Journal Pre-proof

Regenerative endodontic procedure of immature permanent teeth with leukocyte and platelet rich fibrin: a multicenter controlled clinical trial

Nastaran Meschi, Mostafa EzEldeen, Andres Eduardo Torres Garcia, Pierre Lahoud, Gertrude Van Gorp, Wim Coucke, Reinhilde Jacobs, Katleen Vandamme, Wim Teughels, Paul Lambrechts

PII: S0099-2399(21)00565-3

DOI: <https://doi.org/10.1016/j.joen.2021.08.003>

Reference: JOEN 4898

To appear in: *Journal of Endodontics*

Received Date: 6 June 2021

Revised Date: 29 July 2021

Accepted Date: 3 August 2021

Please cite this article as: Meschi N, EzEldeen M, Garcia AET, Lahoud P, Van Gorp G, Coucke W, Jacobs R, Vandamme K, Teughels W, Lambrechts P, Regenerative endodontic procedure of immature permanent teeth with leukocyte and platelet rich fibrin: a multicenter controlled clinical trial, *Journal of Endodontics* (2021), doi: <https://doi.org/10.1016/j.joen.2021.08.003>.

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Copyright © 2021 Published by Elsevier Inc. on behalf of American Association of Endodontists.

Title: Regenerative endodontic procedure of immature permanent teeth with leukocyte and platelet rich fibrin: a multicenter controlled clinical trial

Key words: cone-beam computed tomography, mesenchymal stem cells, dental infection, outcome assessment

Author names and affiliations:

Nastaran Meschi^{a,b}, Mostafa EzEldeen^c, Andres Eduardo Torres Garcia^{a,c}, Pierre Lahoud^c, Gertrude Van Gorp^a, Wim Coucke^d, Reinhilde Jacobs^c, Katleen Vandamme^e, Wim Teughels^f, Paul Lambrechts^{a,b}

- a: Department of Oral Health Sciences, Endodontology, KU Leuven & Dentistry, University Hospitals Leuven, Leuven, Belgium

- b: Department of Oral Health Sciences, BIOMAT – Biomaterials Research Group, KU Leuven & Dentistry, Leuven, Belgium

- c: OMFS IMPATH Research Group, Faculty of Medicine, Department of Imaging and Pathology, KU Leuven & Oral and Maxillofacial Surgery, University Hospitals Leuven, Leuven, Belgium

- d: free-lance statistical consultant, Brugstraat 107, 3001 Heverlee, Belgium

- e: Department of Oral Health Sciences, Restorative Dentistry, KU Leuven & Dentistry, University Hospitals Leuven, Leuven, Belgium

- f: Department of Oral Health Sciences, Periodontology, KU Leuven & Dentistry, University Hospitals Leuven, Leuven, Belgium

nastaran.meschi@kuleuven.be

mostafa.ezeldeen@kuleuven.be

andreseduardo.torresgarcia@kuleuven.be

pierre.lahoud@kuleuven.be

gertrude.vangorp@kuleuven.be

wim.coucke@jewidaco.be

reinilde.jacobs@kuleuven.be

katleen.vandamme@kuleuven.be

wim.teughels@kuleuven.be

paul.lambrechts@kuleuven.be

Corresponding author:

Name: Nastaran Meschi

Address: Biomaterials - BIOMAT, Kapucijnenvoer 7 blok a- box 7001, 3000 Leuven, Belgium

Telephone: 0032476 31 38 89

E-mail: nastaran.meschi@kuleuven.be

Acknowledgements:

The authors deny any conflicts of interest. We would like to express our gratitude to Mr. Jan Berghmans (Megabite & co, Brussels, Belgium), Mr. Koen Ackx (Bitewing, Herent, Belgium), Mr. Mathieu Vandendael (CVORE, Everberg, Belgium) and Mr. Richard Hakse (Endopraktijk Hakse, Oirschot, The Netherlands) for their kind collaboration and referral of patients. Also, many thanks to Dr. Steffen Fieuws (L-BioStat, KU Leuven, Leuven, Belgium) for the statistical help during the set-up of the study protocol.

Regenerative endodontic procedure of immature permanent teeth with leukocyte and platelet rich fibrin: a multicenter controlled clinical trial

Abstract

Aim

The aim of this non-randomized, multicenter controlled clinical trial was to evaluate the impact of leukocyte and platelet rich fibrin (LPRF) on regenerative endodontic procedures (REP) of immature permanent teeth in terms of peri-apical bone healing (PBH) and further root development (RD).

Methodology

Healthy patients between 6-25 years with an inflamed or necrotic immature permanent tooth were included and divided between the test (= REP+LPRF) and control (= REP-LPRF) group, depending on their compliance and the clinical setting (university hospital or private practice). After receiving REP+/-LPRF, the patients were recalled after 3, 6, 12, 24 and 36 months. At each recall session the teeth were clinically and radiographically (by means of a peri-apical radiograph (PR)) evaluated. A cone beam computed tomography (CBCT) was taken pre-operatively, 2 and 3 years post-operatively. PBH and RD were quantitatively and qualitatively assessed.

Results

Twenty-nine teeth with a necrotic pulp were included from which 23 (9 test, 14 control) were analyzed. Three teeth in the test group had a flare-up reaction in the first year post REP. Except for 2 no shows, all the analyzed teeth survived up to 3 years post REP and in case of failure apexification preserved them. Complete PBH was obtained in 91.3% and 87% of the cases, based on PR qualitative and quantitative evaluation respectively, with no significant difference between the groups with respect to (wrt) the baseline. The PR quantitative change in RD at the last recall session, wrt the baseline, was not significant (all p-values >0.05) in both groups. The qualitative assessment of the type of REP root healing was non-uniform. In the test group 55.6% of the teeth presented no RD and no apical closure. Only 50% of the 14 teeth assessed with CBCT presented complete PBH. Regarding

volumetric measurements on RD 3 years post REP, for the change wrt the baseline in root hard tissue volume (RHTV), mean root hard tissue thickness (RHTT) and apical area, the control group performed significantly in favor of RD than the test group ($p= 0.03$, 0.003 and 0.05 respectively). For the volumetric change 3 years post REP wrt the baseline in root length and maximum RHTT, no significant difference ($p= 0.72$ and 0.4 respectively) was found between the groups. The correlation between the PR and CBCT variables assessing RD was weak (root lengthening) to very weak (root thickening).

Conclusion

REP-LPRF seems to be a viable treatment option to obtain PBH and aid further RD of necrotic immature permanent teeth. Caution is needed when evaluating REP with PR.

Introduction

Regenerative medicine is an emerging and exciting field. Regeneration of tissue rather than restoration offers an alternative perspective to endodontic treatment. A regenerative endodontic procedure (REP) is defined as a biologically based treatment of immature permanent teeth designed to replace damaged structures, including dentin and root structures as well as cells of the pulp-dentin complex (1). Mesenchymal stem cells (MSC) are attracted into the root canal by creating bleeding in the peri-apical region and/or chemotactic factors (= cell homing principle) or by transplanting stem cells into the root canal (= cell based principle) (1-5). These undifferentiated stem cells are able to differentiate into several different cell types, when the specific growth factors and scaffold are added (1, 4). The initial differentiation occurs necessarily in a transitory scar matrix formed by fibrin and fibronectin. That is why fibrin is preferentially used as a support matrix for the transplantation of MSC. Several studies have reported that a fibrin matrix is an optimal support to transplanted MSC for obtaining pulp and osseous defect regeneration (6-11).

Amongst the natural scaffolds applied in REP, autologous platelet rich fibrin (PRF) is gaining more interest (12, 13). Namely, platelet-rich preparations have been evaluated with encouraging results to

accelerate tissue healing (14, 15). It is known that platelets contain high quantities of key growth factors, such as platelet derived growth factor, transforming growth factor beta (TGF- β) and vascular endothelial growth factor (VEGF), which are able to stimulate cell proliferation, matrix remodeling and angiogenesis (16). We can divide platelet-rich preparations into two groups: the first-generation platelet rich plasma (PRP) and the second-generation PRF. PRP has been applied in some REP studies with promising results (10, 13, 17). Even if PRP is easily injectable, nevertheless its production demands the addition of bovine thrombin and a double spin in the centrifuge. Furthermore, the cytokines beneficial for wound healing are lost by PRP's loose matrix in less than 14 hours (18). On the other hand, PRF and its leukocyte-rich derivate, leukocyte and platelet rich fibrin (LPRF), present many advantages compared to PRP. It is centrifuged blood without any additives. In the absence of anticoagulants, platelet activation and fibrin polymerization are triggered immediately. Fibrinogen will first appear at the top of the tube whereafter the circulating thrombin transforms soluble fibrinogen into insoluble strands of fibrin. Blood is separated into three components with the formation of a fibrin clot in the middle, acellular plasma at the top and red blood cells at the bottom (Fig. 1) (19). This clot acts as a matrix that contains most light blood components, such as platelets and leukocytes, as well as circulating molecules, such as growth factors and fibronectin. After compression of the LPRF clot, it can be easily used as a membrane (Fig. 1), but has to be cut into pieces in order to insert it with pluggers into a root canal. Additionally, LPRF acts as a reservoir that gradually releases growth factors over a period of 7-14 days (20, 21).

LPRF has been appraised in periodontal literature (22, 23). It has the potential advantage of creating a bioactive construct that stimulates the local environment for differentiation and proliferation of mesenchymal stem and progenitor cells. Of interest for the regenerative endodontic field, PRF was demonstrated to stimulate cell proliferation and differentiation of dental pulp stem cells by upregulating osteoprotegerin and alkaline phosphatase expression (9). Furthermore, the osteo- and odontogenic differentiation of the stem cells of the apical papilla (SCAP) is improved by PRF (24). These PRF matrices have the potential advantage of creating a bioactive construct in which the

provisional fibrin scaffold acts as a conductive matrix for the migration of cells involved in wound repair. The anti-inflammatory cytokines in leukocytes, such as interleukin (IL) 4 and VEGF, aid this wound repair (19). However, they contain pro-inflammatory cytokines as well, such as interleukin IL-1 β , tumor necrosis factor (TNF)- α and IL-6 (19).

The European Society of Endodontology (ESE) and the American Association of Endodontics (AAE) have prescribed evidence-based treatment protocols to standardize REP and to determine utmost outcomes to be achieved by REP (25, 26). A growing body of evidence reports successful treatment outcomes for REP (without autologous platelet concentrates (APC)) (27-29). Due to the above-mentioned favorable properties, APC are being applied and appraised in REP. Nevertheless, these studies seem to have a questionable level of evidence (30). Also, the impact of venipuncture on the compliance of young patients, the user-friendliness of LPRF-application during REP and the potential adverse events when adding immunity modulating scaffolds to REP have nowhere been reported to our knowledge (30). Hence, we have designed this non-randomized, non-commercial, multicenter controlled clinical trial to test the hypothesis that the use of LPRF in REP of immature permanent teeth will accelerate peri-apical bone healing (PBH) and stimulate root development (RD).

Methods

Trial design, registration, randomization and blinding

This study was designed as a multicenter controlled clinical trial with the UZ KU Leuven (Leuven, Belgium) Medical Ethics Committee assigned as the Central Committee (registration number: S56810). The teeth were treated in multiple centers in Belgium by NM (endodontist) or GVG (pedo-endodontist): UZ Leuven (University Hospitals Leuven, Leuven), Megabite & co (Brussels and Herent) and EndoVanGorp (Rotselaer). The patients were divided between the test (REP+LPRF) and control (REP-LPRF) group, depending on their compliance and the clinical setting. Firstly, randomization was not possible, due to non-compliance of the youngest and/or most anxious patients during venipuncture (necessary for LPRF) in the pilot phase of the study. Secondly, no centrifuge nor nurse

(for venipuncture) were available in the included private practices. Only the assessors (ME, ATG and PL) and the statistician were blinded regarding the patients' names and allocation of the patients to the groups. This study was conducted according to the principles of Good Clinical Practice (International Council on Harmonization, 1996), that rely on the ethical principles of the Declaration of Helsinki (World Medical Association, 1964). This trial is registered on ClinicalTrials.gov (ID: NCT02437708) where the protocol can be accessed.

Participants

Healthy patients between 6-25 years with an inflamed or necrotic immature permanent tooth were included. The eligibility criteria are mentioned in Table 1.

Interventions and treatment protocol

Patients in need of a REP were screened for a possible inclusion in this study. The subjects were informed orally and in writing about the study. Written informed consent from the patient/patient's guardian were obtained prior to any examination carried out for study purpose. The screening procedure included collection of demographical, clinical and radiographic data. The teeth were clinically assessed for percussion, periodontal condition (mobility and pocket depth), discoloration, swelling of the mucosa and sensitivity (by means of carbon dioxide snow (CDS) and an electric pulp tester (EPT; Pulppen DP2000 Digital, Dental Electronic, Ballerup, Denmark)). A peri-apical radiograph (PR; Digora and Minray; Soredex, Tuusula, Finland) standardized by means of a silicone guide (Optosil Comfort Putty, Kulzer, Hanau, Germany) was taken pre-operatively. Furthermore, a cone-beam computed tomography (CBCT; Accuitomo, J Morita, Osaka, Japan) was taken as well, if the device was available (not at the above-mentioned private practices). With subjects fulfilling all inclusion criteria and none of the exclusion criteria (Table 1), the treatment plan as well as the costs were discussed.

In the control group REP was performed in 2 sessions following the protocol described by Diogenes A. *et al.* (2013) (4). Briefly, local anesthesia with adrenalin was administered. After rubber dam

isolation and access cavity preparation, the working length measurements were performed with PR if no pre-operative CBCT was available. Afterwards, the root canal was irrigated 1 mm short of working length with 20 mL of 1.5% sodium hypochlorite (NaOCl) and subsequently with 20 mL of saline. The root canal was dried with sterile paper points and calcium hydroxide (UltraCal™ XS, Ultradent Products, Inc., South Jordan, UT) was injected into the root canal (1 mm short of working length). The tooth was temporarily sealed by means of a sterile cotton pellet and glass ionomer cement. During the second session, local anesthesia without adrenalin was administered and the tooth was isolated by means of rubber dam. After rinsing with 30 mL of EDTA 17% 1 mm short of working length, a blood clot was triggered peri-apically. Subsequently, a Collaplug (Zimmer Biomet, Berlin, Germany) was placed upon the blood clot and Pure Portland Cement Med-PZ (MPC; Medcem, Weinfelden, Switzerland) was used instead of mineral trioxide aggregate (MTA), to prevent tooth crown discoloration (31). The tooth was sealed by means of a glass ionomer lining and composite restoration.

In the test group, the treatment was performed similar to the control group with the exception that LPRF was added as a scaffold during the second session. LPRF was collected and produced as described in Fig. 1. The second treatment session was commenced, as described by Diogenes *et al.* (2013), and in the meanwhile the blood samples were centrifuged (4). Fibrin clots were collected after centrifugation and 2 of them were transformed into membranes after 5 minutes pressure under a sterile glass plate (Fig. 1). The exudate of the pressed clots was used as a final rinse of the root canal. Afterwards, a blood clot was triggered peri-apically into the root canal. The fibrin clots and membranes were inserted into the root canal with endodontic pluggers until 3 mm below the cemento-enamel junction (CEJ). No Collaplug was applied below the MPC, as the LPRF-scaffold provided enough resilience to the MPC.

For both groups, the entire treatment was performed under microscopic magnification. Furthermore, in case of flare-up, the treatment provided at the first session was repeated and a double antibiotic

paste (DAP; metronidazole 500 mg, ciprofloxacin 200 mg, Progel) was injected in the root canal, as described by Diogenes A. *et al.* (2013) (4). However, the exact concentration of antibiotic paste was not measured. Furthermore, systemic antibiotics were prescribed as well: amoxicillin or erythromycin (in case of penicillin allergy), dosed depending on the patient's weight.

The patients were recalled at 3, 6, 12, 24 and 36 months. At each recall session the same clinical assessments were performed as during the intake session and 1 PR (with the customized silicone guide) was taken. A CBCT was taken 2 and 3 years post-operatively, if the patient and/or their guardian consented.

Outcomes

The *primary* outcome variable was the amount of PBH post REP, quantitatively measured on PR. The PRs were aligned with a dedicated tool created in MeVisLab (MeVis Research, Bremen, Germany) for image registration, and the peri-apical lesions were outlined and quantified with ImageJ software (National Institutes of Health, Bethesda, MD). Additionally, the full-scale and final peri-apical index (PAI) according to Kirkevang *et al.* (2015) was applied to assess PBH qualitatively (32).

The *secondary* outcome was the amount of RD post REP based on two-dimensional (2D) measurements on PR. The change in root canal wall length (RL) and the radiographic root area (RRA) were measured with ImageJ software following the method described by Flake NM *et al.* (2014) (33). The RL was measured from the apical part of the MPC until the apex.

Qualitative assessment of the REP root healing was performed according to the 5 scores described by Chen MY *et al.* (2012). Furthermore, in case these healing types or a combination of them were not enough, other types of healing were described as well.

Three blinded and independent assessors performed the radiographic measurements. The 2D measurements were performed by ME and ATG. The 3D measurements were performed by ME and PL.

3D analysis of REP outcomes

Based on the three-dimensional (3D) measurements of the CBCTs taken 2 and 3 years post REP, the change in root length (RL), apical area (AA), root hard tissue volume (RHTV), maximum and mean root hard tissue thickness (RHTT) were measured (27, 34). CBCT scans were imported into MeVisLab (MeVis Medical Solutions AG, Bremen, Germany). All teeth (pre- and postoperatively at last follow-up) were then automatically segmented using a novel Artificial intelligence (AI) driven tooth segmentation tool (35) and largely based on the previously validated tool for 3D REP analysis (36). During the segmentation, the AA at the most apical axial slice was measured and the root canal space was subtracted from the segmented image of the tooth pre – and postoperatively. For each tooth the segmented hard tissue preoperative image was then spatially aligned to the segmented postoperative image using the dentin-enamel and the cement-enamel junctions as alignment landmarks and maximization of mutual information as a registration metric (37). The 3D triangle-based surface of the hard tissue was reconstructed. The RHTV and its RL were calculated measuring from the cement-enamel junction (CEJ) to the apex pre- and postoperatively. Morphological quantification was performed in 3matic (Materialise; Leuven, Belgium). The RHT was analyzed to record the mean and maximum RHTT pre- and postoperatively and then expressed as a color-coded map showing the calculated minimal distances from the canal surface to the external root surface. The measurements were then normalized and expressed as the percentage of change related to the pre-operative form.

Sample size calculations

As observed during the pilot phase of this study, the addition of venipuncture (to obtain LPRF) burdened the youngest patients and some of them even refused further compliance. Thus, adding LPRF to the REP-protocol would be appreciated only if it would have a significant positive influence on the healing process. Based on a two-sample pooled *t*-test of a mean ratio with lognormal data, there were 9 teeth per group needed to have minimal 80% power to detect a doubling of the PBH

(primary outcome) assuming a coefficient of variation (CV) equal to 0.5. This CV was obtained from Nagy MM *et al.* (2014) (38). Taking account of possible dropouts, 20 teeth (10 per group) in total were included in the study. The 8 teeth included during the pilot phase of this study remained included. If the pre-set required number of 20 teeth was achieved in less than two years, the inclusion process in this study would not stop until September 2016. As such, the precision of the estimates for the primary and secondary endpoints was increased.

Statistical methods

The assessment of the outcome variables was executed by a statistician (CW) under blinded conditions, namely the patients' names and groups to which the teeth were allocated, were encoded. Before analysis, percentage changes between the measurements at distinct time points and the measurement at baseline were calculated for every tooth individually. For assessing differences between treatments, the percentage changes with respect to (wrt) baseline were subject to a linear mixed model with treatment and time as crossed fixed factors, and patient and tooth, nested in patient as random factors. Residual analysis by means of a normal quantile plot and a residual dot plot showed that the assumptions of normality of residuals were met. Only for 2D PBH, a $\log(x+40)$ transformation was needed. The value of 40 had to be added to render all data strictly positive. Differences between treatments were assessed by constructing the relevant contrasts based on the coefficients of the fixed effects of the linear mixed model and their variance-covariance matrix. P-values for those contrasts were corrected for simultaneous hypothesis testing according to Sidak. The relation between the measurements on 2D images and those on 3D images was assessed by means of calculating the simple linear regression line and inference about its slope, and by means of the Spearman rank correlation coefficient.

Results

Baseline data, recruitment, numbers analyzed and participant flow

From September 2014 until September 2016, 29 teeth (from 27 patients) were recruited and assessed for eligibility (Table 1). The teeth were not randomized but allocated to a group (REP+/-LPRF) depending on the location and the patient's compliance. More specifically, all teeth recruited in private practice were allocated to the REP-LPRF group (due to lack of centrifuge and nurse) and all teeth recruited at the UZ Leuven were allocated to the REP+LPRF group, unless the patient did not allow venipuncture. The number of teeth treated and analyzed over time are mentioned in a flow diagram (Fig. 2). The patients' baseline and demographic data are presented in Table 2. Fifteen male and 14 female patients were recruited, from which 6 and 5 respectively in the test group. The patients were between 6 and 19 years old. In 2 patients 2 teeth were treated (cases 12 and 13; cases 22 and 23). In the test group the age range was 7-19 years, amongst which 64% were older than 10 years. In the control group the age range was 6-18 years, amongst which 28% were older than 10 years. Regarding the type of teeth: 1 upper lateral incisor, 1 lower first premolar and 27 upper central incisors were treated. Regarding the etiological factor: 25 teeth received REP due to trauma, 2 due to an anatomical anomaly (dens invaginatus) and 1 due to caries. Regarding the pulpal status: all teeth were necrotic. Five teeth were symptomless, 1 was percussion sensitive, 4 were discolored, 8 presented a fistula and 12 an abscess. Twenty teeth were treated in the UZ Leuven (Leuven, Belgium) and 9 in private practice. NM treated 27 teeth and GVG 2. The follow-up period ranged between 7 and 54 months. Post-operatively only 9 teeth revealed a positive reaction on CDS and/or EPT on one specific time point. Discoloration post REP was seen in 14 cases: 7 yellow, 4 grey, 1 yellow-grey and 2 brown. However, from these cases, 3 were already discolored pre-operatively.

Outcomes and adverse events

Outcomes and estimation

Three cases from the REP-LPRF group are presented in Fig. 3 and two cases from the REP+LPRF group are presented in Fig. 4. For the 2D qualitative and quantitative analyses, the intra- and interobserver values for parametric data as well as for categoric data varied between 0.6 and 1 (moderate to almost perfect agreement). Therefore, the assessors performed the assessments only once.

Regarding the *primary outcome*, the quantitative and qualitative measurements on PR of the peri-apical lesion area are reported in Table 3. From the 23 teeth analyzed, 9 were from the test group and 14 from the control group. Noticeable is that the mean peri-apical bone lesion at baseline in the test group ($65.3\text{mm}^2 \pm 59 \text{mm}^2$) was threefold of that in the control group ($20.8\text{mm}^2 \pm 36.6\text{mm}^2$). Furthermore, in Table 3 it is noticeable that in 9 teeth (1 test, 8 control) all peri-apical lesion areas on all time points were equal to zero. All teeth in the control group healed completely or remained unchanged (zero values on each time point). In the test group 1 tooth (nr. 24) presented bone loss 1 year post REP, 2 teeth (nr. 1 and 22) incomplete PBH and 6 teeth complete PBH or remained unchanged (zero values on each time point). In total, 87% of all the cases presented complete PBH on the last recall time point when quantitatively assessed with PR. Regarding the change in PBH in time wrt the baseline (Graph 1A), the following tendency is visible: most of the PBH occurred during the first year in both groups. However, no significant difference ($p(3 \text{ months})=1$; $p(6 \text{ months})=1$; $p(1 \text{ year})=0.53$; $p(2 \text{ years})=0.9$; $p(3 \text{ years})=0.9$) was found between both groups wrt the baseline.

Qualitatively, 16 teeth (4 test, 12 control) presented a PAI (final) score of 1 and 5 cases (3 test, 2 control) presented a PAI (final) score of 2. This implies that 91.3% of the cases presented qualitatively successful PBH. In the test group two cases scored PAI 3 and one 4.

Regarding the *secondary outcome*, the RL and RRA measurements of the treated teeth and their contralaterals are presented in Table 3. Noticeable is that for some teeth (1, 2, 3, 10, 11 and 18) the root dimensional changes (Δ in Table 3) scored negatively irrespective of the follow-up time, group, and type of measurement (RL or RRA). Regarding the RL (Graph 1B) the following tendency is visible: from the 12 months recall onwards (positive and negative) changes were more prominent than prior to that period. The RL change in time wrt the baseline was not significant ($p(3 \text{ months})=1$; $p(6 \text{ months})=0.5$; $p(1 \text{ year})=0.9$; $p(2 \text{ years})=1$; $p(3 \text{ years})=0.9$) in both groups.

Regarding the RRA (Graph 1C) a slow and steady change in time is visible. Similar to the RL, the RRA change in time wrt the baseline was not significant ($p(3 \text{ months})=0.4$; $p(6 \text{ months})=1$; $p(1 \text{ year})=1$; $p(2 \text{ years})=0.2$; $p(3 \text{ years})=0.4$) in both groups.

The 2D qualitative assessment of type of REP root healing is presented in Table 3. The 5 types described by Chen MY *et al.* (2012) (39) occurred, but also a combination of these types was visible (cases 8, 9, 20, 28 and 29). Furthermore, other types of healing were seen as well: no RD and no apical closure and ingrowth of hard tissue (cases 1-4, 12, 13, 22, 23). The type of root healing was non-uniform in the control group: all above-mentioned root healing types occurred. Nevertheless, in the test group 55.6% of the cases presented no RD and no apical closure (Table 3).

It is remarkable that in all cases and in all types of 2D measurements (PBH, PAI, RL and RRL) fluctuations (alternating increasing and decreasing) are seen in time (Table 3).

Ancillary analyses

The volumetric assessments are presented in Table 4. From 14 cases (8 test, 6 control) a CBCT was taken at least 1 year post REP. The peri-apical lesions 1 to 3 years post REP were in 50% of the cases fully healed. Regarding the change in RHTV (Table 4) 3 years post REP and wrt the baseline (Graph 2A), the control group presented significantly ($p= 0.03$) more RHTV than the test group. For the change in RL 3 years post REP (Table 4) and wrt the baseline (Graph 2B) there was no difference between both groups ($p= 0.72$). Regarding the change in AA, 3 years post REP (Table 4) and wrt the baseline (Graph 2C) there was a significant difference between both groups ($p= 0.05$). The same conclusions were drawn if the outlier (case 4) was omitted from the statistical calculations. For the change in maximum RHTT 3 years post REP (Table 4) and wrt the baseline (Graph 2D) there was no difference between both groups ($p= 0.4$). Regarding the change in mean RHTT (Table 4) 3 years post REP and wrt the baseline (Graph 2E) the control group performed with a value of 76.8% significantly ($p= 0.003$) different than the test group (-2.3%).

Additionally, the correlation between the 2D and 3D outcomes was investigated via linear regression. Firstly, the change in RHTV (3D) was compared to the change in RRA (2D) wrt the baseline (Graph 3A). The slope-value of 0.06 $[-0.32; 0.43]$ 95% confidence interval, $p= 0.75$) and the Spearman rank correlation of -0.094 ($p= 0.74$) showed a very weak correlation between both variables. Secondly, the change in RL (3D) was compared to the change in RL (2D) wrt the baseline (Graph 3B). The slope-value of 0.20 $[-0.1; 0.5]$ 95% confidence interval, $p= 0.17$) and the Spearman rank correlation of 0.28 ($p= 0.34$) showed a weak correlation between both variables.

Adverse events

Cases number 5, 16 and 23 (Table 2) presented a flare-up reaction within the year post REP+LPRF (Fig. 5). Consequently, case 5 received an apexification treatment 6 months post REP+LPRF. Cases 16 and 23 were moved to the control group 4.5 months and one week post REP+LPRF, respectively (Fig. 2).

Case number 19 presented aggressive replacement resorption due to sequelae of trauma (avulsion) and was extracted 5.5 months post REP. The immunohistological analysis of this tooth was reported in Meschi N. *et al.* (2019) (40).

Harms

Case numbers 10 and 17 (both in the control group and in 8-years-old patients) were performed under nitrous oxide sedation due to anxiety. That is also why case 17 was not added to the test group. Adding nitrous oxide sedation during an endodontic treatment is an extra burden for the patient (costs) and the endodontist (extra assistance and treatment time needed; requires adequate training, know-how and equipment; the nose coverage reduces the space to work in the mouth, especially in the upper front region).

In the REP+LPRF group, for 1 patient a vasovagal syncope occurred post venipuncture (case 1) and 4 patients between 7 and 13 years old wept during venipuncture (cases 4, 22, 25 and 27).

Discussion

The ESE and the AAE have prescribed success criteria for the position statement on revitalization procedures and the clinical considerations on REP respectively (25, 26). In the current study the impact of LPRF on these success criteria was investigated.

Generalisability

Regarding the primary outcome successful PBH was obtained qualitatively (91.3%) and quantitatively (87%) in the majority of the cases. This is in accordance with other trials (at least case series) where REP was applied with (11-13, 41, 42) or without APC (27-29). However, the CBCT outcome regarding PBH of 14 cases is less promising (Table 4): half of the lesions did not heal within 1 to 3 years post REP, irrespective of the application of LPRF. Similarly, in another study based on CBCT analysis of REP treated teeth, it was concluded that the presence of a peri-apical lesion prior to treatment negatively impacts the outcome (43).

~~It should be emphasized that the baseline peri-apical infection of the test group was worse than the control group, what could have led to less favorable results. When PBH failed, apexification preserved the teeth (Fig. 2). It has been shown that apexification with calcium silicate cement plugs is a viable treatment option to obtain PBH and preserve teeth up to 15 years post treatment. A systematic review and meta-analysis presented high (>90%) pooled survival and success rates for MTA-apexification and REP, without significant difference between these 2 groups (29). The cases in Fig. 3 A and B are good examples for the latter: equal survival and success of REP and apexification in the same patient, withstanding the test of time even after orthodontic therapy (Fig. 3B). Hence, except for the 2 no shows (Fig. 2), all the analyzed teeth survived until 3 years post treatment.~~

Regarding further RD based on PR, no significant difference was found between the control and the test group. However, the change in RHTV 3 years post REP was more favorable for the control group in comparison with the test group. Noticeable is that most of the negative values in root dimensional

changes in time, more specifically cases 2, 4, 22 and 25 mentioned in Table 4, were cases of the test group. Regarding the two-dimensional RRA mentioned in other studies, a great variability exists. In a systematic review, a pooled rate for RD in randomized clinical trials (REP+/- APC) of 79% was calculated, with rate variation between 21% and 100% (29). Moreover, assuming that a 20% increase in RRA is a clinically significant change, then only 2 cases in the test group (cases 24 and 25) and 4 in the control group (cases 16, 23, 28 and 29) have met this rule (Table 3) (44). It has been reported that the further RD in REP is a slow process: 30% of increase in RL in 3 years and 30% of root canal wall thickening in 1 year (45). However, also this is not consistent with the current study: concerning RL this would apply to only case 29 (control group).

In a retrospective case series in REP, the untreated contralaterals were assessed, to compare physiologic and post-REP root maturation (27). This comparison was possible due to a baseline equivalency in age between all the patients (6-11 years) and consequently the contralaterals were not fully developed on baseline. However, in the current study, 35% of the patients were older than 11 and most of them were allocated to the test group.

The qualitative assessment of the type of REP root healing was overall not predictable and non-uniform, which is in line with other studies (27, 39). ~~Relying on the CBCT images (Figs. 3-5) the root lengthening and thickening seems to be rather of a reparative nature, as seen in previous clinical and immunohistological studies (27, 36, 40, 46).~~ It should be emphasized that all cases had at the baseline a necrotic pulpal status (Table 2), hence the chance to have an at least partially vital pulp tissue was low. Furthermore, prior to treatment, 20 out of the 29 included teeth presented symptoms of an infection (abscess/fistula; Table 2), implying that the microbial load was high at the baseline. Additionally, as there is a lack of mechanical debridement in REP, an *in vitro* study reported the detrimental role of a residual biofilm on the release of TGF- β 1 after dentin conditioning (47). Moreover, in REP cases with a persistent infection, longer periods of disinfection may clinically lead to success but histologically to repair rather than regeneration (48).

A systematic review and meta-analysis failed to show any evidence for difference in outcomes between these etiological factors (49). However, a study published after that systematic review reported that the age adjusted success percentage for REP was the lowest with caries, higher with an anomaly and the highest with trauma (28). In the current study, most of the teeth (25 out of 29) received REP due to trauma, only 1 tooth had caries and 3 others an anatomical anomaly (Table 2). Hence, it is not possible to draw any accurate conclusions on this basis.

Regarding pulp sensitivity, only one third of the cases reacted positive on CDS and/or EPT and that only on one specific follow-up time point. Noticeable is that despite the presence of a persistent apical periodontitis 1 year post REP+LPRF for cases 22, 23 and 24, still a positive reaction was registered on that time point (Table 2). Furthermore, if there would be a partially vital pulp in REP treated teeth, there is still the issue that the neuronal access to the coronal dentinal tubules is blocked by restorative material (50-52). On the one hand, the latter questions the reliability of the clinical tests as well as the patient's response to such tests. On the other hand, possible explanations for the detection of cold sensibility could be the expression of some thermosensitive ion channels of dental afferent neurons and the release of adenosine triphosphate from pulp cells (53). This inconsistency is also present in scientific literature. Some previous studies with (12, 54) and without (27, 40, 46) APC have reported a total lack of or no significant positive response on pulp sensitivity tests. However, other studies with (13, 30) and without (28) APC reported significant positive reactions on CDS and/or EPT. Chrepa *et al.* showed that teeth with positive sensitivity responses were associated with greater values for RD than the teeth with negative responses (28), emphasizing the role of peripheral sensory neurons in hard tissue regulation (55). However, in the current study 55.6% of the test group reacted (on one time point) positive on CDS and/or EPT and 35.7% of the control group, even if the control group obtained significantly more RHTV.

MPC was used to avoid discoloration post REP. Nevertheless, postoperative discoloration was seen in both groups and in 14 teeth in total, most probably due to the presence of a blood and/or LPRF clot

(56). However, the discolorations were less severe than the ones with MTA, due to the lack of bismuth oxide (31).

Limitations

Even if platelet concentrates are being appraised in literature (22, 23), some hurdles exist in their implementation in dental practice. In some countries dentists are legally not allowed to perform venipuncture on patients. For instance, in Belgium only physicians and nurses are allowed to perform venipuncture (Royal Resolution of 1/6/1934 on the regulation of exercising dentistry in Belgium). Furthermore, obtaining APC demands assistance, investment in a centrifuge and the adequate equipment to manipulate the APC properly. Possible complications during or post venipuncture should be considered as well, such as haematoma at the venipuncture site, nerve injury and vasovagal syncope (57, 58). Furthermore, the compliance of young and/or anxious patients might be reduced or lacking (58, 59). Caution is also needed for bleeding disorders (58). Hence, the application of APC in dentistry is not risk-free and demands investment in an adequate setting in terms of equipment and personnel.

Exactly due to these hurdles randomization was not possible in the current study. Hence, an inconsistency exists in the present study between the test and the control group regarding the age of the included patients. Due to anxiety for venipuncture, but also due to lack of equipment and personnel to perform venipuncture in private practice, more patients younger than 10 years were treated in the control group (72%) than in the test group (36%). Furthermore, more cases in the control group (57%) had for PBH a zero value on all follow-up time points, compared to the test group (11%) and the mean baseline peri-apical lesion in the test group was three times larger than in the control group. Knowing that the pool of MSC reduces with age (60, 61) and that infection control during REP and follow-up is directly proportional to the REP outcome (40, 47, 48), then the control group was predestined to obtain more PBH and further RD than the test group. Hence, the inability

to randomize has negatively impacted the LPRF group for all outcomes assessed. ~~Also, it has been reported that age was a negative predictor for success (28).~~

Another drawback of the current study is the small sample size, even if a sample size calculation and power analysis have been performed. It does not provide room for correlations between other factors involved in the treatment that might be of interest, such as the impact of the REP-etiology or the operator (NM *versus* GVG) on the outcome. However, it should be emphasized that the study was not designed for such evaluations, as they might inflate the type-I error.

The occurrence of the many zero values in the PR assessment of PBH might be due to inaccuracy of 2D radiographic assessment (27, 36). For instance, case 4 presents zero values on all time points (Table 2). However, in Fig. 4A the pre-operative CBCT of case 4 presents a peri-apical lesion that is not visible on the baseline PR. Furthermore, the fluctuations in some root dimensional changes (Table 2) might be due to distortion in the PR as the patients and their changeable dentition grew out of the silicone guides during the 3 years of follow-up. ~~In another trial platelet rich plasma was applied in REP and the follow up was performed with PR (with individualized silicone guides) and CBCT during one year (54). The results of both assessment techniques seemed consistent. However, relying on a short term follow up (1 year) and knowing that the patients had a growing dentition (mean age of 12.66 ± 4.47), it is to our opinion obvious that less distortions would occur (instead of follow up of 3 years) and hence it is not correct to discourage the routinely use of CBCT in REP (27, 36, 54). Furthermore, the term “routinely” should have been better defined in that study, as it is obvious that especially in young patients a CBCT should be taken only for a well indicated reason (36, 62-64).~~

A critical note is that the application of MPC might have introduced some bias in the PR RL measuring techniques, due to the fact that the radiopacity of MPC is difficult to differentiate from root dentin (65, 66). Firstly, the 2D RL is measured in the current study from the apical part of MPC, but the 3D RL is measured from the CEJ. Secondly, if there would be a calcified bridge formed under the MPC due to the cemento-and osteo-inductive property of calcium silicate cements (46, 67), then the 2D RL

measurement would be falsely smaller. Hence, this might explain why the correlation between the 2D and 3D RL (Graph 3B) was weak.

Another critical note on the application of APC in general is that, even if healthy patients are included in a trial, the blood cell and growth factors content per patient are different and are furthermore age and sex related (68-70). Consequently, this will introduce bias in the analyses. Furthermore, the flare-ups appeared only in the test group (Fig. 5) and amounted up to 25% of the included cases in that group. This high flare-up rate has not been reported yet in other trials concerning REP with or without APC to our knowledge (6, 10, 12, 13, 41). On the one hand the pro-inflammatory cytokines in LPRF, such as IL-1 β , TNF- α and IL-6, might have induced the flare-up reaction (19). On the other hand, the amount of LPRF applied, might have played a role as well. Dohan Ehrenfest DM *et al.* (2010) have shown that the proliferation of human jawbone MSC is promoted by PRF in a directly proportional and dose-dependent manner (71). However, this is not the case for the SCAP. Bi J *et al.* (2020) reported that the most optimal microenvironment to stimulate SCAP for osteo/odontogenic differentiation was to apply only 1/8 of a PRF-clot. The bigger the clot, the more this effect was reduced (24). These *in vitro* studies do not tell if the findings are immediately translational to the clinical reality. However, the current findings and the fact that the mechanism of how PRF regulates the function of MSC is still unclear, are a wake-up call to rationally apply LPRF in REP. Additionally, another factor that might have influenced or impeded further RD in the test group is that the concentration of DAP applied might have exceeded the prescribed 1-5 mg/mL (26), which could have been lethal for the SCAP (72). Hence, introduction of an *in vitro* analysis of a LPRF-clot per included patient and an age-related cohort in the study design might partially shed some light on the above-mentioned issues.

Regarding the user-friendliness of the LPRF-application as a scaffold in REP: a fibrin clot/membrane, even if cut into pieces, is difficult to introduce into a root canal. Hence, other APC such as injectable PRF for instance, might be easier to apply (73).

Conclusions

REP (without LPRF) seems to be a viable treatment option to obtain PBH, cure clinical symptoms and aid further RD of necrotic immature permanent teeth. Nevertheless, all the analyzed teeth survived up to 3 years post REP and in case of failure apexification helped to preserve them. Caution is needed when evaluating REP with PRs.

Funding

No funding was applied for this study.

Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Wigler R, Kaufman AY, Lin S, Steinbock N, Hazan-Molina H, Torneck CD. Revascularization: a treatment for permanent teeth with necrotic pulp and incomplete root development. *J Endod* 2013;39:319-26.
2. Lovelace TW, Henry MA, Hargreaves KM, Diogenes A. Evaluation of the delivery of mesenchymal stem cells into the root canal space of necrotic immature teeth after clinical regenerative endodontic procedure. *J Endod* 2011;37:133-8.
3. Hilkens P, Meschi N, Lambrechts P, Bronckaers A, Lambrechts I. Dental Stem Cells in Pulp Regeneration: Near Future or Long Road Ahead? *Stem Cells Dev* 2015;24:1610-22.
4. Diogenes A, Henry MA, Teixeira FB. An update on clinical regenerative endodontics. *Endod Top* 2013;28:2-23.
5. Gronthos S, Mankani M, Brahimi J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. *Proc Natl Acad Sci U S A* 2000;97:13625-30.
6. Bezgin T, Yilmaz AD, Celik BN, Sonmez H. Concentrated platelet-rich plasma used in root canal revascularization: 2 case reports. *Int Endod J* 2014;47:41-9.
7. Bensaid W, Triffitt JT, Blanchat C, Oudina K, Sedel L, Petite H. A biodegradable fibrin scaffold for mesenchymal stem cell transplantation. *Biomaterials* 2003;24:2497-502.
8. Yamada T, Mori Y, Mishima K, Sugahara T. Nasolabial and alveolar morphology following presurgical orthopaedic treatment in complete unilateral clefts of lip, alveolus and palate. *J Craniomaxillofac Surg* 2003;31:343-7.
9. Huang FM, Yang SF, Zhao JH, Chang YC. Platelet-rich fibrin increases proliferation and differentiation of human dental pulp cells. *J Endod* 2010;36:1628-32.
10. Torabinejad M, Turman M. Revitalization of tooth with necrotic pulp and open apex by using platelet-rich plasma: a case report. *J Endod* 2011;37:265-8.
11. Jadhav G, Shah N, Logani A. Revascularization with and without platelet-rich plasma in nonvital, immature, anterior teeth: a pilot clinical study. *J Endod* 2012;38:1581-7.
12. Bakhtiar H, Esmaeili S, Fakhr Tabatabayi S, Ellini MR, Nekoofar MH, Dummer PM. Second-generation Platelet Concentrate (Platelet-rich Fibrin) as a Scaffold in Regenerative Endodontics: A Case Series. *J Endod* 2017;43:401-08.

13. Ulusoy AT, Turedi I, Cimen M, Cehreli ZC. Evaluation of Blood Clot, Platelet-rich Plasma, Platelet-rich Fibrin, and Platelet Pellet as Scaffolds in Regenerative Endodontic Treatment: A Prospective Randomized Trial. *J Endod* 2019;45:560-66.
14. Marx RE. Platelet-rich plasma: evidence to support its use. *J Oral Maxillofac Surg* 2004;62:489-96.
15. Wirz S, Dietrich M, Flanagan TC, Bokermann G, Wagner W, Schmitz-Rode T, et al. Influence of platelet-derived growth factor-AB on tissue development in autologous platelet-rich plasma gels. *Tissue Eng Part A* 2011;17:1891-9.
16. Dohan Ehrenfest DM, Rasmusson L, Albrektsson T. Classification of platelet concentrates: from pure platelet-rich plasma (P-PRP) to leucocyte- and platelet-rich fibrin (L-PRF). *Trends Biotechnol* 2009;27:158-67.
17. Gaviño Orduña JF, Caviedes-Bucheli J, Manzanares Céspedes MC, Berástegui Jimeno E, Martín Biedma B, Segura-Egea JJ, et al. Use of Platelet-rich Plasma in Endodontic Procedures in Adults: Regeneration or Repair? A Report of 3 Cases with 5 Years of Follow-up. *J Endod* 2017;43:1294-301.
18. Dohan DM, Choukroun J, Diss A, Dohan SL, Dohan AJ, Mouhyi J, et al. Platelet-rich fibrin (PRF): a second-generation platelet concentrate. Part I: technological concepts and evolution. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2006;101:e37-44.
19. Dohan DM, Choukroun J. Platelet-rich fibrin (PRF): A second-generation platelet concentrate. Part III: Leucocyte activation: A new feature for platelet concentrates. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2006:E51-5.
20. Dohan DM, Choukroun J. Platelet-rich fibrin (PRF): A second-generation platelet concentrate. Part II: Platelet-related biologic features. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2006;101:E45-50.
21. Arnoczky SP, Sheibani-Rad S. The basic science of platelet-rich plasma (PRP): what clinicians need to know. *Sports Med Arthrosc Rev* 2013;21:180-5.
22. Castro AB, Meschi N, Temmerman A, Pinto N, Lambrechts P, Teughels W, et al. Regenerative potential of leucocyte- and platelet-rich fibrin. Part A: intra-bony defects, furcation defects and periodontal plastic surgery. A systematic review and meta-analysis. *J Clin Periodontol* 2017;44:67-82.
23. Castro AB, Meschi N, Temmerman A, Pinto N, Lambrechts P, Teughels W, et al. Regenerative potential of leucocyte- and platelet-rich fibrin. Part B: sinus floor elevation, alveolar ridge preservation and implant therapy. A systematic review. *J Clin Periodontol* 2017;44:225-34.
24. Bi J, Liu Y, Liu XM, Lei S, Chen X. Platelet-rich Fibrin Improves the Osteo-/Odontogenic Differentiation of Stem Cells from Apical Papilla via the Extracellular Signal-regulated Protein Kinase Signaling Pathway. *J Endod* 2020;46:648-54.
25. Galler KM, Krastl G, Simon S, Van Gorp G, Meschi N, Vahedi B, et al. European Society of Endodontology Position Statement: Revitalisation Procedures. *Int Endod J* 2016;49:717-23.
26. American Association of Endodontists: AAE Clinical Considerations for a Regenerative Procedure- Revised 4/1/2018. . https://www.aae.org/specialty/wp-content/uploads/sites/2/2018/06/ConsiderationsForRegEndo_AsOfApril2018.pdf.
27. Meschi N, EzEldeen M, Torres Garcia AE, Jacobs R, Lambrechts P. A Retrospective Case Series in Regenerative Endodontics: Trend Analysis Based on Clinical Evaluation and 2- and 3-dimensional Radiology. *J Endod* 2018.
28. Chrepa V, Joon R, Austah O, Diogenes A, Hargreaves KM, Ezeldeen M, et al. Clinical Outcomes of Immature Teeth Treated with Regenerative Endodontic Procedures-A San Antonio Study. *J Endod* 2020;46:1074-84.
29. Torabinejad M, Nosrat A, Verma P, Udochukwu O. Regenerative Endodontic Treatment or Mineral Trioxide Aggregate Apical Plug in Teeth with Necrotic Pulps and Open Apices: A Systematic Review and Meta-analysis. *J Endod* 2017.
30. Meschi N, Castro AB, Vandamme K, Quirynen M, Lambrechts P. The impact of autologous platelet concentrates on endodontic healing: a systematic review. *Platelets* 2016;27:613-33.

31. Belobrov I, Parashos P. Treatment of tooth discoloration after the use of white mineral trioxide aggregate. *J Endod* 2011;37:1017-20.
32. Kirkevang LL, Orstavik D, Wenzel A, Vaeth M. Prognostic value of the full-scale Periapical Index. *Int Endod J* 2015;48:1051-8.
33. Flake NM, Gibbs JL, Diogenes A, Hargreaves KM, Khan AA. A standardized novel method to measure radiographic root changes after endodontic therapy in immature teeth. *J Endod* 2014;40:46-50.
34. EzEldeen M, Wyatt J, Al-Rimawi A, Coucke W, Shaheen E, Lambrichts I, et al. Use of CBCT Guidance for Tooth Autotransplantation in Children. *J Dent Res* 2019;98:406-13.
35. Lahoud P, EzEldeen M, Beznik T, Willems H, Leite A, Van Gerven A, et al. Artificial Intelligence for Fast and Accurate 3-Dimensional Tooth Segmentation on Cone-beam Computed Tomography. *J Endod* 2021;47:827-35.
36. EzEldeen M, Van Gorp G, Van Dessel J, Vandermeulen D, Jacobs R. 3-dimensional analysis of regenerative endodontic treatment outcome. *J Endod* 2015;41:317-24.
37. Maes F, Collignon A, Vandermeulen D, Marchal G, Suetens P. Multimodality image registration by maximization of mutual information. *IEEE Trans Med Imaging* 1997;16:187-98.
38. Nagy MM, Tawfik HE, Hashem AA, Abu-Seida AM. Regenerative potential of immature permanent teeth with necrotic pulps after different regenerative protocols. *J Endod* 2014;40:192-8.
39. Chen MY, Chen KL, Chen CA, Tayebaty F, Rosenberg PA, Lin LM. Responses of immature permanent teeth with infected necrotic pulp tissue and apical periodontitis/abscess to revascularization procedures. *Int Endod J* 2012;45:294-305.
40. Meschi N, Hilken P, Van Gorp G, Strijbos O, Mavridou A, Cadenas de Llano Perula M, et al. Regenerative Endodontic Procedures Posttrauma: Immunohistologic Analysis of a Retrospective Series of Failed Cases. *J Endod* 2019;45:427-34.
41. Bezgin T, Yilmaz AD, Celik BN, Kolsuz ME, Sonmez H. Efficacy of platelet-rich plasma as a scaffold in regenerative endodontic treatment. *J Endod* 2015;41:36-44.
42. Jadhav GR, Shah N, Logani A. Comparative outcome of revascularization in bilateral, non-vital, immature maxillary anterior teeth supplemented with or without platelet rich plasma: A case series. *J Conserv Dent* 2013;16:568-72.
43. Shetty H, Shetty S, Kakade A, Mali S, Shetty A, Neelakantan P. Three-dimensional qualitative and quantitative analyses of the effect of periradicular lesions on the outcome of regenerative endodontic procedures: A prospective clinical study. *Clin Oral Investig* 2020.
44. Saoud TM, Zaazou A, Nabil A, Moussa S, Aly HM, Okazaki K, et al. Histological observations of pulpal replacement tissue in immature dog teeth after revascularization of infected pulps. *Dent Traumatol* 2015;31:243-9.
45. Bose R, Nummikoski P, Hargreaves K. A retrospective evaluation of radiographic outcomes in immature teeth with necrotic root canal systems treated with regenerative endodontic procedures. *J Endod* 2009;35:1343-9.
46. Meschi N, Hilken P, Lambrichts I, Van den Eynde K, Mavridou A, Strijbos O, et al. Regenerative endodontic procedure of an infected immature permanent human tooth: an immunohistological study. *Clin Oral Investig* 2016;20:807-14.
47. Cameron R, Claudia E, Ping W, Erin S, Ruparel NB. Effect of a Residual Biofilm on Release of Transforming Growth Factor β 1 from Dentin. *J Endod* 2019;45:1119-25.
48. Lui JN, Lim WY, Ricucci D. An Immunofluorescence Study to Analyze Wound Healing Outcomes of Regenerative Endodontics in an Immature Premolar with Chronic Apical Abscess. *J Endod* 2020;46:627-40.
49. Koç S, Del Fabbro M. Does the Etiology of Pulp Necrosis Affect Regenerative Endodontic Treatment Outcomes? A Systematic Review and Meta-analyses. *J Evid Based Dent Pract* 2020;20:101400.
50. Närhi M, Hirvonen T, Huopaniemi T. The function of intradental nerves in relation to the sensations induced by dental stimulation. *Acupunct Electrother Res* 1984;9:107-13.

51. Ahlquist M, Franzén O, Coffey J, Pashley D. Dental pain evoked by hydrostatic pressures applied to exposed dentin in man: a test of the hydrodynamic theory of dentin sensitivity. *J Endod* 1994;20:130-4.
52. Diogenes A, Ruparel NB, Shiloah Y, Hargreaves KM. Regenerative endodontics: A way forward. *J Am Dent Assoc* 2016;147:372-80.
53. Diogenes A. Trigeminal Sensory Neurons and Pulp Regeneration. *J Endod* 2020;46:S71-s80.
54. ElSheshtawy AS, Nazzal H, El Shahawy OI, El Baz AA, Ismail SM, Kang J, et al. The effect of platelet-rich plasma as a scaffold in regeneration/revitalization endodontics of immature permanent teeth assessed using 2-dimensional radiographs and cone beam computed tomography: a randomized controlled trial. *Int Endod J* 2020;53:905-21.
55. Austah O, Joon R, Fath WM, Chrepa V, Diogenes A, Ezeldeen M, et al. Comprehensive Characterization of 2 Immature Teeth Treated with Regenerative Endodontic Procedures. *J Endod* 2018;44:1802-11.
56. Możyńska J, Metlerski M, Lipski M, Nowicka A. Tooth Discoloration Induced by Different Calcium Silicate-based Cements: A Systematic Review of In Vitro Studies. *J Endod* 2017;43:1593-601.
57. Galena HJ. Complications occurring from diagnostic venipuncture. *J Fam Pract* 1992;34:582-4.
58. *WHO Guidelines Approved by the Guidelines Review Committee*. In: *WHO Guidelines on Drawing Blood: Best Practices in Phlebotomy*. Geneva: World Health Organization Copyright © 2010.
59. Eder AF, Dy BA, Kennedy JM, Notari IV EP, Strupp A, Wissel ME, et al. The American Red Cross donor hemovigilance program: complications of blood donation reported in 2006. *Transfusion (Paris)* 2008;48:1809-19.
60. Jussila M, Juuri E, Thesleff I. Tooth morphogenesis and renewal. In: Huang JTG, Thesleff I, eds. *Stem cells in craniofacial development and regeneration*. New Jersey: Wiley-Blackwell; 2013:109-34.
61. Schultz MB, Sinclair DA. When stem cells grow old: phenotypes and mechanisms of stem cell aging. *Development* 2016;143:3-14.
62. Farman AG. ALARA still applies. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2005;100:395-7.
63. Patel S, Brown J, Semper M, Abella F, Mannocci F. European Society of Endodontology position statement: Use of cone beam computed tomography in Endodontics: European Society of Endodontology (ESE) developed by. *Int Endod J* 2019;52:1675-78.
64. EzEldeen M, Stratis A, Coucke W, Codari M, Politis C, Jacobs R. As Low Dose as Sufficient Quality: Optimization of Cone-beam Computed Tomographic Scanning Protocol for Tooth Autotransplantation Planning and Follow-up in Children. *J Endod* 2017;43:210-17.
65. Camilleri J, Montesin FE, Curtis RV, Ford TR. Characterization of Portland cement for use as a dental restorative material. *Dent Mater* 2006;22:569-75.
66. Islam I, Chng HK, Yap AU. X-ray diffraction analysis of mineral trioxide aggregate and Portland cement. *Int Endod J* 2006;39:220-5.
67. Torabinejad M, Parirokh M, Dummer PMH. Mineral trioxide aggregate and other bioactive endodontic cements: an updated overview - part II: other clinical applications and complications. *Int Endod J* 2018;51:284-317.
68. Taniguchi Y, Yoshioka T, Sugaya H, Gosho M, Aoto K, Kanamori A, et al. Growth factor levels in leukocyte-poor platelet-rich plasma and correlations with donor age, gender, and platelets in the Japanese population. *J Exp Orthop* 2019;6:4.
69. Miron RJ, Dham A, Dham U, Zhang Y, Pikos MA, Sculean A. The effect of age, gender, and time between blood draw and start of centrifugation on the size outcomes of platelet-rich fibrin (PRF) membranes. *Clin Oral Investig* 2019;23:2179-85.
70. Yajamanya SR, Chatterjee A, Babu CN, Karunanithi D. Fibrin network pattern changes of platelet-rich fibrin in young versus old age group of individuals: A cell block cytology study. *J Indian Soc Periodontol* 2016;20:151-6.
71. Dohan Ehrenfest DM, Doglioli P, de Peppo GM, Del Corso M, Charrier JB. Choukroun's platelet-rich fibrin (PRF) stimulates in vitro proliferation and differentiation of human oral bone mesenchymal stem cell in a dose-dependent way. *Arch Oral Biol* 2010;55:185-94.

72. Althumairy RI, Teixeira FB, Diogenes A. Effect of dentin conditioning with intracanal medicaments on survival of stem cells of apical papilla. *J Endod* 2014;40:521-5.
73. Miron RJ, Fujioka-Kobayashi M, Hernandez M, Kandam U, Zhang Y, Ghanaati S, et al. Injectable platelet rich fibrin (i-PRF): opportunities in regenerative dentistry? *Clin Oral Investig* 2017;21:2619-27.

Journal Pre-proof

Table 1: Eligibility criteria of the controlled clinical trial. REP= regenerative endodontic procedures.

Inclusion	Exclusion
Provision of Informed Consent	Unlikely to be able to comply with the study procedures, as judged by the principal investigator
Permanent immature teeth with weak root canal walls	Patients older than 25 years
Patients younger than 25 years	Deciduous teeth
	Permanent immature teeth that can be treated by a conventional root canal treatment/ apexification
	Known or suspected current malignancy
	History of chemotherapy within 5 years prior to study
	History of radiation in the head and neck region
	History of other metabolic bone diseases
	A medical history that makes REP unfavorable
	Involvement in the planning and conduct of the study

Table 2: Demographic patients' data of the controlled clinical trial. The pulpal status of all cases was necrosis. pink= test group; blue= control group; *= drop-out; C= cold test; DE= dens evaginatus; DI= dens invaginatus; EPT= electric pulp tester; F= female; M= male; m= months; n.a.= non-applicable; nr.= number; PP= private practice; UZL= University Hospital Leuven; y= years.

Case nr.	Sex	Age (y)	Tooth nr.	Etiology	Symptoms	Center treated	Practitioner	Follow-up (m)	EPT and C post REP	Discoloration at last recall session
1	M	19	9	trauma	discoloration	UZL	NM	36	all-	yellow
2	M	18	9	caries	-	UZL	NM	38	3 m: EPT+, C+	-
3	M	14	8	trauma	fistula	UZL	NM	24	2 y: EPT+, C-	-
4	F	7	9	trauma	fistula	UZL	NM	37	all -	grey
5*	F	16	9	DI	fistula	UZL	NM	n.a.	n.a.	n.a.
6*	F	15	9	trauma	fistula	UZL	NM	n.a.	n.a.	n.a.
7	M	13	9	trauma	-	PP	NM	40	3 m: C+, 1 y: all+	grey
8	M	9	9	trauma	discoloration	PP	NM	34	all-	-
9	M	8	9	trauma	-	PP	NM	34	3 y: EPT+	grey
10	F	8	9	trauma	discoloration and abscess	PP	NM	33	all-	yellow
11	F	11	9	trauma	abscess	PP	NM	34	2 y: C+, EPT-	-
12	F	7	8	trauma	abscess	PP	NM	38	all-	-
13	F	7	9	trauma	abscess	PP	NM	41	all-	yellow
14*	F	7	8	trauma	abscess	PP	NM	n.a.	n.a.	n.a.
15*	M	7	9	trauma	fistula	PP	NM	n.a.	n.a.	n.a.
16	M	15	9	trauma	discoloration	UZL	NM	36	all-	yellow
17	M	8	8	trauma	-	UZL	NM	54	all-	yellow

18	M	18	8	trauma	-	UZL	NM	42	all-	-
19*	F	6	8	trauma	abscess	UZL	NM	7	all-	yellow
20	F	11	21	DE	abscess	UZL	GVG	38	all-	-
21	M	8	9	trauma	fistula	UZL	NM	34	1 y: C+, EPT-	yellow
22	F	8	8	trauma	abscess	UZL	NM	17	1 y: EPT+, C-	brown
23	F	8	9	trauma	abscess	UZL	NM	17	1 y: EPT+, C-	brown
24	M	12	7	DI	fistula	UZL	NM	12	1 y: C+, EPT-	-
25	F	13	8	trauma	abscess	UZL	NM	37	all-	-
26*	F	10	8	trauma	abscess	UZL	NM	n.a.	n.a.	n.a.
27	M	8	8	trauma	abscess	UZL	NM	36	all-	yellow-grey
28	M	8	8	trauma	fistula	UZL	NM	36	all-	grey
29	F	8	9	trauma	percussion pain	UZL	GVG	36	1 y: C+, EPT+	-

Table 3: Two-dimensional quantitative and qualitative assessment of the teeth (case numbers in Table 2) treated by REP. The peri-apical lesions were qualitatively assessed with the full-scale peri-apical index (PAI) scores and final PAI scores according to Kirkevang *et al.* (2015) (22), giving the same results for both expect for 1 case (case number 21, baseline). Full-scale PAI scores: 1= normal peri-apical structures, 2= small changes in bone structure, 3= changes in bone structure with some mineral loss, 4= apical periodontitis with well-defined radiolucent area, and 5= severe apical periodontitis with exacerbating features. PAI final scores: 1 and 2= success or healthy and 3–5= failure or diseased. The change in RRA is also mentioned in percentage. Baseline indicates directly after REP. The 5 types of REP healing as described by Chen *et al.* (2012) (24) are as follows: 1= increased thickening of the root canal walls and continued root maturation; 2= no significant continuation of root development with the root apex becoming blunt and closed; 3= continued root development with the apical foramen remaining open; 4= severe calcification (obliteration) of the root canal space; and 5= a hard tissue barrier formed in the canal between the coronal MPC plug and the root apex.

*= no root development and no apical closure; **= ingrowth of hard tissue.

C= contralateral tooth; CEJ= cemento-enamel junction; m= month; MPC= Pure Portland Cement Med-PZ (Medcem, Weinfelden, Switzerland); PAI= peri-apical index; PR= peri-apical radiograph; REP= regenerative endodontic procedures; RRA= radiographic root area; y= year; Δ= change between baseline and the last recall time point.

Case number	Time point PR after REP	Peri-apical lesion area (mm ²)	PAI and final score	Length from apical part MPC to apex (mm)	RRA (mm ²)	Type of REP healing
1	baseline	135.8	5	13.7	40.7	*
	3 m	133.5	5	13.9	37.7	
	6 m	77.7	5	13.9	44.5	
	1 y	61.8	3	12.9	38.8	
	2 y	22.9	3	11.2	35.7	
	3 y	34.8	3	12.8	39.2	
	Δ	101 (74.4%)	2	-0.9 (-6.6%)	-1.5 (-3.7%)	
2	baseline	102.2	5	11.4	43.1	*
	3 m	0	2	12.2	48.2	
	6 m	14.6	3	13.8	46.4	
	1 y	0	2	11.9	39.2	
	2 y	0	2	13.4	44.9	
	Δ	102.2 (100%)	3	2 (17.5%)	1.8 (4.2%)	
	baseline	58.9	5	11	40.4	

3	3 m	17.4	3	10.5	38.4	1
	6 m	0	2	11.7	39.5	
	1 y	0	1	11.4	37.8	
	2 y	0	1	11.6	38.8	
	Δ	58.9 (100%)	4	0.6 (5.5%)	-1.6 (-4%)	
4	baseline	0	1	11.3	22.4	* + **
	3 m	0	1	11.1	22.5	
	6 m	0	1	10.6	23.6	
	1 y	0	1	10.2	25.2	
	2 y	0	1	10	23.2	
	3 y	0	1	8.4	20.9	
	Δ	0	0	-2.6 (-23%)	-1.5 (-6.7%)	
7	baseline	0	2	8	39.9	3
	3 m	0	3	8.3	55.2	
	6 m	0	2	8.6	43.7	
	1 y	0	1	10.1	40.6	
	2 y	0	1	10.6	39.6	
	Δ	0	1	2.6 (32.5%)	-0.3 (-4.3%)	
8	baseline	0	1	11.9	57.1	1+5
	3 m	0	1	11.1	51.7	
	6 m	0	1	12.2	60	
	1 y	0	1	10.3	52.7	
	2 y	0	1	11.3	56	
	3 y	0	1	10.7	55.1	
	Δ	0	0	-1.2 (-10.1%)	-2 (-3.5%)	
9	baseline	0	1	9	60.1	1+4
	3 m	0	1	9.4	60	
	6 m	0	1	9.2	61	
	1 y	0	1	8.7	57.5	
	2 y	0	1	9	56.8	
	3 y	0	1	9.9	63.4	
	Δ	0	0	0.9 (10%)	3.3 (5.5%)	

10	baseline	15.8	4	10.6	70	2
	3 m	0	1	11.2	70.6	
	1 y	0	1	9.1	53.8	
	2 y	0	1	9.7	59	
	3 y	0	1	10.4	60.9	
	Δ	15.8 (100%)	3	-0.2 (-9.4%)	-9.1 (-13%)	
11	baseline	65.4	5	13.3	100.1	2
	3 m	51.3	5	13.5	100.4	
	6 m	0	2	13.8	104.8	
	2 y	0	2	13.2	103.1	
	3 y	0	1	12.4	93.5	
	Δ	65.4 (100%)	4	-0.9 (-6.8%)	-6.6 (-6.6%)	
12	baseline	0	3	10	55.5	*+**
	3 m	0	1	12.1	59	
	6 m	0	1	10.2	59.3	
	1 y	0	1	9.8	59.7	
	3 y	0	1	9.6	61.8	
	Δ	0	2	-0.4 (-4%)	6.3 (11.4%)	
13	baseline	0	1	4.2	51.6	*+**
	3 m	0	1	4.4	59.6	
	6 m	0	1	4.1	58.7	
	1 y	0	1	4.8	52.3	
	3 y	0	1	3.8	60.4	
	Δ	0	0	-0.4 (-9.5%)	8.6 (16.7%)	
16	baseline	119.4	5	6.6	64	1
	3 m	59.5	5	6.6	68.2	
	6 m	56.1	5	6.4	63.4	
	1 y	0	2	6.4	69.7	
	2 y	0	1	5.5	64.2	
	3 y	0	1	5.4	77.1	
	Δ	119.4 (100%)	4	-1.2 (-18.2%)	13.1 (20.5%)	
	baseline	0	1	10.3	72.5	

17	3 m	0	1	8.3	67	1
	6 m	0	1	10	76.6	
	1 y	0	1	11.4	86.8	
	2 y	0	1	10.1	80.4	
	Δ	0	0	-0.2 (-1.9%)	7.9 (11%)	
18	baseline	65.8	5	8.3	65.4	3
	3 m	54.7	5	8.1	66.5	
	6 m	79	5	8.7	74	
	1 y	9.7	3	8.4	68.7	
	2 y	0	2	8.2	71.5	
	3 y	0	2	8.4	69	
	Δ	65.8 (100%)	3	0.1 (1.2%)	-3.6 (-5.5%)	
20	baseline	9.1	4	9.4	66.7	2+5
	3 m	9	4	9	77	
	6 m	0	1	8.1	63.5	
	1 y	2.2	3	7	66.5	
	15 m	2	3	7.3	65.1	
	27 m	0	2	9.7	69.4	
	Δ	9.1 (100%)	2	0.3 (3.2%)	2.7 (4.1%)	
21	baseline	13.6	3 (final score: 4)	15.3	41.9	3
	3 m	0	2	15.4	43.9	
	6 m	0	2	15.7	40.4	
	1 y	0	2	16.3	51	
	2 y	0	2	16	41.4	
	3 y	0	1	16.7	45.2	
	Δ	13.6 (100%)	2 (3)	1.4 (9.2%)	3.1 (7.4%)	
22	baseline	43.7	4	10.3	37.3	*
	3 m	0	2	9.8	34.3	
	1 y	7.3	2	11.1	27.1	
	18 m	5.4	2	10.2	33.4	
	Δ	38.3 (87.6%)	2	-0.1 (-1%)	-3.9 (-10.5%)	

23	baseline	15.3	4	9.5	22.1	*
	3 m	0	2	9.3	39.5	
	1 y	0	2	8.1	28.6	
	18 m	0	1	8.2	43.1	
	Δ	15.3 (100%)	3	-1.3 (-13.7%)	21 (95%)	
24	baseline	62.3	5	11.4	30.1	1
	3 m	68.4	5	10.9	31.7	
	6 m	3.6	3	10.3	38.5	
	1 y	70.1	4	10.4	42.7	
	Δ	-7.8 (-12.5%)	1	-1 (-8.8%)	12.6 (41.9%)	
25	baseline	37.6	4	15.7	62.8	*
	3 m	17.5	3	16.2	58.8	
	6 m	12.5	3	16.9	67	
	1 y	12.7	2	18.3	68.1	
	2 y	7.8	2	16.3	65.4	
	3 y	0	2	16.4	75.2	
	Δ	37.6 (100%)	2	0.7 (4.5%)	12.4 (19.8%)	
27	baseline	14.6	4	15.1	42.7	2
	3 m	0	1	15.4	47.7	
	6 m	0	1	19.4	76.1	
	1 y	0	1	15.6	43.7	
	2 y	0	1	16	45	
	3 y	0	1	16.3	44.7	
	Δ	14.6 (100%)	3	1.2 (8%)	2 (4.7%)	
28	baseline	0	2	7.5	17.2	1+2
	3 m	0	1	8	46	
	6 m	0	1	6.9	21.7	
	1 y	0	1	8	19.6	
	2 y	0	1	7.6	21.7	
	3 y	0	1	11.3	39.9	
	Δ	0	1	3.8 (50.7%)	22.7 (132%)	
	baseline	0	1	10	34.2	

29	3 m	0	1	10.4	34.1	1+2
	6 m	0	1	11	40.4	
	1 y	0	1	11.3	33.9	
	18 m	0	1	13	48.8	
	25 m	0	1	13.2	48	
	Δ	0	0	3.2 (32%)	13.8 (40.4%)	

Case number	Change (%) in root hard tissue volume	Change (%) in root length	Change (%) in apical area	Change (%) in maximum root hard tissue thickness	Change (%) in mean root hard tissue thickness	Complete healing peri-apical lesion 1-3 years post REP
1	23.0	7.5	-38.1	2.9	22.2	No (3)
2	-15.9	0.0	0.0	2.0	-9.1	No (3)
3	11.6	1.9	0.0	11.7	8.3	Yes (2)
4	-27.7	-27.8	490.9	-30.8	-18.2	Yes (3)
16	28.1	0.9	-100	-1.4	158.3	Yes (3)
18	17.0	1.8	-36.8	5.1	38.5	Yes (3)
20	13.0	1.0	-66.7	9.9	58.3	Yes (3)
22	37.1	-9.4	-100.0	8.6	300.0	No (1)
23	52.6	1.2	-100.0	-9.4	172.7	No (1)
24	0.9	0.0	-47.1	2.0	7.7	No (1)
25	-7.8	4.8	-6.3	1.8	-6.3	No (3)
27	3.3	6.7	-40.0	-16.2	0.0	Yes (3)
28	38.8	2.0	-100.0	6.7	92.3	No (3)
29	31.2	4.6	-75.4	1.9	36.4	Yes (3)

Table 4: Volumetric measurements of teeth treated with a regenerative endodontic procedure (REP). For all measurements, the percentage of change was related to the preoperative measurement. The last column presents the peri-apical bone healing on the last cone beam CT taken post REP (numbers in brackets= 1, 2 or 3 years).

Fig. 1: Production of leukocyte and platelet rich fibrin (LPRF). After venipuncture, 10 ml of blood is collected in a sterile tube without any additives (a). The tubes are centrifuged (Intra-Lock® International, Florida, USA) for 12 minutes at 2700 rpm (b). Blood is separated into three components with the formation of a fibrin clot in the middle, acellular plasma at the top and red blood cells at the bottom (c). The clot itself (d) can be transformed into a membrane by means of pressure under a sterile glass plate during 5 minutes (e). The exudate of the pressed clot can be collected as well (f).

Fig. 2: Flow diagram of a multicenter controlled clinical trial in regenerative endodontic procedures (REP) with leukocyte and platelet rich fibrin (LPRF). The case numbers refer to the cases in Table 2. n= number of teeth; AP= apical periodontitis.

Fig. 3: Cases of the REP-LPRF group. Due to an enamel-dentin fracture of both central upper permanent incisors an endodontic intervention was required.

- A. Case nr. 8 of Table 2: The right upper central incisor received an apexification treatment with an apical MTA-plug, resin backfill and a fiber glass post. On the left upper central incisor a REP was performed. Peri-apical radiograph: baseline (a), follow-up: 3 m (b), 6m (c), 12m (d), 24m (e), 36m (f). Cone beam CT post 36 m (g), clinical image at 36 m (h).
- B. Case nr. 7 of Table 2: The right upper central incisor received an apexification treatment with Biodentine (Septodont, Saint-Maur-des-Fossés, France) and the left upper central incisor a REP. Peri-apical radiograph: baseline (a), follow-up: 3m (b), 6m (c), 1,5 y (d), 3y (e). Cone beam CT (f-h) and clinical images (i,j) at 36m: the right upper central incisor presented on root level regions of inflammatory resorption and ankylosis and coronally a palatal abfraction of the enamel due to sequelae of trauma and/or collateral damage due to orthodontic treatment.
- C. Case nr. 20 of Table 2: REP of an infected immature premolar due to an evagination on the buccal cusp.
 - I. *Clinical images.* (a-g) during the first REP session: (a) jaw swelling, (b) arrow points at evagination, (c,d) necrotic pulp tissue below the evagination, (e) inflammatory bleeding, (f) after disinfection, (g) calcium hydroxide inside the root canal; (h-n) second REP-session: (h) peri-apical blood is triggered after disinfection, (i,j) Collaplug (Zimmer Biomet, Berlin, Germany) is placed upon the blood clot, (k) Portland cement (Medcem, Weinfelden, Switzerland) is placed upon the Collaplug, (l) glass ionomer cement is placed upon the Portland cement, (m) composite restoration; (o,p) 3 years post REP.
 - II. *Peri-apical radiographs.* (a) baseline, follow-up: (b) 3 m, (c) 6m, (d) 12m, (e) 24m, (f) 36m.
 - III. *CBCT.* (a-d) prior to treatment, (e-g) 2 years post REP, (h-j) 3 years post REP.
 - IV. *Volumetric part comparison analysis* (a-c): assessment of the root surface remodeling by superimposing the pre-REP images on post-REP. The distance between the pre and post 3D surfaces is measured and expressed as a surface distance mask (= color coded map on the 3D surface post-REP), showing where the root has changed (negative-> positive values: blue-> green->yellow-> red).

CT= computed tomography; LPRF= leukocyte and platelet rich fibrin; m= months; REP= regenerative endodontic procedures; y= year

Fig. 4: Cases of the REP+LPRF group.

A. Case nr. 4 of Table 2 .

- I. *Clinical images.* (a) Enamel-dentin fracture of both upper central permanent incisors. The left upper central incisor was also intruded and presented alveolar bone loss. Both teeth were covered with calcium hydroxide, glass ionomer lining and a composite restoration. A rigid splint was placed for 4 weeks. (b,c) After splint removal, the left upper central incisor presented a vestibular abscess and received a REP treatment. (d,e) First REP session: the root canal was disinfected and calcium hydroxide was placed (e). (f-h) Second REP-session: (f) after rinsing with EDTA, the root canal was rinsed with LPRF exudate (g), a peri-apical blood clot was triggered and the root canal was filled with a LPRF clot (i,j). The LPRF was covered with Portland cement (Medcem, Weinfelden, Switzerland) (k), glass ionomer lining and a composite restoration (l). (m) 3 years post REP: the left upper central incisor is ankylotic and in infra-occlusion, that was corrected by means of a composite restoration (n).
- II. *Peri-apical radiographs.* baseline (a), follow-up: 3 m (b), 6m (c), 12m (d), 24m (e), 36m (f). During the second year post trauma, the right upper central incisor presented pain symptoms and received an apexification treatment with an apical Portland cement plug, resin backfill and a fiber glass post. REP was not performed on this tooth, as at this stage the root was quite mature.
- III. *CBCT.* (a-c) prior to treatment, (d-f) 2 years post REP, (g-i) 3 years post REP.
- IV. *Volumetric hard tissue thickness analysis:* morphological comparison between the untreated tooth 8 (a, c) and the REP-tooth 9 (b, d): a and b: baseline; c and d: 3 years later. Volumetric part comparison analysis of teeth 8 (e) and 9 (f): assessment of the root surface remodeling by superimposing the pre on post images. The distance between the pre and post 3D surfaces is measured and expressed as a surface distance mask (= color coded map on the 3D surface post-REP), showing where the root has changed (negative -> positive values: blue-> green->yellow-> red).

B. Case nr. 27 of Table 2.

- I. *Clinical images.* (a) Symptomatic right upper central permanent incisor due to concussion and enamel-dentin fracture. A REP was performed. (b,c) Three years post REP: the treated tooth is greyish discolored but asymptomatic.
- II. *Peri-apical radiographs.* baseline (a), follow-up: 3 m (b), 6m (c), 12m (d), 24m (e), 36m (f).
- III. *CBCT.* (a-c) prior to treatment, (d-f) 2 years post REP, (g-i) 3 years post REP. (e and h) periapically: calcium hydroxide puf.
- IV. *Volumetric hard tissue thickness analysis:* morphological comparison between the REP- tooth 8 (a, c) and the untreated contralateral 9 (b, d): a and b: pre-REP; c and d: 3 years later. Volumetric part comparison analysis of teeth 8 (e) and 9 (f): assessment of the root surface remodeling by superimposing the pre on post images. The distance between the pre and post 3D surfaces is measured and expressed as a surface distance mask (= color coded map on the 3D surface post-REP), showing where the root has changed (negative-> positive values: blue-> green->yellow-> red).

CT= computed tomography; LPRF= leukocyte and platelet rich fibrin; m= months; REP= regenerative endodontic procedures; y= year.

Fig. 5: Test group cases 22 and 23 of Table 2.

- I. *Clinical images.* (a) Intruded right and left upper central permanent incisor presenting a severe vestibular swelling. (b) A REP was performed. After preparation of the access cavity (by means of a ninja access) the right upper central incisor presented spontaneous inflammatory bleeding and pus came out of the left incisor. (c-f) Second REP session. (c,d) The vestibular swelling and clinical symptoms disappeared. (e) LPRF was placed as a scaffold. (f) Portland cement (Medcem, Weinfelden, Switzerland) was placed upon LPRF to seal the root canal. (g-i) A week after the second REP session, the left upper central incisor presented a vestibular abscess (g). A REP without LPRF was performed on this tooth. After removal of the composite restoration and glass ionomer, the Portland cement was drilled out with a long neck bur (h). Below the Portland cement the LPRF was intact (h). One year later, the patient presented without any symptoms. However, as there was not enough peri-apical bone healing obtained prior to orthodontic treatment, apexification was performed on both teeth. (j,k) After emptying both root canals, the apex of the right (j) and left (k) upper central incisor was visible through the microscope. (l) Portland cement was placed in both root canals.
- II. *Peri-apical radiographs.* baseline (a), 3 m post REP for the right upper central incisor and baseline post flare-up for the left upper incisor (b), 12 m for the left upper incisor and 18 m for the right incisor (c), post apexification of both teeth (d).
- III. *CBCT.* (a-d) prior to treatment, (e-h) 1 year post REP, (i-l) 1 year post apexification.

CT= computed tomography; L= left upper central incisor; LPRF= leukocyte and platelet rich fibrin; m= months; R= right upper central incisor; REP= regenerative endodontic procedures.

- REP *without* LPRF is a viable treatment to preserve immature permanent teeth with pulp necrosis;
- Pro-inflammatory cytokines in LPRF may lead to flare-up when applied in REP;
- From diagnosis to follow-up: CBCT is more accurate than PR;
- Compliance of young patients during REP is paramount and defines the study design;
- In order to apply untreated contralaterals as control in REP, baseline equivalency in the patients' age and undeveloped contralaterals are necessary.

Statement of Clinical Relevance

A total pulp necrosis and presence of a peri-apical infection pre-operatively negatively impact cell homing-based REP in terms of pulp-dentin complex regeneration. Caution is needed when adding leukocyte-containing APC as a scaffold and interpreting the REP-outcome by PRs.

A **B** **C**

A **B**

C
I

II

III

IV

A

I

II

III

IV

B

I

II

III

IV