Di-calcium-phosphate and phytosphingosine as an innovative acid resistant treatment to occlude dentine tubules

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ABSTRACT. This study evaluated the ability of an experimental di-calcium-phosphate desensitising agent (DCP) used alone or combined with phytosphingosine (PHS) to occlude dentine tubules and resist a citric acid (CA) or artificial saliva (AS) challenge. Three groups of human dentine specimens (DS) were treated with 1) PHS alone, 2) DCP or 3) a combination of PHS and DCP. Dentine hydraulic conductance was evaluated using a digital flow sensor at 6.9 kPa. The fluid volume average of each treated-DS was used to calculate the total dentine permeability reduction (P%) prior to and following CA immersion for 1 min or 4 weeks in AS. Treated-DS were submitted to SEM and FTIR spectroscopy analysis. Statistically significant differences (P%) were identified between the groups by ANOVA and Fisher's multiple comparison test ($P < 0.05$). Interestingly, PHS and DCP appeared to work synergistically. DS treated with

DCP or PHS/DCP demonstrated a significant reduction (P%) prior to and following CA or AS challenge ($P < 0.05$). SEM and FTIR analysis showed consistent brushite crystals occluding the dentine tubules. Conversely, the application of PHS alone failed to demonstrate any significant reduction of dentine permeability ($P > 0.05$) or show any evidence of occlusion of the dentine tubules. DCP can however, be used alone or combined with PHS to decrease the dentine permeability as well as resisting an acid and artificial saliva challenge. This may therefore represent a suitable treatment for dentine hypersensitivity.

INTRODUCTION

Dentine hypersensitivity (DH) represents a common clinical condition within the young and adult population in western countries [West *et al.*, 2013] mainly due to gastric and dietary acids revealing underlying dentine [Lussi *et al.,* 2004]. DH develops in two phases [Dowell and Addy, 1983]: i) lesion localisation: subsequent loss of enamel caused by tooth wear or to gingival recession; ii) lesion initiation and DH symptomatology, which occurs after the protective smear layer is removed and the underlying dentine tubules are exposed. According to the hydrodynamic theory, the movement of fluid within the dentine tubules following either physical or osmotic stimulation may cause pain [Brannstrom *et al.,* 1968]. The main treatment for DH is based on the reduction of the fluid flow through the physical occlusion of the dentine tubules [Pashley, 1986]. Although several products are currently available, there is still the need to develop innovative acid resistant desensitising agents. Acidic di-calcium phosphates (e.g., brushite) have been widely used as a hard tissue substitute due to their bioactivity and biocompatibility [Cama *et al.* 2009]. Moreover, it has been recently reported that pre-treatment of experimental hydroxyapatite discs (HAp) with sphingoid bases such as sphingosine, phytosphingosine (PHS), PHS phosphate and sphinganine significantly protected HAp against acid demineralisation *in vitro* [Valentijn-Benz *et al*., 2015]. Cukkemane *et al.,* (2015) revealed using atomic force measurement that PHS and other sphingoid bases can form diffusion barriers against H^+ ions and bacteria. In principle, the reported anti-erosive properties would suggest that PHS could be included in oral care products for DH treatment. The aim of the present study was to evaluate the ability of experimental desensitising agents based on an acid di-calcium-phosphate (DCP) alone or in combination with PHS to occlude exposed dentine tubules. This aim was accomplished by quantitatively evaluating the reduction of the hydraulic conductance following the application of the tested materials and a subsequent citric acid (CA) or artificial saliva (AS) challenge. SEM and FTIR spectroscopy analysis were also conducted. The null hypotheses tested were: 1) the application of DCP onto exposed dentine when used alone or in combination with PHS would not reduce the hydraulic conductance of EDTA-treated dentine; 2) the citric acid (CA) or artificial saliva (AS) challenge would reduce their ability to maintain the occlusion of the dentine tubules (longevity of treatment).

MATERIALS AND METHODS.

Preparation of specimens. Thirty sound human molars were extracted for surgical reasons under institutional ethical approval (granted by the research ethics committee) and used to create mid coronal dentine discs (DS) as described by Sauro *et al.*, [2006]. In brief, occlusal enamel was removed using a slow-speed, water-cooled diamond saw (RS-70300; Struers, Copenhagen, Denmark). A second parallel cut was performed 1.5 mm beneath the cementum-enamel junction in order to remove the roots. A standard smear layer was created using a 180-grit silicon-carbide paper (30 s) and subsequently removed using 17% EDTA (pH 7.4) for 1 min followed by ultrasonic bath containing distilled water (5 min). DS were randomly divided into two main groups based on the challenge storage (n=15/ group): i) CA: citric acid; ii) AS: artificial saliva. Each main group was then divided in three sub groups (n=5/sub-group) based on the desensitising treatment: A) PHS: 4-hydroxysphinganine; B) DCP: Di-calcium-Phosphate (Brushite); C) PHS/DCP: phytosphingosine + Brushite. A Tris-Tween/ethanol solution (5 mg/ml) of PHS was prepared as described by Valentijn-Benz *et al.*, [2015].

***Desensitising dentine treatment*.** Specimens were rinsed with deionised water prior to the pre-treatment with PHS. PHS (0.1 ml) was gently brushed onto the dentine surface of all the specimens in Group A using a micro-brush (20 s), in triplicate (60 s; 0.3 ml) and then rinsed with deionised water (10s). The DCP was prepared as described by Cama *et al.*, [2009] by mixing equimolar quantities of β-tricalcium phosphate (β-TCP, Sigma-Aldrich, Gillingham, UK) and monocalcium phosphate monohydrate (MCPM, Sigma-Aldrich) in deionised water (R= 3 g/ml). The DCP specimens (Group B) were treated by an application of DCP (0.3 g) on the EDTA-treated dentine. Two consecutive layers of a semi-fluid paste (30s each; ~0.15 g) were gently brushed onto the dentine surface using a micro-brush (60s) and left undisturbed for a further 30 s. Finally, the specimens were rinsed with deionised water (10s) and the excess of DCP was removed from the dentine surface using the tip of a soft paint brush. The PHS/DCP specimens (Group C) also received the same PHS treatment, immediately followed by application of DCP as described above.

Dentine permeability evaluation. All DS were cemented (ROCKET Heavy DVA, USA) to Plexiglass blocks penetrated with an 18 Gauge stainless steel tube. Each specimen was finally connected to a hydraulic pressure device (Fig. 1) under a constant hydraulic pressure of 6.9 kPa (Sauro *et al.,* 2007; Pashley *at al.,* 1986) for the measurement of the fluid volume (FV) through a digital sensor with a resolutions of ~100 nl/min and a response reading frequency of 1.56 Hz (ASL 1600, Sensirion, Staefa, Switzerland). The highest hydraulic conductance of each specimen was recorded (Lp-max = 100% was arbitrarily assigned); specimens with a fluid flow rate less than 3µl/min were excluded and replaced with discs with a higher flow rate. Lp-max permits an evaluation of the changes in dentine permeability following the application of the test treatments. Each specimen was treated with the test materials as described above, and based on observations obtained during a pilot study, five FV readings were performed every 3 minutes for 15 minutes. These readings were then averaged and used to calculate the permeability reduction (P %) of each specimen using the following equation:

$$\%P = \frac{fluid\ filtration\ rate\ of\ the\ treated\ dentine}{fluid\ filtration\ rate\ of\ EDTA{-}etched\ dentine}\ x\ 100$$

The specimens were subsequently tested according to two different ageing protocols (CA or AS). DS were immersed in CA (6 wt%; pH 1.5) and then left undisturbed for 60 s or in AS for 4 weeks (37°C). The composition of the AS was 1.5 mmol/L $CaCl_2$, 50 mmol/L KCl, 0.9 mmol/L KH_2PO_4, 20 mmol/L Tris, pH 7.4. This solution (25 ml) was replaced every 72 h. The means (P %) and standard deviations of each group were calculated and any significant differences were observed between the groups by One-way ANOVA and Fisher's least test ($P < 0.05$).

ATR/FTIR Spectroscopy and SEM evaluation. Two further DS were prepared for each sub-group and subsequently treated and challenged as previously described. These were analysed using a ATR/FTIR Spectrometer (Perkin-Elmer, Beaconsfield, UK) with a resolution of 4 cm^{-1} to characterise the chemical composition of the dentine prior to and following each product application and challenge protocol (*i.e*. CA or AS). The same specimens were then dried overnight in a silica-containing desiccator at 37ºC, gold sputter-coated (SCD004 Bal- Tec, Vaduz, Liechtenstein) and examined using SEM (S-3500; Hitachi, Wokingham, UK).

RESULTS.

The results of dentine permeability reduction (P%) are illustrated in Figure 2. The application of DCP or PHS/DCP onto the EDTA-etched dentine significantly reduced dentine permeability ($P < 0.05$). However, the specimens treated with PHS/DCP demonstrated an ability to reduce dentine permeability by 92.2% after CA attack (Fig. 2A) and 83.1% after AS immersion (Fig. 2B). There was no significance reduction ($P > 0.05$) prior to and following CA or AS challenge in any group. PHS induced the lowest Lp reduction (10%) and no significant change ($P > 0.05$) was observed following CA or AS challenge. These results were confirmed by the SEM analysis, which showed a demineralised dentine surface with patent dentine tubules and exposure of collagen fibrils following EDTA etching (Fig. 3A), PHS application (Fig. 3B) and after CA attack (Fig. 3C). The FTIR analysis showed demineralised dentine (Amide I and II) both after PHS application (Fig. 3D) and after AS aging (Fig. 3E).

Conversely, dentine treated with DCP or PHS/DCP showed dentine tubules that remained occluded following CA (Fig. 4A and 4B, respectively) or AS challenge (Fig. 4C). Conversely, the EDTA-etched specimens treated with PHS alone and subsequently immersed in AS presented only very few mineral deposits on the outer surface and patent dentine tubules (Fig. 4D). The FTIR analysis revealed that the mineral crystallites precipitated on the dentine surface following DCP or PHS/DCP application was brushite (Fig. 4E). The brushite’s crystals (size < 2µm) that precipitated within the tubules and on the dentine surface (Fig. 4A and 4B), converted into a more complex apatite-like calcium phosphate following AS immersion (Fig. 4F), although the size and the morphology of such latter crystals presented no clear change over time (Fig. 4C). Conversely, the EDTA-etched specimens treated with PHS and immersed in AS presented a very low PO peak at 1019 cm^{-1} and a clear demineralised dentine surface (Amide I and II), (Fig. 4G).

DISCUSSION

An ideal dentine desensitiser should be easy to apply, act rapidly, cause no alteration to the tooth structure and/or irritation to pulp, and last as long as possible [Grossman, 1935].

However, in order to reduce the symptomatology of DH, it is of key importance to decrease dentine permeability (Lp), but also maintain the occlusion of the dentine tubules following subsequent acid and saliva challenges [Wang *et al.,* 2010]. The risk for DH may increase with the presence of dietary acids, as these remove the smear layer and open the underlying dentine tubules [Sauro *et al.,* 2007]. Citric acid is a common component of both fruit and soft drinks, and it is widely used in *in vitro* studies to simulate the oral environment and test the resistance of desensitisers to an acid challenge [Wiegand *et al.*, 2007]. Saliva can also solubilise materials adhering to teeth and contains calcium and phosphate ions that can interact with surfaces [Arrais *at al.,* 2003]. Therefore, it is essential to evaluate whether novel desensitising agents have the potential to effectively occlude the dentine tubules under circumstances similar to the oral environment. The results of the present study would therefore appear to reject both of the two null hypotheses since the DCP paste alone or in combination with PHS caused a significant ($P < 0.05$) permeability reduction before and after a CA challenge due to the precipitation of brushite both within the dentine tubules and on the dentine surface (Fig. 4A, 4B and 4E) or after AS storage, where this brushite converted to a different and probably more complex calcium-phosphate (Fig. 4 F) thereby maintaining the status of tubular occlusion over a period of 4 weeks (Fig. 4C). Indeed, Jiang *et al.*, (2009) demonstrated that brushite may convert to stable hydroxyapatite when immersed in a calcium-rich solution at a slightly alkaline pH.

Similarly, a novel calcium phosphate desensitising agent (TEETHMATE™, Kuraray corp., Japan), consisting of tetracalcium phosphate and di-calcium phosphate anhydrous (i.e. Monetite) has been demonstrated both in clinical [Mehta et al., 2014] and *in vitro* [Thanatvarakorn *et al.*, 2013] studies to be efficacious as dentine desensitising agent. This product contains di-calcium phosphate as one of the main constituent, whereas the DCP paste used in this study was made of equimolar quantities of β-TCP and mono-calcium phosphate-monohydrate that precipitate as brushite (Cama *et al.,* 2009) during application (Fig. 4). Conversely, TEETHMATE appears to precipitate as an apatite-like mineral [Brown and Chow, 1983]; its solubility in acid solutions [pH <5.0] may be much lower than that of brushite [Jiang *et al.*, 2009]. The precipitation of brushite however, is not a new issue in dental research. For instance, dentine acid-etching induces the release of calcium and phosphate which may precipitate as either brushite or octacalcium phosphate depending on the environmental pH. However, Shellis *et al.,* (1997) demonstrated that at a pH below 4, as in the DCP paste (Fig. 4E), brushite is mainly precipitated. Moreover, the acidic environment created by the CA challenge induced further precipitation of monetite [Şahin and Çiftçioğlu, 2014] and tubules occlusion (Fig. 4C). Indeed, crystals of di-calcium phosphates may increase and create a mechanical interlocking in acidic pH, thereby providing a more structural resistance to further hard tissue loss [Wang and Nancollas, 2008].

Although PHS was not able to suitably occlude the dentine tubules (Fig. 3B) even after prolonged AS immersion (Fig. 4D), it appears to work synergistically in combination with DCP, forming an effective DH desensitiser. These specific results were probably due to the anti-erosive characteristic of PHS. Indeed, PHS may capture ionised phosphate and have a protection effect against demineralisation by binding any remaining HAp crystals [Kosoric *et al.*, 2007]. However, due to its amphipathic character, in solution, PHS has the tendency to assemble into highly positively-charged aggregates or micelles, with the fatty acid tails buried inside and the positively charged head groups exposed to the bulk of the solution. Hence, the high density of positive charges on such aggregates will more likely produce a high avidity for negatively charged phosphate-rich surfaces such as HAp [Valentijn-Benz *et al*., 2015].

It is also acknowledged that erosion initiated by dietary acids may exacerbate DH and cause demineralisation of the collagen matrix. Demineralised dentine is characterised by unprotected collagen fibrils (Fig. 3A) that can be degraded by endogenous enzymes e.g., metalloproteases and cysteine cathepsins [Zarella *et al.*, 2015]. Moreover, further collagen degradation can be also induced by salivary esterases and/or bacteria proteases [Park *et al.*, 2008]. However, it has been demonstrated that mineral precipitation induced by bioactive substances e.g., calcium-phosphates and bioactive glasses may also reduce the enzymatic-mediated collagen degradation [Tezvergil-Mutluay *et al.*, 2014] and the risk for further wear of hard tissues (e.g. dentine) [Zarella *et al.*, 2015].

In conclusion, this experimental *in vitro* study demonstrated that the use of DCP paste alone or in combination with PHS may represent a suitable treatment for DH. The formation of acid resistant crystals within the dentine tubules produced by the experimental materials evaluated in the present study would suggest that they may be useful as potential long-term desensitisers for the treatment of DH. Further evaluation however, would be required in order to define and create more suitable clinical formulations for commercial products and their subsequent application *in vivo*.

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FIGURES AND CAPTIONS

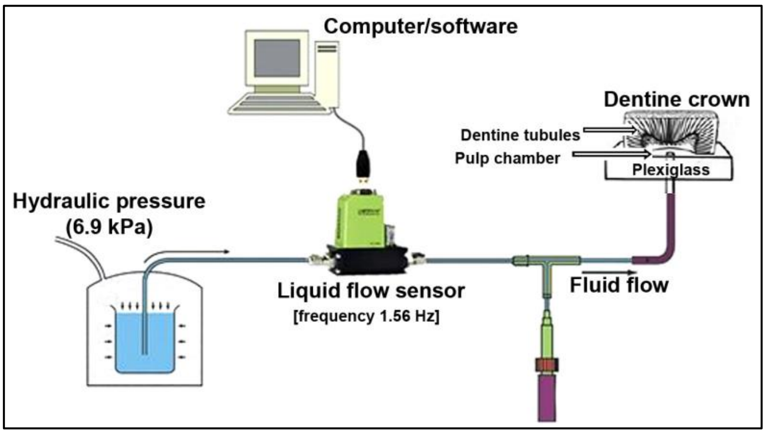
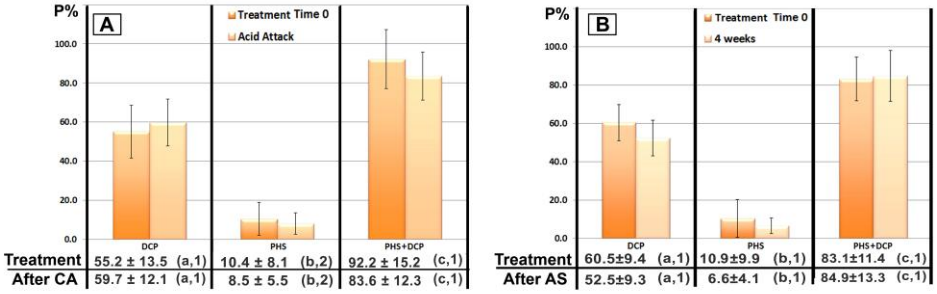


Figure 1. Schematic illustration of how the dentine specimens were connected to a hydraulic pressure device under a constant hydraulic pressure (6.9 KPa) and the measurements of the fluid volume (FV) were attained via a digital sensor.



	DCP	PHS	PHS+DCP
Treatment	55.2 ± 13.5 (a,1)	10.4 ± 8.1 (b,2)	92.2 ± 15.2 (c,1)
After CA	59.7 ± 12.1 (a,1)	8.5 ± 5.5 (b,2)	83.6 ± 12.3 (c,1)

	DCP	PHS	PHS+DCP
Treatment	60.5±9.4 (a,1)	10.9±9.9 (b,1)	83.1±11.4 (c,1)
After AS	52.5±9.3 (a,1)	6.6±4.1 (b,1)	84.9±13.3 (c,1)

Figure 2. Mean and standard deviations of %P (dentine permeability reduction) values before and after a citric acid **A)** and AS **B)** challenge. In rows, different superscript letters indicate significant differences between the three experimental desensitising agents following application or following a CA or AS challenge (P<0.05). In columns, different superscript numbers indicate significant differences in the same desensitising agent, between application and CA or AS challenge (P<0.05).

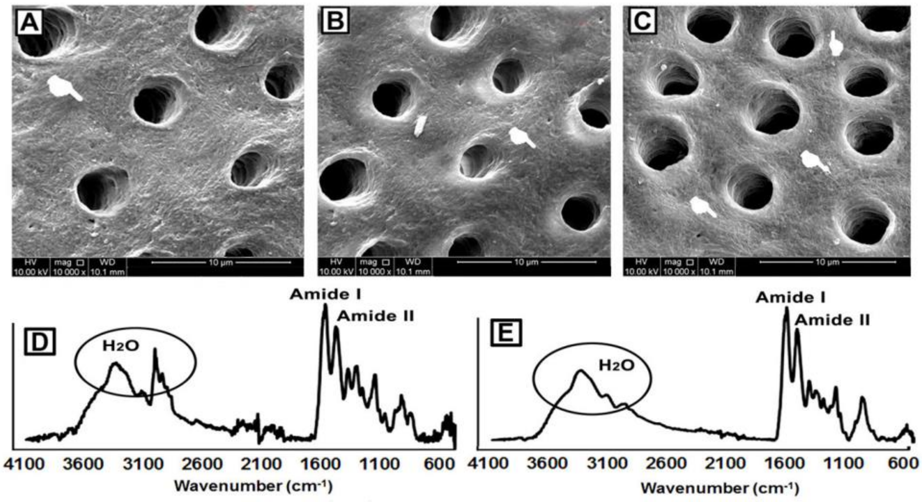


Figure 3. SEM micrograph of EDTA-etched dentine showing several patent tubules and collapsed collagen fibrils (pointer). **B:** EDTA-etched dentine following application of PHS showing no tubules occlusion, but only collapsed collagen fibrils (pointer). **C:** EDTA-etched dentine surface following application of PHS and subsequent CA attack. Note the presence of patent tubules and collapsed collagen fibrils (pointer). **D:** Spectra of EDTA-etched dentine treated with PHS. Note the bands at 3200–3400 cm^{-1} due to the O–H stretching of water (H_2O) and amide bands of collagen (1200–1725 cm^{-1}) in dentine. The same spectra was also observed following PHS application and after CA attack **(E).** EDTA-etched dentine that received no desensitising treatment shows the same FTIR

features observed in figure-(E).

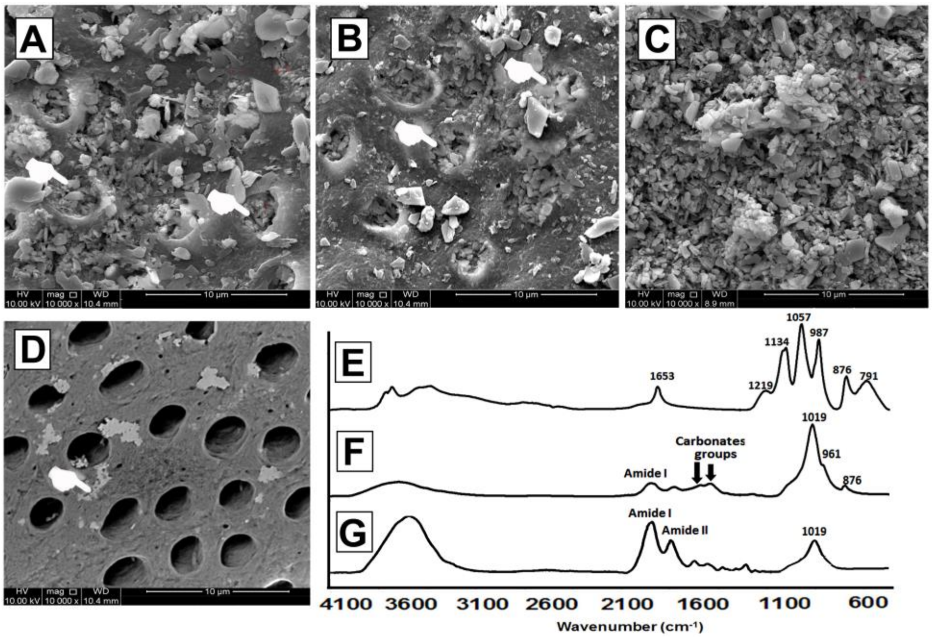


Figure 4. A: SEM micrograph of the EDTA-etched dentine following application of DCP and subsequent CA attack. Note the presence of mineral crystals (size <2 µm) inside the dentine tubules (pointer)**. B:** EDTA-etched dentine treated with PHS/DCP and exposed to CA; mineral crystals are still present inside dentine tubules (pointer)**. C:** EDTA-etched dentine following application of PHS/DCP and AS immersion. Note the greater amount of crystals covering the dentine surface; similar features were also observed in the specimens treated with DCP and immersed in AS**. D:** SEM micrograph of EDTA-etched dentine treated with PHS and immersed in AS. Note the presence of very few mineral deposits on the dentine surface (pointer) **E:** FTIR spectra obtained from EDTA-etched dentine treated with DCP and submitted to CA attack. Note bands at 3200–3400 cm^{-1} (O–H stretching of water in dentine). Water in brushite can be observed at 1653 cm^{-1} (bending mode), O–H in-plane bending at 1219 cm^{-1} and H_2O oscillating motion at 791 cm^{-1}. The PO stretching peaks of the brushite is observed at 1134, 1057, and 987 cm^{-1}. The same spectra was attained after application of PHS/DCP on EDTA-treated dentine and subsequent CA attack. **F:** FTIR spectra specimens treated with PHS/DCP and immersed in AS. Note the PO peaks at 961 cm^{-1} (v_1), 1019 cm^{-1} (v_3 – asymmetric stretching mode of hydroxyapatite) and carbonate bands at 1400–1500 cm^{-1}. These peaks were also present in mineralised dentine and in the specimens treated with DCP and submitted to AS ageing. **G:** FTIR spectra of EDTA-etched dentine treated with PHS and immersed in AS. In this case, only a low PO peak at 1019 cm^{-}could be detected. Whereas, amide bands from organic components (1200–1725 cm^{-1}) were clearly visible, indicating that dentine was still demineralised.

Role of all authors:

Sauro S: Corresponding author. He wrote the entire manuscript. He elaborated of the experimental project and performed part of the dentine permeability assessment.

Lin CY: He contributed to perform the experimental project, specifically in the preparation of specimens and dentine permeability assessment.

Bikker FJ: He contributed to revise the manuscript. He was also involved in the elaboration of the experimental project. He formulated the experimental phytosphingosine used in this study.

Cama G: He contributed to revise the manuscript. He was also involved in the elaboration of the experimental project. He formulated the experimental Di-calcium phosphate used in this study.

Dubruel P: He performed the entire experimental part about FTIR analysis and he was involved in the interpretation of the results**.**

Soria JM: He was involved in the interpretation of the statistical analysis.

D’Onofrio A: She performed the SEM analysis and interpretation the results.

Gillam D: Head of the group. He contributed to write and revise the entire manuscript. He was also involved in the elaboration of the experimental project and interpretation of results.