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Influence of different pre-treatments on the resin infiltration depth into enamel of teeth affected by molar-incisor hypomineralization (MIH)

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ABSTRACT

Objectives: This *in vitro* pilot study aimed to evaluate whether different pre-treatments (demineralization, deproteinization, (chemo-)mechanical reduction of the surface layer) influence the penetration depth of a resin infiltrant into MIH-affected enamel compared to initial carious lesions.
Methods: Thirty extracted human permanent molars with non-cavitated initial carious lesions ($n = 5$) or MIH ($n = 25$) were chosen and randomly assigned to six experimental groups: IC: initial caries; M: MIH; MN: MIH, 5.25% sodium hypochlorite; MM: MIH, microabrasion; MA: MIH, air abrasion; MAN: MIH, air abrasion and 5.25% sodium hypochlorite. A modified indirect dual fluorescence staining method was adopted to assess the penetration depth (PD) of the resin infiltrant and the lesion depth (LD) by confocal laser scanning microscopy (CLSM). Exemplarily, scanning electron microscopic (SEM) images were captured. The relationship between group assignment and penetration/lesion depth was estimated using a linear mixed model incorporating the tooth as random effect (two observations/tooth). The significance level was set at $p < 0.05$.
Results: For MIH-affected molars, the mean PD (in µm; median, [minimum-maximum]) were M (178.2 [32.5–748.9]), MN (275.6 [105.3–1131.0]), MM (48.7 [0.0–334.4]), MA (287.7 [239.4–491.7]), and MAN (245.4 [76.1–313.5]). Despite the observed differences in PD between the groups, these could not be statistically verified (Bonferroni, $p = 0.322$). The percentage penetration was significantly higher for IC than for MIH groups (Bonferroni, $p < 0.05$).
Significance: Compared to IC, resin infiltration into MIH-affected enamel ist more variable. Different pre-treatments influence the resin penetration into developmentally hypomineralized enamel to a fluctuating level.

1. Introduction

In 2001, molar-incisor hypomineralization (MIH) was defined as a qualitative enamel defect of systemic origin diagnosed in one to four first permanent molars with permanent incisors also being frequently affected by this developmental defect of enamel [1]. Over time, the definition has been expanded to include other teeth with demarcated opacities such as second permanent molars, premolars, canines, and second primary molars, which has also been defined as hypomineralized second primary molars (HSPM) for the latter [2–5].

The estimated global prevalence of MIH is about 13.5% according to two meta-analyses published in 2018 and in 2021 [6–8]. Despite the fact that MIH is highly prevalent around the globe [6–8], the reason for this hypomineralization has not been conclusively clarified yet. A recently published systematic review underlines the multifactorial etiology of MIH with different systemic and/or (epi-)genetic factors synergistically influencing the amelogenesis of affected teeth, especially peri- and postnatally [9].

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Though several etiological factors may be involved, MIH-affected teeth share the following clinical characteristics: demarcated opacities of creamy-white to yellow-brown color being prone to post-eruptive enamel breakdown (PEB), atypical restorations being placed to reconstruct teeth after PEB, and extractions of severely hypomineralized teeth [10]. Additionally, 34.7% of MIH-affected molars present with hypersensitivities [11]. These might result from a bacterial penetration into the porous MIH-affected enamel leading to a colonization of dentinal tubules and inflammatory reactions of the coronal pulp [12]. Apart from hypersensitivities, the vulnerability for PEB [13–15] and the increased caries susceptibility [16] are other problems associated with MIH-affected teeth. A prospective cohort study by *Fragelli et al.* (2015) showed that PEB of MIH-associated opacities is more likely to occur in molars (7%) over 12 months compared to permanent incisors (1%). Besides the type of the tooth [13,14], the color of the opacity influences the outcome, as yellow and brown opacities are at a higher susceptibility for PEB [13–15].

Reasons for the susceptibility to PEB can be found in the morphological peculiarities of MIH-affected enamel. Compared to sound enamel, the enamel of MIH-affected teeth is characterized by a reduction in the mineral quality, the mineral quantity, the hardness, and the elastic modulus. On the other hand, the protein and carbon/carbonate content are increased and the microstructure is more porous [17]. Especially yellow and brown opacities, which are extending through the whole enamel thickness, are more porous compared to demarcated opacities of creamy-white color whose porosities are located in enamel areas close to the enamel-dentin junction [18].

The general paradigm shift towards minimum intervention dentistry [19,20] is also reflected in the recently published updated treatment recommendations for MIH, the so-called “Würzburg concept”, which considers noninvasive and microinvasive treatments for MIH-affected teeth [21]. In the literature, resin infiltration has been examined as a treatment modality for MIH-affected teeth to stabilize the porous enamel and to improve its mechanical properties [22–26]. Originally, the concept of resin infiltration has been introduced as ultraconservative treatment approach for non-cavitated carious enamel lesions by having a low viscous, light-curing, TEGDMA-based resin with a high penetration coefficient fill the porosities of the lesion body to hamper the caries progression [19,27]. Several studies have proven the suitability of this microinvasive treatment “closing the gap between oral hygiene and minimally invasive dentistry” for initial carious lesions, especially those located on proximal surfaces [19,20,28–31]. It is well known that the approximately 40 μm thick surface layer of non-cavitated carious enamel lesions acts as a diffusion barrier impeding a successful resin infiltration, which can be effectively demineralized by 15% hydrochloric acid applied for 90–120 s to gain access to the lesion body for an improved resin penetration as compared to 37% phosphoric acid [27,32]. In addition to the choice of the acid for demineralization, the penetration of the resinous infiltrant is also influenced by the caries lesion activity; however, surface conditioning with 15% hydrochloric acid proved to be effective for resin infiltration of active and inactive lesions [33]. Since MIH-affected enamel is also covered by a pseudointact surface layer of about 58 μm thickness in 95% of cases [25], effective demineralization or removal of the latter could be important to gain access to the subsurface porosities. Adding to this, deproteinization with 1% sodium hypochlorite before demineralization with 15% hydrochloric acid has been recommended for an improved infiltrability of non-cavitated carious enamel lesions [34].

For the porous MIH-affected enamel, *in vitro* studies have shown variable infiltration results for different treatment protocols so far [22–24]. It was shown that the resin infiltrant penetrates MIH-affected enamel to a mean depth of about 670 μm; however, the pattern has been described as “*erratic*” after pre-treatment by etching with 15% hydrochloric acid, either with or without additional deproteinization with 0.95% w/v sodium hypochlorite [24]. The results of another study confirmed the inconsistency in resin penetration into MIH-affected enamel with penetration depths ranging from 0 μm to 900 μm after pre-treatment with 15% hydrochloric acid, 5% sodium hypochlorite, or 30% hydrogen peroxide [23]. The impact of resin infiltration on the mechanical properties remains inconclusive with one *in vitro* study reporting about an increased Vickers hardness even though only 23% of the lesions were penetrated [24], while another study could not find a significant change in Knoop hardness for resin-infiltrated MIH-affected enamel. Improved mechanical properties could be a reason for the reduced risk of PEB over 18 months when MIH-affected molars were treated with resin infiltration compared to mineralization procedures with fluoride varnish [26]. However, little is known about the influence of additional pre-treatments (e.g., microabrasion, air abrasion) on the infiltrability of MIH-affected enamel.

Against this background, the objective of the present *in vitro* pilot study was to assess whether the following pre-treatment protocols increase the infiltrability of MIH-affected enamel: (i) demineralization with hydrochloric acid alone or combined with (chemo-)mechanical pre-treatment (microabrasion, air abrasion) to reduce the surface layer thickness and expose the porous subsurface lesion, and/or (ii) deproteinization with sodium hypochlorite to reduce the organic content. Non-cavitated initial carious enamel lesions served as control to compare the findings to those of MIH-affected enamel because resin infiltration was originally invented for this entity. Even though both entities differ regarding the location, the depth, and the width of the lesions, they share some characteristics such as a mineral-rich surface layer and porosities allowing for a certain comparability [17,19,20,25,35,36].

It was hypothesized that the (chemo-)mechanical reduction of the surface layer combined with the demineralization with hydrochloric acid or the demineralization combined with deproteinization would lead to a deeper resin penetration into MIH-affected enamel. Therefore, the following null hypotheses were formulated: (i) Resin penetration into MIH-affected enamel presenting demarcated opacities does not vary compared to initial carious lesions. (ii) The different pre-treatment protocols for MIH-affected enamel opacities have no impact on the penetration depth of the resinous infiltrant.

2. Materials and methods

2.1. Specimen selection

This laboratory research was carried out in accordance with the ethical principles for medical research involving human subjects outlined in the WMA Declaration of Helsinki (2013). The local ethics committee of the Justus-Liebig-University Giessen (Germany) endorsed the study protocol (reference number: 143/09) and parents/patients obtained their informed consent for tooth collection. Thirty teeth (5 permanent (pre-)molars with active, non-cavitated initial carious lesions located on proximal surfaces (International Caries Detection and Assessment System (ICDAS) code 1–2 [37,38]); 25 first permanent molars with non-cavitated demarcated opacities assigned to MIH) freshly extracted for medical reasons (e.g., orthodontic reasons, endodontic reasons) were stored in 0.5% chloramine T solution (Chloramin T Trihydrat; Carl Roth, Karlsruhe, Germany) at room temperature for up to 28 days.

Based on the criteria published by the European Academy of Paediatric Dentistry (EAPD) in 2003 [10], first permanent molars with MIH presenting demarcated opacities of creamy-white to yellow-brown color with intact enamel surfaces in the region of interest were included. Teeth presenting other types of developmental defects of enamel were excluded.

Whereas initial carious lesions were diagnosed by visual caries detection and classified according to ICDAS, MIH was visually detected and classified according to the EAPD criteria. A consensus-based decision was reached on the diagnosis by three examiners (S.S., S.A., N.K.), with one of them being an experienced epidemiologist (N.K.).

Organic tissue residues were carefully removed with a scaler (Hu Friedy everedge SH 6/79; Hu Friedy, Rotterdam, The Netherlands). The teeth were placed in distilled water and frozen at − 18 °C until a sufficient number of MIH-affected teeth was collected for further processing. Following thawing, the teeth were cleaned with the powder-water stream (Clinpro Glycine Prophy Powder; 3 M Oral Care, Seefeld, Germany) of an air polishing system (PROPHYflex 3; KaVo Dental, Biberach/Riß, Germany). The cleaned teeth stored in sample containers (Probenbehälter 40 ml; VWR International, Leuven, Belgium) filled with distilled water were placed in a sealed opaque box for blinding purposes and then randomly allocated by adding one tooth after the other to each of the treatment groups.

2.2. Calibration training

Resin infiltration was performed by one previously calibrated operator (S.S.). For calibration purposes, additional 13 extracted human premolars and molars with active, non-cavitated initial carious lesions (ICDAS-II 1–2 [37,38]) were resin infiltrated by one operator (S.S.) according to the protocol described below (paragraph 2.4) with (n = 9) and without (n = 4) additional pre-treatment (paragraph 2.3). The penetration and lesion depths were measured independently by two examiners (S.A., S.S.) to assess the inter-rater reliability and repeatedly by the operator (S.S.) after 3 months for the assessment of the intra-rater reliability.

2.3. Pre-treatments

The enamel surfaces were subjected to different pre-treatment protocols attempting to enhance the resin infiltration according to the group assignment (Table 1 and Table 2).

Specimens of group IC (initial caries, control group) and M (MIH) were only dried for 30 s with an oil-free and anhydrous air stream. MIH-affected molars of group MN were exposed to 5.25% sodium hypochlorite (Hypochlorit-SPEIKO; SPEIKO-Dr. Speier, Bielefeld, Germany) for 2 min, rinsed with water spray for 2 min, and dried with oil-free and anhydrous air stream for 30 s. In group MM, MIH-affected enamel was microabraded using a slurry for chemical (6.6% hydrochloric acid) and mechanical abrasion (silicon carbide microparticles; Opalustre; Ultradent Products, Cologne, Germany) for 60 s according to the manufacturer's specifications. The slurry was rinsed off with water spray for 2 min and specimens were dried with an oil-free and anhydrous air stream for 30 s. Another pre-treatment for demarcated enamel opacities of MIH-affected teeth (group MA) was air abrasion (RONDOflex plus 360, KaVo Dental) with 27 µm Al_2O_3 particles (RONDOflex Pulver 27 µ, KaVo Dental) used at 4.5 bar in 1 mm distance to the enamel surface for 5 s. Again, enamel surfaces were rinsed with water spray for 30 s and air-dried for 30 s. In the last group (MAN), MIH-affected molars were pre-treated with a combination of the protocols from group MA and MN, meaning air abrasion was followed by deproteinization with 5.25% sodium hypochlorite (Hypochlorit-SPEIKO; SPEIKO-Dr. Speier).

Table 1
Composition and batch numbers of products used in this study according to the manufacturers' specifications on safety data sheets.

Product name; manufacturer, company headquarter	Batch number	Composition
Hypochlorit-SPEIKO; SPEIKO-Dr. Speier, Bielefeld, Germany	13179 13187	100 ml contain 6% (M/M) sodium hypochlorite solution (83.283 ml) in purified water (final concentration 5.25% (M/M) sodium hypochlorite)
Opalustre; Ultradent Products, Cologne, Germany	BGP4L	< 10 wt% hydrochloric acid, < 45 wt% silicon dioxide
RONDOflex Pulver 27 µ; KaVo Dental, Biberach/Riß, Germany	-	Aluminum oxide particles (particle size 27 µ)
Icon-Etch; DMG Chemisch-Pharmazeutische Fabrik, Hamburg, Germany	805650	15-20% hydrochloric acid
Icon; DMG Chemisch-Pharmazeutische Fabrik	805650	70-95% TEDMA, < 2.5% camphor quinone

Abbreviations: TEDMA: triethylene glycol dimethacrylate

Table 2
Specimen preparation according to group assignment.

Group	Tooth tissue	Pre-treatment	Resin infiltration and modified dual fluorescence staining method[29]
IC	Initial caries (ICDAS-II 1-2)	Drying with oil-free and anhydrous air stream for 30 s.	Application of 15% HCl gel (Icon-Etch; DMG Chemisch-Pharmazeutische Fabrik, Hamburg, Germany) for 2 min, rinsing with water spray for 30 s, drying with oil-free and anhydrous air stream for 30 s. Staining with 0.1% Rhodamine B isothiocyanate solution (Rhodamine B isothiocyanate CAS 36877-69-7; Santa Cruz Biotechnology, Dallas, TX, USA) for 12 h, drying with oil-free and anhydrous air stream for 30 s. Resin infiltration (Icon; DMG Chemisch-Pharmazeutische Fabrik) for 5 min, polymerization for 60 s. Storage of halved specimens in 30% hydrogen peroxide (Wasserstoffperoxid 30%; Merck, Darmstadt, Germany) for 12 h, rinsing with distilled water for 30 s, drying with oil-free and anhydrous air stream for 30 s. Staining with 100 µM sodium fluorescein (Fluorescein sodium C. I.45350; SIGMA-ALDRICH Chemie, Steinheim, Germany) for 3 min, rinsing with distilled water for 10 s, drying with oil-free and anhydrous air stream for 30 s.
M	MIH, enamel with demarcated opacity	Drying with oil-free and anhydrous air stream for 30 s.	
MN	MIH, enamel with demarcated opacity	Storage in **5.25% NaOCl** (M/M; Hypochlorit-SPEIKO; SPEIKO-Dr. Speier, Bielefeld, Germany) for 2 min, rinsing with water spray for 2 min, drying with oil-free and anhydrous air stream for 30 s.	
MM	MIH, enamel with demarcated opacity	**Microabrasion** (Opalustre; Ultradent Products, Cologne, Germany) for 60 s, rinsing with water spray for 2 min, drying with oil-free and anhydrous air stream for 30 s.	
MA	MIH, enamel with demarcated opacity	**Air abrasion** (RONDOflex plus 360; KaVo Dental, Biberach/Riß, Germany; 4.5 bar; 1 mm distance to the enamel surface) with 27 µm Al_2O_3 (RONDOflex Pulver 27 µ; KaVo Dental) for 5 s, rinsing with water spray for 30 s, drying with oil-free and anhydrous air stream for 30 s.	
MAN	MIH, enamel with demarcated opacity	**Air abrasion** (RONDOflex plus 360; KaVo Dental; 4.5 bar; 1 mm distance to the enamel surface) with 27 µm Al_2O_3 (RONDOflex Pulver 27 µ; KaVo Dental) for 5 s, rinsing with water spray for 30 s, drying with oil-free and anhydrous air stream for 30 s. Storage in **5.25% NaOCl** (M/M; Hypochlorit-SPEIKO; SPEIKO-Dr. Speier) for 2 min, rinsing with water spray for 2 min, drying with oil-free and anhydrous air stream for 30 s.	

2.4. Resin infiltration and fluorescence staining

After the additional pre-treatment, the specimens' enamel surfaces were resin infiltrated and stained by adopting a modified version of the dual fluorescence staining method described by *Paris et al.* (2009) [39].

Fifteen percent hydrochloric acid in gel form (Icon-Etch; DMG Chemisch Pharmazeutische Fabrik, Hamburg, Germany) was applied extending the lesion borders by 2 mm for 2 min. The gel was continuously moved with the application tip on the enamel to demineralize the surface layer. After having sucked off the excess gel, specimens were rinsed with water spray for 30 s and air-dried for 30 s. The exposed porosities within the lesion body were stained by 12 h storage in 0.1% Rhodamine B isothiocyanate solution (Rhodamine B isothiocyanate CAS 36877–69-7; Santa Cruz Biotechnology, Dallas, TX, USA) dissolved in 99% ethanol (Ethanol; SIGMA-ALDRICH Chemie, Steinheim, Germany) at room temperature protected from light. Specimens stained with the red fluorescence dye were dried with oil-free and anhydrous compressed air for 30 s to evaporate the solvent. The resin infiltrant (Icon-Infiltrant; DMG Chemisch Pharmazeutische Fabrik) was applied for 5 min by moving the applicator tip continuously on the enamel surface, excess was soaked up with foam pellets (Pele Tim No. 2; Voco, Cuxhaven, Germany), and the resin infiltrant was polymerized for 60 s with a light curing device (Bluephase g2; Ivoclar Vivadent, Schaan, Liechtenstein) producing a light intensity of 1200 mW/cm^2.

Then, specimens were cut into halves with a diamond-coated saw blade running perpendicularly to the lesion surface in a water-cooled precision saw (IsoMet 1000; Buehler, ITW Test & Measurement, Esslingen, Germany) working at a cutting speed of 975 rpms. Exposed residues of pulp tissue were removed with a college forceps (PLULINE 43083; Pluradent, Offenbach, Germany). A subsequent storage in 30% hydrogen peroxide (Wasserstoffperoxid 30%; Merck, Darmstadt, Germany) for 12 h at 37 °C aimed to bleach the surplus of red fluorescence dye outside the resin infiltrated area.

Following bleaching, specimens were rinsed with distilled water for 30 s, cut surfaces were polished with silicon carbide paper (Beta GRINDER-POLISHER and fine grit silicon carbide paper P4000; both Buehler, ITW Test & Measurement) for 10 s under water cooling, again rinsed with distilled water for 30 s, and dried with an oil-free and anhydrous air stream for 30 s. To stain non-infiltrated porosities with a green fluorescence dye, specimens were immersed in 100 μM sodium fluorescein solution (Fluorescein sodium C.I.45350; SIGMA-ALDRICH Chemie) dissolved in 50% ethanol (Ethanol; SIGMA-ALDRICH Chemie) for 3 min, rinsed with distilled water for 10 s, and air-dried for confocal laser scanning microscopy.

2.5. Light microscopy and confocal laser scanning microscopy

Prior to resin infiltration, light microscopic images (AZ100M; Nikon, Tokyo, Japan; using AZ Plan Apo 1 × objective lens combined with 1 × zoom factor, image resolution 1024 × 1024 pixels) were taken of the MIH-affected enamel to record the lesion color (white-opaque vs. yellow-brown) and exemplarily of the initial carious lesions.

In the following, a confocal laser scanning microscope (CLSM; AZ100M; Nikon; using AZ Plan Apo 4 × objective lens combined with 1.5 × to 2 × zoom factor, image resolution 1024 × 1024 pixels) was used in dual fluorescence mode (D-ECLIPSE C1; Nikon) to record images of the stained specimens. According to manufacturer's specifications, the absorption maximum of Rhodamine B isothiocyanate (Rhodamine B isothiocyanate CAS 36877–69-7; Santa Cruz Biotechnology) was at 543 nm and the emission maximum at 571 nm. Therefore, a helium-neon laser (CVI Melles Griot, Carlsbad, CA, USA) of 543.5 nm wavelength was used to excite the fluorescence dye. For sodium fluorescein (Fluorescein sodium C.I.45350; SIGMA-ALDRICH Chemie), the manufacturer specified an absorption maximum of 490 nm and an emission maximum of 515 nm. The excitation was performed with an argon laser (CVI Melles Griot) working at an excitation wavelength of 488 nm. Emission filters for the wavelengths 408/488/543 nm and bandpass filters (485–535 nm, 530–680 nm) were built in the beam path during image capturing.

2.6. Assessment of penetration and lesion depth

The penetration depth of the resin infiltrant (PD; red fluorescence signal) and the lesion depth (LD; green fluorescence signal) of initial caries as well as MIH were measured on the images obtained by CLSM (AZ100M; Nikon) at five defined measuring points per tooth half.

The first measurement was performed from the lesion surface to the point of deepest resin infiltration following the orientation of enamel prisms. On each side of the first measurement, two further measurements were made in the same direction after having divided the infiltrated area into three parts. The lesion depth was also measured at each of the five measuring points. Means of the penetration/lesion depth were calculated based on the five measurements per specimen half. Additionally, the measurement for the maximum and minimum penetration/lesion depth was recorded. The penetration depth in percent was computed by the following formula:

$$\text{Penetration depth}[\%] = \frac{\textit{mean penetration depth}}{\textit{mean lesion depth}} * 100. \quad (1)$$

2.7. Scanning electron microscopy

One specimen per group was prepared to capture exemplary images of the resin-infiltrated areas using a scanning electron microscope (SEM; Philips XL30; Philips Electron Optics, Eindhoven, The Netherlands; FEI XL30; FEI, Hillsboro, OR, USA) at 1000 × - 10,000 × magnification and an acceleration voltage of 15 kV in the Institute of Anatomy and Cell Biology at Justus-Liebig-University Giessen (Germany).

Specimens were demineralized by storage in 10% hydrochloric acid (Salzsäure 10%; SIGMA-ALDRICH Chemie) for 60 s according to *Paris et al.* (2012) [39]. After rinsing with distilled water, specimens were stored in ethanol (Ethanol; SIGMA-ALDRICH Chemie) of ascending concentration (70 – 100%; 2 h each) for dehydration purposes. Again, specimens were rinsed with distilled water and air-dried at room temperature for 24 h. Then, they were mounted to aluminum sample trays (Stiftprobenteller für Amray; Plano, Wetzlar, Germany) using double-sided, adhesive carbon tabs (Leit-Tabs; Plano). Finally, specimens were gold sputter-coated (Sputter Coater SC502; Polaron, Fisons Instruments, Mainz-Kastel, Germany) prior to SEM-imaging.

2.8. Statistical analysis

The tooth half ($n = 10$/group) served as unit for the statistical analysis, which was done using a statistical analysis software (IBM SPSS Statistics 26; IBM Statistics, Armonk, NY, USA) for Windows operating system (Windows 11; Microsoft, Redmond, WA, USA).

For calibration, the intraclass correlation coefficient (ICC) was determined using a two-way random effects model with a definition of absolute agreement and an interpretation of single measurements.

For descriptive statistics, minima, medians, percentiles, maxima, means, and 95% confidence intervals (CIs) were computed. Normal distribution of residuals was interpreted graphically by QQ-plots and box-and-whisker plots. Robustness analyses were conducted in case of substantial statistical outliers by checking the results through calculation with and without outliers. If the outcome was comparable, results considering outliers were reported. Moreover, variance homogeneity of residuals was considered and in case of variance heterogeneity, it was modelled and admitted. A linear mixed-effects model (LMM) with the group assignment as fixed effect and the tooth as random effect was designed. By this, tooth dependency was considered within a variance-components model. Penetration/lesion depth and percentage of penetration (penetration depth divided by lesion depth) were dependent

variables. The restricted maximum likelihood (REML) estimation was chosen to fit the linear mixed-effects model. A calculation of stratified residuals followed. Bonferroni correction was applied to account for a feasible α inflation resulting from multiple pairwise comparisons during post hoc tests. The level of significance was set at $p < 0.05$.

2.9. Post hoc sensitivity analysis

A post hoc sensitivity analysis was performed to assess the robustness of results of this pilot study using a statistical analysis software (Prozedur -power; Stata 16.1, StataCorp, College Station, TX, USA). Based on a power of 0.8 and $\alpha = 0.05$, effect sizes within the population of at least Cohen's $d = 1.73$ (large population effect size according to Cohen (1992) [40]) would be needed to detect differences between two of the experimental groups with statistical reliability considering the study design with 5 teeth per group, 2 observations per tooth, and the dependence of data due to multiple measurements on tooth level (intra-class coefficient ICC = 0.9).

3. Results

3.1. Color of the demarcated opacities

The demarcated opacities of 8 MIH-affected tooth halves were of creamy-white color. The remaining 42 tooth halves were affected by demarcated opacities of yellow-brown color. Except for the MIH-group being pre-treated with air abrasion (Group MA), in which the demarcated opacities of all specimens were yellow to brown ($n = 10$), the other groups of MIH-affected teeth were composed of 2 specimens with creamy-white demarcated opacities and 8 tooth halves presenting with demarcated opacities of yellow-brown color. The mean lesion depth was 1600.0 ± 225.7 µm for lesions of creamy-white color, and 1535.1 ± 294.7 µm for lesions of yellow-brown color with a small influence of the lesion color on the mean lesion depth (Cohen's $d = 0.23$). The mean penetration depth irrespective of the pre-treatment chosen was 258.9 ± 58.2 µm for lesions of creamy-white color, and 264.7 ± 223.9 µm for lesions of yellow-brown color with an effect size of Cohen's $d = 0.03$. The influence of the lesion color on the mean penetration depth of creamy-white of yellow-brown demarcated opacities was small.

3.2. Lesion depth

Estimated means of the lesion depth (LD) were significantly higher for all groups with MIH-affected teeth compared to initial carious lesions (Bonferroni, $p < 0.001$; Table 3; Fig. 1 and Fig. 2). On average, initial carious lesions (Group IC) showed a LD of 356.4 µm, 95% CI [122.4, 590.3]. The corresponding values for hypomineralized enamel of MIH-affected teeth ranged from 1422.7 µm, [1176.1, 1669.2] in Group MA to 1739.9 µm, [1511.2, 1968.7] in Group M. Likewise, the estimated maximum LD and minimum LD were significantly higher for all MIH-Groups (M, MN, MM, MA, MAN) in comparison to teeth with initial carious lesions (Group IC; Bonferroni, $p < 0.001$). The deepest lesions in terms of signs of hypomineralization were observed in Group M, where maximum values of 1813.0 µm, [1571.0, 2055.1] were measured.

Table 3
Estimated means [95% CI] of the lesion depth in µm (mean, maximum, minimum) according to group assignment for initial carious lesions (IC) or MIH-affected teeth after different pre-treatment procedures (M = no pre-treatment; MN = 5.25% sodium hypochlorite, MM = microabrasion; MA = air abrasion; MAN = combination of air abrasion and 5.25% sodium hypochlorite). Different lower case superscript letters indicate statistically significant differences among groups (Bonferroni, p < 0.001).

Group	Lesion depth (LD, in µm)		
	Mean	Maximum	Minimum
IC	356.4 [122.4, 590.3][a]	480.6 [230.8, 730.4][c]	243.3 [− 0.251, 486.9][e]
M	1739.9 [1511.2, 1968.7][b]	1813.0 [1571.0, 2055.1][d]	1683.5 [1441.8, 1925.2][f]
MN	1423.1 [1178.2, 1668.1][b]	1570.0 [1317.3, 1822.8][d]	1253.4 [987.3, 1519.5][f]
MM	1488.1 [1259.1, 1717.1][b]	1594.0 [1351.4, 1836.7][d]	1341.3 [1096.9, 1585.7][f]
MA	1422.7 [1176.1, 1669.2][b]	1537.8 [1281.5, 1794.2][d]	1276.3 [1005.5, 1547.1][f]
MAN	1653.6 [1395.6, 1911.6][b]	1718.9 [1451.6, 1986.2][d]	1578.4 [1304.5, 1852.3][f]

3.3. Penetration depth

Medians were reported for this parameter because means were skewed due to single outliers. The robustness analysis revealed that the results with all cases were confirmed by the statistical analysis without outliers, therefore, all specimens were included in the analysis ($n = 10$). The resin infiltration measured as penetration depth (PD) of the low viscous resinous infiltrant into the porous enamel exhibited no statistically significant differences among groups, irrespective of the tooth tissue under investigation ($p > 0.05$; Table 4; Fig. 1 and Fig. 2). However, a clear scattering of mean, maximum, and minimum PD was evident for MIH-affected enamel within each group and between the groups. In MIH-affected molars pretreated with microabrasion (Group MM), the lowest mean PD of the resinous infiltrant was achieved (48.7 µm, [0.0–334.4]). The most favorable mean PD was achieved in specimens where the demarcated opacities were pretreated with either sodium hypochlorite (Group MN; 275.6 µm, [105.3–1131.0]), air abrasion (Group MA; 287.7 [239.4–491.7]), or a combined pre-treatment with air abrasion followed by sodium hypochlorite (Group MAN; 245.4 [76.1–313.5]). Despite the observed differences in the mean penetration depth between the groups (Cohen's $d = 1.73$), these could not be statistically verified ($p = 0.322$).

3.4. Percentage penetration depth

Estimated mean percentage penetration depth (%PD) was significantly higher for initial carious lesions (Group IC) with 67%, [54.2,

Fig. 1. CLSM image of an initial carious lesion after almost complete resin infiltration at 8 × magnification. I: infiltrated area (red), remaining porosities (green); E: enamel; D: dentin.

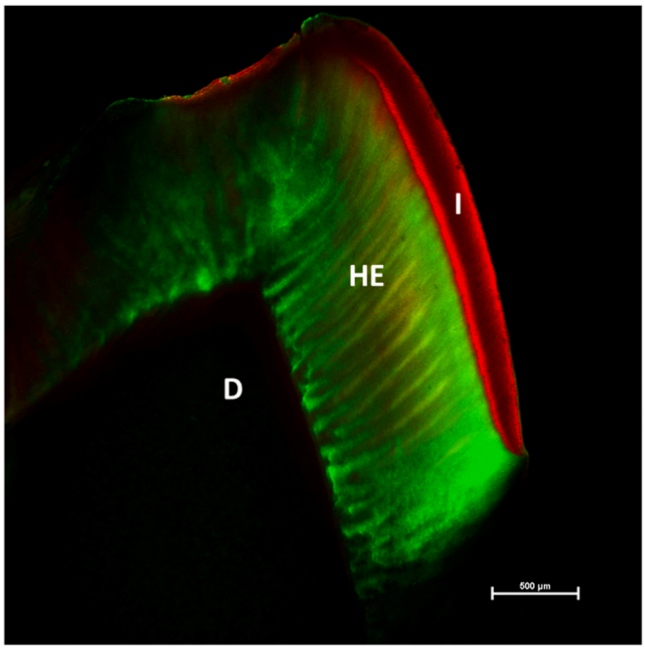

Fig. 2. CLSM image of an MIH-affected lesion after pre-treatment with air abrasion and 5.25% sodium hypochlorite followed by resin infiltration (group MAN) at 6 × magnification. The infiltrated area is located in the outer part of the MIH-affected enamel (red), whereas the hypomineralization (green) extends through the entire thickness of enamel. I: infiltrated area (red), HE: hypomineralized enamel (green); D: dentin.

Table 4
Medians [Minima – Maxima] of the resin infiltrant's penetration depth (mean, maximum, minimum) according to group assignment for initial carious lesions (IC) or MIH-affected teeth after different pre-treatment procedures (M = no pre-treatment; MN = 5.25% sodium hypochlorite, MM = microabrasion; MA = air abrasion; MAN = combination of air abrasion and 5.25% sodium hypochlorite).

Group	Penetration depth (PD, in µm)		
	Mean	Maximum	Minimum
IC	194.1 [100.6-329.6]	269.7 [127.8-603.2]	120.3 [42.1-207.5]
M	178.2 [32.5-748.9]	268.9 [39.2-936.8]	109.5 [21.9-624.3]
MN	275.6 [105.3-1131.0]	387.2 [195.5-1745.6]	197.3 [42.9-611.9]
MM	48.7 [0.0-334.4]	64.7 [0.0-401.1]	36.7 [0.0-255.1]
MA	287.7 [239.4-491.7]	373.7 [301.7-577.8]	206.0 [144.7-395.2]
MAN	245.4 [76.1-313.5]	303.6 [117.3-409.3]	143.2 [51.4-230.5]
p	0.322	0.217	0.143

Table 5
Estimated means [95% CI] of the penetration depth in % (mean, maximum, minimum) according to group assignment for initial carious lesions (IC) or MIH-affected teeth after different pre-treatment procedures (M = no pre-treatment; MN = 5.25% sodium hypochlorite, MM = microabrasion; MA = air abrasion; MAN = combination of air abrasion and 5.25% sodium hypochlorite). Different lower case superscript letters indicate statistically significant differences among groups (Bonferroni, $p < 0.05$).

Group	Percentage penetration depth (%PD)		
	Mean	Point of maximum PD	Point of minimum PD
IC	67.0 [54.2, 79.7]^{a}	68.7 [51.6, 85.7]^{c}	72.7 [46.9, 98.4]^{e}
M	14.4 [2.0, 26.7]^{b}	18.5 [7.0, 30.0]^{d}	8.9 [0.5, 17.3]^{f}
MN	24.5 [11.0, 38.0]^{b}	35.6 [16.5, 54.7]^{c,d}	16.5 [8.2, 24.9]^{f}
MM	9.4 [– 2.9, 21.8]^{b}	10.3 [– 1.1, 21.8]^{d}	8.3 [1.4, 15.2]^{f}
MA	22.6 [10.3, 35.0]^{b}	25.6 [14.1, 37.1]^{d}	19.4 [12.0, 26.8]^{f}
MAN	13.9 [1.6, 26.3]^{b}	16.2 [4.8, 27.7]^{d}	9.9 [2.9, 16.9]^{f}

79.7] compared to all MIH-groups (Bonferroni, $p \leq 0.001$; Table 5). Among these, the lowest amount of resin infiltration was achieved in Group MM with 9.4% [– 2.9, 21.8] of the developmentally hypomineralized enamel being penetrated by the resinous infiltrant after pre-treatment with microabrasion. Highest estimated mean %PD were observed after sodium hypochlorite pre-treatment (Group MN) with about one quarter of the lesion (24.5% [11.0, 38.0]) being penetrated by the resin infiltrant in comparison to the MIH-group with the lowest estimated %PD, which was pre-treated by microabrasion (Group MM; 9.4% [−2.9, 21.8]). Even though differences in the percentage penetration depth could be observed between the different pre-treatment protocols for MIH-affected enamel (Cohen's $d = 1.73$), these could not be statistically verified (Bonferroni, $p = 1.000$). Among all groups with MIH-affected teeth, maximally 35.6% [16.5, 54.7] of the hypomineralized lesion were filled by resin infiltration.

3.5. Scanning electron microscopic results

In the infiltrated areas, the enamel prisms appeared to be covered by a layer of resin inflitrant on the SEM images (Fig. 3 and Fig. 4). Compared with initial carious lesions, the infiltrated area of MIH-affected enamel appeared to be structured more irregularly. Interestingly, a rather amorphous, non-porous intermediate zone could be observed in all MIH-affected specimens between the infiltrated and non-infiltrated areas. In the non-infiltrated zone, the characteristic prismatic microstructure of enamel was visible.

4. Discussion

Resin infiltration is a well-established treatment procedure to slow down the progression of proximal non-cavitated initial carious lesions [30]. It can also be successfully integrated with smooth surface carious lesions, thus providing "a time-effective treatment option for esthetically camouflaging [white spot lesions]", which would not be achieved by other treatment regimens (e.g., oral hygiene, remineralization with fluoride) likewise [41]. The penetration of a low viscous resinous infiltrant occludes the porosities within the lesion body of non-cavitated initial carious lesions on a three-dimensional basis, thus creating a diffusion barrier for organic acids and inhibiting further demineralization [20,42]. It has been shown, however, that the microporosities located in proximity to the enamel-dentin junction might not be completely penetrated by the resin, thereby leaving a certain degree of porosity within the infiltrated carious enamel lesion [20]. Like the subsurface lesion body of an initial carious lesion shows an increased pore volume of about 25% [17,43], the porosity within the microstructure of MIH-affected enamel is also increased by up to 5 to more than 25% in comparison to sound enamel [17,18,36]. Creamy-white lesions and those without PEB are less porous than yellow-brown opacities and those with PEB [36]. An influence of the lesion color on the penetration of the resinous infiltrant could not be observed in this study, however, it has to be mentioned that 84% of specimens were diagnosed with yellow-brown opacities and only 16% were creamy-white. Furthermore, MIH-affected enamel is characterized by an increase in organic content (especially proteins) [44], which can also be observed at the enamel surface [19,20] and within the dark zone of initial carious lesions [45], possibly occluding the porosities and thus impeding a successful resin infiltration [33].

Apart from these similarities characterizing initial carious lesions and demarcated opacities assigned to MIH, the lesion depth of the latter was found to be significantly deeper. This is in accordance with other studies focusing on morphological characteristics of developmentally hypomineralized enamel in which the hypomineralization was described as extending throughout the entire enamel thickness [17,36,46].

Since there were no statistically significant differences among the groups regarding the resin infiltrant's penetration depth into initial

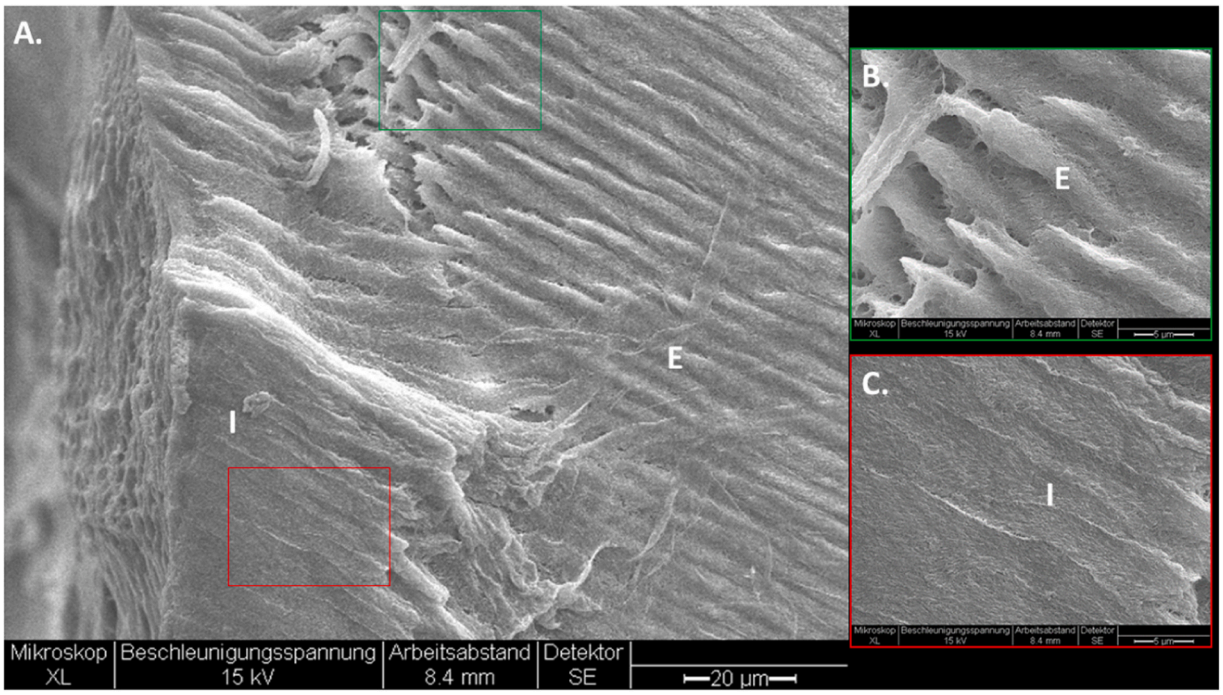

Fig. 3. SEM image of an initial carious lesion after almost complete resin infiltration (group IC) at 2000 × magnification (A.). Higher magnification of the enamel adjacent to the infiltrated area (B.) and the infiltrated area (C.) at 10,000 × magnification. I: infiltrated area, E: enamel.

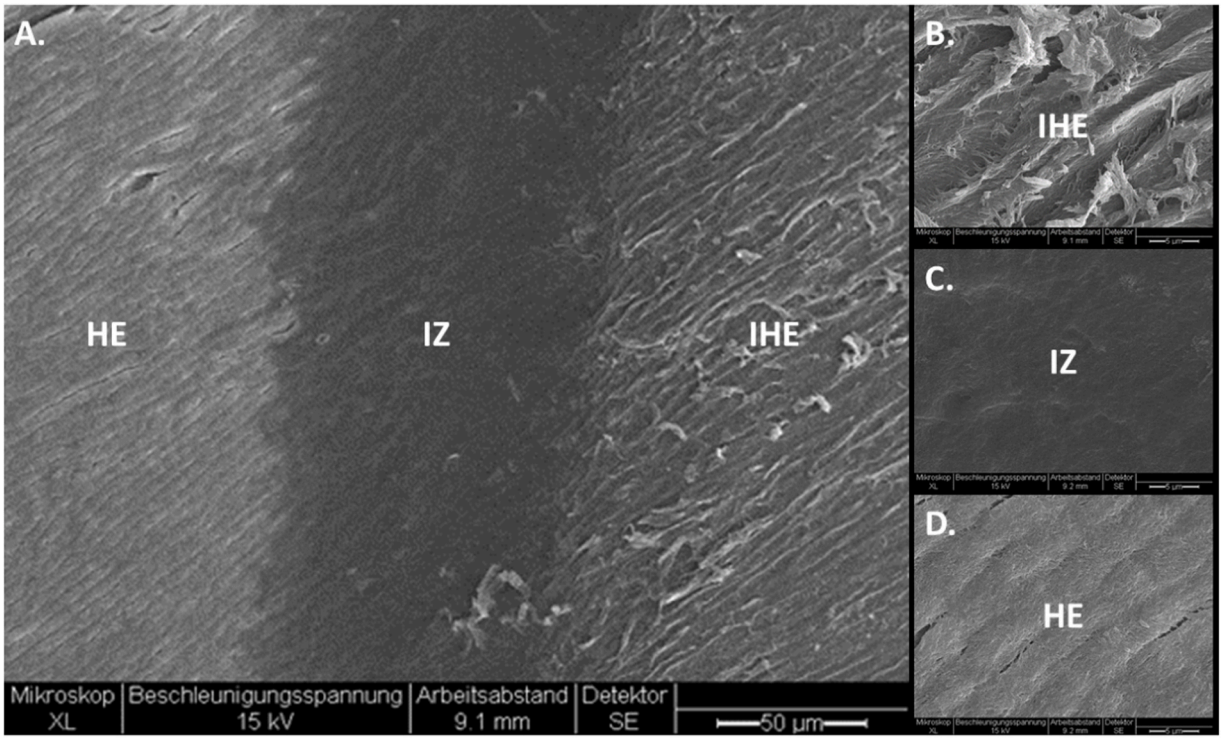

Fig. 4. SEM image of an MIH-affected lesion after pre-treatment with air abrasion and 5.25% sodium hypochlorite followed by resin infiltration (group MAN) at 1000 × magnification (A.). Higher magnification of the infiltrated area (B.), an amorphous, non-porous intermediate zone (C.), and the hypomineralized enamel adjacent to the infiltrated area (D.) at 10,000 × magnification. HE: hypomineralized enamel; IZ: intermediate zone; IHE: infiltrated hypomineralized enamel.

carious and MIH-affected enamel, the first null hypothesis – resin penetration into MIH-affected enamel presenting demarcated opacities does not vary compared to initial carious lesions – could not be rejected. The results have to be interpreted with caution because of the small sample size per group. Differences in the mean penetration depths were observed, although they did not reach statistical significance. When resin infiltration was conducted with demineralization by 15% hydrochloric acid, the mean penetration depth was higher and more variable for MIH-affected enamel (M: 275.9 ± 255.2 µm) as compared to initial carious lesions (IC: 202.3 ± 69.2 µm), which could be associated with

the increased lesion depth of MIH-affected enamel. This indicates that a demineralization with 15% hydrochloric acid for 120 s can reduce the surface layer of MIH-affected enamel thereby facilitating the resin infiltration into the microporosities. *Kumar et al.* (2016) [25] showed that etching MIH-affected enamel with 15% hydrochloric acid for 120 s resulted in an incomplete erosion of the surface layer in 40% of cases. A partial erosion of the surface layer of MIH-affected enamel could be an explanation for the variability in penetration depth of the resinous infiltrant in this study. When correlating the penetration depth of the resinous infiltrant to the lesion depth a significantly higher percentage of penetration could be observed for initial carious enamel given the shallower lesion morphology compared to MIH-affected enamel. It may be argued that 5 min application time of the resinous infiltrant may be sufficient to completely penetrate initial carious lesions yet insufficient to penetrate the entire thickness of developmentally hypomineralized enamel. An influence of the application time on the resinous infiltrant's penetration was reported in an *in vitro* study by *Meyer-Lueckel et al.* (2011) [28], in which initial carious enamel with a maximum lesion depth in lesions > 500 μm showing a median of 661 μm (25% percentile: 525 μm; 75% percentile: 731 μm) was almost completely penetrated by a pre-product of the infiltrant after application times of 3 min and 5 min. Since testing different application times was not within the scope of this study, no comments can be made to the question as to whether longer application would have increased the resin penetration depth. An application time of 5 min was chosen because it appeared to be realistic that paediatric patients could manage to cooperate for this time span, which might not hold true in case of an extension of the application time.

Further pre-treatments were investigated in this study to improve the resin infiltration into MIH-affected enamel, since it would be desirable to find a method that fills the porosities of MIH-affected enamel, thus reducing the susceptibility to PEB, dental caries [13], and alleviating hypersensitivities [11]. At the same time, several techniques have been described aiming at an improvement of the treatment outcome for developmentally hypomineralized teeth, such as deproteinization with sodium hypochlorite [22,24] and the "bleach-etch-seal" technique [47].

Deproteinization with 1% sodium hypochlorite is well known from resin infiltration into initial carious lesions, where it has been used to clean the enamel surface prior to surface conditioning with hydrochloric acid to im improve the infiltrability [34]. A pre-treatment with sodium hypochlorite, which has an unspecific proteolytic activity and antimicrobial properties [48], has also been used *in vitro* to deproteinize MIH-affected enamel with variable results [22,24,47]. The protein content of hypomineralized enamel is three to fifteen times higher compared with sound enamel [44]. These proteins may negatively influence the success of adhesive dentistry for MIH-affected teeth, which is why several attempts have been undertaken to remove the organic matter [22,24,47]. On the one hand, it was shown by *Chay et al.* (2013) [22] that the pre-treatment with 5.25% sodium hypochlorite for 60 s increased the microshear bond strength of a composite resin to MIH-affected enamel. This was independent of whether resin infiltration was additionally performed or not [22]. The use of sodium hypochlorite for the removal of organic matter on the enamel surface prior to resin infiltration combined with composite resin for pit and fissure sealing of initial subsurface lesions has been recommended for the clinical control of occlusal carious lesions due to an enhanced initial quality of the pit and fissure sealing [49]. On the other hand, *Gandhi et al.* (2012) [47] found no advantage when MIH-affected molars were solely pre-treated with 5% sodium hypochlorite for 60 s prior to pit and fissure sealing. The omission of etching in this group genuinely impaired the sealing quality. The implementation of a "bleach-etch-seal" technique, meaning the application of 5% sodium hypochlorite for 60 s followed by etching with 35% phosphoric acid for 30 s and pit and fissure sealing, revealed a tag quality comparable to the control group, in which pit and fissures were etched and sealed. All in all, the use of 5% sodium hypochlorite did not reduce the overall high probability of obtaining insufficient sealant tags [47]. In another study, the microtensile bond strength of composite resin to MIH-affected enamel could not be increased by deproteinization with 5.25% sodium hypochlorite and resin infiltration [49]. *Crombie et al.* (2014) [24] showed that the deproteinization with 0.95% (w/v) sodium hypochlorite prior to or after etching with 15% sodium hypochlorite did not significantly improve the results for resin infiltration into MIH-affected enamel. According to the authors of the study, a possible explanation for this could have been that solely surface proteins were removed or that the surface proteins were cross-linked by the storage medium formaldehyde thus inhibiting the proteolytic activity of sodium hypochlorite [24]. Though the effectiveness of deproteinization with sodium hypochlorite to improve the infiltrability into MIH-affected enamel could not be statistically verified in the present study, it was observed that the penetration depth increased after this pre-treatment indicating that there could be a positive effect of deproteinization prior to resin infiltration.

In addition, two methods for a (chemo-)mechanical and mechanical enamel surface layer removal were evaluated in this study. Microabrasion has been introduced as microinvasive treatment to remove superficial enamel discoloration by a combination of erosion and abrasion when using a 18% hydrochloric acid-containing abrasive pumice, which is brushed onto the tooth surface with a rotary cup working in a low-speed handpiece [50,51]. In this study, microabrasion as pre-treatment before resin infiltration resulted in the lowest penetration of the resin infiltrant into MIH-affected enamel. Despite the more aggressive pre-treatment approach employed by microabrasion to remove the hypermineralized surface layer of MIH-affected enamel, the resin infiltration could not be enhanced. It was even inferior to the pure demineralization with 15% hydrochloric acid. It can only be hypothesized that the hydrochloric acid-containing abrasive pumice and the dissolved anorganic and organic matter might have occluded the microporosities of the MIH-affected enamel which is why the resinous infiltrant failed to penetrate the subsurface lesion body. Air abrasion with aluminum oxide particles being applied to enamel under pressure via an air stream has been used for enamel removal during cavity preparation [52]. The pre-treatment with air abrasion resulted an increased penetration of the resinous infiltrant into MIH-affected enamel as compared to the sole demineralization with hydrochloric acid. A possible reason for this might have been the improved removal of the surface layer covering the MIH-affected enamel. It is well known from initial carious lesions that the erosion of the surface layer is important for a successful penetration of the resinous infiltrant [27,32] and this could be the same for MIH. The combination of air abrasion and deproteinization with sodium hypochlorite failed to improve the penetration of the resinous infiltrant even further, although it could have been expected that the mechanical reduction of the surface layer might have led to an improved dissolution of the organic matter within the MIH-affected enamel through deproteinization. Theoretically conceivable, chlorine and oxygen dissociated from sodium hypochlorite could have been incompletely removed from the exposed subsurface porosities, which might have impeded a deeper resin infiltration through interaction with these molecules [34]. However, the observed differences between the groups could not be statistically verified in this study. Based on these results, the second null hypothesis – different pre-treatment protocols for MIH-affected enamel opacities have no impact on the penetration depth of the resinous infiltrant – could not be rejected. Again, the results need to be interpreted with caution due to the small sample size, which might have prevented the observed differences between the groups from becoming statistically significant.

Several limitations have to be taken into consideration when it comes to interpreting the findings. First, the sample size of 10 tooth halves per group was small in the present study. The reason for this small sample size was associated with difficulties in obtaining extracted MIH-affected first permanent molars with intact enamel surfaces given the prevalence of about 13.5% [6–8] and the fact that tooth extractions are the *ultima ratio* for severely hypomineralized teeth frequently affected by extensive enamel breakdown [53]. Unlike for caries, it has not been possible to

create artificial MIH-affected enamel in the laboratory so far ruling out this option to increase the sample size. This might explain why other published studies on resin infiltration of MIH-affected enamel also included a small number of teeth [23–25]. Notwithstanding, some laboratory studies on resin infiltration of natural caries lesions included 8 to 10 tooth halves per group [27,32] which indicates that finding teeth with natural non-cavitated carious lesions meeting the inclusion criteria for *in vitro* studies might also be difficult despite the high global prevalence of dental caries [54]. It was therefore decided to conduct a pilot study carefully considering that relevant, yet statically not significant differences might be observed. Since MIH is diagnosed on the basis of clinical findings [10] extracted first permanent molars were selected for the present study that had been exposed to the oral environment before extraction. Therefore, a possible treatment with mineralizing agents (*i.e.* fluoride, CPP-ACP) of the MIH-affected teeth during the time span within the oral cavity cannot be ruled out, which may have influenced the results due to changes of the enamel surface properties [24]. The heterogeneity of MIH-lesions may have also influenced the results impeding a standardization of the experiment [24]. In comparison to the *in vitro* study on resin infiltration of developmentally hypomineralized teeth by *Crombie et al.* (2013) [24], in which the authors reported on an insufficient number of included brown lesions, it was rather *vice versa* in this study. However, the heterogeneity of developmentally hypomineralized enamel included this study may mimic the clinical situation more realistically. Finally, the results do neither allow any statements to be made about the microhardness of MIH-affected enamel after resin infiltration, nor the clinical applicability in children suffering from hypersensitivities in MIH affected molars [24].

5. Conclusion

The resinous infiltrant penetrates the microporosities of non-cavitated initial carious lesions confined to enamel and MIH-affected enamel to comparable depths after the demineralization of the surface layer with 15% hydrochloric acid for 120 s, even though MIH-lesions are considerably deeper. Additional pre-treatments, such as a deproteinization with 5.25% sodium hypochlorite for 120 s or the mechanical reduction of the surface layer by air abrasion for 5 s, could allow for a deeper resin penetration into MIH-affected enamel. Nevertheless, it has to be considered that resin infiltration into developmentally hypomineralized enamel is more variable, leaving a higher percentage of porous enamel behind given the more extensive lesion body of MIH-affected enamel.

Authors' contributions

N.K. and S.A. conceived the idea and developed the design for the *in-vitro*-study. S.S. conducted the resin infiltration and collected the data. S. L. supported the test executions and data acquisition. S.A., S.S., N.K., and R.F. performed the data analysis and interpretation. U.G. and A.S. performed the SEM imaging. S.A. supported the SEM evaluation and drafted the manuscript. S.S., N.K., and R.F. critically revised the manuscript.

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