Official title: Effectiveness of hyaluronic acid application in Coronally Advanced Flap Combined with a subepithelial connective tissue graft Versus Coronally Advanced Flap Combined with a subepithelial connective tissue graft alone: A clinical trial

Date: 02-03-2026

Clinical trial registration: NCT to be assigned

Document type: Study Protocol

1. INTRODUCTION AND RATIONALE

1.1. Introduction

Gingival recession (GR) is a frequent mucogingival deformity characterized by the apical displacement of the gingival margin with exposure of the root surface beyond the cemento-enamel junction (CEJ) [1–3]. Its prevalence increases with age and may lead to dentin hypersensitivity, root caries, plaque retention, aesthetic impairment, and patient discomfort [4,5]. Among the available treatment modalities, the coronally advanced flap (CAF), particularly when combined with a subepithelial connective tissue graft (SCTG), is a well-documented and widely adopted approach for the management of single and multiple recession-type defects, providing predictable root coverage, gain of keratinized tissue, and long-term stability [6–8]. However, CAF + SCTG procedures may still be associated with postoperative morbidity, donor-site discomfort, and incomplete root coverage in some cases [9,10]. Therefore, adjunctive strategies aimed at enhancing wound healing and increasing the probability of complete root coverage (CRC) remain of significant clinical interest.

Hyaluronic acid (HA), a high-molecular-weight, non-sulfated glycosaminoglycan, is a key component of the periodontal extracellular matrix and plays a pivotal role in angiogenesis, cell migration, proliferation, and differentiation during tissue healing [11–13]. HA has been shown to accelerate granulation tissue formation, modulate inflammatory responses, and stimulate fibroblast and keratinocyte activity [14–16]. Preclinical studies demonstrated that HA enhances vascularization, supports osteoinduction, and upregulates osteogenic markers such as Runx2, alkaline phosphatase (ALP), and osteocalcin [17,18]. In vitro studies further confirm that HA promotes fibroblast proliferation, migration, and extracellular matrix synthesis [19,20], while cross-linked formulations prolong its bioactivity in surgical wounds [21]. Moreover, HA exhibits bacteriostatic properties against periodontopathogens such as *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* [22], potentially reducing early contamination of surgical sites.

The adjunctive use of HA in periodontal regenerative and mucogingival surgery has been investigated in several clinical studies. Randomized controlled trials (RCTs) demonstrated significantly greater clinical attachment level (CAL) gain, mean root coverage (MRC), and CRC when

HA was used in combination with CAF compared to CAF alone [23,24]. Case series also reported high root coverage percentages and enhanced esthetic outcomes when HA was combined with SCTG in tunnel and minimally invasive techniques [25,26]. Beyond recession coverage, clinical studies on intrabony defects showed that HA, particularly when combined with xenografts, promoted the formation of new bone, cementum, and periodontal ligament [27]. Moreover, HA application has been associated with reduced postoperative pain and improved palatal wound healing in donor sites following free gingival grafting [28]. A recent systematic review and meta-analysis concluded that HA may provide additional clinical benefit in both non-surgical and surgical periodontal therapy, although heterogeneity across studies highlights the need for further high-quality trials [29].

Parallel to clinical findings, mechanistic studies provide biological plausibility for the observed effects. Fibroblasts play a central role in periodontal wound healing through extracellular matrix synthesis, wound contraction, and secretion of growth factors [30]. In vitro investigations demonstrated that HA enhances fibroblast proliferation, stimulates type I and III collagen synthesis, and modulates cytokine and matrix metalloproteinase activity [19,20]. Furthermore, periodontal ligament (PDL) cell and cementoblast studies confirmed that HA supports cell viability and induces the expression of mineralization-related genes [16,31]. Culturing fibroblasts derived from SCTGs obtained in clinical settings offers a unique translational approach, allowing donor-specific responses to HA to be studied under standardized conditions and correlated with clinical outcomes [32,33]. This bench-to-bedside model strengthens the biological rationale for HA-mediated wound healing and regeneration.

Based on this rationale, the present randomized controlled clinical trial was designed to evaluate the potential benefits of adjunctive HA application in CAF combined with SCTG for the treatment of single RT1 and RT2 gingival recession defects. In parallel, an in vitro study using fibroblasts isolated from SCTGs of the same patients will investigate cell proliferation, migration, and expression of wound healing–related genes in the presence or absence of HA. Together, these complementary investigations aim to provide a comprehensive understanding of the clinical and biological effects of HA, potentially paving the way for optimized regenerative protocols in periodontal plastic surgery.

1.2. Study Rationale

The rationale for this study is to evaluate whether the adjunctive application of cross-linked hyaluronic acid gel (hyaDENT BG®) enhances clinical outcomes of coronally advanced flap combined with subepithelial connective tissue graft (CAF + SCTG) in single RT1/RT2 gingival recessions. By promoting fibroblast migration, proliferation, and angiogenesis, HA may increase complete root coverage rates, improve patient-reported outcomes, and reduce donor-site morbidity. A parallel in vitro study using fibroblasts from the same patients will explore the cellular and molecular mechanisms underlying the clinical findings, strengthening the biological plausibility of the results.

2. STUDY PRODUCT DESCRIPTION

The investigational product is hyaDENT BG®, a sterile, transparent, highly viscous gel containing highly purified hyaluronic acid of non-animal origin with specifically adjusted molecular weight.

2.1 Study Product Description

The hyaDENT BG® gel (Fig. 1) is a sterile, resorbable, cross-linked hyaluronic acid preparation specifically designed for dental applications. It is primarily used to support soft tissue healing and regeneration, particularly in mucogingival procedures such as coronally advanced flap (CAF) surgery combined with connective tissue grafting. Its biocompatibility and prolonged residence time at the surgical site enable sustained stimulation of fibroblast migration, angiogenesis, and extracellular matrix synthesis, which are critical for optimal wound healing outcomes.
In periodontal plastic surgery, hyaDENT BG® serves as an adjunctive biomaterial to enhance root coverage predictability and keratinized tissue gain, while reducing patient morbidity associated with donor-site harvesting. Evidence supports its clinical efficacy in improving complete root coverage, reducing postoperative pain and edema, and accelerating the healing process. In addition, its bacteriostatic properties may limit early microbial colonization of the surgical wound.

The product is supplied as a ready-to-use 1.2 ml glass cartridge, containing a transparent, highly viscous gel with the following composition per 1 ml:

- 16 mg cross-linked hyaluronic acid
- 2 mg natural hyaluronic acid
- 6.9 mg sodium chloride
- Water for injection up to 1.0 ml

Fig 1: hyaDENT BG®

Manufacturer:

BioScience GmbH

Walsmühler Straße 18, 19073 Dümmer, Germany

www.bio-science.org

BioScience GmbH is a leading manufacturer specializing in the development and production of medical devices based on hyaluronic acid. All manufacturing activities are carried out under a rigorous Quality Management System, certified according to ISO 13485, ISO 10993, and in compliance with European Medical Device Regulation (MDR). The product is CE-marked (CE 2797), reflecting its adherence to the highest quality and regulatory standards.

Characteristics:

hyaDENT BG® is a cross-linked hyaluronic acid gel produced through a proprietary stabilization process that ensures a prolonged residence time in the surgical site while maintaining the biological activity of native HA. This cross-linking technology provides controlled, slow degradation, allowing sustained bioactivity during the critical phases of wound healing.

The product exhibits excellent biocompatibility and tissue integration, forming a viscoelastic scaffold that supports fibroblast migration, angiogenesis, and collagen deposition. Its rheological

properties ensure that the gel remains localized at the application site, offering mechanical stability and protection of the underlying graft or connective tissue during the early healing phase.
Additionally, hyaDENT BG® exerts a bacteriostatic effect against common periodontal pathogens and modulates the inflammatory response, contributing to a favorable environment for regeneration. Its hydrophilic and hygroscopic nature facilitates moisture retention and enhances cell–matrix interactions, guiding orderly tissue regeneration and reducing the risk of scar tissue formation.

The study product will be used for its indication. hyaDENT BG® is a sterile medical device intended for single use. When applied in the following dental fields, hyaDENT BG® supports wound healing:

- In the treatment of gingival recession using the coronally advanced flap technique.
- After tooth extraction, due to its positive effect on postoperative swelling, pain, and trismus.
- In the context of aesthetic dentistry, hyaDENT BG®, when injected into a receded interdental papilla, provides a regenerative effect.

Contraindications:

hyaDENT BG® must not be used in patients with:

- Autoimmune diseases or those undergoing immunotherapy
- Known hypersensitivity to hyaluronic acid
- Severe allergy predisposition
- Acute inflammatory or infectious processes
- Coagulation disorders or patients undergoing anticoagulant therapy
- Hormonal disorders

There are no clinical data regarding the use of this product in pregnant or breastfeeding women, or in adolescents under 18 years of age.

Adverse Events:

The use of hyaDENT BG® may, in very rare cases, lead to tissue reactions. As with any similar procedure, the use of hyaDENT BG® carries an inherent risk of infection.

During papilla reconstruction, temporary pressure pain may occur as a result of the injection. No interactions of hyaDENT BG® with other medications are known.

2.1. Product Registration Status

All articles used in this clinical trial are CE marked.

2.2. Instruction for Use, Handling, Labelling Packaging Type, Contents and Storage

2.2.1. Instruction for Use

Remove the glass cartridge from the blister and insert it into a cartridge syringe. For application of the product to the treatment area, blunt needles (23G) are recommended. For papilla reconstruction, sharp needles (27G) are recommended. The product is completely degraded over time.

2.2.2. Handling

REGEDENT AG will provide the study site with the required number of hyaDENT BG® cartridges for use exclusively in patients enrolled in the study and strictly according to the approved protocol. Each unit is supplied as a single-use, sterile 1.2 ml glass cartridge packaged in a sealed blister.
The product must not be used if the sterile packaging is opened or damaged prior to use. In such cases, the cartridge shall be discarded following institutional biohazard procedures or returned to the manufacturer for documentation. hyaDENT BG® is intended for single use only and must not be re-sterilized or reused, as reuse may pose a risk of patient infection.
Before application, the glass cartridge is inserted into a compatible cartridge syringe. For application to the recipient site, blunt cannulas (23G) are recommended to deliver the gel directly into the surgical area. In the test group, immediately after harvesting, the subepithelial connective tissue graft is fully soaked in sterile hyaluronic acid gel prior to its placement at the recipient site, as described in detail in Section 7.4 (Surgical procedure). hyaDENT BG® is applied immediately after flap elevation and prior to suturing, ensuring direct contact with the connective tissue graft and the recipient bed.

The gel should be dispensed slowly to achieve an even distribution, creating a thin, homogeneous layer covering the graft. Once applied, the flap is coronally advanced and sutured to ensure complete coverage of the treated site, minimizing the risk of contamination and optimizing healing.

2.2.3. Labelling

The study product is not relabeled specifically for use in this clinical study, since it is a product already available in the market.

2.2.4. Packaging Type and Contents

hyaDENT BG® is supplied ready-to-use in a 1.2 ml glass cartridge, packaged in a blister for single use. The syringe content of hyaDENT BG® is heat sterilized.
The instructions for use, as well as labels with the batch number and expiry date, are included inside the box together with the blister. One of the labels is handed over to the patient to ensure product traceability.
hyaDENT BG must not be resterilized for reuse. If the packaging is open or damaged, or if the blister is compromised, the product must not be used, as sterility can no longer be guaranteed and the risk of infection is increased. One package contains two glass cartridges.

2.2.5. Storage

Do not freeze and do not expose to extreme heat. Store at room temperature (2 °C – 25 °C).

2.2.6. Important Information

The scale on the glass cartridge serves as a guide for the user and refers to the final volume. It is not intended for measurement purposes but merely indicates the amount used relative to the nominal content of 1.2 ml.

2.3. Device Accountability

The Investigator must maintain an accurate and up-to-date accountability record of all hyaDENT BG® cartridges received, used, discarded (opened but unused), and returned throughout the duration of the study. This information shall be documented in the Study Product Accountability Log, including product name, lot number, expiration date, date of use, and patient identification code.

At each monitoring visit, the study monitor will verify the completeness and accuracy of the accountability records and cross-check them with source documentation. At study close-out, the monitor or designated delegate will ensure full reconciliation of all investigational product units, confirming that all cartridges have been either used, appropriately discarded, or returned to the sponsor according to Good Clinical Practice (GCP) requirements

2.4. Return of Study Product

After treatment of the last enrolled patient, any remaining unopened hyaDENT BG® cartridges at the study site must be returned to REGEDENT AG. The sponsor will issue an acknowledgement of receipt, which must be filed in the Investigator Site File (ISF) as part of the essential study documentation.

3. RISKS AND BENEFITS OF THE STUDY PRODUCT AND CLINICAL STUDY

According to the hyaDENT BG® Clinical Evaluation Report (CER) and post-market clinical follow-up (PMCF) data, the product has demonstrated a favorable safety profile and proven performance for its intended use in periodontal and mucogingival procedures. Reported adverse events are rare and limited to mild, transient local reactions such as temporary pressure pain or localized swelling, with no serious device-related events documented.

There are no new or unforeseen risks identified that require additional clinical assessment. The overall risk–benefit ratio remains highly favorable, and the residual risk acceptability, as documented in the Risk Management Report (RMR), is unchanged.

The potential benefits of adjunctive hyaDENT BG® include increased probability of complete root coverage, enhanced soft tissue healing, reduced postoperative discomfort, and decreased donor-site morbidity, which outweigh the minimal associated risks.

3.1. Risks Linked to the Product

Risk management for hyaDENT BG® is conducted in accordance with ISO 14971. A comprehensive description of the risk analysis, identified hazards, and control measures is presented in the Risk Management Report (RMR) provided by the manufacturer.

For both the investigational and control interventions, residual and overall risks are considered acceptable, and no additional risks were identified that would require further clinical investigation. Residual risks cannot be further mitigated by the manufacturer; additional risk reduction relies on proper handling and clinical use by trained dental professionals. Therefore, the product must be used exclusively by well-trained clinicians and strictly following the Instructions for Use (IFU).

Relevant side effects identified in the risk analysis and IFU include:

- Allergy or hypersensitivity to hyaluronic acid or excipients
- Localized edema
- Hematoma or transient swelling
- Gingival irritation or inflammation
- Postoperative pain or discomfort
- Localized or systemic infection
- Esthetic dissatisfaction due to partial root coverage
- Delayed healing at donor site (if SCTG used)
- Transient sensory alterations (paresthesia/dysesthesia)

Except for allergic or hypersensitivity reactions to hyaluronic acid, these potential adverse events are considered inherent to mucogingival surgical procedures and may occur regardless of whether hyaDENT BG® is used.

All identified residual risks have been minimized to the lowest achievable level and are considered acceptable when weighed against the expected clinical benefits, in accordance with Essential Requirement 6 of the Medical Device Directive (MDD) 93/42/EEC and MDR requirements.

To further mitigate risks, all investigators will receive training in the correct handling and application of hyaDENT BG® prior to the initiation of the study.

Alternatives to the use of hyaDENT BG® include:

- Standard CAF + SCTG procedure without adjunctive material
- Other soft tissue substitutes or biomaterials
- No surgical treatment (maintaining the recession defect)

3.2. Risks Linked to the Study

This clinical investigation is conducted as part of the ongoing **Post-Market Clinical Follow-Up (PMCF)** activities for hyaDENT BG®, designed to detect any previously unreported risks and to further confirm the clinical performance and safety profile documented in the Risk Management Report (RMR).

The investigational product will be applied in conjunction with the **coronally advanced flap and subepithelial connective tissue graft (CAF + SCTG)** technique, which represents a standard and well-documented surgical procedure in periodontal plastic surgery. Therefore, no additional clinical risks are anticipated beyond those normally associated with routine mucogingival surgery.

Compared to current practice, the only specificities introduced by this study are the **patient selection criteria** (inclusion/exclusion), standardized **follow-up schedule**, and additional **data collection procedures** (photographs, questionnaires, and in vitro cell analyses). These activities pose minimal risk to participants and are not expected to have any negative impact on patient safety.

3.3. Benefits

Patients participating in this study may benefit from adjunctive application of hyaDENT BG® during CAF + SCTG surgery for the treatment of single RT1/RT2 gingival recessions. The use of cross-linked hyaluronic acid has the potential to enhance soft tissue healing, increase the probability of complete root coverage, and reduce donor-site morbidity by accelerating wound healing and minimizing postoperative pain and swelling.

By improving the quality and stability of the soft tissue seal around teeth, this approach may contribute to better plaque control, reduced gingival inflammation, and improved long-term periodontal stability. Enhanced aesthetic outcomes and greater patient satisfaction are also expected.

Taken together, these data support a favorable benefit–risk ratio for the use of hyaDENT BG® as an adjunct in CAF + SCTG procedures for the proposed clinical indication.

4. OBJECTIVES

4.1. Primary Objectives

- Complete Root Coverage (CRC) at 12 months (proportion of sites with RD = 0).

4.2. Secondary Objectives

- Mean Root Coverage (MRC) – percentage of root coverage achieved at 12 months.
- Recession Reduction (RecRed) – reduction in gingival recession depth (mm) from baseline to follow-up.
- Clinical Attachment Level (CAL) gain – improvement in CAL (mm) from baseline to 12 months.
- Probing Depth (PD) reduction – decrease in PD (mm) from baseline to 12 months.
- Keratinized Tissue Width (KT) gain - increase in the width of keratinized gingiva (mm) from baseline to follow-up.
- Operative and postoperative factors – total procedure time (minutes) and postoperative morbidity, evaluated via Visual Analog Scale (VAS) scores for pain and discomfort and by tracking analgesic use in the first week.
- Early wound healing – Early Wound Healing Score (EHS) at early time points (3 days, 14 days, 6 weeks) to assess initial healing quality.
- Esthetic outcome – Root Coverage Esthetic Score (RES) at 12 months to appraise the esthetic success of root coverage.

Periodontal health indices (FMPS, FMBS, BOP) will be monitored throughout the study to ensure consistent oral hygiene and periodontal stability. These variables are not subject to formal hypothesis testing.

4.3. Safety Objectives

This study aims to confirm the safety profile of hyaDENT BG® when used as an adjunct to coronally advanced flap combined with subepithelial connective tissue graft (CAF + SCTG).

Through continuous adverse event (AE) and serious adverse event (SAE) monitoring, the study seeks to:

- Identify any previously unknown or unexpected side effects and continuously monitor those already documented in the Instructions for Use (IFU).
- Analyse emerging risks using factual clinical data to enable timely intervention and risk mitigation.
- Detect any potential systematic misuse or off-label application of hyaDENT BG®, allowing for corrective measures or additional training if required.

By systematically documenting and reviewing adverse events throughout the trial, this study ensures the ongoing verification of product safety and contributes to the overall post-market clinical follow-up (PMCF) activities of the manufacturer.

By closely monitoring adverse events and analysing emerging risks, we are committed to ensuring the ongoing safety and efficacy of the device.

4.4. Hypotheses in both the clinical and the in vitro sections

4.4.1. Clinical trial

Primary Hypothesis/Alternative Hypothesis (H1)

H1: Adjunctive application of hyaDENT BG® during CAF + SCTG surgery results in a higher proportion of sites achieving complete root coverage (CRC) at 12 months compared to CAF + SCTG alone.

Secondary Hypotheses (H2-H9)

H2: Adjunctive hyaDENT BG® leads to greater mean root coverage (MRC) and greater recession reduction (RecRed) at 12 months versus control.

H3: Adjunctive hyaDENT BG® yields greater CAL gain and PD reduction compared to control sites at 12 months.

H4: Adjunctive hyaDENT BG® results in greater height of keratinized tissue (HKT) gain compared to CAF + SCTG alone.

H5: Patients receiving hyaDENT BG® report lower postoperative pain, swelling, and discomfort (VAS)

H6: Adjunctive hyaDENT BG® improves oral health–related quality of life, as measured by the OHIP-14 questionnaire at early postoperative time points (3, 5, 14 days, 6 weeks).

H7: Adjunctive hyaDENT BG® improves early wound healing score (EHS) at 3, 5, 14 days, and 6 weeks compared to control.

H8: Adjunctive hyaDENT BG® provides better aesthetic outcomes (Root Coverage Esthetic Score, RES) compared to CAF + SCTG alone.

H9: Adjunctive hyaDENT BG® is associated with a lower incidence of surgical complications (e.g., flap dehiscence, infection, graft necrosis).

Null Hypothesis (H_0): No statistically significant differences are observed between test and control groups in any of the evaluated outcomes

Multiplicity Control: To manage the risk of Type I error across multiple endpoints, a hierarchical testing strategy will be applied to the following prioritized hypotheses: **H2 (MRC), H3 (CAL and PPD), H4 (HKT), H5 (VAS), and H8 (RES).** Each hypothesis in this sequence will be tested at the $\alpha = 0.05$ level, and testing will proceed only if the previous hypothesis in the hierarchy is statistically significant. Remaining secondary outcomes (H6, H7, H9) will be analyzed descriptively.

4.4.2. In vitro study

Primary Hypothesis/Alternative Hypothesis (H1)

H1-in vitro: *Exposure of primary human gingival fibroblasts to hyaDENT BG® (4 mg/mL) enhances cell proliferation, migration, and extracellular matrix production compared to untreated control cells, supporting its role in soft tissue healing and regeneration.*

Secondary Hypotheses (H2-H5)

H2-*in vitro*: hyaDENT BG® treatment results in higher cell viability (CellTiter-Blue assay) compared to control.

H3-*in vitro*: hyaDENT BG® induces upregulation of wound-healing-related genes, including COL1A1, COL3A1, TGFB1, TGFB3, PDGFB, FGF2, EGF, relative to untreated control fibroblasts.

H4-*in vitro*: hyaDENT BG® modulates inflammatory mediator expression (IL1A, IL1B, TNF, IL-8) and

MMP activity (MMP1, MMP2, MMP3, MMP8) toward a profile compatible with controlled matrix remodeling.

H5-*in vitro*: hyaDENT BG® activates key intracellular signaling pathways involved in cell proliferation and migration (p-AKT, p-ERK1/2, p-p38) as shown by Western blot analysis.

Null Hypothesis (H_0): Exposure to hyaDENT BG® does not significantly affect proliferation, migration, ECM synthesis, inflammatory profile, or signaling activity of fibroblasts compared to untreated controls.

5. STUDY DESIGN

5.1. Type and Design of the Study

This is a prospective, randomized, controlled, post-market clinical follow-up (PMCF) clinical trial designed to compare the outcomes of CAF + SCTG with adjunctive hyaDENT BG® application versus CAF + SCTG alone for the treatment of single RT1/RT2 gingival recessions.

The study is planned to last approximately 2 years, including a patient enrolment phase and a follow-up period of 12 months for each participant, starting from the date of surgery. Periodical follow-up visits will be scheduled as outlined in the study flow chart (Figure 2), ensuring standardized clinical measurements, photographic documentation, and patient-reported outcomes collection throughout the study timeline.

During each appointment, among the clinical procedures, several data will be recorded to accomplish the study goals.

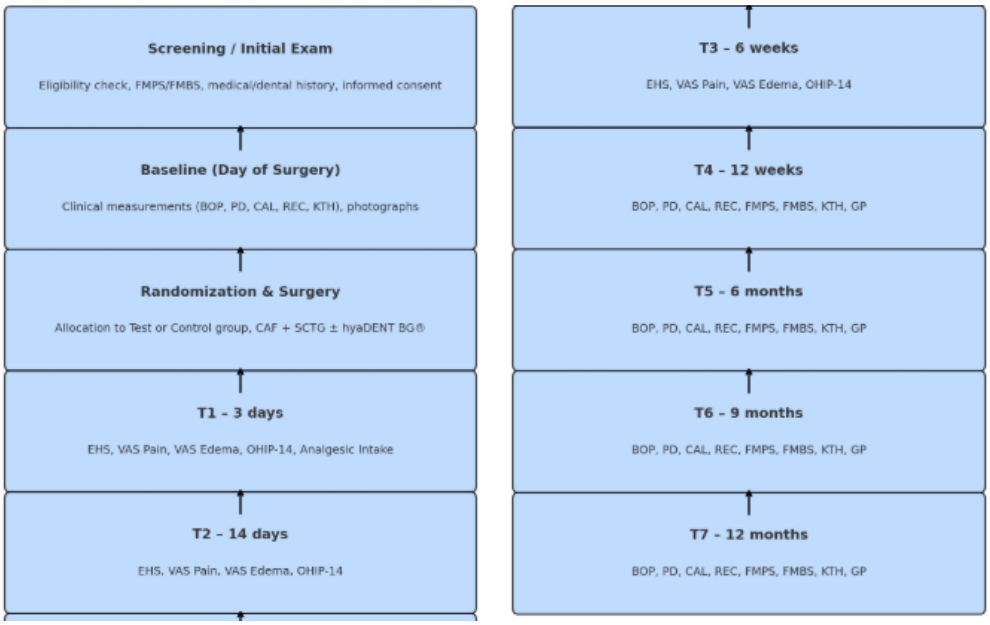

Figure 2: Study Flow Chart

5.1.1. Endpoints

5.1.1.1. Primary Endpoint

Complete Root Coverage (CRC) at 12 months, defined as the proportion of treated recession sites presenting Recession Depth (RD) = 0 mm relative to the cemento-enamel junction (CEJ). Measurements will be performed with a CP-15 calibrated periodontal probe by a blinded examiner, using the CEJ as a fixed reference point.

Photographic documentation will be standardized using the same digital camera model, lens, and magnification settings. All images will be taken at a fixed distance with the aid of a custom acrylic stent and include a millimeter scale for calibration. Consistent lighting and angulation will be maintained across all time points to ensure reliable visual assessment of root coverage.

5.1.1.2 Secondary Endpoints

- **Mean Root Coverage (MRC)** and **Recession Reduction (RecRed**) at 12 months (T7), expressed in millimeters and as a percentage of baseline RD.
- **Clinical Attachment Level (CAL) gain** and **Probing Depth (PD) reduction** from baseline (T0) to 12 months (T7).
- **Height of Keratinized Tissue (HKT) gain**, measured from the gingival margin to the mucogingival junction (MGJ) at T4 (12 weeks), T5 (6 months), T6 (9 months), and T7 (12 months).
- Periodontal parameters: **Full-Mouth Plaque Score (FMPS), Full-Mouth Bleeding Score (FMBS), and Bleeding on Probing (BOP)** at T4, T5, T6, T7 to evaluate maintenance of periodontal health.
- **Patient-Reported Outcomes (PROMs):**
  - Visual Analog Scale (VAS) for pain, swelling, and discomfort at T1 (3 days), T2 (14 days), and T3 (6 weeks).
  - Oral Health Impact Profile-14 (OHIP-14) questionnaire at the same time points to assess oral health-related quality of life.
  - Number of analgesics and days of medication recorded at T1.
- **Early Wound Healing Score (EHS):** assessed at T1 (3 days), T2 (14 days), and T3 (6 weeks) to monitor soft tissue healing dynamics.
- **Surgical and postoperative complications:** any intraoperative or postoperative complications (e.g., flap dehiscence, graft necrosis, infection) will be recorded in the adverse event log, including clinical management and outcome.
- **Aesthetic outcomes:** standardized intra-oral photographs will be analyzed to calculate the Root Coverage Esthetic Score (RES) at T7 (12 months) using fixed camera-to-subject distance, identical lighting conditions, and reference positioning. **At T7 (12 months), the esthetic outcome will be assessed using the Root Coverage Esthetic Score (RES)**, as described by ***Cairo et al. 2009*** [34].
  - The RES system evaluates five parameters:
    - **Gingival margin (GM):** 0 points = failure of root coverage (gingival margin apical or equal to baseline recession), 3 points = partial root coverage, and 6 points = complete root coverage (CRC).
    - **Marginal tissue contour (MTC):** 0 = irregular/flat margin, 1 = scalloped, physiologic contour.
    - **Soft tissue texture (STT):** 0 = scar formation or keloid-like surface, 1 = normal texture without scarring.

- **Mucogingival junction (MGJ) alignment:** 0 = misaligned MGJ, 1 = MGJ aligned with adjacent teeth.
- **Gingival color (GC):** 0 = color mismatch with adjacent gingiva, 1 = normal, harmonious color.

- The total RES ranges from 0 to 10, with higher scores indicating better esthetic integration and soft tissue harmony.
- All photographs will be evaluated by a single calibrated examiner blinded to treatment allocation to minimize assessment bias and ensure consistency across all cases. The examiner will undergo a calibration session using reference images prior to study initiation to standardize scoring criteria.

5.2. Study Population

The patient population targeted by this clinical trial comprises adult patients (≥18 years old) presenting with single RT1 or RT2 gingival recessions in maxillary or mandibular teeth, requiring surgical root coverage with coronally advanced flap and subepithelial connective tissue graft (CAF + SCTG).

Patients must meet **all inclusion criteria** and **none of the exclusion criteria** listed below.

5.2.1. Inclusion Criteria

Patients will be considered eligible for enrolment if they meet all the following criteria:

- Age ≥ 18 years, in good general health (ASA I or II).
- Presence of a single gingival recession defect classified as RT1 or RT2 according to the Cairo classification, located on a maxillary or mandibular tooth.
- Recession depth ≥ 2 mm, with a clearly identifiable cemento-enamel junction (CEJ).
- Presence of at least 1 mm of keratinized tissue at the site of the recession defect.
- No active periodontal disease (probing depth ≤ 4 mm and no bleeding on probing at the selected tooth).
- Adequate periodontal support of adjacent teeth.
- Full-Mouth Plaque Score (FMPS ≤ 20%) and Full-Mouth Bleeding Score (FMBS ≤ 20%) at screening and immediately prior to surgery.

- Tooth vitality confirmed and absence of caries, non-carious cervical lesions, or defective restorations in the area to be treated.
- Patient demonstrates good oral hygiene and is motivated to maintain plaque control throughout the study period.
- Patient is able and willing to comply with all study requirements, including scheduled follow-up visits and completion of patient-reported outcome measures (PROMs).
- Patient has provided written informed consent before any study-related procedure.

5.2.2. Exclusion Criteria

- Patients will be excluded from participation if they meet any of the following criteria:
- Uncontrolled systemic diseases or conditions that contraindicate periodontal surgery (e.g., uncontrolled diabetes mellitus (HbA1c ≥7.0), severe cardiovascular disease, immunodeficiency).
- Pregnant or breastfeeding women.
- History of autoimmune disease or current immunosuppressive therapy.
- Known hypersensitivity or allergy to hyaluronic acid or any component of the investigational product.
- Heavy smokers (>10 cigarettes/day) or users of smokeless tobacco/nicotine products within the last 6 months.
- Untreated periodontal disease or presence of probing depth ≥ 4 mm with bleeding on probing at the study tooth.
- Caries, non-carious cervical lesions, root fractures, defective restorations, or endodontic lesions in the study tooth.
- History of mucogingival surgery or previous root coverage procedure at the study tooth.
- Use of systemic antibiotics, corticosteroids, or anti-inflammatory drugs within 3 months prior to surgery (unless considered safe by the investigator).
- Coagulation disorders or current use of anticoagulant/antiplatelet medication that cannot be discontinued for surgery.
- Hormonal disorders significantly affecting wound healing.
- Active acute infections or inflammatory processes in the oral cavity at the time of surgery.

- Any medical, psychological, or social condition that, in the opinion of the investigator, could interfere with study participation or adherence to the follow-up schedule.

Patients may be informed about the study by any member of the department. However, after verifying the inclusion and exclusion criteria, the principal investigator will ultimately validate the patient's inclusion in the study.

5.2.3. Sample Size

This randomized, controlled PMCF study will enroll a total of 34 patients, allocated 1:1 to:

- Test group (n = 17): CAF + SCTG with adjunctive hyaDENT BG®
- Control group (n = 17): CAF + SCTG without hyaDENT BG®

The slight increase from the initially planned 30 patients is intended to accommodate an approximate 10% drop-out rate, ensuring that about 30 patients (15 per group) complete the study as evaluable. The sample size calculation remains based on the primary endpoint (CRC at 12 months) with 30 evaluable patients, while the additional 4 subjects provide a margin of safety for potential losses to follow-up.

The sample size was determined to reliably detect a clinically meaningful difference in the primary outcome. Previous randomized studies (e.g. Pilloni et al. 2019) have reported CRC rates of roughly 80% with CAF+HA versus 33% with CAF alone, an absolute difference of ~47 percentage points. Using this large effect size (47 pp), a two-sided $\alpha = 0.05$, and power ($1–\beta$) of 80%, a minimum of 15 patients per group was deemed sufficient to demonstrate a statistically significant difference in CRC. By enrolling 17 per group (34 total), the study preserves this power assumption while accounting for possible drop-outs, thereby maintaining at least 15 analyzable subjects in each arm.

Sensitivity Analysis of Power: The sample size is powered to detect a large CRC difference (47 pp), based on prior data. However, if the true effect is smaller (e.g. 20–30 pp), the study may be underpowered to reach statistical significance. This highlights a potential risk of type II error for modest but clinically relevant differences. Exact power estimates for these scenarios will be calculated by the statistician using final parameters. Overall, the sample size represents a practical balance between feasibility and detecting a meaningful effect

5.2.4. Early Withdrawal / Removal of Patients from Treatment

Any patient may withdraw from the study at any time, without prejudice to their ongoing dental care. If a patient fails to attend a scheduled follow-up visit, the investigator (or designee) will attempt to contact the patient to reschedule the appointment and document the reason for non-attendance.

If a patient states that he/she no longer wishes to participate, the reason for withdrawal will be recorded whenever possible (e.g., lack of interest, relocation, change of dentist, adverse event). Patients will be reminded of the importance of attending follow-up visits for their own clinical care and to allow collection of safety data.

Alternative treatment options appropriate for the patient's condition will be offered in case of withdrawal before treatment completion.

The investigator may withdraw a patient from the study in the following cases:

- Non-compliance with the protocol requirements.
- Repeated failure to attend follow-up visits.
- Development of a medical condition, SAE, or AE which, in the opinion of the investigator, contraindicates further participation.
- Identification of a new exclusion criterion (or one not previously recognized) that precludes continued participation.
- Withdrawal of informed consent.

The investigator must withdraw any patient in case of tooth loss or if the site becomes unsuitable for evaluation of the primary endpoint.

All patient withdrawals will be documented in the study termination case report form (eCRF), including the reason for withdrawal. Whenever possible, the investigator will make every effort to collect the primary endpoint data (CRC at 12 months) prior to final withdrawal.

In case of premature study withdrawal after surgery, the patient will continue to receive follow-up care according to current clinical practice.

5.2.4.1. Procedures for Replacement of Patients

Patients who withdraw from the study after randomization will not be re-enrolled. Their data will be included in the analysis following the intention-to-treat (ITT) principle whenever possible.

If a patient withdraws during the enrolment phase and prior to surgery, the investigator will make all reasonable efforts to recruit a new eligible patient in order to maintain the planned sample size (30 patients, 15 per group). This approach is intended to preserve the statistical power of the study and the validity of the comparative analysis between the test and control groups.

5.2.5. Screening Failures

Any patient that has signed the informed consent form and does not receive the study product is considered a screening failure. In the event of a screening failure, the eCRF page should be completed up to the visit when the patient was determined to be a screening failure. The study termination eCRF page should also be completed.

5.3. Study treatment

5.3.1. Concomitant Interventions

Any concomitant Interventions at the intervention area of the study are allowed and must be recorded (date and specify intervention).

5.4. Study Duration

The total study duration is expected to be approximately 2 years, calculated from the inclusion of the first patient to the completion of the 12-month follow-up visit of the last enrolled patient.

For each patient, the individual study participation will last approximately 13 months, including the baseline screening, surgery, and all scheduled follow-up visits up to 12 months post-surgery.

For the purpose of study planning, monitoring, and reporting, the following definitions are applied:
• First Patient First Visit (FPFV): the date on which the first eligible patient undergoes the baseline visit and is enrolled in the study.
• Last Patient First Visit (LPFV): the date on which the last eligible patient undergoes the baseline visit and is enrolled in the study.

• Last Patient Last Visit (LPLV): the date on which the last enrolled patient completes the final (12-month) follow-up visit.
• Final report / evaluation: the period following LPLV during which data cleaning, statistical analysis, and preparation of the final study report are performed.

5.5. Discussion of Study Design

This investigation is designed as a post-market clinical follow-up (PMCF) study, since both the test and control interventions (CAF + SCTG with or without adjunctive hyaDENT BG®) are established techniques in periodontal plastic surgery. A prospective, randomized, controlled parallel-arm design was chosen to generate high-level clinical evidence on the adjunctive use of cross-linked hyaluronic acid versus the conventional gold-standard alone. To minimize bias and account for baseline variability, the randomization will be *stratified by recession type* (Cairo RT1 vs RT2 classification), ensuring that defects of differing initial severity are evenly distributed between the test and control groups. Allocation concealment is maintained via sealed, sequentially numbered opaque envelopes, and blinding is upheld for outcome examiners, which together strengthen the internal validity of the trial.

The sample size and power considerations have been tailored to balance scientific rigor with practical feasibility. The trial plans to recruit 34 patients to allow for a ~10% drop-out rate, aiming for 30 patients to complete the 12-month follow-up. This enrollment strategy preserves the statistical power of the study by compensating for potential attrition. The sample size is driven by the primary outcome (complete root coverage at 12 months), which is a critical clinical and esthetic benchmark in mucogingival surgery. Detecting a significant difference in the proportion of sites with complete root coverage between groups will directly address the primary objective and could validate the added value of hyaluronic acid in enhancing surgical outcomes.

In addition to the primary endpoint, the study will evaluate multiple secondary endpoints covering clinical efficacy (such as mean root coverage, CAL gain, and keratinized tissue gain), patient-centered outcomes (pain/discomfort levels and oral health-related quality of life), and esthetic results (RES score). A hierarchical statistical testing plan has been incorporated for these secondary endpoints to allow a confirmatory interpretation of the most relevant

outcomes while controlling the overall Type I error. In practice, this means the analysis will formally test secondary outcomes in a prioritized sequence, enabling the trial to make stronger evidence-based claims about secondary benefits (e.g., improvements in mean root coverage or patient-reported pain) if the data support them. By pre-specifying this hierarchy, the study design addresses multiplicity and increases the reliability of conclusions drawn from secondary analyses.

Overall, the chosen design – including stratified randomization, an adequate sample with drop-out accommodation, and a controlled approach to multiple endpoints – is intended to ensure that the findings are both scientifically robust and applicable to real-world clinical practice. The results of this study will provide valuable clinical data on the potential benefits of adjunctive hyaDENT BG® in periodontal surgery, which can be compared against published outcomes in the literature and used to guide evidence-based decision-making in periodontal therapy.

6. MATERIALS AND METHODS

6.1. Study outline

A prospective, experimental, controlled, randomized study will be performed on 30. patients divided into two groups. In the test group, patients will undergo coronally advanced flap surgery combined with connective tissue graft with application of hyaluronic acid. In the control group, patients will undergo the same surgery without the application of hyaluronic acid.

The study will be complemented by an in vitro component in which a sample of the connective tissue graft will be taken from each patient to isolate fibroblast cells, culture them, and evaluate their viability, proliferation, differentiation, and mediation in cell signaling in the presence or absence of hyaluronic acid.

7. CLINICAL TRIAL

7.1. Sample Size and Randomization

Each patient will be randomly assigned to one of the two study groups (test or control) in a 1:1 ratio. The randomization sequence will be generated using a computer program (e.g., IBM SPSS) with stratification by recession type (Cairo classification RT1 vs RT2) and using permuted

blocks of random sizes. This stratified, blocked randomization ensures that patients with RT1 and RT2 recession defects are equally balanced across the two groups, preventing any imbalance in baseline recession severity. Allocation concealment will be maintained through the use of opaque, sealed, sequentially numbered envelopes. An independent investigator (not involved in the treatment) will open the next envelope in sequence immediately after flap elevation, thereby revealing the group assignment. The treating surgeon will thus be unblinded at the point of graft placement (since they must know whether to apply the HA gel), but the patients will not be explicitly informed of their group allocation. (Patients might potentially guess their group based on postoperative symptoms or the presence of the HA application, so complete patient blinding cannot be guaranteed; however, all outcome assessors remain fully blinded to group assignment.)

Based on the sample size calculations and previous study data, approximately 17 patients per group (34 total) will be enrolled in this trial. This enrollment target includes a buffer for potential drop-outs, with the goal of ending up with at least 15 evaluable patients in each arm at study completion. The groups are defined as follows:

- Test group (n ≈ 17): CAF + SCTG with adjunctive hyaDENT BG® (HA applied at the recipient site).
- Control group (n ≈ 17): CAF + SCTG without the adjunctive hyaDENT BG® (no HA application).

By employing stratified randomization and concealing the allocation, the study design mitigates selection bias and ensures that both groups are comparable with respect to key prognostic factors (notably the recession type). The 1:1 allocation with an effective sample of ~30 completers (15 per group) provides sufficient power to detect the expected difference in the primary endpoint, while the slight over-enrollment to 34 accounts for attrition and preserves statistical integrity.

7.2. Study population

All patients undergoing coronally advanced flap combined with a subepithelial connective tissue graft, at the clinical centre CEPI, Porto, will be screened for this study.

7.3. Ethical considerations

The research project will be submitted to the Ethics Committee. All patients signed a written informed consent before participation. The identification of all data is replaced by a code in order to ensure complete confidentiality and anonymity.

7.4. Surgical procedure

All patients receive basic periodontal therapy and oral hygiene instructions prior to treatment. The periodontal surgery is performed by a single experienced periodontist. Hyaluronic acid (HA) application is performed by a third examiner who is not involved in the surgical treatment sequence. Surgical chair time was measured using a chronometer from the first incision to the last suture in both groups. Coronally Advanced Flap Combined with a subepithelial connective tissue graft was performed in both patient groups to accomplish root coverage.

Surgical technique:

- Local infiltrative anesthesia (2% lidocain with epinephrine at a concentration of 1:100,000)
- Incision design is performed with a microblade (USM-6700) or with a 15c blade
- After flap preparation, a gentle root planing is performed using gracey curettes /mini-five
- Coronal advanced flap is obtained by cutting the superficial muscle fibers using a blade parallel to the external surface of the mucosa.
- Anatomical papillae are de-epithelialized with the scalpel blade and microsurgery scissors.
- The width of the graft was chosen according to the amount of tissue required to cover the exposed root and 3 mm of connective tissue mesial and distal to it.
- Immediately after harvesting, the subepithelial connective tissue graft (SCTG) is placed in a sterile dish
- In the test group, the SCTG is completely immersed (fully soaked) in sterile hyaluronic acid gel
- The graft remains immersed for approximately 1–3 minutes to ensure full surface contact and to prevent dehydration
- Excess hyaluronic acid is allowed to drain passively without compression prior to graft placement

- In the control group, the SCTG is maintained under sterile conditions and hydrated with sterile saline solution for a comparable time interval, without contact with hyaluronic acid

Suture:

- Suture for connective grafts should be resorbable Vicryl 7-0 with a 6,6 mm P1 needle (3/8 of circumference).
- Suture for the flap is 6-0 resorbable PGA with 11 mm C3 (3/8 circumference) cutting needle or 6-0 resorbable Vicryl with 13 mm C3 (3/8 circumference) cutting needle.

7.5. Postoperative protocol

Post-operative pain and edema were controlled with ibuprofen. Patients received 600 mg at the beginning of the surgical procedure. Subsequent doses were taken only if necessary to control pain. Patients had to record the quantity of analgesics taken during the first week post-surgery.

All patients were instructed to intermittently apply an ice bag on the operated area. Patients were instructed to discontinue tooth brushing and avoid any trauma at the surgical site. A 60-second rinse with 0.12% chlorhexidine digluconate was prescribed twice daily, starting on the third day after surgery, and continued for the first two weeks.

Fourteen days after the surgical treatment, the sutures were removed. Plaque control in the surgically treated area was maintained by chlorhexidine rinsing for an additional 1 week after suture removal. After this period, patients were again instructed in mechanical tooth cleaning of the treated tooth using an ultra-soft toothbrush and a roll technique for 1 month. During this month, chlorhexidine rinsing was used twice a day. Then the patient started to use a soft- toothbrush and chlorhexidine once a day for another month. All patients were recalled for prophylaxis 2 and 4 weeks after suture removal and, subsequently, once every 2 months until the final examination (12 months).

Patients were recalled for follow-up (and professional oral hygiene/maintenance procedures, clinical measurements as needed and photographs) on days 4, 8, 15 and every 2 months until 12 months.

7.6. Clinical parameters

The following clinical measurements are taken at baseline and at 12 months after surgery for each tooth by a blinded calibrated examiner: full-mouth plaque score (FMPS), full-mouth bleeding score (FMBS), bleeding on probing (BOP), probing depth (PD), and clinical attachment level (CAL), using a CP-15 mm graduated periodontal probe.

Photographic recording is done at every visit in order to compare the healing process between the two groups. Photographic record: before treatment, during surgery and follow up at days 4, 8, 15 and every 2 months until 12 months.

Clinical measurements will be performed under standardized clinical conditions (adequate lighting, gentle probing force, and dry field). To ensure measurement standardization and reproducibility, an individualized acrylic stent will be fabricated for each participant to serve as a fixed reference point for all clinical measurements, as described by *Zucchelli et al. 2010* [35]**.** The stent will be positioned on the occlusal surfaces of adjacent teeth and will include a vertical guiding groove aligned with the mid-buccal aspect of the treated tooth, ensuring consistent probe angulation and insertion point at each timepoint. All measurements will be performed by a single calibrated examiner to eliminate inter-examiner variability and ensure consistency across all study visits.

Prior to the beginning of the study, an intra-examiner calibration process will be performed in order to assess measurement reproducibility. For this purpose, clinical measurements will be recorded in a sample of 10 patients not included in the study population.
The same clinical parameters will be reassessed by the same examiner after an interval of approximately 15 days. Intra-examiner reliability will be evaluated by calculating the Cohen's kappa coefficient (κ) for categorical variables and/or the intraclass correlation coefficient (ICC) for continuous variables, as appropriate. A kappa or ICC value ≥ 0.75 will be considered indicative of acceptable intra-examiner agreement. Only after achieving adequate intra-examiner calibration will patient recruitment and data collection for the study commence.

7.6.1. Full-mouth plaque score (FMPS)

A simplified variant of the Plaque score (Visible Plaque score), proposed by Ainamo and Bay (1975), is used. In this case, the classification criterion is binominal and is based on the presence/absence of plaque on tooth surfaces: 0- Absence of plaque and 1- Presence of visible plaque

Calculation of the Full-mouth plaque score:

$$FMPS(\%) = \frac{\text{no. of surfaces with plaque}}{\text{nº of surfaces evaluated}} \times 100$$

7.6.2. Full-mouth bleeding score (FMBS)

For the evaluation of gingival inflammation, a simplified variant, also proposed by Ainamo and Bay (1975), is used. In this case the classification criterion is also binominal: 0- Absence of bleeding and 1- Presence of bleeding from the gingival margin up to 10s after probing

Calculation of the Full-mouth bleeding score:

$$FMBS\ (\%) = \frac{\text{no. of surfaces with bleeding}}{\text{no. of evaluated surfaces}} \times 100$$

7.6.3. Gingival recession depth (RD)

Measured from the CEJ to the most apical extension of the gingival margin using a calibrated UNC-15 periodontal probe, with readings rounded to the nearest 0.5 mm.

7.6.4. Height of keratinized tissue (HKT)

Measured as the distance (in millimeters) between the gingival margin and the mucogingival junction (MGJ) using a calibrated UNC-15 periodontal probe, with readings rounded to the nearest 0.5 mm.

7.6.5. Probing Depth

The probing depth is defined as the distance, in millimeters, from the gingival margin to the bottom of the gingival sulcus/pocket. All tooth surfaces (mesio-buccal, centro-buccal, disto-buccal, disto-lingual, centro-lingual and mesio-lingual) are evaluated with a periodontal probe, which is graded every 3 mm. The measurements are recorded to the nearest milimeter. By summing all the PD values it is possible to calculate the average PD value for each patient at the various evaluation times.

7.6.6. Bleeding on probing (BOP)

Bleeding after probing is a common means of assessing the presence of subgingival inflammation, although its negative predictive value is higher, i.e. when there is no bleeding. In this way, it is possible to check whether a pocket is in its active or inactive state. The measurement is made concurrently with the evaluation of the PD. A simplified variant of the bleeding on probing was proposed by Mühlemann & Son, (1971).

In this case the classification criterion is binominal:

0- Absence of bleeding within 15s;

1- Presence of bleeding from the gingival margin within 15s.

The calculation is obtained by dividing the areas where there was bleeding on probing by the total number of areas evaluated.

7.6.7. Clinical attachment level (CAL)

The clinical attachment level is defined as the distance in millimeters from the cementoenamel junction (CEJ) to the bottom of the gingival sulcus or pocket. All tooth surfaces (mesio-buccal, centro-vestibular, disto-buccal, disto-lingual, centro-lingual and mesio-lingual) are evaluated with a periodontal probe, which is graded every 3 mm. The measurements are recorded to the nearest milimeter. By summing all the PD values, it is possible to calculate the average value for each patient at the various evaluation times. The clinical evaluation requires measuring the distance from the free gingival margin to the CEJ.

Thus, the level of insertion can be calculated as follows:

In the presence of gingival recession: Pocket Depth + distance from the gingival margin to the CEJ.

7.6.8. Patient related outcome measures

Postoperative pain, number of analgesic pills taken, and number of days the pills were taken were assessed using questionnaires.

Postoperative questionnaires evaluated the subjects' postoperative pain, interference with daily life, and the patient's perception of swelling using the 100-mm visual analog scale (VAS).

The questionnaire was given to the patients 1 week after surgery. The subjects were also asked to indicate the location of the pain: donor site, recipient site, or elsewhere in the mouth.

8. IN VITRO

8.1 Cell culture and HA preparations

8.1.1 Hyaluronic acid formula

HA (hyaDENT BG) is a formula containing 1000 kDa monomers of HA cross-linked to butanediol diglycidyl ether (BDDE) complexes and natural HA. (1.6% cross-linked hyaluronic acid and 0.2% natural hyaluronic acid).

8.1.2. Concentration of the hyaluronic acid preparations

HA will be used at a final concentration of 4 mg/ml in DMEM medium supplemented with 0.3% FBS, as described in the literature. The final concentration of 4 mg/mL was selected to balance biological relevance, experimental feasibility, and clinical translatability. First, previous experiments demonstrated that HA diluted to 4 mg/mL elicited biological responses comparable to those observed with undiluted commercial HA preparations, while allowing improved handling and homogeneous distribution in cell culture conditions [14]. Second, concentrations in the range of 1–5 mg/mL are commonly reported in the literature for in vitro studies evaluating the effects of hyaluronic acid on periodontal and oral cells, particularly under low-serum conditions, supporting the biological validity of this concentration range [36-38]. Third, from a clinical perspective, commercially available HA formulations applied during periodontal surgery are rapidly diluted in situ by blood, gingival crevicular fluid, and saliva. Therefore, the use of a 4 mg/mL concentration in vitro more closely reflects the effective HA concentration at the surgical site after application, rather than the nominal concentration of the undiluted product.

Taken together, these considerations support the use of 4 mg/mL as a biologically active, clinically relevant, and methodologically justified concentration for the present study, with this concentration

range being widely supported by the existing literature on in vitro and translational periodontal models involving hyaluronic acid.

8.1.3. Cell culture

Primary human connective tissue fibroblasts are obtained from each patient undergoing clinical treatment. Tissue samples will be collected from subepithelial palatal connective tissue grafts obtained from systemically and periodontally healthy individuals undergoing periodontal root coverage surgery. Each donor tissue sample will be minced into 1 mm pieces and then pipetted into a 25 cm3 tissue culture flask and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

8.2. Cell viability assay

Cell viability can be assessed by CellTiter-Blue viability assay (e.g.) Procedure: After 24 hours of starvation, cells are seeded in triplicate at 5 × 103 cells/well in 96-well plates coated with HA at the indicated concentrations (between 0 and 4 mg/mL) prepared in 0.3% FCS/DMEM. 2 hours after seeding, CellTiter-Blue® Reagent (20 µL/well) is added to the cells for 4 hours before recording fluorescence using a luminometer. Experimental values are normalized to the values for untreated cells.

8.3. Cell proliferation assay

The proliferation rates of HA-treated cells are determined using the 5-bromo-2'- deoxyuridine (BrdU) chemiluminescent ELISA (enzyme-linked immunosorbent assay). Procedure: After 24 h of starvation, cells are seeded in triplicate at 2 × 103 cells/well in 3% FBS/DMEM in black 96-well microtiter plates coated with HA at a final concentration of 4 mg/ml. Cells were allowed to proliferate for 0, 24, 48, 72 and 96 h before labeling with BrdU for 2 h. The BrdU uptake in the newly synthesized DNA is determined according to the manufacturer's instructions using a luminometer. Experimental values are normalized to the values of untreated control cells at time point 0.

8.4. Cell migration assay

Cell migration is tested using Transwell® Polycarbonate Membrane Inserts. Procedure: After 24 hours of starvation, 5 × 104 cells are seeded in the upper insert chamber in serum-free DMEM. The lower chamber is coated with HA at a final concentration of 4 mg/mL in 10% FCS/DMEM. Cells are allowed to migrate through the filter for 18 hours at 37°C before fixation and staining with crystal violet. Images of duplicate inserts are acquired using a microscope. Migration is quantified using ImageJ's Fiji distribution.

8.5. RNA analysis by qRT-PCR

For oral fibroblast cells quantitative RT-PCR is used to investigate the expression of COL1A1, COL3A1, TGFB1, TGFB3, PDGFB, FGF2, EGF, IL1A, IL1B, TNF, MMP1, MMP2, MMP3 and MMP8 genes. Procedure: Total RNA from cells treated with HA or growth factor is isolated using the RNeasy Mini Kit. RNA is reverse transcribed and the relative transcripts of the listed genes, normalized to GAPDH, are measured using FastStart Universal SYBR Green Master ROX and primer sequences are shown in the table. Real-time PCR is performed using a 7500 Real-Time PCR system. Data are analyzed using the ΔΔCt efficiency method. All samples are run in duplicate.

8.6. Protein analysis by Western Blot

Complete cell extracts of HA-treated oral fibroblasts are prepared by lysis in RIPA buffer. Proteins of interest are visualized using anti-phospho-akt, anti-akt, anti-phospho-erk1/2, anti-erk, anti-phospho-p38, anti-p38 and anti-vinculin antibodies followed by horseradish peroxidase-conjugated secondary antibodies for detection with the SuperSignalTM West Dura Substrate protein immunodetection reagent. The expression of phospho-Akt, phospho-Erk1/2 or phospho-p38 protein relative to the respective total protein control is quantified by densitometry.

9. RESULTS

9.1. Clinical Trial

We intend to evaluate the effect of HA, compared to the control group, on clinical parameters:

1. Full-mouth plaque score (FMPS)
2. Full-mouth bleeding score (FMBS)
3. Bleeding on probing (BOP)
4. Probing Depth (PD)
5. Clinical attachment level (CAL)

Evaluation times: before treatment, days 4, 8, 15 and every 2 months until 12 months

9.2 In-vitro study

It is intended to evaluate the effect of HA preparation, compared to control cells, on oral fibroblast cells (obtained from gingival grafts) regarding the:

1. Viability of primary cells. Ability to adhere and assume a fibroblast-specific fusiform morphology (Fig.1);
2. Proliferative and migratory capacity of the cells;
3. Expression of COL3A1 and TGFB3 genes (characterize wound healing without scarring);
4. Expression of genes encoding growth factors and cytokines involved in wound healing;
5. Expression of MMP2 and MMP3 (matrix metalloproteinases) genes
6. Phosphorylation of Akt, Erk1/2 and p38 kinases.

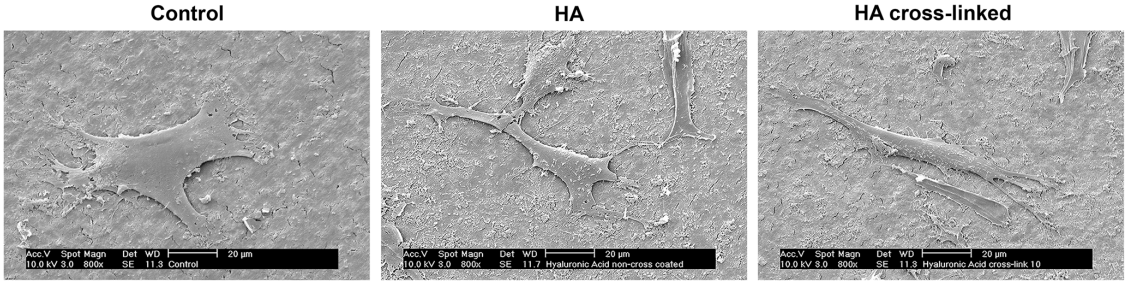

Fig. 3: Scanning electron microscope images (SEM) of primary human PDL cells seeded onto dentin discs in control, HA and HA cl samples. PDL cells seeded in cross-linked HA

demonstrated qualitatively more elongated cell morphology when compared to control and HA.

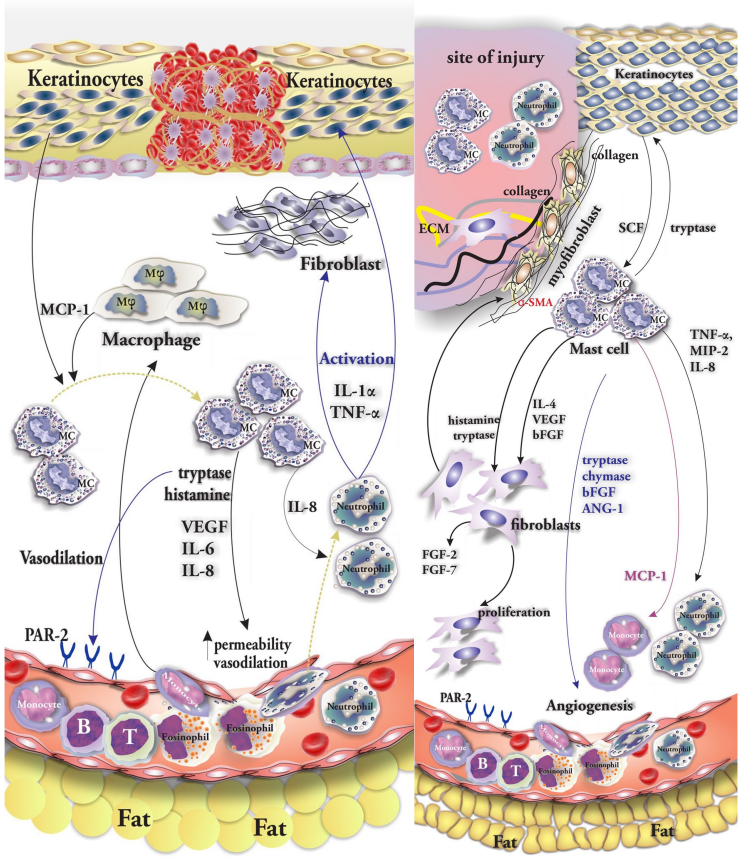

Fig 4: Left image: Neutrophils in humans are recruited by IL-8 to injury sites where they release IL-1α and TNF-α and activate fibroblasts and keratinocytes to facilitate wound healing. Right image: Cellular and molecular interactions at the wound site. Fibroblast proliferation and differentiation into myofibroblasts are essential to produce ECM, collagen and α-SMA.Macrophages release mediators that facilitate angiogenesis in order to nourish infiltrated and newly proliferated cells at the wound site (Komi et al).

Table 1: General properties and effects of mast cell-derived mediators in the wound healing process (Komi et al.).

Table 1 General properties and effects of mast cell-derived mediators in wound healing process

Mediator	General properties and effects in wound healing process	Ref
TGF-β1	• Profibrotic multifunctional growth factor • Stimulates fibroblast proliferation and matrix formation • Some mast cell mediators including protease and chymase through activation of latent TGF-1 contribute in the regulation of extracellular matrix formation	[56]
TGF-β2	• Is involved in: 1. Marked collagen deposition 2. Increasing mechanical strength of regenerated tissue 3. Accelerating the rate of wound healing	[56]
Histamine	• Capable of inducing proliferation and collagen synthesis of fibroblasts • Causes the dilatation of arterials and increases permeability of venules	[22] [39]
Chymase	• Possesses mitogenic effect on fibroblasts • Promotes myocardial and skin fibroblast proliferation • Regulates angiotensin (Ang) II generation • Regulates degradation of procollagen protein which participates in tissue remodeling and inflammation	[22, 77] [77]
IL-4	• Increases collagen production • Stimulates accumulation of extracellular matrix macromolecules • Stimulates synthesis of the extracellular matrix proteins including type I and III collagen and fibronectin in fibroblast culture	[22] [78] [22]
mMCP-6	• Stimulates dermal fibroblasts, inducing DNA synthesis in quiescent cells • Increases α-1 collagen production • Stimulates fibroblast and smooth muscle proliferation	[63] [79]
MMP-9	• Facilitates tissue remodeling by degradation of extracellular matrix (ECM)	[80]
mMCP-4	• Human homolog: chymase • Increases fibroblast proliferation • Directly proangiogenic by angiotensin I conversion • Indirectly proangiogenic by modulating ECM, releasing VEGF, and activating progelatinase B • Participates in neutrophil recruitment during the inflammatory phase	[79] [79] [81]
mMCP-5	• Human homolog: elastase • Induces the release of TGF-β1	[79]
Tryptase	• Contributes to angiogenesis through directly degrading the ECM components • Releases matrix-bound growth factors owing to its proteolytic activity • Activates latent matrix metalloproteases • Induces endothelial cell proliferation in a dose-dependent manner • Participates in inflammation by leukocyte recruitment • Induces α-SMA expression • Mitogenic for fibroblasts, smooth muscle cells • Possesses chemotactic and mitogenic effects on fibroblasts • Stimulates collagen synthesis • Involved in contraction, and fibroblast differentiation into myofibroblasts	[82] [82] [82] [82] [83] [84] [85] [21] [21] [21]
Carboxypeptidase A	• Increases fibroblast proliferation, and type I collagen production	[53]
bFGF	• Mitogenic factor for fibroblasts, endothelial cells	[86]

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