THE INFLUENCE OF CONNECTIVE TISSUE GRAFT IN DETERMINING EPITHELIAL DIFFERENTIATION AFTER PERIODONTAL PLASTIC SURGICAL PROCEDURES: A THREE-YEARS CLINICAL AND HISTOLOGICAL STUDY.

Di Martino M, Di Domenico GL, Arrigoni G, Aroca S, de Sanctis M.

Department of Periodontology, Università Vita-Salute San Raffaele, Milano, Italy.

Abstract

Objectives: investigation of the influence of Connective Tissue Graft (CTG) on gingival phenotype changes following Multiple Coronally Advanced Flap with selective use of CTG. A 3-year prospective clinical and histological evaluation.

Materials and methods: Twenty patients treated with MCAF+CTG were available for the 3-year follow-up. Outcome measures included Complete Root Coverage, Recession Reduction, Keratinized Tissue Width, Marginal Tissue Thickness and Primary Flap Position. Biopsies were harvested at one of treated sites with MCAF+CTG for semiquantitative analysis.

Results: A statistically significant greater KTW increase was observed in MCAF+CTG group, when compared to baseline. Both initial KTW and Primary Flap Position were statistically significant associated factors with KTW changes. The 47% of sites treated with MCAF+CTG presented an apical shift of primary flap and showed a statistically significant greater KTW increase. The histological examination demonstrated a separation between the chorion of the gingival flap and the CTG with no effect on modification of connective tissue density of the flap and thus no induction of keratinization.

Conclusions: The selective use of CTG +MCAF is effective in reducing recession depth. The CTG cannot exert any differentiation of the overlying epithelium when the flap maintains its original position. Only, the flap contraction over the CTG shows an evident increase in KTW.

Sintesi

Obiettivi: Studio dell'influenza dell'innesto di tessuto connettivo (CTG) sui cambiamenti del fenotipo gengivale in seguito a Lembo Avanzato Coronalmente Multiplo con uso selettivo di CTG. Valutazione clinica e istologica prospettica a 3 anni.

Materiali e metodi: Venti pazienti trattati con MCAF+CTG erano disponibili per il follow-up a 3 anni. Le misure registrate: Copertura Radicolare Completa, Riduzione Recessione, Ampiezza Tessuto Cheratinizzato, Spessore Tessuto Marginale, Posizione Lembo Primario. Le biopsie sono state raccolte in uno dei siti trattati con MCAF+CTG per l'analisi semi-quantitativa.

Risultati: è stato osservato un aumento maggiore statisticamente significativo di KTW nel gruppo MCAF+CTG. KTW iniziale e Posizione del Lembo Primario erano fattori statisticamente significativi associati ai cambiamenti del KTW. Il 47% dei siti trattati con MCAF+CTG ha presentato uno spostamento apicale del lembo primario ed un maggiore aumento di KTW statisticamente significativo. Tutti i campioni di biopsie hanno mostrato marcata separazione tra corion del lembo gengivale e CTG senza effetto sulla modifica della densità del tessuto connettivo del lembo e quindi senza induzione di cheratinizzazione.

Conclusioni: L'uso selettivo di CTG + MCAF è una terapia efficace. Il CTG non può esercitare alcuna

differenziazione dell'epitelio sovrastante se il lembo mantiene la sua posizione originale. Solamente la contrazione del lembo sul CTG mostra un evidente aumento del KTW.

Manuscript

1. Introduction

Gingival recessions can be successfully treated by several surgical approaches. Many studies attested that pedicle flaps, free soft tissue grafts or combination of pedicle flaps with grafts, barrier membranes or enamel matrix derivates are all effective procedures to cover the exposed root surfaces. [1-4]

The multiple coronally advanced flap (MCAF), proposed by Zucchelli and de Sanctis (2000), has become the most widely used root coverage procedure for multiple recession defects. It is the first-choice surgical technique in presence of an adequate keratinized tissue width (KTW) apical to the recession and it is very effective with advantages for the patients in terms of aesthetics and morbidity. [5,6]

The use of a connective tissue graft (CTG) under MCAF has been introduced to increase the predictability of the procedure. The application of CTG is associated with the highest probability to obtain complete root coverage[1,2,4,7], as well as with an increase of gingival thickness (GT) and of the KTW over time.[6,8,9]

The increased dimension in KTW was reported with different amount by several authors. Cairo et al.[6] in a randomized controlled clinical trial found that the addition of a CTG under MCAF was associated with a significant increase in both KT (1.8 □0.6mm) and GT (0.66 □0.17mm) at 1-year, when compared to MCAF per se'.

Since very often teeth adjacent to each other present different phenotype, Stefanini et al. (2018)[8] in a case series proposed MCAF with site-specific application of CTG, reporting a significant increase in KTW in the MCAF+CTG treated-sites compared to baseline (3.14 □0.48mm).

Various explanations have been proposed to justify the increase in KT width following bilaminar techniques, such as genetically predetermined position of mucogingival line [8,10-12], inductive potential of a dense connective graft on epithelial phenotype[12-14] or repositioning of the primary flap in an apical direction during wound healing.[15,16]

When discussing the effect of connective tissue to induce epithelial keratinization, several authors reported that the dense connective tissue of the graft could exert its influence over the epithelium, even when covered by the chorion of the covering flap.[12-14] Nevertheless, it should be taken in to consideration that the previous studies demonstrated that the stimulus which determine the histodifferentiation of the epithelium occurs when the connective tissue is left uncovered[17, 18] while it is unclear how the differentiation of the mucosa epithelium into keratinized one, could occur when utilizing a bilaminar technique, that is covering the connective tissue with the coronal advanced flap.

On the other hand, some authors have indicated that the post-surgical instability of the flap, which leads to the exposure of marginal part of the underlying connective tissue, could explain the epithelial differentiation and the increase in keratinization[15,16] Moreover, this localized increase in keratinization due to the shrinkage of the covering flap is reported by some authors as keloid formation[19] or as an unaesthetic result.[6]

The main objective of the present investigation was to prospectively evaluate the influence of Connective Tissue Graft (CTG) on the gingival phenotype and the increase of keratinized tissue following a mini-invasive bilaminar technique, the multiple coronally advanced flap (MCAF) plus selective use of CTG.
In order to identify the factors related to these changes, selective biopsies were taken and a semiquantitative analysis of the specimens was conducted.

2. Material and methods

2.1. Study design and population

This study reported the 3-year follow-up of a prospective trial on the treatment of multiple gingival recessions. All subjects were treated with multiple coronally advanced flap (MCAF) with selective use of CTG, where CTG was applied only to the sites showing a KTW less than 2mm or when the phenotype was evaluated as thin (<1mm), in order to reduce the invasiveness of the technique. (Figure 1) Details of the study protocol were presented in a previous paper reporting 1-year results.[20]

This study was approved by the Ethical Committee of San Raffaele Hospital (with number of protocol "KT"— EC Reg. N. 157/INT/2019) and performed in accordance with the Helsinki Declaration of Human Studies.

The study population was enrolled at the Department of Periodontology, San Raffaele Hospital (Milan, Italy), between January 2022 and March 2022. Details of inclusion and exclusion criteria have been reported in the previous paper. [20] All subjects were informed about all pertinent aspects of the study and gave written informed consent.

2.2. Clinical evaluation

The following clinical parameters were collected at the treated sites:

- *Recession Depth (REC)* – measured at the mid-buccal site from CEJ to the gingival margin
- *Keratinized Tissue Width (KTW)* – measured as the distance from the gingival margin to the mucogingival junction at the middle buccal point. The measurements were repeated a second time with the use of iodine solution (Lugol's solution 5%).[21]
- *Marginal Tissue Thickness (MTT)* measured using a short needle for anesthesia with a silicon disk stop 2 mm apical to the gingival margin.
- *Primary Flap Position (PFP)* – that is the position of the primary flap (the flap covering the CTG) following the healing phases. This was evaluated dichotomously as "coronal" when the original position was maintained and the connective tissue remained covered, and "apical" when an apical shift of the flap was observed, thus exposing part of the connective tissue graft. The use of iodine solution (Lugol's solution 5%) evidenced the apical shift of the margin of the flap. (Figure 2)

All clinical measurements were performed by two calibrated examiners (MDM and GLDD) using a manual probe (PCP-UNC 15 probe tip, Hu-Friedy). In order to demonstrate an acceptable level of intra- and inter-examiner agreement, duplicate measurements for probing depth and recession depth of 5 subjects presenting multiple gingival recessions, were repeated after 24 hours following the first measurements. The intra-examiner intra-class correlation coefficient was 0.96 (95% CI 0.932–0.976) for MDM and 0.9775 (95% CI 0.957–0.985) for GLDD. The inter-examiner intra- class correlation coefficient was 0.97 (95% CI 0.954–0.98).

2.3. Biopsy and sample preparation

Informed consent was obtained by 9 patients to execute a biopsy at one of the sites treated with the adjunct of CTG for histologic evaluation. In addition, two biopsies of normal palatal and gingival mucosa were obtained as control.

Gingival tissue samples were harvested by one operator (MDM) using a 3-mm punch biopsy pen under local anesthesia. Incision was performed positioning the handheld disposable punch perpendicular to the tooth at 2 mm apical to the gingival margin at the edge between the exposed connective tissue and the residual covering flap, performing simultaneous rotational movements under gentle pressure (Figure S1).

Each specimen was positioned on blotting paper and it was fixed in 10% neutral buffered formalin solution with the correct orientation, by one examiner (GA) of the Pathological Anatomy Laboratory (San Raffaele Hospital, Milan, Italy). Following dehydration, the specimens were embedded in paraffin, with proper positioning (at buccal-lingual direction) and oriented for sectioning as perpendicularly to the surface plane as possible. Five-micrometer-thick sections were collected serially and thaw-mounted on glass slides.

All sections were stained with hematoxylin and eosin (H&E) for morphological evaluation and Masson's trichrome (M&T), Verohoeff-van Gieson and Alcian Blue stain for evaluation of the collagen framework, elastic fibers and extracellular matrix mucopolysaccharides, respectively. For immunohistochemical studies, the slides were stained with mouse monoclonal anti-tenascin (clone BC-24, Sigma-Aldrich, St Louis, USA), performed in the ULTRA Benchmark automated immunostainer (Ventana Medical Systems/Roche, Tucson, AZ, USA) using standard procedures.

2.4. Histologic, histochemical and immunohistochemical analysis

From each sample, one histological section was randomly chosen and examined by a blinded investigator (AG) using a light microscope at x 50 magnification (AxioVision System SE64 - Zeiss, Oberkochen, Germany).

The images were captured at an original magnification of ×20 using Aperio ImageScope software (Leica Biosystems Srl, Buccinasco, Milan, Italy).

Semiquantitative analysis was performed to detect the presence of specific staining on each section to record information relative to the overall structure and composition of the soft tissues, including both the graft and the flap.[35] The intensity of staining (intensity score) or the degree of staining (proportion score) were calculated.

2.5. Statistical analysis

Statistical analysis was performed using SPSS software. Mean values and standard deviations were calculated for continuous variables, such as REC, KTW, MTT, recession reduction (RecRed), percentage of root coverage (%RC), KTW change (ΔKTW), MTT change (ΔMTT), and frequencies and percentage for ordinal ones, such as PFP and complete root coverage (CRC).

Intergroup analysis (MCAF *versus* MCAF+CTG and Apical PFP *versus* Coronal PFP) of continuous variables were performed using paired t-test, while ordinal ones were compared using chi-square test. Significance was set at 0.05.

A linear regression model was used to explore the possible relationship between the KTW changes at 3-year follow-up and some variables of interest (REC0, KTW0, MTT0, CTG thickness, CTG height, PFP).

3. Results

3.1. Clinical evaluation

Experimental population, patients and defects characteristics at baseline

A total of 23 patients (11 women and 12 men; mean age: 46.2 ± 10.6 years) with multiple recession defects were included in the study, and 93 recession defects were treated. Of these, 59 recessions (63%, 14 patients) were located in the maxilla and 34 (37%, 9 patients) in the mandible.

The surgical treatment involved MCAF+CTG for 54 sites and MCAF alone for 39 sites.

At MCAF sites, the mean REC at baseline was statistically significantly lower than MCAF+CTG group (MCAF: 1.97±0.87mm; MCAF+CTG: 2.91±1.01mm; $p<0.001$), while mean KTW was statistically significantly higher (MCAF: 2.39±1.02mm; MCAF+CTG: 1.74±0.89mm; $p=0.002$). (Table S1)

1-year outcomes

At 1-year follow-up, CRC was observed in 90% of all treated sites (84 of 93 gingival recessions), with no statistically significant differences between MCAF and MCAF+CTG sites (MCAF: 3.67±1.52mm; MCAF+CTG: 3.85±1.51mm; $p=0.26$), while a statistically KTW increase was observed in both groups when compared to baseline ($p=0.01$). (Table 1)

3-years outcomes

Between 1- and 3-years follow-up, a total of three dropouts was registered.

Complete root coverage was detected in 86% of sites treated with MCAF alone and 81% of sites treated with MCAF+CTG, without any significative difference. (Table 1)

No statistically significant difference was observed in KTW at 3-years between groups (3.19 ± 1.21mm at MCAF-treated sites and 3.33 ± 1.2mm at MCAF+CTG treated sites) (Table S1), while a statistically significant greater KTW increase was observed in MCAF+CTG group, when compared to baseline. (Table 1)

Linear regression showed a significant association between KTW change and the initial KTW, in both MCAF and MCAF+CTG-treated sites. In addition, in the last group also the position of primary flap was a statistically significant associated factor (Table 2).

The apical PFP was observed in 28 (47%) of the sites treated with MCAF+CTG.

The subgroup analysis showed a statistically significant difference between teeth presenting apical and coronal PFP in terms of mean KTW at 3 years (Apical PFP: 3.82 ± 1.14 mm, Coronal PFP: 2.8 ± 1.07 mm; *p*=0.0027) (Table 3), and a statistically significant greater KTW increase was observed in sites presenting an apical PFP (Table 1).

When comparing MCAF+CTG sites showing coronal PFP with MCAF-treated sites, no statistically significant differences were observed both for mean KTW (Coronal PFP: 2.8 ± 1.07mm, MCAF: 3.19 ± 1.21mm; *p*=0.212) and KTW change at 3 years (Coronal PFP: 1.02 ± 1.59mm, MCAF: 0.72 ± 1.11mm; *p*=407).

3.2. Histologic evaluation

Eleven biopsies from nine different patients were examined. Nine of these biopsies were performed in MCAF+CTG-treated sites; the other two biopsies were performed in normal gingival mucosa and in normal palatal mucosa.

In all the biopsies of treated sites examined, the differences in connective tissue composition between chorion of the flap and graft are conspicuous. As evidenced by the different stains used, there is always a marked and clear contrast between the gingival flap and the palatal graft.

Masson's trichrome (Figure 3a, f)

In the sections stained with Masson's trichrome, as expected, a net differentiation between the two layer was evident: collagen fibers are denser in palatal graft than in gingival flap; only in two cases a significant difference in collagen fiber density between the two areas could not be observed (Table S2). This increase in collagen fiber is explained by the transformation of palatal connective stromal tissue into a scar tissue due to remodeling processes after surgery.

Verhoeff-Van Gieson's stain (Figure 3b, g and S2)

Elastic fibers are present in both flap and graft connective tissue; in 6 out of 9 biopsies they are abundant (++) in flap and mild (+) in graft tissue (Table S3).

Alcian blue (Figure 3c, h)

In 7 out of 9 histological sections from treated sites the expression of Alcian blue was significantly higher in palatal graft than in gingival flap. In fact, in all gingival flaps the stain is mild, while in palatal graft can vary from mild (2/9) and moderate (2/9) to abundant (5/9) (Table S4). Moreover, it is interesting to notice that in both biopsies taken from normal palate and normal gingiva there was no expression of Alcian blue (Figure S3).

We can hypothesize that the insertion of the graft under the flap determine an inflammatory status, which is responsible of inflammatory modulators expression. These molecules, in fact, can modulate the production of all members of the metallo-proteinases family (that is metalloproteinases and tissue inhibitors of metalloproteinases), which are involved in the stability of connective tissue. This mechanism is involved both in tissue development and tissue remodeling. [22] Moreover, in hyalinized and fibrous tissue the number of glicosamminoglicans (GAGs) and hyaluronic acid is increased[23] and so this could explain our observation of higher intensity of stain in palatal graft connective tissue compared to adjacent stromal tissue.

Tenascin (Figure 3d,i)

Tenascin is an extracellular matrix glycoprotein of 1900 kD. [24,25] In human skin and oral mucosa, tenascin is contained in the papillary connective tissue and near the basement membrane.[26]

In all specimens from treated sites, tenascin expression is increased in connective tissue of palatal graft, while its expression remains confined at the basement membrane and around vessels in gingival flaps (Table S5).

Tenascin expression is significantly induced and increased in areas where tissue remodeling is taking place, that is areas with high rates of cell turnover and migration, such as in sites of neovascularization, wound healing and tumorigenesis .[26-28]

The presence of tenascin however did not correlate with the presence of cellular inflammatory infiltrate or granulation tissue[26] Its expression is also increased during different pathological conditions. Different expressions of tenascin are modulated by a variety of growth factors such as transforming growth factor-β (TGFβ), tumour necrosis factor-α (TNFα) and interleukin-1 (IL-1).[26,28,29]

According to our observations, in scar tissue (palatal graft) the expression of tenascin remains high for years after surgery. This is the first study that evidence gingival tenascin expression after surgery with a so long follow-up (three years) and for this reason we couldn't compare our data with existing literature.

Hematoxilin-eosin (Figure 3e, j)

Likewise, some differences in the epidermal layers between the flap and the epithelium formed after insertion of the palatal graft were revealed by hematoxilin-eosin stain. The epithelium covering the exposed graft, indeed, is hyperplastic, with elongation of rete ridges.

All these modifications could be determined by the type of tissue in the lamina propria below. The density of the connective tissue, in fact, is different between the graft and the flap: palatal connective tissue has a greater density than gingival connective tissue.

Moreover, after surgery, the graft undergoes some modifications related to wound healing and inflammatory status that ends in a transformation of the connective tissue in scar tissue.

4. Discussion

The present study has investigated clinical and histological results at three years following a multiple coronally advanced flap with site-specific application of connective tissue graft for the treatment of multiple RT1 recession type defects.

Sites treated with MCAF and MCAF+CTG were compared. Results obtained from this study showed that both approaches were effective in reducing recession depth, recording no statistically significant difference at the final assessment at 3 years postoperatively. Furthermore, the results showed no statistically significant difference between groups both at 1-year and 3-years assessments in terms of CRC.

It is important to underline that the sites where coronally advanced flap was utilized without the connective tissue graft, were selected on the basis of thick phenotype and the presence of at least 2 mm of KTW at baseline. The present data suggest that these successful outcomes achieved with both surgical approaches can be maintained in the long term, as per the previous literature.[19,30]

In terms of post-surgical increase in KTW, the results showed that the variation was statistically significant higher in sites treated with MCAF+CTG (MCAF: 0.74 ± 0.21 mm, MCAF+CTG: 1.23 ± 0.2 mm, *p*=0.025), in agreement with previous studies.[7,8,19]

The higher efficacy in augmenting KTW is often described in the literature as the result of the induction of the dense connective tissue graft on the epithelium of the flap, thus inducing keratinization. [12-14] This effect was described by Karring et al. (1971, 1975)[17,18] in a fundamental experiment to evaluate epithelia-mesenchimal interaction. Nevertheless, one has to keep in mind that this effect was observed only when the connective tissue, transplanted in an area of alveolar mucosa, was left uncovered.

In the present article, the primary flap position was evaluated, highlighting that in almost 50% of the sites it was possible to evidence an apical shift of the flap, leaving the most coronal part of the connective tissue exposed. These exposed areas were re-epithelized during the healing phases: being the dense connective tissue exposed, the induction of keratinization could occur, by this augmenting the KTW. On the contrary, sites where the primary flap was maintained at the coronal position showed a minor amount of KTW increase. In fact, when comparing sites with CTG, where the position of the flap was maintained coronal, and sites without CTG (MCAF), there was not statistically difference in terms of KTW. It could be speculated that augmented KTW did not depend by the presence of dense connective tissue, but from the repositioning of mucogingival line, such as for the MCAF[8,12], or by the inclusion of the periosteum in the flap, being the technique split-full-split elevated as described by Zucchelli & de Sanctis (2000)[5].

To explain the relative high percent of sites where flap was showing an apical shift, it could be speculated that the areas where a connective tissue graft was utilized were composed of a thin or very thin gingiva phenotype thus more prone to recede[1,7] and the interposition of the connective graft could also impair the vascular exchange between the covering flap and the underlying receiving bed.[19,31]

At the histological examination, the areas of exposed graft showed an increase in collagen fibers, transforming the palatal connective stromal tissue into a scar tissue due to remodeling processes after surgery.

Clinically many authors have evidenced this change to occur when utilizing a connective tissue graft, defining these areas as a keloid formation.[19] The formation of a localized area of increased keratinization (keloid) is also responsible for less esthetic results, as described by Zucchelli et al. (2003, 2014) [19,31] and Cairo et al. (2010)[32] previously.

The findings of the present study confirm that the quality of connective tissue determines the quality of the epithelium[17,18] nevertheless, in order for this phenomenon to occur, following a bilaminar technique, the connective tissue graft must be exposed.[16,33,34] When the flap is covering the graft, this effect cannot be seen and the increase in KTW is similar to MCAF alone.[36]

Furthermore, the histological observation allows us to speculate that the quality and density of the connective tissue graft maintain its characteristic, nevertheless it does not influence the quality and composition of the overlaying connective tissue of the flap. In fact, with all the stain utilized, a net separation between the two layers (the connective tissue graft and the flap) is always well evident. In other words, the chorion of the flap retains always its original characteristics in composition and density so, when covering the graft, it is acting as a “buffer zone”, impeding the dense connective tissue of the graft to induce any induction of keratinization to the overlying epithelium.[19,31] Only in instances where the connective tissue is coronal to the margin of the flap, the epithelial modifications become evident.

5. Conclusion

From the clinical stand point, our data support that the selective use of connective tissue together with a MCAF is effective in reducing recession depth. No statistically significant difference between sites with and without the graft was assessed, both for root coverage and KTW at the final evaluation at 3 years postoperatively.

From a biological stand point, it can be concluded that the connective tissue graft cannot exert any differentiation of the overlying epithelium in instances where the flap maintains its original position at CEJ. Only where a recession of the flap over the connective tissue is observed, there is an evident increase in the amount of KTW.

Also, it should be taken in to account that a thick connective tissue positioned under a MCAF could impede a normal vascularization of the overlying flap, augmenting the risk of flap contraction, that will explain areas of increased keratinization.

Tables

Table 1 Changes in clinical parameters at 3-years follow-up

	MCAF (n=36)	**MCAF+CTG (n=48)**	***p*[a]**	**Apical PFP (n=25)**	**Coronal PFP (n=23)**	***p***
RecRed (mm)	1.69 ± 0.81	2.69 ± 1.08	0.000018*	3 ± 0.98	2.35 ± 1.09	0.0037[a]*
%RC (%)	93 ± 21	93 ± 16	0.95	96 ± 10	89 ± 20	0.118[a]
CRC	86	81	0.76	88	74	0.38
ΔKTW (mm)	0.72 ± 1.11	1.69 ± 1.59	0.0029*	2.3 ± 1.34	1.02 ± 1.59	0.0049[a]*
ΔMTT (mm)	0.22 ± 0.89	1.47 ± 0.77	<0.001	1.62 ± 0.83	1.31 ± 0.67	0.182[a]

Abbreviation: MCAF: multiple coronally advanced flap; MCAF+CTG: multiple coronally advanced flap plus connective tissue graft; PFP: primary flap position; RecRed: recession reduction; %RC: percentage of root coverage; CRC: complete root coverage; ΔKTW: keratinized tissue width changes; ΔMTT: marginal tissue thickness changes.
[a] Student T-test, [b] Chi-square test, * Statistically significance

Table 2 Linear regression with KTW changes (measured as difference between KTW at 3-years follow-up and KTW at baseline) of MCAF and MCAF+CTG-treated sites as outcome variable.

	MCAF			**MCAF+CTG**		
	Estimate	**Standard Error**	***p***	**Estimate**	**Standard Error**	***p***
KTW0	-0.40	0.186	0.038*	-1.09	0.19	<0.001*
REC0	-0.14	0.228	0.546	0.26	0.18	0.152
MTT0	0.80	0.841	0.952	-0.97	1.38	0.487
CTG height	na	na	na	0.15	0.30	0.619
CTG thickness	na	na	na	0.51	0.99	0.607
PFP	na	na	na	0.81	0.37	0.04*

Abbreviation: MCAF: multiple coronally advanced flap; MCAF+CTG: multiple coronally advanced flap plus connective tissue graft; REC0: recession depth at baseline; KTW0: keratinized tissue width at baseline; MTT0: marginal tissue thickness at baseline; CTG: connective tissue graft; PFP: primary flap position.
[a] Student T-test, * Statistically significance.

Table 3 Clinical parameters (mean ± sd, mm) of MCAF+CTG-treated sites with apical and coronal primary flap position (PFP) at baseline and 3 years.

	Apical PFP (n=25)		**Coronal PFP (n=23)**		***p*[a]**	
	Baseline	**3-year**	**Baseline**	**3-year**	**Baseline**	**3-year**
REC (mm)	3.12 ± 0.97	0.12 ± 0.33	2.65 ± 1.07	0.3 ± 0.56	0.12	0.167
KTW (mm)	1.52 ± 0.82	3.82 ± 1.14	1.78 ± 0.96	2.8 ± 1.07	0.31	0.0027*
MTT (mm)	0.38 ±0.16	2 ± 0.8	0.46 ± 0.17	1.78 ± 0.66	0.08	0.321

Abbreviation: MCAF: multiple coronally advanced flap; MCAF+CTG: multiple coronally advanced flap plus connective tissue graft; REC: recession depth; KTW: keratinized tissue width; MTT: marginal tissue thickness.
[a] Student T-test, * Statistically significance.

Figures

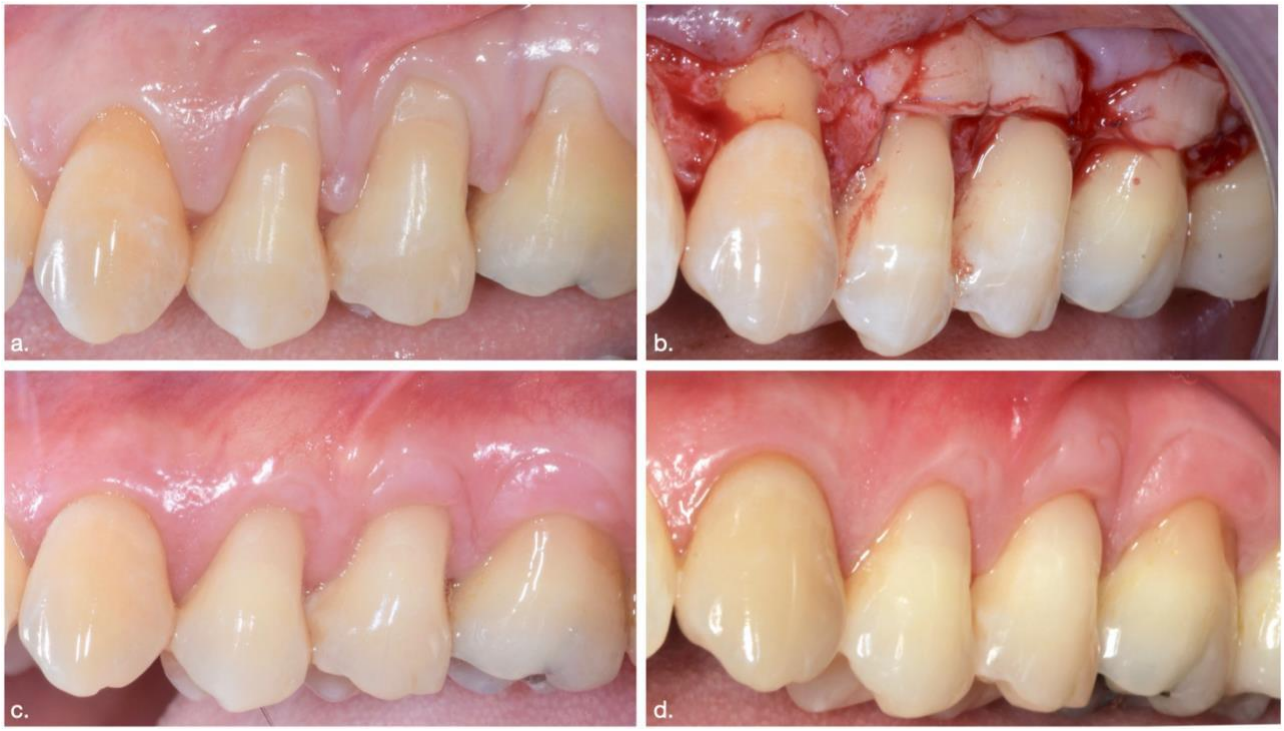

Figure 1 (a) Baseline situation showing multiple gingival recessions in the maxilla. **(b)** The CTG was positioned at the level of the first and second premolars and first molar. **(c)** Clinical outcome at 1 year. **(d)** Clinical outcome at 3 years.

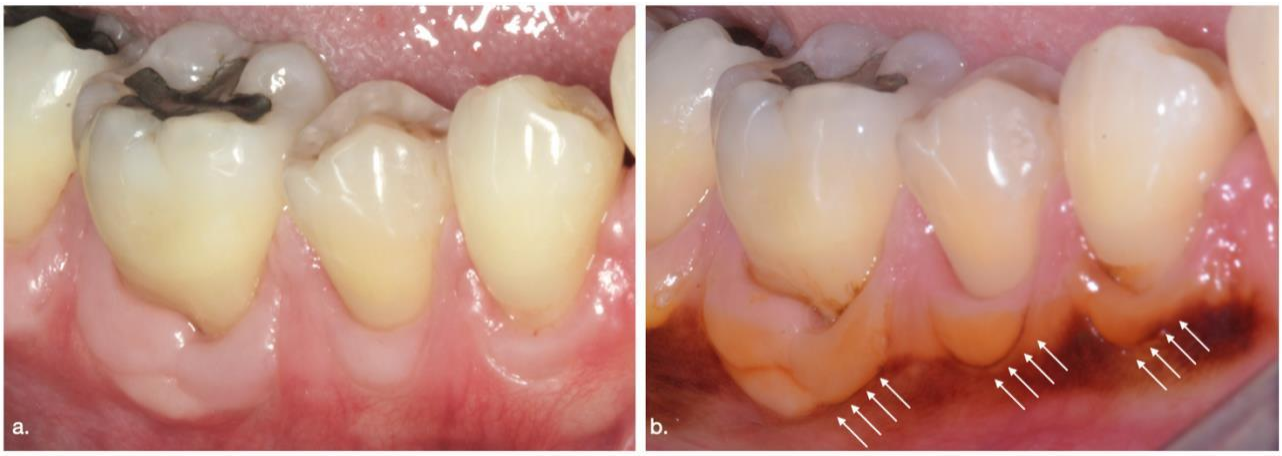

Figure 2 Identification of the primary position flap (PFP): the use of iodine solution (b) evidenced the apical shift of the margin of the flap (arrows).

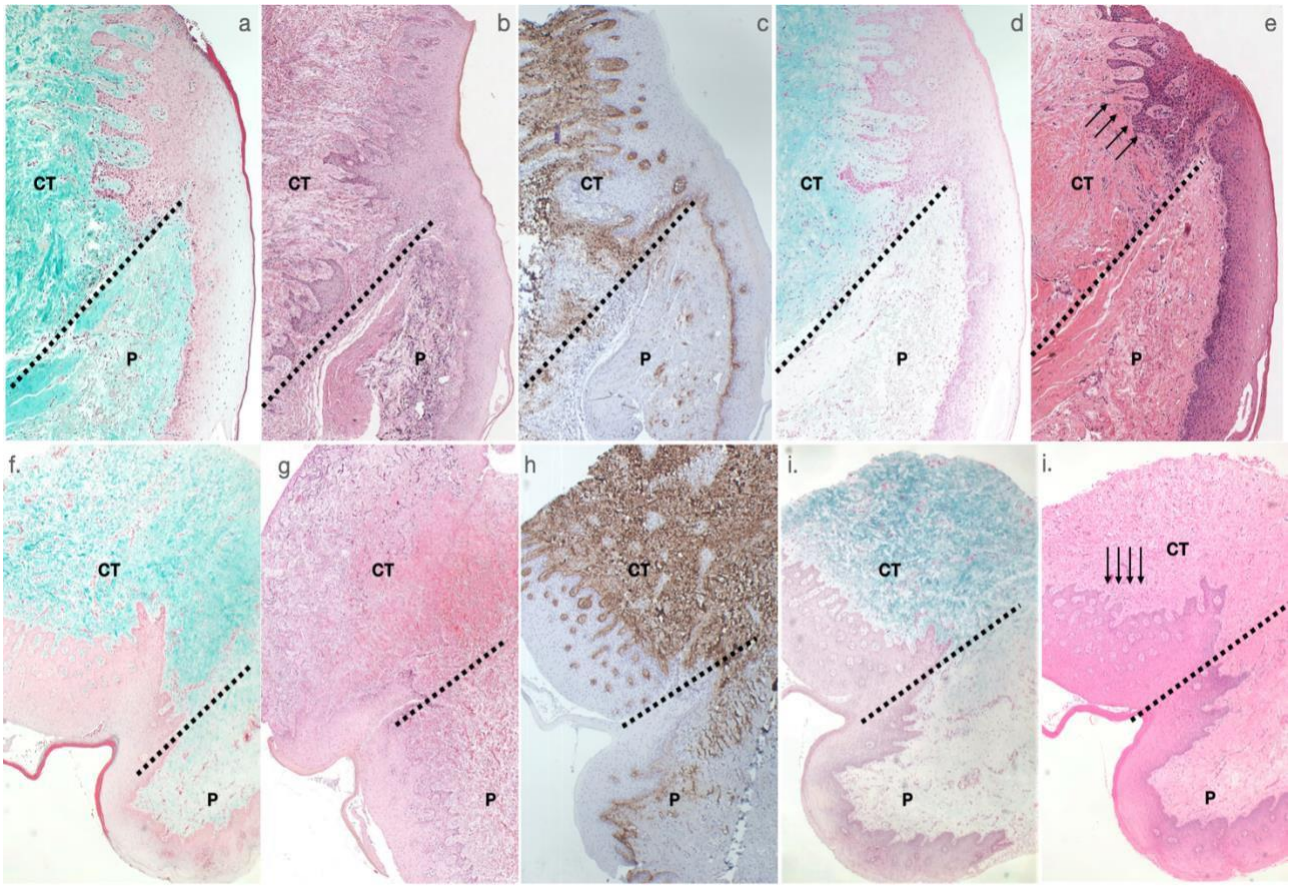

Figure 3 Histologic sections showing **(a, f)** Masson's trichrome stain: collagen fibers are denser in CTG than in PF; **(b, g)** Verohoeff-van Gieson stain: the number of elastic fibers is higher in PF than in CTG; **(c, h)** Tenascin: please note that its expression is increased in CTG; **(d, i)** Alcian Blue: the stain is abudant in CTG, while mild in PF; **(e, j)** Hematoxylin and eosin (H&E) stain: please note as the epithelium covering the exposed CTG, is hyperplastic, with elongation of rete ridges (arrows).

References

1. Cairo, F., Pagliaro, U., Nieri, M. (2008) Treatment of gingival recession with coronally advanced flap procedures: a systematic review. *Journal of Clinical Periodontology, 35*(8 Suppl):136-62. doi: 10.1111/j.1600-051X.2008.01267.x. PMID: 18724847.

2. Graziani, F., Gennai, S., Roldán, S., Discepoli, N., Buti, J., Madianos, P., & Herrera, D. (2014). Efficacy of periodontal plastic procedures in the treatment of multiple gingival recessions. *Journal of Clinical Periodontology, 41* Suppl 15, S63–S76. https://doi.org/10.1111/jcpe.12172

3. Chambrone, L., & Tatakis, D. N. (2015). Periodontal soft tissue root coverage procedures: a systematic review from the AAP Regeneration Workshop. *Journal of Periodontology, 86*(2 Suppl), S8–S51. https://doi.org/10.1902/jop.2015.130674

4. Chambrone, L., Salinas Ortega, M. A., Sukekava, F., Rotundo, R., Kalemaj, Z., Buti, J., & Pini Prato, G. P. (2018). Root coverage procedures for treating localised and multiple recession-type defects. *The Cochrane database of systematic reviews*, 10(10), CD007161. https://doi.org/10.1002/14651858.CD007161.pub3

5. Zucchelli, G., & De Sanctis, M. (2000). Treatment of multiple recession-type defects in patients with esthetic demands. *Journal of Periodontology, 71*(9), 1506–1514. https://doi.org/10.1902/jop.2000.71.9.1506

6. Cairo, F., Cortellini, P., Pilloni, A., Nieri, M., Cincinelli, S., Amunni, F., Pagavino, G., & Tonetti, M. S. (2016). 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), 849–856. https://doi.org/10.1111/jcpe.12590

7. Pini Prato, G. P., Baldi, C., Nieri, M., Franseschi, D., Cortellini, P., Clauser, C., Rotundo, R., & Muzzi, L. (2005). Coronally advanced flap: the post-surgical position of the gingival margin is an important factor for achieving complete root coverage. *Journal of Periodontology, 76*(5), 713–722.

8. Stefanini, M., Zucchelli, G., Marzadori, M., & de Sanctis, M. (2018). Coronally Advanced Flap with Site-Specific Application of Connective Tissue Graft for the Treatment of Multiple Adjacent Gingival Recessions: A 3-Year Follow-Up Case Series. *The International Journal of Periodontics & Restorative Dentistry, 38*(1), 25–33.

9. Cortellini, P., & Bissada, N. F. (2018). Mucogingival conditions in the natural dentition: Narrative review, case definitions, and diagnostic considerations. *Journal of Periodontology, 89* Suppl 1, S204–S213.

10. Ainamo, A., Bergenholtz, A., Hugoson, A., & Ainamo, J. (1992). Location of the mucogingival junction 18 years after apically repositioned flap surgery. *Journal of clinical periodontology*, *19*(1), 49–52. https://doi.org/10.1111/j.1600-051x.1992.tb01148.x

11. Romanos, G. E., Bernimoulin, J. P., & Marggraf, E. (1993). The double lateral bridging flap for coverage of denuded root surface: longitudinal study and clinical evaluation after 5 to 8 years. *Journal of Periodontology, 64*(8), 683–688. https://doi.org/10.1902/jop.1993.64.8.683

12. Wennström, J. L., & Zucchelli, G. (1996). Increased gingival dimensions. A significant factor for successful outcome of root coverage procedures? A 2-year prospective clinical study. *Journal of Clinical Periodontology, 23*(8), 770–777. https://doi.org/10.1111/j.1600-051x.1996.tb00608.

13. Bokan I. (1997). Potential of gingival connective tissue to induce keratinization of an alveolar mucosal flap: a long-term histologic and clinical assessment. Case report. *Quintessence International* (Berlin, Germany: 1985), *28*(11), 731–736.

14. Gholami, G. A., Saberi, A., Kadkhodazadeh, M., Amid, R., & Karami, D. (2013). Comparison of the clinical outcomes of connective tissue and acellular dermal matrix in combination with double papillary flap for root coverage: A 6-month trial. *Dental Research Journal*, 10(4), 506–513.

15. Borghetti, A., & Louise, F. (1994). Controlled clinical evaluation of the subpedicle connective tissue graft for the coverage of gingival recession. Journal of Periodontology, 65(12), 1107–1112. https://doi.org/10.1902/jop.1994.65.12.1107

16. Cordioli, G., Mortarino, C., Chierico, A., Grusovin, M. G., & Majzoub, Z. (2001). Comparison of 2 techniques of subepithelial connective tissue graft in the treatment of gingival recessions. *Journal of Periodontology, 72*(11), 1470–1476. https://doi.org/10.1902/jop.2001.72.11.1470

17. Karring, T., Ostergaard, E., & Löe, H. (1971). Conservation of tissue specificity after heterotopic transplantation of gingiva and alveolar mucosa. *Journal of Periodontal Research, 6*(4), 282–293. https://doi.org/10.1111/j.1600-0765.1971.tb00619.x

18. Karring, T., Lang, N. P., & Löe, H. (1975). The role of gingival connective tissue in determining epithelial differentiation. *Journal of Periodontal Research, 10*(1), 1–11. https://doi.org/10.1111/j.1600-0765.1975.tb00001.

19. Zucchelli, G., Mounssif, I., Mazzotti, C., Stefanini, M., Marzadori, M., Petracci, E., & Montebugnoli, L. (2014). Coronally advanced flap with and without connective tissue graft for the treatment of multiple gingival recessions: a comparative short- and long-term controlled randomized clinical trial. *Journal of Clinical Periodontology, 41*(4), 396–403.

20. de Sanctis, M., Di Domenico, G. L., Bandel, A., Pedercini, C., & Guglielmi, D. (2020). The Influence of Cementoenamel Restorations in the Treatment of Multiple Gingival Recession Defects Associated with Noncarious Cervical Lesions: A Prospective Study. *The International Journal of Periodontics & Restorative Dentistry, 40*(3), 333–342. https://doi.org/10.11607/prd.4639.

21. Guglielmoni, P., Promsudthi, A., Tatakis, D. N., & Trombelli, L. (2001). Intra- and inter-examiner reproducibility in keratinized tissue width assessment with 3 methods for mucogingival junction determination. *Journal of Periodontology, 72*(2), 134–139. https://doi.org/10.1902/jop.2001.72.2.134

22. Borsani, E., Salgarello, S., Mensi, M., Boninsegna, R., Stacchiotti, A., Rezzani, R., Sapelli, P., Bianchi, R., & Rodella, L. F. (2005). Histochemical and immunohistochemical evaluation of gingival collagen and metalloproteinases in peri-implantitis. *Acta histochemica, 107*(3), 231–240. https://doi.org/10.1016/j.acthis.2005.06.002

23. Augustine, D., Rao, R. S., & Patil, S. (2021). Hyalinization as a histomorphological risk predictor in oral pathological lesions. Journal of oral biology and craniofacial research, 11(3), 415–422. https://doi.org/10.1016/j.jobcr.2021.05.002

24. Mane, D. R., Kale, A. D., & Naik, V. V. (2011). Immunohistochemical expression of Tenascin in embryogenesis, tumorigenesis and inflammatory oral mucosa. *Archives of Oral Biology, 56*(7), 655–663. https://doi.org/10.1016/j.archoralbio.2010.11.020

25. Taylor, H. C., Lightner, V. A., Beyer, W. F., Jr, McCaslin, D., Briscoe, G., & Erickson, H. P. (1989). Biochemical and structural studies of tenascin/hexabrachion proteins. *Journal of cellular biochemistry, 41*(2), 71–90. https://doi.org/10.1002/jcb.240410204

26. Latijnhouwers, M. A., Bergers, M., Van Bergen, B. H., Spruijt, K. I., Andriessen, M. P., & Schalkwijk, J. (1996). Tenascin expression during wound healing in human skin. *The Journal of Pathology, 178*(1), 30–35. https://doi.org/10.1002/(SICI)1096-9896(199601)178:1<30::AID-PATH442>3.0.CO;2-7

27. Grünheid, T., & Zentner, A. (2005). Extracellular matrix synthesis, proliferation and death in mechanically stimulated human gingival fibroblasts in vitro. *Clinical Oral Investigations*, *9*(2), 124–130. https://doi.org/10.1007/s00784-004-0279-y

28. Jones, F. S., & Jones, P. L. (2000). The tenascin family of ECM glycoproteins: structure, function, and regulation during embryonic development and tissue remodeling. *Developmental dynamics: an official publication of the American Association of Anatomists, 218*(2), 235–259.

29. Rettig, W. J., Erickson, H. P., Albino, A. P., & Garin-Chesa, P. (1994). Induction of human tenascin (neuronectin) by growth factors and cytokines: cell type-specific signals and signalling pathways. *Journal of cell science, 107* (Pt 2), 487–497.

30. Zucchelli, G., & De Sanctis, M. (2005). Long-term outcome following treatment of multiple Miller class I and II recession defects in esthetic areas of the mouth. *Journal of periodontology, 76*(12), 2286–2292. https://doi.org/10.1902/jop.2005.76.12.2286

31. Zucchelli, G., Amore, C., Sforza, N. M., Montebugnoli, L., & De Sanctis, M. (2003). Bilaminar techniques for the treatment of recession-type defects. A comparative clinical study. *Journal of Clinical Periodontology, 30*(10), 862–870.

32. Cairo, F., Nieri, M., Cattabriga, M., Cortellini, P., De Paoli, S., De Sanctis, M., Fonzar, A., Francetti, L., Merli, M., Rasperini, G., Silvestri, M., Trombelli, L., Zucchelli, G., & Pini-Prato, G. P. (2010). Root coverage esthetic score after treatment of gingival recession: an interrater agreement multicenter study. *Journal of Periodontology, 81*(12), 1752–1758.

33. Bouchard, P., Etienne, D., Ouhayoun, J. P., & Nilvéus, R. (1994). Subepithelial connective tissue grafts in the treatment of gingival recessions. A comparative study of 2 procedures. *Journal of Periodontology, 65*(10), 929–936. https://doi.org/10.1902/jop.1994.65.10.929

34. Trombelli, L., Scabbia, A., Tatakis, D. N., & Calura, G. (1998). Subpedicle connective tissue graft versus guided tissue regeneration with bioabsorbable membrane in the treatment of human gingival recession defects. *Journal of Periodontology, 69*(11), 1271–1277. https://doi.org/10.1902/jop.1998.69.11.1271

35. Wei, P. C., Laurell, L., Lingen, M. W., & Geivelis, M. (2002). Acellular dermal matrix allografts to achieve increased attached gingiva. Part 2. A histological comparative study. Journal of periodontology, 73(3), 257–265. https://doi.org/10.1902/jop.2002.73.3.257

36. Cummings, L. C., Kaldahl, W. B., & Allen, E. P. (2005). Histologic evaluation of autogenous connective tissue and acellular dermal matrix grafts in humans. *Journal of periodontology*, *76*(2), 178–186. https://doi.org/10.1902/jop.2005.76.2.178.