duttaroy, A. K. & Jørgensen, A. (2004) Effects of kiwi fruit consumption on platelet aggregation and plasma lipids in healthy human volunteers. *Platelets* **15**, 287-92.

Ellulu, M. S., Rahmat, A., Patimah, I., Khaza'ai, H. & Abed, Y. (2015) Effect of vitamin C on inflammation and metabolic markers in hypertensive and/or diabetic obese adults: a randomized controlled trial. *Drug, Design, Development and Therapy* **1**, 3405-12.

Gammon, C. S., Kruger, R., Brown, S. J., Conlon, C. A., von Hurst, P. R. & Stonehouse, W. (2014) Daily kiwifruit consumption did not improve blood pressure and markers of cardiovascular function in men with hypercholesterolemia. *Nutrition Research* **34**, 235-40.

Gammon, C. S., Kruger, R., Minihane, A. M., Conlon, C. A., von Hurst, P. R. & Stonehouse, W. (2013) Kiwifruit consumption favourably affects plasma lipids in a randomised controlled trial in hypercholesterolaemic men. *British Journal* of *Nutrition* **28**, 2208-18.

Gokhale, N.H., Acharya, A. B., Patil, V. S., Trivedi, D. J. & Thakur, S. L. (2013) A short-term evaluation of the relationship between plasma ascorbic acid levels and periodontal disease in systemically healthy and type 2 diabetes mellitus subjects. *Journal of Dietary Supplements* **10**, 93-104.

Graziani, F., Cei, S., Tonetti, M., Paolantonio, M., Serio, R., Sammartino, G., Gabriele, M. & D'Aiuto, F. (2010) Systemic inflammation following non-surgical and surgical periodontal therapy. *Journal of Clinical Periodontology* **37**, 848-54.

Goh, C.J., Waite, I.M., Groves, B.J. & Cornick, D.E. (1986) The influence of gingival inflammation and pocketing on the rate of plaque formation during non-surgical periodontal treatment. *British Dental Journal* **161**, 165-169.

Hampl, J. S., Taylor, C. A. & Johnston, C. S. (2004) Vitamin C deficiency and depletion in the United States: the Third National Health and Nutrition Examination Survey, 1988 to 1994. *American Journal* of *Public Health* **94**, 870-5.

Helmersson, J., Arnlöv, J., Larsson, A, & Basu, S. (2009) Low dietary intake of beta-carotene, alpha-tocopherol and ascorbic acid is associated with increased inflammatory and oxidative stress status in a Swedish cohort. *The British Journal of Nutrition* **101**, 1775-82.

Jacob, R. A., Skala, J. H., Omaye, S. T. & Turnlund, J. R. (1987) Effect of varying ascorbic acid intakes on copper absorption and ceruloplasmin levels of young men. *Journal of Nutrition* **117**,2109-15.

Juraschek, S. P., Guallar, E., Appel, L. J. & Miller, E. R. 3rd. (2012) Effects of vitamin C supplementation on blood pressure: a meta-analysis of randomized controlled trials. *American Journal of Clinical Nutrition* **95**, 1079-88.

Karlsen, A., Svendsen, M., Seljeflot, I., Laake, P., Duttaroy, A. K., Drevon, C. A., Arnesen, H., Tonstad, S. & Blomhoff, R. (2013) Kiwifruit decreases blood pressure and whole-blood platelet aggregation in male smokers. *Journal of Human Hypertension* **27**, 126-30.

Karlsen, A., Svendsen, M., Seljeflot, I., Sommernes, M. A., Sexton, J., Brevik, A., Erlund, .I, Serafini, M., Bastani, N., Remberg, S. F., Borge, G. I., Carlsen, M. H., Bøhn, S. K., Myhrstad, M. C.,Dragsted, L. O., Duttaroy, A. K., Haffner, K., Laake, P., Drevon, C. A., Arnesen, H., Collins, A., Tonstad, S. & Blomhoff, R. (2011) Compliance, tolerability and safety of two antioxidant-rich diets: a randomised controlled trial in male smokers. *British Journal of Nutrition* **106**, 557-71.

Schneider, H. G. & Knieknecht, I. (1986) Reduction of dental plaque by apples and chewing gum. *Nahrung* **30**, 907-912.

Kuzmanova, D., Jansen, I. D., Schoenmaker, T., Nazmi, K., Teeuw, W. J., Bizzarro, S., Loos, B. G. & van der Velden, U. (2012) Vitamin C in plasma and leucocytes in relation to periodontitis. *Journal of Clinical Periodontology* **39**, 905-12.

Lang NP, Cumming BR, Loe H. (1973) Toothbrushing frequency as it relates to plaque development and gingival health. *Journal of Periodontology* **44**, 396-405.

Leggott, P. J., Robertson, P. B., Rothman, D. L., Murray, P. A. & Jacob, R. A. (1986) The effect of controlled ascorbic acid depletion and supplementation on periodontal health. *Journal of Periodontology* **57**, 480–485.

Leggott, P. J., Robertson, P. B., Jacob, R. A., Zambon, J. J., Walsh, M. & Armitage, G. C. (1991) Effects of ascorbic acid depletion and supplementation on periodontal health and subgingival microflora in humans. *Journal of Dental Research* **70**, 1531-6.

Levine, M., Conry-Cantilena, C., Wang, Y., Welch, R. W., Washko, P. W., Dhariwal, K. R., Park, J. B., Lazarev, A., Graumlich, J. F., King, J. & Cantilena, L. R. (1996) Vitamin C pharmacokinetics in healthy volunteers: evidence for a recommended dietary allowance. *Proceedings of the National Academy of Sciences of the United States of America.* **16**, 3704-9.

Ma, T., Sun, X., Zhao, J., You, Y., Lei, Y., Gao, G. & Zhan, J. (2017) Nutrient compositions and antioxidant capacity of kiwifruit (Actinidia) and their relationship with flesh color and commercial value. *Food Chemistry* **218**, 294-304.

Melnick, S. L., Alvarez, J. O., Navia, J. M., Cogen, R. B. & Roseman, J. M. (1998) A case-control study of plasma ascorbate and acute necrotizing ulcerative gingivitis. *Journal of Dental Research* **67**, 855-60.

O'Leary, T. J., Drake, R. B. & Naylor, J. E. (1972) The plaque control record. *Journal of Periodontology* **43**, 38.

Panjamurthy, K., Manoharan, S. & Ramachandran, C. R. (2005) Lipid peroxidation and antioxidant status in patients with periodontitis. *Cellular & Molecular Biology Letters.* **10**, 255-64.

Quirynen, M., Dekeyser, C. & van Steenberghe, D. (1991) The influence of gingival inflammation, tooth type, and timing on the rate of plaque formation. *Journal of Periodontology* **62**, 219-222.

Ramberg, P., Lindhe, J., Dahlen, G. & Volpe, A.R. (1994) The influence of gingival inflammation on de novo plaque formation. *Journal of Clinical Periodontology* **21**, 51-56.

Recio-Rodriguez, J.I., Gomez-Marcos, M.A., Patino-Alonso, M.C., Puigdomenech, E., Notario-Pacheco, B., Mendizabal-Gallastegui, N., de la Fuente Ade, L., Otegui-Ilarduya, L., Maderuelo-Fernandez, J.A., de Cabo Laso, A., Agudo-Conde, C. & Garcia-Ortiz, L., EVIDENT Group (2015). Effects of kiwi consumption on plasma lipids, fibrinogen and insulin resistance in the context of a normal diet. *Nutrition Journal* **14**, 97-106.

Rowshani, B., Timmerman, M.F. & Van der Velden, U. (2004) Plaque development in relation to the periodontal condition and the bacterial load of the saliva. *Journal of Clinical Periodontology* **31**, 214-218.

Santini, A., Tenore, G.C. & Novellino, E. (2016) Nutraceuticals: A paradigm of proactive medicine. *European Journal of Pharmaceutical Sciences* **96**, 53-61.

Svendsen, M., Tonstad, S., Heggen, E., Pedersen, T. R., Seljeflot, I., Bøhn, S. K., Bastani, N. E., Blomhoff, R., Holme, I. M. & Klemsdal, T. O. (2015) The effect of kiwifruit consumption on blood pressure in subjects with moderately elevated blood pressure: a randomized, controlled study. *Blood Pressure* **24**, 48-54.

Staudte, H., Kranz, S., Völpel, A., Schütze, J. & Sigusch, B. W. (2012) Comparison of nutrient intake between patients with periodontitis and healthy subjects. *Quintessence International* **43**, 907-16.

Staudte, H., Sigusch, B. W. & Glockmann, E. (2005) Grapefruit consumption improves vitamin C status in periodontitis patients. *British Dental Journal* **199**, 213-7.

Tonetti, M. S. & Claffey, N. (2005) European Workshop in Periodontology group C. Advances in the progression of periodontitis and proposal of definitions of a periodontitis case and disease progression for use in risk factor research. Group C consensus report of the 5th European Workshop in Periodontology. *Journal of Clinical Periodontology* **32**, 210-3.

Torfason, T., Kiger, R., Selvig, K.A. & Egelberg, J. (1979) Clinical improvement of gingival conditions following ultrasonic versus hand instrumentation of periodontal pockets. *Journal of Clinical Periodontology* **6**, 165-176.

Van der Velden, U., Kuzmanova, D. & Chapple, I. L. (2011) Micronutritional approaches to periodontal therapy. *Journal of Clinical Periodontology* **38**, 142-58.

Vogel, R. I., Lamster, I. B., Wechsler, S. A., Macedo, B., Hartley, L. J. & Macedo, J. A. (1986) The effects of megadoses of ascorbic acid on PMN chemotaxis and experimental gingivitis. *Journal of Periodontology* **57,** 472-9.

Table 1. Periodontal and systemic characteristics of the study sample at baseline, 2 and 5 months (unadjusted p-values).

Variable	*Test Group (N=25)*			*Control Group (N=25)*		
	BL	**2M**	**5M**	**BL**	**2M**	**5M**
FMBS, %	54.3±16.4	47.0±17.9**	12.1±9.3*	62.9±20.4	65.3±19.0	13.3±5.6*
FMPS, %	74.8±23.8	70.1±23.3	19.3±15.4*	83.0±19.1	84.6±15.6	18.0±11.1*
Recession, mm	0.4±0.5	0.4±0.6	0.8±0.6*	0.4±1.5	0.4±0.5	0.9±0.5*
Probing depth, mm	3.8±0.5	3.7±0.5	2.6±0.3*	3.8±0.5	3.8±0.5	2.5±0.3*
Probing depth ≥ 5mm	5.8±0.4	5.8±0.5	4.9±1.2*	5.7±0.5	5.7±0.4	5.3±0.3*
Number of pockets ≥ 5 mm	48.9±17.3	45.8±21.6†	6.4±10.1*	54.3±23.5	53.6±20.1	6.1±6.3*
Clinical attachment level, mm	4.0±0.6	4.0±0.8	3.3±0.7*	4.2±0.7	4.3±0.8	3.4±0.6*
BMI	23.9±4.4		23.8±4.4	24.4±3.6		24.6±3.3
Vitamin C, mg/l	2.2±3.2	4.3±5.2	6.8±6.4**	3.2±5.2	3.0±5.1	5.6±6.0
CRP, mg/l	2.3±2.8	2.4±2.4	2.0±2.6	1.5±1.6	1.6±2.3	1.1±1.2
Triglycerides, mmol/l	94.6±42.4	82.4±24.4	101.1±46.4	87.4±34.2	92.7±41.0	89.2±30.2
Total Cholesterol, mmol/L	205.2±35.3	205,0±41.1	209.0±40.0	216.6±36.9	207.6±33.7	213.0±30.0
HDL, mmol/L	59.5±15.7	60.2±15.7	58.8±15.2	61.1±13.6	60.0±12.6	63.4±13.3
HbA1c, mmol/mol	38.9±4.7	39.0±5.0	39.0±4.6	35.8±3.6	36.4±3.6	36.0±3.6
Systolic BP, mmHg	125.0±20.5	125.2±25.3	122.0±18.7	120.8±18.7	119.0±19.3	122.7±18.6
Diastolic BP, mmHg	80.8±10.5	77.2±10.8	77.0±10.1	76.0±11.3	77.4±13.1	78.3±12.0

Body temperature °C	36.5±0.5	36.6±0.4	36.7±0.4	36.7±0.6	36.5±0.5	36.5±0.5
Heartrate, beat/minute	72.6±8,8	73.8±10.9	73.0±12.7	72.4±7.5	71.3±7.1	70.8±8.7

* p<0.001 from baseline & 2M

** p<0.01 from baseline

† p<0.05 from baseline

FMPS, Full Mouth Plaque Score; FMBS, Full Mouth Bleeding Score; Rec, PD, Probing depth; BP, blood pressure; BMI, body mass index; CRP, C reactive protein; HbA1C= glycated hemoglobin

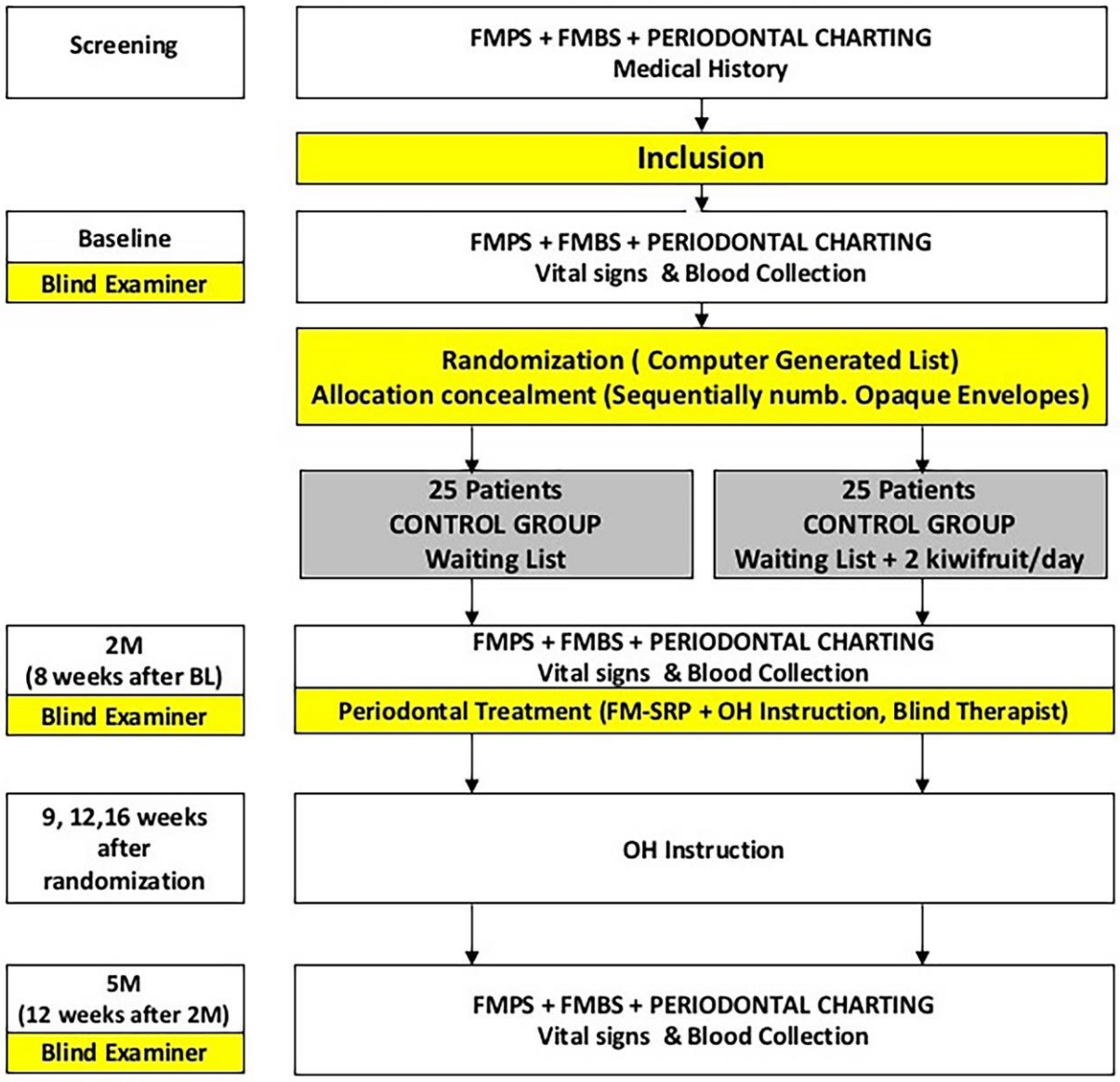
Table 2. Clinical periodontal parameters of groups at various time points and differences between groups analysed by linear mixed model analysis (adjusted for baseline values of age, gender, smoking status, vitamin C, BMI and HbA1c)

Variable (mean ± SD)	Time	**Within Test Group Difference**	**Within Control group Difference**	**Differences Test vs Control p-value**
FMBS, %	BL – 2M	6.67 ± 11.90	-2.68 ± 8.23	<0.001
	2M – 5M	36.16 ± 20.39	51.49 ± 15.99	0.009
FMPS, %	BL-2M	3.95 ± 12.95	-2.25 ± 11.1	0.001
	2M-5M	51.40 ± 20.92	65.10 ± 13.97	0.014
Recession, mm	BL-2M	-0.03 ± 0.52	-0.02 ± 14.9	0.09
	2M-5M	-0.40 ± 0.62	-0.46 ± 0.36	0.19
Probing depth, mm	BL-2M	0.04 ± 12.95	-0.01 ± 0.03	0.38
	2M- 5M	1.14 ± 0.44	1.29 ± 0.48	0.15
Probing depth ≥5mm	BL – 2M	-0.03 ± 0.18	0.03 ± 0.15	0.42
	2M – 5M	0.96 ± 1.18	0.46 ± 0.54	0.10
Number of pockets ≥5mm	BL – 2M	3.04 ± 7.25	0.68 ± 9,25	0.059
	2M – 5M	39.48 ± 21.39	47.56 ± 19.88	0.16
Clinical attachment level, mm	BL- 2M	0.03 ± 0.41	-0.06 ± 0.39	0.039
	2M – 5M	0.66 ± 0.46	0.89 ± 0.83	0.031
Vitamin C, mg/l	BL – 2M	-1.98±5.67	0.16±6.52	0.87

	2M – 5M	-2.62±7.24	-2.58±6.67	0.78
CRP, mg/l	BL – 2M	-0.3±2.4	0.4±1.8	0.86
	2M – 5M	-0.2±1.9	0.5±1.9	0.20
Tryglicerids, mmol/l	BL – 5M	8.67 ± 30.68	-0.68 ± 31.64	0.15
	2M – 5M	-15.88 ± 34.54	1.40 ± 39.10	0.008
Total Cholesterol, mmol/l	BL – 2M	-2.96 ± 15.35	9.54 ± 18.42	0.81
	2M – 5M	-2.39 ± 25.71	-7.67 ± 18.15	0.88
HDL, mmol/l	BL – 2M	-3.25 ± 15.10	2.30 ± 6.10	0.48
	2M – 5M	3.33 ± 14.51	-4.37 ± 7.41	0.024
HbA1c, mmol/l	BL – 2M	0.09 ± 1.74	-0.83 ± 2.31	0.14
	2M – 5M	-0.09 ± 1.68	0.72 ± 1.90	0.059
Systolic BP, mmHg	BL – 2M	-0.20 ± 13.81	0.00 ± 12.99	0.034
	2M – 5M	3.20 ± 20.09	-1.46 ± 14.93	0.30
Diastolic BP, mmHg	BL – 2M	3.60 ± 10.05	0.40 ± 9.35	0.36
	2M – 5M	0.20 ± 11,32	-2.25 ± 11.49	0.41
Body temperature °C	BL – 2M	-0.11 ± 0.52	0.24 ± 0.10	0.38
	2M – 5M	-0.12 ± 0.47	0.03 ± 0.71	0.42
Heartrate, beat/minute	BL – 2M	-1.20 ± 9.63	1.08 ± 7.84	0.27
	2M – 5M	0.78 ± 12.49	0.58 ± 9.33	0.70

CRP, C-reactive Protein; BP, blood pressure; HDL, high-density lipoprotein BL, baseline, 2M; 2 months; 5M, 5 months.

Figure 1. Flowchart of the study



PERIODONTAL THERAPY INFLUENCES DNA METHYLATION OF INFLAMMATORY GENES IN CHRONIC PERIODONTITIS

LA TERAPIA PARODONTALE INFLUENZA LA METILAZIONE DEL DNA DI GENI INFIAMMATORI NELLA MALATTIA PARODONTALE CRONICA

Farah Asa'ad [1], Giulio Rasperini [1], Valentina Bollati [2,3], Giorgio Pagni [1], Rogerio M. Castilho [4,5], Eleonora Rossi [1], Francesca Pomingi [6], Letizia Tarantini [2], Dario Consonni [3], William V. Giannobile [5]

1 Department of Biomedical, Surgical and Dental Sciences, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, University of Milan, Milan, Italy

2 EPIGET-Epidemiology, Epigenetics and Toxicology Lab, Department of Clinical Sciences and Community Health, University of Milan, Milan, Italy

3 Epidemiology Unit, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, University of Milan, Milan, Italy

4 Laboratory of Epithelial Biology, University of Michigan School of Dentistry, Ann Arbor, MI, USA

5 Department of Periodontics and Oral Medicine School of Dentistry & Department of Biomedical Engineering, College of Engineering, University of Michigan, Ann Arbor, MI, USA

6 Dental Hygienist, Private Dental Practice, Piacenza, Italy

Running title: Effect of Periodontal Therapy on Epigenetic Modifications

Abstract

Aim: To evaluate the influence of periodontal therapy on epigenetic modifications in chronic periodontitis patients compared to healthy individuals.

Methods: Ten patients with healthy periodontium and ten patients with chronic moderate periodontitis were enrolled. Gingival biopsies were collected at baseline for both groups and at 2 & 8 weeks post-periodontal therapy for the disease group (from normal and periodontitis sites). Random-intercept linear regression models were applied to evaluate methylation levels across groups at baseline and to assess changes in the disease group overtime, separately for normal and periodontitis sites.

Results: Periodontal therapy restored methylation levels of TNF-α, IFN-γ and COX-2 genes in periodontitis sites after 2 & 8 weeks to levels reported in normal sites. A DNA methylation gradient of COX-2 promoter region was observed at baseline for healthy group and periodontitis group (percentage mean in normal sites = 8.8 ± 5.7, p= 0.47 vs healthy; percentage mean in periodontitis sites = 13.2 ± 7.3, p = 0.03 vs healthy). Maintenance of high LINE-1 methylation on periodontitis sites was observed throughout all data points, suggesting up-regulation of methyltransferase in chronic disease.

Conclusions: Periodontal therapy resets the DNA methylation of inflammatory genes to levels observed in normal sites. Maintenance of high levels of DNA methylation was higher in periodontitis sites, reflecting methyl-transferase up-regulation in chronic disease.

Keywords: Periodontal Diseases/therapy, Epigenetics, DNA Methylation, Inflammatory genes, Biomarkers

Abstract

Obiettivo dello studio: Valutare l'influenza della terapia parodontale sulle modifiche epigenetiche in pazienti con parodontite cronica rispetto a pazienti sani.

Metodi: Sono stati reclutati 10 pazienti parodontalmente sani e 10 affetti da parodontite cronica moderata. Lo studio si è articolato raccogliendo campioni di tessuto gengivale in tre momenti: baseline (per entrambi i gruppi), 2 settimane dopo la terapia parodontale (solo per il gruppo malato, eseguiti due prelievi per paziente: sito sano "**SS**" e sito malato "**SM"**), ad 8 settimane (solo per il gruppo test, rieseguiti i due prelievi). Sono stati usati "random-intercept linear regression models" per valutare le variazioni di metilazione tra gruppi al baseline ed eventuali cambiamenti nel gruppo malato, nei siti sia sani che malati, nel corso dello studio.

Risultati: La terapia parodontale ha riportato, dopo 2 e 8 settimane, i livelli di metilazione dei geni di TNF-α, IFN-γ e COX-2 dei SM ai livelli osservati nei SS.

Per COX-2, al baseline, è stato riscontrato una differenza di metilazione della regione promotrice del DNA tra pazienti sani e parodontopatici (percentuale media in SS= 8.8 ± 5.7, p= 0.47 rispetto ai pazienti sani. Percentuale media in SM= 13.2 ± 7.3, p = 0.03 rispetto ai pazienti sani).

Inoltre, nei siti con parodontite, è sempre risultato, un alto livello di metilazione di LINE-1, indice di iperespressione dell'enzima DNA metil-transferasi che si verifica in caso di malattia cronica.

Conclusioni: La terapia parodontale è in grado di riportare i livelli di metilazione dei geni analizzati nei SM ai livelli dei SS. Inoltre, l'ipermetilazione del genoma dei SM riflette l'upregulation dell'enzima DNAmetil-transferasi tipico della malattia cronica.

Keywords: Periodontal Diseases/therapy, Epigenetics, DNA Methylation, Inflammatory genes, Biomarkers

Introduction

Periodontitis is a multifactorial disease caused predominantly by gram-negative, anaerobic and microaerophilic bacteria that can colonize the subgingival regions **(Page, 1991; Page & Kornman, 1997),** and eventually trigger an inflammatory host response influenced by environmental, genetic and epigenetic factors **(Borrell & Papapanou, 2005; Takashiba & Naruishi, 2006; Kornman, 2008)**. Of interest to this study, epigenetic modification can further regulate gene expression of an individual's immune response **(Schulz et al, 2016),** and despite the well-established influence of epigenetic modification in cancer and inflammatory diseases **(Fitzpatrick & Wilson, 2003; Adcock et al, 2007; El Gazzar et al, 2007; Ngollo et al, 2014),** little is known in the context of oral health **(Lod et al, 2014).**

In general, epigenetics embodies the modifications of gene expression, without changing the DNA

sequence, **(Bird, 2002; Adcock et al, 2007)** through chemical alterations of DNA and associated proteins **(Barros & Offenbacher, 2014)** that evoke chromatin remodeling and a successive rapid inactivation (turn off) or activation (turn on) of genes **(Larsson et al, 2015).** Notably, such alterations occur through two major mechanisms in human cells **(Shaw, 2006);** DNA methylation and histone modifications **(Wilson, 2008)** that are reversible **(Lod et al, 2014),** and linked **(Jenuwein & Allis, 2001; Robertson & Wolffe, 2000).** Nevertheless, DNA methylation offers a more stable form of gene regulation **(Bäckdahl et al, 2009)** characterized by the addition of a methyl group, through DNA methyl-transferase enzymes, to the fifth carbon on the cytosine base within CpG islands, which are frequently found in the promoter region of the gene **(Sanders, 2006)**, consequently inhibiting gene expression.

Accordingly, most of the studies exploring the link between epigenetics and periodontal disease have investigated the changes in the *DNA methylation* of genes **(Baptista et al, 2014; Andia et al., 2015),** involved in the regulation of cytokine production, since these signaling molecules play a key role in periodontal tissue breakdown; for reviews: see **(Barros & Offenbacher, 2014; Lod et al, 2014; Larsson et al, 2015).** Emerging studies are also demonstrating the activation of inflammatory pathways like the NFkB signaling pathway by histone modifications **(Martins et al, 2016).**

Various studies demonstrated a lower level of DNA methylation of genes expressing pro-inflammatory cytokines in periodontitis patients, either chronic or aggressive, compared to those with a healthy periodontium, leading to over-expression of these mediators in inflamed tissues **(Oliveira et al, 2009; Andia et al, 2010; Zhang et al, 2010a; Ishida et al, 2012).** Nevertheless, hypermethylation has been interestingly described for certain genes in the chronic state of periodontal disease, as a down-regulatory mechanism to prevent unrestricted tissue destruction **(Zhang et al, 2010b).**

Although several epigenetic alterations have been described in periodontal disease, little is known about the efficacy of periodontal therapy in reestablishing normal DNA methylation levels in patients. The prospective positive effects of periodontal therapy on the methylation profile of DNA and specific genes came from the study of **Andia & colleagues (2015)** that found no changes between healthy tissues and periodontitis after a three months follow-up. These findings suggest that periodontal therapy can influence epigenetic modifications. However, variations in the methylation level between both groups were not assessed at baseline (before therapy), and samples of inflamed tissues were not investigated.

Based on these observations, we conducted a clinical study to monitor the changes in DNA methylation of LINE-1, TNF-α, IFN-γ and COX-2 inflammatory genes in periodontitis patients following periodontal therapy in comparison to disease free subjects at both the site and patient levels.

Materials and Methods

This study was approved by the ethical committee of the University of Milan, Italy and was conducted in the period between October 2015 and June 2016.

- Study Participants & Inclusion Criteria:

From the pool of patients attending a private dental practice in Piacenza- Italy, twenty individuals were enrolled equally in two groups: "*disease free*" including ten patients with healthy

periodontium and a "*periodontal disease group*" including ten chronic moderate periodontitis patients. All participants were voluntarily included in the study after explaining its objectives and obtaining their verbal and written informed consent.

Study participants fit the following inclusion criteria:

1. Patients older than 18 years of age.
2. Patients without any systemic diseases.
3. Non-smokers.
4. Patients of the same Caucasian origin (Italian descent).
5. **For the disease free group:** patients who showed no clinical signs of gingival inflammation and had no history of periodontitis were included.
6. **For the periodontal disease group:** patients diagnosed with chronic periodontitis, based on the American Academy of Periodontology (AAP) Workshop **(Armitage, 1999)** were included. Patients in this group must have had at least a normal site (PD < 4 mm with no signs of bleeding on probing, mobility and inflammation) and a periodontitis site (PD ≥ 5 mm) for later biopsy harvesting.

The exclusion criteria were the following:

1. Current pregnancy
2. Patients who reported the use of antibiotics and/or non-steroidal anti-inflammatory drugs (NSAIDs), for at least one month before enrolment in the study.

- **Study timeline & Periodontal Parameters:**

* ***Baseline:*** For all study participants, probing pocket depth (PPD), clinical attachment level (CAL), bleeding on probing (BOP), full mouth plaque score (FMPS) and full mouth bleeding score (FMBS) were recorded at six sites around each tooth. Next, gingival biopsies, using a 3 mm of diameter punch, were obtained from the disease free group during crown lengthening procedures or surgical removal of wisdom teeth. In the periodontal disease group, biopsies were harvested from two sites: a normal site and a periodontitis site with a purpose to compare methylation levels between both. Following biopsy collection, chronic periodontitis patients underwent conventional periodontal therapy; full mouth scaling and root planing with ultrasonic and manual instruments. Chlorhexidine mouthwash (0.2%) was prescribed for daily use (twice a day for twenty days).
* ***Two weeks after periodontal therapy:*** PPD, CAL & BOP, FMPS & FMBS were registered and gingival biopsies were harvested for the disease group only, from a normal and periodontitis site, to analyze the influence of periodontal therapy on epigenetic modifications, in comparison to baseline.
* ***Eight weeks after periodontal therapy:*** As at the 2 weeks time point, PPD, CAL, BOP, FMPS & FMBS were registered and gingival biopsies were harvested for the disease group only (from two sites), to analyze the influence of periodontal therapy on epigenetic changes, in comparison to those observed among disease free and periodontal disease groups at baseline and in the periodontal disease group at 2 weeks.

- **Sample Collection, DNA Extraction, and Bisulfite Treatment:**

Briefly, freshly harvested tissues were collected in vials and submerged in Allprotect Tissue Reagent (Qiagen, USA) to stabilize DNA immediately, and were stored at 2-8 °C for up to 6 months. DNA was extracted using the QIAamp DNA Mini Kit (Qiagen) following the manufacturer's recommendations.

EZ DNA Methylation-Gold™ Kit (Zymo Research, Orange, CA, USA) was used to treat 500 ng DNA (concentration 25 ng/µl) according to the manufacturer's protocol. Bisulfite-treated DNA was eluted in 300 µl of M-Elution Buffer.

- Analysis of DNA Methylation:

Analysis of DNA methylation was performed using previously published methods, **(Bollati et al. 2007; Tarantini et al. 2013)** with minor modifications. Briefly, a 50 µl PCR was carried out in 25 µl of GoTaq Hot Start Green Master Mix (Promega, Madison, WI, USA), 1 pmol of the forward primer, 1 pmol of the biotinylated reverse primer, 25 ng of bisulfite-treated genomic DNA and water. Primers for DNA methylation analysis and PCR cycling conditions are shown in **table 1.**

The biotin-labeled primers were used to purify the final PCR product using Sepharose beads. The PCR product was bound to Streptavidin Sepharose HP (Amersham Biosciences, Uppsala, Sweden) and the Sepharose beads containing the immobilized PCR product were purified, washed, denatured using a 0.2 M NaOH solution, and washed again using the Pyrosequencing Vacuum Prep Tool (Pyrosequencing, Inc., Westborough, MA), as recommended by the manufacturer. Then, 0.3 µl Pyrosequencing primer was annealed to the purified single-stranded PCR product, and Pyrosequencing was performed using the PyroMark MD System (Pyrosequencing, Inc.). The degree of methylation was expressed as a percentage of methylated cytosines divided by the sum of methylated and unmethylated cytosines (%5mC).

- Statistical Analysis:

In order to take into account correlations within subjects, random-intercept linear regression models were applied to evaluate methylation levels across groups at baseline and to evaluate methylation changes over time in the periodontal disease group, separately for the normal and periodontitis sites. Statistical analyses were performed with Stata 13 (StataCorp. 2013) **(Rabe-Hesketh et al, 2008).**

Results

Twenty participants (8 males, 12 females) were included in this study, distributed into "disease free" (7 males, 3 females) and "periodontal disease" groups (3 males, 7 females). The age in the disease free group was between 25 and 69 years old (mean = 53.3 ± 12.3 years), while the range was 26-60 years old in the periodontal disease group (mean = 46.6 ± 10 years). Nineteen participants completed the study, as demonstrated in the CONSORT flow diagram **(figure 1).**

Success of periodontal therapy. We only reported the improvement of periodontal pocket depth in the disease group overtime, because this parameter provides an assessment of disease presence in respect to attachment loss/bone loss measurements which reflect past experience of periodontitis **(Tonetti & Claffey, 2005).** Periodontal therapy in the periodontal disease group was successful based on the changes in the mean of PPD over time. Mean PPD at baseline was 4.2 ± 0.4 mm indicating a moderate periodontal disease. After treatment mean PPD was 2.9 ± 0.4 mm ($p < 0.001$)

at 2 weeks and 2.5 ± 0.3 ($p < 0.001$) at 8 weeks. In the disease free group PPD at baseline in all sites was ≤ 3mm. Demographic and clinical characteristics of the study sample are shown in **table (2).**

Periodontal disease patients present reduced methylation of TNF-α in normal and diseased sites compared with healthy population. The promoter region was more methylated in disease free individuals (36.6% ± 9.2), compared to normal & periodontitis sites of the periodontal disease group (34.1% ± 5.2 and 31.4% ± 7.8 respectively), indicating a relatively more active gene expression of TNF-α in the state of disease. Methylation status remained almost stable in normal sites throughout the evaluation period. In the periodontitis sites, however, methylation level was almost stable up to 2 weeks. Afterward, it started to rise reaching 33.7% ± 7.4 at 8 weeks, which is quite close to that reported in normal sites at baseline. Nonetheless, these results were not statistically significant (P value > 0.05), neither among groups nor among sites.

IFN-γ present increased methylation within periodontitis tissues. The promoter region of IFN-γ gene was "*hypermethylated*" among all entities, with the disease free group displaying the highest methylation percentage among all (88.6% ± 1.4) reflecting a decreased gene expression of IFN-γ in the state of periodontal health. Regarding the periodontal disease group, the level of methylation in periodontitis sites was almost comparable to disease free individuals (88.3% ± 2.1), while normal sites exhibited a slightly lower methylation (85.8% ± 6.5), implying a down-regulatory mechanism in periodontitis sites to cease further destruction of periodontal tissues. Through time, DNA methylation in periodontitis sites started to decline, reaching at 8 weeks a percentage almost equivalent to that reported in normal sites at baseline (86.2% ± 3.8). On the other hand, methylation levels started to increase in normal sites, approaching a level at 8 weeks a comparable to the disease free group (88.8% ± 2.8). None of these findings was statistically significant (P value > 0.05).

Periodontal therapy reduces the methylation of COX-2 promoter. Unlike TNF-γ and IFN-γ, COX-2 promoter region was highly "*hypomethylated*" in periodontium of disease free patients (6.7% ± 7.6). DNA methylation was nearly as twice as high in the periodontitis sites (13.2% ± 7.3), while on normal tissues from periodontal disease patients it was 8.8% ± 5.7. High levels of COX-2 methylation in periodontitis sites can render an intrinsic protective mechanism capable of preventing the unrestrained breakdown of the periodontium. Such discrepancies in the mean of methylation between normal and periodontitis sites in comparison to free disease group at baseline were statistically significant (P=0.47 for normal sites vs disease free group, P=0.03 for periodontitis sites vs disease free group). Following periodontal treatment, methylation percentage decreased dramatically in periodontitis sites, at 2 weeks, almost to half (5.8% ± 3.2, P <0.001 vs baseline), with a very slight rise from 2 weeks to 8 weeks (7.2% ± 4.3, P=0.004 vs baseline), which is still higher than the methylation level reported in the disease free group. On the contrary, the mean percentage of methylation steadily declined in normal sites, reaching 5% ± 1.9 at 8 weeks, P=0.03 vs baseline). High levels of COX-2 methylation at baseline in the periodontal disease group can render an intrinsic protective mechanism capable of preventing the unrestrained breakdown of the periodontium

LINE-1 promoter methylation in periodontal disease. To evaluate the DNA methylation at a broader level, we explored the methylation profile of LINE-1 promoter region. We observed that the mean percentage of methylation was similar between the disease free group (69.5% ± 3.5) and normal sites in diseased patients (69.4% ± 2.1), and remained fairly stable during the observation period. Nevertheless, periodontitis sites underwent increased methylation of the promoter region (70.2% ± 2.8), showing an increase in the methylation percentage at 8 weeks (71.7% ± 2.8). The

variations in the methylation profile between different entities were not statistically significant (P value > 0.05).

Mean methylation percentages of the selected genes and p values are summarized in **table 3.** DNA methylation patterns for each gene among different groups and in different points of time are demonstrated in **figure 2.**

Conclusions

Here we provide for the first time evidence of epigenetic modifications mediated by periodontal therapy. Due to the well-established association of methylation with stable gene regulation, we chose to evaluate the status of DNA methylation instead of the acetylation of genes related to inflammation **(Bäckdahl et al, 2009).**

Our study presents the expression pattern of methylated genes before periodontal therapy and during the two months follow-up. Since alterations in the methylation patterns might vary from site to site within the same individual **(Barros & Offenbacher, 2014),** we assessed the epigenetic modifications in normal and periodontitis sites within the same chronic periodontitis patient. We also compared our data to tissue samples from healthy periodontium. Our study gave emphasis to the methylation status of the tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), and the cyclooxygenase-2, (COX-2, also known as prostaglandin-endoperoxide synthase 2 or PTGS2) as key genes associated with periodontal disease progression. We also carefully selected the long interspersed nuclear element-1 (LINE-1) gene as a genome-wide readout for methylation. Of note, our cohort of patients includes Caucasians of Italian descent, and careful interpretation of the results must take into consideration the potential ethnicity influence over epigenetic modifications **(Kwabi-Addo et al, 2010; Straughen et al, 2015).**

Based on the findings of the present study, we can conclude that; (i) periodontal treatment does influence the DNA methylation pattern of various inflammatory genes, (ii) in periodontitis sites, the promoter region of different inflammatory genes was either hypo- or hypermethylated, depending on the activation of a down-regulatory mechanism to limit ongoing destruction of periodontal tissues, (iii) periodontal therapy is not sufficient to reset the methylation levels of inflammatory genes in periodontitis sites to health levels of periodontitis-free patients, at least on the short term. However, DNA methylation in periodontitis sites was restored to that of normal sites. (iv) The methylation profile from normal tissues was either restored or not to healthy patient levels, depending on the inflammatory gene, which suggests that unaffected sites from periodontal disease patients are more prone to periodontal tissue breakdown with the presence of other factors and thus should frequently be monitored within a tailored maintenance phase of periodontal therapy. In fact, such information might help to better elucidate disease development in healthy sites in susceptible periodontitis patients, which needs to be further investigated for implementing appropriate intervention plans **(Mdala et al, 2014).**

On a global methylation perspective, periodontally-diseased sites showed a different DNA methylation profile in comparison to normal sites and disease free patients, even after periodontal treatment. These findings suggest that the local effects of the disease have an influence on the epigenetics of the tissues that may be modulated by environmental factors, including the microbiota. In this context, future investigations should further explore the identification of specific factors that affect the local epigenetics of periodontal soft and hard tissues.

Despite these findings, the results of the study must be interpreted with caution as it has certain limitations. Gingival biopsies of normal and inflamed tissues were harvested from different sites and not the same one during different points of time. Sample collection from the same site is preferable when evaluating the influence of periodontal therapy over time, however, that wasn't possible because 2 weeks after periodontal therapy soft tissues at the biopsy site were not completely healed. Also, findings of this study represent data on the short-term. Thus, epigenetic changes still need to be monitored on the long-term to understand if the methylation status in chronic periodontitis could turn out similar to that in healthy individuals with no history of periodontitis, following an effective periodontal therapy, maintenance, and compliance.

References

Adcock, I. M., Tsaprouni, L., Bhavsar, P. and Ito, K. (2007) Epigenetic regulation of airway inflammation. *Current Opinion in Immunology* **19**, 694–700

Andia, D. C., de Oliveira, N. F. P., Casarin, R. C. V, Casati, M. Z., Line, S. R. P. and de Souza, A. P. (2010) DNA methylation status of the IL8 gene promoter in aggressive periodontitis. *Journal of Periodontology* **81**,1336–1341

Andia, D. C., Planello, A. C., Portinho, D., da Silva, R. A., Salmon, C. R., Sallum, E. A., Junior, F. H. N. and de Souza, A. P. (2015) DNA methylation analysis of SOCS1, SOCS3, and LINE-1 in microdissected gingival tissue. *Clinical Oral Investigations* **19**, 2337–2344

Bäckdahl, L., Bushell, A. and Beck, S. (2009) Inflammatory signalling as mediator of epigenetic modulation in tissue-specific chronic inflammation. *International Journal of Biochemistry and Cell Biology*. **41**, 176–184

Baptista, N. B., Portinho, D., Casarin, R. C. V, Vale, H. F., Casati, M. Z., De Souza, A. P. and Andia, D. C. (2014) DNA methylation levels of SOCS1 and LINE-1 in oral epithelial cells from aggressive periodontitis patients. *Archives of Oral Biology*. **59**, 670–678

Barros, S. P. and Offenbacher, S. (2014) Modifiable risk factors in periodontal disease: epigenetic regulation of gene expression in the inflammatory response. *Periodontology 2000* **64**, 95–110

Bird, A. (2002) DNA methylation patterns and epigenetic memory. *Genes and Development* **16**, 6–21

Borrell, L. N. and Papapanou, P. N. (2005) Analytical epidemiology of periodontitis. *Journal of Clinical Periodontology* **32**, 132–158

Bollati, V., Baccarelli, A., Hou, L., Bonzini, M., Fustinoni, S., Cavallo, D., Byun, H. M., Jiang, J., Marinelli, B., Pesatori, A. C., Bertazzi, P. A. and Yang, A. S. (2007) Changes in DNA methylation patterns in subjects exposed to low-dose benzene. *Cancer Research* **67**, 876–880

El Gazzar, M., Yoza, B. K., Hu, J. Y.-Q., Cousart, S. L. and McCall, C. E. (2007) Epigenetic silencing of tumor necrosis factor alpha during endotoxin tolerance. *The Journal of biological chemistry* **282**, 26857–26864

Fitzpatrick, D. R. and Wilson, C. B. (2003) Methylation and demethylation in the regulation of genes, cells, and responses in the immune system, *Clinical Immunology* **109**, 37–45

Ishida, K., Kobayashi, T., Ito, S., Komatsu, Y., Yokoyama, T., Okada, M., Abe, A., Murasawa, A. and Yoshie, H. (2012) Interleukin-6 Gene Promoter Methylation in Rheumatoid Arthritis and Chronic Periodontitis. *Journal of Periodontology* **83**, 917–925

Jenuwein, T. and Allis, C. D. (2001) Translating the histone code. *Science (New York, N.Y.)* **293**, 1074–1080

Kornman, K. S. (2008) Mapping the pathogenesis of periodontitis: a new look. *The Journal of periodontology* **79**, 1560–1568

Kwabi-Addo, B., Wang, S., Chung, W., Jelinek, J., Patierno, S. R., Wang, B. D., Andrawis, R., Lee, N. H., Apprey, V., Issa, J. P. and Ittmann, M. (2010) Identification of differentially methylated genes in normal prostate tissues from African American and Caucasian men. *Clinical Cancer Research* **16**, 3539–3547

Larsson, L., Castilho, R. M. and Giannobile, W. V. (2015) Epigenetics and its role in periodontal diseases: a state-of-the-art review. *Journal of periodontology* **86**, 556–568

Lod, S., Johansson, T., Abrahamsson, K. H. and Larsson, L. (2014) The influence of epigenetics in relation to oral health. *International Journal of Dental Hygiene* **12**, 48–54

Martins, M. D., Jiao, Y., Larsson, L., Almeida, L. O., Garaicoa-Pazmino, C., Le, J. M., Squarize, C. H., Inohara, N., Giannobile, W. V. and Castilho, R. M. (2016) Epigenetic Modifications of Histones in Periodontal Disease. *Journal of Dental Research* **95**, 215–222

Mdala, I., Olsen, I., Haffajee, A. D., Socransky, S. S., Thoresen, M. and De Blasio, B. F. (2014) Comparing clinical attachment level and pocket depth for predicting periodontal disease progression in healthy sites of patients with chronic periodontitis using multi-state Markov models. *Journal of Clinical Periodontology* **41**, 837–845

Ngollo, M., Dagdemir, A., Karsli-Ceppioglu, S., Judes, G., Pajon, A., Penault-Llorca, F., Boiteux, J.-P., Bignon, Y.-J., Guy, L. and Bernard-Gallon, D. J. (2014) Epigenetic modifications in prostate cancer. *Epigenomics* **6**, 415–426

Oliveira, N. F. P., Damm, G. R., Andia, D. C., Salmon, C., Nociti, F. H., Line, S. R. P. and De Souza, A. P. (2009) DNA methylation status of the IL8 gene promoter in oral cells of smokers and non-smokers with chronic periodontitis. *Journal of Clinical Periodontology* **36**, 719–725

Page, R. C. (1991) The role of inflammatory mediators in the pathogenesis of periodontal disease. *Journal of Periodontal Research* **26**, 230–242

Page, R. C. and Kornman, K. S. (1997) The pathogenesis of human periodontitis: an introduction. *Periodontology 2000* **14**, 9–11

Robertson, K. D. and Wolffe, A. P. (2000) DNA methylation in health and disease. *Nature reviews. Genetics* **1**, 11–19

Sanders, V. M. (2006) Epigenetic regulation of Th1 and Th2 cell development. *Brain, Behavior, and Immunity* **20**, 317–324

Schulz, S., Immel, U. D., Just, L., Schaller, H. G., Gläser, C. and Reichert, S. (2016) Epigenetic characteristics in inflammatory candidate genes in aggressive periodontitis. *Human Immunology* **77**, 71–75

Shaw, R. (2006) The epigenetics of oral cancer. *International Journal of Oral and Maxillofacial Surgery* **35**, 101–108

Straughen, J. K., Sipahi, L., Uddin, M., Misra, D. P. and Misra, V. K. (2015) Racial differences in IGF1 methylation and birth weight. *Clinical Epigenetics* **7**, 47

Takashiba, S. and Naruishi K. (2006) Gene polymorphisms in periodontal health and disease. *Periodontology 2000* **40**, 94–106

Tarantini, L., Bonzini, M., Tripodi, A., Angelici, L., Nordio, F., Cantone, L., Apostoli, P., Bertazzi, P. A. and Baccarelli, A. A. (2013) Blood hypomethylation of inflammatory genes mediates the effects of metal-rich airborne pollutants on blood coagulation. *Occupational and Environmental Medicine* **70**, 418–25

Tonetti, M.S. and Claffey, N. (2005) European Workshop in Periodontology group C. Advances in the progression of periodontitis and proposal of definitions of a periodontitis case and disease progression for use in risk factor research. Group C consensus report of the 5th European Workshop in Periodontology. *Journal of Clinical Periodontology* **32**, 210-213

Wilson, A. G. (2008) Epigenetic regulation of gene expression in the inflammatory response and relevance to common diseases. *Journal of Periodontol*ogy **79**, 1514–1519

Zhang, S., Crivello, A., Offenbacher, S., Moretti, A., Paquette, D. W. and Barros, S. P. (2010 a) Interferon-gamma promoter hypomethylation and increased expression in chronic periodontitis. *Journal of Clinical Periodontology* **37**, 953–961

Zhang, S., Barros, S. P., Niculescu, M. D., Moretti, A. J., Preisser, J. S. and Offenbacher, S. (2010 b) Alteration of PTGS2 promoter methylation in chronic periodontitis. *Journal of Dental Research* **89**, 133-137

Table 1. Primers Used for DNA methylation analysis and PCR cycling conditions

Sequence ID	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Sequencing Primer (5' to 3')	Sequence analyzed* (5' to 3')	Annealing conditions	Fragment size (bp)	Chr and CpG positions
Repetitive element methylation analysis							
LINE-1	TTTTGAGTTAGGTGTGGGATATA	Biotin-AAAATCAAAAAATTCCCTTTC	AGTTAGGTGTGGGATATAGT	TTC/TGTGGTGC/TGTC/TG	50°C for 30 seconds	146	
Gene-specific methylation analysis							
COX-2	GGAGATTAGTTTAGAATTGGTTTT	Biotin-AATCCCCACTCTCCTATCTAATCC	AAGAAGAAAAGATATTTGG	C/TGGAAATTTGTGC/TGTTTGGGGC/TGGTGGAATTC/TGGGG	59°C for 60 seconds	139	Chr1 Pos1: 186649540 Pos2: 186649552 Pos3: 186649561 Pos4: 186649570
IFN-γ	Biotin-GTTTTTTGGATTTGATTAGTTTGA	CAATAACAACCAAAAAAACCCA	TATAACTTATATATTTCATC	G/ATTTCCG/AAAAAAATTAAACC	54°C for 60 seconds	143	Chr12 Pos1: 66840192 Pos2: 66840186
TNF-α	Biotin-TGAGGGGTATTTTTGATGTTTGT	CCAACAACTACCTTTATATATCCC	ATAAACCCTACACCTTCTAT-	CTCA/GATTTCTTCTCCATCA/GCA/GAAAACA/GAAAA	57°C for 60 seconds	208	Chr 6 Pos1:31651172; Pos2:31651157; Pos3:31651155; Pos4: 31651149

* Nucleotides at which DNA methylation was measured are underlined

* Chr = Chromosome, Pos= Position

Table 2. Demographic and clinical characteristics of the study sample

Demographic/Clinical characteristics	**Disease Free Group (n =10)**	**Periodontal Disease Group (n =10)**		
Males/Females	7/3	3/7		
Age (years) (Mean ± SD)	53.3 ± 12.3	46.6 ± 10.0		
Probing Depth (mm) (Mean ± SD)	*At baseline*	*At baseline*	*Two weeks after periodontal treatment*	*Eight weeks after periodontal treatment*
	≤ 3	4.2 ± 0.4	2.9 ± 0.4	2.5 ± 0.4
		Reference	P <0.001	P <0.001

*P-values from random intercept linear regression model.

Table 3. Methylation of selected genes across groups and over time. Results of random-intercept linear regression models

		Methylation percentage (Mean ± SD)		
Genes	***Group***	***At baseline***	***Two weeks after periodontal treatment***	***Eight weeks after Periodontal treatment***
TNF-α	Disease Free	36.6 ± 9.2	-	-
	Periodontal Disease/Normal Sites	34.1 ± 5.2	33.9 ± 6.5	34.0 ± 9.3
		P=0.44 vs Disease free	*P=0.92 vs baseline*	*P=0.90 vs baseline*
	Periodontal Disease/ Periodontitis Sites	31.4 ± 7.8	31.5 ± 8.5	33.7 ± 7.4
		P=0.11 vs Disease free	*P=0.97 vs baseline*	*P=0.50 vs baseline*
IFN-γ	Disease Free	88.6 ± 1.4	-	-
	Periodontal Disease/Normal Sites	85.8 ± 6.5	87.7 ± 3.5	88.8 ± 2.8
		P=0.10 vs Disease free	*P=0.33 vs baseline*	*P=0.14 vs baseline*
	Periodontal Disease/ Periodontitis Sites	88.3 ± 2.1	88.4 ± 2.2	86.2 ± 3.8
		P=0.85 vs Disease free	*P=0.89 vs baseline*	*P=0.08 vs baseline*
COX-2	Disease Free	6.7 ± 7.6	-	-
	Periodontal Disease/Normal Sites	8.8 ± 5.7	6.6 ± 3.6	5.0 ± 1.9
		P=0.47 vs Disease free	*P=0.18 vs baseline*	***P =0.03 vs baseline***
	Periodontal Disease/ Periodontitis Sites	13.2 ± 7.3	5.8 ± 3.2	7.2 ± 4.3

		P =0.03 vs Disease free	***P <0.001 vs baseline***	***P=0.004 vs baseline***
LINE-1	Disease Free	69.5 ± 3.5	-	-
	Periodontal Disease/ Normal Sites	69.4 ± 2.1	70.0 ± 2.3	69.5 ± 1.4
		P=0.92 vs Disease free	*P=0.36 vs baseline*	*P=0.91 vs baseline*
	Periodontal Disease/ Periodontitis Sites	70.2 ± 2.8	70.7 ± 2.8	71.7 ± 2.8
		P=0.58 vs Disease free	*P=0.61 vs baseline*	*P=0.22 vs baseline*

*P-values calculated with random-intercept linear regression models.

Figure 1. CONSORT Flow Diagram of the Study Sample

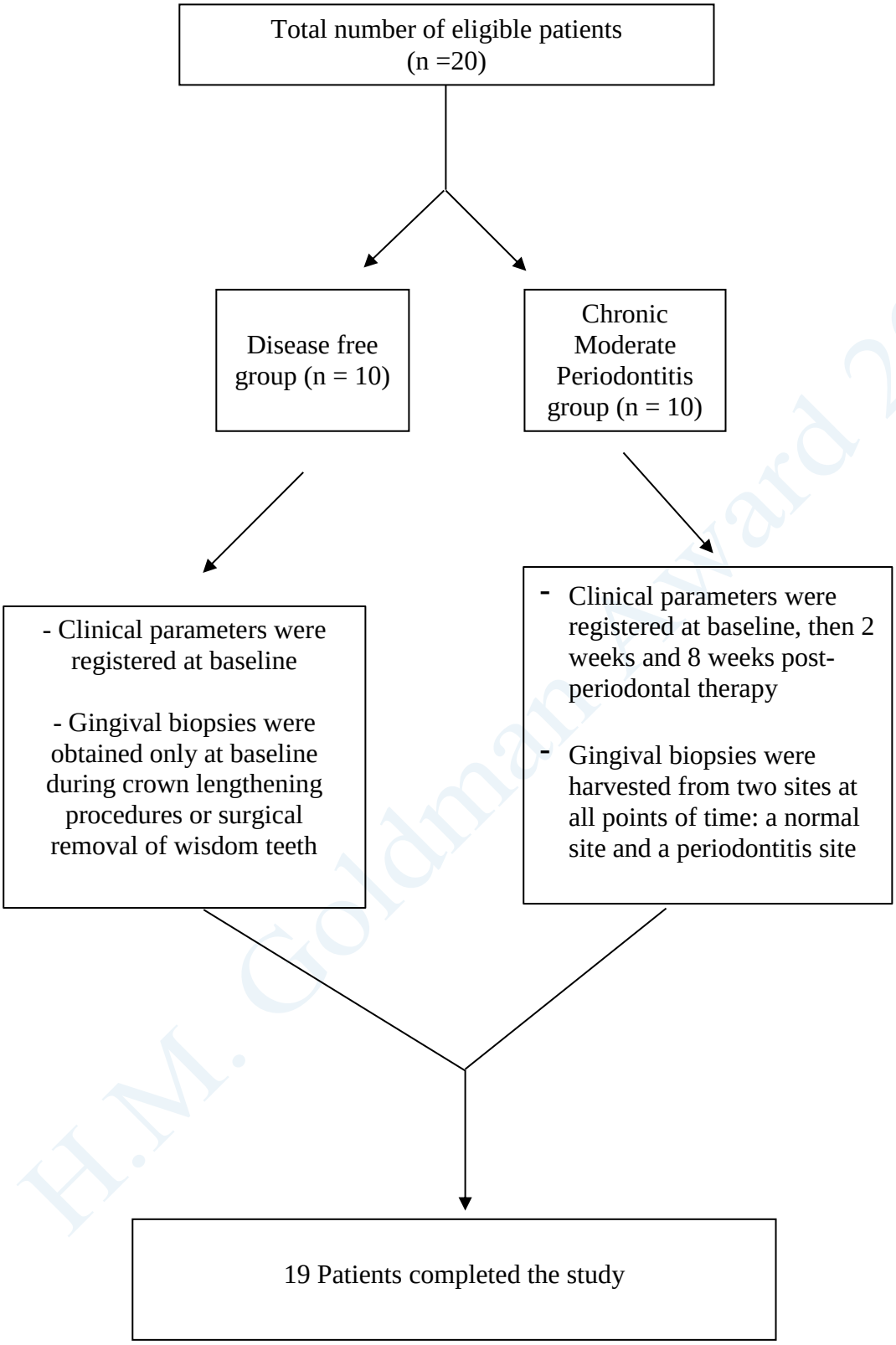
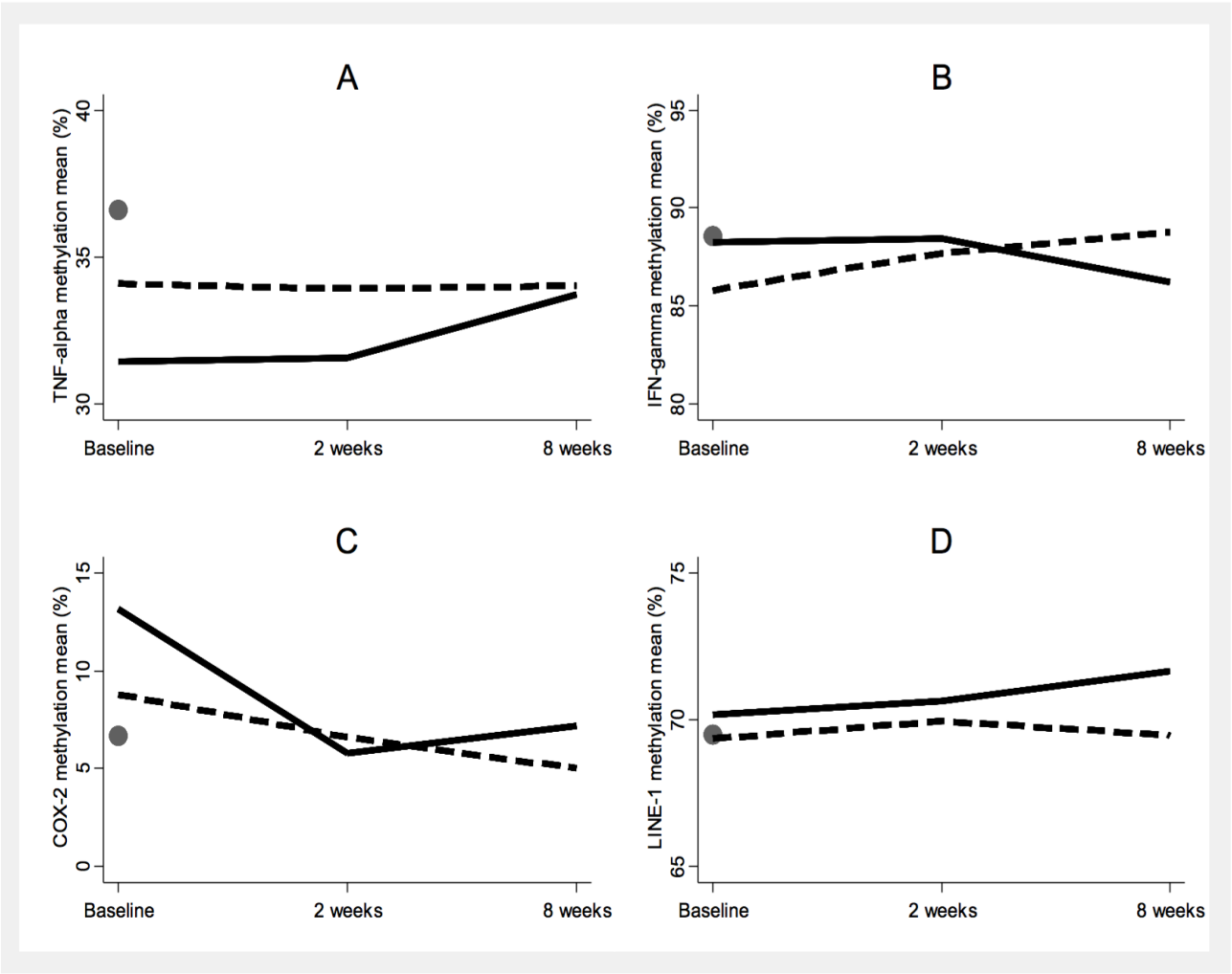


Figure 2. DNA methylation patterns among different groups and at different points of time, for TNF-α, IFN- γ, COX-2 and LINE-1



Circle: Disease-free group

Dashed line: Periodontal disease group, normal sites

Continuous line: Periodontal disease group, periodontitis sites

EFFECT OF AN ENAMEL MATRIX DERIVATIVE ON WOUND HEALING FOLLOWING GINGIVAL RECESSION COVERAGE: A RANDOMIZED, CONTROLLED, CLINICAL STUDY

Effetto dell'amelogenina sulla guarigione dei tessuti molli dopo copertura di recessioni gengivali: lavoro pilota randomizzato e controllato

Jean-Claude Imber*, Alexandra Stähli*, Elena Raptis, Giovanni E. Salvi, Sigrun Eick, Anton Sculean

med. dent. Jean-Claude Imber, Department of Periodontology, University of Bern, Switzerland
Dr. med. dent. Alexandra Stähli, Department of Oral Biology, Medical University of Vienna, Austria
med. dent. Elena Raptis, Department of Periodontology, University of Bern, Switzerland
Prof. Dr. med. dent. Giovanni E. Salvi, Professor and Vice Chairman, Department of Periodontology, University of Bern, Switzerland
Prof. Dr. med. dent. Sigrun Eick, Laboratory of Oral Microbiology, Department of Periodontology, University of Bern, Switzerland
Prof. Dr. med. dent. Anton Sculean, Professor and Chair, Department of Periodontology, University of Bern, Switzerland
**these authors contributed equally*

Running head: Gingival Recession Coverage and Emdogain
Key words: gingival recession, enamel matrix derivative, mucogingival surgery

Abstract

Aim: The aim of this randomized, controlled, single blinded clinical study was to investigate clinically and immunologically the potential effects of Emdogain on early wound healing and clinical results following treatment of gingival recessions.

Materials and Methods: A total of 40 healthy patients with Miller class I to III single or multiple gingival recessions were treated with the modified coronally advanced tunnel technique (MCAT) + subepithelial connective tissue (CTG) with or without Emdogain. Patients were consecutively enrolled and randomly assigned to Emdogain or control. Inflammatory markers and were measured at baseline, 2 days, and 1 week postoperatively. Early wound healing was expressed by a newly described Recession Healing Index (RHI). Clinical parameters were assessed at baseline, 2 weeks and 6 months postoperatively. Patient-reported outcomes were analyzed with a visual analogue scale.

Results: No statistically significant differences were detected between the 2 groups in terms of RHI, inflammatory markers and patient-reported outcomes during early wound healing. At six months following treatment, mean root coverage amounted to 78% for the test and 77% for the control group, respectively, without any statistically significant differences.

Conclusion: Within their limits, the present data have failed to show an influence of EMD on clinical and immunological parameters related to wound healing following recession coverage surgery using MCAT and CTG.

Riassunto

Scopo: Lo scopo di questo lavoro randomizzato e controllato fu di verificare se l'uso di amelogenina (Emdogain, EMD) in aggiunta alla tecnica di tunnel modificata (TM) con innesto di connettivo (TC) abbia degli effetti sulla guarigione dopo copertura di recessioni gengivali.

Materiale e metodi: In 40 pazienti con recessioni gengivali di classe Miller I-III, la tecnica di TM con innesto di TC fu utilizzata con o senza l'aggiunta di EMD. I pazienti furono arruolati e randomizzati nel gruppo test (EMD) oppure controllo (senza EMD). Marcatori infiammatori furono prelevati dal solco gengivale al giorno 0 (chirurgia) e dopo 2 e 7 giorni. I parametri clinici furono analizzati 2 settimane e 6 mesi dopo l'intervento. Un nuovo indice di guarigione dopo copertura di recessioni gengivali fu introdotto (Recession Healing Index, RHI). I parametri riportati dai pazienti furono analizzati tramite scala visuale analoga fino a 6 mesi.

Risultati: Dopo 6 mesi, la copertura media delle recessioni gengivali ammontava a 78% nel gruppo test e a 77% nel gruppo controllo ($p>0.05$). Nessuna differenza significativa ($p>0.05$) fu trovata nei marcatori infiammatori durante la guarigione tra gruppo test e gruppo controllo. L'analisi del RHI non rivelò nessuna differenza significativa tra i due gruppi.

Conclusione: I risultati di questo lavoro pilota indicano che l'aggiunta di EMD non influenza positivamente i parametri clinici ed infiammatori durante la guarigione dopo copertura di recessioni gengivali.

Introduction

Gingival recession is the exposure of the root surface due to displacement of the gingival margin apical to the cementoenamel junction and can affect the labial, lingual and/or interproximal areas (Wennstrom, 1996). This root exposure is

frequently associated with "wedge-shaped" defects at the crevicular area (Sangnes and Gjermo, 1976), aesthetic impairment, predilection to root caries, root hypersensivity, and difficulties to achieve optimal plaque control (Susin et al., 2004), (Daprile et al., 2007), (Serino et al., 1994), (Lovegrove and Leichter, 2004), (Allen and Miller, 1989).

Single and multiple gingival recessions can be successfully treated by means of coronally advanced flap (CAF) or the modified coronally advanced tunnel (MCAT) in combination with subepithelial connective tissue grafts (CTG) (Aroca et al., 2013, Cairo et al., 2014, Graziani et al. 2014, Sculean et al., 2014, 2016)) while recent evidence indicates that both techniques can lead to comparable outcomes (Azaripour et al., 2016).

An enamel matrix derivative (EMD) has been shown to promote periodontal regeneration by mimicking the embryonic development of the periodontal tissues (Hammarstrom, 1997), (Gestrelius et al., 2000), (Bosshardt, 2008). Clinically, EMD is used for periodontal regeneration at teeth affected by periodontitis (i.e., 2- or 3-wall intrabony defects, Class II

furcation defect), root coverage procedures, and tooth replantation (Miron et al., 2016). Histologically, the additional use of EMD with CTG in root coverage procedures results primarily in connective tissue adhesion to the root surface and a short junctional epithelium (Miron et al., 2016). Interestingly, a plethora of clinical observations and data from experimental studies have indicated that the application of EMD in conjunction with flap surgery may result in an accelerated wound healing and less inflammation compared to placebo treated sites thus pointing to its clinical relevance in modulating early wound healing (Okuda et al., 2001, Miron et al., 2016).

However, according to the best of our knowledge, until now, no randomized controlled study has evaluated the potential effects of EMD following recession coverage with CTG focusing on clinical and immunological parameters related to wound healing.

The aim of this prospective, randomized, controlled, clinical study was therefore, to characterize clinically and immunologically the early wound healing events and clinical outcomes following treatment of Miller Class I, II or III recessions by means of MCAT with and without application of EMD.

Materials and Methods

Study population

Forty patients with single or multiple Miller Class I, II or III (Miller 1985) gingival recessions were enrolled in this study. All the included patient signed an informed consent. This study protocol was in accordance with the moral, ethical and scientific principles governing clinical research as set out in the current version of the Declaration of Helsinki. Exclusion criteria comprised age < 16 years, plaque score over 25% (O'Leary 1972), history of chronic infectious or inflammatory diseases (i.e. rheumatoid arthritis, systemic lupus erythematodes, Crohn's disease, or HIV-, HCV- infection etc.), any clinical signs of an acute infection, renal failure (GFR < 30ml/min), current smoking (> 5 cigarettes per day).

Study Design

This prospective, randomized, single blinded clinical trial (Trial registration number: NCT02230787) was conducted and included a total of 40 patients undergoing elective root coverage. Upon approval of the local ethics committee (KEK-186-13- PRR-2015079) patients were randomly assigned to test (EMD + CTG) or control (CTG). The individuals who were included in the study were de-personalized for evaluation of all data, the analysis of crevicular samples and assessment of clinical data were performed in a blinded fashion.

Clinical data were recorded at baseline, at 2 days, 7 days, 14 days and 6 months after surgery, gingival crevicular fluid (GCF) was obtained at baseline, 2 and 7 days.

Surgical procedure

All surgeries were performed by one experienced periodontist (A.S.). All controls and samplings were performed by two investigators (A.St + J.-C. I.) who were blinded to the test or control group. There were examinations right before surgery, at 2 days, 7 days, 14 days and 6 months. The patients were treated with (MCAT) in combination with CTG (Fig. 1). In the test group the CTG, donor site and graft site were covered with EMD (Emdogain, Straumann AG, Switzerland), Before graft insertion into the tunnel, the roots of the test

group were treated with a pH neutral, 24% EDTA root surface conditioner (Straumann® PrefGel, Straumann AG, Switzerland) for 2 minutes. In the control group, no EMD was used. Follow-up visits will be performed as described below. The flow chart of the study is summarized in Figure 2.

Effectiveness parameters: measuring and times

The baseline examination included measuring of the periodontal probing depth (PPD) at the involved tooth, periodontal screening record (PSR) (AAP, ADA 1992), recession from the CEJ (mm), recording of fillings at the involved tooth). Furthermore, we assessed whether remnants of EMD were still detectable in the wound fluid at two days after surgery.

Wound healing after root coverage procedure was assessed (2 days, 7 days, 14 days) using the following, newly proposed early wound healing index (Recession Healing Index – RHI) (Fig. 3).

Wound healing was quantified and graded into 4 stages: 0:
complete wound closure, no fibrin, no suppuration

1: complete wound closure, thin fibrin-layer on the wound, no suppuration

2: incomplete wound closure, thick fibrin-layer on the wound, slight suppuration 3: incomplete wound closure, suppuration or abscess formation

At the 6 months control, the examinators measured the distance from the Gingiva to the CEJ and the PPD at the involved tooth and calculated the percentage of root coverage (Fig. 4). Additionally, the post-operative healing process was judged by a patient questionnaire.

GCF samples

GCF was sampled by using the extracrevicular method to avoid traumatization. Paper strips (Periopaper, Oraflow Inc., Smithtown, NY, USA) were overlaid placed at the gingival crevice region and left in place for 30 s. Immediately after collection, samples were stored at -80°C until analyzed.

Before analyzing, GCF samples were eluted at 4°C overnight into 750 µl phosphate-buffered saline containing proteinase inhibitors (Sigma-Aldrich, Buchs, Switzerland). From the eluates, the levels of interleukin (IL)-1β, IL-8, IL10, matrixmetalloprotease (MMP)-8 and TGF-β1 were determined by using commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems Europe Ltd., Abingdon, UK) according to the manufacturer's instructions. Both active and total TGF-β1 were measured. For determination of total TGF-β1, samples were preheated at 99°C for 1 min. The detection levels of the kits were 1 pg/site for IL-1β, IL-8, IL-10, TGF-β1 and 100 pg/site for MMP-8.

Statistical methods

The primary outcome was the progress of wound healing assessed by the "Recession Healing Index (RHI) at day 2, 7 and 14 after surgery.

The GCF levels of the biomarkers IL- 8, IL-10, IL-1beta, MMP-8 and TGF beta-1 at

2 days and 1 week after the surgery. Moreover, the traceability of EMD in the operation field was investigated two days after surgery. The patients' post-operative comfort assessed after 2 days, 1 and 2 weeks and 6 months postoperatively (documented by questionnaire) was analyzed. The root coverage in mm assessed 6 months after surgery completed the secondary outcomes.

Normality of the distribution was evaluated assessing skewness and kurtosis and applying the Kolmogorov-Smirnov test. All continuous variables were presented as means ±SD when normally distributed and as medians and interquartile ranges when not normally distributed. Categorical variables were given as frequencies and percentages. Continuous variables were tested for differences with the Wilcoxon signed-rank test. Categorical variables were tested by the Pearson s 2 test or the Fisher s exact test as appropriate. The differences between patients in the treatment groups were determined at each time point using the Mann-Whitney U test.

Surgical results were correlated to complications and inflammatory markers using Spearman's rank correlation, if appropriate. All statistical analyses were performed with the use of JMP (SAS Institute Inc., Cary, NC). For all tests, a two-sided $p < 0.05$ was considered statistically significant.

Results

Study participants

Patient recruitment started in September 2014 and ended in June 2016. A baseline screening was performed in totally 42 patients of whom 40 (29 females, 11 males) were entered in the study all fulfilling the inclusion criteria. The eligible patients were randomized equally into test (14 females, 6 males) and control groups (15 females, 5 males). One patient in the control group was lost during the follow up and only 2 weeks follow up data were available for this individual. Thus, only 39 patients completed the 6 months follow-up. All included surgeries were performed by the same experienced clinician (A.S.) using MCAT and CTG either with or without the adjunctive use of EMD. No systemic side effects were recorded. A total of 6 patients (3 in each treatment group) received systemic antibiotic (Amoxicillin) therapy in the first 2 weeks following surgery. The reason for the prescription of antibiotics was suppuration or abscess formation during the first postoperative week.

Baseline characteristics

Description of patient demographics and baseline data for all patients in both study groups are depicted in table 1. There were no significant differences between groups regarding age and ethnicity. There were no statistical significant differences in the periodontal screening index (PSI), recession dimensions and Miller classes. Mean PSI values for test and control group were 1.35 (⍰0.75) and 0.99 (⍰0.75). Mean recession length before surgery was 4.07 mm (⍰1.28) for the test and 4.47 (⍰2.08) for the control group. Ten patients showed gingival defects in the maxilla and 30 patients had defects in the mandible. Of the involved sites 36 were located at anterior teeth and 4 at bicuspids (3 in the mandible, 1 in the maxilla).

Recession healing index

The recession healing index (RHI) was taken at the recession site as well as at the donor site of the CTG in the palate. At 2 days the average RHI at the recession site for test and control group was 2.10 (±0.72) and 1.85 (±0.67) respectively. At 7 and 14 days the values had decreased to 1.20 (±0.89) and 0.40 (±0.50) for the test, respectively to 1.30 (±0.80) and 0.55 (±0.61) for the control group with no statistically significant difference among the groups. For the palate the respective values at 2, 7 and 14 days were 1.42 (±0.51), 1.00 (±0.86), and 0.40 (±0.68) for the test group. The corresponding values for the control group were 1.70 (±0.83), 1.45 (±0.83), and 0.35 (±0.59) (Table 2). Data analysis by the Mann-Whitney-U test revealed no statistically significant variations among the groups (2 days recession: p=0.259, palate p= 0.083; 7 days recession: p=0.676, palate: p=0.078; 14 days recession: p=0.456 and palate: p=0.920).

Biomarkers

Gingival crevicular samples were harvested at baseline, 2 and 7 days after surgery and assessed for IL 8, IL-1β, IL 10, and MMP 8. In both groups IL 8 and IL-1β levels increased statistically significantly at 2 days when compared to baseline values. They decreased at 7 days being still statistically significantly higher than at baseline. In the test group, the IL10 levels decreased slightly (p=0.047) at day 7 when compared with day 2, in the control group they were lower at day 2 than at baseline (p=0.046). In both groups MMP8 levels were increased at day 2 compared to baseline (test group: p<0.001, control group: p=0.015). In the test group MMP8 levels were decreased at day 7 when compared to day 2 (p=0.044), but still higher than at baseline (p=0.023). There was never a statistically significant difference between test and control group at any time and for any biomarker. Results are presented in Figure 4.

GCF samples were further analyzed for active and total TGF-β1 levels. Positive results were assessed only at day 2, total TGFβ1 was detectable in five samples (among them four in the test group) and active TGF-β1 in one sample of the test group.

Soft tissue parameters

The values of the soft tissue parameters at baseline and the 6 months follow-up are shown in table 3. Both groups demonstrated statistically significant improvement in the coverage of the recession compared to baseline values with no significant intergroup differences. The mean recession length at baseline was 4.475 mm for the control group and 4.075 mm for the test group, the recession width 2.85 mm and 3.4 mm. Recession length after recession coverage shrunk to 1.00 and 0.944 mm respectively. The respective values expressed as percentage of root coverage at 6 months revealed an overall root coverage rate of 78.0% (±22.6) with 78.7% (±26.8) for the test and 77.4% (±18.1) for the control group, whereby complete root coverage was obtained in 8 test and 3 control subjects. Mean width of keratinized tissue at baseline was 1.125 mm for the control and 1.075 mm for the test group. The changes between BL and 6 month follow up showed a mean increase of 0.822 and 0.814 mm. No statistically significant differences were found between the test and the control group for the changes between baseline and the follow up period.

Patient-reported outcomes

VAS scores were evaluated at 2, 7 and 14 days after surgery. Mean VAS scores at the palate for day 2 were at 2.7 for the test and 4.0 for the control group. Hereby no statistical significant difference was demonstrated. Values further declined to the 7 and 14 day follow ups for both groups (0.7 and 0.6 for the test and 1.4 and 1.1 for the control site). Mean VAS scores at the tooth site were 2.9, 2.9 and 1.1 for the test and 5.1, 3.5 and 1.6 for the control site (Fig.6).

Discussion

The present randomized controlled clinical trial has failed to show any additional effect of using EMD as an adjunct to MCAT/CTG on wound healing as assessed through clinical and inflammatory parameters. In some cases (e.g. 3 patients in each group) suppuration and/or abscesses occurred during the first two postoperative weeks but disappeared immediately following the systemic administration of antibiotics.

Periodontal wound healing/regeneration requires adequate infection control, undisturbed early wound healing and implies adhesion, migration and proliferation of inflammatory cells in order to establish a sufficient blood supply to support the healing process. In this respect it was hypothesized that the use of EMD may enhance early wound healing and, in the same time, decrease post-operative complication rates. To assess early wound healing, a novel clinical index, the RHI, was proposed. The RHI includes parameters such as wound closure, fibrin formation, suppuration, and abscess formation and was assessed at 2, 7, and 14 days postoperatively representing a modification of the early wound healing index described by Wachtel et al. (2003) to describe early wound healing features following regenerative surgery in intrabony defcts (Wachtel et al., 2003). The aim of the newly proposed index was to more accurately characterize early wound healing than simply by evaluating primary and secondary wound closure.

Numerous in vitro studies have extensively investigated in vitro cell responses to enamel matrix derivative (EMD) and have demonstrated a plethora of beneficial effects on periodontal wound healing and regeneration. EMD has been demonstrated to influence wound healing favouring the wound fill rates in vitro (Bosshardt, 2008, Miron et al. 2016), stimulating cell growth and metabolism as well as proliferation and migration of periodontal ligament cells (Haase and Bartold, 2001), (Gibson, 2008). Furthermore, EMD has been shown to increase the attachment rate of periodontal ligament cells by interfering with specific integrins (Hoang et al., 2000), (Suzuki et al., 2001), (Rincon et al., 2005) and to promote angiogenesis by enhancing mesenchymal and microvascular cell differentiation (Miron et al.,2016). In an oral mucosa wound model in the rat, the injection of EMD led to increased formation of blood vessels and collagen production thus improving early wound healing (Maymon- Gil et al., 2016). Although in vitro studies have provided evidence for a beneficial effect of EMD on wound healing and regeneration, it has been difficult to corroborate these findings in clinical studies. Wennström and Lindhe 2002 evaluated the application of EMD versus a carrier in a split mouth RCT in a group of patients receiving scaling and root planing. Patient-reported outcomes of up to 3 weeks favoured the application of EMD (Wennstrom and Lindhe, 2002). Tonetti et al. 2004 showed earlier gains in soft tissue densities after EMD application as well as high patient comfort. Here, soft tissue healing and patient morbidity at deep intrabony defects were evaluated(Tonetti et al., 2004). On the other hand, other studies could not show any

effects on wound healing following the use of EMD (Hagenaars et al., 2004).

Gingival crevicular fluid was sampled and analyzed for IL8, IL10, MMP8, IL1 β and TGF- β1. Frequency of detection and interleukin levels were compared both between the different time-points and the two groups. For IL8 and IL1β a statistically significant postoperative increase was noted in both groups, with a peak at day 2. Postoperative MMP8 levels were significantly increased in the EMD treated group, but remained unchanged in controls. Interleukin 10 was not increased at any time- point. The changes of inflammatory markers showed similar tendencies for both groups and can be interpreted as response to the surgical trauma with no clear tendencies among the two groups. TGF- β1 was statistically significantly increased after the procedure in only 3 samples (e.g. one test and two controls). Consistent with these findings, previous studies have shown an increase of TGF- β1 levels after EMD application. Maymon-Gil et al 2016 showed in the rat wound healing model an increase of TGF- β1 and β2, vascular endothelial growth factor, IL-1β, matrix- metalloproteinase-1, versican, and fibronectin.

Recently, microarray analyses have been performed shedding light onto cellular responses to EMD. These studies appear to support the assumption that EMD effects are partly mediated through TGF- β activity (Brett et al., 2002); (Parkar and Tonetti, 2004); (Kapferer et al., 2011); (Stahli et al., 2014).

The evaluation of soft tissue parameters 6 months after surgery revealed a similar gain of keratinized tissue for both groups while the mean coverage rates of about 80% compare well with those obtained in other studies (Cairo et al. 2014, Graziani et al. 2014).

The use of EMD related to mean root coverage and gain of keratinized tissue has also been investigated in several clinical studies.

Some of the available data appear to indicate improved clinical results in terms of root coverage following the combination of EMD + CTG (Hagewald et al., 2002), (Cueva et al., 2004). It has been shown that EMD resulted in increased gain of keratinized tissue and improved long-term stability when compared to CAF alone (Castellanos et al., 2006). Other studies failed to show superiority of Emdogain when used with CTG and CAF after one year (Hagewald et al., 2002), (Chambrone et al., 2008). However, when looking at the 2 year-results the EMD group reached 53% of root coverage compared to 23% in the control group (Spahr et al., 2005). Other investigations based on Miller's class III gingival recession defects suggested that, the use of EMD is beneficial in augmenting the effects of the CTG in terms of root coverage, gain in clinical attachment, probing depth reduction (Sato et al., 2006), (Henriques et al., 2010). Aroca et al 2010 in a split mouth study failed to show superiority of EMD when treating multiple type III recession defects (Aroca et al., 2010). However, a recent systematic review has provided evidence that the adjunctive application of EMD onto denuded root surfaces in combination with CAF may result more often in complete root coverage (CRC) and increased keratinized tissue gain (Cairo et al., 2014).

When interpreting the present findings, it should be kept in mind that the used surgical approach (e.g. MCAT) does not completely detach the flap from the underlying bone and tooth surfaces, which in turn may additionally stabilize the blood clot. On the other hand, it cannot be ruled out that a blood contamination of the root surfaces can more easily occur when this surgical approach is adopted thus making the precipitation and persistence of EMD on the root surfaces and in the would area unpredictable. It has been previously demonstrated that plasma proteins from blood, may alter the ability of EMD to

adsorb to root surfaces thus negatively affecting cell attachment, differentiation and proliferation (Miron et al. 2012).

In conclusion, within their limits, the present study has failed to demonstrate an influence of EMD on clinical and immunological parameters related to wound healing following recession coverage surgery using MCAT and CTG.

Conflict of interest

The authors report no conflict of interest regarding the study or products used for this trial. The study was funded by the Department of Periodontology, University of Bern, and partly by Straumann, Basel, Switzerland.

References

Agudio, G., Nieri, M., Rotundo, R., Franceschi, D., Cortellini, P. & Pini Prato, G. P. (2009) Periodontal conditions of sites treated with gingival-augmentation surgery compared to untreated contralateral homologous sites: a 10- to 27-year long- term study. *J Periodontol* **80,** 1399-1405. doi:10.1902/jop.2009.090122.

Allen, A. L. (1994) Use of the supraperiosteal envelope in soft tissue grafting for root coverage. I. Rationale and technique. *Int J Periodontics Restorative Dent* **14,** 216- 227.

Allen, E. P. & Miller, P. D., Jr. (1989) Coronal positioning of existing gingiva: short term results in the treatment of shallow marginal tissue recession. *J Periodontol* **60,** 316-319. doi:10.1902/jop.1989.60.6.316.

Armitage, G. C. (1999) Development of a classification system for periodontal diseases and conditions. *Ann Periodontol* **4,** 1-6. doi:10.1902/annals.1999.4.1.1.

Aroca, S., Keglevich, T., Nikolidakis, D., Gera, I., Nagy, K., Azzi, R. & Etienne, D. (2010) Treatment of class III multiple gingival recessions: a randomized-clinical trial. *J Clin Periodontol* **37,** 88-97. doi:10.1111/j.1600-051X.2009.01492.x.

Aroca, S., Molnar, B., Windisch, P., Gera, I., Salvi, G. E., Nikolidakis, D. & Sculean, A. (2013) Treatment of multiple adjacent Miller class I and II gingival recessions with a Modified Coronally Advanced Tunnel (MCAT) technique and a collagen matrix or palatal connective tissue graft: a randomized, controlled clinical trial. *J Clin Periodontol* **40,** 713-720. doi:10.1111/jcpe.12112.

Azaripour, A., Kissinger, M., Farina, V. S., Van Noorden, C. J., Gerhold-Ay, A., Willershausen, B. & Cortellini, P. (2016) Root coverage with connective tissue graft associated with coronally advanced flap or tunnel technique: a randomized, double-blind, mono-centre clinical trial. *J Clin Periodontol* **43,** 1142-1150. doi:10.1111/jcpe.12627.

Azzi, R., Etienne, D. & Carranza, F. (1998) Surgical reconstruction of the interdental papilla. *Int J Periodontics Restorative Dent* **18,** 466-473.

Bosshardt, D. D. (2008) Biological mediators and periodontal regeneration: a review of enamel matrix proteins at the cellular and molecular levels. *J Clin Periodontol* **35,** 87-105. doi:10.1111/j.1600-051X.2008.01264.x.

Brett, P. M., Parkar, M., Olsen, I. & Tonetti, M. (2002) Expression profiling of periodontal ligament cells stimulated with enamel matrix proteins in vitro: a model for tissue regeneration. *J Dent Res* **81,** 776-783.

Cairo, F., Nieri, M. & Pagliaro, U. (2014) Efficacy of periodontal plastic surgery procedures in the treatment of localized facial gingival recessions. A systematic review. *J Clin Periodontol* **41 Suppl 15,** S44-62. doi:10.1111/jcpe.12182.

Castellanos, A., de la Rosa, M., de la Garza, M. & Caffesse, R. G. (2006) Enamel matrix derivative and coronal flaps to cover marginal tissue recessions. *J Periodontol* **77,** 7-14. doi:10.1902/jop.2006.77.1.7.

Chambrone, L., Chambrone, D., Pustiglioni, F. E., Chambrone, L. A. & Lima, L. A. (2008) Can subepithelial connective tissue grafts be considered the gold standard procedure in the treatment of Miller Class I and II recession-type defects? *J Dent* **36,** 659-671. doi:10.1016/j.jdent.2008.05.007.

Cueva, M. A., Boltchi, F. E., Hallmon, W. W., Nunn, M. E., Rivera-Hidalgo, F. & Rees, T. (2004) A comparative study of coronally advanced flaps with and without the addition of enamel matrix derivative in the treatment of marginal tissue recession. *J Periodontol* **75,** 949-956. doi:10.1902/jop.2004.75.7.949.

Daprile, G., Gatto, M. R. & Checchi, L. (2007) The evolution of buccal gingival recessions in a student population: a 5-year follow-up. *J Periodontol* **78,** 611-614. doi:10.1902/jop.2007.060277.

Gestrelius, S., Lyngstadaas, S. P. & Hammarstrom, L. (2000) Emdogain--periodontal regeneration based on biomimicry. *Clin Oral Investig* **4,** 120-125.

Gibson, C. W. (2008) The amelogenin "enamel proteins" and cells in the periodontium.

Crit Rev Eukaryot Gene Expr **18,** 345-360.

Haase, H. R. & Bartold, P. M. (2001) Enamel matrix derivative induces matrix synthesis by cultured human periodontal fibroblast cells. *J Periodontol* **72,** 341-348. doi:10.1902/jop.2001.72.3.341.

Hagenaars, S., Louwerse, P. H., Timmerman, M. F., Van der Velden, U. & Van der Weijden,

G. A. (2004) Soft-tissue wound healing following periodontal surgery and Emdogain application. *J Clin Periodontol* **31,** 850-856. doi:10.1111/j.1600- 051X.2004.00571.x.

Hagewald, S., Spahr, A., Rompola, E., Haller, B., Heijl, L. & Bernimoulin, J. P. (2002) Comparative study of Emdogain and coronally advanced flap technique in the treatment of human gingival recessions. A prospective controlled clinical study. *J Clin Periodontol* **29,** 35-41.

Hammarstrom, L. (1997) The role of enamel matrix proteins in the development of cementum and periodontal tissues. *Ciba Found Symp* **205,** 246-255; discussion 255-260.

Henriques, P. S., Pelegrine, A. A., Nogueira, A. A. & Borghi, M. M. (2010) Application of subepithelial connective tissue graft with or without enamel matrix derivative for root coverage: a split-mouth randomized study. *J Oral Sci* **52,** 463-471.

Hoang, A. M., Oates, T. W. & Cochran, D. L. (2000) In vitro wound healing responses to enamel matrix derivative. *J Periodontol* **71,** 1270-1277. doi:10.1902/jop.2000.71.8.1270.

Hofmanner, P., Alessandri, R., Laugisch, O., Aroca, S., Salvi, G. E., Stavropoulos, A. & Sculean, A. (2012) Predictability of surgical techniques used for coverage of multiple adjacent gingival recessions--A systematic review. *Quintessence Int* **43,** 545-554.

Kapferer, I., Schmidt, S., Gstir, R., Durstberger, G., Huber, L. A. & Vietor, I. (2011) Gene- expression profiles of epithelial cells treated with EMD in vitro: analysis using complementary DNA arrays. *J Periodontal Res* **46,** 118-125. doi:10.1111/j.1600- 0765.2010.01321.x.

Kennedy, J. E., Bird, W. C., Palcanis, K. G. & Dorfman, H. S. (1985) A longitudinal evaluation of varying widths of attached gingiva. *J Clin Periodontol* **12,** 667-675.

Lovegrove, J. & Leichter, J. (2004) Exposed root surface: a review of aetiology, management and evidence-based outcomes of treatment. *N Z Dent J* **100,** 72-81.

Maymon-Gil, T., Weinberg, E., Nemcovsky, C. & Weinreb, M. (2016) Enamel Matrix Derivative Promotes Healing of a Surgical Wound in the Rat Oral Mucosa. *J Periodontol* **87,** 601-609. doi:10.1902/jop.2016.150567.

Miron, R. J., Bosshardt, D. D., Laugisch, O., Katsaros, C., Buser, D. & Sculean, A. (2012) Enamel matrix protein adsorption to root surfaces in the presence or absence of human blood. *J Periodontol* **83,** 885-92. doi:10.1902/jop.2011.110404.

Miron, R. J., Chandad, F., Buser, D., Sculean, A., Cochran, D. L. & Zhang, Y. (2016) Effect of Enamel Matrix Derivative Liquid on Osteoblast and Periodontal Ligament Cell Proliferation and Differentiation. *J Periodontol* **87,** 91-99. doi:10.1902/jop.2015.150389.

Muller, H. P., Eger, T. & Schorb, A. (1998) Alteration of gingival dimensions in a complicated case of gingival recession. *Int J Periodontics Restorative Dent* **18,** 345-353.

Okuda, K., Miyazaki, A., Momose, M., Murata, M., Nomura, T., Kubota, T., Wolff, L. F. & Yoshie, H. (2001) Levels of tissue inhibitor of metalloproteinases-1 and matrix metalloproteinases-1 and -8 in gingival crevicular fluid following treatment with enamel matrix derivative (EMDOGAIN). *J Periodontal Res* **36,** 309-316.

Parkar, M. H. & Tonetti, M. (2004) Gene expression profiles of periodontal ligament cells treated with enamel matrix proteins in vitro: analysis using cDNA arrays. *J Periodontol* **75,** 1539-1546. doi:10.1902/jop.2004.75.11.1539.

Pini-Prato, G. (2011) The Miller classification of gingival recession: limits and drawbacks. *J Clin Periodontol* **38,** 243-245. doi:10.1111/j.1600- 051X.2010.01655.x.

Rincon, J. C., Xiao, Y., Young, W. G. & Bartold, P. M. (2005) Enhanced proliferation, attachment and osteopontin expression by porcine periodontal cells exposed to Emdogain. *Arch Oral Biol* **50,** 1047-1054.

Sangnes, G. & Gjermo, P. (1976) Prevalence of oral soft and hard tissue lesions related to mechanical toothcleansing procedures. *Community Dent Oral Epidemiol* **4,** 77-83.

Sato, S., Yamada, K., Kato, T., Haryu, K. & Ito, K. (2006) Treatment of Miller Class III recessions with enamel matrix derivative (Emdogain) in combination with subepithelial connective tissue grafting. *Int J Periodontics Restorative Dent* **26,** 71- 77.

Sculean, A., Cosgarea, R., Stahli, A., Katsaros, C., Arweiler, N. B., Brecx, M. & Deppe, H. (2014) The modified coronally advanced tunnel combined with an enamel matrix derivative and subepithelial connective tissue graft for the treatment of isolated mandibular Miller Class I and II gingival recessions: a report of 16 cases.

Quintessence Int **45,** 829-835. doi:10.3290/j.qi.a32636.

Sculean, A., Cosgarea, R., Stahli,A., Katsaros, C., Arweiler, N.B., Miron, R. J., & Deppe, H. (2016) Treatment of multiple adjacent maxillary Miller Class I, II, and III gingival recessions with the modified coronally advanced tunnel, enamel matrix derivative, and subepithelial connective tissue graft: A report of 12 cases.

Quintessence Int **47,** 653-9. doi:10.3290/j.qi.a36562

Serino, G., Wennstrom, J. L., Lindhe, J. & Eneroth, L. (1994) The prevalence and distribution of gingival recession in subjects with a high standard of oral hygiene. *J Clin Periodontol* **21,** 57-63.

Spahr, A., Haegewald, S., Tsoulfidou, F., Rompola, E., Heijl, L., Bernimoulin, J. P., Ring, C., Sander, S. & Haller, B. (2005) Coverage of Miller class I and II recession defects using enamel matrix proteins versus coronally advanced flap technique: a 2-year report. *J Periodontol* **76,** 1871-1880. doi:10.1902/jop.2005.76.11.1871.

Stahli, A., Bosshardt, D., Sculean, A. & Gruber, R. (2014) Emdogain-regulated gene expression in palatal fibroblasts requires TGF-betaRI kinase signaling. *PLoS One* **9,** e105672. doi:10.1371/journal.pone.0105672.

Susin, C., Haas, A. N., Oppermann, R. V., Haugejorden, O. & Albandar, J. M. (2004) Gingival recession: epidemiology and risk indicators in a representative urban Brazilian population. *J Periodontol* **75,** 1377-1386. doi:10.1902/jop.2004.75.10.1377.

Suzuki, N., Ohyama, M., Maeno, M., Ito, K. & Otsuka, K. (2001) Attachment of human periodontal ligament cells to enamel matrix-derived protein is mediated via interaction between BSP-like molecules and integrin alpha(v)beta3. *J Periodontol* **72,** 1520-1526. doi:10.1902/jop.2001.72.11.1520.

Tonetti, M. S., Fourmousis, I., Suvan, J., Cortellini, P., Bragger, U., Lang, N. P. & European Research Group on, P. (2004) Healing, post-operative morbidity and patient perception of outcomes following regenerative therapy of deep intrabony defects. *J Clin Periodontol* **31,** 1092-1098. doi:10.1111/j.1600- 051X.2004.00615.x.

Wachtel, H., Schenk, G., Bohm, S., Weng, D., Zuhr, O. & Hurzeler, M. B. (2003) Microsurgical access flap and enamel matrix derivative for the treatment of periodontal intrabony defects: a controlled clinical study. *J Clin Periodontol* **30,** 496-504.

Wennstrom, J. L. (1996) Mucogingival therapy. *Ann Periodontol* **1,** 671-701. doi:10.1902/annals.1996.1.1.671.

Wennstrom, J. L. & Lindhe, J. (2002) Some effects of enamel matrix proteins on wound healing in the dento-gingival region. *J Clin Periodontol* **29,** 9-14.

Tables

Table 1 Patient characteristics

	Control group	Test group
Gender		
n (female)	15	14
n (male)	5	6
Age		
Mean	30.8	32.8
SD	9.9	11.1
Ethnicity		
Caucasian	19	19
Afro-American	1	1
Miller class		
I	2	4
II	10	10
III	8	6
Smoking	0	3
PSI		
Mean	0.9	1.3
SD	0.7	0.9

PSI, periodontal screening index; SD, standard deviation

Table 2 Recession healing index (RHI) and graft exposure

	recession			palate				
	RHI 2 d	RHI 7 d	RHI 14 d	RHI 2 d	RHI 7 d	RHI 14 d	GE 2 d (mm)	GE 7 d (mm)
control	1.8⍰0.6	1.3⍰0.8	0.5⍰0.6	1.7⍰0.4	1.4⍰0.8	0.3⍰0.5	0.3⍰0.6	0.8⍰1.4
test	2.1⍰0.7	1.2⍰0.8	0.4⍰0.5	1.4⍰0.5	1.0⍰0.8	0.4⍰0.6	0.1⍰0.4	0.2⍰0.9
p-value	0.485	0.838	0.538	0.079	0.244	0.801		

RHI, recession healing index; d, days; GE, graft exposure

Table 3 Change of parameters at recession sites between baseline and 6 months po

	Test Mean ± SD	Control Mean ± SD
Recession length		
Baseline	4.0 ± 1.2	4.4 ± 2.0
After surgery	0.9 ± 1.3	1.0 ± 1.0
p-value	< 0.001	< 0.001
Recession width		
Baseline	3.4 ± 1.0	2.8 ± 0.7
Width of KT		
Baseline	1.0 ± 0.9	1.1 ± 0.8
After surgery	1.8 ± 1.2	1.9 ± 1.0
p-value	0.001	0.001

po, postoperatively; SD, standard deviation; KT, keratinized tissue

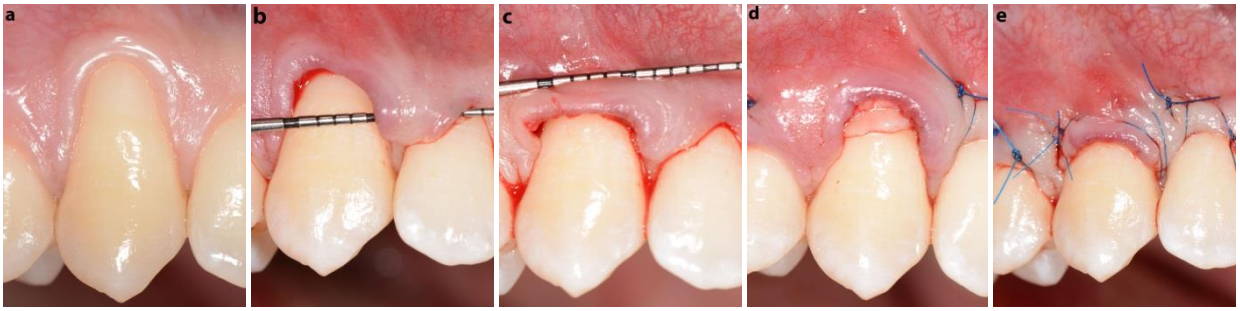


Fig. 1. *Surgical technique.*

a: baseline, b: tunneled flap, c: mobilisation , d: connective tissue graft, e: coronally sutured tunnel

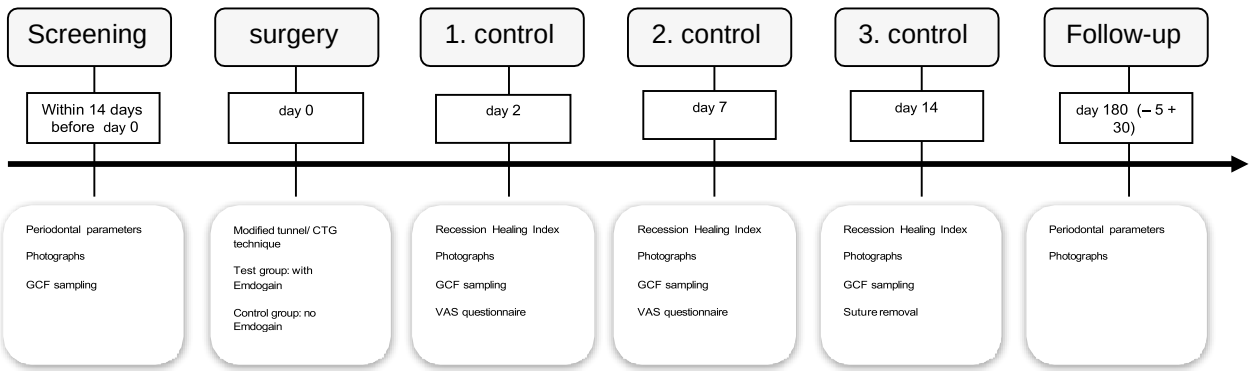


Fig. 2. *Flow chart*

Flow chart showing the timeline of visits with assessed parameters. GCF, gingival crevicular fluid; CTG, connective tissue graft.

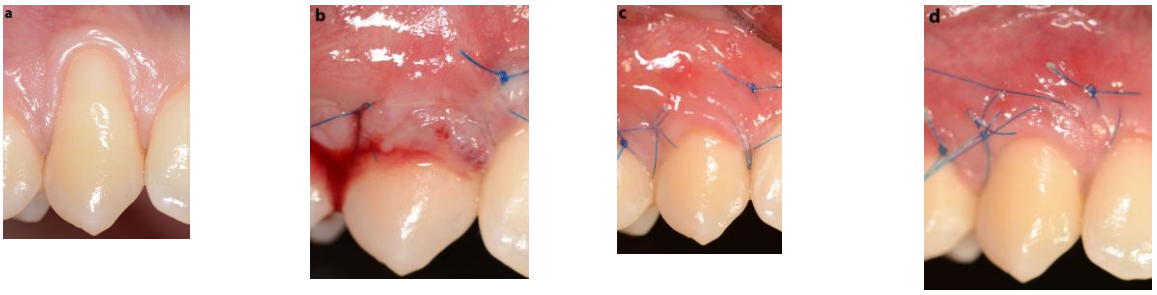


Fig. 3. *Early wound healing (example)*

a: baseline, b: 2 days post operation, c: 7 days post operation, d: 14 d post operation

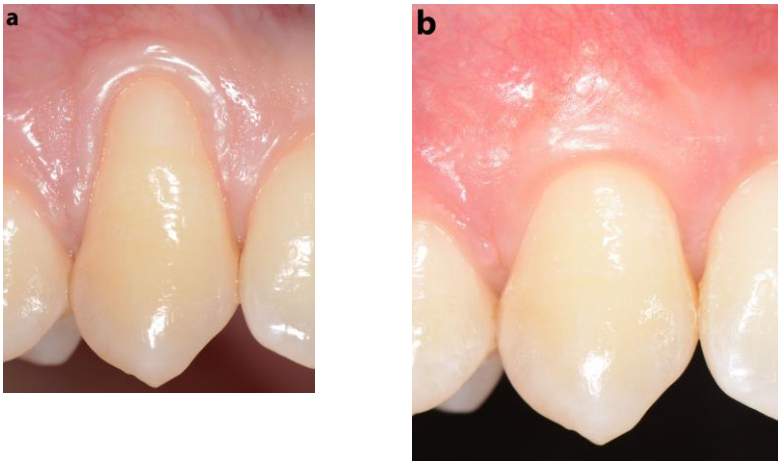


Fig. 4. *Case example*

a: baseline, b: 6 month control

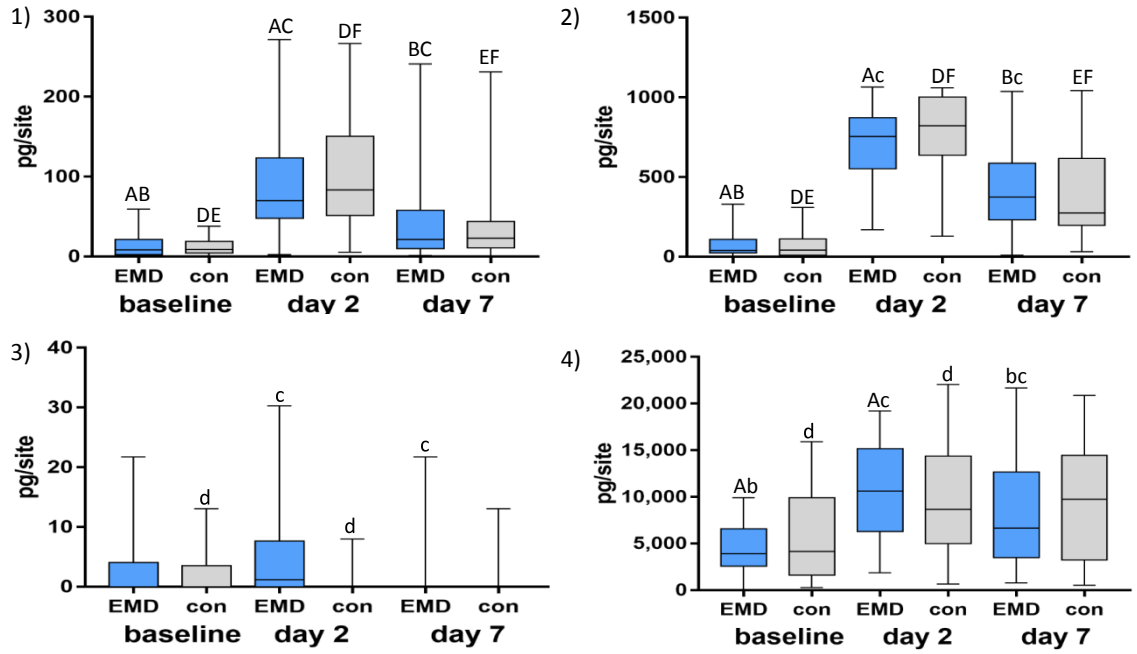


Fig. 5. *Change in inflammatory markers*

1) values for IL-8; 2) IL-10; 3) MMP-8; 4) IL-1

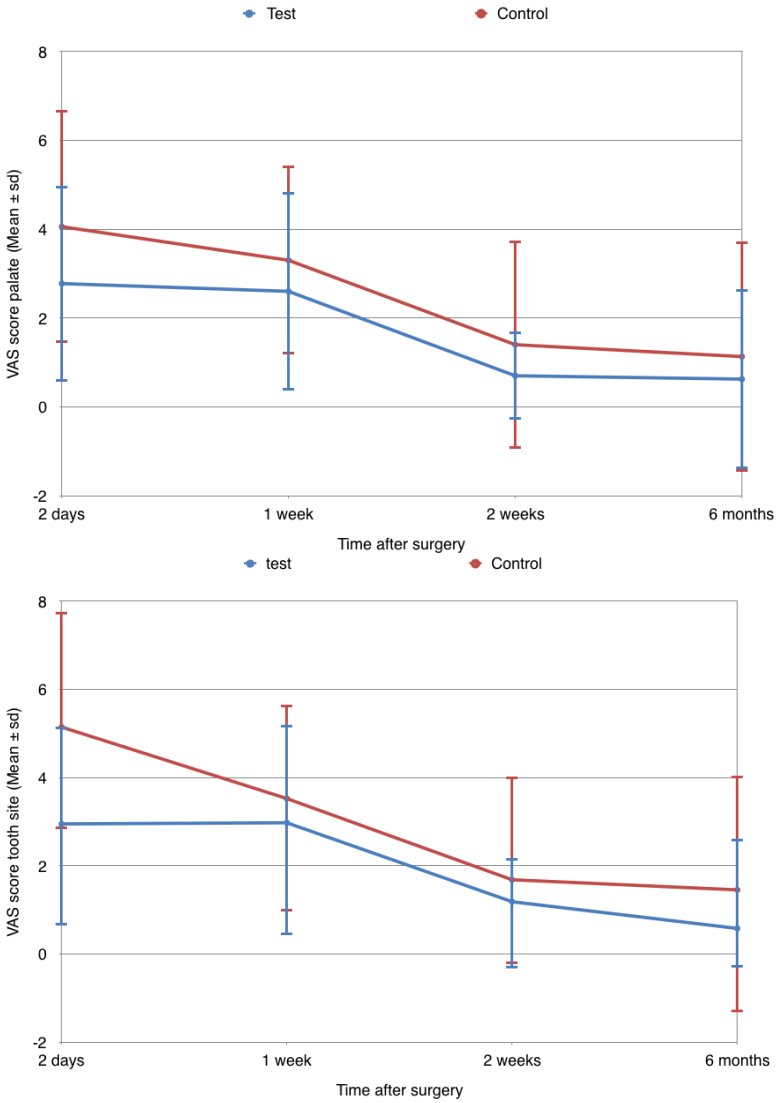


Fig. 6. *VAS scores*

VAS scores for pain as reported by the patients for different timepoints depicted as mean and standard deviations.

A SYSTEMATIC REVIEW AND META-ANALYSIS OF EPIDEMIOLOGIC OBSERVATIONAL EVIDENCE ON GLYCAEMIC CONTROL AND RISK OF DEVELOPING DIABETES IN HEALTHY PATIENTS

Revisione sistematica e metanalisi dell'evidenza epidemiologica-osservazionale sul controllo glicemico ed il rischio di sviluppare il diabete in pazienti sani

Filippo Graziani[1,2], Stefano Gennai[1,2], Luca Lillo[1,2], Morena Petrini[1,2]

1. Department of Surgical, Medical and Molecular Pathology and Critical Care Medicine, University of Pisa, Pisa, Italy

2. Sub-Unit of Periodontology, Halitosis and Periodontal Medicine, University Hospital of Pisa, Pisa, Italy

Running title: Effect of periodontitis on glycaemic control

Key words: Periodontal disease, diabetes, epidemiology

Abstract

Aim: to evaluate the impact of periodontal disease on glycaemic control, and new diabetes development in healthy patients.

Methods: Observational studies (cross-sectional, case-control and cohort design) on periodontitis effect on glycaemic control, published until January 2017, were identified through electronic databases and hand- searched journals. Findings were summarized by evidence tables, using PRISMA statement. Quality of the included studies was evaluated through the Newcastle Ottawa scale. Meta-analysis was performed with random approach when feasible.

Results: healthy subjects with periodontitis show a worse glycaemic control: 0.29 % of Hb1AC (0.20-0.37 %, 95% C.I. p<0.01) and a higher risk of 29% (1,11-1-46, 95% CI , p<0.0001) of developing diabetes.

Conclusions: Periodontitis has a significant impact on diabetes incidence and glycaemic control in healthy patients. Nevertheless, additional evidence is needed to further re-enforce such knowledge.

Introduction

Diabetes and periodontitis are two common chronic diseases that affect people worldwide.

The recent report published by WHO in 2016 has stated that in 2014 about 422 million adults were living with diabetes (World Health Organization 2016); this data is very alarming because the global prevalence of this condition increased from 4.7% in 1980 to 8.5% in 2014, in the adult population. Another important aspect is that diabetes increment was particularly marked in low- and middle-income countries than in high-income ones, as a consequence of such risk factors increment, like obesity and sedentary behaviours (Lear et al. 2014). In 2012 diabetes and poor

glycaemic control have caused globally about 3.7 million of deaths, affecting the risks of cardiovascular and other diseases and 43% of these occurred in people younger than 70 years old.

Periodontitis affects about the 50 % of word population, with severe forms incidence ranging between 5-10% (Petersen et al. 2005, Eke & Dye 2009, Mattila et al. 2010, Hu et al. 2011).

Currently there is an increasing interest in the literature in links between periodontitis and inflammatory systemic diseases, like hypertension, diabetes and cerebro-cardiovascular conditions

(Ylöstalo et al. 2010, Lockhart et al. 2012, Borgnakke et al. 2013). The persistence of bacterial biofilm in periodontal sites and the consequent inflammatory reaction, could lead to a cumulative inflammatory burden in the host, predisposing the patients to the development of other systemic chronic conditions. Loos et al. have shown that people affected by periodontitis are characterized by altered total numbers of leukocytes and plasma levels of C-reactive protein (CRP)(Loos et al. 2000).

The aim of this work is to perform a systematic review and meta-analysis of epidemiologic observational evidence on glycaemic control and risk of developing diabetes in healthy patients

Materials and Methods

Protocol development and eligibility criteria

The present study aimed to review observational studies published until January 2017 that analysed the effect of periodontitis on glycaemic control. In particular we have performed an update of the systematic review published by Borgnakke in 2013 and that have analysed article published until the January 2013 (Borgnakke et al. 2013).

A detailed protocol was designed according to the PRISMA (Preferred Reporting Items for Systematic Review and Meta-Analyses) statement (Moher et al. 2009, Liberati et al. 2009). The systematic review was designed to answer the following focused questions:

1. Do people with not known diabetes, who have poorer periodontal health, have poorer glycaemic control than those with better periodontal health?

2. Do people without known diabetes, who have poorer periodontal health, have greater risk for developing (incident) type 2 diabetes than those with better periodontal health?

Studies to be included had to be non-intervention, observational studies such as cohort, case-control (cases represented by periodontally-affected subjects and controls by non periodontally – affected subjects) or cross-sectional in design. In the selected studies, *exposure* had to be periodontal status (measures of inflammation, sings of disease such as pocketing and attachment level excluding tooth loss/edentulousims) of the included subjects and *outcome* one parameter related to diabetes such as measures of glucidic metabolism (glycated hemoglobin (Hb1Ac), fasting and 2-h 75 g plasma glucose (FBG) level and oral glucose tolerance test (OGT)), incidence of new cases, diabetes-related complications. Only studies in English language were selected.

Information sources and Search

We conducted a search on electronic databases using the same MeSH terms and free text words,

up to January 2017. Words searched were: ("periodontal diseases"[mh] OR periodontium[mh] OR periodon- tics[mh] OR periodont*[tiab]) AND ("diabetes mellitus"[mh] OR "diabe- tes insipidus"[mh] OR diabet*[tiab] OR "dm 1"[tiab] OR "dm i"[tiab] OR "dm 2"[tiab] OR "dm ii"[tiab] OR "hemoglobin a, glycosylated"[mh] OR a1c[tiab] OR "hb a1c"[tiab] OR hba1c[tiab] OR "blood glu- cose"[mh] OR "blood sugar"[tiab] OR ((glucose[ti] OR sugar[ti]) AND (level[ti] OR control[ti])) OR hyperglycemia[mh] OR hypoglyce- mia[mh] OR glycemi*[tiab] OR glycaemi*[tiab] OR hyperglyc*[tiab] OR hypoglyc*[tiab]). Filters used were: Humans and English language.

Presence of duplicates was assessed through Mendeley software.

Study selection and Data Collection

Eligibility assessment was performed through titles, abstract analysis, and full text analysis. Titles and abstracts of the search results were initially screened by the two reviewers (F.G. and M.P.), for possible inclusion in the review. Each round of calibration consisted of a duplicate, independent validity assessment of 20 titles and abstracts from the search. After two rounds of calibration, a consistent level of agreement was found.

In order to avoid excluding potentially relevant articles, abstracts providing unclear results were included in the full text analysis. The full text of all studies of possible relevance was then obtained for independent assessment against the stated inclusion criteria. Any disagreement was resolved by discussion among the reviewers.

Excluded articles were classified according to a hierarchical scale according to the main reasons for exclusion (Borgnakke et al. 2013): N1. Not original study (review, guidelines, comment); N3. Original, but not epidemiologic study; N4. Original, but interventional study; N2. Original study, but not on effect of periodontal disease on glycaemic control; N5. Other reasons.

Manuscripts not to be excluded were categorized into the following groups: E1. Glycaemic control in not known diabetes, E2. Incident type 2 diabetes (new diabetes developed in individuals without diabetes at baseline).

Data of the included articles were extrapolated through an "ad hoc" extraction sheet.

Data Items

Risk of bias across studies

Heterogeneity among the studies was tested when feasible and evaluated through Q and I^2 test. A p value of Q statistic <0.05 was defined as an indicator of heterogeneity and data were considered heterogeneous for I^2 value higher than 40%.

Risk of bias in individual studies

The quality of each cohort and case- control study according to NOS for Assessing the Quality of Non-randomized Studies (Wells et al. 2011). Evaluation of cross-sectional studies was made according to scale suggested by Borgnakke and co-workers (Borgnakke et al. 2013). Using these forms, we rated each report at both the study and outcome levels.

Summary measures and synthesis of the results

Outcomes considered were odds ratios (OR), hazard ratios (HR) and hazard rate ratios (HRR), risk ratios, rate ratios or relative risks (RR). Results were presented as the manuscript presented by Needleman and co-workers in a recent EFP workshop highlighting results of the previous

evidence, summary of the new evidence and overall synthesis (Needleman et al. 2015). Data were collected in evidence tables and results of the meta-analysis were summarized with Forest plots.

Additional analysis

Meta-analysis was performed when outcome data were homogenous and available from at least three studies. HbA1c was expressed as whited mean differences (WMD) and 95% CI for continuous outcomes using random model. The patient was the unit of the analysis. Analyses were performed with OpenMeta[Analyst]

http://www.cebm.brown.edu/open_meta/open_meta/open_meta.

Hazard ratios (HR) was expressed as mean effect size and 95% CI for dichotomous data using random model and was calculated with Meta-Essentials: Workbooks for meta-analysis (Version 1.1) (http://www.erim.eur.nl/research-support/meta-essentials/downloads/).

Results

Study selection

A total of 696 studies, published between 2013 and 2017, were identified for inclusion in the review (Fig. 1). Screening of titles and abstracts led to rejection of 595 papers and thus the full text of the remaining 101 papers was obtained. After full-text analysis and the exclusion of further 80 articles, the remaining 21 articles were analysed for methodological quality and availability of data for systematic review or meta-analysis (Tab.1). The final evidence was obtained including the 6 articles selected in the meta-analysis of Borgnakke et al., for what concerning the period until the January 2013.

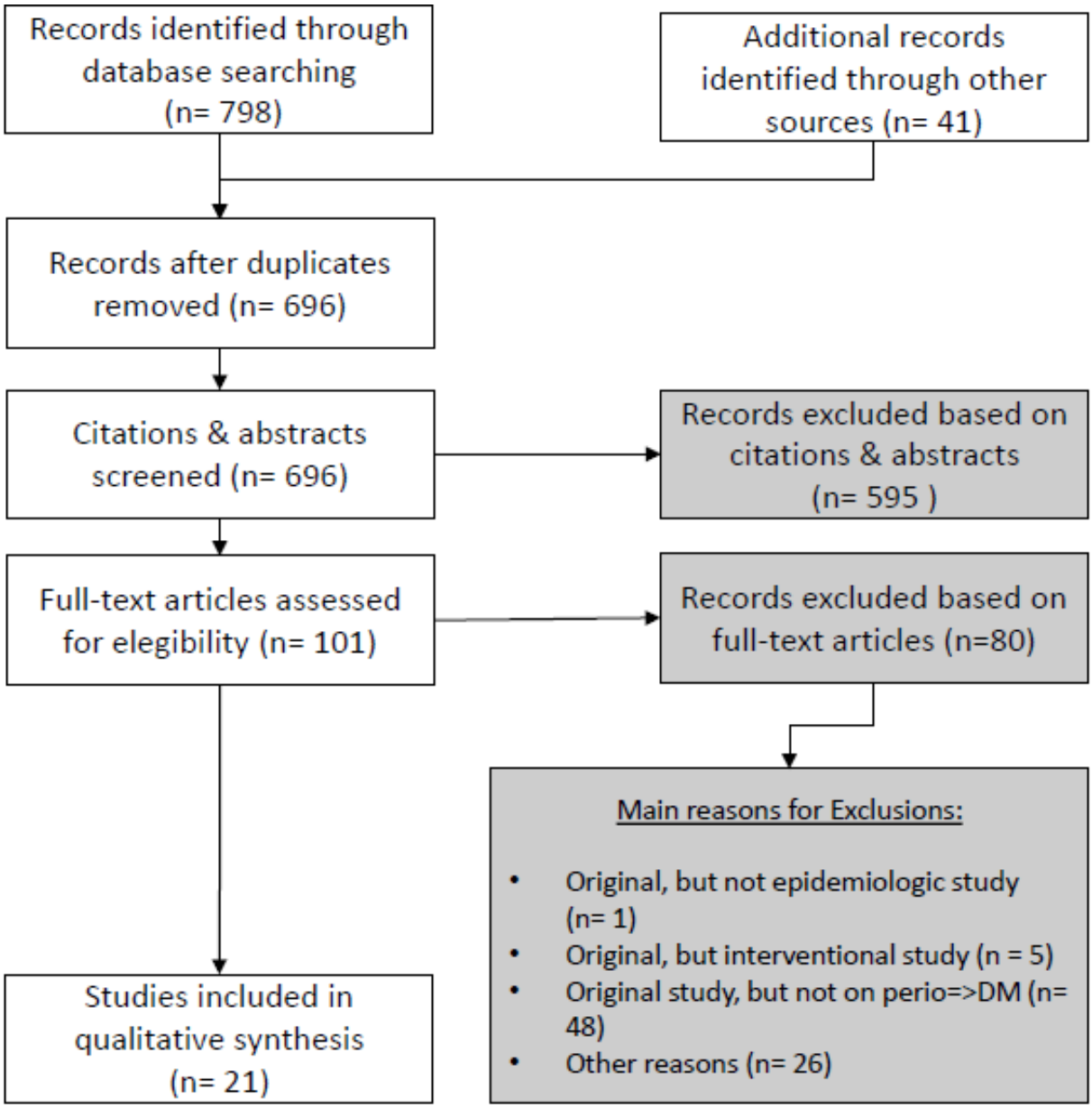
Table 1. The 27 reports included in the final review: citations; the 6 article in red have been included from Borgnakke et al. (Borgnakke et al. 2013)

1. Arora, N., Papapanou, P.N., Rosenbaum, M., Jacobs, D.R., Desvarieux, M. & Demmer, R.T. (2014) Periodontal infection, impaired fasting glucose and impaired glucose tolerance: results from the Continuous National Health and Nutrition Examination Survey 2009-2010. Journal Of Clinical Periodontology 41, 643–52.
2. Banu, S., Jabir, N.R., Mohan, R., Manjunath, N.C., Kamal, M.A., Kumar, K.R.V., Zaidi, S.K., Khan, M.S. & Tabrez, S. (2015) Correlation of Toll-like receptor 4, interleukin-18, transaminases, and uric acid in patients with chronic periodontitis and healthy adults. Journal Of Periodontology 86, 431–9.
3. Chang, J.-F., Yeh, J.-C., Chiu, Y.-L., Liou, J.-C., Hsiung, J.-R. & Tung, T.-H. (2017) Periodontal Pocket Depth, Hyperglycemia, and Progression of Chronic Kidney Disease: A Population-Based Longitudinal Study. The American Journal Of Medicine 130, 61–69.e1.
4. Chiu, S.Y.-H., Lai, H., Yen, A.M.-F., Fann, J.C.-Y., Chen, L.-S. & Chen, H.-H. (2015) Temporal sequence of the bidirectional relationship between hyperglycemia and periodontal disease: a community-based study of 5,885 Taiwanese aged 35-44 years (KCIS No. 32). Acta Diabetologica 52, 123–31.
5. Choi, Y.-H., McKeown, R.E., Mayer-Davis, E.J., Liese, A.D., Song, K.-B. & Merchant, A.T. (2014) Serum C-reactive protein and immunoglobulin G antibodies to periodontal pathogens may be effect modifiers of periodontitis and hyperglycemia. Journal Of Periodontology 85, 1172–81.
6. Demmer, R. T., Desvarieux, M., Holtfreter, B., Jacobs, D. R., Jr., Wallaschofski, H., Nauck, M., Volzke, H. & Kocher, T. (2010) Periodontal status and A1C change: longitudinal results from the study of

health in Pomerania (SHIP). Diabetes Care 33, 1037–1043. doi:10.2337/dc09-1778.
7. Demmer, R. T., Jacobs, D. R., Jr. & Desvarieux, M. (2008) Periodontal disease and incident type 2 diabetes: results from the First National Health and Nutrition Examination Survey and its epidemiologic follow-up study. Diabetes Care 31, 1373–1379. doi:10.2337/dc08-0026.
8. El-Beshbishy, H.A., Maria, R.A. & Bardi, F.A. (2014) Biochemical and C-reactive protein alterations in myocardial infarction periodontitis patients. The American Journal Of The Medical Sciences 348, 181–5.
9. Flores, M.F., Montenegro, M.M., Furtado, M. V, Polanczyk, C.A., Rösing, C.K. & Haas, A.N. (2014) Periodontal status affects C-reactive protein and lipids in patients with stable heart disease from a tertiary care cardiovascular clinic. Journal Of Periodontology 85, 545–53.
10. Garcia, D., Tarima, S. & Okunseri, C. (2015) Periodontitis and glycemic control in diabetes: NHANES 2009 to 2012. Journal Of Periodontology 86, 499–506.
11. Gokhale, N.H., Acharya, A.B., Patil, V.S., Trivedi, D.J., Setty, S. & Thakur, S.L. (2014) Resistin Levels in Gingival Crevicular Fluid of Patients With Chronic Periodontitis and Type 2 Diabetes Mellitus. Journal Of Periodontology 85, 610–617.
12. Hong, J.W., Noh, J.H. & Kim, D.-J. (2016) The Prevalence and Associated Factors of Periodontitis According to Fasting Plasma Glucose in the Korean Adults: The 2012-2013 Korea National Health and Nutrition Examination Survey. Medicine 95, e3226.
13. Ide, R., Hoshuyama, T., Wilson, D., Takahashi, K. & Higashi, T. (2011) Periodontal disease and incident diabetes: a seven-year study. J Dent Res 90, 41–46. doi:10.1177/0022034510381902.
14. Islam, S.K.M.A., Seo, M., Lee, Y.-S. & Moon, S.-S. (2015) Association of periodontitis with insulin resistance, β-cell function, and impaired fasting glucose before onset of diabetes. Endocrine Journal 62, 981–9.
15. Javed, F., Ahmed, H.B., Saeed, A., Mehmood, A. & Bain, C. (2014) Whole salivary interleukin-6 and matrix metalloproteinase-8 levels in patients with chronic periodontitis with and without prediabetes. Journal Of Periodontology 85, e130-5.
16. Kapellas, K., Skilton, M., Maple-Brown, L., Do, L., Bartold, P., O'Dea, K., Brown, A., Celermajer, D. & Jamieson, L. (2014) Periodontal disease and dental caries among Indigenous Australians living in the Northern Territory, Australia. Australian Dental Journal 59, 93–99.
17. Lee, K.-S., Lee, S.G., Kim, E.-K., Jin, H.-J., Im, S.-U., Lee, H.-K., Merchant, A.T., Song, K.-B. & Choi, Y.-H. (2015) Metabolic syndrome parameters in adolescents may be determinants for the future periodontal diseases. Journal Of Clinical Periodontology 42, 105–12.
18. Lin, S.-Y., Lin, C.-L., Liu, J.-H., Wang, I.-K., Hsu, W.-H., Chen, C.-J., Ting, I.-W., Wu, I.-T., Sung, F.-C., Huang, C.-C. & Chang, Y.-J. (2014) Association between periodontitis needing surgical treatment and subsequent diabetes risk: a population-based cohort study. Journal Of Periodontology 85, 779–86.
19. Longo, P.L., Artese, H.P.C., Rabelo, M.S., Kawamoto, D., Foz, A.M., Romito, G.A., Dib, S.A. & Mayer, M.P.A. (2014) Serum levels of inflammatory markers in type 2 diabetes patients with chronic periodontitis. Journal Of Applied Oral Science : Revista FOB 22, 103–8.
20. Morita, I., Inagaki, K., Nakamura, F., Noguchi, T., Matsubara, T., Yoshii, S., Nakagaki, H., Mizuno, K., Sheiham, A. & Sabbah, W. (2012) Relationship between periodontal status and levels of glycated hemoglobin. J Dent Res 91, 161–166. doi:10.1177/0022034511431583.
21. Morita, T., Yamazaki, Y., Mita, A., Takada, K., Seto, M., Nishinoue, N., Sasaki, Y., Motohashi, M. & Maeno, M. (2010) A cohort study on the association between periodontal disease and the development of metabolic syndrome. J Periodontol 81, 512–519. doi:10.1902/jop. 2010.090594.
22. Perayil, J., Suresh, N., Fenol, A., Vyloppillil, R., Bhaskar, A. & Menon, S. (2014) Comparison of glycated hemoglobin levels in individuals without diabetes and with and without periodontitis before and after non-surgical periodontal therapy. Journal Of Periodontology 85, 1658–66.
23. Rao Deepika, P.C. & Saxena, R.M. (2013) Comparison of glycosylated hemoglobin levels in severe periodontitis patients and healthy controls: a study in an Indian population. Quintessence International (Berlin, Germany : 1985) 44, 319–25.

24. Saito, T., Shimazaki, Y., Kiyohara, Y., Kato, I., Kubo, M., Iida, M. & Koga, T. (2004) The severity of periodontal disease isassociated with the development of glucose intolerance in non-diabetics: the Hisayama study. J Dent Res 83, 485–490. doi:10.1177/154405910408300610.
25. Srinivasa, T.S., Agrawal, P., Goyal, P., Farista, S., Sowmya, N.K. & Deonani, S. (2015) Comparative clinical evaluation of glycosylated haemoglobin level in healthy and chronic periodontitis patients: A chairside diagnostic method. Indian Journal Of Dental Research : Official Publication Of Indian Society For Dental Research 26, 504–7.
26. Tu, Y.-K., D'Aiuto, F., Lin, H.-J., Chen, Y.-W. & Chien, K.-L. (2013) Relationship between metabolic syndrome and diagnoses of periodontal diseases among participants in a large Taiwanese cohort. Journal Of Clinical Periodontology 40, 994–1000.
27. Xiong, X., Elkind-Hirsch, K.E., Xie, Y., Delarosa, R., Maney, P., Pridjian, G. & Buekens, P. (2013) Periodontal disease as a potential risk factor for the development of diabetes in women with a prior history of gestational diabetes mellitus. Journal Of Public Health Dentistry 73, 41–9.

Fig.1. Flow of studies during review



Description of characteristics, results and quality of each study

The findings from this review are described in the following for each of the originally posed questions. For each topic, a table displays the characteristics and findings from each study, and a brief summary is provided of only the longitudinal results, that is, any cross-sectional findings at baseline are not shown. Importantly, all confounders for which the analyses are controlled are displayed for each outcome or model, respectively, in Tables 2–3 under the heading "Confounders Controlled." In consideration of space and readability, these confounders will not be re-cited in the text. Risk of bias within and across studies is addressed briefly and the consensus NOS quality scores for each study are displayed in the online Appendix, with such tables corresponding by topic to the results tables included in this main report. All studies were conducted among adults.

Do people without manifest type 2 diabetes, who have poorer periodontal health, have poorer glycaemic control than those with better periodontal health?

Findings of New Evidence

Non-diabetic subjects, in a follow-up ranging from 4 to 10 years, showed a greater deterioration of glycaemic control as showed by an increase of Hb1Ac, impaired glucose tolerance or metabolic syndrome incidence associated with higher values of periodontal parameters such as PPD or CAL (Saito et al. 2004, Demmer et al. 2010b, Morita et al. 2010). Each additional millimeter of PPD determined a 0,13% increase of Hb1Ac (Saito et al. 2004). Overall, it appeared that subjects with periodontits have a higher risk of worse glycaemic control.

An important increase of studies was noted in the last four years. The actual evidence can be drawn on twenty manuscripts, involving a total of 117077 non-diabetic subjects, ranging from 30 to 67284, conducted over 4 continents except Europe (Javed et al. 2013, Tu et al. 2013, Xiong et al. 2013, Rao Deepika & Saxena 2013, Boland et al. 2013, Longo et al. 2014, Garcia et al. 2014, Kapellas et al. 2014, Flores et al. 2014, Gokhale et al. 2014, Arora et al. 2014, Choi et al. 2014, El-Beshbishy et al. 2014, Perayil et al. 2014, Banu et al. 2015, Islam et al. 2015, Lee et al. 2015, Srinivasa et al. 2015, Hong et al. 2016, Chang et al. 2017) (Tab.2). The total evidence almost unanimously reported a worse glycemic control in subjects with periodontitis. Subjects with periodontal disease showed greater level of fasting blood glucose (Xiong et al. 2013, Islam et al. 2015), Hb1aC (Javed et al. 2013, Arora et al. 2014, Perayil et al. 2014, Srinivasa et al. 2015, Hong et al. 2016, Chang et al. 2017) and prevalence of pre-diabetes/diabetes (Kapellas et al. 2014, Choi et al. 2014, El-Beshbishy et al. 2014).

The data allowed meta-analysis of the Hb1Ac values based on 47,781 subjects. Subjects with periodontitis showed a weighted mean (WM) of 5.64 % (5.54-5.74 %, 95% C.I.). Whilst non-affected subjects showed a WM 5.31 % (5.18-5.44 %, 95% C.I.), showing a statistically significant ($p<0.01$) WM difference of 0.29 % Hb1AC (0.20-0.37 %, 95% C.I.).

Quality Assessment

The quality of the included study is depicted in the Table S1-3. A significant heterogeneity was noted among the included studies. Periodontal outcomes were taken in full-mouth examination (Javed et al. 2013, Xiong et al. 2013, Arora et al. 2014, Perayil et al. 2014, Banu et al. 2015, Hong et al. 2016, Chang et al. 2017), partial-mouth (Taylor et al. 1996, Saito et al. 2004, Morita et al. 2010, Demmer et al. 2010a, Longo et al. 2014, Kapellas et al. 2014, Choi et al. 2014, Lee et al. 2015) or gathered from electronic clinical notes/database (Boland et al. 2013). The majority of the studies were of cross-sectional design.

Some of the included articles were performed in selected populations such as adolescents (Lee et al. 2015) or young adults (Chiu et al. 2015a) and thus not generalizable. Some of the data are gathered form cross-sectional studies which primary intention was not to compare periodontitis versus non-periodontitis affected subjects. Moreover, these studies may highlight association rather than causality.

Fig.2. Forrest Plot from random effect of meta-analysis evaluating the difference in Hb1Ac among periodontitis affected subjects (cases) and non-affected ones (control) as gathered from observational studies (weighted mean difference (WMD), 95% Confidence Interval (C.I.).

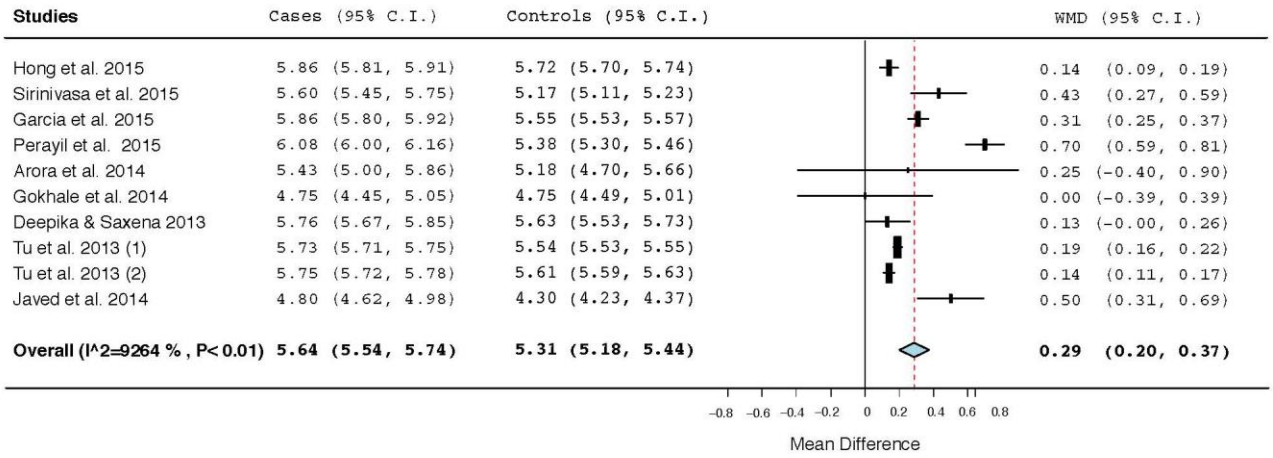


Table 2. Effect of Periodontal disease on metabolic control in subjects without diabetes. Articles in red have been included from Borgnakke et al. (Borgnakke et al. 2013)

Author Year Country Study Design BL DM Typer	**A) Subjects: a. Perio Cases b. Comparison groups B) Age C) Study Duration**	**EXPOSURE**	**OUTCOME**	**Effect on Metabolic Control? & Generalizable?**	**Effect size: Odds Ratio (OR), Trend, HR, HRR & Significance (95%CI)**	**Effect on Metabolic Control/Conclusion**	**Confunders Controlled**
Saito et al. 2004 Japan Retrospective Cohort* No DM	All without DM @BL in 1988 A) @ FU in 1998: N1=961 (377M+584F) N2=591=those among N1 aged >40yrs in 1988 N3=545 w/HbA1c values both at BL and FU A) 40-79 yrs B) 10 yrs	Partial mouth** • PPD • CAL PD-1: Mean DDP: a1) Intermediate: 1.3-2.0mm a2) Deep/High: >2.0mm b) Shallow/Low: <1.3mm PD-2: Mean CAL: a1) Intermediate: 1.5-2.5mm a2) High: >2.5mm b) Low: <1.5mm	• 2hr 75g OGTT (BL) • IGT • HbA1c • Incident Glucose Intolerance: NGT in 1988& IGT in 1998 • Glucose Intolerance progression= HbA1c (1998) – HbA1c (1988) >0.2% (=difference after 10 years)	Yes, stat.sign. in Japanese (Hisayama) 40-79yrs community dwellers Not generalizable	1) High vs.Low PPD categories: OR=2.4 (1.4-2.6; p= 0.009) for risk of IGT 2) No sign. increase in IGT with mean CAL	1) Proportion w/IGT increased significantly w/mean PPD 2) Those w/normal BL GT who developed IGT over 10 years were sign. more likely to have deep PPD, but not CAL, at FU 3) Each additional mm mean PPD corresponded to 0.13% HbA1c increase (p=0.007) 4) Severity of perio-dontal disease expressed as PPD, but not CAL, was sign. associated with development of glucose intolerance	• age • sex • smoking • BMI • exercise • alcohol
COMMENTS: *: May be regarded as 1998 cross-sectional exam plus 1988 OGGT data, i.e., oral health data only from 1998 (not from BL 1988); **NHANES III protocol (1 max. + 1 mand. quadrant); 4 "trained" examiners; No calibration reported							
Demmer et al. 2010 Germany (Pomerania)	A) N=2,793 (47%M+53%F) a1) 488 a2) 463	Perio Exam: Partial mouth* Tooth count:	HbA1c	Yes, stat. sign. in Caucasians in Pomerania in former East Germany Not generalisable	1) BL # teeth was not consistently associated with 5yr change in HbA1c (ptrend=0.84) 2) Those perio healthy at	5- year change in mean CAL (but not in mean PPD) was associated with HbA1c change	•age •waist/hip ratio systolic •BP triglycerides physical activity •white blood cell count

Cohort No DM	a3) 479 a4) 241 b) 1,122 B) 48(+15)yrs [20-81yrs] C) 5yrs	Full mouth <28 teeth PPD CAL # teeth PD groups based on% BL sites w/CAL>5mm: a1) 1-8% a2) 9-33% a3) 34-100% a4) Edentulous b) 0% Used 3 additional PD groupings based on: 1) BL PPD 2) BL # teeth 3) 5yr change in % sites w/CAL>5mm		BL & FU had less 5yr HbA1c change than those w/poor BL perio health and 5yrs perio deterioration: 0.005 vs. 0.143% (p=0.003)		•fibrinogen hsCRP •sex region smoking •education •family DM history •Multivariat linear regression

COMMENTS: Study of Health In Pomerania (SHIP); *right or left side of mouth; Good to excellent agreement in intra- and inter-examiner periodontal exam calibration

Morita et al. 2010 Japan Cohort No DM No MetS	A) N=1,023 (727M+296F) B) 37.3yrs [20-56yrs] C) 4yrs	Partial mouth (sextants) CPI Codes: 0: healthy 1: bleeding 2: calculus 3: >1 PPD 4-5mm 4:>1 PPD> 6mm PD Groups: CPI < 2 vs. CPI > 3	Incidence of metabolic syndrome (association w/PD) HbA1c fasting glucose OGTT	Yes, stat. sign. in (71% male) Japanese employees under 60 years Not generalisable	OR=1.6 (1.1-2.2; p<0.05) for >1 positive MetS component vs. no positive MetS component in PD; OR=1.4 (1.0-2.1) for >1; OR=2.2 (1.1-4.1) for >2 MetS components	In initially healthy individuals, periodontal disease is associated in a dose-response manner with development of >1 components of metabolic syndrome over 4 years	•age •gender •cigarette •smoking •exercise •eating btw. •meals •weight at BL •Multivariate

COMMENTS No examiner calibration; Dose-response effect stat. sign for trend; CPI is a poor measure of PD

Tu et al. 2013 Taiwan Cross -sectional NO DM	A) N= 33,740 (18,469 F + 15,271 M) Perio a1) 10381 b1) G=4729 b2) H=18536 B) CP: M= 53.22 yrs (dev.st 11.15) F= 54.15 yrs (dev.st 11.08) H: M=50.55 yrs (dev.st 12.93) F=49.96 yrs (dev.st 12.43) G: M=47.06 yrs (dev.st 11.78) F= 48.57 yrs (dev.st 11.48) C) NA	Gingivitis group: at least one tooth with the diagnosis of gingivitis but not periodontitis; Periodontitis group: at least one tooth with the diagnosis of periodontitis;	• FBG • PC • HbA1c (%) • Insulin resistance • Metabolic syndrome	• Yes, periodontal patients had statistically significant higher FBG, PC and HbA1c, compared to the reference control group. • Periodontitis is highly associated with insulin resistence and metabolic syndrome in female subjects. A weaker relation was noted for men (insulin resistance and periodontitis) Not generalizable	• <u>OR for MetS (significant only for Women)</u> Gingivitis 1.424 (1.301-1.559) p <0.001 Periodontitis 1.517 (1.413-1.628) p <0.001 • <u>OR for Insulin Resistance</u> <u>Women</u> Gingivitis 1.499 (1.368-1.643) p <0.001 Periodontitis 1.606 (1.494-1.726) p <0.001 <u>Men</u> Periodontitis 1.129 (1.039-1.227) p =0.004	Subject with periodontitis were characterized by higher level of Fasting glucose, postprandial glucose and HbA1c respect gingivitis and reference group. There is an increased prevalence of MetS in women affected by gingivitis and periodontitis. Effect even stronger in non smokers.	•Patients' occupations •Age •medications taken •major medical •conditions •smoking history (current, former or non-smokers) •number of cigarettes smoked per day
COMMENTS: no radiographs or periodontal charting was taken. No calibration reported. Only private patients coming from upper and middle class only.							
Xiong et al. 2013 USA Case-Control NO DM	A) N=39 Perio a1) 13 b1)26 B) prior GDM •<25 years old= 1	Full-mouth periodontal examination at six sites per tooth: • Probing depth (PD) • Gingival margin level • Clinical attachment loss (CAL) • Bleeding on probing (BOP)	• FBG • 1⁄2-hour glucose (mg/dL) • 1-hour glucose (mg/dL) • 2-hour glucose (mg/dL) • Fasting insulin (uU/mL) • 1⁄2-hour insulin (uU/mL) • 1-hour insulin (uU/mL) • 2-hour insulin (uU/mL) • HOMA-IR	Yes, worse blood glucose levels, 1-hour insulin and IS-SI were recorded in periodontal subjects Women with both prior GDM	• 1⁄2-hour glucose (mg/dL): 112.48 ± 1.25 (NPD) 138.41 ± 1.30 (PD) p<0.05 • 1-hour glucose (mg/dL): 91.62 ± 1.33 (NPD) 132.50 ± 1.34 (PD) <0.01 • 2-hour glucose (mg/dL): 90.66 ± 1.25 (NPD) 98.15 ± 1.41 (PD) NS	Periodontal disease alone is also associated (to a lesser extent) with lower insulin sensitivity, poorer b-cell function,and hyperglycemia. Even more significant if associate dwith history of GDM.	• Age • Race/ethnicity • Education • BMI

	•25-35 years old= 14 •>35 years old= 5 B2) no prior GDM •<25 years old= 2 •25-35 years old= 11 •>35 years old =6 C) not reported	26= Women without periodontal disease (NPD) 13= Women with periodontal disease (PD)	• Matsuda index (ISOGTT) • IGI/HOMA-IR • IS-SI	and periodontal disease had the most impaired glucose metabolism; Not Generalizable	• 1-hour insulin (uU/mL): 45.86 ± 1.64 (NPD) 73.83 ± 1.84 (PD) <0.01 • IS-SI: 620.61 ± 2.51 (NPD) 315.86 ± 3.51 (PD) P = 0.06		
COMMENTS: limited and peculiar sample (history of GDM: N=19 with a history of GDM , N=20 without GDM history), results hardly generalizable							
Arora et al. 2014 USA Cohort* NO DM	A) N= 1165 B) 30–80 years C) 1 year	Full-mouth examination 6 sites per tooth By a trained hygienist • PD • AL	• IFG or IGT • FPG • Two hour glucose (OGTT) (mg/dl) • Insulin levels (IU/ml) • HOMA-IR • HbA1c% (mmol/mol) Impaired fasting glucose (IFG) and Impaired glucose tolerance (IGT) IFG = fasting plasma glucose ≥100 mg/dl and <126 mg/dl; IGT = 2-h post-challenge glucose values ≥140 mg/dl and <200 mg/dl.	Yes, severe periodontitis was associated with a 93% increase in the odds of impaired glucose tolerance after multivariable adjustment. Findings were similar for mean PD. Associations between measures of periodontal infection and IFG were weak and not statistically significant. Genralizable	IGT prevalence: controls 12% cases 31% OR varied according to the model of adjustment from 1.75 (1.16–2.62) to 2.90 (1.80–4.68) highly significant. IFG prevalence controls 38% cases 59% OR varied according to the model of adjustment from 1.05 (0.56-1.99) to 2.01 (1.28–3.14). Only few models of adjustment reached significance Multivariable logistic regression models	Clinical measure of periodontal infections are associated with pre-diabetes	• Age • Race/ethnicity • Sex • Education • Physical activity level • Cigarette Smoking • Alcohol consumption Caloric intake • Height • Weight Blood pressure measures • BMI • Triglycerides Total cholesterol • HDL-cholesterol C-reactive protein (CRP) White blood cell count (WBC)

					• Periodontal status Mean PD ≥75th percentile: Isolated-IFG, OR= 0.91 [0.57, 1.45]; • Isolated-IGT, OR= 1.85 [0.73, 4.66]; • Combined IFG+IGT, OR= 2.06 [0.91, 4.66]; p= 0.05;		
COMMENTS: part of the NHANES, may be regarded as cross-sectional; Periodontal examination were made by trained, registered hygienist whose received intense training followed by periodic monitoring and recalibration against a reference examiner.							
Choi et al. 2014 USA Cross-sectional NO DM	A) N= 5,731 (2,575 M + 3,156 F) Periodontal quartiles: a1) Q1=1462 a2) Q2=1416 a3) Q2=1420 a4) Q2=1433 B) 20-65 yrs (43±16.9 yrs) C) 3 yrs	Periodontal examination (NHANES protocol) • CAL • PD measured at two sites (mid-buccal and mesiobuccal) on every tooth in each of two randomly chosen quadrants, one in the maxilla and the other in the mandible IgG antibodies to A. actinomycetemcomitans (cut off 156 EU) and P. gingivalis (cut-off 168) Median CAL: • Q1= 0.27 • Q2= 0.59 • Q3=1.00 • Q4= 2.19	Fasting plasma glucose (FPG) • normal; FPG <100 mg/dL (5.6 mmol/L) • prediabetes:100<FPG<126mg/dL (7.0 mmol/L), • diabetes: FPG >126 mg/dL or self-reported	Yes, there was a statistically significant association between IFG and diabetes with CAL, in particular Q3 and Q4; for what concerning PD, Q4 was associated with IFG and Q2, Q3 and Q4 were associated with diabetes.	Confounding Effect (OR [95% confidence interval]) of CRP and Antibodies on CAL and Prediabetes and Diabetes -Quartile for CAL IFG • Q2=0.97 (0.83 to 1.13)* ; 0.96 (0.83 to 1.13)**; 0.97 (0.83 to 1.13)***; 0.97 (0.83 to 1.13)**** • Q3=1.34 (1.25 to 1.44)*; 1.34 (1.25 to 1.43)**; 1.35 (1.26 to 1.45)***; 1.34 (1.25 to 1.43)**** • Q4=1.74 (1.62 to 1.86)*; 1.75 (1.64 to 1.87)**; 1.76 (1.65 to 1.88)***; 1.73 (1.62 to 1.85)**** DIABETES • Q2=1.22 (0.99 to 1.51)*; 1.28 (1.04 to 1.59)**; 1.22 (0.99 to 1.51)***; 1.20 (0.97 to 1.48)**** • Q3=1.43 (1.16 to 1.76)*;	A strong association was noted among periodontits and diabetes in individuals with high levels of CRP and P.gingivalis.	• Age • Sex • Education • Income • Race • Smoking • alcohol intake • missing teeth • frequency of dental visits • BMI • central adiposity • physical activity **Additionally adjusted for: • Inflammation history • CRP ***First model additionally adjusted for • A. actinomycetemcomitans ****First model additionally adjusted for

					1.48 (1.20 to 1.83)**; 1.48 (1.20 to 1.83)***; 1.40 (1.15 to 1.71)**** • Q4=3.65 (2.84 to 4.69)*; 3.86 (3.01 to 4.97)**; 3.98 (3.03 to 5.22)***; 3.46 (2.73 to 4.39)**** -Quartile for PPD: IFG • Q2=0.94 (0.79 to 1.10)*; 0.93 (0.79 to 1.10)**; 0.94 (0.79 to 1.10)***; 0.93 (0.79 to 1.10)**** • Q3=1.12 (0.93 to 1.35)*; 1.12 (0.93 to 1.35)**; 1.12 (0.93 to 1.35)***; 1.12 (0.93 to 1.35)**** • Q4=1.27 (1.12 to 1.45)*; 1.27 (1.12 to 1.46)**; 1.27 (1.12 to 1.45)***; 1.26 (1.10 to 1.44)**** DIABETES • Q2=1.66 (1.45 to 1.91)*; 1.61 (1.38 to 1.87)**; 1.64 (1.43 to 1.88)***; 1.63 (1.40 to 1.90)**** • Q3=1.28 (1.08 to 1.52)*, 1.27 (1.07 to 1.51)**; 1.29 (1.09 to 1.53)***; 1.25 (1.03 to 1.51)**** • Q4=1.66 (1.33 to 2.08)*; 1.61 (1.29 to 2.02)**; 1.66 (1.31 to 2.09)***; 1.53 (1.21 to 1.94)****	• P. gingivalis
COMMENTS: Limitation of the NHANES in terms of oral measurement; calibration not reported							
El-Beshbishy et al. 2014 Saudi Arabia Cross-Sectional NO DM	A) N=60 a1) 5 periodontitis systemically healthy a2) 13 periodontitis + acute myocardial	Presence of Periodontitis	% of diabetes cases	% of diabetes was higher in PD affected subjects no statistical difference was reported.	% of diabetes: 13.3 % NO PD vs 20% PD 5.9% (AMI NO PD), vs 38.5%(AMI+PD)	Among AMI patients, the % diabetes is higher in people affected by periodontitis	None

	infarction (AMI) b1) H=25 perio and systemically healthy b2) H+ AMI= 17 perio and AMI B) 35-70 yrs C)NA				Not generalizable		

COMENTS: limited sample, no statistical analysis in metabolic outcome, no adjustments. No information on periodontal examination.

Flores et al. 2014 Brazil Cross-sectional NO DM	A) N=93 (57 M; 63F) a1) 51 previous myocardial infarction a2) 42 other major cardiovascular events B) 63.5±9.8 yrs C) NA	Full mouth Periodontal examination at six sites per tooth: • VP • GR • PPD • BOP	• FPG • HbA1c	Statistically significant differences for FPG between periodontitis and controls; No statistically significant differences for HbA1c Not generalizable	• FPG=126.8±48.4 (P); 109.8±37.5 (NO-P), p=0.03 • HbA1c= 7.2±2.3 (P); 6.4±1.1 (NO-P), p=0.17	Patients affected by periodontitis were characterized by higher level of fasting plasma glucose	• Sex • Age • Smoking exposure • Diabetes status • Toothbrushing frequency • Dental visits • CRP • triglycerides (TGs) • very-low-density • lipoprotein cholesterol

COMMENTS: Limited sample on cardio-vascular subjects. Two trained and calibrated examiners, but calibration modalities are not reported.

Gokhale et al. 2014 India Case-control DM 1 + DM 2	A) N=60 (24 M, 36F) Perio a1) DMCP=15 a2) CP=15 b1) DMPH=15 b2) H =15 B) 36-60 yrs (44.2 yrs) C) 2 months	Full mouth Periodontal examination (4 sites/tooth) Periodontal clinical examination • GI • PD Radiographic evidence of bone loss Periodontitis= GI >1, >3 teeth with PD> 5mm, BOP+RX bone loss	• RBS • glycated hemoglobin (HbA1c) Diabetes= HbA1c>6,5 %, RBS >200 mg/dL	No statistically significant differences for HbA1c and RBS between periodontitis and no periodontitis in systemically healthy patients Not generalizable	Consolidated Pairwise Comparison (P value) Among H vs CP for: RBS: p= 0.9993 HbA1c: p= 1.0000 One-way analysis of variance and Tukey multiple post hoc procedures	The presence of periodontitis in healthy patients, did not statistically influenced glycaemic control	None to assess metabolic control
COMMENTS: Limited sample; limited sample; Performed to analyze the resistin level; Examiner calibration not reported; Type of perio exam: not reported							
Javed et al. 2014 Pakistan Cross-sectional NO DM	A) N=88 (M) a1) 28 patients with CP and prediabetes a2) 30 patients with CP and without prediabetes a3) 30 controls B) 39-51 yrs C) NA	Periodontal clinical parameters: • plaque index • bleeding on probing • probing depth • attachment loss • number of missing teeth Radiological parameter: • marginal bone loss CP was defined as clinical AL ≥ 3 mm,25,26 PD ≥ 5 mm,25,26 and MBL ≥ 3 mm 6,27 in > 30% of the sites.	• Fasting blood glucose (FBG) • hemoglobin A1c (HbA1c) prediabetes= fasting blood glucose [FBG] 100-125 mg/dL [5.6-6.9 mmol/L] and hemoglobin A1c [HbA1c] 5.7%-6.4% Health was self-reported	No effect was noted Not generalizable;	•FBG (mg/dL): CP= 80.1 ± 3.5; NO CP= 75.3 ± 2.2 •HbA1c (%): CP=4.8 ± 0.5; NO CP= 4.3 ± 0.2; Not significant	No effected noted.	None
COMMENTS: Study conducted only in male subjects; Trained and calibrated examiner but calibration modalities are not reported. Digital panoramic radiographs were viewed on a calibrated computer screen using a software program for analysis of MBL.							
Kapellas et al. 2014 Australia Cross-sectional	A) N=310 a1) S-PD=83 a2) M-PD= 188	Partial Periodontal examination: -For 6 index teeth:	No. self-reported diabetes	Yes, there are statistically significant differences for self reported diabetes,	No. self-reported diabetes – (Yes) p<0.01 • Non-cases:0 • M-PD= 21 (11.2) • S-PD= 20 (24.4)	Participants with severe periodontal disease were more likely to be self –report diabetes	None

DM	b1) 39=non cases B) 22-73 yrs C)NA	• Oral plaque score • Dental calculus -At 4 sites for every other tooth: • PPD • GR • CAL • Moderate Periodontitis: >2 interprox sites with CAL>4mm, or >2 sites with PD>5 mm • Severe Periodontitis: >2 interprox sites with CAL>6mm and >1 site with PD>5 mm		among different periodontal groups sample of Indigenous Australian adults Not generalizable			
COMMENTS: Calibrated examiners, but calibration modalities are not specified							
Longo et al. 2014 Brazil Case-Control DM	A) N=42 (23 M, 19 F) Perio a1) P=6 (5 M, 5 F) a2) DMI+P=10 (4M, 6 F) a3) DMA+P=10 (6M, 4 F) b1) H=6 (2M, 4F) b2) DMPH=10 (6M, 4 F) B) B) >35 yrs C) 15 months	Full mouth Periodontal examination (6 sites/tooth) • PI • Ging/BOP • PPD • CAL Periodontitis (American Academy of Periodontology, 1998) >30% of sites with PD >4 mm + BOP	HbA1c	No, there were no statistically significant differences for HbA1c %	HbA1c % • H=5.18±0.60 • P=5.43±0.54 mean±standard deviation	The presence of periodontitis in healthy patients, did not statistically influenced glycaemic controls	None
COMMENTS: Underpowered sample per comparison; Intraexaminer reliability for detecting PDs within 1 mm was >90%.							
Perayil et al. 2014	A) N=60 (26 M + 34 F)		HbA1c level	Yes, there were significant	HbA1c level of group PD (6.08% ±0.23%) was higher	HbA1c level was higher in PD group	None

India Case-control NO DM	a1) PD=30 b1) H=30 B) 35 -65 yrs c)	Full mouth periodontal assessment on 6 sites • oral hygiene index simplified(OHI-S) score • gingival index (GI) • probing depth (PD) • clinical attachment level (CAL) Periodontitis: PD ≥5 mm and CAL >3 mm in ≥5 teeth		differences between group H and group PD in regard to baseline OHI-S, GI, PD, and HbA1c (P <0.05). Not Generalizable	than that of group H (5.38% ±0.22%), (P <0.001)		
COMMENTS: Single, trained examiner, but calibration modalities are not reported.							
Rao Deepika & Saxena 2013 India Case-Control NO DM	N=60 a1) PD=30 b1) H=60 3 months 35-65 yrs	Examiner trained and calibrated CAL,BOP, PD · PD= CAL >30% + BOP in more than 30% sites · Controls: PPD<4 mm + BOP< 15%, no previous PD treatment, no CAL	HbA1c level	There was a slight increase of HbA1c level in periodontitis group (5,76%) in comparison to controls (5,63%), p=0,071 Among women, HbA1c was statistically significant higher (p=0,024) in cases (5,81%) vs controls (5,54%) Not generalizable	significant (p=0.01) positive correlation (r=0,349) between HbA1c and the plaque scores of the entire sample.	the level of HBA1Ac was not statistically significant between PD and controls	None
COMMENTS: Undajusted data. No info on calibration of the examiners.							
Banu et al. 2015 India Case-Control	A) N=60 (23 M + 37 F) a1) 40 CP b1) 20 H B) 40-65 yrs C) 15 months	Periodontal examination: • PI • PPD Bitewing radiographs: • interproximal bone loss	• FPG • IU	No statistically significant differences among H and CP for FBG and Insulin level The differences in	• FPG (mg/dl): H=88.75±7.05; CP=89.70±6.56; p=0.662 • IU: H=5.90±1.70; CP=7.16±5.92: p=0.367	The presence of periodontitis did not statistically influenced glycaemic controls	None

		from the cemento-enamel junction of the tooth to the bone crest for each patient Chronic periodontitis • radiographic evidence of interproximal bone loss (>50% alveolar bone loss in >2 quadrants of the dentition • >4 teeth should be involved in each jaw, >5mm PD, >4 mm clinical attachment level, and 80% BOP of the proximal sites.		Not generalizable	continuous variables between groups were compared by the use of Kruskal–Wallis test.		
COMMENTS: Examiners number not specified and calibration modalities are not reported							
Garcia et al. 2015 USA Cohort DM & NO DM	A) N=7,042 (3506 M; 3536 F) Perio a1) PD=3161 a2) PD+DM=707 b1) H=2860 b2) DM=314 B) 52yrs (30- 80 yrs) C) 3 yrs	Periodontal examination based on the FMPE protocol Total number of teeth	DM status as self-reported DM glycemic control was stratified using Hb1AC cut- off points of 7.0%, 7.5%, 8.0%, 8.5%, and 9.0%.	Yes, there was a statistically significant association between Hb1AC level and periodontitis in United States adults ages ‡30 years Generalizable	Periodontitis Status by Self-Reported DM Status and Glycemic Control Adjusted OR for Periodontal Status By Hb1AC cut-off: 1) DM<7,0%=0,98 (0,70-1,361); DM> 7,0%=1,33 (1,01-1,750) 2) DM<7,5%=0,96 (0,71-1,29); DM> 7,5%=1,58 (1,10-2,28) 3) DM<8,0%=1,00 (0,75-1,32); DM> 8,0%=1,65 (1,17-2,33) 4) DM<8,5%=0,98 (0,75-1,29); DM> 8,5%=2,17 (1,52-3,11) 5) DM<9,0%=1,00 (0,77-1,32); DM> 9,0%=2,22 (1,41-3,51) Glycoemoglobin % OR=1,14 (1,08-1,22)	The mean Hb1AC levels for individuals with and without periodontitis were 5.9% and 5.6%, respectively	Demographic factors: • Age • Gender • Educational Level • Marital Status, • Race/Ethnicity • Smoking Status • Federal Poverty Level, Number of Teeth Behavioral and dental • BMI • Smoking status

COMMENTS: Full mouth periodontal examination data from NHANES 2009-2012. Examiner calibration not reported. Definition of total periodontitis from the American Academy of Periodontology (AAP)							
Islam et al. 2015 South Korea Cohort DM + NO DM	A)N=19122 (8248 M + 10874 F) a1) CP=5070 a2) CP+DM=922 b1) H=12108 b2) DM= 1022 B)>20yrs C)3 yrs	Partial mouth CPI normal (CPI=0), gingival bleeding (CPI=1), calculus (CPI=2), a shallow periodontal pocket of 3.5~5.5 mm (CPI=3) or a deep periodontal pocket of 5.5 mm or more (CPI=4). PD=CPI ≥ 3.	• glucose • insulin resistance • HbA1c IFG diabetes mellitus was defined based on physician diagnosis or those with a fasting blood glucose ≥ 126 mg/dL, taking insulin or antidiabetic medication.	No, there were no statistically significant differences (p=0,172) although higher levels of HbA1c were found in periodontitis participants as compared to those without In logistic regression analysis periodontitis showed a significant association with IFG as an independent variable after adjustment for potential confounding factors in every model (p<0.001).	HbA1c, %b : • No Periodontitis=7.3±1.6 • Periodontitis= 7.4±1.6 NS Adjusted ORs and 95% CIs of prevalence of IFG for periodontitis among participants without diabetes • Model-1:(OR, 1.302; 95% CI,1.199~1.413); p<0.001 • Model-2:(OR, 1.282; 95% CI, 1.180~1.393); p<0.001 • Model-3: (OR, 1.301; 95% CI,1.193~1.418); p<0.001	Higher mean HbA1c levels were found in periodontitis patients rather than subjects without periodontitis, although the result was statistically insignificant. In patients without diabetes, the prevalence of IFG was higher in periodontitis patients as compared to subjects without periodontitis (28.5% vs. 17.7%).	• Model-1 for: Age, Sex, BMI • Model-2 for: Model 1 + SBP •T-chol • Model-3: Model 2+ region smoking •alcohol consumption •exercise
COMMENTS: no info on calibration of the examiner; CPI is a poor measure of PD							
Lee et al. 2015 Korea Cross-Sectional NO DM	A=941 (590 M+ 351 F) a1) Code CPI 0 (N = 579) a2) Code 1 (N = 125) a3) Code 2 (N = 228) B) 12-18 yrs C) 1 year	Partial mouth Periodontal examination CPI • 0=healthy • 1=bleeding following probing • 2=presence of dental calculus • 3=4 >PD<5 mm • 4=PD ≥6 mm Subjects divided in healthy gingiva and gingivitis (CPI ≥1)	FBG (mg/dL) Risk of MetS: three or more of these parameters: abdominal obesity; FPG ≥110 mg/dl; elevated blood pressure including treatment for hypertension; hypertriglyceridemia: serum triglyceride level ≥110 mg/dl; and low HDL cholesterol: serum HDL cholesterol ≤40 mg/dl.	No statistically significant differences for fasting blood glucose (mg/dl) among the CPI groups Not generalizable (adolescents)	FBG: 89.19±0.29 md/dl (no gingivitis); 88.82±0.45 md/dl (gingivitis), p=0.526 OR for gingivitis for High fasting glucose: crude=0.07 (0.01–0.70); adjusted= 0.07 (0.00–0.81)	The presence of periodontitis did not statistically influence glycaemic control, nor risk of MetS	•age •gender •income •dental check-up •frequency of brushing •frequency of eating between meals •physical activity

COMMENTS: Trained and calibrated examiner. Conducted in adolescents cohort. Only 8 subjects with FBG ≥ 110 mg/dl. CPI is a poor measure of PD.							
Srinivasa 2015 India Case-control General population	A)N=40 (22 M + 18F) a1) severe PD=20 b1) H=20 B1)H= 40.1±14.4 yrs B2) PD= 38.9±13.4 yrs	Clinical parameters: • PPD • BoP • CAL H = PDs ≤4 mm and BoP ≤15% and no CAL Severe PD = at least five teeth with PD ≥5 mm, BOP and CAL>1 mm on >5 teeth or radiographic bone loss	HbA1c	Yes, there were statistically significant differences (p = 0.003) for HbA1c between the two groups Not generalizable	HbA1c (%)Mean: Periodontitis=±SD 5.66±0.35 %; No Periodontitis= 5.17±0.3 %; p=0.003*	HbA1c levels were slightly elevated in chronic periodontitis cases than in controls.	None
COMMENTS: limited sample. No adjustment. Not specific data about the periodontal examination and calibration modalities are not reported							
Chang et al. 2016 Taiwan Chronic Kidney Disease Cohort	A) N=2831 B) 53.1± 8.4 C) 2.4 – 7.3 yrs	PD The 2015 updated classifications from the American Academy of Periodontology, periodontal diseases are classified into gingivitis and periodontitis	• fasting blood glucose, • HbA1c	Yes, patients with higher periodontal pocket depth (>4.5 mm) showed FBG and and HbA1c Not generalizable	Bivariate Correlation Coefficients Between Baseline Periodontal Pocket Depth and: • fasting blood glucose=0.28, p<0.01 • hemoglobin A1c (HbA1c)=0.26, p<0.01	Patients affected by periodontitis were characterized by higher level of FBG and HbA1c	• age •sex •diabetes mellitus •PPD • hypertension •smoke •betel nut • albuminuria •creatinine
COMMENTS: Subjects with chronic kidney disease; Not specific data about the periodontal examination and calibration modalities are not reported							
Hong et al. 2016 Republic of Korea Cross-sectional DM + NO DM	A) N= 9977 a1) CP=2728 b1) H=7249 B) 19 yrs C)NA	CPI Full mouth Periodontal Examination Periodontitis (CP) was defined as a community periodontal index score of ≥ 3	• NFG • IFG • HbA1c (%) • Anti-diabetes medication (%) • Diabetes (%) • NFG 1hrs (<90 mg/dL) • NFG 2hrs (90–99 mg/dL) • IFG 1hrs (100–110 mg/dL) • IFG 2hrs (111–125 mg/dL), and diabetes (>126 mg/dL)	Statistically significant differences among CP and NO CP for FPG and HbA1c and diabetes prevalence Not generalizable	• FBG mg/dL: H=96.8 (96.3–97.3); CP=100.4 (99.0–101.8), p<0.001 • HbA1c (%): H=5.72 (5.70–5.74); CP=5.86 (5.81–5.91), p<0.001 • Anti-diabetes medication (%): H=4.9 (4.3–5.4); CP=6.2 (4.9–7.4), p=0.072 • Diabetes (%): H=7.60	People affected by periodontitis were characterized by higher level of HbA1c and FPG	age, sex, smoking history, heavy alcohol drinking, college graduation, household income, waist circumference, serum TG level, serum HDL-cholesterol level, and the presence of hypertension

							(6.90–8.30); CP=12.0 (10.3–13.7), p <0.001 OR for FBG 111-125 mg/dl 1.33 (1.01-1.75) p=0.044 in periodontitis subjects		

COMMENTS: Oral examination of periodontal health was conducted by trained dentists; CPI is a screening test of PD.

AMI= acute myocardial infarction; BP, Blood Pressure; BOP= Bleeding on Probing; BPE=Basic Periodontal Examination; CKD, chronic kidney disease; C-PPD, cumulative periodontal probing depth; CPI,Community Periodontal Index; CRAE/CRVE, Central retinal arteriolar/venular equivalents; Excl.,Excluding/Excluded; MetS, Metabolic Syndrome; Perio, Periodontal/Periodontally; #, Number (of); &, and; BL, Baseline/Beginning of Study Period; CAL, Clinical Attachment Loss; CI, Confidence Interval; CPI, Community Periodontal Index; DM, Diabetes Mellitus; DM2, Type 2 Diabetes Mellitus; DMCP=Diabetes + chronic periodontitis; DMA+P= Diabetics with periodontitis and adequate glycaemic control DMI+P= Diabetics with periodontitis and inadequate glycaemic control; DMG=Diabetes+ Gingivitis; DMPH= Diabetes+periodontal health; F, Female; FBG= Fasting Blood Glucose; FU, Follow-Up/End of Study Period; G= gingivitis; GR= gingival recession; GT, Glucose Tolerance; H= Systemic and periodontal healthy; HbA1c, Glycosylated(Glycated) Haemoglobin; HOMA-IR: Homeostasis Model Assessment of Insulin Resistance; hr,hour; HR, Hazard Ratio; hrs=hours hsCRP, high-sensitivity C-reactive protein; IGI/HOMA-IR: insulin secretion Index; IGT, Impaired Glucose Tolerance; IU: Insuline; M, Male; MBL=marginal bone loss; MGI= Modified Gingival Index; MI= myocardial infarction; M-PD=moderate periodontitis; NFG, normal fasting glucose; NGT, Normal Glucose Tolerance; NHANES, National Health and Nutrition Examination Survey; OGTT, Oral Glucose Tolerance Test; OR, Odds Ratio; Perio/PD, PC, post-challenge glucose; Periodontal Disease; PI= Plaque index; PPD, Periodontal Probing (Pocket) Depth; RBS=Random blood sugar; R-DM2=retinopathy+ diabetes; RR, Risk Ratio; RX= X rays; SBP, systolicblood pressure; S-PD= severe periodontitis; Stat. sign., statistically significant; T-chol, total cholesterol; VP= visible plaque; vs., versus; yr(s), Year(s).

Appendix. Tab. S1 NOS scale for quality rating of Case-Control Study

Author, Year	A) SELECTION (Max. 4 Stars)				B) COMPARABILITY (Max. 2 Stars)	C) EXPOSURE: PERIODONTAL DISEASE (Max. 3 Stars)			TOTAL OF STAR
	Is the case definition adequate? (Max 1 Star)	Representativeness of the cases (Max 1 Star)	Selection of Controls (Max 1 Star)	Definition of Controls (Max 1 Star)	Comparability of cases and controls on the basis of the design or analysis (Max 2 Stars) a) Study controls for age (= most important factor) b) Study controls for smoking (=additional	Ascertainment of Exposure (Max 1 Star) a) Secure record, e.g. surgical/dental records/oral clinical examination b) Structure interview where blind to case/control status (self-reported periodontal disease) c) Interview not blinded to	Same method of ascertainment for cases and controls (Max 1 Star)	Non-response rate (Max 1 Star)	

					important factor)	**case/control status** **d) Written self-report or medical/dental record only** **e) No description**			
Rao Deepika et al.2013	★	★	★	★	★★	★	★	★	9
Xiong et al. 2013	★		★	★		★	★	★	6
El-Beshbishy et al. 2014					★			★	2
Srinivasa et al. 2015	★	★		★	★	★	★	★	7

Appendix. Tab. S2 NOS scale for quality rating of Cross-Sectional Study

Author, Year	**A) SELECTION (Max. 4 Stars)** **(Max. 4 Stars)**				**B) COMPARABILITY (Max. 2 Stars)**	**C) OUTCOME (max 1 star)**	**TOTAL OF STAR**
	Representativeness of the exposed subjects (Periodontal infection/Periodontitis) (Max 1 Star)	**Selection of non-exposed subjects (No/Only mild periodontal infection/periodontitis) (Max 1 Star)**	**Ascertainment of exposure (Periodontal infection/Periodontitis) (Max 1 Star)**	**Ascertainment of outcome (Glycemic control/Diabetes) (Max 1 Star)**	**Comparability of exposed & non-exposed groups on the basis of the design or analysis (Max 2 Stars)** **a) Study controls for age (= most important factor)** **b) Study controls for smoking (=additional important factor)**	**Assessment of outcome (Glycemic control/Diabetes) (Max 1 Star)**	
Arora et al. 2014	★	★	★	★		★	5

Choi et al. 2014	★	★		★	★★	★	6
Flores et al. 2014	★	★	★	★	★★	★	7
Gokhale et a. 2014	★	★	★	★	★	★	6
Javed et al. 2014	★	★	★	★	★★	★	7
Kapellas et al. 2014	★	★	★	★	★	★	6
Banu et al. 2015	★	★	★	★	★★	★	7
Garcia et al. 2015	★	★	★	★	★★	★	7
Islam et al. 2015	★	★		★	★★	★	6
Lee et al. 2015	★	★	★		★	★	5
Hong et al. 2016	★	★		★	★★	★	6
Chang et al. 2017	★	★	★	★	★★		6

Appendix. Tab. S3 NOS scale for quality rating of Cohort Studies

Author, Year	A) SELECTION (Max. 4 Stars) (Max. 4 Stars)				B) COMPARABILITY (Max. 2 Stars)	C) OUTCOME (max 3 star)			TOTAL OF STAR
	Representativeness of the exposed cohort (Max 1 Star)	Selection of non-exposed cohort (Max 1 Star)	Ascertainment of exposure (Max 1 Star)	. Demonstration that outcome was not present at start of study (Max 1 Star)	Comparability of cohorts on the basis of the design or analysis (Max 2 Stars) Study controls for age (= most important factor) Study controls for smoking (=additional important factor)	Assessment of outcome (Max 1 Star) Independent or blind assessment stated in the paper, or confirmation of the outcome by reference to secure records (X-rays, medical/dental records, etc.) Record linkage Self-report only (no confirmation by secure records) No description	Was follow-up long enough for outcomes to occur (Max 1 Star)	Adequacy of follow-up of cohorts (ensuring losses are not related to exposure or outcome) (Max 1 Star)	
Morita et al. 2010	★	★		★	★★	★	★	★	8
Demmer et al. 2010	★	★	★	★	★★	★	★	★	9
Saito et al. 2004	★	★	★	★	★★	★	★	★	9

E2. Do people without known diabetes, who have poorer periodontal health, have greater risk for developing (incident) type 2 diabetes than those with better periodontal health?

Findings of New Evidence

Current evidence on the influence of periodontal disease on the risk of developing (incident) type 2 diabetes is based only on few studies that analyzed specified populations. The included study were predominantly conducted in Japan (3 out of 4 studies) for a total of 22,230 individuals (Saito et al. 2004, Demmer et al. 2010b, Ide et al. 2011, Morita et al. 2012). An increased risk of developing DMT2 was noted in subjects with poorer periodontal health was thoroughly noted, as measured by increase in PPD, and remained after adjustment in the majority of the study (Saito et al. 2004, Demmer et al. 2008a, Morita et al. 2012) . The presence of PD showed increased odds for developing diabetes of 50% (Demmer et al. 2008a).

Additional evidence are gathered form two Taiwanese studies involving a total of 50486 participants followed for 5 (Chiu et al. 2015a) and 13-years (Lin et al. 2014a). In both cases an increased risk to develop diabetes was registered in PD-affected subjects. Over 5-year, young adults (aged 35-44 years) with community periodontal index score of at least 3 presented a 33 % increased risk of incident hyperglycemia (including diabetes) [adjusted hazard ratio (aHR) = 1.33 (95 % CI 1.09–1.63)] after controlling for potential confounding factors. A larger retrospective study on 22299 PD-affected subjects and 22302 periodontally healthy subjects found that DMT2 incidence, over 13-year period, was 1.24-fold higher in the PD cohort than in the control group, with an adjusted hazard ratio of 1.19 (95% confidence interval = 1.10 to 1.29). Interestingly, patients requiring periodontal surgery, i.e. probable higher severity of PD, showed a higher risk in the first 6 year.

Although the majority of the included studies have been conducted on Asiatic populations, results appear robust enough to state that subjects affected by PD show a higher chance to develop diabetes when compared to non-PD affected ones. The meta-analytic data for adjusted hazard ratio shows a value of 1.29 (95% CI 1,11-1-46, $p<0.0001$).

Quality assessment

The quality of the included study is depicted in the Table S4. Five of the six studies included in the study have been conducted in Asia: 3 in Japan (Saito et al. 2004, Ide et al. 2011, Morita et al. 2012) and 2 in Taiwan (Lin et al. 2014b, Chiu et al. 2015b). The other study have been conducted in USA (Demmer et al. 2008b). Consequently results are not generalizable despite the selected participants were representative of the general population. One study was of retrospective design. In one study periodontitis was measured with Community Periodontal Index (Chiu et al. 2015a) or computerized medical notes (Lin et al. 2014a).

These large cohort studies of high quality according to the NOS scale.

Fig.3. Forest plot from random effects of meta-analysis evaluating the aHR (adjusted Hazard Ratio) among periodontitis cases in terms of incident diabetes/hyperglycemia as gathered from longitudinal observational studies (weighted mean difference (WMD), 95% Confidence Interval (C.I.)).

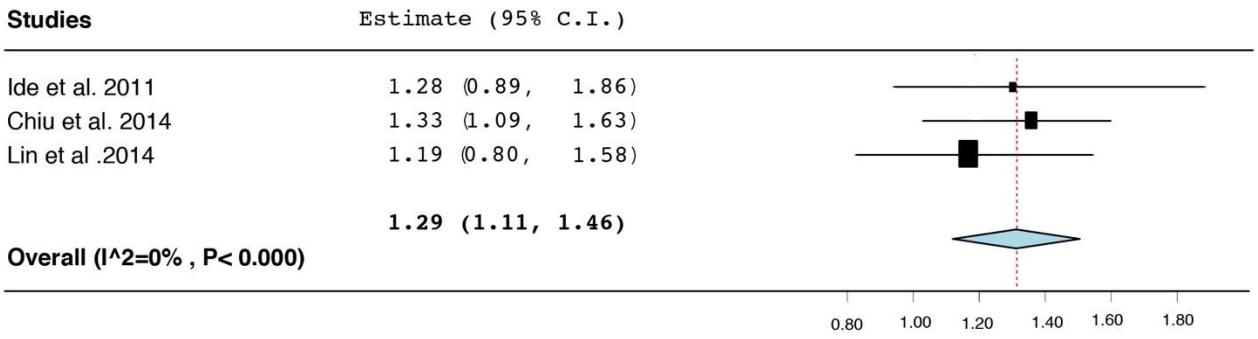


Table 3. Effect of Periodontal disease on the risk of incident diabetes

Author Year Country Study Design BL DM Typer	**A)Subjects: a. Perio Cases b. Comparison groups B) Age C) Study Duration**	**EXPOSURE**	**OUTCOME**	**Effect on Metabolic Control? & Generalisable?**	**Effect size: Odds Ratio (OR), Trend, HR, HRR & Significance (95%CI)**	**Effect on Metabolic Control/Conclusion**	**Confunders Controlled**
Saito et al. 2004 Japan Retrospective Cohort* No DM	All without DM @BL in 1988 A)@ FU in 1998: N1=961 (377M+584F); N2=591=those among N1 aged >40yrs in 1988; N3=545 w/HbA1c values both at BL and FU B) 40-79yrs C)10yrs	Partial mouth** PPD CAL PD-1: Mean DDP: a1) Intermediate: 1.3-2.0mm a2) Deep/High: >2.0mm b) Shallow/Low: <1.3mm PD-2: Mean CAL: a1) Intermediate: 1.5-2.5mm a2) High: >2.5mm b) Low: <1.5mm	2hr 75g OGTT @BL HbA1c	Yes, stat.sign. in Japanese (Hisayama) 40-79yrs community dwellers Not generalisable	1) High vs. Low PPD categories: Risk of DM:OR=2.6 (1.3-5.0;p=0.004) 2) Sign. increase in DM with mean CAL	1) Proportion w/DM increased significantly w/mean PPD 2) Each additional mm mean PPD corresponded to 0.13% HbA1c increase (p=0.007) 3) Severity of periodontal disease (expressed as either PPD or CAL) was sign. associated with development of manifest diabetes	age sex smoking BMI exercise alcohol
COMMENTS: *May be regarded as 1998 cross-sectional exam plus 1988 OGGT data, i.e., oral health data only from 1998 (not from BL 1988); **NHANES III protocol (1 max.+ 1 mand. quadrant) 4 "trained" examiners; No calibration reported							
Demmer et al. 2008 USA Prospective Cohort	A) N @ BL =11,375 (40%M+60%F) n @ FU =9,296 PD Groups@BL: PD-1#(PI)=Periodontal Index: a1) 762 (>0-0.87) a2) 761 (0.88-1.60) a3) 759 (1.61-2.44) a4) 759 (2.45-5.07) a5) 760 (5.08-8.0)	NHANES I protocol gingival inflammation extent presence or absence of periodontal pockets tooth mobility PDGroups@BL PD-1#(PI): "Periodontal	death certificates DM discharge diagnosis from health care facility self-reported DM requiring medication	Yes, stat. sign. in US adults Generalisable to US adults	1) Compared to those periodontally healthy (PI=0), the risk of incident DM were: 1a) PD-1#: OR for PI quintiles w/increasing PD: a1) 1.10(0.73-1.64) a2) 1.03(0.65–1.63) a3) 2.08(1.51-2.87) a4) 1.71(1.19-2.45) a5) 1.50(0.98–2.27)	1) The extent of periodontal disease (using PD-1) and periodontitis (using PD-2) were associated with incident diabetes 2) The association of periodontal disease (PD-1) and periodontitis (PD-	age sex race education smoking status BMI subscapular skinfold physical activity hypertension total cholesterol total caloric intake total protein

	a6) 2,127edentulous b) 3,368 (PI=0) (healthy) PD-2&#: a1) 2,135 gingivitis a2) 1,662 perio b) 3,372 healthy B) 50+19yrs [25-74yrs] C) 17(+4)yrs [1-22yrs]	Index"(Mean Score (0-8) for Dentition): a1) lowest PI to a5) highest PI quintile a6) edentulous b) healthy: PI=0 PD-2&#: a1) 2,135 gingivitis a2) 1,662 perio b) 3,372 healthy PD-3: # Natural Teeth: a1) 18-23 a2) 8-17 a3) 1-7 b) 24-32		1b) PD-2#&: Those with gingivitis had 40% and those with periodontitis 50% increased odds of developing DM (p< 0.05 for both) 1c) Edentulous: OR=1.3(1.00-1.70) 2) PD-3: Dentate with advanced tooth loss (25-31 teeth missing) had OR=1.70 (P<0.05) relative to those with minimal tooth loss (0-8 teeth)	2) with incident diabetes was found also in normo-weight andin never-smoking partecipants	total carbohydrate total fat poverty index white blood cell count
COMMENTS: Data from NHANES 1 [1971-1976 (BL)] & NHEFS 1982-1992 (FU); n = 817 incident DM cases were reported (cumulative incidence = 9%); &) *Wu et al. 2000*; #) *Hujoel et al. 2000*							
Ide et al. 2011 Japan Retrospective Cohort	A) # w/oral exams:N=8,752@BL /5,848@FU (3,883M+1,965F) Perio: a1) Moderate: 2,167 (37.1%) (1,511M+656F) a2) Severe:490 (8.4%) (384M+106F) b) No:3,191 (54.6%) (1,988M+1,203F) B) 30-59yrs; At FU: M:43.4(+7.5)yrs; F:43.9(+7.5)yrs C) 6.5yrs [2-7yrs]	Partial mouth (sextants) CPI Codes: 0: healthy 1: bleeding 2: calculus 3: >1 PPD 4-5mm 4:>1 PPD> 6mm BL CPI Scores: a1) Mod.: 3 a2) Sev.: 4 b) No: 0, 1, or 2	FPG>125mg/dL @ FU	1) Unadjusted: Yes, in employed 30-59yrs old Japanese 2) Adjusted: No Not generalisable	1) Unadjusted: HR for trend <0.0001 DM Incidence: a) No Perio (4.0% DM): HR=1(Referent) b) Mod.Perio(5.4% DM): HR=1.38(1.08-1.78) c) Sev.Perio(8.4% DM): HR=2.23(1.57-3.17) 2) Fully Adjusted: Females only: Mod.Perio: HR=2.3(1.30-4.08)	1) Moderate&severe perio sign. associated w/DM risk (Unadjusted only) 2) Tendency for increased risk, but not sign. after adjustment 3) Females w/mod. perio. have sign. higher risk for DM	age sex smoking BMI triglycerides hypertension HDL cholesterol gammaglutamyl-transpeptidase
COMMENTS: 7 "trained" examiners; Intra- & inter-examiner calibration done, but not recorded; Oral exam at BL only; FPG measured 1-6 times 2000-2007; No FU data on n = 2,904 w/oral exam@BL = 33.2% of original study population; No adjustment for education, income, exercise, medication, co-morbidities							

Morita et al. 2012 Japan Cohort	A) # @ BL unknown* N @ FU=6,125 (76.6%M+23.4%F) w/BL HbA1c<6.5% Nby CPI Code: a1) 4,114 (3,383M+731F) a2) 1,634 (1,424M+210F) b) 377 (240M+137F) B) [30-69yrs] C) 4-5.5yrs	Partial mouth CPI CPI Code 0: Healthy gingiva CPI Code 3: >1 PPD= 4-5mm CPI Code 4: >1 PPD> 6mm PD by CPI Code: a1) 3 a2) 4 b) 0	HbA1c>6.5% @ FU	Yes, stat. sign. in employed 30-69 years old (76.6% male) Japanese (Nagoya) Not generalisable	Relative risk (RR) for HbA1c≥6.5% at 5yr FU in groups w/PPD of 4-5mm was 2.47 (0.78-7.79; p=0.122) and for those w/PPD of >6mm: 3.45 (1.08-11.02; p=0.037)	Periodontal disease (pockets >6mm) leads to increased inci-dence of type 2 diabetes (HbA1c>6.5%) in 5 years	BMI alcohol smoking status sex age
COMMENTS: *No FU of those who left the workplace during study period; Kappa statistics 0.7-0.9 for calibration of 7 dentist periodontal examiners; Dose-response effect % of BL CPI codes 0, 3, & 4 w/5yr HbA1c>6.5%: 0.8%, 2.5%, and 3.9% (p = 0.001)							
Lin et al. 2014 Taiwan Retrospective cohort study 13 years DM-2	A) N=44601 a1) S-PD=22299 a2) M-PD=22302 B) age>40 yrs, mean 53.0 yrs C) 5.47 ± 3.54 years	Periodontitis Needing Surgical Treatment (YES or NOT)	• Incidence of DM2= patients who have been diagnosed with ICD- 9-CM codes 250 at least two times and concomitantly received antidiabetes medications.	Incidence of DM2 was 1.24-fold higher in the periodontitis cohort than in the control cohort, with an adjusted hazard ratio of 1.19 (95% confidence interval = 1.10 to 1.29); The elevated risk disappeared after being followed up for 6 years. Not generalizable	Diabetes Compared Between Periodontitis Cohorts With and Without Surgical Treatment: • IRR=1.24 (1.18 to 1.30) • aHR=1.19 (1.10 to 1.29)	Patients with periodontitis needing dental surgery have increased risk of future diabetes within 2 years compared with those participants with periodontitis not requiring dental surgery.	• Age • Sex • Urbanization • Income • Comorbidity • Hypertension • Hyperlipidemia • coronary artery disease • Obesity
COMMENTS: diagnosis according to ICD-9 codes. Comparison is among difference type of periodontits cohorts (no periodontally healthy controls)							
Chiu et al 2015 Taiwan Cohort	A) N= 5,885 a1) 1341 b1) 4033 B) 35–44 yrs C) 5 yrs	- CPI - PD= CPI ≥3	• FPG • 8 hr FBG	The cumulative incidence rates of hyperglycemia show that PD with CPI > 3 led to a dramatic increase in the risk of	•aHR of incident hyperglycemia (including diabetes)= 1.33 (95 % CI 1.09–1.63	Periodontal disease increases the risk (33%) of hyperglycemia	• Demographic features • Life style (betel quid chewing, smoking, and drinking), • Personal, and family disease history (DM2, hypertension,

	incident hyperglycemia compared with CPI <3 Not generalizable		cardiovascular and cerebrovascular disease, hyperlipidemia, and stroke) • Anthropometric measurements: tape measure, and weight scales. • Waist size • BP • Frequency of the dietary pattern classified into 5 levels: never or seldom, 1–2, 3–4, 5–6, and more than 7 times/per week over the past 6 months • TG • TC • HDL

COMMENTS: calibrated examiners. No HR for diabetes only presented.

Appendix. Tab. S4 NOS scale for quality rating of Cohort Studies

Author, Year	A) SELECTION (Max. 4 Stars) (Max. 4 Stars)				B) COMPARABILITY (Max. 2 Stars)	C) OUTCOME (max 3 star)			TOTAL OF STAR
	Representativeness of the exposed cohort (Max 1 Star)	Selection of non-exposed cohort (Max 1 Star)	Ascertainment of exposure (Max 1 Star)	. Demonstration that outcome was not present at start of study (Max 1 Star)	Comparability of cohorts on the basis of the design or analysis (Max 2 Stars) Study controls for age (= most important factor) Study controls for smoking (=additional important factor)	Assessment of outcome (Max 1 Star) Independent or blind assessment stated in the paper, or confirmation of the outcome by reference to secure records (X-rays, medical/dental records, etc.) Record linkage Self-report only (no confirmation by secure records) No description	Was follow-up long enough for outcomes to occur (Max 1 Star)	Adequacy of follow-up of cohorts (ensuring losses are not related to exposure or outcome) (Max 1 Star)	
Saito et al. 2004	★	★		★	★★	★	★	★	8
Demmer 2008	★	★	★	★	★★	★	★	★	9
Ide et al. 2011	★	★		★	★★	★	★		7
Morita 2012	★	★	★	★	★★	★	★	★	9
Lin et al. 2014	★	★	★	★	★★	★	★		8
Chiu et al. 2015	★	★	★	★	★★	★	★	★	9

Discussion

Summary of the evidence

- The available evidence suggests that:
- Subjects affected by periodontal disease and not affected by diabetes are associated to higher level of HB1Ac, fasting blood glucose or pre-diabetes/diabetes prevalence. In particular, these subjects show a statistically significant increase of 0.29 % of Hb1AC (0.20-0.37 %, 95% C.I.).
- Subjects affected by periodontal disease show a 29% significant higher risk (adjusted hazard ratio 1.29 95% CI 1,11-1-46) of developing incident diabetes when affected by severe periodontitis compared to periodontal healthy subjects.

Limitations

Most studies suffered of intrinsic limitations that render the overall applicability of the results. Samples were sometimes limited and not generalizable. Most importantly, some of the evidence was indirectly drawn from manuscripts which primary intention was not to assess the effect of periodontal disease on glycaemic control. Heterogeneity in terms of adjustment was important and, in multifactorial pathologies such as the ones we are dealing with, it might have an impact. Another limitation is that only article in English were searched and publication bias cannot be excluded.

Conclusions

Periodontal disease has a negative influence on glycaemic control of people with not known diabetes; in particular subjects affected by severe periodontitis are characterized by higher level of HB1Ac, fasting blood glucose and present a 29 % higher risk of developing diabetes, respect those people with healthy periodontium.

References

Arora, N., Papapanou, P.N., Rosenbaum, M., Jacobs, D.R., Desvarieux, M. & Demmer, R.T. (2014) Periodontal infection, impaired fasting glucose and impaired glucose tolerance: results from the Continuous National Health and Nutrition Examination Survey 2009-2010. *Journal Of Clinical Periodontology* **41**, 643–52.

Banu, S., Jabir, N.R., Mohan, R., Manjunath, N.C., Kamal, M.A., Vinod Kumar, K.R., Zaidi, S.K., Khan, M.S. & Tabrez, S. (2015) Correlation of toll-like receptor 4, interleukin-18, transaminases, and uric Acid in patients with chronic periodontitis and healthy adults. *Journal Of Periodontology* **86**, 431–9.

Boland, M.R., Hripcsak, G., Albers, D.J., Wei, Y., Wilcox, A.B., Wei, J., Li, J., Lin, S., Breene, M., Myers, R., Zimmerman, J., Papapanou, P.N. & Weng, C. (2013) Discovering medical conditions associated with periodontitis using linked electronic health records. *Journal Of Clinical Periodontology* **40**, 474–82.

Borgnakke, W.S., Ylöstalo, P. V., Taylor, G.W. & Genco, R.J. (2013) Effect of periodontal disease on diabetes: Systematic review of epidemiologic observational evidence. *Journal Of Clinical Periodontology* **40**, S135-52.

Chang, J.-F., Yeh, J.-C., Chiu, Y.-L., Liou, J.-C., Hsiung, J.-R. & Tung, T.-H. (2017) Periodontal Pocket Depth, Hyperglycemia, and Progression of Chronic Kidney Disease: A Population-Based Longitudinal Study. *The American Journal Of Medicine* **130**, 61–69.e1.

Chiu, S.Y.H., Lai, H., Yen, A.M.F., Fann, J.C.Y., Chen, L.S. & Chen, H.H. (2015a) Temporal sequence of the bidirectional relationship between hyperglycemia and periodontal disease: a community-based study of 5,885 Taiwanese aged 35???44??years (KCIS No. 32). *Acta Diabetologica* **52**, 123–131.

Chiu, S.Y.-H., Lai, H., Yen, A.M.-F., Fann, J.C.-Y., Chen, L.-S. & Chen, H.-H. (2015b) Temporal sequence of the bidirectional relationship between hyperglycemia and periodontal disease: a community-based study of 5,885 Taiwanese aged 35-44 years (KCIS No. 32). *Acta Diabetologica* **52**, 123–31.

Choi, Y.-H., McKeown, R.E., Mayer-Davis, E.J., Liese, A.D., Song, K.-B. & Merchant, A.T. (2014) Serum C-reactive protein and immunoglobulin G antibodies to periodontal pathogens may be effect modifiers of periodontitis and hyperglycemia. *Journal Of Periodontology* **85**, 1172–81.

Demmer, R.T., Desvarieux, M., Holtfreter, B., Jacobs, D.R., Wallaschofski, H., Nauck, M., Völzke, H. & Kocher, T. (2010a) Periodontal status and A1C change: longitudinal results from the study of health in Pomerania (SHIP). *Diabetes Care* **33**, 1037–43.

Demmer, R.T., Desvarieux, M. & Jacobs, D.R. (2008a) Periodontal Disease and Incident Type 2 Diabetes. *Diabetes Care* **31**, 1373–1379.

Demmer, R., Desvraieux, M., Holtfreter, B., Jacobs, D., Wallaschofski, H., Nauck, M., Volzke, H. & Kocher, T. (2010b) Periodontal Status and A1c Change. *Diabetes Care* **33**, 1037–1043.

Demmer, R.T., Jacobs, D.R. & Desvarieux, M. (2008b) Periodontal disease and incident type 2 diabetes: results from the First National Health and Nutrition Examination Survey and its epidemiologic follow-up study. *Diabetes Care* **31**, 1373–9.

Eke, P.I. & Dye, B. (2009) Assessment of self-report measures for predicting population prevalence of periodontitis. *Journal Of Periodontology* **80**, 1371–9.

El-Beshbishy, H.A., Maria, R.A. & Bardi, F.A. (2014) Biochemical and C-reactive protein alterations in myocardial infarction periodontitis patients. *The American Journal Of The Medical Sciences* **348**, 181–5.

Flores, M.F., Montenegro, M.M., Furtado, M. V, Polanczyk, C.A., Rösing, C.K. & Haas, A.N. (2014) Periodontal status affects C-reactive protein and lipids in patients with stable heart disease from a tertiary care cardiovascular clinic. *Journal Of Periodontology* **85**, 545–53.

Garcia, D., Tarima, S. & Okunseri, C. (2014) Periodontitis and Glycemic Control in Diabetes: NHANES 2009-2012. *Journal Of Periodontology*, 1–10.

Gokhale, N.H., Acharya, A.B., Patil, V.S., Trivedi, D.J., Setty, S. & Thakur, S.L. (2014) Resistin Levels in Gingival Crevicular Fluid of Patients With Chronic Periodontitis and Type 2 Diabetes Mellitus. *Journal Of Periodontology* **85**, 610–617.

Hong, J.W., Noh, J.H. & Kim, D.-J. (2016) The Prevalence and Associated Factors of Periodontitis According to Fasting

Plasma Glucose in the Korean Adults. *Medicine* **95**, e3226.

Hu, D., Hong, X. & Li, X. (2011) Oral health in China – trends and challenges. *International Journal Of Oral Science* **3**, 7–12.

Ide, R., Hoshuyama, T., Wilson, D., Takahashi, K. & Higashi, T. (2011) Periodontal disease and incident diabetes: a seven-year study. *Journal Of Dental Research* **90**, 41–6.

Islam, S.K.M.A., Seo, M., Lee, Y.-S. & Moon, S.-S. (2015) Association of periodontitis with insulin resistance, β-cell function, and impaired fasting glucose before onset of diabetes. *Endocrine Journal* **62**, 981–9.

Javed, F., Ahmed, H.B., Saeed, A., Mehmood, A. & Bain, C. (2013) Whole Salivary Interleukin-6 and Matrix Metalloproteinase-8 Levels in Chronic Periodontitis Patients With and Without Prediabetes. *Journal Of Periodontology* **85**, e130–e–135.

Kapellas, K., Skilton, M., Maple-Brown, L., Do, L., Bartold, P., O'Dea, K., Brown, A., Celermajer, D. & Jamieson, L. (2014) Periodontal disease and dental caries among Indigenous Australians living in the Northern Territory, Australia. *Australian Dental Journal* **59**, 93–99.

Lear, S.A., Teo, K., Gasevic, D., Zhang, X., Poirier, P.P., Rangarajan, S., Seron, P., Kelishadi, R., Tamil, A.M., Kruger, A., Iqbal, R., Swidan, H., Gomez-Arbelaez, D., Yusuf, R., Chifamba, J., Kutty, V.R., Karsidag, K., Kumar, R., Li, W., Szuba, A., Avezum, A., Diaz, R., Anand, S.S., Rosengren, A., Yusuf, S. & Prospective Urban Rural Epidemiology (PURE) study. (2014) The association between ownership of common household devices and obesity and diabetes in high, middle and low income countries. *Canadian Medical Association Journal* **186**, 258–266.

Lee, K.S., Lee, S.G., Kim, E.K., Jin, H.J., Im, S.U., Lee, H.K., Merchant, A.T., Song, K.B. & Choi, Y.H. (2015) Metabolic Syndrome Parameters in adolescents may be determinants for the future periodontal diseases. *Journal Of Clinical Periodontology* **42**, 105–112.

Liberati, A., Altman, D.G., Tetzlaff, J., Mulrow, C., Gøtzsche, P.C., Ioannidis, J.P.A., Clarke, M., Devereaux, P.J., Kleijnen, J. & Moher, D. (2009) The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate health care interventions: explanation and elaboration. *Journal Of Clinical Epidemiology* **62**, e1-34.

Lin, S.-Y., Lin, C.-L., Liu, J.-H., Wang, I.-K., Hsu, W.-H., Chen, C.-J., Ting, I.-W., Wu, I.-T., Sung, F.-C., Huang, C.-C. & Chang, Y.-J. (2014a) Association between periodontitis needing surgical treatment and subsequent diabetes risk: a population-based cohort study. *Journal Of Periodontology* **85**, 779–86.

Lin, S.-Y., Lin, C.-L., Liu, J.-H., Wang, I.-K., Hsu, W.-H., Chen, C.-J., Ting, I.-W., Wu, I.-T., Sung, F.-C., Huang, C.-C. & Chang, Y.-J. (2014b) Association between periodontitis needing surgical treatment and subsequent diabetes risk: a population-based cohort study. *Journal Of Periodontology* **85**, 779–86.

Lockhart, P.B., Bolger, A.F., Papapanou, P.N., Osinbowale, O., Trevisan, M., Levison, M.E., Taubert, K.A., Newburger, J.W., Gornik, H.L., Gewitz, M.H., Wilson, W.R., Smith, S.C. & Baddour, L.M. (2012) Periodontal Disease and Atherosclerotic Vascular Disease: Does the Evidence Support an Independent Association?: A Scientific Statement From the American Heart Association. *Circulation* **125**, 2520–2544.

Longo, P., Artese, H., Rabelo, M., Kawamoto, D., Foz, A., Romito, G., Dib, S. & Mayer, M.P.A. (2014) Serum levels of inflammatory markers in type 2 diabetes patients with chronic periodontitis. *Journal Of Applied Oral Science* **57**, 1–6.

Loos, B.G., Craandijk, J., Hoek, F.J., Wertheim-van Dillen, P.M. & van der Velden, U. (2000) Elevation of systemic markers related to cardiovascular diseases in the peripheral blood of periodontitis patients. *Journal Of Periodontology* **71**, 1528–34.

Mattila, P.T., Niskanen, M.C., Vehkalahti, M.M., Nordblad, A. & Knuuttila, M.L.E. (2010) Prevalence and simultaneous occurrence of periodontitis and dental caries. *Journal Of Clinical Periodontology* **37**, 962–967.

Moher, D., Liberati, A., Tetzlaff, J. & Altman, D.G. (2009) Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *BMJ* **339**, b2535–b2535.

Morita, I., Inagaki, K., Nakamura, F., Noguchi, T., Matsubara, T., Yoshii, S., Nakagaki, H., Mizuno, K., Sheiham, A. & Sabbah, W. (2012) Relationship between periodontal status and levels of glycated hemoglobin. *Journal Of Dental Research* **91**, 161–6.

Morita, T., Yamazaki, Y., Mita, A., Takada, K., Seto, M., Nishinoue, N., Sasaki, Y., Motohashi, M. & Maeno, M. (2010) A

cohort study on the association between periodontal disease and the development of metabolic syndrome. *Journal Of Periodontology* **81**, 512–9.

Needleman, I., Nibali, L. & Di Iorio, A. (2015) Professional mechanical plaque removal for prevention of periodontal diseases in adults - Systematic review update. *Journal Of Clinical Periodontology* **42**, S12–S35.

Perayil, J., Suresh, N., Fenol, A., Vyloppillil, R., Bhaskar, A. & Menon, S. (2014) Comparison of glycated hemoglobin levels in individuals without diabetes and with and without periodontitis before and after non-surgical periodontal therapy. *Journal Of Periodontology* **85**, 1658–66.

Petersen, P.E., Bourgeois, D., Ogawa, H., Estupinan-Day, S. & Ndiaye, C. (2005) The global burden of oral diseases and risks to oral health. *Bulletin Of The World Health Organization* **83**, 661–9.

Rao Deepika, P.C. & Saxena, R.M. (2013) Comparison of glycosylated hemoglobin levels in severe periodontitis patients and healthy controls: a study in an Indian population. *Quintessence International (Berlin, Germany : 1985)* **44**, 319–25.

Saito, T., Shimazaki, Y., Kiyohara, Y., Kato, I., Kubo, M., Iida, M. & Koga, T. (2004) The severity of periodontal disease is associated with the development of glucose intolerance in non-diabetics: the Hisayama study. *Journal Of Dental Research* **83**, 485–90.

Srinivasa, T.S., Agrawal, P., Goyal, P., Farista, S., Sowmya, N.K. & Deonani, S. (2015) Comparative clinical evaluation of glycosylated haemoglobin level in healthy and chronic periodontitis patients: A chairside diagnostic method. *Indian Journal Of Dental Research : Official Publication Of Indian Society For Dental Research* **26**, 504–7.

Taylor, G.W., Burt, B.A., Becker, M.P., Genco, R.J., Shlossman, M., Knowler, W.C. & Pettitt, D.J. (1996) Severe Periodontitis and Risk for Poor Glycemic Control in Patients with Non-Insulin-Dependent Diabetes Mellitus. *Journal Of Periodontology* **67**, 1085–1093.

Tu, Y.K., D'Aiuto, F., Lin, H.J., Chen, Y.W. & Chien, K.L. (2013) Relationship between metabolic syndrome and diagnoses of periodontal diseases among participants in a large Taiwanese cohort. *Journal Of Clinical Periodontology* **40**, 994–1000.

World Health Organization. (2016) *Global Report on Diabetes*.

Xiong, X., Elkind-Hirsch, K.E., Xie, Y., Delarosa, R., Maney, P., Pridjian, G. & Buekens, P. (2013) Periodontal disease as a potential risk factor for the development of diabetes in women with a prior history of gestational diabetes mellitus. *Journal Of Public Health Dentistry* **73**, 41–9.

Ylöstalo, P., Anttila, S., Rajala, U., Päivänsalo, M., Keinänen-Kiukaanniemi, S., Sakki, T. & Knuuttila, M. (2010) Periodontal infection and subclinical atherosclerosis: the role of high-density lipoprotein as a modifying factor. *Journal Of Clinical Periodontology* **37**, 617–24.

Conflict of Interest and Sources of Funding Statement

The study was partly funded by the Italian Ministry Health and the Tuscan Region (Grant # GR-2009-1592229 "Periodontal disease as emergent systemic pathology: development of a new clinical unit for patient global health care") and partly self-supported by the Unit of Dentistry and Oral Surgery of the Department of Surgical and Medical Pathology of the University of Pisa. Prof. Graziani has previously received a research grant from Straumann AG despite not related to this article. The authors have stated explicitly that there are no conflicts of interest in connection with this article.

VOLUMETRIC AND HISTOLOGIC ANALYSIS OF BONE REMODELLING PATTERN AFTER IMMEDIATE AUGMENTATION OF COMPROMISED EXTRACTION SOCKETS IN PERIODONTITIS PATIENTS: A 1-YEAR RANDOMIZED CONTROLLED STUDY

Analisi volumetrica ed istologica del pattern di rimodellamento osseo dopo ricostruzione immediata di alveoli con pareti riassorbite in pazienti affetti da malattia parodontale: studio controllato e randomizzato con 1 anno di follow-up

Valeria Manavella *, Federica Romano*, Marilisa Toma, Claudia Dellavia**,**

Mario Aimetti *

*Department of C.I.R. Dental School, Surgical Sciences, University of Turin, Turin, Italy

**Department of Biomedical, Surgical and Dental Sciences, University of Milan, Milan, Italy

Abstract

Objectives: To evaluate linear and volumetric hard tissue changes and new bone formation in severely resorbed alveolar sockets after ridge augmentation procedure compared with spontaneous healing.

Material and methods: Thirty hopeless teeth for advanced periodontitis were randomly allocated to test or control group. After extraction, test sites were grafted with collagenated bovine-derived bone xenograft (DBBM-C) and covering collagen membrane, while control sites had spontaneous healing. Linear and volumetric measurements were calculated on superimposed cone bean computed tomography images obtained after tooth extraction and 12 months later before implant placement. At this time, bone core biopsies were harvested for histological analysis.

Results: Horizontal dimensions decreased by 1.97 mm and by 3.83 mm in the test and control group, respectively. A rebuilding of the buccal wall was observed in both groups, although most pronounced in the grafted sockets (2.50 ± 2.12 mm *versus* 0.51± 1.02 mm). A significant difference was also registered in volume loss between grafted and non-grafted sites (9.14% *versus* 35.16%, P <0.0001), and in new lamellar bone formation.

Conclusion: Immediate augmentation using DBBM-C and collagen membrane is effective in improving alveolar ridge shape and dimensions, reducing the need for further bone regenerative procedures at the time of implant placement.

Riassunto

Obiettivo: comparare le variazioni lineari e volumetriche e la neoformazione ossea in alveoli con pareti riassorbite dopo procedura di aumento di cresta rispetto alla guarigione naturale.

Materiali e Metodi: Trenta elementi dentali da estrarre per motivi parodontali sono stati randomizzati in un gruppo test o controllo. Dopo l'estrazione, gli alveoli test sono stati trattati con inserimento di xenoinnesto bovino collagenato (DBBM-C) e membrana in collagene stabilizzata e in doppio strato, mentre quelli controllo sono andati incontro a guarigione naturale. Le misure lineari e volumetriche sono state calcolare sulle immagini Cone-Beam sovrapposte, ottenute dopo l'estrazione e a 12 mesi. Biopsie di tessuto osseo sono state prelevate per l'analisi istologica.

Risultati: La dimensione orizzontale si è ridotta di 1.97 mm nel gruppo test e di 3.83 mm nei controlli. Si sono osservati un aumento verticale, più pronunciato nel gruppo test, a livello della parete vestibolare (2.50 ± 2.12 mm verso 0.51± 1.02 mm), mentre una maggiore riduzione volumetrica (9.14% verso 35.16%, *P* <0.0001) e neoformazione di osso lamellare nei controlli.

Conclusioni: La ricostruzione immediata mediante DBBM-C e membrana in collagene si è dimostrata efficace nel migliorare la morfologia della cresta ossea, riducendo la necessità di ulteriori procedure rigenerative al momento dell'inserimento implantare.

Introduction

Following tooth extraction the residual alveolar bone undergoes marked qualitative and quantitative changes.[1,2] Schropp et al. described a mean vertical reduction of 0.8 mm and a collapse of more than 50% of the bucco-lingual ridge dimension during the first year after tooth extraction.[3] A similar magnitude of dimensional bone loss was reported in recent systematic reviews on post-extraction sockets with intact 4-wall configuration.[4,5]
Data on the healing pattern of sockets with compromised bony walls are limited. A decrease of about 60% in the horizontal dimensions was observed in buccal-bone-deficient alveolar sockets within the first 8 weeks of healing[6], compared with a 35% reduction in intact extraction sites over a 6-month period in the animal model[7]. Therefore, it can be expected that compromised extraction sites undergo more pronounced additional atrophy than intact sockets as a result of the natural remodelling process.
In such cases critical-sized alveolar ridge defects are most likely to occur leading to increased difficulty in placing the implant fixture at a prosthodontically suitable position. To restore the lost volume and facilitate implant insertion several ridge reconstructive techniques, including guided bone regeneration (GBR), distraction osteogenesis, and use of particulate and block grafting materials, have been proposed.[8-10] Most of these demanding and technically sensitive procedures could not be required if the shape and dimensions of the compromised extraction sockets are restored at the time of tooth extraction.[11] Since damaged extraction sockets are uncontained defects, the GBR technique is recommended to ensure clot protection and space maintenance by positioning a barrier membrane instead of the compromised bony wall.[12]
Recent clinical studies in humans reported favourable outcomes when fresh extraction sockets with a bone dehiscence > 5 mm at the buccal wall or showing partial buccal wall deficiency were treated with the insertion of hydroxyapatite or corticocancellous bone and collagen membrane.[13,14]
No information is available on the immediate reconstruction of alveolar sockets with severely resorbed buccal/lingual plate in patients with severe periodontitis. The association of a

secured resorbable collagen membrane with a bovine xenograft may be a promising combination. Collagen barriers show chemotactic effect over gingival fibroblasts, proangiogenic qualities, and early wound stabilization but require bone graft materials to maintain space for regeneration.[15,16] Deproteinized bovine bone mineral coated with 10% porcine derived collagen (DBBM-C) seems to be an appropriate material for alveolar reconstruction.[17,18] Collagen would seem to render the bone mineral surface more attractive for cell adhesion.[19,20] However, mixed histologic data arise from the literature. While some Authors reported improved new bone formation in intact alveolar sockets, others failed to confirm it.[21,22]

In view of these considerations, the aim of the present randomized controlled trial was to analyse 12-month volumetric and histological tissue modifications of severely resorbed alveolar sockets grafted with DBBM-C and a collagen membrane compared with spontaneous healing in periodontitis patients.

Material and methods

This single-centre randomized controlled clinical study was approved by the Institutional Ethical Committee (Protocol n° 695/2015). Adult patients requiring tooth extraction for advanced periodontitis were consecutively recruited between January and June 2015 at the Section of Periodontology, C.I.R. Dental School, University of Turin (Italy). Each patient signed informed consent form.

Main inclusion criteria were at least one hopeless tooth in the maxillary or mandibular anterior or premolar region to be extracted for periodontal reasons in patients with chronic periodontitis[23] who had completed the etiological periodontal therapy. Only sockets with severely resorbed buccal wall were included. Preliminary screening was performed on the basis of clinical examination and intraoral radiography.

Exclusion criteria were as follows: systemic diseases precluding surgical procedures (such as bone, metabolic disorders, uncontrolled diabetes), current use of steroids and bisphosphonates, smoking > 10 cigarettes/day, pregnancy and lactation, active periodontal disease, full-mouth plaque score (FMPS) and full-mouth bleeding score (FMBS) >20% at the time of tooth extraction.[12] Hopeless teeth due to trauma, endodontic problems or prosthetic reasons were excluded from the study.

Randomization and allocation concealment

Each participant was randomly assigned to receive either the test (DBBM-C and collagen membrane) or the control procedure (natural healing) by a computer-generated table. A balanced randomly permuted block was used to prepare the randomization table to avoid unequal balance between two treatments. Forms with the treatment modality were put into identical and opaque envelopes with the patient corresponding number on the outside and placed into the custody of a clinician not involved in diagnosis or treatment delivery. He opened the envelope just after tooth extraction and informed the surgeon which treatment had to be performed. The operators involved in the radiographic and histological analysis, as well as the statistician were blinded.

Surgical protocol

Prophylactic antibiotic therapy (2 g amoxicillin and clavulanic acid) was administered 1 h prior to surgery. Intra-oral antisepsis was performed with 0.2% chlorhexidine digluconate (CHX) rinse for 2 minutes. After extraction, sockets were thoroughly curetted and copiously irrigated with sterile saline solution. On test sites, two vertical releasing incisions were made beyond the mucogingival junction and a buccal mucoperiosteal flap was elevated (Fig. 1). Lingual tissues were undermined at least 10 mm beyond the alveolar crest margin. The socket was augmented by means of DBBM-C (BioOss® Collagen, Geistlich Pharma AB, Wolhusen, Switzerland) with light compaction to the most coronal bone peak level and covered by a double layer of collagen membrane (Bio-Gide®, Geistlich Pharma AB) secured with pins. The flap was sutured in the presurgical position by horizontal mattress sutures. On the control sites, no augmentation procedure was performed (Fig. 2). Healing was by secondary intention in both test and control sites. A resin bonded provisional pontic was used to replace front teeth taking care to avoid any pressure on the underlying tissue.

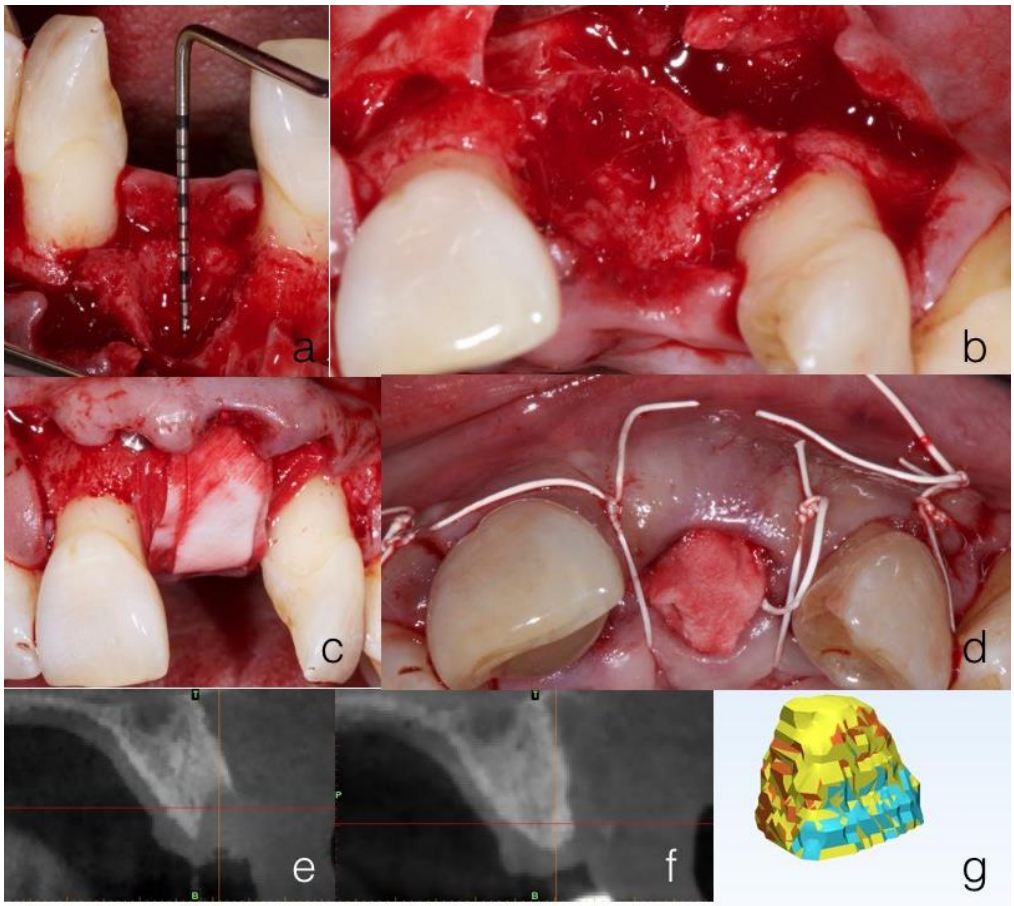


Figure 1. *Pictures of clinical case of the augmentation technique (test group): (a-b) intraoperative view of buccal deficiency following following extraction of hopeless maxillary central incisor, (c) membrane placement, (d) sutures, (e-f) CBCT images of the alveolar socket at baseline and 12 months after the reconstructive procedure, (g) 3D rendering of the augmented alveolar socket. Changes in the socket volume from baseline (yellow) are represented as a 3D coloured-mapped model (augmentation in orange, and resorption in cyan).*

Post-operative care

Patients were prescribed amoxicillin 1g and ibuprofen 600 mg to be taken after the surgical session, and 0.12% CHX mouthwash to be used twice daily for 2 weeks. Sutures were removed at day 14. Patients were recalled weekly within the first month and every three months for the maintenance periodontal treatment.

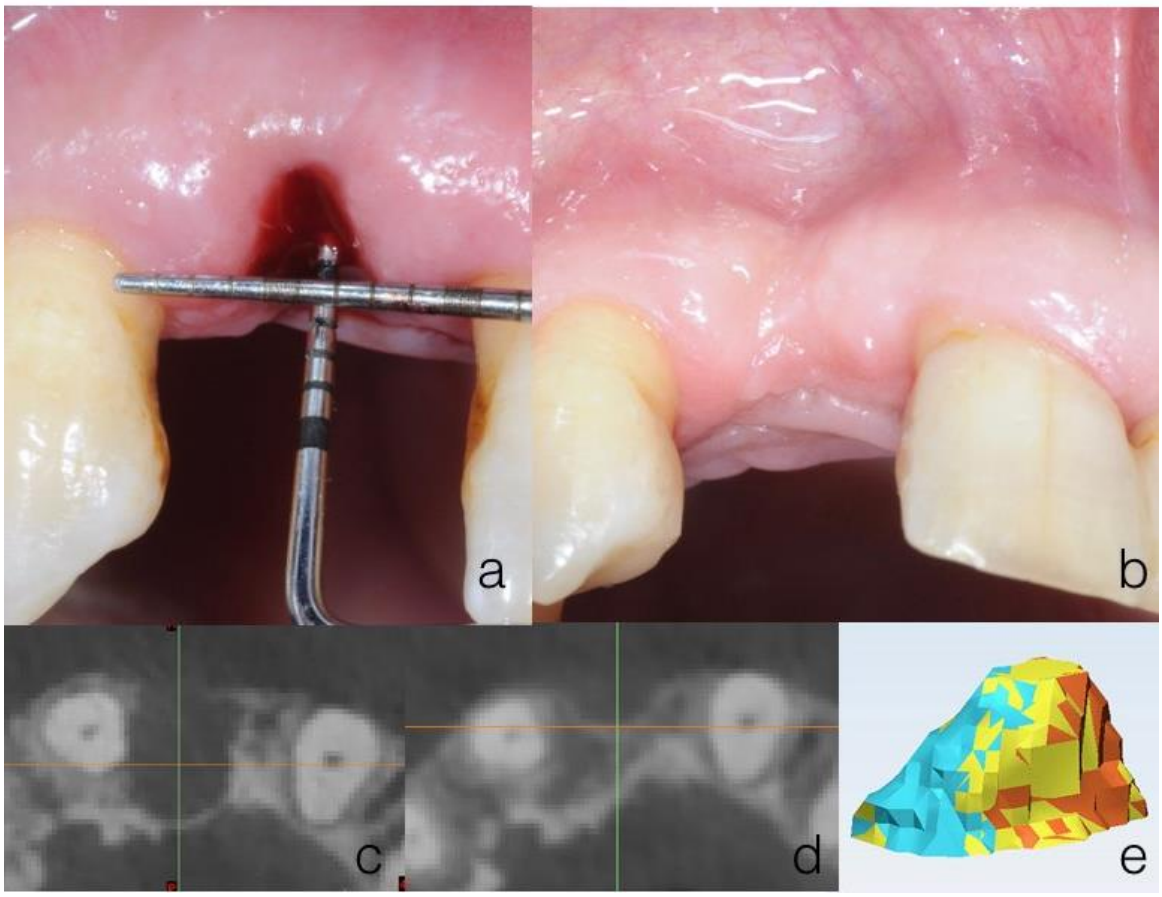


Figure 2. *Pictures of a clinical case in the control group: (a) clinical view following extraction of hopeless maxillary lateral incisor, (b) 12 months after extraction, (c-d) CBCT images of the alveolar socket at baseline and 12 months later, (e) 3D rendering of the alveolar socket.*

Radiographic volumetric and linear measurements

CBCT scans (New Tom/NTVG; field of view = 153.60 mm; thickness = 0.4 mm slice; pixels size = 0.3 mm; voltage = 110 kV; 2 mA; 10 s) were obtained immediately after tooth extraction and 12 months later just before implant placement (Fig. 1 e-f). Patients were asked to wear a custom-made template in radiotransparent acrylic resin with aluminium radiopaque markers (high-precision balls, diameter 5 mm, volume 65.5 mm^3, Martin & C. Srl, Italy). All DICOM (Digital Imaging and Communications in Medicine) images were imported into Mimics 17.0 software (Mimics Innovation Suite®, Materialise NV, Belgium) where bone and teeth were segmented by a mask creation tool, using thresholds corresponding to the greyscale values of these anatomic structures. The 3D-rendered images of the volumetric reconstructions of the alveolar sockets were then generated and exported as STL files. The superimposition of the

volumes of interest (VOI), corresponding to the alveolar sockets, was obtained by inserting some reference points on the pre- and postoperative CBCT images that were appropriately realigned and fused. The middle and posterior reference points were identified according to Alsufyani et al.[23], while the anterior landmarks were placed in the centre of the aluminium spheres. By means of the rendering operation it was possible to calculate 3D volumetric measurements of the VOI (Figs. 1g, 2e) The volume and the percentage of mineralized bone tissue loss were calculated at three zones: 1) from the most coronal part of the preoperative crest to 1 mm apically (0-1 mm zone); 2) from 1 to 3 mm below the alveolar crest (1-3 mm zone); 3) from 3 to 5 mm below the alveolar crest (3-5 mm zone).
On the same CBCT images linear measurements were made according to Jung et al.[17]. Three reference lines were drawn: a vertical line, parallel to the long axis of the socket, from the apex to the centre of the sockets (C–C) and two horizontal lines, perpendicular to line C-C, projecting from the most coronal portion and the most apical point of the alveolar socket. The following parameters were recorded: thickness of the vestibular bone plate at three levels (1 mm, 3 mm and 5 mm) below the lingual bone crest (VT-1, VT-3, VT-5) (only at baseline); height of the socket at the mid-vestibular (HV) and mid-lingual (HL) aspect; height of the socket at the vertical references line (H); horizontal ridge width measured at the horizontal coronal reference line (W); and horizontal ridge width at three levels (1 mm, 3 mm, 5 mm) below the most coronal aspect of the crest (HW-1, HW-3, HW-5).
Radiographic measurements were recorded by a masked engineer. In order to assess the intra-examiner reproducibility, 30% of the cases were randomly reanalysed in two different occasions. The duplicate measurements differed by <6%.

Histological analysis

Histological specimens were obtained from the centre of prior extraction sockets 12 months after extraction, at the time of implant placement, by means of a trephine bur. Bone biopsies were prepared according to the method by Donath & Breuner.[24] Briefly, they were fixed in 10% formalin/0.1 M phosphate buffer saline solution for 10 days, dehydrated in increasing ethanol concentrations and embedded in acrylic resin (Kulzer Technovit 7200 VLC, Bio-Optica, Milan, Italy). Each sample was sectioned along the major axis of the biopsy, and grounded to 70 μm. Sections were stained with toluidine blue/pyronine G (Sigma–Aldrich) and observed using a Nikon light microscope (Eclipse E600) equipped with a calibrated digital camera (DXM1200, Nikon). Histomorphometrical measurements were performed at a magnification of 10x using a stereological method and separate quantifications of areas of mineralized tissue, graft particles, and non-mineralized tissue were performed.

Sample size calculation

Each patient provided one alveolar socket to be treated. The horizontal width was set as the primary outcome. A sample size of 12 patients per group was calculated to detect a minimum difference of 1.5 mm between test and control treatment procedures at 1-year follow-up with an expected standard deviation (SD) of 1.2 mm, a two-sided alpha error of 0.05 and a power of 80%.[25] For compensation of possible dropouts, 30 individuals were recruited.

Statistical analysis

The statistical unit was the patient. The Shapiro–Wilk test confirmed the gaussian distribution of the volumetric and linear variables, except for HV. Pair-wise comparisons were performed by the Wilcoxon-signed-rank test or by the paired *t*-test for matched samples and by the Mann-Whitney *U*-test or the Student *t*-test for independent samples. Differences between groups in qualitative variables were assessed by means of the Chi-square test. All statistical tests were two-tailed and conducted at a 5% level of significance. All analyses were conducted using a statistical tool package (SPSS version 19, IBM, Chicago, IL, USA).

Results

Forty-two subjects were assessed for their eligibility. Of these 12 were excluded: 8 did not meet the inclusion criteria, while the other four refused tooth extraction. As a result, a total of 30 patients with advanced chronic periodontitis (18 females and 12 males, mean age 53.2 ± 6.3 years, range 45-68 years) were enrolled in the study and randomly assigned to the test or control procedures. All 30 participants (15 [test] and 15 [control]) received the allocated procedure and were included in the statistical analyses.

Patient characteristics at baseline were not significantly different ($P > 0.05$) between groups (Table 1). The distributions of hopeless teeth were: 53.3% incisive, 6.7% canine, and 40% premolar for the test group and 60% incisive, and 40% premolar, for the control group.

Table 1. Baseline characteristics of patients

Variables	Test Group (*n*=15)	Control Group (*n*=15)	*P*-value
Age *(years)*	53.6 ± 7.4	52.9 ± 5.1	0.765 [a]
Males/females *(n)*	7/8	5/10	0.709[b]
Light smokers *(n)*	1/15	3/15	0.598[b]
Maxilla/Mandible *(n)*	13/2	11/4	0.651[b]
Incisors/Canines/Premolars *(n)*	8/1/6	9/0/6	0.792[b]

[a]Unpaired *t*-test

[b]Chi-square test

Radiographic measurements

As reported in Tables 2 and 3, no statistically significant difference was detected for any of the baseline defect dimensions between treatment modalities. At baseline the buccal bone plate was detectable on CBCT images in only 4 test and 5 control sockets at -3 mm level, whereas it appeared relatively intact at the more apical portion in most of the extraction sites.

Both groups showed a significant horizontal width reduction from baseline to the 12-month follow-up (Table 2) with an average shrinkage of 3.83 ± 1.49 mm in the control group and 1.97 ± 1.55 mm in the test group (all $P < 0.0001$). The differences between the treatment groups

were statistically significant (*P* <0.0001) and more pronounced in the cervical alveolar region (4.92 ± 2.45 mm *versus* 2.60 ± 1.24 mm, HW-1). At 12 months, no statistically significant vertical changes were observed with respect to the lingual crest (LH) in both test and control sites, whereas it was possible to recognize a height gain on the buccal wall (HV) with values ranging from 0.51 ± 1.02 mm in the control group to 2.50 ± 2.12 mm in the test group. The difference reached statistical significance (*P* < 0.0001).

Table 2. Changes in ridge height and width between baseline and 12 months based on CBCT measurements (mean ± SD).

Variables	**Test Group (*n*=15)**	**Control Group (*n*=15)**	***P*-value**
H *(mm)*			
Baseline	6.92 ± 1.54	6.68 ± 1.05	0.680
12 months	8.26 ± 1.59	6.22 ± 1.12	<0.0001
Difference (mm)	1.34 ± 1.45	-0.46 ± 1.35	
Difference (%)	22.11 ± 24.18	-5.31 ± 19.81	
P-value	0.003	0.182	
HV *(mm)*			
Baseline	2.89 ± 2.27	2.74 ± 1.49	0.902
12 months	5.39 ± 1.35	3.25 ± 1.53	<0.0001
Difference (mm)	2.50 ± 2.12	0.51 ± 1.02	
Difference (%)	82.12 ± 18.73	27.97 ± 38.47	
P-value	0.002	0.046	
HL *(mm)*			
Baseline	6.96 ± 1.60	6.04 ± 1.11	0.078
12 months	7.23 ± 1.13	5.83 ± 1.01	<0.0001
Difference (mm)	0.27 ± 1.31	-0.21 ± 0.78	
Difference (%)	7.89 ± 25.83	-1.88 ± 16.97	
P-value	0.435	0.326	
W *(mm)*			
Baseline	8.62 ± 1.57	7.82 ±1.64	0.105
12 months	6.65 ± 1.41	3.99 ± 1.30	<0.0001
Difference (mm)	-1.97 ± 1.55	-3.83 ± 1.49	

Difference (%)	-21.63 ± 16.25	-46.32 ± 17.61	
P-value	<0.0001	<0.0001	
HW_1 *(mm)*			
Baseline	8.27 ± 1.50	7.63 ± 1.48	0.249
12 months	5.68 ± 1.03	2.72 ± 2.52	<0.0001
Difference (mm)	-2.60 ± 1.24	-4.92 ± 2.45	
Difference (%)	-30.51 ± 12.05	-64.38 ± 31.88	
P-value	<0.0001	<0.0001	
HW_3 *(mm)*			
Baseline	7.84 ± 1.29	7.04 ± 1.09	0.077
12 months	6.98 ± 1.24	5.03 ± 1.39	<0.0001
Difference (mm)	-0.86 ± 0.82	-2.01 ± 0.97	
Difference (%)	-10.67 ± 9.48	-28.93 ± 13.86	
P-value	0.001	<0.0001	
HW_5 *(mm)*			
Baseline	6.91 ± 2.20	6.13 ± 1.52	0.654
12 months	7.37 ± 1.32	5.52 ± 1.38	<0.0001
Difference (mm)	0.34 ± 0.87	-0.61 ± 1.14	
Difference (%)	4.38 ± 11.16	-8.46 ± 17.31	
P-value	0.160	0.061	

H = total height; HL = mid-lingual height; HV = mid-buccal height; W = total (bucco-lingual) horizontal width; HW_1_3_5 = horizontal width at 1-3-5 mm from the top of the crest. Positive values = gain of tissue; negative values = loss of tissue.

The volumetric analysis (Table 3) showed a statistically significant decrease in volume (*P* = 0.001) in both groups with a mean difference of 18.61 ± 17.93 mm^3 (9.14%) in the grafted sockets that increased to 62.09 ± 25.81 mm^3 (35.16%) in the non-grafted sites. The most pronounced differences were observed in the first millimetre below the bone crest (zone 1) where the control group displayed an average loss of bone volume of 18.14 ± 12.13 mm^3 (42.96%) compared to 6.20 ± 6.34 mm^3 (16.24%) in the test group (*P* = 0.001). No additional bone regenerative procedures were needed at the time of implant placement in the grafted sites.

Table 3. Changes in ridge volume between baseline and 12 months based on CBCT measurements (mean ± SD).

Variables	**Test Group (*n*=15)**	**Control Group (*n*=15)**	***P*-value**
Volume *(mm^3)*			
Baseline	208.80 ± 83.53	183.65 ± 69.32	0.595
12 months	190.19 ± 82.05	121.56 ± 66.96	<0.0001
Difference (mm^3)	18.61 ± 17.93	62.09 ± 25.81	
Difference (%)	9.14 ± 8.44	35.16 ± 10.92	
P-value	0.001	0.001	
Zone 1 *(mm^3)*			
Baseline	42.24 ± 16.27	40.20 ± 12.49	0.672
12 months	36.05 ± 18.29	22.06 ± 10.49	0.001
Difference (mm^3)	6.20 ± 6.34	18.14 ± 12.13	
Difference (%)	16.24 ± 17.50	42.96 ± 21.78	
P-value	0.002	<0.0001	
Zone 2 *(mm^3)*			
Baseline	85.76 ± 36.63	78.54 ± 30.96	0.564
12 months	77.05 ± 35.91	55.38 ± 25.21	0.066
Difference (mm^3)	8.72 ± 8.09	23.15 ± 15.41	
Difference (%)	10.14 ± 8.96	29.26 ± 12.71	
P-value	0.001	<0.0001	
Zone 3 *(mm^3)*			
Baseline	56.26 ± 24.74	46.17 ± 22.57	0.253
12 months	53.36 ± 23.67	36.26 ± 21.22	0.046
Difference (mm^3)	2.89 ± 4.92	9.91 ± 12.48	
Difference (%)	4.87 ± 6.82	20.41 ± 22.79	
P-value	0.059	0.008	

Zone 1-2-3 = volume at 0-1 mm, 1-3 mm and 3-5 mm from the top of the crest.

Histomorphometrical measurements

All samples had a normal structure without evident presence of inflammatory infiltrate at 12 months of healing (Figs 3a, 4). In the test group, grafted particles were still present and were surrounded by new woven bone, confirming the process of osseointegration of the graft. At low magnification, lamellar bone tissue appeared organized in trabeculae, and the biomaterial was well integrated with woven bone that was completely organized. At higher magnification, no gaps between regenerated bone and biomaterial particles still existed (Fig. 3b). Histomorphometrical analysis revealed a significant difference only for lamellar bone values between groups (P = 0.02). Overall, a mean of 30.2 ± 8% lamellar bone, 20.8 ± 9.7% woven bone, and 22.2 ± 12.7% connective tissue were detected in the grafted sites compared to 52.8 ± 13.9%, 21.4 ± 9.9% and 23.8 ± 13.8%, respectively, in the non-grafted sites. The residual graft material was 26.8 ± 10.4%.

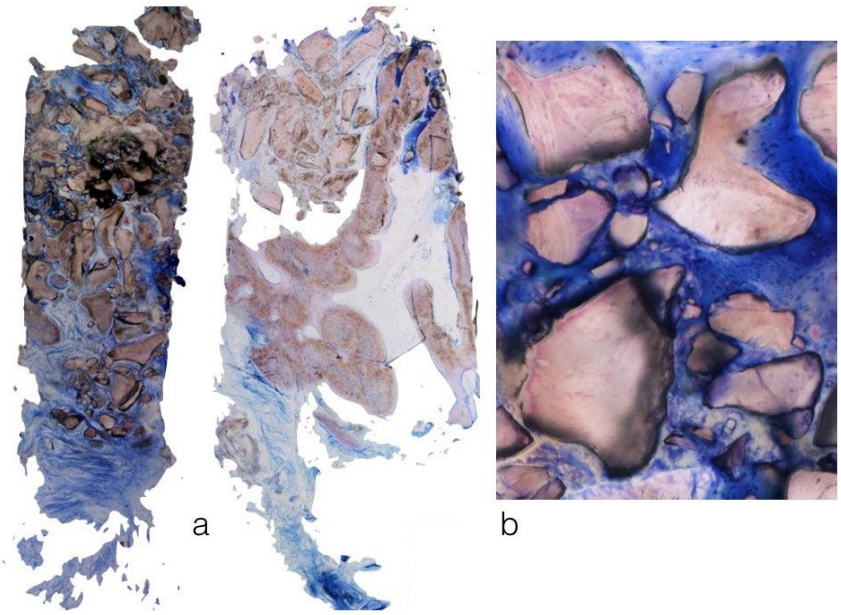


***Figure 3**. Histological analysis at 12 months of healing in test group: (a) overview of two samples. Biomaterial blocks (in dark brown) appeared surrounded by new woven bone and bone marrow (blue) (Toluidine Blue and Pyronine Y staining, total magnification 100x), (b) detail showing that the interface between biomaterial and new bone matrix was closed (total magnification 200x).*

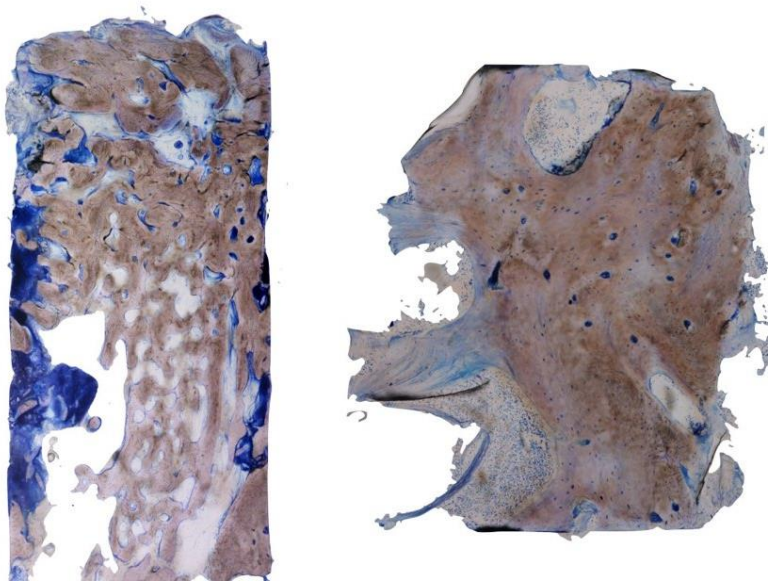


Figure 4. *Histological analysis at 12 months of healing in control group: overview of two samples.*

Discussion

The aim of this randomized controlled trial was to evaluate linear and volumetric hard tissue changes and new bone formation in augmented alveolar sockets with DBBM-C and covering collagen membrane during 12 months of follow-up compared with spontaneous healing. Only fresh alveolar sockets in the upper and lower anterior region with extensive buccal bone loss as a result of advanced chronic periodontitis were enrolled.

Unlike abundant data for peri-implant buccal bone defects demonstrating that they do not repair themselves completely without the use GBR procedures or grafting materials[26,27], there is scant information on dimensional changes that occur in compromised alveolar sockets following immediate augmentation procedures[13,14,28]. Furthermore, these studies mostly relied on direct intra-surgical or linear measurements on periapical radiographs on CBCT images or on volumetric analysis of scanned study cast and provided clinicians only with approximate estimates for volumetric bone ridge changes.[29]

In the present study, the VOI of the experimental sockets in the preoperative CBCT images were segmented and digitally superimposed on the corresponding postoperative VOI, allowing a qualitative and quantitative evaluation of 3D changes of the hard tissue volume and contour on 3D overlay images.[30] As suggested by Lagravère et al.[31], we considered 6 cranial-base landmarks and the centre of the spherical markers to optimize images superimposition. In addition, Mimics software ensures that the angles and distances between landmark points are maintained for different images. Of note, the baseline CBCT images were taken immediately after tooth removal. This eliminated the need of digital subtraction of pre-extraction target teeth, and had the advantage to avoid the superimposition of bone and dental tissue.

The 3D ridge morphology analysis in the present study showed a dome-shaped configuration of the edentulous crest after 12 months of healing as described in previous histological studies

on dogs.[32,33] The placement of Bio-Oss collagen in fresh extraction sockets seemed to counteract the bone remodelling process resulting in a less pronounced change of the buccal profile of the alveolar crest and a better maintenance of the hard tissue volume when compared to the naturally healing control. The non-grafted sockets resulted in a much narrower and irregular shape.[32]

A significant 3D volumetric bone loss of about 35% occurred in the non-grafted sockets. In agreement with previous studies on post-extraction sockets with buccal dehiscence defects, bone loss occurred primarily in the 0-3 mm zone apical to the alveolar crest (from 29% to 43%).[28,34] Conversely, the augmented sockets exhibited a volume deficiency of about 9% with respect to the maximum volume for regeneration. It was slightly more pronounced in the more coronal portion of the crest (10%) and decreased to 5% in its middle part. These data are consistent with findings by Araujo & Lindhe[1] who reported a 12% reduction in the coronal portion, and an increase of 4% in the middle and apical portion of intact sockets grafted with DBBM-C after a healing period of 6 months in the canine model.

With regard to linear hard tissue changes, the proposed ridge augmentation procedure was effective not only in limiting the physiological ridge reduction but also in repairing significant portion of the buccal wall as compared with tooth extraction alone. The mean differences between grafted and non-grafted sockets at 12 months were 1.86 mm in terms of buccolingual width (P <0.0001) and 2.00 mm for midbuccal height (P <0.0001), whereas the midlingual height was nearly unchanged in both groups. Interestingly, these findings were in line with those observed after alveolar ridge preservation techniques demonstrating that, in spite of the unfavourable anatomy, augmented sockets exhibit a pattern and a degree of bone resorption comparable with grafted intact sockets.[35]

Of note, all the sockets observed in this study presented a significant vertical increase of the buccal bone plate that was more pronounced in the augmented group. A vertical buccal bone growth was previously described by Crespi et al.[28] after 3 months of spontaneous healing in unfilled extraction sockets covered only with a collagen sponge. The authors hypothesized the role of the blood clot as a physical matrix amplifying and regulating the migration, proliferation and differentiation of cells involved in angiogenesis and subsequent new bone formation.[36,37] However, it should be considered the peculiar anatomy of compromised sockets that are uncontained defects in the coronal portion, but provide a bone envelope in the most apical part with high regenerative potential.

The present encouraging results may be also due to the long degradation time of the grafting material and to the subsequent lower turnover of the remodelling process in the extraction sockets. The uncontained anatomy of the socket defects requires the placement, in association with a secured membrane barrier, of a biomaterial capable of maintaining stable shape and dimensions in the early healing phase.[12] The fixation of the membrane plays a pivotal role in optimizing bone regeneration from a biological point of view.[6,38,39] Furthermore, the membrane was applied in a double layer to prolong its barrier function.[40,41]

Mixed data are available in the literature on time and amount of new bone formation with DBBM-C in humans. The initial histomorphometrical data on DBBM remodelling in fresh extraction sockets were described by Becker et al.[42] The authors reported that after 3–7 months of healing the bovine bone particles were surrounded by connective tissue with

marginal presence of new woven bone. Lindhe et al. found comparable results at 6-month evaluation. DBBM-C particles were not resorbed but surrounded by a provisional connective tissue in the central part of the grafted site, but the peripheral part showed new bone formation.[43] Conversely, Alkan et al. observed remaining DBBM-C particles mainly in the coronal area of grafted intact sockets after 3 months of healing. The quality and quantity of the bone was clinically sufficient for a correct implant placement.[44]

Due to the anatomy of alveolar sockets and the delayed healing of DBBM-C grafted sites, implants were placed 12 months after the reconstructive procedure to optimize primary stability.[45] The histomorphometrical analysis in this study revealed a high percentage of deproteinized bovine bone particles still remaining in site at 12 months. Despite the high presence of deproteinized bovine bone, a process of osteointegration was evident, and woven bone was observed surrounding grafts, with bridges of newly formed bone between xenograft particles. These findings proved the good osseointegration of graft material and the absence of inflammatory response.

Conclusion

Results from the present study suggest that 3D volumetric dimensional alterations of the hard tissues in severely resorbed alveolar sockets can be quite extensive. The application of a slow resorbing xenograft with a secured covering collagen membrane may prove effective not only in limiting post-extraction crestal ridge bone loss but also in improving alveolar ridge shape and dimensions with the advantage to simplify later implant insertion and to be less technically demanding than more complex GBR techniques. The present study permits also to confirm the osteoconductive properties of DBBM-C, at 12 months of healing, providing an explanation by a biological point of view.

References

Araújo, M.G. & Lindhe, J. (2005) Dimensional ridge alterations following tooth extraction. An experimental study in the dog. *Journal of Clinical Periodontology* **32**: 212-218.

Pietrokovski, J., Starinsky, R., Arensburg, B. & Kaffe, I. (2007) Morphologic characteristics of bony edentulous jaws. *Journal of Prosthodontics* **16**: 141-147.

Schropp, L., Wenzel, A., Kostopoulos, L. & Karring, T (2003) Bone healing and soft tissue contour changes following single-tooth extraction: a clinical and radiographic 12-month prospective study. *International Journal of Periodontics & Restorative Dentistry* **23**: 313-323.

Van der Weijden, F., Dell'Acqua, F., Slot, D.E. (2009) Alveolar bone dimensional changes of post-extraction sockets in humans: a systematic review. *Journal of Clinical Periodontology* **36**: 1048–1058.

Tan, W.L., Wong, T.L.T., Wong, M.C.M. & Lang, N.P. (2012) A systematic review of post-extractional alveolar hard and soft tissue dimensional changes in humans. *Clinical Oral Implants Research* **23**: 1-21.

Lee, J.S., Jung, J.S., Im, G.I., Kim, B.S., Cho, K.S., Kim, C.S. (2015) Ridge regeneration of damaged extraction sockets using rhBMP-2: an experimental study in canine. *Journal of Clinical Periodontology* **42**: 678-687.

Araújo, M.G. & Lindhe, J. (2009) Ridge preservation with the use of Bio-Oss Collagen: a 6-month study in the dog. *Clinical Oral Implants Research* **20:** 433-440.

Chiapasco, M., Zaniboni, M. & Rimondini, L. (2007) Autogenous onlay bone grafts vs. alveolar distraction osteogenesis for the correction of vertically deficient edentulous ridges: a 2-4-year prospective study on humans. *Clinical Oral Implants Research* **18**: 432-440.

Sbordone, L., Toti, P., Menchini-Fabris, G.B., Sbordone, C., Piombino, P. & Guidetti, F. (2009) Volume changes of autogenous bone grafts after alveolar ridge augmentation of atrophic maxillae and mandibles. *The International Journal of Oral and Maxillofacial Surgery* **38**: 1059-1065.

Sbordone, C., Toti, P., Guidetti, F., Califano, L., Santoro, A. & Sbordone, L. (2012) Volume changes of iliac crest autogenous bone grafts after vertical and horizontal alveolar ridge augmentation of atrophic maxillas and mandibles: a 6-year computerized tomographic follow-up. *Journal of Oral and Maxillofacial Surgery* **70**: 2559-2565.

McAllister, B.S. & Haghighat, K. (2007) Bone augmentation techniques. *Journal of Periodontology* **78**: 377-396.

Hämmerle, C.H.F., Araújo, M.G., Simion, M. & Osteology Consensus Group 2011 (2012) Evidence-based knowledge on the biology and treatment of extraction sockets. *Clinical Oral Implants Research* **23**(Suppl. 5): 80-82.

Sisti, A., Canullo, L., Mottola, M.P., Covani, U., Barone, A. & Botticelli, D. (2012) Clinical evaluation of a ridge augmentation procedure for the severely resorbed alveolar socket: multicenter randomized controlled trial, preliminary results. *Clinical Oral Implants Research* **23**: 526-535.

Barone, A., Ricci, M., Romanos, G.E., Tonelli, P., Alfonsi, F. & Covani, U. (2015) Buccal bone deficiency in fresh extraction sockets: a prospective single cohort study. *Clinical Oral Implants Research* **26**: 823-830.

Rothamel, D., Schwarz, F., Sculean, A., Herten, M., Scherbaum, W. & Becker, J. (2004) Biocompatibility of various collagen membranes in cultures of human PDL fibroblasts and human osteoblast-like cells. *Clinical Oral Implants Research* **15**: 443-449.

Rothamel, D., Schwarz, F., Sager, M., Herten, M., Sculean, A. & Becker, J. (2005) Biodegradation of differently cross-linked collagen membranes: an experimental study in the rat. *Clinical Oral Implants Research* **16**: 369-378.

Heberer, S., Al-Chawaf, B., Hildebrand, D., Nelson, J.J. & Nelson, K. (2008) Histomorphometric analysis of extraction sockets augmented with Bio-Oss Collagen after a 6-week healing period: A prospective study. *Clinical Oral Implants Research* **19**: 1219-1225.

Jung, R.E., Philipp, A., Annen, B.M., Signorelli, L., Thoma, D.S., Hämmerle, C.H.F., Attin, T. & Schmidlin, P. (2013) Radiographic evaluation of different techniques for ridge preservation after tooth extraction: a randomized controlled clinical trial. *Journal of Clinical Periodontology* **40**: 90–98.

Sculean, A., Chiantella, G.C., Windisch, P., Arweiler, N.B., Brecx, M. & Gera, I. (2005) Healing of intra-bony defects following treatment with a composite bovine-derived xenograft (Bio-Oss Collagen) in combination with a collagen membrane (Bio-Gide PERIO). *Journal of Clinical Periodontology* **32**: 720-724.

Schwartz, Z., Goldstein, M., Raviv, E., Hirsch, A., Ranly, D.M. & Boyan, B.D. (2007) Clinical evaluation of demineralized bone allograft in a hyaluronic acid carrier for sinus lift augmentation in humans: a computed tomography and histomorphometric study. *Clinical Oral Implants Research* **18**: 204-211.

Araújo, M.G., Linder, E. & Lindhe, J. (2011) Bio-Oss® Collagen in the buccal gap at immediate I mplants: a 6-month study in the dog. *Clinical Oral Implants Research* **22**: 1-8.

Heberer, S., Al-Chawaf, B., Jablonski, C., Nelson, J.J., Lage, H. & Nelson, K. (2011) Healing of ungrafted and grafted extraction sockets after 12 weeks: a prospective clinical study. *International Journal of Oral and Maxillofacial Implants* **26**: 385-392.

Armitage, G.C. (1999) Development of a classification system for periodontal diseases and conditions. *Annals of Periodontology* **4**: 1-6.

Alsufyani, N.A., Dietrich, N.H., Lagravère, M.O., Carey, J.P. & Major, P.W. (2014) Cone beam computed tomography registration for 3-D airway analysis based on anatomic landmarks. *Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology* **118**: 371-383.

Donath, K. & Breuner, G. (1982) A method for the study of undecalcified bones and teeth with attached soft tissues. The Säge-Schliff (sawing and grinding) technique. *Journal of Oral Pathology* **11**: 318-326.

Barone, A., Aldini, N.N., Fini, M., Giardino, R., Calvo Guirado, J.L. & Covani, U. (2008) Xenograft versus extraction alone for ridge preservation after tooth removal: a clinical and histomorphometric study. *Journal of Periodontology* **79**: 1370-1377.

Nevins, M. & Mellonig, J. (1992) Enhancement of damaged edentulous ridge to receive dental implants: A combination of allografts and the Gore-Tex membrane. *International Journal of Periodontics & Restorative Dentistry* **12**: 97-111.

Hürzeler, M.B., Kohal, R.J., Naghshbandi, J., Mota, L.F., Conradt, J., Hutmacher, D. & Caffesse, R.G. (1998) Evaluation of a new bioresorbable barrier to facilitate guided bone regeneration around exposed implant threads. An experimental study in the monkey. *The International Journal of Oral and Maxillofacial Surgery* **27**:315-320.

Crespi, R., Capparè, P. & Gherlone, E.F. (2014) Bone recontouring in fresh sockets with buccal bone loss: a cone beam computed tomography study. *The International Journal of Oral and Maxillofacial Implants* **29**: 863-868.

Horváth, A., Mardas, N., Mezzomo, L.A., Needleman, I.G. & Donos, N. (2012) Alveolar ridge preservation. A systematic review. *Clinical Oral Investigations* **17**: 341-363.

Linderup, B.W., Küseler, A., Jensen, J. & Cattaneo, P.M. (2015) A novel semiautomatic technique for volumetric assessment of the alveolar bone defect using cone beam computed tomography. *Cleft Palate Craniofacial Journal* **52**: e47-55.

Lagravère, M.O., Secanell, M., Major, P.W. & Carey, J.P. (2011) Optimization analysis for plane orientation in 3-dimensional cephalometric analysis of serial cone-beam computerized tomography images. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology* **111**: 771-777.

Araújo, M.G., Linder, E., Wennstrom, J. & Lindhe, J. (2008) The influence of Bio-Oss Collagen on healing of an extraction socket: an experimental study in the dog. *The International Journal of Periodontics & Restorative Dentistry* **28**: 123-135.

Araújo, M.G., Liljenberg, B. & Lindhe, J. (2010) Dynamics of Bio-Oss® Collagen incorporation in fresh extraction wounds: an experimental study in the dog. *Clinical Oral Implants Research* **21**: 55-64.

Abdelhamid, A., Omran, M., Bakhshalian, N., Tarnow, D. & Zadeh, H.H. (2016) Preservation and repair using SocketKAP™ and SocketKAGE™: part 2- Three-dimensional alveolar bone volumetric analysis of CBCT imaging. *Clinical Oral Implants Research* **27**:631-639.

Avila-Ortiz, G., Elangovan, S., Kramer, K.W.O., Blanchette, D. & Dawson, D.V. (2014) Effect of alveolar ridge preservation after tooth extraction: a systematic review and meta-analysis. *Journal of Dental Research* **93**: 950-958.

Amler, M.H. (1969) The time sequence of tissue regeneration in human extraction wounds. *Oral Surgery, Oral Medicine, Oral Pathology* **27**: 309-318.

Evian, C.I., Rosenberg, E.S., Coslet, J.G. & Corn, H. (1982) The osteogenic activity of bone removed from healing extraction sockets in humans. *Journal of Periodontology* **53**: 81-85.

Hämmerle, C.H.F., Jung, R.E., Yaman, D. & Lang, N.P. (2008) Ridge augmentation by applying bioresorbable membranes and deproteinized bovine bone mineral: a report of twelve consecutive cases. *Clinical Oral Implants Research* **19**: 19–25.

Kim, J.J., Schwarz, F., Song, H.Y., Choi, Y., Kang, K.R. & Koo, K.T. (2016) Ridge preservation of extraction sockets with chronic pathology using Bio-Oss® Collagen with or without collagen membrane: an experimental study in dogs. *Clinical Oral Implants Research* May 18, doi:10.1111/clr.12870.

Von Arx, T. & Buser, D. (2006) Horizontal ridge augmentation using autogenous block grafts and the guided bone regeneration technique with collagen membranes: a clinical study with 42 patients. *Clinical Oral Implants Research* **17**: 359-366.

Kim, S.H., Kim, D.Y., Kim, K.H., Ku, Y., Rhyu, I.C. & Lee, Y.M. (2009) The efficacy of a double-layer collagen membrane technique for overlaying block grafts in a rabbit calvarium model. *Clinical Oral Implants Research* **20**: 1124-1132.

Becker, W., Clokie, C., Sennerby, L., Urist, M.R. & Becker, B.E. (1998) Histologic findings after implantation and evaluation of different grafting materials and titanium micro screws into extraction sockets: case reports. *Journal of Periodontology* **69**: 414-421

Lindhe, J., Cecchinato, D., Donati, M., Tomasi, C. & Liljenberg, B. (2014) Ridge preservation with the use of deproteinized bovine bone mineral. *Clinical Oral Implants Research* **25**: 786-790.

Alkan, E.A., Parlar, A., Yildirim, B. & Sengüven, B. (2013) Histological comparison of healing following tooth extraction with ridge preservation using enamel matrix derivatives versus Bio-Oss Collagen: a pilot study. *International Journal of Oral and Maxillofacial Surgery* **42**: 1522-1528.

Rasperini, G., Canullo, L., Dellavia, C., Pellegrini, G. & Simion, M. (2010) Socket grafting in the posterior maxilla reduces the need for sinus augmentation. *International Journal of Oral and Maxillofacial Surgery* **30**: 265-273.

Epigenetics and its role on implant therapy: a review

L'epigenetica e il suo ruolo nella terapia implantare: review

Riccardo Di Gianfilippo*; **Carmine Di Gianfilippo***; **GiovanPaolo Pini Prato°**

** Private practice, Avezzano, Italy*

° Tuscan Academy of Dental Research, Florence, Italy

Short running title: Epigenetics and implant treatment

One sentence summary of key findings: Implant surfaces influence gene expression in osteoblasts

Key words: epigenomics, microRNAs, implant dentistry, osseointegration, review

ABSTRACT

Aim: To analyse all scientific articles investigating the relations between DNA methylations, histone modifications or micro-RNA production and implant therapy.
Methods: A systematic bibliographical electronic research was carried out on PubMed/Medline, selecting all potentially relevant publications dealing with the influences of implant surface features on gene activation and the influence of epigenetic changes on implant therapy outcomes. Review was conducted according to Cook's principles, with a pre-planned method and using explicit and reproducible criteria. Potentially relevant articles were investigated in a comprehensive search. All presented data were appraised, synthesized, interpreted and discussed.
Results: Of sixty-seven articles found, seventeen met the inclusion criteria. Both in vitro and in vivo studies were included. New implant treatments like KOH alkali-etching, electrolytic etching, ionization, functionalization with miRNAs or anti-microRNAs or osteogenic peptides seem to enhance osteoblast differentiation and genes activation by regulating miRNA production. However, due to the heterogeneity of methodologies and types of cells used, a meta-analysis was not possible to achieve. Besides, epigenetic changes on peri-implant cells induced by smoking still remain unclear.
Conclusions: New titanium implants, functionalized with gene activators, could contribute to develop a new generation of devices, cutting-edge of faster osseointegration. More investigations with other osteoblast-like cell lines, primary cultures, different time points and surfaces functionalized with genetic molecules are needed to get a global comprehension of the epigenetic influence on peri-implant biological mechanisms.

INTRODUCTION

Even though the rehabilitation of partial and total edentulism using dental implants has shown highly satisfactory clinical outcomes[1], 80% of subjects and 50% of implants exhibit mucositis or other biological problems[2,3]. Biological complications around dental implants have been attributed to several factors, from the establishment of a pathogenic microflora[4] to the presence of inflammatory cells close to the implant-abutment interface[5,6]. Despite the amount of existing hypothesis, the genetic mechanisms controlling the peri-implant biological processes remains largely unexplored.

In 2008, at a Cold Spring Harbor meeting, epigenetics was defined as a "stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence"[7]. Epigenetic modifications alter the structure of chromatin and influence gene expression without alterations in the sequence of bases. Moreover, epigenetic inheritance influences phenotypes over multiple generations in the absence of any apparent genetic mutation. Epigenetic changes are potentially reversible and can lead to the development and maintenance of cancer, immune or inflammatory diseases. On the other hand, epigenetic influences may play a protective role silencing parasitic DNAs, inactive X chromosome and imprinted genes[8].

Environmental stressors including toxins and microbial exposures[9], adrenaline and psychological stress[10], diet[11], hormones and toxicants[12] can change epigenetic patterns and thereby effect changes in gene activation and cell phenotype.

Epigenetic mechanisms mainly involve DNA methylation, histone modification and mRNA regulation by non-coding RNAs called microRNAs[13] (Fig.1.).

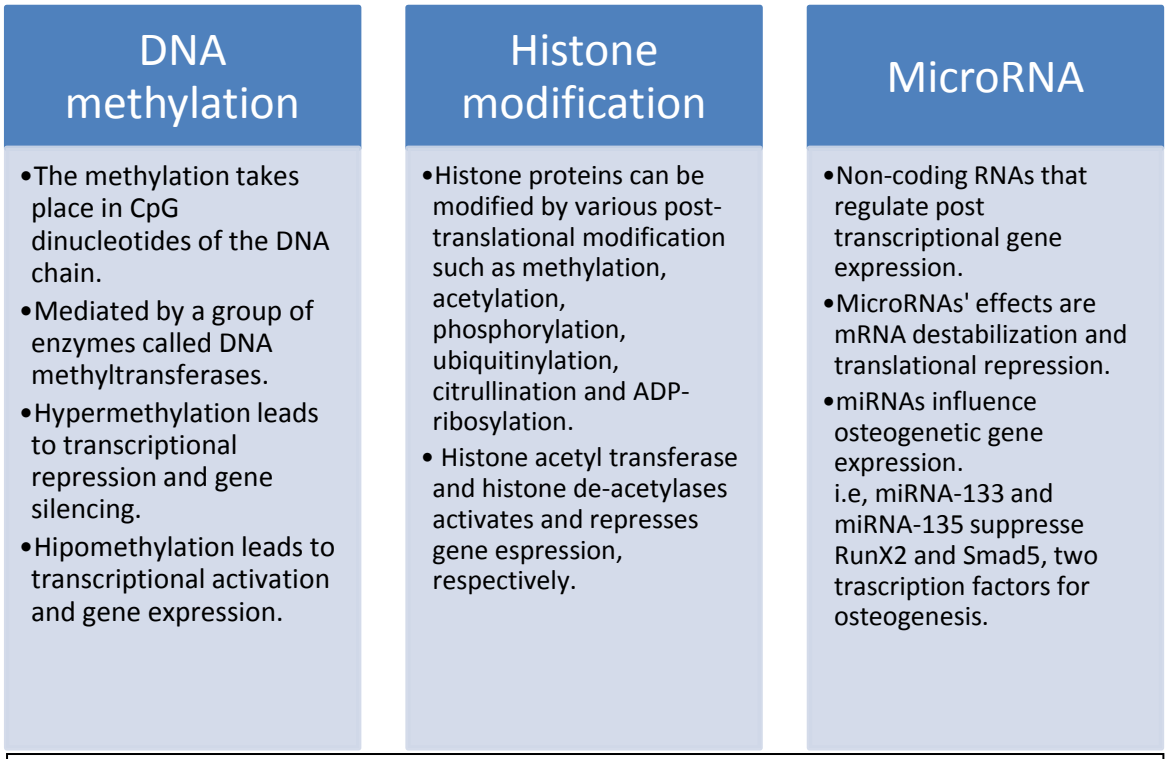


Fig.1. DNA methylation, histone modification and miRNA production influence the DNA expression through chromatin modification and post transcriptional gene repression.

DNA methylation takes place in Cytosine-phosphate-Guanine (CpG) dinucleotides of the DNA chain [14] and consists in a covalent enzymatic transfer of a methyl group from S-Adenosyl Methionine to the C-5 position of a cytosine residues in the CpG island of the promoter region. The methylated DNA sequence in CpG sites causes a more condensed DNA structure leading to transcriptional repression and gene silencing[15].The most known enzyme involved in the DNA methylation is the DNA methyltransferases. When DNA becomes methylated, those methyl groups protrude from the cytosine nucleotides into the major groove of the DNA to displace transcription factors that normally bind to the DNA[16].

Histones can be modified by various post-translational modifications. Acetylation of a core histone results in a more open chromatin's structure that facilitates gene expression. On the other hand, histone deacetylation brings to condensation of chromatin and inhibits gene transcription. Histone methylation can either results in an activated or repressed chromatin state[17]. Has been established that the processes of DNA methylation and histone modification are deeply linked. When the CpG island of a promoter becomes methylated, methyl-CpG-binding-proteins recruit histone deacetylases[18]. A strong electrostatic interaction occurs between the positively charged acetylated histone residues and the negatively charged DNA causing the deacetylation of histone proteins. Due to the condensed nucleosome particle, the gene expression is repressed**.**

One of the most documented epigenetic modifications is the post-transcriptional repression due the production of microRNAs (miRNAs or miR), brief sequences of non-coding RNAs composed by 18-22 nucleotides[19]. They are crucial in regulation of development, proliferation, differentiation, apoptosis and response to different extracellular signals and stress. Besides, they seem to be related with the expression of osteogenic genes like Runx2 and Smad5[20]. MicroRNA pathways regulate gene expression by inducing degradation and/or translational repression of target mRNAs. MicroRNAs influence gene expression by translational repression and gene silencing. If the miRNA production increases, levels of target mRNAs decrease and, therefore, the gene expression is repressed. In the same way, if the miRNA levels decrease, the gene expression is up-regulated. In addition, messengerRNA can bind the promoter of specific microRNAs activating an auto-regulatory feedback loop; thus, when a specific mRNA is up-regulated, the related miRNA is also over-expressed[21]. Each miRNA may target hundreds of mRNAs, and some targets are affected by multiple miRNAs. Probably, miRNAs are fundamental in the maintenance of pluripotency and undifferentiation of adult stem cells; indeed, several miRNAs appear to significantly modulate the differentiation of mesenchymal precursors in osteoblast cells, regulating the activity of transcription factors[19, 22].

Several authors investigated the role of epigenetics in chronic or aggressive periodontitis[23, 24, 25, 26]. Authors demonstrated that the expression levels of cytokines and chemokines [27, 28], toll-like receptors[29], protease-activated receptor[30], interleukin-8[31] and cyclooxygenase-2[24] could be affected by oral bacteria. A recent study documented that the presence of different oral bacteria resulted in differential methylation profile in gingival epithelia[32] while another article demonstrated a hypomethylated oral epithelia in patients with generalized aggressive periodontitis[23].

Despite some available data regarding epigenetics and periodontology, the knowledge on epigenetics related to implant dentistry lack of evidence. Only a few experimental in vivo and in vitro studies are available in literature. Therefore, aim of this review was to evaluate the available evidence investigating the potential effects of DNA methylations, histone modifications or micro-RNA production on implant survival, osseointegration, peri-implant mucositis, perimplantitis or implant-abutment leakage.

MATERIALS AND METHODS

This review was conducted according to Cook's principles, with a pre-planned method and using explicit and reproducible criteria[33]. Potentially relevant articles were investigated in a comprehensive search. All presented data were appraised, synthesized, interpreted and discussed[33].

A systematic bibliographical electronic research was carried out on PubMed/Medline, selecting all potentially relevant publications dealing with the influences of implant surface features on gene activation and the influence of epigenetic changes on implant therapy outcomes.

Search strategy

The systematic search was performed using the following terms and boolean connectors: *((epigenetics) OR (dna methylation) OR (dna methyl transferase) OR (histone deacetylation) OR (histone deacetylase) OR (histone methyl transferase) OR (histone demethylase) OR (micro-rna)) AND ((dental implant) OR (dental implants) OR (implantology) OR (implant dentistry) OR (implant failure) OR (platform shifting) OR (platform switching) OR (implant-abutment connection) OR (osseointegration) OR (mucositis) OR (perimplantitis)).*

The electronic outcome was: *(("epigenomics"[MeSH Terms] OR "epigenomics"[All Fields] OR "epigenetics"[All Fields]) OR ("dna methylation"[MeSH Terms] OR ("dna"[All Fields] AND "methylation"[All Fields]) OR "dna methylation"[All Fields]) OR ((("dna"[MeSH Terms] OR "dna"[All Fields]) AND methyl[All Fields] AND ("transferases"[MeSH Terms] OR "transferases"[All Fields] OR "transferase"[All Fields])) OR (("histones"[MeSH Terms] OR "histones"[All Fields] OR "histone"[All Fields]) AND deacetylation[All Fields]) OR ("histone deacetylases"[MeSH Terms] OR ("histone"[All Fields] AND "deacetylases"[All Fields]) OR "histone deacetylases"[All Fields] OR ("histone"[All Fields] AND "deacetylase"[All Fields]) OR "histone deacetylase"[All Fields]) OR ((("histones"[MeSH Terms] OR "histones"[All Fields] OR "histone"[All Fields]) AND methyl[All Fields] AND ("transferases"[MeSH Terms] OR "transferases"[All Fields] OR "transferase"[All Fields])) OR (("histones"[MeSH Terms] OR "histones"[All Fields] OR "histone"[All Fields]) AND demethylase[All Fields]) OR ("micrornas"[MeSH Terms] OR "micrornas"[All Fields] OR ("micro"[All Fields] AND "rna"[All Fields]) OR "micro rna"[All Fields])) AND (("dental implants"[MeSH Terms] OR ("dental"[All Fields] AND "implants"[All Fields]) OR "dental implants"[All Fields] OR ("dental"[All Fields] AND "implant"[All Fields]) OR "dental implant"[All Fields]) OR ("dental implants"[MeSH Terms] OR ("dental"[All Fields] AND "implants"[All Fields]) OR "dental implants"[All Fields]) OR implantology[All Fields] OR ("Implant Dent"[Journal] OR ("implant"[All Fields] AND "dentistry"[All Fields]) OR "implant dentistry"[All Fields]) OR (implant[All Fields] AND failure[All Fields]) OR (platform[All Fields] AND shifting[All Fields]) OR (platform[All Fields] AND switching[All Fields]) OR (implant-abutment[All Fields] AND connection[All Fields]) OR ("osseointegration"[MeSH Terms] OR "osseointegration"[All Fields]) OR ("mucositis"[MeSH Terms] OR "mucositis"[All Fields]) OR perimplantitis[All Fields])*

Selection of studies

Inclusion criteria: All scientific in vivo and in vitro publications investigating the impact of genetic expression levels on implant rehabilitations were included. According to Mulrow[34], studies with both direct and indirect evidence were also included. No filters like language or time limitation were applied.

Exclusion criteria: Published studies not meeting the inclusion criteria and all those not providing any information concerning dental implant therapy, up-regulation or down-regulation of genes or their products were excluded. All scientific articles resulted from the use of confounding words and not meeting the inclusion criteria were excluded; in example, articles aimed to investigate oncologic issues, mucositis different from peri-implant mucositis, or orthopaedic prosthetic joint were excluded.

Development of the review

This study was conducted over three phases. The first phase was the screening of titles and abstracts. The second phase consisted in the screening of full-text articles. Finally, the third phase consisted in the review of included articles. Two authors (RDG, CDG) worked independently and compared their results at the end of each phase. One author (GPP) supervised each phase in the role of quality control.

Two authors (RDG, GPP) worked on the critical analysis of included studies. For each included study, several data regarding activated genes, influences of implant materials on genes expression, types of implant materials, types of surface modifications, number of patients or types of cells studied were re-wrote.

RESULTS

The electronic search found sixty-seven articles (Fig.2.). During the first phase, which consists in the screening of titles and abstracts, eighteen studies were excluded because aimed to study oral mucositis during radiotherapy or chemotherapy, mucositis in patients with neoplastic diseases like cancer or leukaemia, tolerability of temsirolimus, graft-versus-host disease, fluorouracil toxicity, hypomethylating agent therapy for neoplastic diseases and radiation-induced tissue damages.

Forty-nine articles were downloaded and studied (Fig.2.). During the second phase, which consists in the full-text examination, thirty-two articles were excluded because not investigating the effect of gene expression changing on implant therapy, but evaluating data regarding: oral or neck cancer (seven articles), papilla stem cells (one article), nano-hydroxyapatite (one article), endometrial epithelial cells (one article), systemic influence of titanium and zirconia (one article), chemical drugs for systemic diseases (one article), osteogenic peptides but not associated with implant surfaces (two articles), muscle cells (one article), immunoglobulins (one article), myelodysplastic syndromes (one article), orthopaedic defects (five articles), critical limb ischemia (one article), cementoblasts (one article), bone-ligament cells (one article), calvarial bone defect (one article), genetic effects of adrenaline (one article), evaluation of systemic miRNAs (one article), or publications without information regarding gene expression (one article), or aimed to discuss different methods to detect RNAs from cells (three articles).

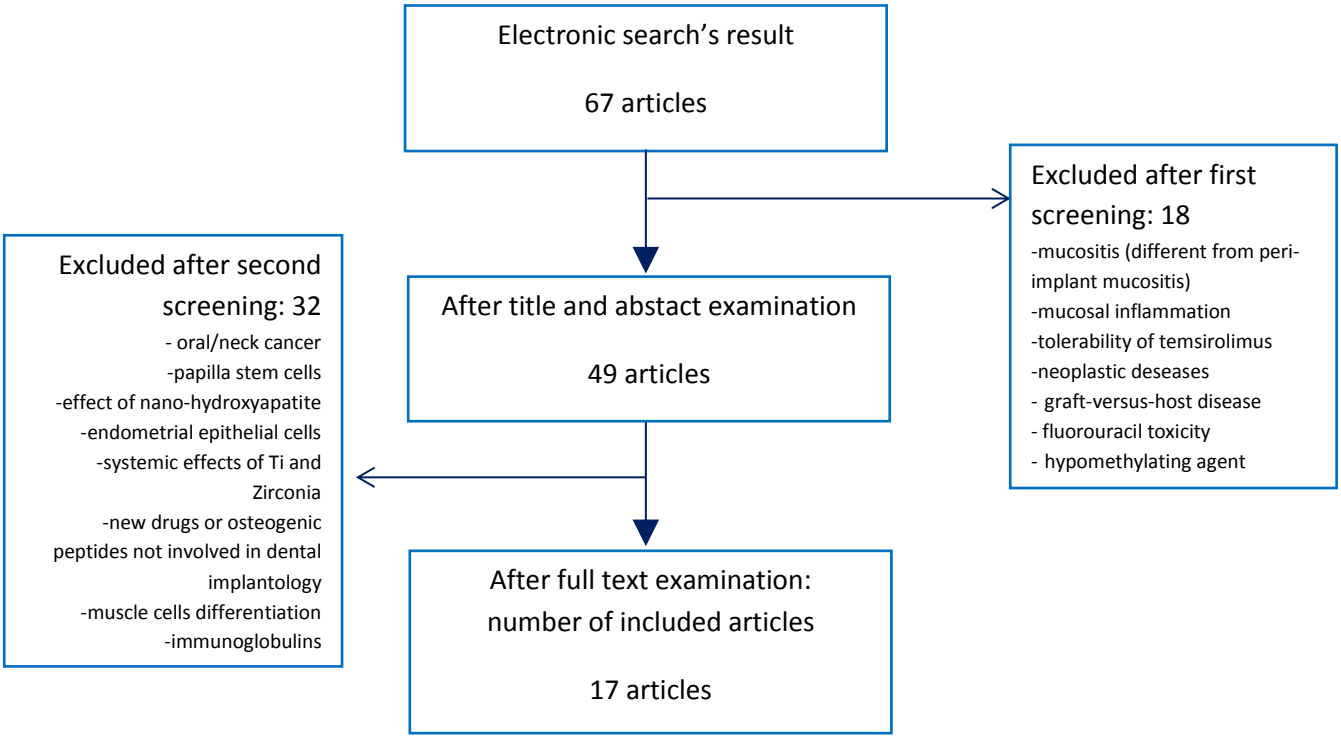


Fig.2. Flow-chart. Search strategy, screening for eligibility and final number of included publications: The electronic search found 67 studies regarding changing in gene expression and implantology. After title and abstract screening, 18 of them were excluded because focussed mainly on mucositis after radio/chemotherapy, mucosal inflammation, neoplastic deseases, hypomethylating agent therapy for neoplastic diseases and radiation-induced tissue damages. 49 articles were downloaded and studied. 32 of them were excluded because not investigating the effect of DNA methylations, histone modifications or micro-RNA production on implant survival, osseointegration, peri-implant mucositis, perimplantitis or implant-abutment leakage. Thus, this systematic review was finalized with

Thus, this review was finalized on seventeen scientific articles (Fig.3.) which were evaluated during the phase three. Fifteen of seventeen articles were in vitro or in vivo studies [35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49]. One was a randomized clinical trial with immune-histochemical analysis [50]. One article was a narrative review [51].

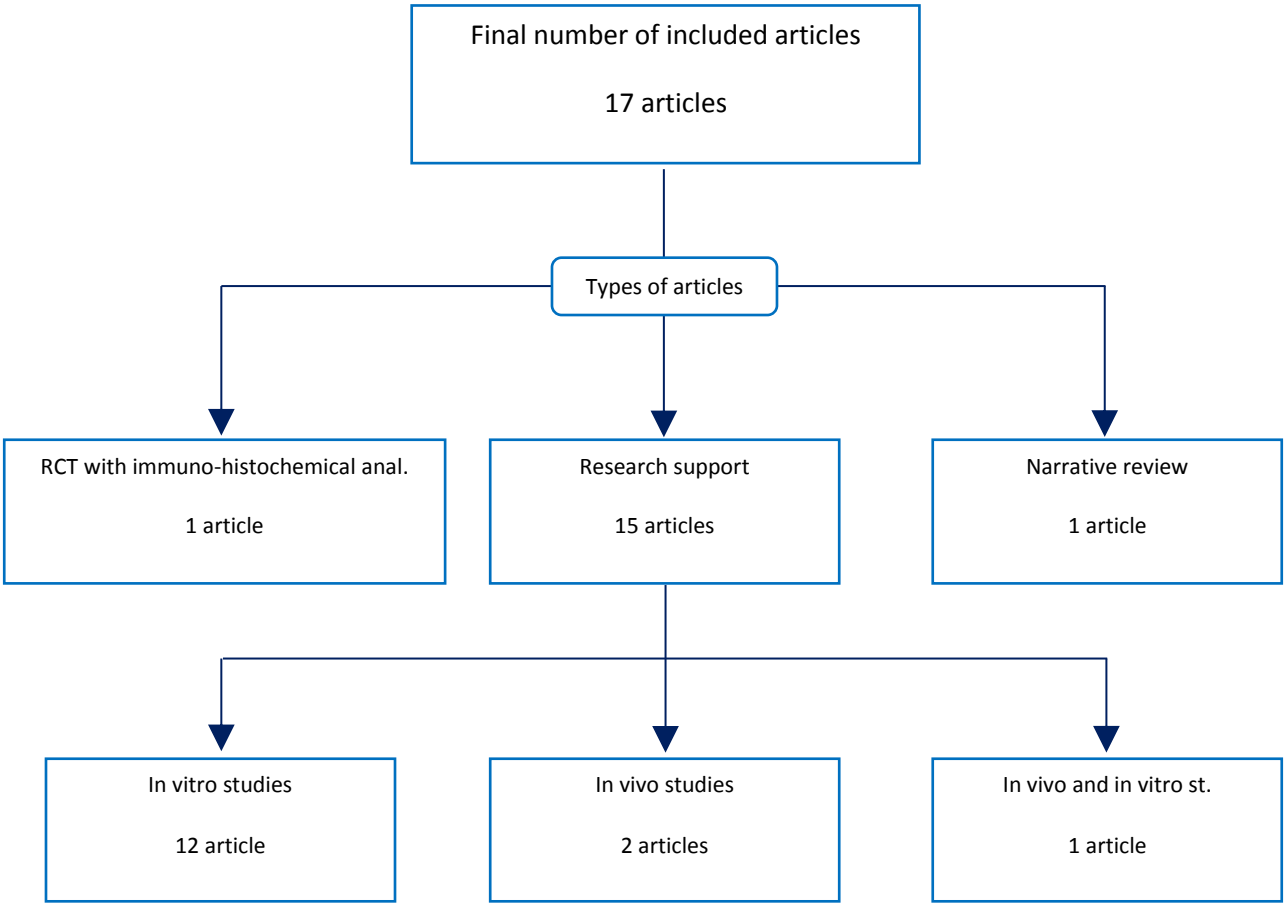


Fig.3. Types of included articles. Out of 17 included articles: 12 were in vitro studies, 2 were in vivo studies, 1 was both an in vivo and in vitro study, 1 was a RCT with immune-histocemical and RNA analyses, 1 was a narrative review. All 17 articles were reviewed.

In vivo and in vitro studies

Fifteen of the seventeen included publications aimed to compare changing in gene expression profiles of cells cultured on different implant surfaces[35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49]. (Tab. 1.)

Eight articles documented the miRNA production[36, 40, 41, 42, 44, 45, 46, 47]. Seven articles didn't provided information on miRNA production[35, 37, 38, 39, 43, 48, 49]. No one of the included articles reported information on chromatin alteration due to DNA methylation or histone modification.

Cell population/Implant surfaces

A large variability of cells selection was tested. Out of fifteen studies, twelve were in vitro studies: four articles used MG-63 osteoblast like cells for cell culture[37, 42, 45, 46] while other four studies used human alveolar stem cells from human donors[40, 41, 44, 48]. Human mesenchymal stem cells[49], dental pulp stem cells[36], rat bone marrow cells[47], marrow stromal cells obtained from iliac crest[35] were used in one study each.

Two of fifteen articles were in vivo studies aimed to investigate genetic expression levels of implant-adherent cells taken from rats' tibia[43] and from humans[38].

One of fifteen articles was both an in vitro and in vivo study using MG-63 cells and implant adherent cells from beagle dogs' tibias[39].

Iliac crest cells grown on KOH alkali-etched, NaOH alkali-etched or not-etched surfaces

One article challenged experimental titanium alkali-etched surfaces with human bone marrow stromal cells[35]. Cells were cultured on KOH alkali-etched, on NaOH alkali-etched or on not-etched surfaces. Bone sialo protein and matrix metalloprotease 2 levels were found enhanced on alkali-etched metals compared to not-etched surfaces. Higher expression of osteogenic genes was found in cells grown on KOH alkali-etched surfaces.

Dental pulp stem cells grown on ionized or not ionized SLA surfaces

One study evaluated the differentiation and gene activation of dental pulp stem cells grown on ionized SLA (sandblasted acid etched) surfaces, or on not ionized surfaces[36]. Authors found that ionized surfaces induced a more marked cell differentiation. Besides, higher levels of Runx-2, Smad5 and osteocalcin were found in cells grown on ionized surfaces.

MG-63 cells grown on electrolytic etched, sandblasted acid etched or machined surfaces

One article cultured MG-63 cells on electrolytic etched, sandblasted acid etched or machined surfaces[37]. Levels of alkaline phosphatase, osteocalcin, Runx-2, ospeopontin and collagen type Iα1 were higher in electrolytic group.

Human alveolar bone cells grown on SLA, modified SLA or smooth surfaces

Human alveolar bone cells were grown on SLA (saldblasted acid etched), hydrophilic SLA or smooth surfaces[40]. Authors found that the majority of miRNAs were down-regulated in response to the SLA and modSLA surfaces compared to the SMO one, with only relatively changes found between SLA and modSLA.

Alveolar stem cells grown on microarc oxidated surface or chitosan/hyaluronic acid surface with miRNA-21

Human bone marrow mesenchymal cells were cultured on microarc oxidated surface or on CS/HA/miR-21 surfaces[41]. After PCR analysis, levels of collagen type IIIα1, osteocalcin, Runx-2, ospeopontin and collagen type Iα1 were higher in cells grown in contact with CS/HA/miR-21 surfaces.

MG-63 cells grown on zirconia or machined titanium surfaces

One article evaluated miRNA production and gene expression of MG-63 cells grown on zirconia or machined titanium surfaces[42]. Osteogenic gene activation were higher in machined titanium group and bone morphogenic protein-4 and -7 were more expressed in cells grown on titanium surfaces.

Human alveolar bone cells grown on nanotextured, nano-submicrotextured, rough microtextured or smooth surfaces

In one article, human alveolar cells were cultured on titanium surfaces with different rough scale[44]. It was not possible to define a certainly better surface. However, the nanotextured surface group showed the highest alkaline phosphatase production while the microtextured surface group had the greatest amount of calcium and mineralized nodules.

MG-63 cells grown on zirconia surfaces

In one article, MG-63 cells were cultured on zirconia wells and, than, their miRNA production was evaluated[45]. Eighteen miRNAs involved in the repression of osteogenic genes were found up-regulated while only three miRNAs were down-regulated. Authors did not provide information about control groups.

MG-63 cells grown on anatase surfaces

One study evaluated the osteogenic gene activation and differentiation of MG-63 cells grown on anatase surfaces[46]. Authors found that nine miRNAs were up-regulated and ten down-regulated. Due to down-regulated miRNAs, three genes were more expressed and they are fibrillin 1, insulinlike growth factor-binding protein4 and calcitonin. Due to up-regulated miRNAs, three genes were repressed and they are collagen 9α2, ADAMTS4 member of metalloproteinases and alkaline phosphatase. Authors did not provide information about control group.

Rat bone marrow cells grown on surfaces functionalized with miRNA-29b, antimiRNA138 or without functionalization

One article cultured rat bone cells on microporus titanium oxide surfaces with or without functionalization[47]. For bone morphogenic protein, osteocalcin, osterix, and Runx2, the antimiR-138 functionalized surface induced higher expression. For collagen type Iα1, the miR-29b functionalized surface induced higher expression than using antimiR-138, whereas this trend is reversed after 14 days of culture. The miR-29b functionalized surface induces higher expression of alkaline phosphatase.

Human alveolar bone cells grown on microsandblasted, macrosandblasted or machined surfaces

In one article, primary cultures of osteoblasts derived from human mandibular bone were cultured on titanium microsandblasted, macrosandblasted or machined surfaces[48]. Gene activation was evaluated with RT-PCR analysis. All blasted surfaces showed higher DNA activation than the machined surfaces. However, TGFβ2, osteopontin, Runx-2 and bone sialoprotein levels were higher in macrosandblasted groups.

Human mesenchymal stem cells and human osteoblasts grown on titanium-alluminium-vanadium surfaces with different rough scale or on polystyrene surface

One study evaluated gene activation of human mesenchymal stem cells and human osteoblasts grown on titanium-alluminium-vanadium surfaces[49]. Ti6A14V #9 surface showed greater ALP, osteocalcin, VEGF-A, FGF-2, bone morphogenic protein and osteoprotegerin production. Integrin expression also varied with the surface. MessengerRNAs for all integrin subunits except ITGA-5 were higher when cells were cultured on Ti6A14V substrates than on polystyrene ones.

Human cells adherent to surgically placed tioblast or osseospeed implants

One article was performed placing tioblast and osseospeed implants in human smoker and nonsmoker patients. Implants were harvested after two and four days of submerged healing, and implant adherent cells were studied[38]. Interestingly, authors found that the variable of time influences gene expression more that the effect of different surfaces or nicotine. Similar trends in gene expression were noted in implant-adherent cells regardless of implant surface and smoking status.

Rat cells adherent to surgically placed nano- or micro-rughened implants

In one in vivo study, nano- or micro roughened implants were placed in rats' tibias and were harvested after two and four days of submerged healing[43]. Implant adherent cells were studied in order to investigate the influence of different surfaces on gene expression. Significant differences at the gene level were not noted when comparing the two implant surfaces at each timepoint. However, genes were differentially regulated at different days for both implant surfaces.

MG-63 cells and beagle cells grown on implants with or without nanotubes

Only one study evaluated the genetic effect of implant surfaces both in vivo and in vitro[39]. The in vitro experiment was conducted with MG-63 cells while the in vivo phase was performed placing and harvesting implants in beagle tibias. The surfaces compared were SLA without nanotubes, SLA with nanotubes of 30nm, SLA with nanotubes of 50nm and SLA with nanotubes of 80nm. SLA+80nm showed to induce the highest gene expression compared with the other sizes of nanotubes. SLA+80nm also showed to induce the formation of the higher number of filopodia, lamellipodia and cellular extensions.

Comparative analysis

Nine studies found that specific surfaces induced osteoblast differentiation, expression of osteogenic genes or repression of those miRNAs which down-regulate osteogenic genes[35, 36, 37, 39, 41, 42, 47, 48, 49] while six articles did not indicate a better surface[38, 40, 43, 44, 45, 46]. Two articles documented similar trends in gene expression regardless of implant surface; they found that the effect of time influences gene expression more than the surfaces[38, 43]. Only two publications studied the genetic effect of zirconia surfaces. In the first study, zirconia surfaces were associated with worse genetic activation than machined titanium ones[42]. In the second one the number of miRNAs up-regulated was much higher than the number of miRNAs down-regulated[45].

Randomized clinical trial

One article out of the seventeen evaluated in the present review was a randomized clinical trial[50]. Purpose of the study was to investigate the expression levels of osteogenic genes after two different treatments (Tab.2.). Twenty-six patients with one buccal implant dehiscence defect each were randomly treated with particulate allograft bone (control) or particulate allograft bone and pericardium membrane (test). After 6 months of healing, analysis of bone volume and gene expression was performed. After bone volume evaluation, greater volume levels were found in the test group (treatment with bone and membrane) than in control group (treatment with bone without membrane). Then, bone biopsies were harvested and processed. Genetic expression of osteogenic genes was evaluated with an immune-histochemical analysis. Positive periostin (POSTN), sclerostin, and runt-related transcription factor-2 (Runx2) immunoreactivities were detected in both the control and test groups without statistically differences. Tartrate-resistant acid phosphatase (TRAP) positive was mostly noted in the control group. Analysis of DNA methylation, histone modifications and miRNA production was not provided.

Narrative review

A narrative review, investigating the epigenetic effect of smoking and diabetes on osseointegration, was also included and evaluated in the present study[51] (Tab.3.). It resulted that global DNA methylation is

influenced by smoking behaviour. Smoking resulted to have an impact on bone metabolism and estrogen production, leading to a phenotype of low-bone mineral density. In fact, gene expression of bone matrix proteins, including osteopontin, Type II collagen, bone morphogenetic protein-2 and osteoprotegerin resulted significantly down-regulated by smoking components.

Diabetes was associated with decreased gene expression of bone matrix proteins (osteocalcin, PTHrP), of transcription factors involved in osteoblast differentiation (Runx2, osterix) and of osteoprotegerin through the receptor activator of nuclear factor-kappaB ligand mRNA ratio[52]. Histone lysine methylation and other post transcriptional changes, has been implicated in aberrant gene regulation associated with the pathology of diabetes and its complications. Moreover, serum osteocalcin levels have been found to be significantly increased in patients with Type II diabetes[53]. As a result, alteration of bone healing in diabetes and epigenetic modifications are deeply connected.

DISCUSSION

Genetic information is encoded not only by the linear sequence of DNA, but also by epigenetic modifications[9].

Some miRNAs are key regulators for the development of osteoblasts by targeting anti-osteogenic factors such as histone deacetylase-4 and modulating bone extracellular matrixproteins (ECM)[54]. MicroRNAs induce degradation and translational repression of target mRNAs. Therefore, production of miRNAs and post-transcriptional gene expression are inversely correlated. Indeed, antimiR-138 has been shown to enhance in vivo bone formation by inhibiting miRNA-138[55].

To better understand the epigenetic influence on implant therapy, this review was conducted to evaluate the available evidence investigating the potential effects of DNA methylations, histone modifications or micro-RNA production on implant survival, osseointegration, peri-implant mucositis, perimplantitis or implant-abutment leakage.

It has been demonstrated that surface roughness, cellular attachment, and osteoblast activity are directly correlated[56]. Sandblasted and acid etched surfaces (SLA), largely used in today's implants, influence positively genetic expression if compared with smooth surfaces, machined surfaces and zirconia surfaces. To achieve even better clinical results and to accelerate the healing period required for the prosthetic loading, several innovative methods for implant surface modification were studied, including alkali-etching[35], ionization[36], electrolytic etching[37], surfaces with nanotubes[39], isotonic solution and N_2 treatments [40], surfaces functionalized with miRNAs[41, 47], hydrofluoric treatments[43], anatase coating[46] and others. Most of the authors documented better results with processed surfaces in terms of osteoblast differentiation, expression of osteogenic genes or repression of those miRNAs which down-regulate osteogenic genes [35, 36, 37, 39, 41, 43, 44, 45]. In light of these findings, surface presented could represent a pivotal advancement to obtain a faster osseontegration, more predictable post-extractive implants or immediate loading. However, the reviewed articles are not immune to bias.

Type of studied cells

First, most of the reported data derived from studies performed on MG-63 cells or animal cells, which are not normal human osteoblasts. Notwithstanding this, the advantages of using a cell line, like MG-63, are

related to the fact that the reproducibility of the data is higher because there is not the variability in the patient studied. Primary cell cultures provide a source of normal cells, but they also contain contaminating cells of different types and cells in variable differentiation states.

Second, most of the reviewed studies are in vitro studies[35, 36, 37, 40, 41, 42, 44, 45, 46, 47, 48, 49]. On one hand, in vitro studies are not influenced by systemic interindividual variability of human patients; indeed, adrenaline released after a psychological stress may inhibit osteogenic differentiation through histone acetylation and down-regulation of miR-21[10]. On the other hand, in vivo human studies, with a large sample and long follow-up are absolutely required for a better comprehension of epigenetic influences.

Effect of time

The influence of time affects gene expression more than different surfaces[38, 43]. Recently, in an in vivo study, similar trends in gene expression were noted in implant-adherent cells regardless of implant surface and smoking status only if they were evaluated at the same early time point. However, when the time-course was evaluated, statistically differences in genetic patterns was identified.

In the light of these considerations, all further studies have to include two or more time points to evaluate how big is the influence of time if compared with the influence of different surfaces.

Smoking and nicotine

Thalji and co-workers, in their in vivo study, noted that the impact of smoking did not occur at early time points[38]. Potentially, detrimental effects were likely to occur at a later stage and upon exposure of the implants to the oral environment. Implants submerged and never exposed to the oral environment prior to retrieval did not suffer the negative effect of nicotine. The absorption of nicotine through the oral mucosal tissues is pH dependent. Since the pH of tobacco smoke in most cigarettes is acidic, nicotine is primarily ionized resulting in minimal absorption of nicotine from cigarette smoke. Thalji's data indicate that gene expression profiles of submerged implant adherent cells were similar among smokers and non-smokers.

Interestingly, the effects of smoking may have been negated or delayed by the implant surface topography. In a long-term retrospective study, Balshe et al. compared the survival rates of smooth and rough surface dental implants among smokers and non-smokers[57]. Smoking was identified as significantly associated with implant failure only in the smooth surface group. Similar results were reported by Sayardoust et al. in patients with periodontitis, where the smokers' likelihood ratio for implant failure was 6.40 for smooth surface implants and 0 for oxidized implants[58].

Yamano et al, in a rat model, showed that while no differences were noted on bone-to-implant contact after 2 weeks of systemic nicotine exposure, significant differences were observed after 4 weeks[59]. They noted significantly decreased expression of Bmp2, Bsp, Opn, Col2, Cbfal in peri-implant tissues in rats exposed to nicotine compared with controls at 4 weeks. This demonstrates that the systemic effects of nicotine on peri-implant healing occur at later stages. Therefore, future studies should include a period longer than 2 weeks to evaluate the smoking's effects on osseointegration.

All original articles investigating the role of genetic changes on implant therapy outcomes present an indirect evidence[34]. Besides, due to the heterogeneity of methods, of types of cells used and of evaluation

time points, a meta-analysis was not possible to achieve. Even if all included articles agreed with the idea that implant features influence osteoblast differentiation, only a weak evidence could be obtained in the present systematic qualitative review.

CONCLUSION

This systematic qualitative review shows that positive genetic stimulation is associated with surface treatments like alkali-etching, ionization, electrolytic etching, surfaces with nanotubes, isotonic solution and N_2 treatments; osteogenic inhibition was found around zirconia surfaces and anatase coating.

Micro and nanoporous surfaces may provide a larger surface area for loading miRNAs, anti-miRNAs, peptides or other osteogenic drugs. Implant surface could be used like a carrier to position functional groups or biomolecules contributing to achieve a faster osseointegration.

Treated surfaces, early checkpoints and submerged healing seem to be less related to the negative epigenetic effects of smoking.

More investigations with other osteoblast-like cell lines, primary cultures, different time points and surfaces functionalized with genetic molecules are needed to get a global comprehension of the epigenetic influence on peri-implant biological mechanisms.

Conflict of interest: The author declared no conflict of interest.

Tab.1. EFFECTS OF DIFFERENT SURFACES ON GENETIC EXPRESSION							
Author	**Year**	**Type**	**Surfaces tested**	**Type of cells**	**Evaluation of miRNAs**	**Gene expression and molecular mechanism**	**Conclusion**
Giannoni P.	2009	In vitro, research support.	KOH alkali-etched; NaOH alkali-etched; not etched surface.	Bone marrow stromal cells from iliac crest.	not provided	High expression of BSP was found on KOH etched surfaces.	KOH modifications seem to allow the best osteogenic differentiation of human mesenchymal stromal cells.
Iaculli F.	2016	In vitro, research support.	SLA surface without ionization (control); Ionized SLA surface (test).	Dental pulp stem cells.	provided	miRNA-133a, miRNA-133b and miRNA-135 influenced the expression of Runx2 and Smad5 genes.	Ionized sandblasted and acid-etched surface seemed to markedly enhance the development and differentiation of osteoblast cells.
Meng W.	2013	In vitro, research support.	EE; SLA; M.	MG-63 osteosarcoma cells.	n.p.	Gene expression is related to features of implant surface and the level of osteoblast differentiation.	Hierarchical micro-/nanostructured titanium surface treated by EE enhanced the ALP, OCN, Runx2, OPN activity, and COL1 mRNA gene expression of osteoblast.
Thalji G.	2015	In vivo (human).	TiO versus OS; smoker versus non-smoker.	Implant adherent cells (alveolar bone cells).	n.p.	The variable of time influences the gene expression more that the effect of nicotine. Modified surfaces could soften the negative effect of nicotine. High number of genes has been investigated.	At early time points, similar trends in gene expression were noted in implant-adherent cells regardless of implant surface and smoking status.
Ding X.	2015	In vitro and in vivo (animal, beagle dog).	SLA + 30nm; SLA + 50nm; SLA + 80nm; SLA without nanotubes.	(in vitro study) MG-63 osteosarcoma cells. (in vivo study) beagles' tibias cells.	n.p.	Nanotube diameters influence cell phenotype (filopodia and lamellipodia) and ALP, Runx2 and OCN genes expression.	SLA + 80 nm surface is the most favourable for promoting the activity of osteoblasts and early bone bonding.
Chakravorty N.	2012	In vitro, research support.	SLA; ModSLA; SMO.	Human alveolar bone cells.	provided	Highest level of bone expression in modSLA and SLA. 35 different types of miRNA were downregulated in modSLA, and 32 in SLA surfaces. High number of genes and miRNAs has been investigated.	The majority of miRNA were down-regulated in response to the SLA and modSLA surfaces compared to the SMO one, with only relatively changes found between SLA and modSLA.
Wang Z.	2015	In vitro, research support.	MAO; CS/HA/miR-21.	Human bone marrow mesenchymal stem cells (alveolar cells).	Provided only miRNA-21	miRNA-21 induces upregulation of osteogenesis-related genes like COL1, COL3, Runx2, OPN, and OCN.	Titanium surfaces functionalized with miRNA-21 presented a significantly higher expression of osteogenic genes.
Palmieri A.	2008a	In vitro, research support.	Machined Ti; Zirconia.	MG-63 cells.	provided	Six miRNAs were found up-regulated in zirconia compared to Titanium (miR-214,miR-337,miR-423,miR-339,miR377, and miR-193b), and four down-regulated	Ti surfaces could provide some advantages to earlier osteogenesis useful for immediate loading.

						(miR-143, miR-17-5p, miR-24, and miR-22). Bone related genes BMP4 and 7 were more expressed in osteoblasts exposed to Ti surface.	
Thalji G.	2013	In vivo (animal, rat tibia model).	AT-1; AT-2.	Rat's tibia cells.	n.p.	Significant differences at the gene level were not noted when comparing the two implant surfaces at each timepoint. However, genes were differentially regulated at day 4 vs.day 2 for both implant surfaces. High number of genes and miRNAs has been investigated.	The number of genes that were associated with the inflammatory or immune response category was greater for AT-1 than AT-2.
Wimmers Ferreira M.R.	2016	In vitro, research support.	Nanotextured; NanoSubmicrotextured; Rough microtextured; Smooth surface.	Human alveolar bone cells.	provided	The nanotextured surface group showed the highest alkaline phosphatase activation. The rough microtextured surface group had the greatest amount of calcium produced. NOTCH1 gene increased its expression in nanosubmicrotexture surfaces.	Oxidative nanopatterning of titanium surfaces induces changes in the metabolism of osteoblastic cells and cell responses.
Palmieri A.	2008b	In vitro, research support.	Zirconium Oxide; Control group not provided.	MG-63 cells.	provided	Zirconia disks up-regulated 18 miRNAs and down-regulated 3 miRNAs related to osseogenetic genes. The most notable osseogenic genes influences by zirconia are NOG, SHOX, IGF1, BMP1 and FGFR1.	Zirconium oxide surfaces influence genic expression, however speculations about clinical outcomes of zirconia implants were not provided.
Palmieri A.	2008c	In vitro, research support.	Anatase coating; Control group not provided.	MG-63 cells	provided	There were 9 up-regulated miRNAs and 10 downregulated miRNAs. PRDX1, COL9A2, ADAMTS4, SHOX and ALPL, AMBN, TUFT1 were up-regulated. PHEX, FBN1, IGFBP4, CALCA, TFIP1 and PTH were down-regulated.	Anatase colloidal solution regulates osteogenic genes and miRNAs, however clinical speculations were not provided.
Wu K.	2013	In vitro, research support.	MAO+miR29b; MAO+antimiR138; Nacked MAO surface.	Rat bone marrow cells.	provided	For the genes BMP, OCN, OSX, and Runx2, the antimiR-138 functionalized surface induces higher expression. For COL1, the miR-29b functionalized surface induces higher expression than using antimiR-138, whereas this trend is reversed after 14 days of culture. The miR-29b functionalized surface induces higher expression of ALP.	MicroRNA-29b enhances osteogenic activity and antimicroRNA-138 inhibits miR-138, inhibitors of endogenous osteogenesis. Clear stimulation of osteogenic process was observed, in terms of up-regulating osteogenic expression and enhancing alkaline phosphatase production, collagen secretion and mineralization.
Marinucci L.	2006	In vitro, research support.	Machined; Microsandblasted; Macrosandblasted.	Human alveolar bone cells.	n.p.	All blasted surfaces showed significantly higher DNA synthesis than the machined surfaces. Other mRNA transcripts were increased in osteoblasts cultured on rough	Macro-sandblasted titanium showed best results in favouring osteoblast differentiation.

						titanium surfaces, particularly the macrosandblasted surface.	
Olivares-Navarette R.	2014	In vitro, research support.	TCPS; Ti6A14V #5; Ti6A14V #9; Ti6A14V #12.	Human mesenchymal stem cells; human osteoblasts.	n.p.	Test #9 showed greater ALP activity, OCN and osteoprotegerin production. BMP2 and BMP4 were highest in cultures grown on #9, as were VEGF-A and FGF-2. Integrin expression also varied with the surface. mRNAs for all integrin subunits except ITGA-5 were higher when cells were cultured on test substrates than on TCPS.	Osteoblast lineage cells are sensitive to specific micro/nanostructures.

List of abbreviated surfaces:

SLA: sandblasted acid-etched Titanium surface
modSLA: SLA surface with an N_2 protection and stored in an isotonic saline solution
MAO: microarch-oxidated titanium
CH/HA/mR-21: chitosan/hyaluronic acid surface with miRNA-21
Ti6A14V: Micron-scale rough Titanium alloy
EE: micro/nanostructured surface electrolytic etched
M: Machined surface
TiO: TiOBlast; surface blasted with TiO_2
OS: Osseospeed; surface blasted with TiO_2 then treated with hydrofluoric acid
SMO: smooth polished surface
AT-1: oxalic acid and hydrofluoric acid treated surface
AT-2: oxalic acid treated surface
Ti6A14V: Micron-scale rough titanium alloy (# indicates different dimension of roughness parameters).
TCPS: polystyrene surface

List of abbreviated genes and proteins:

ALP: alkaline phosphatase
BMP: bone morphogenic protein
BSP: bone sialoprotein
Runx: runt-related transcription factor
OCN: osteocalcin
PTHrp: Parathyroid hormone-related protein
PTH: Parathyroid hormone
BIC: bone-implant contact
POSTN: periostin related factor
VEGF: vascular endothelial growth factor
COL1: collagen type Iα1
COL3: collagen type IIIα1
OPN: osteopontin
FGF: fibroblast growth factor
ITGA: integrin subunit
SHOX: Short stature HOmeoboX-containing gene
IGF: insulin-like grow factor
NOG: noggin gene
PRDX: peroxiredoxin
ADAMTS: gene encoding for disintegrin and metalloproteinase with thrombospondin motifs
AMBN: ameloblastin
PHEX: Phosphate-regulating neutral endopeptidase
FBN: fibrillin
CALCA: Calcitonin Related Polypeptide Alpha
TFIP: Tissue factor pathway inhibitor
OSX: Osteoblast-specific transcription factor, osterix

Tab.1. Effects of different surfaces on genetic expression. Types of cells used, tested surfaces, gene influenced and conclusions of different authors are presented.

Tab.2. COMPARISON OF CLINICAL TREATMENTS								
Author	**Year**	**Type**	**N° of patients**	**Bone defect**	**Clinical treatments tested**	**Evaluat. of miRNAs**	**Results**	**Conclusion**
Fu J.H.	2015	RCT with immuno-histochemical and RNA analyses.	26 patients 13 (test) 13 (contr)	Buccal implant dehiscences in maxilla.	Defects treated with bone particulate allograft (control) or bone and pericardium membrane (test).	Not provided.	No significant differences in POSTN, Runx2 and VEGF expressions between test and control groups were found. Epigenetic mechanism was not provided.	Bone preserved with the membrane was bigger in volume but less mineralized and more fibrous. No significant differences in mRNA expression between the two groups were found.

Tab.2. Comparison of genetic stimulation of two clinical treatments for implant dehiscence. Dehiscences treated with both bone and membrane showed bigger bone volume and immature bone. Dehiscences treated with only bone showed fasted healing and lower bone volume. No significant differences were found in RNA analyses.

Tab.3. NARRATIVE REVIEW			
Author	**Year**	**Type**	**Mechanism**
Razzouk S.	2013	Narrative review.	Smoking down-regulates osteopontin, Type 2 collagen, BMP-2 and osteoprotegerin. Diabetes influences the expression of PTHrP, OCN, Runx2 and OSX.

Tab.3. Narrative review about the epigenetic influence of smoking and diabetes on osseointegration. Smoking and diabetes can lead to a low-quality bone and altered microarchitecture through the histone deacetylation and DNA methylation of different genes.

Corresponding Author:
Riccardo Di Gianfilippo
Via Leone Marsicano, 41
Avezzano (AQ) 67051, Italy
phone number: (+39) 339 1149700
email: rdigianfilippo@hotmail.it

REFERENCES

1. Berglundh T, Persson L, Klinge B. A systematic review of the incidence of biological and technical complications in implant dentistry reported in prospective longitudinal studies of at least 5 years. J Clin Periodontol. 2002;29 Suppl 3:197-212.
2. Roos-Jansåker A-M, Lindahl C, Renvert H, Renvert S. Nine to fourteen year follow-up of implant treatment. Part I: implant loss and associations to various factors. J Clin Periodontol 2006a;33:283–9.
3. Roos-Jansåker A-M, Lindahl C, Renvert H, Renvert S. Nine to fourteen year follow-up of implant treatment. Part II: presence of peri-implant lesions. J Clin Periodontol 2006b; 33:290–5.
4. Lindhe J, Meyle J. Peri-implant diseases: Consensus Report of the Sixth European Workshop on Periodontology. J Clin Periodontol. 2008 Sep;35(8 Suppl):282-5.
5. Broggini N, McManus LM, Hermann JS, Medina R, Schenk RK, Buser D, Cochran DL. Peri-implant inflammation defined by the implant-abutment interface. J Dent Res. 2006 May;85(5):473-8.
6. Piattelli A, Vrespa G, Petrone G, Iezzi G, Annibali S, Scarano A. Role of the microgap between implant and abutment: a retrospective histologic evaluation in monkeys. J Periodontol. 2003 Mar;74(3):346-52.
7. Berger SL, Kouzarides T, Shiekhattar R, Shilatifard A. An operational definition of epigenetics. Genes Dev. 2009;23(7):781-83.
8. Fazzari MJ, Greally JM (2004). Epigenomics: beyond CpG islands. Nat Rev Genet 5:446-455.
9. Barros SP, Offenbacher S. Epigenetics: Connecting environment and genotype to phenotype and disease. J Dent Res. 2009;88:400-08.
10. Chen D, Wang Z. Adrenaline inhibits osteogenesis via repressing miR-21 expression. Cell Biol Int. 2017 Jan;41(1):8-15.
11. Bishop KS, Ferguson LR. The interaction between epigenetics, nutrition and the development of cancer. Nutrients. 2015 Jan 30;7(2):922-47. doi: 10.3390/nu7020922.
12. Martos SN, Tang WY, Wang Z. Elusive inheritance: Transgenerational effects and epigenetic inheritance in human environmental disease. Prog Biophys Mol Biol. 2015 Jul;118(1-2):44-54. doi: 10.1016/j.pbiomolbio.2015.02.011. Epub 2015 Mar 16.
13. Egger G, Liang G, Aparicio A, Jones PA. Epigenetics in human disease and prospects for epigenetic therapy. Nature. 2004; 429:457-63.
14. Ji-Yun Seo, Yoon-Jung Park, Young-Ah Yi, Ji-Yun Hwang, In-Bog Lee, Byeong-Hoon Cho, et al. Epigenetics: General characteristics and implications for oral health. Restor Dent Endod. 2015;40(1):14-22.
15. Jones PA, Liang G. Rethinking how DNA methylation patterns are maintained. Nat Rev Genet. 2009;10:805-11.

16. Hark AT, Schoenherr CJ, Katz DJ, Ingram RS, Levorse JM, Tilghman SM (2000). CTCF mediates methylation-sensitive enhancer-blocking activity at the H19/Igf2 locus. Nature 405:486-489.

17. Bayarsaihan D. Epigenetic mechanisms in inflammation. J Dent Res. 2011;90:917.

18. Ari G, Cherukuri S, Namasivayam A. Epigenetics and Periodontitis: A Contemporary Review. J Clin Diagn Res. 2016 Nov;10(11):ZE07-ZE09.

19. Shookhoff JM, Gallicano GI. The emerging role of microRNAs in adult stem cells. Turksen K, editor. Stem cell biology and regenerative medicine, vol 1, Humana Press: Springer; 2011. pp 57–94.

20. Fakhry M, Hamade E, Badran B, Buchet R, Magne D. Molecular mechanisms of mesenchymal stem cell differentiation towards osteoblasts. World J Stem Cells 2013;5:136–148.

21. Sylvestre Y, De Guire V, Querido E, et al. An E2F/miR-20a autoregulatory feedback loop. J Biol Chem 2007; 282:2135– 2143.

22. Gordeladze JO, Reseland JE, Duroux-Richard I, Apparailly F, Jorgensen C. From stem cells to bone: phenotype acquisition, stabilization, and tissue engineering in animal models. LAR J 2009; 51:42–61.

23. Andia DC, de Oliveira NF, Casarin RC, Casati MZ, Line SR, de Souza AP. DNA methylation status of the IL-8 gene promoter in aggressive periodontitis. J Periodontol. 2010;81:1336-41.

24. Zhang S, Barros SP, Niculescu MD, Moretti AJ, Preisser JS, Offenbacher S. Alteration of PTGS2 promoter methylation in chronic periodontitis. J Dent Res. 2010;89:133–7.

25. Zhang S, Crivello A, Offenbacher S, Moretti A, Paquette DW, Barros SP. Interferon-gamma promoter hypomethylation and increased expression in chronic periodontitis. J Clin Periodontol. 2010;37:953-61.

26. Miao D, Godovikova V, Qian X, Seshadrinathan S, Kapila YL, Fenno JC. Treponema denticola upregulates MMP-2 activation in periodontal ligament cells: interplay between epigenetics and periodontal infection. Arch Oral Biol. 2014 Oct;59(10):1056-64.

27. Yin L, Sanson B, An J, Hacker BM, Silverman GA, Dale BA, et al. Differential effects of peripopathogens on host protease inhibitors SLP1, elafin, SCCA1, SCCA 2. J Oral Microbiol. 2010;2:1–12.

28. Chung WO, An JY, Yin L, Hacker BM, Rohani MG, Dommisch H, et al. Interplay of proteaseactivated receptors and NOD pattern recognition receptors in epithelial innate immune responses to bacteria. Immunol Lett. 2010;131:113–9.

29. Darveau RP, Pham TT, Lemley K, Reife RA, Bainbridge BW, Coats SR, Howald WN, Way SS, Hajjar AM. Porphyromonas gingivalis lipopolysaccharide contains multiple lipid A species that functionally interact with both toll-like receptors 2 and 4. Infect Immun. 2004 Sep;72(9):5041-51.

30. Chung WO, Hansen SR, Rao D, Dale BA. Protease-activated receptor signaling increases epithelial antimicrobial peptide expression. J Immunol. 2004;173:5165–70.

31. Oliveira NF, Damm GR, Andia DC, Salmon C, Nociti FH, Jr, Line SR, et al. DNA methylation status of the IL8 gene promoter in oral cells of smokers and non-smokers with chronic periodontitis. J Clin Periodontol. 2009;36:719–25.

32. Yin L, Chung WO. Epigenetic regulation of human ß-defensin 2 and CC chemokine ligand 20 expression in gingival epithelial cells in response to oral bacteria. Mucosal Immunol. 2011;4:409– 19.

33. Cook DJ, Mulrow CD, Haynes RB. Systematic reviews: synthesis of best evidence for clinical decisions. Ann Intern Med. 1997 Mar 1;126(5):376-80.

34. Mulrow C, Langhorne P, Grimshaw J. Integrating heterogeneous pieces of evidence in systematic reviews. Ann Intern Med. 1997 Dec 1;127(11):989-95.

35. Giannoni P, Muraglia A, Giordano C, Narcisi R, Cancedda R, Quarto R, Chiesa R. Osteogenic differentiation of human mesenchymal stromal cells on surface-modified titanium alloys for orthopedic and dental implants. Int J Artif Organs. 2009 Nov;32(11):811-20.

36. Iaculli F, Di Filippo ES, Piattelli A, Mancinelli R, Fulle S. Dental pulp stem cells grown on dental implant titanium surfaces: An in vitro evaluation of differentiation and microRNAs expression. J Biomed Mater Res B Appl Biomater. 2016 Feb 9. doi: 10.1002/jbm.b.33628.

37. Meng W, Zhou Y, Zhang Y, Cai Q, Yang L, Wang B. Effects of hierarchical micro/nano-textured titanium surface features on osteoblast-specific gene expression. Implant Dent. 2013 Dec;22(6):656-61.

38. Thalji G, Cooper LF, Nares S. Gene Expression Profiles of Early Implant Adherent Cells in Smokers and Nonsmokers. J Oral Implantol. 2015 Dec;41(6):640-5.

39. Ding X, Zhou L, Wang J, Zhao Q, Lin X, Gao Y, Li S, Wu J, Rong M, Guo Z, Lai C, Lu H, Jia F. The effects of hierarchical micro/nanosurfaces decorated with TiO2 nanotubes on the bioactivity of titanium implants in vitro and in vivo. Int J Nanomedicine. 2015 Nov 9;10:6955-73.

40. Chakravorty N, Ivanovski S, Prasadam I, Crawford R, Oloyede A, Xiao Y. The microRNA expression signature on modified titanium implant surfaces influences genetic mechanisms leading to osteogenic differentiation. Acta Biomater. 2012 Sep;8(9):3516-23. doi: 10.1016/j.actbio.2012.05.008.

41. Wang Z, Wu G, Feng Z, Bai S, Dong Y, Wu G, Zhao Y. Microarc-oxidized titanium surfaces functionalized with microRNA-21-loaded chitosan/hyaluronic acid nanoparticles promote the osteogenic differentiation of human bone marrow mesenchymal stem cells. Int J Nanomedicine. 2015 Oct 27;10:6675-87.

42. Palmieri A, Pezzetti F, Brunelli G, Lo Muzio L, Scarano A, Scapoli L, Martinelli M, Arlotti M, Guerzoni L, Rubini C, Carinci F. Short-period effects of zirconia and titanium on osteoblast microRNAs. Clin Implant Dent Relat Res. 2008a Sep;10(3):200-5.

43. Thalji G, Gretzer C, Cooper LF. Comparative molecular assessment of early osseointegration in implant-adherent cells. Bone. 2013 Jan;52(1):444-53.

44. Wimmers Ferreira MR, Rodrigo Fernandes R, Freire Assis A, Dernowsek JA, Passos GA, Variola F, Fittipaldi Bombonato-Prado K. Oxidative Nanopatterning of Titanium Surface Influences mRNA and MicroRNA Expression in Human Alveolar Bone Osteoblastic Cells. Int J Biomater. 2016;2016:9169371.

45. Palmieri A, Pezzetti F, Brunelli G, Zollino I, Lo Muzio L, Martinelli M, Scapoli L, Arlotti M, Masiero E, Carinci F. Zirconium oxide regulates RNA interfering of osteoblast-like cells. J Mater Sci Mater Med. 2008b Jun;19(6):2471-6.

46. Palmieri A, Pezzetti F, Brunelli G, Arlotti M, Lo Muzio L, Scarano A, Rubini C, Sollazzo V, Massari L, Carinci F. Anatase nanosurface regulates microRNAs. J Craniofac Surg. 2008c Mar;19(2):328-33.

47. Wu K, Song W, Zhao L, Liu M, Yan J, Andersen MØ, Kjems J, Gao S, Zhang Y. MicroRNA functionalized microporous titanium oxide surface by lyophilization with enhanced osteogenic activity. ACS Appl Mater Interfaces. 2013 Apr 10;5(7):2733-44.

48. Marinucci L, Balloni S, Becchetti E, Belcastro S, Guerra M, Calvitti M, Lilli C, Calvi EM, Locci P. Effect of titanium surface roughness on human osteoblast proliferation and gene expression in vitro. Int J Oral Maxillofac Implants. 2006 Sep-Oct;21(5):719-25. Erratum in: Int J Oral Maxillofac Implants. 2007 Jan-Feb;22(1):19.

49. Olivares-Navarrete R, Hyzy SL, Berg ME, Schneider JM, Hotchkiss K, Schwartz Z, Boyan BD. Osteoblast lineage cells can discriminate microscale topographic features on titanium-aluminum-vanadium surfaces. Ann Biomed Eng. 2014 Dec;42(12):2551-61.

50. Fu JH, Rios H, Al-Hezaimi K, Oh TJ, Benavides E, Wang HL. A randomized clinical trial evaluating the efficacy of the sandwich bone augmentation technique in increasing buccal bone thickness during implant placement. II. Tomographic, histologic, immunohistochemical, and RNA analyses. Clin Oral Implants Res. 2015 Oct;26(10):1150-7. doi: 10.1111/clr.12481.

51. Razzouk S, Sarkis R. Smoking and Diabetes Epigenetics Involvement in Osseointegration. N Y State Dent J. 2013 Mar;79(2):27-30.

52. Lozano D, de Castro LF, Dapia S, Andrade-Zapata, Manzarbeitia F, Alvarez-Arroyo MV, Gómez-Barrena E, Esbrit P. Role of parathyroid hormone-related protein in the decreased osteoblast function in diabetes-related osteopenia. Endocrinology 2OO9;15O:2O27-35.

53. Ferron M, Wei J, Yoshizawa T, Del Fattore A, DePinho RA, Teti A, Ducy P, Karsenty G. Insulin signaling in osteoblasts integrates bone remodeling and energy metabolism. Cell 2010, 142:296-308.

54. Li Z, Hassan MQ, Jafferji M, Aqeilan RI, Garzon R, Croce CM, van Wijnen AJ, Stein JL, Stein GS, Lian JB. Biological functions of miR-29b contribute to positive regulation of osteoblast differentiation. J Biol Chem. 2009 Jun 5;284(23):15676-84.

55. Eskildsen T, Taipaleenmäki H, Stenvang J, Abdallah BM, Ditzel N, Nossent AY, Bak M, Kauppinen S, Kassem M. MicroRNA-138 regulates osteogenic differentiation of human stromal (mesenchymal) stem cells in vivo. Proc Natl Acad Sci U S A. 2011 Apr 12;108(15):6139-44.

56. Mustafa K,Wennerberg A,Wroblewski J, Hultenby K,Lopez BS, Arvidson K.Determining optimal surface roughness of TiO blasted titanium implant material for attachment,proliferation and differentiation of cells derived from human mandibular alveolar bone. Clin Oral Implants Res 2001;12:515–525.

57. Balshe AA, Eckert SE, Koka S, Assad DA, Weaver AL. The effects of smoking on the survival of smooth- and rough-surface dental implants. Int J Oral Maxillofac Implants. 2008;23:1117-1122.

58. Sayardoust S, Grondahl K, Johansson E, Thomsen P, Slotte C. Implant survival and marginal bone loss at turned and oxidized implants in periodontitis-susceptible smokers and never-smokers: a retrospective, clinical, radiographic case-control study. J Periodontol. 2013;84:1775-1782.

59. Yamano S, Al-Sowygh ZH, Gallucci GO, Wada K, Weber HP, Sukotjo C. Early peri-implant tissue reactions on different titanium surface topographies. Clin Oral Implants Res. 2011;22:815-819.

XENOGENEIC COLLAGEN MATRIX VERSUS CONNECTIVE TISSUE GRAFT FOR SOFT TISSUE AUGMENTATION AT IMPLANT SITE. A RANDOMIZED, CONTROLLED CLINICAL TRIAL

MATRICE XENOGENICA IN COLLAGENE VERSUS INNESTO DI TESSUTO CONNETTIVO PER L'INCREMENTO DEI TESSUTI MOLLI PERI-IMPLANTARI: STUDIO CLINICO CONTROLLATO RANDOMIZZATO

Lorenzo Landini[1], Chiara Gori[1], Tommaso Giuntoli[1], Giulia Bertini[1], Luigi Barbato[1], Michele Nieri[1], Francesco Cairo[1],

[1]Section of Periodontology, Department of Surgery and Translational Medicine, A.O.U. Careggi, University of Florence, Florence, Italy

Key words:

Collagen matrix, connective tissue graft, keratinized tissue, soft tissue augmentation, randomized clinical trial, dental implants.

Running title:

Soft tissue augmentation at implants

Abstract

Background: The purpose of the present randomized clinical trial (RCT) is to compare Xenogeneic Collagen Matrix (XCM) versus Connective Tissue Graft (CTG) for soft tissue augmentation at implant site.

Material and methods: Soft tissue augmentation procedure with XCM (test) or CTG (control) was performed at 60 implants in 60 patients at the time of implant uncovering. Measurements were performed by a blind and calibrated examiner. Outcome measures included soft tissue thickness (GT), apico-coronal Keratinized Tissue (KT), peri-implant bone levels (BLs), chair time and post-operative discomfort. Visual Analogue Scale (VAS) was used to evaluate patient satisfaction.

Results: After 6 months, CTG yielded to significantly higher GT increase than XCM (0.3 mm; p=0.0001). Both procedures resulted also in a significant KT width increase compared with baseline with no significant difference between treatments. XCM was associated with significant less chair-time (p<0.0001), less post-operative pain (p<0.0001), painkillers intake (p<0.0001) and higher final satisfaction than CTG (p=0.0195). There was no significant difference for final BLs.

Conclusions: Both procedures increased amount of KT compared with baseline. CTG was more effective than XCM to increase peri-implant soft tissue thickness.

Clinical Relevance

Scientific rationale for the study

The effect of XCM or CTG for soft tissue augmentation at implant site is poorly investigated in RCTs.

Principal findings

CTG resulted in higher soft tissue thickness than XCM. Both procedures yielded similar apico-coronal KT compared with baseline. XCM was associated with significant better patient-reported outcomes including less post-operative pain and higher final satisfaction than CTG.

Practical implications

CTG should be preferred when increase in thickness is the primary treatment goal, while XCM is a viable alternative when the increase in apico-coronal KT and the reduction of patient morbidity is the primary target of therapy.

Background

Emerging evidence suggested that keratinized tissue (KT) around dental implant might be critical to prevent plaque accumulation, mucosal recession and peri-implant inflammation (Lin et al. 2013, Gobbato et al. 2013, Brito et al. 2014). An adequate volume of peri-implant soft tissue seems to be also critical in preventing crestal bone resorption that may occur when thin peri-implant mucosa is detectable (Linkevicious et al. 2010). Therefore, the careful management of soft tissue around implants is considered a key factor in order to obtain aesthetic outcomes and to support long-term maintenance in implant dentistry (Cairo et al. 2008). Different surgical strategies may be used to improve the amount of KT around dental implants, including the use of pedicle flaps and soft tissue grafts (Cairo et al. 2008, Thoma et al. 2014).

Xenogeneic Collagen Matrix (XCM), a two-layer product of porcine origin favoring blood vessels ingrowth, was recently introduced. Clinical studies showed that XCM may be a viable alternative for gingival augmentation purpose (Sanz et al 2009), for improving root coverage outcomes (Jepsen et al. 2013) and may represent a possible alternative to CTG for limiting morbidity due to harvest procedure (Cairo et al. 2014).

The aim of this RCT is to compare Xenogeneic Collagen Matrix versus Connective Tissue Graft for soft tissue augmentation at implant site.

Material and methods

Participants

The present study is a parallel, randomized single-center clinical trial according to the CONSORT statement (http://www.consort-statement.org/). Two different treatment modalities for obtaining soft tissue augmentation at the time of implant uncovering were compared: Xenogenic Collagen

Matrix (XCM) (Test group) and Connective Tissue Graft (CTG) (control group). The flowchart of the study is presented in figure 1.

The study protocol was approved by the Ethical Board (Ref. Prot.12/P CESM del 17.06.2013). Informed consent was obtained from all subjects included in the study. Experimental procedures were conducted according to the principles outlined in the Declaration of Helsinki on study involving human subjects, as revised in 2004. Experimental procedures were performed from July 2013 until March 2016.

Participants satisfying the following entry criteria were recruited:

- Age ≥18 years
- No systemic diseases or pregnancy.
- Self reported smoking ≤ 10 cigarettes/day.
- No probing depths ≥ 5mm
- Full-mouth plaque score (FMPS) and full-mouth bleeding score (FMBS) ≤ 15% (measured at four sites per tooth).
- Single dental implant treatment with a scheduled second stage surgery for implant uncovering at both upper and lower jaw
- Need of soft tissue augmentation for aesthetic purpose and/or functional reasons.
- No previous soft tissue augmentation procedure at experimental site.

Exclusion criteria were:

- Disease affecting connective tissue metabolism
- Diabetes
- Allergy to the collagen
- Pregnant or lactating women
- Participation in an investigational device, drug or biologic study within the last 6 months prior to the study start
- Untreated periodontal disease
- Dental implants previously uncovered or applied with a single-stage procedure
- No residual keratinized tissue (KT) at experimental area.

Interventions/Operator/Investigators

All surgical procedures were performed by a single expert periodontist (F.C.) with more than 10 years of experience in periodontal plastic surgery and implant dentistry. The examiner, blinded with respect to the surgical procedures, assessed all the clinical outcomes of treatments and attended a preliminary calibration session reporting intra-class correlation coefficient of 0.87 (CI 95% 0.82; 0.91).

Clinical measurements before surgery (T0)

After data collection regarding age, gender, medications, smoking habits, number of cigarettes/day, the following measurements were taken at baseline (before implant uncovering) for each treated implant by a blind examiner, using a periodontal probe (PCP UNC 15, Hu-Friedy):

- KT 0: Baseline buccal Keratinized Tissue at baseline measured as the distance between mucogingival junction (MGJ) to the most coronal point of the ridge using a periodontal probe (PCP UNC 15, Hu-Friedy)
- GT 0: Buccal Gingival thickness at baseline measured 1,0 mm coronal to the MGJ using an injection needle, perpendicular to the tissue surface, with a silicon stop over the gingival surface. The silicon disk stop was then placed in tight contact with the soft tissue surface and fixed with a drop of cyanocrylic adhesive. After needle removal, the distance between needle tip and the silicon stop was measured using a digital caliper with 0.01mm of accuracy (Cairo et al. 2016a).

All variations in the soft tissue were monitored considering the position of the gingival margin (GM) and MGJ at further follow-ups.

Intra-operatory measurements

The following measurement was taken immediately after the end of surgical procedure at each experimental implant.

- KT1: the amount of KT immediately after the end of surgery evaluated as the distance between MGJ and the most coronal point of KT stabilized at the healing abutment
- Bone levels: distance in mm between bone levels at mesial (mBL) and distal (dBL) site of the experimental implant using the first thread as reference point. This measurement was obtained after the application of healing abutment using an intra-oral x-ray obtained with parallel technique. The measurements were rated positive when the bone crest (BC) was placed coronal to the first implant thread and negative when BC was below the reference point.

When patient was allocated in the control group (CTG), the type of harvesting procedure was described in the charting. Chair-time of the surgical procedure was also measured from the end of local anaesthesia until the completion of the last suture. In addition, data regarding patient-reported outcomes measures (PROMs) regarding hardship perception of procedure and pain during surgery were measured by Visual Analogue Scale-VAS from 0 to 100 after surgery.

Clinical measurements to monitor early healing

The amount of buccal KT was evaluated at 7 (time for suture removal) and 14-days. In addition, data on possible soft tissue complications (necrosis, edema, bleeding) were also collected. General discomfort and pain using VAS were also registered at the 7-days follow-up. A further evaluation of KT amount was performed at 1-month follow-up.

Clinical measures at the 3 (T1) and 6-months (T2) follow-ups

- KT: Buccal Keratinized Tissue measured as the distance between mucogingival junction (MGJ) and the gingival margin.
- GT: Buccal Gingival thickness measured 1,0 mm coronal to the MGJ using the same method reported above.
- Rec: Recession depth at 6 points for implant
- PD: Probing depth at 6 points for implant
- Bop: Bleeding on probing as yes/no in 6 points/implant
- PI: Plaque as yes/no in 6 points/implant

At the 6-month follow-up only (T2), data regarding final bone levels (BLs 6m) were collected using the same procedure reported above. In addition, patient outcomes regarding aesthetic and overall satisfaction with VAS scale was collected. In case of drop out, the related reason was registered.

Treatment procedures

Before surgery, a careful evaluation of residual buccal, crestal ad lingual KT was performed. After local anaesthesia, a crestal or slightly lingual horizontal incision was performed. Care was taken to preserve an adequate amount of lingual KT. A split-thickness buccal flap was gently raised-up beyond the MGJ. Implant screw was then removed and healing abutment was applied. The randomization sealed and opaque envelope was then opened and patient allocated to test or control group. In the test group a double layer of XCM (Mucograft®, Geistlich Pharma AG, Wolhusen, Switzerland) was applied. The first layer was applied and secured over supra-periosteal tissue while the second one was gently applied over the first one. Both layers were carefully sutured at supra-periosteal buccal tissue with resorbable sutures. In the control group a CTG was harvest at palatal side using trap door approach or de-ephiteliazed free gingival graft procedure. A standard 1-mm thick CTG was sutured at buccal supra-periosteal tissue. The flap was then carefully sutured to completely cover XCM or CTG. Complete clinical cases regarding test and control group were presented in Fig. 2 and 3.

Post-surgical instructions and Prosthetic treatments

Patients were instructed to avoid mechanical trauma and tooth brushing for 2 weeks and to intermittently apply an ice bag for the first 4 hours. Patients received ibuprofen 600 mg at the end of the surgical procedure and were instructed to take another tablet 6 hours later; they were also instructed to take additional doses if needed. Chlorhexidine mouthrinsings (0.12%) were prescribed twice daily for 1min. Smokers were reminded to quit smoking in the first 2 weeks after surgery. Seven days after surgery, sutures were removed. Two weeks after surgery, patients were instructed to resume mechanical tooth-cleaning. Prosthetic treatment including the application of a temporary crown and the final rehabilitation was performed according to the specific treatment plan. The prosthodontist was blind in respect to the type of soft tissue augmentation procedure perfomed. Professional oral hygiene procedures were performed at 3- and 6- months follow-up

Sample size

The sample dimension was calculated using difference in GT, $\alpha = 0.05$, power of 80%, and standard deviation 0.63mm (Wiesner et al. 2010). The minimum clinically significant difference in GT (δ) was 0.5mm.

On the basis of these data, the needed number of patient to be enrolled to conduct this study has been calculated as 26 for the test group (XCM) and 26 for the control group (CTG). In order to compensate for 10% of potential dropouts, the final sample was 30 patients for each group.

Randomization/Allocation concealment/Masking of examiners

Each experimental subject was randomly assigned to one of the two treatment regimens. A blocked randomization in order to obtain the same number of patients in each arm was used. Treatment assignment was noted in the registration and treatment assignment form that was kept by the study registrar (M.N., statistician). Allocation concealment was performed by opaque sealed envelopes, sequentially numbered. The statistician generated the allocation sequence by means of a computer-generated random list and instructed a different subject to assign a sealed envelope containing the treatments (XCM and CTG). The opaque envelope was opened after flap elevation and treatment assignment communicated to the operator. Blinding of examiners was maintained throughout all experimental procedures.

Statistical analysis

Descriptive statistics were performed using mean ± standard deviation for quantitative variables and frequency and percentage for qualitative variables. The statistical unit was the patient. Primary outcomes variable was soft tissue thickness (GT) difference, considered as the difference between thickness at T0 (GT T0) and thickness at 6 months follow-up (GT 6m). An analysis of covariance was performed for this outcome variable using treatment as explicative variable and GT T0 as a covariate. The interaction term Treatment and GT T0 was added to the model if significant.

The analysis of covariance was also performed for difference in apico-coronal KT amount and bone levels difference comparing baseline and 6 months of follow-up; t-tests were performed for chair time, number of postoperative painkillers, number of days with discomfort, PD, Rec, number of BoP sites, VAS regarding final aesthetic and overall satisfaction. Fisher exact tests were performed for complications and the presence of postoperative oedema. This analysis was defined a priori. Intention to treat analysis was applied. Statistical analysis was performed with JMP 11.0 SAS Institute Inc.

Results

Experimental population, patients and defects characteristics at baseline

An original sample of 68 patients satisfying the entry criteria was identified; 8 of 68 declined to participate into the experimental procedures. A total of 60 patients were enrolled in the study; 30 patients were allocated to XCM treatment (test group) and 30 to CTG (control group). In the test group, 27 (90%) Straumann Bone levels implants and 3 (10%) Astra Tech dental implants were used, while in the control group 22 (73%) Straumann Bone levels and 8 (27%) Astra Tech dental implants.

In XCM group, twenty out of 30 were females (67%), and the mean age was 50.3 ±12.4 years [Minimum: 21; Maximum: 73]. Nine patients were smokers (30%). A total of 17 implant at upper jaw and 13 at the lower jaw were treated. The baseline mean width of KT (KT 0) was 3.1±1.2mm while the mean baseline GT (GT 0) was 2.1±0.63 mm. In CTG group, twenty-four out of 30 were females (24%), and the mean age was 48.3 ±11.8 years [Minimum: 22; Maximum: 69]. Six patients were smokers (20%). A total of 27 implant at upper jaw and 3 at the lower jaw were treated. The baseline mean width of KT (KT 0) was 3.5±1.1mm while the mean baseline GT (GT 0) was 2.1±0.59 mm. Regarding the harvesting procedure, in 23 cases was used the trap door approach, in 4 the single incision technique, in 2 the de-epithelized graft and 1 from maxillary tuberosity. There was no clinical difference at baseline between the two groups.

Evaluation of the surgical procedure and post-operative period (1, 2, 4 weeks)

Immediately after surgery the apico-coronal amount of KT was 4.7±1.2mm for XCM and 5.0±1.6mm for CTG. At this time BLs were 0.7±0.2mm for test group and 0.8±0.3mm for control group. The mean duration of the surgical procedure was 35.5 ± 9.4 minutes for the test group and 51.7 ± 7.0 minutes for the control group (difference: -16.2 min; 95%CI from -20.5 to -11.9; p<0.0001). Hardship perception of the procedure in term of VAS value was 17 ± 13 in the test group and 35 ± 23 in the control group (difference: -18; 95%CI from -28 to -8; p=0.0008). No significant difference was reported for perceived pain (difference: -4; 95%CI from -8 to 1; p=0.0940). After 7 days, patients from the XCM group reported an intake of 2.2 ± 0.8 anti-inflammatory tablets compared with 3.9±0.7 for the CTG group (difference -1.7; 95%CI from -2.1 to -1.3; p<0.0001). Patients allocated in the test group experienced also significantly lower intensity of post-surgical pain than the control group (13.0 ±10 vs 37.0 ± 15 VAS values, difference -24; 95%CI from -31 to -17; p<0.0001) and lower number of unconformable days (1.2 ± 0.7 vs 2.4 ± 0.7; difference -1.2 days, 95%CI from -0.9 to -1.6, p<0.0001). At 2 weeks, the only significant difference was the higher number of sites with edema (20 *vs* 7 sites) in the control group (relative risk 0.35; 95%CI from 0.17 to 0.70; p=0.0016). No other significant difference was detected in the post-operative period.

Clinical outcomes

Descriptive statistics of peri-implant soft tissue for test and control group is presented in table 1. Furthermore, KT changes at each interval are presented in fig. 4.

At the final follow-up, two dropouts in the test group were registered. Both patients did not comply with the recall visits for the distance from their house after the 4 weeks visits, but successfully completed prosthetic treatment at the referral dentist office. All remaining 58 patients attended to all follow-up visits and no significant complication was reported. No implant failure was registered. At the final visit all patients were highly satisfied, with 95 ± 5 mean VAS value in the test group and 91 ± 9 in the control group. The difference was significant (difference 4; 95%CI from 1 to 8; p=0.0195). Furthermore, patients were also satisfied in term of aesthetic outcomes with no difference between groups (90 ± 8 for test group *vs* 90 ± 9 for control group, difference 0.1; 95%CI from -4 to 5; p= 0.9715).

At the 6-month follow-up visit, both procedures resulted in a significant increase in KT width compared with baseline (p<0.0001). In the XCM group the final KT was 4.3 ± 1.2 mm while the CTG group was 4.4. ± 1.5mm. There was no significant difference between treatments (difference 0.1 mm 95%CI from -0.3 to 0.5; p=0.4754). In addition, both procedures yielded to a significant increase of gingival thickness (GT) compared with baseline (p<0.0001). The final soft tissue thickness was 3.0 ± 0.7 in the XCM group and 3.4 ± 0.6mm in the CTG group. The increase in KT thickness was significantly lower in the test group (difference -0.3 mm; 95%CI from -0.5 to -0.2; p=0.0001). After 6 months, only a single case of 1-mm soft tissue recession was identified in the

test group. No significant difference was also reported in term of mean bone levels at the last follow-up (difference 0.1 mm; 95%CI from -0.1 to 0.3; p=0.3022). Inferential statistics comparing test and control group is presented in table 2.

Discussion

Recent information supports the importance of KT around implant to improve aesthetic outcomes, soft tissue stability and to prevent peri-implant inflammation (Tonetti et al. 2014). The present RCT was performed to test XCM versus CTG for soft tissue augmentation at implant site. A total of 60 dental implants on 60 patients were treated at the time of second stage surgery.

The outcomes of the study showed that CTG was more effective than XCM for improving soft tissue thickness at implant site, leading to a mean 0.3 mm higher increase in soft tissue thickness. Modern clinical research in implant dentistry suggest that gingival thickness may play a significant role in preventing bone resorption, showing that less bone loss may occur at thick mucosal tissue compared with thin soft tissue (Puisys & Linkevicious 2015). A possible explanation may be related to the higher capability of thick soft tissue in counteracting the inflammation process related to infection at microgap level, limiting its widespread at bone level (Ericsson et al. 1995, Herman et al. 2000). In addition, when minimal amount of soft tissue is coronal to the bone crest, a bone resorption occurs for allowing the formation of biological dimension around implant (Berglundh & Lindhe 1996). A recent SR suggests also that thick peri-implant soft tissue is associated with 0.8 mm less bone loss than thin tissue in the short term (Suarez-Lopez Del Amo 2016). In the present RCT, both procedures resulted in a significant improvement of soft tissue thickness compared with baseline (1.2mm for CTG and 0.9mm for XCM); interestingly, 79% of XCM-treated sites and 93% of CTG-treated sites achieved final soft tissue thickness ≥ 2.5 mm. The magnitude of thickness increase was similar for that obtained with CTG (Weisner et al. 2010) or XCM (Froum et al. 2015) in pilot studies testing soft tissue increase at implant site. Furthermore, in another pilot RCT a similar effect in soft tissue augmentation was described comparing CTG to a stable collagen matrix prototype (Thoma et a. 2016).

In the present RCT a significant increase in apico-coronal KT dimension was reported for both techniques, with no significant difference between procedures. The importance of KT dimension was pointed out in an early experimental study in the animal model demonstrating that minimal amount of KT was associated with higher gingival recession/bone loss under experimental peri-implantitis conditions (Warrer et al. 1995). More recently, clinical studies applying modern implant systems showed significant benefits in preserving KT at dental implants, reporting that higher amount of KT prevented plaque accumulation, mucosal recession and peri-implant inflammation (Lin et al. 2013, Gobbato et al. 2013, Brito et al. 2014). In the present study, both procedures were associated with ~1mm increase of final KT compared with baseline. This finding is similar to that reported in another RCT where CTG and XCM were used for pure gingival augmentation proposal at implant site and sutured over the recipient bed without flap coverage (Lorenzo et al. 2012). In

the present study both XCM and CTG were completely submerged under a split-thickness buccal flap in order to maximize the blood supply that has shown to be the critical factor during healing process (Guhia 2001). Furthermore, the increased amount of KT is in accordance to that observed when using CTG (Cairo et al. 2012, Cairo et al. 2016a) or XCM (Jepsen et al. 2013) under coronally advanced flap for root coverage purpose. Interestingly, specific time-frame change in buccal KT amount was described in this RCT for both techniques (fig. 4). In fact, after a visible tissue augmentation due to the inflammatory phase at 1-week, a tissue shrinkage trend due to the resolution of the inflammation was detectable in the 2th/4th week. Between the 1- and 3-month follow-ups a significant KT increase was shown; this finding was probably due to augmented amount of well-organized collagen fibers originating from the grafted connective tissue (Nobuto et al. 1988). It can be speculated that this may be similar when applying a collagen matrix under the split-thickness flap: after the blood clot stability, an ingrowth of blood vessel into the matrix occurred leading to a subsequent collagen fibers maturation (Thoma et al. 2011). This may explain the final increase in KT detectable after the application of XCM.

The present study confirms that the use of CTG is associated with longer chair-time and greater morbidity (Cortellini et al. 2009; Cairo et al. 2012) than XCM. Surgical-time was 16 minutes longer than XCM ($p<0.0001$) and patients experienced significantly higher post-surgical discomfort ($p<0.0001$) and reported greater anti-inflammatory tablets consumption ($p=0.0001$). All treated patients were highly satisfied in terms of final aesthetic outcomes after both treatments with no significant difference between groups, thus confirming that soft tissue reconstruction is associated with high patient satisfaction at the final follow-up (Cairo et al. 2016b). Interestingly, when assessing overall final patient satisfaction, higher VAS values was reported for XCM treated than controls, thus supporting the detrimental effect of harvesting procedure on patient opinion (McGuire et al. 2003).

Within the limits of this study, the following conclusions can be drawn:

- CTG was more effective than XCM for improving soft tissue thickness at implant site.
- XCM and CTG obtained similar amount of apico-coronal KT after 6 months.
- XCM is associated with shorter surgical time, lower post-operative morbidity, less anti-inflammatory tablets consumption and higher final patient satisfaction than CTG.

References

Berglundh, T., Lindhe, J. (1996). Dimension of the periimplant mucosa. Biological width revisited. *Journal of Clinical Periodontology* **23**:971-973.

Brito, C., Tenenbaum, H.C., Wong, B.K., Schmitt, C., Nogueira-Filho, G. (2014). Is keratinized mucosa indispensable to maintain peri-implant health? A systematic review of the literature.

Journal of Biomedical Materials Research part B Applied Biomaterials 102(3):643-50.

Cairo, F., Pagliaro, U., Nieri, M. (2008). Soft tissue management at implant sites. *Journal of Clinical Periodontology* **35**(8 Suppl):163-7.

Cairo, F., Cortellini, P., Tonetti, M., Nieri, M., Mervelt, J., Cincinelli, S. & Pini-Prato, G. (2012) Coronally advanced flap with and without connective tissue graft for the treatment of single maxillary gingival recession with loss of inter-dental attachment. A randomized controlled clinical trial. *Journal of Clinical Periodontology* **39**(8):760-8.

Cairo, F., Nieri, M. & Pagliaro, U. (2014). Efficacy of periodontal plastic surgery procedures in the treatment of localized facial gingival recessions. A systematic review. *Journal of Clinical Periodontology* **41** (Suppl 15):S44-62. doi: 10.1111/jcpe.12182.

Cairo, F., Cortellini, P., Pilloni, A., Nieri, M., Cincinelli, S., Amunni, F., Pagavino, G., Tonetti, M.S. (2016a). Clinical efficacy of coronally advanced flap with or without connective tissue graft for the treatment of multiple adjacent gingival recessions in the aesthetic area: a randomized controlled clinical trial. *Journal of Clinical Periodontology* 43(10):849-56.

Cairo, F., Pagliaro, U., Buti, J., Baccini, M., Graziani, F., Tonelli, P., Pagavino, G., Tonetti, M.S. (2016b). Root coverage procedures improve patient aesthetics. A systematic review and Bayesian network meta-analysis. *Journal of Clinical Periodontology* 43(11):965-975.

Cortellini, P., Tonetti, M., Baldi, C., Francetti, L., Rasperini, G., Rotundo, R., Nieri, M., Franceschi, D., Labriola, A., Pini-Prato, G.P. (2009) Does placement of a connective tissue graft improve the outcomes of coronally advanced flap for coverage of single gingival recessions in upper anterior teeth? A multi-centre, randomized, double-blind, clinical trial. *Journal of Clinical Periodontology* **36**:68-79.

Ericsson, I., Persson, L.G., Berglundh, T., Marinello, C.P., Lindhe, J., Klinge, B. (1995). Different types of inflammatory reactions in peri-implant soft tissues. *Journal of Clinical Periodontology*

22:255-261.

Froum, S.J., Khouly, I., Tarnow, D.P., Froum, S., Rosenberg, E., Corby, P., Kye, W., Elian, N., Schoor, R., Cho, S.C. (2015). The use of a xenogeneic collagen matrix at the time of implant placement to increase the volume of buccal soft tissue. *International Journal Periodontics Restorative Dentistry* 35(2):179-89

Gobbato, L., Avila-Ortiz, G., Sohrabi, K., Wang, C.W., Karimbux, N. The effect of keratinized mucosa width on peri-implant health: a systematic review. (2013). *International Journal of Oral Maxillofacial Implants* 28(6):1536-45.

Guiha, R., el Khodeiry, S., Mota, L. & Caffesse, R. (2001) Histological evaluation of healing

and revascularization of the subepithelial connective tissue graft. *Journal of Periodontology* 72, 470–8.

Jepsen, K., Jepsen, S., Zucchelli, G., Stefanini,M., de Sanctis, M., Beldini, N., Greven, B., Heinz, B., Wennstrom, J., Cassel, B., Vignoletti, F. & Sanz, M. (2013) Treatment of gingival recession defects with a coronally advanced flap and a xenogeneic collagen matrix: a multicenter randomized clinical trial. *Journal of Clinical Periodontology* 40, 82–89.

Hermann, J.S., Buser, D., Schenk, R.K., Cochran, D.L. (2000) Crestal bone changes around titanium implants. A histometric evaluation of unloaded non-submerged and submerged implants in the canine mandible. *Journal of Periodontology* 71:1412-1424.

Lin, G., Chan, H., Wang, H.L. (2013). The significance of keratinized mucosa on implant health: systematic review. *Journal of Periodontology* 44: 1755-1767.

Linkevicius, T., Apse, P., Grybauskas, S., Puisys, A. (2010). Influence of thin mucosal tissues on crestal bone stability

around implants with platform switching: a 1-year pilot study. *Journal of Oral Maxillofacial Surgery* 68(9):2272-7.

Lorenzo R, García V, Orsini M, Martin C, Sanz M. (2012). Clinical efficacy of a xenogeneic collagen matrix in augmenting keratinized mucosa around implants: a randomized controlled prospective clinical trial. *Clinical Oral Implants Research* 23(3):316-24.

McGuire, M.K., Nunn, M. (2003). Evaluation of human recession defects treated with coronally advanced flaps and either enamel matrix derivative or connective tissue. Part 1: Comparison of clinical parameters. *Journal of Periodontology* 74(8):1110-25.

Nobuto T, Imai H, Yamaoka A. (1988). Microvascularization of the free gingival autograft.

Journal of Periodontology 59(10):639-46.

Puisys, A., Linkevicius, T. (2015).The influence of mucosal tissue thickening on crestal bone stability around bone-level implants. A prospective controlled clinical trial. *Clinical Oral Implants Research* 26(2):123-9.

Sanz, M., Lorenzo, R., Aranda, J.J., Martin, C., Orsini, M. (2009). Clinical evaluation of a new collagen matrix (Mucograft prototype) to enhance the width of keratinized tissue in patients with fixed prosthetic restorations: a randomized prospective clinical trial. *Journal of Clinical Periodontology* 36(10): 868-76.

Suárez-López Del Amo, F., Lin, G.H., Monje, A., Galindo-Moreno, P., Wang, H.L. (2016). Influence of Soft Tissue Thickness on Peri-Implant Marginal Bone Loss: A Systematic Review and Meta-Analysis. *Journal of Periodontology* 87(6):690-9

Thoma DS, Hämmerle CH, Cochran DL, Jones AA, Görlach C, Uebersax L, Mathes S, Graf-Hausner U, Jung RE. (2011). Soft tissue volume augmentation by the use of collagen-based matrices in the dog mandible - a histological analysis. *Journal of Clinical Periodontology* 38(11):1063-70.

Thoma, D.S., Buranawat, B., Hämmerle, C.H., Held, U., Jung, R.E. (2014). Efficacy of soft tissue augmentation around dental implants and in partially edentulous areas: a systematic review.

Journal of Clinical Periodontology 41 (Suppl 15):S77-91.

Thoma, D.S., Zeltner, M., Hilbe, M., Hämmerle, C.H., Hüsler, J., Jung, R.E. (2016). Randomized controlled clinical study evaluating effectiveness and safety of a volume-stable collagen matrix compared to autogenous connective tissue grafts for soft tissue augmentation at implant sites.

Journal of Clinical Periodontology 43(10):874-85.

Tonetti, M.S., Jepsen, S.; Working Group 2 of the European Workshop on Periodontology. (2014)

Clinical efficacy of periodontal plastic surgery procedures: consensus report of Group 2 of the 10th European Workshop on Periodontology. *Journal of Clinical Periodontology* **41** (Suppl 15):S36-43. doi: 10.1111/jcpe.12219.

Warrer, K., Buser, D., Lang, N.P., Karring, T. (1995). Plaque-induced peri-implantitis in the presence or absence of keratinized mucosa. An experimental study in monkeys. *Clinical Oral Implants Research* 6(3):131-8.

Wiesner, G., Esposito, M., Worthington, H., Schlee, M. (2010) Connective tissue grafts for thickening peri-implant tissues at implant placement. One-year results from an explanatory split-mouth randomised controlled clinical trial. *European Journal Oral Implantology* 3(1):27-35.

Table 1: Descriptive statistics at baseline, 1, 3 and 6 months regarding peri-implant soft tissue conditions.

Variable	XCM (baseline) N=30	CTG (baseline) N=30	XCM (1 month) N=29	CTG (1 month) N=30	XCM (3 months) N=28	CTG (3 months) N=30	XCM (6 months) N=28	CTG (6 months) N=30
KT (mm)	3.1 (±1.2)	3.5 (±1.7)	4.5 (±1.2)	4.7 (±1.7)	3.7 (±1.1)	4.0 (±1.7)	4.3 (±1.2)	4.4 (±1.5)
GT (mm)	2.1(±0.6)	2.1 (±0.6)	-	-	2.8 (±0.7)	3.1 (±0.5)	3.0 (±0.7)	3.4 (±0.6)

Legend:

KT = width of keratinized tissue; GT = Gingival Thickness; XCM= Xenogenic Collagen Matrix; CTG= Connective Tissue Graft.

Table 2: Inferential statistics comparing clinical outcomes for test and control group at 1(1m), 3 (3m), and 6 (6m) months.

Variable	XCM	CTG	Difference	95% CI	p-value
KT 6m (mm)	4.3 (±1.2)	4.4 (±1.5)	0.1	-0.3; 0.5	p=0.4754
KT 6m-KT 0 (mm)	1.1 (±0.8)	0.9 (±0.8)	0.1	-0.3; 0.5	p=0.4754
GT 6m (mm)	3.0 (± 0.7)	3.4 (± 0.6)	-0.3	-0.5;-0.2	p<0.0001
GT 6m –GT 0 (mm)	0.9 (± 0.2)	1.2 (± 0.3)	-0.3	-0.5;-0.2	p<0.0001
Bone Levels 6 m (mm)	-0.2 (±0.4)	-0.2 (±0.4)	0.1	-0.1; 0.3	p=0.3022
PD 6 m (mm)	2.8 (± 0.2)	2.9 (± 0.3)	-0.1	-0.2;0.01	p=0.0705
Rec 6 m(mm)	0.04 (± 0.2)	0.0 (± 0.0)	0.04	-0.04;0.1	p=0.3262
Bop (n)	0.1 (± 0.4)	0.3 (± 0.5)	-0.2	-0.4;0.1	p=0.2016
VAS Est 6m (0-100)	90 (±8)	90 (±9)	0.1	-4; 5	p=0.9715
VAS Sat 6m (0-100)	95 (±5)	91 (±9)	4	1; 8	p=0.0195

Legend:

KT = width of keratinized tissue; GT = Gingival Thickness; XCM= Xenogenic Collagen Matrix; CTG= Connective Tissue Graft; PD= mean Probing depth; Bop= Bleeding on Probing; VAS Est 6m= Visual Analogue Scale for Aesthetic satisfaction at 6 months; VAS Sat 6m= Visual Analogue Scale for overall satisfaction at 6 months

Figure 1: CONSORT flowchart of the study

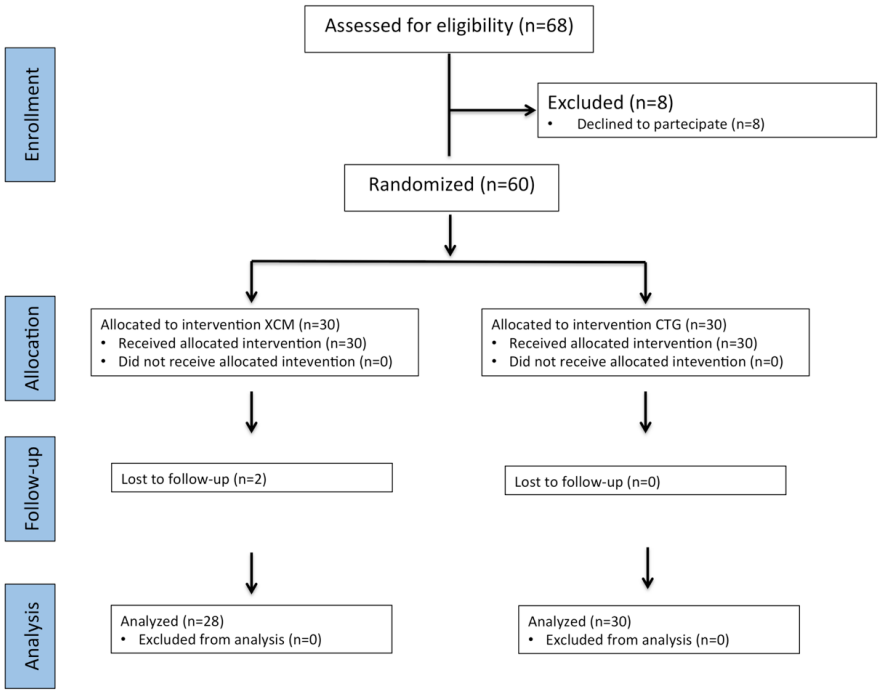
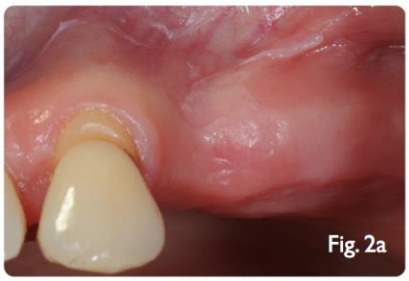
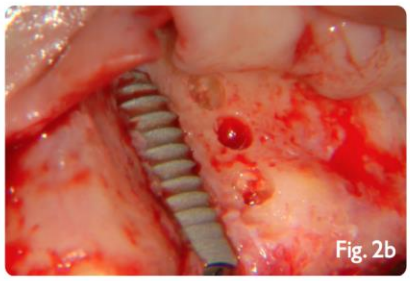
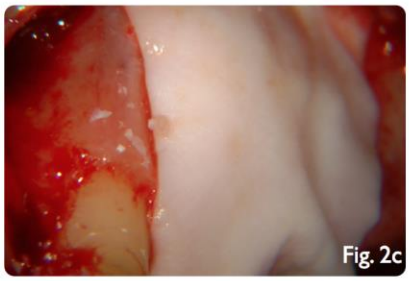


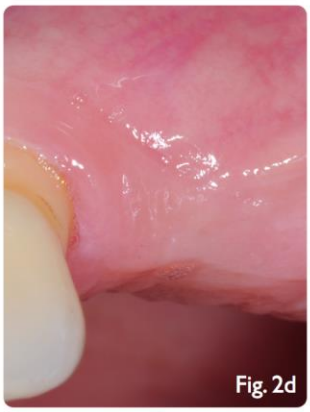
Figure 2

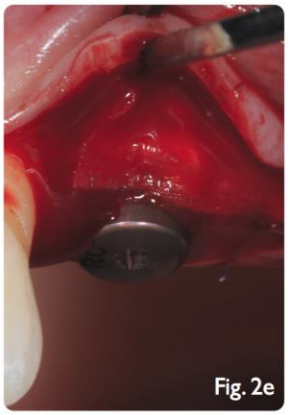
2 a: baseline conditions; 2b: fixture application; 2c: guided bone regeneration; 2d: baseline of experimental procedures, 6 months after implant application; 2e: clinical case allocated to XCM; 2f: final soft tissue healing at the last follow-up; 2g: final x-ray

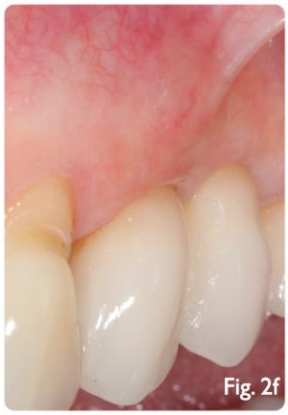












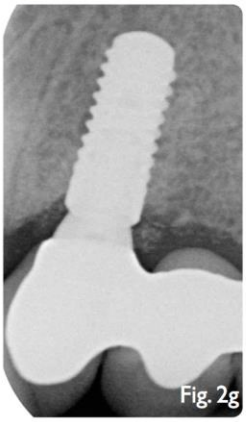


Figure 3

3 a: baseline conditions, tooth 2.1 was scheduled for extraction due to root fracture; 3b: fixture application; 3c: guided bone regeneration; 3d: baseline of experimental procedures, 6 months after implant application; 3e: clinical case allocated to CTG; 3f: final soft tissue healing at the last follow-up; 3g: final x-ray

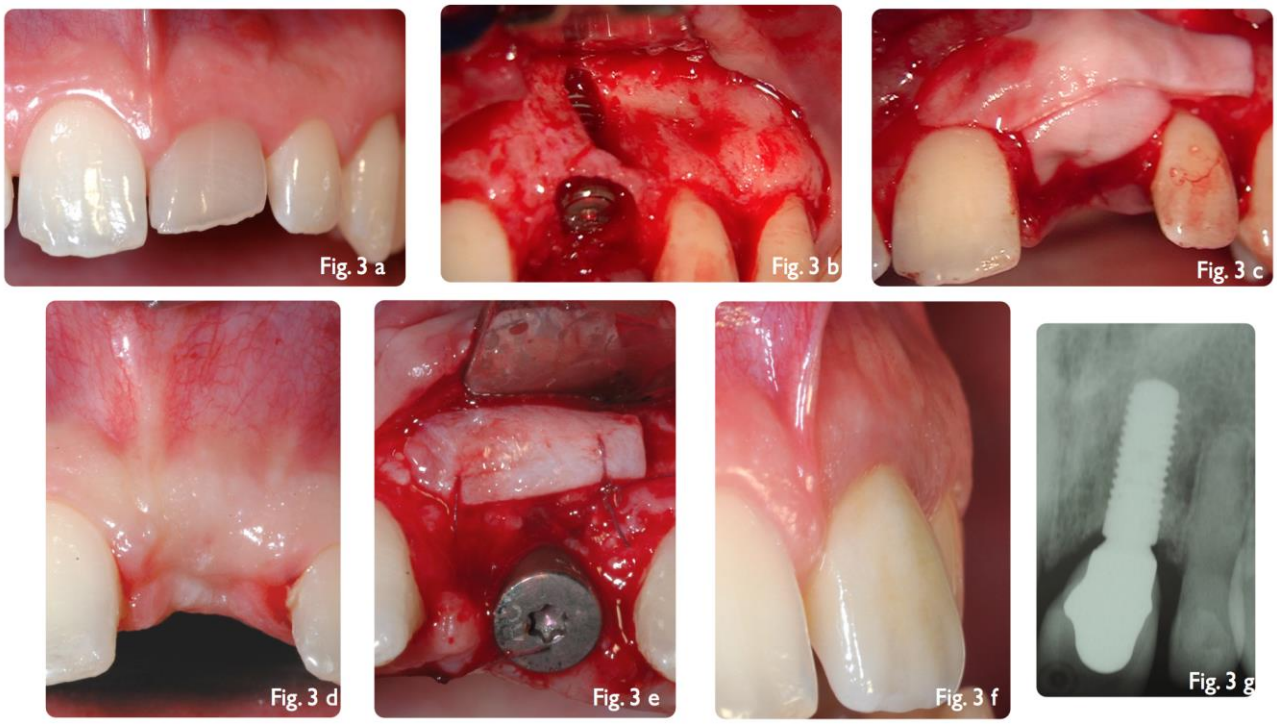
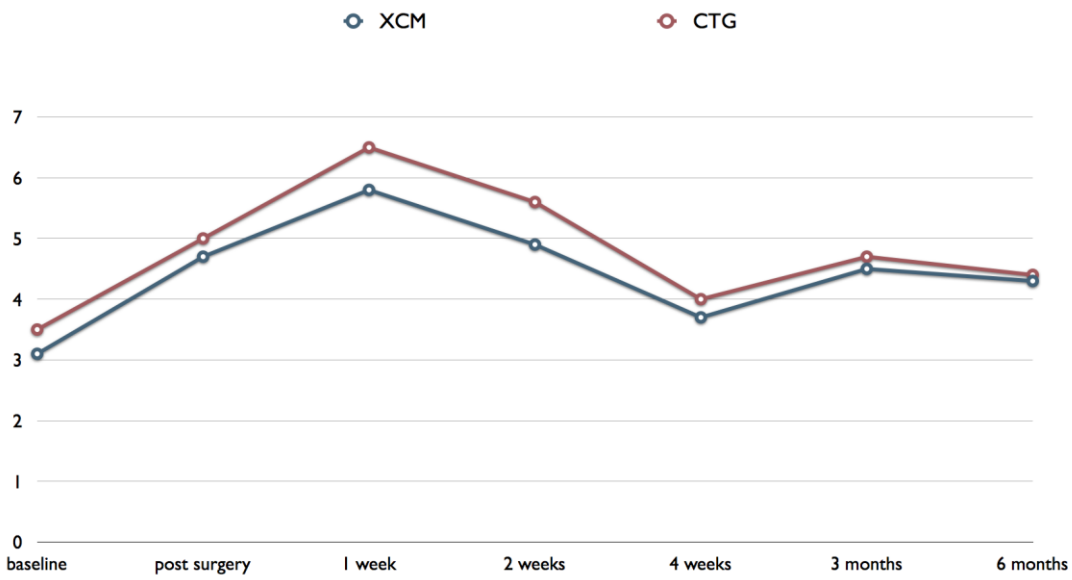


Figure 4

KT changes at each interval (XCM, Xenogenic Collagen Matrix; CTG, Connective Tissue Graft)



Correspondence to:

Dr. Francesco Cairo, E-mail: *cairofrancesco@virgilio.it*

Conflict of interest:

The authors have stated explicitly that there are no conflicts of interest in connection with this article

randomized clinical trial, dental implants.

Source of Funding:

The study was self-funded by the authors and their institution and by a research grant from Geistlich Pharma AG, Wolhusen, Switzerland.

INNOVATIVE SUPER-HYDROPHILIC TITANIUM SELECTIVELY PROMOTES FIBRONECTIN ADSORPTION AND OSTEOBLASTS ADHESION AND PROLIFERATION

Superfici implantari iperidrofiliche favoriscono l'assorbimento selettivo di fibronectina plasmatica e migliorano la risposta osteoblastica

A.Toffoli[1,2], L. Parisi[1,2], M.Bianchi[2], F. Rivara[1,2], E. Calciolari[1,3], S. Lumetti[1,2], E. Manfredi[1,2], C. Galli[1,2,4], G.M. Macaluso[1,2,4]

[1] Centro di Odontoiatria, University of Parma, Via Gramsci 14, 43121 Parma, Italy

[2] Dipartimento di Medicina e Chirurgia, University of Parma, Via Gramsci 14, 43121 Parma, Italy

[3] Centre for Oral Clinical Research, Institute of Dentistry, Barts & The London School of Medicine and Dentistry, Queen Mary University of London, Mile End Road London E1 4NS, UK

[4] MEM-CNR National Research Council, Parco Area delle Scienze 37/A, 43124 Parma, Italy

Abstract

Titanium surface wettability has been shown to affect the adsorption of blood proteins and consequently cell response. The aim of our study was to further investigate the influence of super-hydrophilic surfaces obtained through a proprietary process on the adsorption of specific blood proteins and the corresponding response of osteoblasts.

Fibronectin adsorption on surfaces was monitored over time, and qualitatively assessed by western blot analysis. Subsequently, the influence of super-hydrophilic and fibronectin-enriched surfaces was tested on murine MC3T3-E1 osteoblasts, which were assayed for viability, proliferation, morphology and adhesion.

The super-hydrophilic titanium induced a significant increase of fibronectin adsorption. Osteoblasts showed a faster proliferation, closer adhesion and higher expression of focal adhesions when cultured on super-hydrophilic substrates.

These data showed how the enhanced adsorption of fibronectin influences the response of osteoblasts on titanium surfaces. Moreover, they showed the enhancement of cellular responses that can be achieved by a super-hydrophilic titanium surface modification obtained by a proprietary method

Riassunto

È stato dimostrato che la bagnabilità della superficie degli impianti in titanio giochi un ruolo fondamentale nell'adsorbimento di proteine presenti nel sangue, influenzando positivamente la risposta cellulare primaria successiva al posizionamento dell'impianto. Pertanto, dopo aver messo a punto un metodo per rendere le superfici implantari iperidrofiliche, abbiamo deciso di studiare

l'influenza della topografia superficiale e della bagnabilità sull'adsorbimento di specifiche proteine plasmatiche e sulla risposta cellulare primaria.

L'adsorbimento di fibronectina sulle superfici è stato monitorato nel tempo e successivamente analizzato qualitativamente attraverso un immunoblotting. Successivamente, l'influenza della super-idrofilicità e del conseguente arricchimento superficiale di fibronectina è stato testato su cellule osteoblastiche murine, di cui sono state valutate vitalità, proliferazione, morfologia e adesione.

I risultati che abbiamo ottenuto hanno mostrato come il nostro metodo permetta un sensibile guadagno di idrofilicità del titanio, inducendo altresì un aumento dell'adsorbimento superficiale di fibronectina. Inoltre, i nostri dati supportano l'idea che l'adsorbimento selettivo di fibronectina possa giocare un ruolo centrale nella risposta osteoblastica. Sulle superfici super-idrofiliche gli osteoblasti hanno infatti mostrato una proliferazione più rapida, un'adesione più stretta al substrato ed una maggiore espressione di adesioni focali.

In conclusione i nostri dati supportano l'efficacia del nostro trattamento nell'ottenimento di superfici implantari super-idrofiliche e nell'aumento della selettività di queste superfici per l'adsorbimento di proteine coinvolte nelle risposte cellulari primarie.

Introduction

Since their introduction in the clinical scene, dental implants underwent major modifications in terms of material, shape, surface topography and physical properties. In the last years, many authors focused their research on implant surface properties, to enhance their biological and clinical performances [1,2,3]. Many studies demonstrated that surface hydrophilicity can influence protein adsorption and cellular behaviour, with better osseointegration for increased wettability [4,5]. At a microscopic level, high surface hydrophilicity has been shown to preserve protein conformation upon adsorption and consequently promote the development of a favourable micro-environment [6,7]. Hydrophilic titanium surfaces, in fact, allow lower protein denaturation and misfolding during adsorption, preserving their correct molecular function and enhancing surface bioactivity. As a consequence, this leads to a faster and better osseointegration [4].

Fibronectin plays a crucial role in wound healing [8]. Along with fibrin, plasma fibronectin is deposited at injury sites, contributing to form a blood clot that stops bleeding and protects the underlying tissue. Subsequently, it forms a scaffold to which bone cells can bind and proliferate. Considering this, a modified titanium implant surface with a fibronectin coating was found to enhance osteoblasts differentiation, driving to a faster osseointegration [9,10].

We have recently developed a new implant surface treatment to enhance titanium hydrophilicity. The aim of this study is to compare the effects of topographically different implant surfaces with and without super-hydrophilic treatment by measuring the amount and quality of fibronectin adsorbed and bone cell viability, morphology, adhesion and proliferation.

Materials and Methods

Titanium discs - Commercially pure, grade 4 (ISO5832/2) titanium discs (d=8.0mm) with machined or sand-blasted/acid-etched surface were kindly provided by Sweden&Martina S.P.A. (Due Carrare, Padova, Italy). Discs were treated with a proprietary method, which involves a thermal treatment and which cannot be disclosed because under patent, in order to obtain super hydrophilic titanium surfaces.

The four experimental conditions were as follows:

Machined surface= MAC

Super-hydrophilic - Machined surface= SMAC

Sand-blasted/acid-etched surface= SAE

Super-hydrophilic - Sand-blasted/acid-etched surface= SSAE

Protein adsorption - To investigate whether hydrophilicity influence the adsorption of proteins involved in primary cells responses, a quantitatively and a qualitatively analysis was performed.

For quantitative analysis titanium discs were soaked in 500µl of a 200µg/ml human fibronectin (Sigma-Aldrich) solution for 2 hours. Protein concentration decrement in the supernatant was measured after 5, 15, 30, 60, 120 and 180 minutes through the Bradford (BIO-RAD) mixing 10µl of sample and 200µl of Bradford Working Solution. Sample absorbances were read at 620nm with a Multiskan FC plate reader (Thermo Fisher Scientific) and the amount of adsorbed fibronectin was calculated subtracting the residual concentration in the supernatants.

For qualitative analysis, discs were incubated in 500µl of 2% human serum (Sigma-Aldrich) solution for 1 hour at room temperature (RT). Discs were subsequently rinsed twice in PBS in order to remove unbound proteins and covered with 80µl of Sample Buffer 1X. Adsorbed proteins were then completely recovered by freezing, thawing, sonicating for 15 minutes and boiling samples for 10 minutes. Equal volumes of samples were then run on a 12% polyacrylamide gel (Acrylamide/Bis-Acrylamide 30%, Sigma-Aldrich) for 1 hour and 180 V. Separated proteins were subsequently blotted on a PVDF membrane (Immobilon-P) at 100V for 1 hour. Non-specific sites were blocked with Tris-buffer saline containing 10% of blocking reagent (Roche SpA) for 1 hour at RT and fibronectin revealed with an anti-Fibronectin (Sigma-Aldrich) primary antibody diluted 1:800 in 0.1 % v/v TBS Tween 20 supplemented with 5% BSA (Sigma-Aldrich) and with a HRP-conjugated secondary antibody (Cell Signaling Technology) diluted 1:10000.

Cell culture - *In vitro* assays were performed with murine osteoblasts (MC3T3-E1) obtained from the America Typer Culture Collection (LGC Standards). Cells were cultured in complete Alpha-MEM (Life Technologies) additioned with 10% Fetal Bovine Serum (Life Technologies) and 1% Penicillin and Streptomycin (Life Technologies) at a density of 10000cells/disc.

Cell viability - Cell viability was assessed through LIVE/DEAD assay. After 48 hours of culture, culturing medium was removed and cells stained with a 4µM Calcein AM (Calcein AM, Life

Technologies) and 7.5µM PI (PI, Life Technologies) solution for 10 minutes at RT in dark conditions. Samples were then fixed with 4% PFA (Sigma-Aldrich) for 20 minutes in dark conditions and observed with a fluorescence-equipped stereomicroscope (Nikon). Three representative regions of interest (ROIs) were chosen and viable and dead cells counted through the use of the Documentation D3 software (Nikon).

Cell metabolic activity - Cell metabolic activity was measured through Resazurin Sodium Salt assay 24, 48 and 72 hours after seeding. At each experimental point, 100µl of Resazurin Sodium Salt stock solution (final concentration 0.15mg/ml) were added to 500µl of serum-free culturing medium and samples incubated at 37°C and 5% CO_2 for 4 hours. Fluorescence was finally excited at 560nm and read at 585nm through a Multiskan Ascent microcell plate reader (Thermo Labsystems**).**

Cell morphology - Cell morphology and focal adhesion distribution were studied trough cytofluorescence for actin, vinculin and cell nuclei. After 24 hours of culture, culturing medium was removed and cells fixed in a 4% PFA solution for 10 minutes at RT. After twice rinsed in PBS, cells were permeabilized with 0.1% v/v Triton X-100 (Sigma-Aldrich) solution for 5 minutes at RT and washed twice in PBS. One-% BSA solution was then added to the samples for 30 minutes at RT in order to block non-specific sites. Subsequently, cells were stained for focal adhesion with a primary anti-vinculin monoclonal antibody, clone 7F9 (Merck Millipore) for 1 hour at RT and washed twice in PBS. To reveal primary antibody, a secondary anti-rabbit labeled with the AlexaFluor®488 chromophore (Life Technologies) diluted 1:200 in PBS was used and co-incubated with TRITC-conjugated phalloidin (Merck Milliporere) (dilution 1:200) for actin staining. After three rinses in PBS, nuclei counterstaining was performed with a 1:1000 PBS solution of DAPI (Merck Millipore) for 5 minutes at RT.

Cell adhesion - To study the interactions of cells with the underlying titanium surface, a scanning electron microscopy (SEM) in combination with the orthogonal sample cutting through a Gallium Focused Ion Beam (FIB) source was performed. SEM-FIB preparation was performed 24 hours after seeding at RT. Culturing medium was removed, cells rinsed in PBS, fixed in a 2.5% gluteraldehyde (Sigma-Aldrich) solution in Na-Cacodylate buffer (Sigma-Aldrich) for 30 minutes, washed in Na-Cacodylate buffer for 5 minutes and dehydrated in ethanol at increasing concentrations (Sigma-Aldrich). Finally, samples were critical point dried with liquid carbon dioxide (Balzer Union) and sputtered with a thin layer of gold through a SCD 040 coating device (Balzer Union). Photographs were taken using a dual beam Zeiss Auriga Compact system equipped with a GEMINI Field-Effect SEM column and a Gallium FIB source (Zeiss). SEM analysis was performed at 5keV, while the cross-sectional analysis at 30kV with a current of 10pA.

Statistical analysis - Data were analyzed using Prism 6 (La Jolla). All the values are reported as the mean ± SD of three repeated experiments performed in multiple replicates. Differences between groups were evaluated with the two-way ANOVA statistical test and Tukey's multiple comparison post-test. Differences were considered significant when $p<0.05$.

Results

Super-hydrophilicity increases the amount of stable adsorbed fibronectin - The overall amount of deposited protein onto titanium surfaces was not influenced by hydrophilicity. Bradford assay revealed that after 2 hours a mean of 13.6 ± 0.7µg of fibronectin was present on MAC samples, while 13.9 ± 1.4µg on SMAC discs (fig.1A). Similarly, 12.3 ± 2.5µg of fibronectin were on SAE sample vs the 13.9 ± 1.4 µg on SSAE (fig.1B). Interestingly neither hydrophilicity, nor surface topography seemed to affect this. Surprisingly however, after rinsing with PBS the amount of stable bound proteins was different and heavily influenced by hydrophilicity (fig.1C). As western blot analysis revealed, the amount of adsorbed fibronectin was higher on super-hydrophilic samples than on controls (fig.1D-E).

Super-hyrophilicity enhances cells proliferation - The relationship between live and dead cells was not altered through the groups (fig.2A). However, the number of cells was higher on treated samples than on controls both for machined (fig.2B) and for sandblasted/acid-etched surfaces (fig.2C), with statistically significative differences ($p<0.0001$). In spite of this, proliferation was enhanced on super-hydrophilic surfaces and differences were highly different between non-super-hydrophilic and super-hydrophilic discs both for machined and for sanblasted/acid-etched surface ($p<0.0001$).

Moreover, proliferation was significantly higher on SMAC than on SSAE samples ($p=0.0064$), and interestingly these data linearly fitted with the amount of fibronectin adsorbed on samples as reported in figure 1D-E.

On the other hand, hydrophilicity did not seem to influence cell metabolic activity (fig.3), which was influenced by the surface microtopography in a statistically significative way after 48 hours for super hydrophilic surfaces (SMAC-SSAE $p=0.0199$) and after 72 hours for hydrophobic and for hydrophilic surfaces (MAC-SAE $p<0.0001$; SMAC-SSAE $p=0.0010$).

Super-hydrophilicity influenced cell morphology and focal adhesion expression - Immunostaining revealed the presence of healthy cells on all the samples (fig.4A). In particular, the staining for focal adhesions showed more numerous focal adhesions per cell on treated samples than on controls (fig.4B), with statistically significative differences between the groups (MAC-SMAC $p=0.0100$; SAE-SSAE $p=0.0061$; SMAC-SSAE $p=0.0140$) and a trend that perfectly fits the amount of stably adsorbed fibronectin on surfaces and analyzed in figure 1D-E.

Super-hydrophilicity allows a closer adhesion to titanium of cells - SEM analysis reported in figure 5, revealed a similar cell adhesion to MAC or SMAC samples, where cells displayed a flat and well spread shape. However, a different cell morphology was observed for control and treated sandblasted/acid-etched surfaces: cells adhered more closely to super-hydrophilic surfaces, and showed a flatter shape that allowed to glimpse the underlying micro texture of the underlying titanium profile. FIB analysis confirmed SEM observations, showing how super-hydrophilic surfaces could promote a closer adhesion of cells to the material, and how they appeared thinner, adhering preferentially to surface peaks of SSAE sample.

Discussion

With this study we intended to assess the efficacy of super-hydrophilicity in improving the adsorption of a target blood protein, fibronectin, which severely affected primary osteoblasts response, on the titanium dental implant surfaces.

The process we designed to obtain super-hydrophilic titanium efficiently led to a gain in surface hydrophilicity both on machined and sandblasted/acid-etched surfaces. Furthermore, the gain of hydrophilicity allowed a stable adsorption of fibronectin on the surfaces and this impacted on the subsequent osteoblasts response (fig.1). The number of cells was higher on treated samples than on the controls regardless of surface topography. Higher cell viability was matched by a higher amount of focal adhesions per cell unit (fig.2-4) and, consistently, a tighter cell adhesion, as visible at SEM and FIB sections (fig.5).

Acknowledgements

The authors would like to thank Sweden&Martina S.P.A. for titanium discs manufacturing and Dr. Enrico Babetto (Sweden&Martina S.P.A., Due Carrare, PD, Italy) for his precious advice. The authors would like to thank also Dr. Paola Lagonegro (IMEM-CNR , Parma, Italy) and Dr. Davide Dallatana (Dip. Medicina e Chirurgia, Parma, Italy) for their precious advice in SEM-FIB preparation and analysis.
The authors declare that the treatment method whose effect are described in this work is actually under patent process.

References

Barfeie A., Wilson J., Rees J. “Implant surface characteristics and their effect on osseointegration.” Br Dent J. 2015 Mar 13;218(5):E9.

Wennerberg A., Jimbo R., Stübinger S., Obrecht M., Dard M., Berner S. “Nanostructures and hydrophilicity influence osseointegration: a biomechanical study in the rabbit tibia.” 2014, Clinical Oral Implant Research 25: 1041-1050.

Le Gue’hennec L., Soueidan A., Layrolle P., Amouriq Y. “Surface treatments of titanium dental implants for rapid osseointegration” 2007, Dental Materials 24:844-854.

Lang NP, Salvi GE, Huynh-Ba G, Ivanovski S, Donos N, Bosshardt DD. Early osseointegration to hydrophilic and hydrophobic implant surfaces in humans. 2011, Clinical Oral Implant Research 22(4):349-56.

Schwarz F., Ferrari D., Herten M., Mihatovic I., Wieland M., Sager M, Becker J. “Effects of surface hydrophilicity and microtopography on early stages of soft and hard tissue integration at non-submerged titanium implants: an immunohistochemical study in dogs.” J Periodontol. 2007 Nov;78(11):2171-84.

Rupp A., Gittens R.A., Scheideler L., Marmur A., Boyan B.D., Schwartz Z., Geis-Gerstorfer J.”A review on the wettability of dental implant surfaces I: Theoretical and experimental aspects”. 2014, Acta Biomaterialia 10:2894–2906.

Gittens R.A., Scheideler L., Rupp, F., Hyzy S.L., Geis-Gerstorfer J., Schwartz Z., Boyan B.D. “A review on the wettability of dental implant surfaces II: Biological and clinical aspects”. 2014, Acta Biomaterialia 10:2907-2918.

Grinnell F. “Fibronectin and wound healing.” 1984 Journal of Cellular Biochemistry 26(2):107-116.

Petrie T.A., Reyes C.D., Burns K.L., García A.J. “Simple application of fibronectin-mimetic coating enhances osseointegration of titanium implants.” J Cell Mol Med. 2009 Aug;13(8B):2602-12.

Elkarargy A. “Biological functionalization of dental implants with fibronectin: a scanning electron microscopic study.” 2014, Int J Health Sci (Qassim) 8(1):57-66.

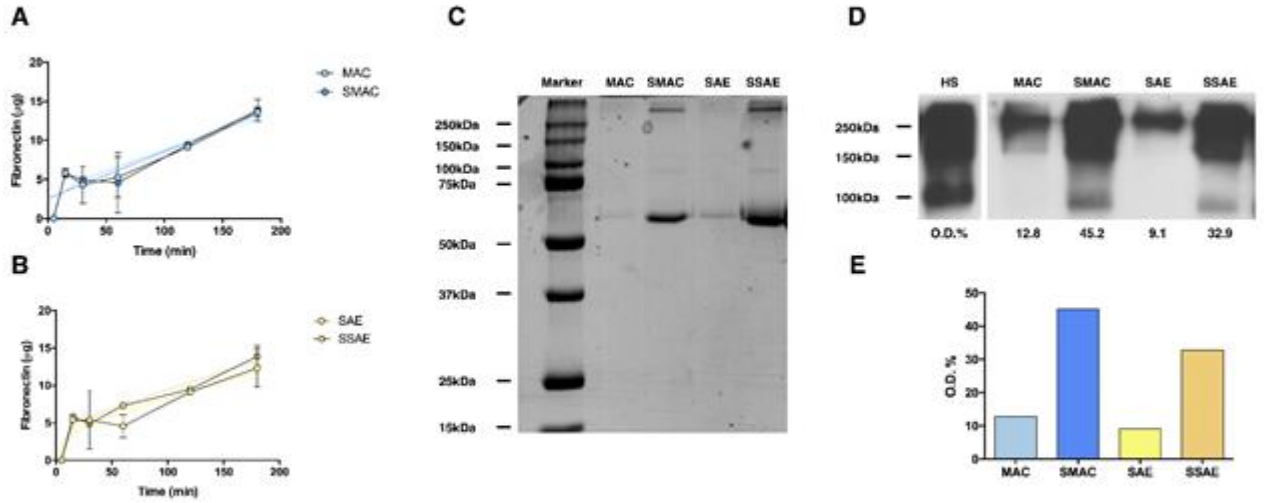


Figure 1 - Protein adsorption. (A) Fibronectin adsorption on MAC and SMAC titanium surfaces and quantitated through the Bradford. (B) Fibronectin adsorption on SAE and SSAE titanium surfaces and quantitated through the Bradford. (C) SDS-PAGE separation of human serum proteins after Coomassie Blue staining. (D-E) Immunoblot for fibronectin and optical density percentual plotting.

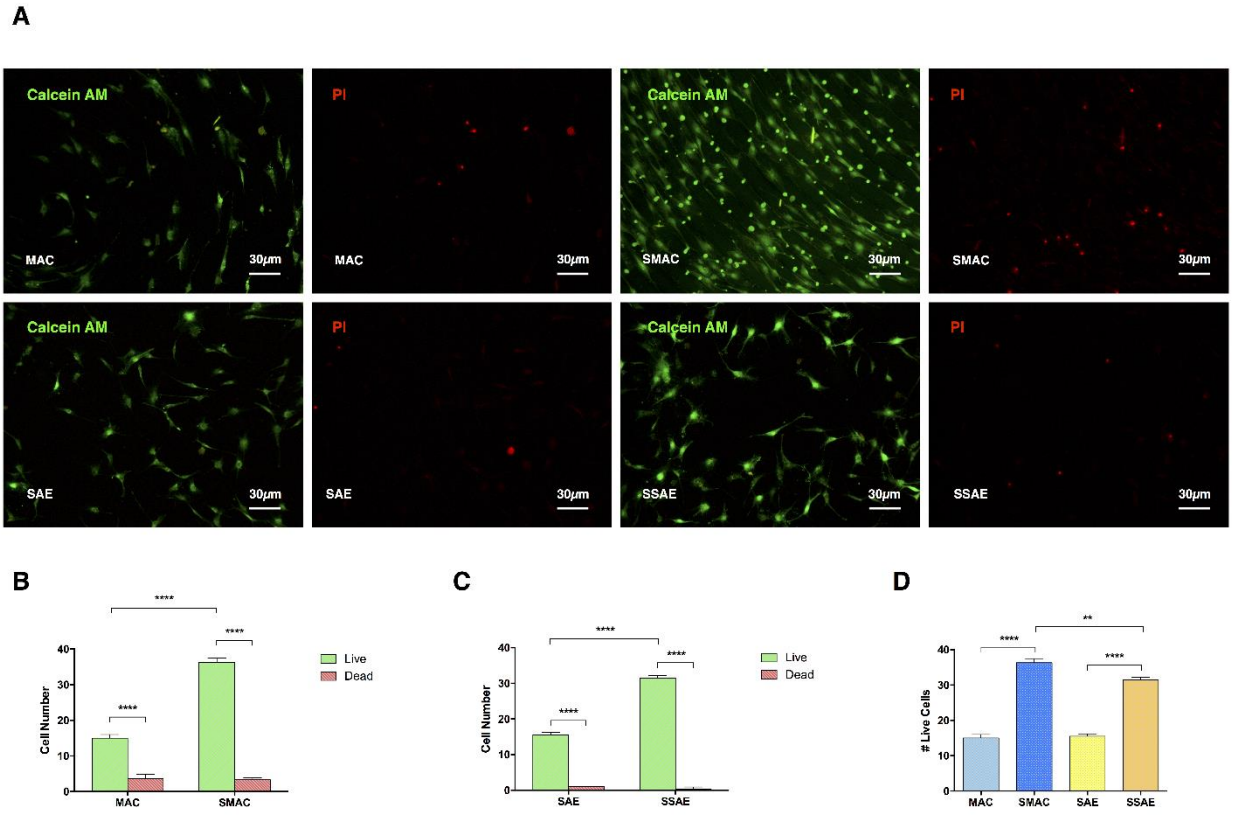


Figure 2 - Cell viability. (A) Murine MC3T3-E1 osteoblasts viability on different titanium surfaces. (B) Quantitation of live (green) and dead (red) cells on MAC or SMAC titanium surfaces. (C) Quantitation of live (green) and dead (red) cells on SAE or SSAE titanium surfaces. (D) Quantitation of live (green) cells on different titanium surfaces.

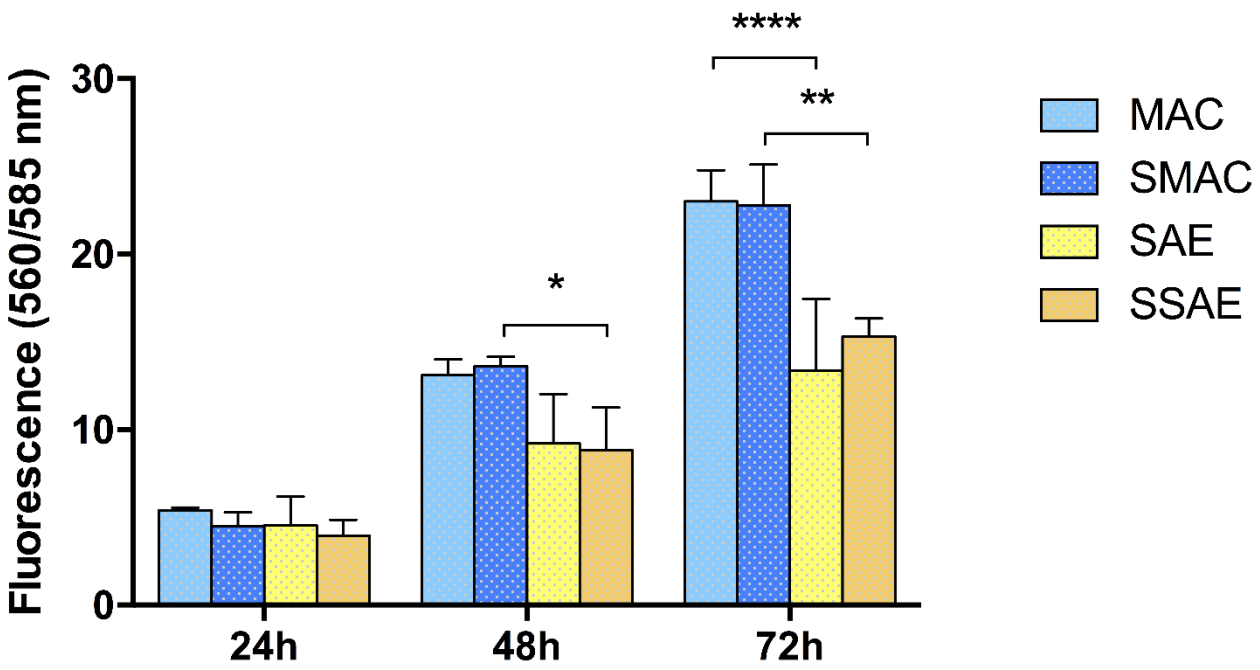


Figure 3 - Cell metabolic activity on different titanium surfaces, measured through Resazurin sodium salt assay after 24, 48 and 72 hours of culturing.

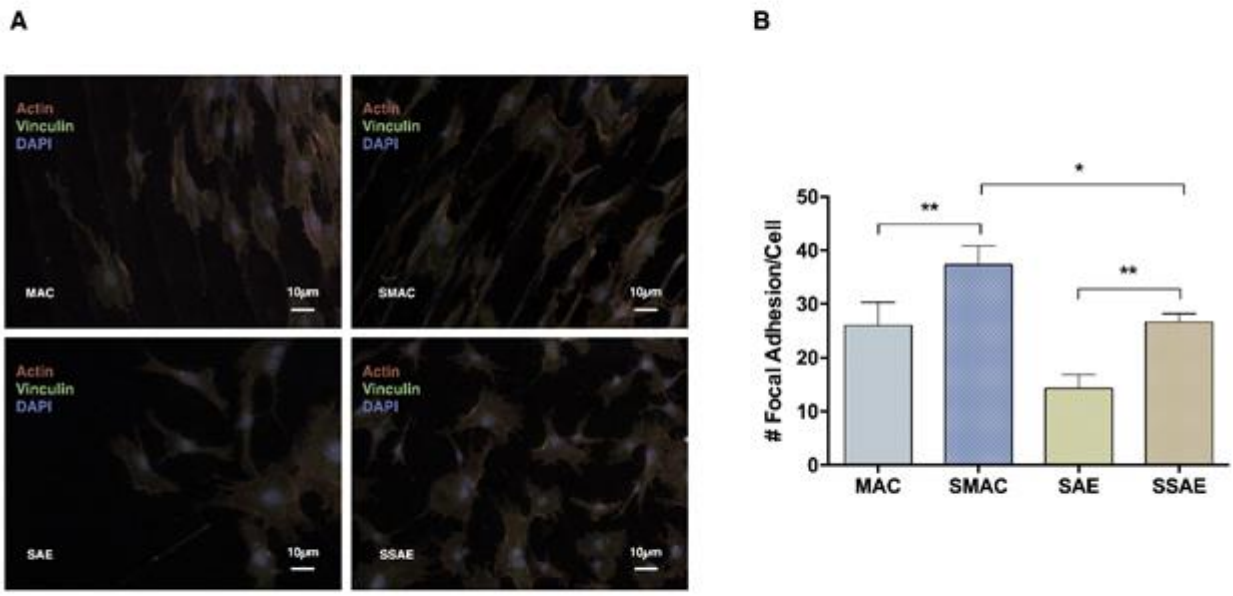


Figure 4 - Cell morphology. (A) Murine MC3T3-E1 osteoblasts morphology different titanium surfaces stained for actin (red), focal adhesion (green spots) and cell nuclei (blue). (B) Quantitation of focal adhesion per cell expressed and quantitated through the Documentation D3 software.

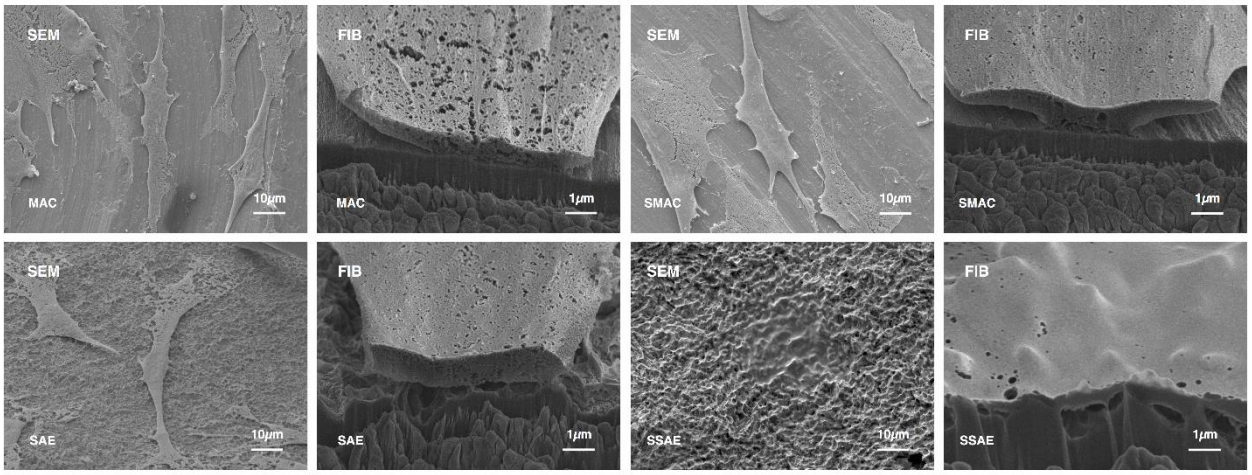


Figure 5 - Cell adhesion. Murine MC3T3-E1 osteoblasts morphology and adhesion on different titanium surfaces fixed and observed through SEM-FIB.