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DOCTORAL THESIS

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DOCTORAL THESIS

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Stomatološki fakultet

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PROCJENA REMINERALIZACIJSKOG UČINKA SUVREMENIH MATERIJALA KOJI OSLOBAĐAJU IONE NA POVRŠINU CAKLINE I DENTINA

DOKTORSKI RAD

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SUMMARY

A new approach in carious lesion therapy is focused on preservation of hard dental tissues and recommends different remineralization procedures. Quick development of the materials prompted the introduction of different materials which release ions to the market, many of which are not investigated enough. Therefore, the aim of this study is to determine the influence of new ion-releasing materials on enamel and dentine remineralization.

Firstly, the influence of topical enamel materials on microhardness, which can be used to assess the depth of remineralization, surface appearance and chemical composition was analysed. Due to the higher share of organic content, dentine reparation is more complex; thus, apart from the microhardness, chemical composition and surface appearance analysis, remineralization effect of new and standard materials was assessed by micro-CT testing, by determining dentine density. Remineralization of all materials was quantified and expressed as a percentage of recovered minerals.

All tested materials increased the mineral content of demineralized lesion. Combination of fluoride and CPP-ACP outperformed other tested materials. Nano-hydroxyapatite-based material proved to be a valid alternative to solely fluoride-based products. Novel glass hybrid material showed the highest potential for remineralizing a mineral-depleted lesion. Micro-CT scanning produced images which confirm mineral deposition and increase in dentine density.

Keywords: demineralization, remineralization, ion-releasing materials, enamel, dentine, microhardness, SEM/EDS, Micro-CT

PROŠIRENI SAŽETAK

Svrha rada

Novi pristup u terapiji karijesne lezije temelji se na maksimalnom očuvanju tvrdih zubnih tkiva te se preporučuju postupci remineralizacije različitim materijalima. U posljednje vrijeme stavlja se naglasak na materijale koji oslobađaju ione te izazivaju specifičan biološki odgovor i stvaraju vezu između tvrdih zubnih tkiva i samog materijala. Danas se fluor smatra glavnim sredstvom za zaustavljanje gubitka minerala iz cakline. Uvođenje korištenja kazein fosfopeptid-amorfnog kalcijevog fosfata (engl. casein phosphopeptides-amorphous calcium phosphate, CPP-ACP) kao remineralizirajućeg agensa veliki je napredak u zaustavljanju ranih karijesnih lezija. CPP-ACP stvara nekariogeni plak koji oslobađa kalcijeve i fosfatne ione, pospješujući remineralizaciju u kiselim sredinama. Nanohidroksiapatit, čija je struktura slična kristalu apatita u tvrdim zubnim tkivima, može zamijeniti prirodni mineralni sadržaj cakline. Budući da su fluoridi prepoznati kao mogući neurotoksin, zanimljivo je vidjeti mogu li proizvodi koji ne sadrže fluor poslužiti kao alternativa. U području restaurativne dentalne medicine materijali koji otpuštaju ione uključuju staklenoionomerne cemente (SIC) i kalcij-silikatne cemente,koji se smatraju standardom. U skladu s nastojanjima da se poboljša kvalitete SIC-a, nedavno je predstavljena nova generacija ove grupe materijala; staklohibridi. Međutim, nema podataka o trajnosti ovih materijala te njihova svojstva nisu dovoljno istražena. Druga skupina široko korištenih bioaktivnih materijala temelji se na kalcijevom silikatu, kao što je mineral-trioksid-agregat (MTA), biodentin ili biokeramika. Kako su indikacije za njihovu upotrebu često slične SIC-u, usporedba njihovih svojstava, kao što su mikrotvrdoća i oslobađanje fluorida i kalcija, smatra se vrlo zanimljivom. Alkaziti su još jedna skupina nedavno predstavljenih materijala, a sadrže alkalno punilo koje otpušta ione neutralizirajuće kiseline zajedno s fluoridima, kalcijem i hidroksidnim ionima kada je pH u usnoj šupljini nizak. Dinamika mineralizacije može se promatrati ispitivanjem mikrotvrdoće uzorka i provođenjem skenirajuće elektronske mikroskopije u kombinaciji sa spektroskopijom rendgenskih zraka s disperzijom energije (SEM/EDS), a sve nabrojane metode korisne su za promatranje površine uzorka i određivanje mineralnog sastava. Osim toga, korištenje mikro-CT-a za određivanje gustoće zubnog tkiva već je dokazana uspješna metoda, stoga je cilj ovog istraživanja utvrditi utjecaj novih materijala koji otpuštaju ione na remineralizaciju cakline i dentina.

Materijali i postupci

U prvoj fazi istraživanja ispitivao se utjecaj triju materijala, koji otpuštaju ione, na mikrotvrdoću i kemijski sastav demineralizirane cakline. Korišteni materijali bili su 3M™ Clinpro™ White, MI Varnish® i Megasonex® pasta za zube, svaki s različitim aktivnim sastojkom. 33 ekstrahirana treća molara korištena su za pripremu uzoraka te su nasumično podijeljeni u tri skupine za analizu mikrotvrdoće (n = 10). Za izradu uzoraka za SEM-EDS analizu koristio se jedan dodatni uzorak za svaku skupinu. Izložena površina cakline demineralizirana je primjenom 37-postottne fosforne kiseline tijekom tri minute. Tri različita sredstva za remineralizaciju primjenjivana su dva puta dnevno po dvije minute te čuvana u fiziološkoj otopini na sobnoj temperaturi tijekom 14 dana. Mikrotvrdoća uzoraka po Vickersovoj metodi mjerila se u tri faze: početna vrijednost, nakon demineralizacije i nakon razdoblja remineralizacije. Opterećenje korišteno za mjerenje bilo je 0,1 kgf (HV 0,1) u trajanju od 10 sekundi. SEM/EDS analiza izvršena je na jednom uzorku za svaki materijal. Nastavak istraživanja uključivao je ispitivanje remineralizacijskog učinka restaurativnih materijala koji oslobađaju ione, koji dolaze u kapsuliranom obliku. Novi staklo-hibridni cementi i alkaziti uspoređeni su sa staklenoionomerima i kalcij-silikatnim materijalom: EQUIA Forte® HT – staklo-hibridni cement, Riva Self Cure – staklo-hibridni cement, Cention Forte – alkazit, Biodentine™ – materijal na bazi trikalcij silikata i GC Fuji TRIAGE – staklenoiomerni cement. U kontrolnoj se skupini koristio konvencionalni kompozitni materijal, 3M™ Filtek™ Universal Composite. 72 ekstrahirana ljudska treća kutnjaka podijeljena su u grupe od pet komada za svaki materijal i kontrolnu skupinu zbog korištenja različitih uzoraka za svako ispitivanje (nakon 2 odnosno 4 tjedna). Kavitet klase I pripremljen je na svakom zubu; jedna polovica šupljine prekrivena je lakom za nokte otpornim na kiseline za izravnu usporedbu površina. Uzorci su zatim demineralizirani uranjanjem svakog pojedinačnog u demineralizacijsku otopinu pri pH 5,0 (37°C) trajanju od dva tjedna. Kaviteti su ispunjeni jednim od ispitanih materijala te su se inkubirali u mješavini umjetne sline i fiziološke otopine u trajanju od 2 odnosno 4 tjedna na sobnoj temperaturi. Nakon razdoblja inkubacije, svi su uzorci rezani dijamantnom pilom okomito na spoj materijala i demineralizirane površine zuba čime se za svaku skupinu dobilo 10 uzoraka za SEM analizu i mikrotvrdoću (n = 10). Dodatna dva uzorka pripremljena su u svakoj skupini za EDS analizu (n = 2). Mikrotvrdoća površine uzorka određena je metodom po Vickersu. Zadnja faza istraživanja obuhvatila je iste materijale korištene u drugoj fazi i njihova je sposobnost remineralizacije dodatno procijenjena pomoću mikro-CT analize. Za pripremu uzoraka koristilo se 15 ekstrahiranih ljudskih trećih kutnjaka.

Okluzalna trećina krune uklonjena je dijamantnom pilom otkrivajući površinu dentina. Na njoj je izrađeno šest kaviteta dok je ostatak površine izoliran lakom za nokte. Preparirani kaviteti demineralizirani su ranije opisanim protokolom. U sljedećem koraku svaki je kavitet ispunjen odgovarajućim materijalom. Mikro-CT analiza provela se prije demineralizacije, nakon demineralizacije te nakon perioda inkubacije od 45 dana.

Rezultati

Srednje vrijednosti mikrotvrdoće (HV 0,1) dobivene za skupinu uzoraka tretiranih MI Varnishom® bile su više u usporedbi s druge dvije skupine ($p = 0,001$ za obje usporedbe), dok se prva i treća skupina nisu međusobno značajno razlikovale ($p = 0,97$). SEM analiza je pokazala različite uzorke i poroznosti na svim testiranim uzorcima. EDS rezultati pokazali su povećanje minerala sadržaja ispitivanih uzoraka, s najvećim sadržajem minerala zabilježenim u MI Varnish® grupi. U drugom dijelu ispitivanja srednje vrijednosti mikrotvrdoće bile su: EQUIA Forte ® HT (26,7 ± 1,45 i 37,74 ± 1,56), Riva Self Cure (19,66 ± 1,02 i 29,58 ± 1,18), Cention Forte (19,01 ± 1,24 i 27,93 ± 1,33), Biodentine™ (23,35 ± 1,23 i 29,92 ± 1,02), GC Fuji TRIAGE (25,94 ± 1,35 i 33,87 ± 5,57) i kontrolna skupina (15,57 ± 0,68 i 15,64 ± 0,82). Rezultati su se značajno razlikovali između većine skupina($p<0,001$). SEM/EDS otkrio je različite uzorke, naslage materijala i različite elementarne varijacije. Mikro-CT analiza otkrila je porast gustoće dentina u zonama tretiranim restaurativnim materijalima. Od svih ispitanih materijala, EQUIA Forte® HT vratila je najveći postotak izgubljenih minerala.

Zaključak

Svi ispitani materijali doveli su do porasta mineralnog sadržaja u demineraliziranim lezijama. Preparati bazirani na nanohidroksiapatitu mogu poslužiti kao alternativa potpuno fluoridnim proizvodima. Od svih ispitanih restaurativnih materijala, staklohibridi su pokazali najveći potencijal za remineralizaciju demineraliziranih lezija. Novi materijali koji otpuštaju ione pokazali su se uspješnima u poticanju stvaranja minerala.

Ključne riječi: demineralizacija, remineralizacija, materijali koji oslobađaju ione, caklina, dentin, mikrotvrdoća, SEM/EDS, mikro-CT

The list of abbreviations:

FDI	World Dental Federation (French: *Fédération Dentaire Internationale*)
IRM	Ion-Releasing Materials
IRB	Ion-Releasing Biomaterials
MIOC	Minimum Intervention Oral Healthcare
HA	Hydroxyapatite
CPP-ACP	Casein Phosphopeptide-Amorphous Calcium Phosphate
FA	Fluorapatite
DEJ	Dentine-Enamel Junction
n-HA	Nano-hydroxyapatite
PAMAM	Poly (amino amine)
GIC	Glass-ionomer cements
RM-GIC	Resin-modified glass ionomer cements
GH	Glass hybrids
TMR	Transversal Microradiography
EDS	Energy Dispersive Spectroscopy
SEM	Scanning Electron Microscopy
Micro-CT	Micro-focus X-ray Computed Tomography
CLSM	Confocal Laser Scanning Microscopy
TEM	Transmission Electron Microscopy
FEG-EPMA	Field Emission Gun Electron Probe Micro-Analysis
PS-OCT	Polarization-sensitive Optical Coherence Tomography
UDMA	Urethane Dimethacrylate

DCP	Dicalcium Phosphate
FAS	Fluoroaluminosilicate
TGF-β1	Transforming Growth Factor Beta 1
VHN	Vickers Hardness Number
FAS	Fluoroaluminosilicate

CONTENTS

1. INTRODUCTION

The World Dental Federation (FDI) estimates that around 2.5 billion people worldwide are affected by tooth decay (1), with other studies suggesting that almost 90% of adults have had active tooth decay at some point in their lives (2). The spread of the disease is uneven and socioeconomically dependent, which has a significant impact on quality of life and high healthcare costs (3). The development of dental caries is caused by a combination of factors: genetic, physiological, environmental and behavioural (4). Although this disease is almost entirely preventable, its prevalence has hardly been reduced in recent decades (5).

Dental caries is increasingly seen as a lifestyle-related disease, with the primary responsibility for treatment and prevention lying with a patient, supported to varying degrees by the whole oral health team (6). New approaches to caries treatment should include stopping lesions, allowing time for remineralization and using selective caries removal techniques (7, 8). Therefore, progress in dentistry is closely linked to the development of materials that are constantly being improved to increase their physical, mechanical and biological properties as well as their clinical performance and durability. The concept of minimum intervention oral healthcare (MIOC) promotes the use of methods and materials at all levels that are biological and include bioactive/biointeractive “smart” materials (9, 10). As a result, the use of ion-releasing materials has been increasingly promoted recently as a means of achieving better lifelong oral health. Ion-releasing materials (IRM) or ion-releasing biomaterials (IRB) refer to substances that trigger a targeted cellular and biological response at the material interface. This response leads to the formation of a bond between the desired tissue and the material. Alternatively, these materials can be described as those that generate a surface layer resembling apatite when exposed to saliva or its substitute (9, 11, 12). In a biological sense, bioactive compounds are considered active substances that potentially interact with viable cells and tissues (11). Since most dental biomaterials do not fall under this description, the term "bio-interactive material" is also used (13, 14). These materials are characterized by their ability to bind with collagen, serving as a matrix for calcium and phosphorus, promoting nucleation of apatite crystallization, safeguarding collagen from degradation, maintaining an optimal pH for the generation of new minerals, and exhibiting anti-cariogenic properties (15). It should be noted that the classification of a particular material as bioactive depends on the field of dentistry in which it is used. For example, in restorative dental medicine, bioactivity usually refers to the ability of a material to induce hydroxyapatite crystals creation (16, 17). In implantology, however, the term bioactive refers to the material’s potential to chemically bond the bone and implant (16).

Minimum intervention concept also includes techniques and materials for topical application that remineralize early, non-cavitated lesions as opposed to traditional excavation treatment (18). Resin-based composite materials are widely used in restorative dentistry for posterior restorations and their high failure rate (19), and the sensitivity of the technique (20) prompted Schwendicke et al. (21) to provide a consensus statement on when restorative IRMs can be used:

a) High caries risk patients (22).

b) Active carious dentine lesion (23).

c) Cavities with little or no enamel at the margins, with active carious lesion (24).

1.1. ENAMEL AND DENTINE

1.1.1. Enamel

Enamel is a layer of the tooth that covers the entire tooth crown (Figure 1). It is, therefore, also the part of the tooth that is exposed to the environment of the oral cavity (25). It also serves as a protective layer over dentine and pulp against mechanical and acidic influences as well as against the penetration of bacteria (26 - 29). Although it is the hardest calcified matrix found in humans, it is severely affected by the acidic environment that usually occurs after the consumption of sugary and acidic foods (30). It consists of approximately 96% minerals, 3% water and just under 1% organic matrix (31). The inorganic portion is largely organized in the form of hydroxyapatite ($Ca_{10}(P0_4)_6(OH)_2$), a crystalline form of calcium phosphate (32).

Enamel is commonly described as being developed in a series of successive stages beginning with the presecretory stage, progressing to the secretory stage, moving briefly to the maturation stage and ending with ameloblast apoptosis, which eventually leads to emergence of the tooth into the oral cavity (33). Various ions, such as strontium, fluoride and magnesium, can be incorporated into the crystal if they are present during amelogenesis. How prone these crystals are to dissolution by acid, forms the chemical basis for dental caries. Hydroxyapatite crystals are tightly packed together, with the organic matrix effectively binding these crystals together (34). The crystals are then arranged into a basic structural unit called an enamel prism or rod, consisting of elongated crystals arranged in parallel rows with their crystallographic c-axes aligned. Their diameter is 2-3 μm, and they are enveloped by a thin layer of organic matrix, the melting rod envelopes (31, 35). Finally, prisms and interprismatic structures combine to form prismatic bands that extend through the entire enamel layer. Although their proportion in mature enamel is almost negligible, the organic matrix plays an important role in strengthening the structure (36, 37).

1.1.2. Dentine

Dentine, the largest component of the human tooth (Figure 1), consists of 70 % (55 % by volume) of minerals, while the proportion of organic matter (mostly collagen type 1) is about 20 % (30 %); the rest is mainly water, which is predominantly located inside dentinal tubules (38). In dentine, the hydroxyapatite crystals have the shape of a platelet with a length of 50 nm, a width of 20 nm and a thickness of 2–5 nm (39). However, the actual composition of dentine varies within the structure (40, 41). Dentine is primarily produced by odontoblasts, originating from embryonic ectomesenchymal connective tissue cells derived from the cranial neural crest (42). The dentine has a tubular structure, with the tubules surrounded by intertubular dentine (43). The diameter of the dentinal tubules varies and ranges from 0.9 µm in the periphery to 2.5 µm on the pulp side. At the same time, the density of these tubules on the pulp side is approximately 59,000–76,000 tubules/mm², which decreases to half this amount near the dentine-enamel junction (DEJ) (44). Despite the considerable differences in structure, five types of dentine are generally distinguished, depending on the phases of their formation: dentine–enamel junction, mantle dentine, primary dentine, secondary dentine and tertiary dentine (45).

1.1.2.1. Dentine-enamel junction

The DEJ or dentine–enamel junction complex is a 7 to 15 mm wide structure characterized by a distinct separation of enamel and dentine. It consists of considerable amounts of organic and mineral substances (46). The DEJ is thought to be a complex of two distinct, thin, contiguous layers. The first layer is the inner aprismatic enamel, which has some variations compared to the prismatic enamel. The second layer is the mantle dentine, which has some similarities with the circumpulpal dentine, but is structurally different (47). Its undulating structure is thought to play a role in the interlocking of enamel and dentine (48 – 50).

1.1.2.2. Mantle dentine

Mantle dentine is a 5 to 30 µm thick layer (40) of the first formed dentine. The most distinct feature of this layer is Korff fibres, which typically consist of collagen type III (51). It does not consist of dentine tubules, but only of tubular branches (52). Different authors believe that the mantle dentine, together with parts of the circumpulpal dentine, plays a significant role in imparting the elastic properties of teeth. This property enables teeth to withstand relatively high occlusal loads without fracturing enamel or dentine (45).

1.1.2.3. Circumpulpal dentine

Circumpulpal dentine consists of primary, secondary and tertiary dentine. The primary dentine is its largest part and gives the tooth its shape (53). It is formed rapidly during tooth formation. Secondary dentine has minor differences from primary dentine, such as slight variations in the curvature of dentinal tubules and a less regular tubular structure. In addition, the deposition of dentine may be uneven, with a notable tendency for greater deposition on the floor and roof of the pulp chamber, which is particularly evident in molars (54).

The formation of tertiary dentine occurs as a response to irritants, usually caries or mechanical injury, to protect the vitality of the pulp. It comes in two types: reactive dentine, which is formed by the original primary odontoblasts, and reparative dentine, which is formed by newly differentiated odontoblasts (55 – 57).

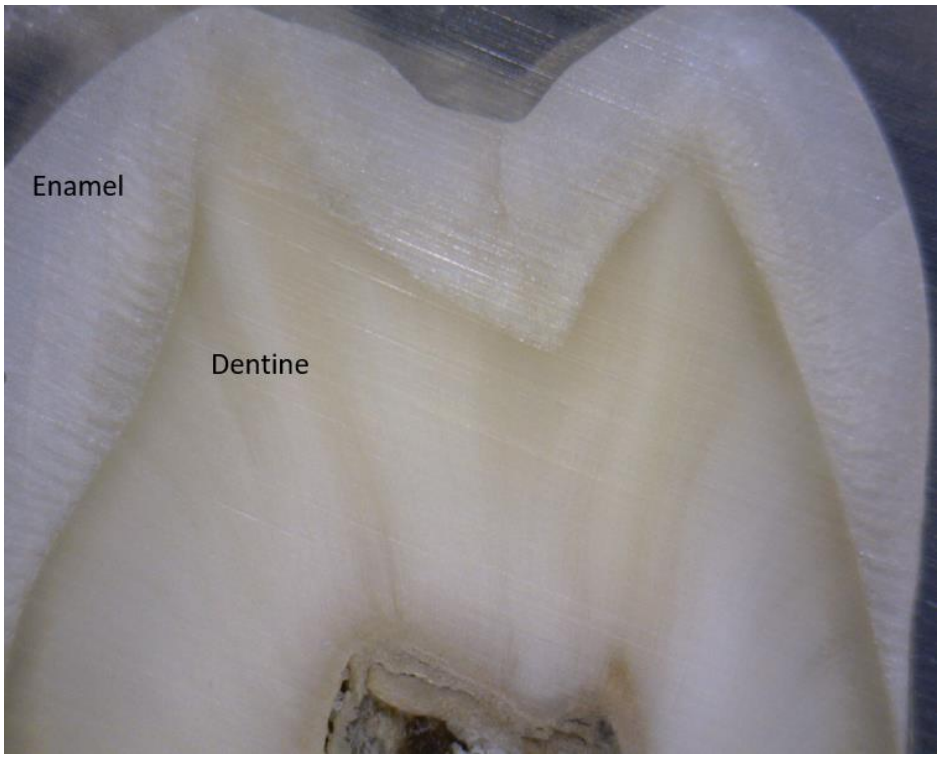


Figure 1. Scanning electron microscopy image of enamel and dentine.

1.2. MINERALIZATION DYNAMICS

Biomineralization is a complex and dynamic lifelong process in which living organisms control the formation of inorganic nanocrystals in organic matrices (39). During this process, different tissues such as enamel, dentine, cementum and bone are formed. Ion substitutions in biological apatite include exchanging calcium ions with magnesium and sodium or replacing hydroxyl sites with fluoride and chloride. These substitutions can notably impact the apatite properties; the introduction of carbonate enhances while the incorporation of fluoride reduces solubility (54). Demineralization is a process in which the mineral ions are removed from the hydroxyapatite (HA) crystals. A pH of <5.5 is considered the critical point at which HA can dissolve (58). When these mineral ions are reincorporated into the HA crystals, the process is referred to as remineralization. Both processes take place on the surface of the tooth. The progression of this process can lead to caries formation (59). Demineralization can be reversed; moderately demineralized HA crystals in teeth can regenerate when conditions in the oral cavity are balanced. Although complete remineralization of a lesion is unlikely, it is usually sufficient for arresting caries progression or improve other symptoms of mineral loss (60).

1.2.1. Demineralization

Caries development is described as an outcome of an imbalance in the resident microflora, where potentially more cariogenic bacteria dominate the microbial composition, resulting in HA crystals dissolution:

$$Ca_{10}(PO_4)_6(OH)_2 + 14H+ \rightarrow 10Ca+ + 6H_2PO_4+ H_2O$$

This is often triggered by frequent instances of low pH conditions in plaque biofilms, such as those arising from dietary changes or a decrease in saliva flow (61).

Enamel demineralization is usually related to the interaction between bacteria and sugars on the tooth surface; the breakdown of fermentable carbohydrates such as glucose, sucrose and fructose by bacteria creates an acidic environment that leads to demineralization and the development of carious lesions (62) (Figure 2). A variety of microorganisms are found in dental plaque, representing a diverse ecosystem on the tooth surface: 15-27 different species are present in a small plaque sample, as well as digestive products, epithelial cells, blood and salivary cells, and glycoproteins from saliva (63, 64). Most of these organisms are essential for

maintaining stability on the tooth surface and are considered non-mutans (65). A low pH environment favours mutans bacteria, which promote demineralization and accelerate the development of lesions. *S. mutans, S. sobrinus* and *lactobacilli* are the primary oral cariogenic pathogens. This distinction is based on their ability to produce high levels of lactic acid through sugar fermentation and their resistance to the damaging effects of a low pH environment (66, 67). However, it has been suggested that under low pH conditions, normally less cariogenic bacteria such as *S. mitis*, *S. oralis* and *Actinomyces* may adopt a more acidogenic phenotype and further promote caries development (65) (Figure 3). Furthermore, even the elimination of *S. mutans* would have insignificant effects on the progression of demineralization, as it is assumed that they would soon be replaced by other acidogenic bacteria (68). A drop in pH leading to demineralization is not only triggered by bacterial activity resulting from the consumption of sugar and acidic beverages; other factors also play an important role. Some medications, such as certain types of asthma inhalers, can lower the pH of saliva (69, 70). In addition, medications can significantly alter salivary flow, reducing the buffering capacity of saliva (71). In some conditions, such as gastroesophageal reflux and bulimia nervosa, gastric acid with a pH of about 1.2 enters the oral cavity (72, 73), leading to rapid demineralization.

Mineral loss begins in the interprismatic areas and then progresses to the enamel prisms (74), resulting in a "white spot lesion" as the enamel surface is initially less affected than the underlying layer (75). Enamel caries manifests itself in four different zones, starting with the advancing front of the lesion. The first is the translucent zone, where the caries progresses and which is characterized by a mineral loss of 1.2%, while the organic material remains largely unaffected. This is followed by the dark zone, which shows a mineral loss of 6% per unit volume of enamel and causes a brown discoloration. The third zone represents the body of the lesion, with a significant mineral loss of 24% per unit volume compared to normal enamel. Finally, the surface zone shows partial demineralization leading to a mineral loss of 10% (76).

The uncontrolled progression of the demineralized enamel lesion usually leads to caries formation, which then reaches the dentine. The progression of the dentine lesion is much faster as the mineral phase is affected, collagen fibres are exposed and degraded and the entire dentine structure is affected (77). The larger surface area of dentine apatite makes it more susceptible to dissolution when exposed to acids (78). This susceptibility is exacerbated by the higher carbonate content of the dentine mineral. Consequently, the dissolution of dentine minerals occurs faster than that of enamel, as the total mineral content in dentine is lower (79). In addition, the critical pH required to initiate demineralization in dentine is between 6.2 and 6.4,

a value higher than that of enamel (80). Caries-infected dentine is characterized by significant decalcification, degenerated collagen fibres and inactive odontoblastic processes and therefore lacks the physiological ability to recalcify. In contrast, caries-affected dentine, which exhibits moderate decalcification, intact collagen fibres and active odontoblastic processes, retain the physiological ability to recalcify (81).

To prevent the spread of the caries lesion into the dentine and avoid a vicious restorative cycle, a biological approach to caries management has been proposed (82), based on the use of different remineralization systems. With this approach, the need for traditional restorative systems can be significantly reduced (83).

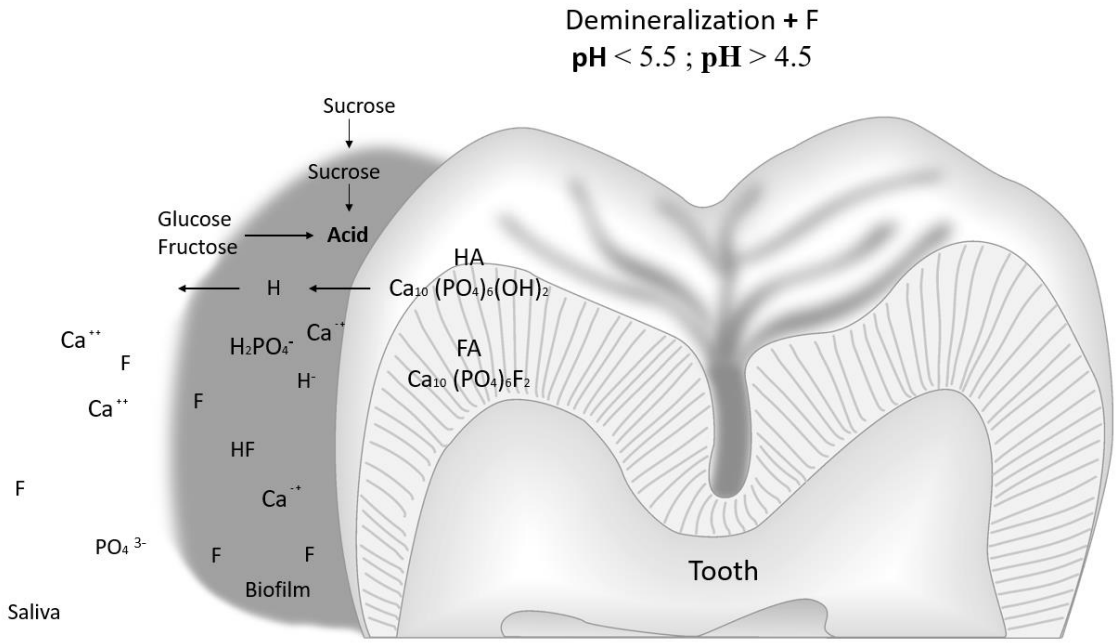


Figure 2. Demineralization process in enamel and dentine [*Cury JA*, adapted from (91)].

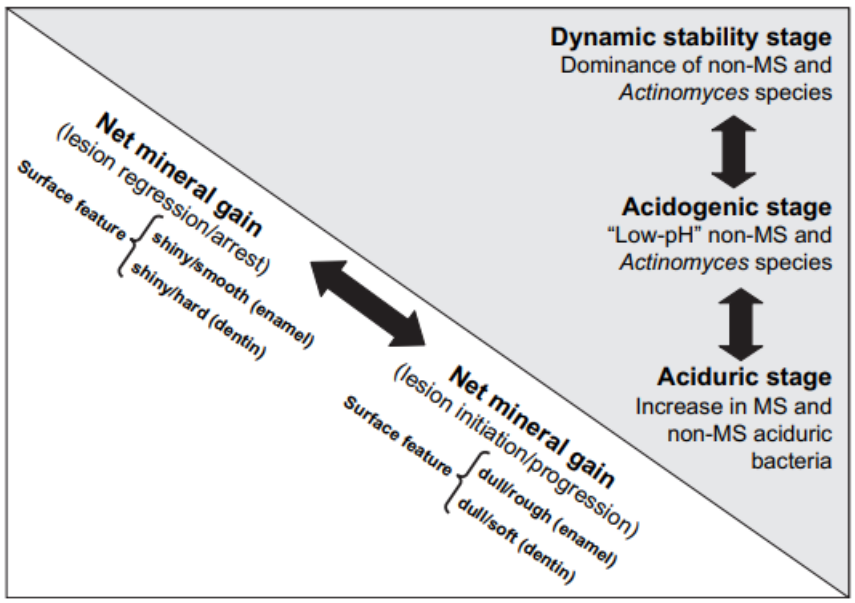


Figure 3. Caries development ecological hypothesis [*Takahashi N et al*, adapted from (65)].

1.2.2. Remineralization

Early detection and prevention of demineralization is currently considered the best approach to the clinical management of early enamel lesions, with saliva rich in calcium and phosphate ions serving as a buffer (84, 85). In this way, the de-/remineralization process can be influenced in favour of remineralization.

The most commonly used agent is fluoride-based products. Fluoride has been shown to be anticariogenic and to reduce the solubility of enamel and dentine through the formation of fluorapatite (FA), lowering the critical value for mineral dissolution to a pH of < 4.5 (86 - 88). However, fluoride concentrations decrease significantly beyond the outermost 10-20 micrometres, and calculations indicate that these levels are well below those capable of providing a substantial reduction in the solubility of HA (89). However, the topical application of fluoride allows it to be present on almost all exposed tooth surfaces, where it can combine with calcium to form CaF deposits that act as a reservoir to protect the underlying minerals. Triggered by a drop in pH, fluorides can be easily released and serve as an inhibitor of demineralization and act as a promoter of remineralization (90) (Figure 4).

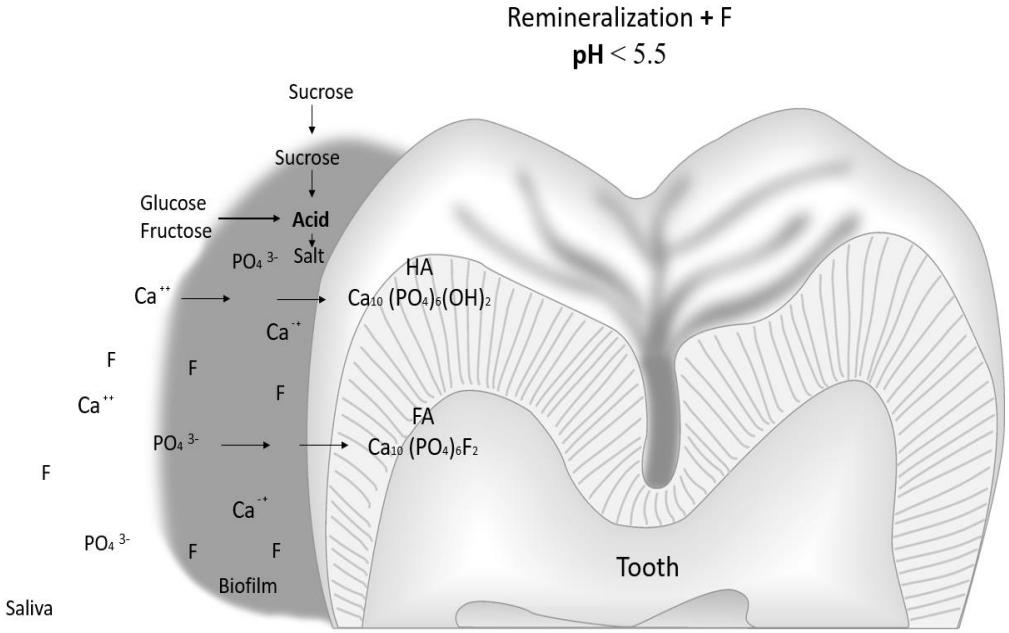


Figure 4. Remineralization process triggered by fluoride presence [*Cury JA*, adapted from (91)].

The concentrations of calcium and phosphate in saliva and plaque play crucial roles in both the demineralization and remineralization processes of teeth. Different researches suggest that elevated levels of calcium in saliva may exert a protective effect against dental caries (92, 93). A significant breakthrough in addressing early carious lesions occurred with the introduction of the casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) complex, derived from milk protein, as an enamel remineralizing agent (94). Studies have demonstrated its effectiveness in diminishing enamel demineralization and fostering remineralization by buffering the activities of free calcium and phosphate ions. This is achieved through the integration of amorphous calcium phosphate (ACP) into the plaque and on the tooth surfaces, thus creating non-cariogenic plaque (95 - 97). There is a hypothesis that the plaque's ability to retain fluoride for extended periods is primarily dictated by the calcium concentration within the plaque; therefore, many commercially available products nowadays contain both fluorides and CPP-ACP or other form of calcium phosphate (98 - 100).

On the other hand, remineralization of demineralized dentine can occur through several processes (101):

a) the odontoblast process, which involves the supply of calcium and phosphate from the vital pulp (102)

b) the diffusion of ions from materials applied to the base of a restored cavity (103)

c) the interaction of saliva with the carious lesion, particularly in root dentine, aided by oral hygiene practices (104).

Complete bacteria removal from carious dentine lesion is nowadays deemed unnecessary; sealing the lesion and cutting the bacteria off from the oral environment diminishes their cariogenic potential as it inhibits their metabolism (101, 105). This can further be boosted with the use of IRMs.

1.3. REMINERALIZATION THERAPIES IN ENAMEL

Dental caries is more than a continuous loss of tooth minerals; it is a dynamic process characterized by alternating phases of demineralization and remineralization (106). The progression or reversal of lesions depends on the balance between factors that promote demineralization (such as cariogenic bacteria, carbohydrates and low saliva flow) and protective factors (including antibacterial agents, adequate saliva and remineralizing ions) (107). IRMs make it possible to restore the inherent characteristics and composition of enamel while preventing the issues linked to dental fillings. These materials play a vital role in enamel repair as they are able to facilitate and organize the deposition of calcium and phosphate ions or modify the solubility of HA. As far as enamel remineralization is concerned, such products are usually offered in the form of toothpastes or varnishes for topical application. In 2014, Naveena et al (108) suggested the following properties that remineralizing agents should have:

- Ability to deliver calcium and phosphate into the subsurface
- Avoids excessive deposition of calcium and calculus formation
- Effectiveness in an acidic pH
- Acts potent in patients with xerostomia
- Enhances the saliva remineralizing efficacy

In 2022, Xu et al (109) suggested the latest classification of enamel-repairing materials:

1.3.1. *Functional inorganic materials*

- Calcium phosphates
- Fluorinated compounds
- Magnesium related materials

These materials are able to stimulate the formation of apatite layers or release ions, facilitating the process of enamel remineralization. Materials from this group are most commonly used because calcium phosphates provide ions for successful remineralization, fluorides have a preventive effect and magnesium-containing products cause highly ordered crystallization (110 - 112). ACP, a calcium phosphate, is a precursor of HA in the teeth. It is rich in Ca^{2+} and PO_4

3− ions, which would aggregate without different stabilizers, such as CPP, which ensure ion supply during remineralization. Nano-hydroxyapatite (n-HA) resembles the structure of natural HA but has a small particle size of 10–20 nm in diameter and 60–80 nm in length (113). Due to its small size, it can penetrate deeper into the lesion and is therefore often used in oral care products today, as it can replace the minerals present in the enamel (114).

1.3.2. Functionalized organic materials

- Amino-acids
- Enamel matrix proteins and proteases
- Functional peptides
- Alendronate

Organic materials, with their hydroxyl and phosphate functional groups, play a role in stabilizing the environment in which remineralization takes place; they initiate the nucleation of inorganic compounds, prevent excessive growth and combine with the hydroxyapatite on the enamel surface, increasing the adsorption capacity (115, 116).

1.3.3. Polymer materials

- Non-collagenous protein analogues
- Poly (amino amine) (PAMAM)
- Polydopamine
- Biopolymers
- Other polymers

Polymer materials are very versatile, and they can replicate the enamel structure. Some of them maintain ion stability, while others can be transformed into gel carriers for transporting functional peptides (117, 118). They can even exhibit antibacterial effect (119).

1.4. REMINERALIZATION THERAPIES IN DENTINE

Dentine has the ability to regenerate itself through the development of secondary and tertiary dentine. In contrast, enamel lacks the ability to regenerate. However, due to the much higher proportion of organic matter in dentine, its remineralization is a much more complex process than in enamel (120). Both the organic collagen type I and the inorganic apatite are in a specific spatial relationship with each other. This means that remineralization alone is not sufficient to completely restore demineralized dentine. Therefore, the structure of the collagen matrix must also be restored and both phases must be connected in a specific way to achieve intrafibrillar mineralization of the collagen (77, 121). Recent evidence suggests that collagen type I plays a crucial role as a template for mineralization by directing the growth and deposition of crystals in the preferred direction (122). This is in contrast with previous perception of collagen as a passive scaffold for mineralization (123, 124).

The main recommendation for the treatment of a decayed tooth is to remove the decayed dentine and to preserve demineralized (formerly called 'infected') dentine and restore the cleaned cavity with an ion-releasing restorative material that has optimal biological and physical properties (101). Classification of restorative IRMs, proposed by Slimani et al in 2022 (9), is shown in Table 1.

Table 1. Restorative IRMs for dentine lesions treatment.

Types of Materials	Bio-Interactive Properties	Biological Effects
Glass ionomer cements	-release of fluoride, calcium and aluminium ions -polyalkenoate salts and calcium polycarbonate creation	-antibacterial effect -anticariogenic effect
Glass hybrid cements	-release of fluoride, calcium and aluminium ions	-antibacterial effect -anticariogenic effect

	-polyalkenoate salts and calcium polycarbonate creation	
Resin-modified glass ionomer cements	-release of fluoride, calcium and aluminium ions -polyalkenoate salts and calcium polycarbonate creation	-antibacterial effect -anticariogenic effect
Resin composites with minerals (ion-releasing)	-release of fluoride ions (others as well if present)	- promote remineralization - reduction of material degradation
Self-adhesive ion-releasing resin composites	- release of fluoride and aluminium ions - set by chemical and light	- promote remineralization - reduction of sensitivity
Giomers	- combination of resin-based composites and glass ionomer	- improvement in fluoride release, but largely similar to conventional resin composites
Alkasites	- Alkaline glass fillers - release of fluorides and hydroxyl ions (pH dependant)	- hydroxyl ions can alter pH values and supress demineralization - buffering ability at pH 5.7 - calcium forms apatite, if phosphates are present
Calcium silicate-based materials	- remineralisation occurs due to an alkaline reaction. - antibacterial effect on caries-affected and dentine	- increased mineralization in the affected zone due to the penetration of calcium ions

Resin-modified MTA (calcium silicate-based)	- similar to regular calcium silicate-based materials	-vital pulp therapy -simplified application
Silver Diamine Fluoride	- well known antimicrobial effects of silver, combined with fluoride effects - diminishes the possibility for staining	-beneficial in high caries risk patients -usually combined with GICs
Resin-modified glass ionomer adhesives	- contains Ionglass™ fillers with fluoro-aluminosilicate glass (fluoride release)	- can be combined with both resin-based composites and
Adhesive with chlorhexidine	- release of chlorhexidine	- antimicrobial effect - stabilization of the hybrid layer

1.4.1. Glass-ionomer cements

Glass-ionomer cements (GIC) or glass-polyalkenoate cements are the most commonly used restorative IRMs that have been on the market since the 1970s (125). They usually consist of glass compounds, polyacrylic acid and water (126). The basic glass usually contains calcium fluoro-alumino-silicate powder, but phosphate, strontium, calcium or other elements may also be included (127). They show outstanding biological characteristics, including biocompatibility, bioactivity, fluoride release, an impressive linear coefficient of thermal expansion/contraction, modulus of elasticity, and the capacity for chemical bonding to teeth (128, 129). However, their poor mechanical properties have prevented them from becoming a material of choice for long-term posterior restorations (130). Many attempts have been made to improve their mechanical properties. Resin-modified glass ionomer cements (RM-GIC), a light-cured resin is added to the structure (131). This changed and improved their mechanical properties compared to the original form; however, they remained inferior to composite materials (132).

1.4.2. Glass hybrids

Glass hybrids (GH) were developed from the glass ionomers, offering a significant improvement in mechanical properties compared to GICs while retaining the advantageous ion release mechanisms. One of the novelties is the introduction of highly reactive silicate glass particles which, combined with other modifications, improve the physical characteristics of the material (133). This indicates that glass hybrids are suitable for long-term Class I and Class II restorations, which has been confirmed by various clinical studies (134 - 136).

1.4.3. Resin-based composites with minerals (ion-releasing composites) and self-adhesive ion-releasing composites

Resin-based composites, the most commonly used material for posterior restorations, have also seen significant improvements, but this time in terms of biological properties, as there are now versions on the market that also release ions, similar to glass ionomer cements. In vitro studies have shown that such materials, especially when releasing calcium and fluoride ions, can remineralize both enamel and dentine lesions (137, 138). However, their remineralization effectiveness seems to be inferior to GICs and GHs (139), but such studies are lacking for a complete conclusion.

Bulk-filled self-adhesive composites have also been recently introduced; their structure is modified with acidic groups to enable bonding with tooth structures, but their usefulness is questioned and still not examined enough (140 - 142).

1.4.4. Giomers

Giomers were introduced as a material that combines the composition and effects of both GICs and resin-based composites by incorporating pre-reacted glass filler particles into the composite matrix (143). They have been found very successful in restoring cervical lesions (144), but their use for other indications is not widespread.

1.4.5. Alkasites

In recent times, a new category of IRMs has emerged, blending the characteristics and composition of glass ionomer cements (GICs) and resin-based composites. Termed alkasites, these materials incorporate an alkaline filler, allowing them to release acid-neutralizing ions along with fluorides, hydroxyl ions, and calcium in low pH oral conditions (145). The first manufacturer listed this these materials as a subgroup of resin-based composite materials (146). Hydroxyl ions are thought to neutralize cariogenic acids, while calcium is necessary for remineralization, with fluorides accelerating the process (147 - 149).

1.4.6. Calcium silicate-based materials

While previously used mostly in endodontics, due to their long setting reaction, tricalcium-silicate based materials are now also used as a dentine replacement material, since they can prompt mineral deposition (15). Such materials are alkaline in nature; when placed on dentine, degradation of the collagen fibrils occurs. This leads to the formation of a porous structure that facilitates the penetration of high concentrations of calcium and carbonate ions, which increases mineralization of the affected zone (150). Variations modified with resins have been introduced to reduce the material's solubility and improve bonding (151).

1.4.7. Silver diamine fluoride

Silver diamine fluoride has been used as a caries-arresting remedy in certain parts of the world, utilizing a century-long knowledge of antimicrobial properties of silver (152). However, its effectiveness is mostly limited to primary dentition (153).

1.4.8. Modified bonding agents

Bonding agents have also been modified to exhibit certain degree of biointeractivity. Riva Bond LC (SDI Limited, Bayswater, VI, Australia) is a light-cure adhesive with an Ionglass™ filler, allowing it to release significant amounts of fluorides. In other cases, adhesives have chlorhexidine in their structure, meant to act antibacterial.

1.5. ENAMEL AND DENTINE REMINERALIZATION ASSESSMENT

Various methods can be used to analyse tooth de- and remineralization, ranging from direct measures like microradiography to indirect techniques such as iodide permeability, with their accuracy depending on specimen preparation, the magnitude of change, and analysis protocols (154).

1.5.1. Transversal microradiography

Transversal microradiography (TMR) is one of the methods which offer quantification of the mineral density of hard dental tissues (155). In this method, thin slices of the tooth structure are prepared, high-resolution X-ray images are taken and compared (156). The mineral density is calculated from the grey values of the images. The main limitation of this method is its susceptibility to destruction, which precludes the possibility of longitudinal studies.

1.5.2. Energy dispersive spectroscopy

Energy dispersive spectroscopy (EDS) is a valuable method that allows us to identify and quantify the main components of enamel and dentine (157, 158). It also allows us to follow changes in composition according to specific protocols. EDS uses the X-ray spectrum emitted when a solid sample is bombarded with a focused electron beam to enable localized chemical analysis. When qualitative analysis is conducted the spectral lines are identified, allowed by the simplicity of the X-ray spectra. In quantitative analysis, the determination of elemental concentrations requires the measurement of line intensities in the sample and in calibration standards of known composition (159).

1.5.3. Hardness evaluation

Numerous studies have evaluated microhardness to assess demineralization and remineralization of tissues (160 - 163). Hardness, an important mechanical property of dental materials, is described as the resistance to permanent indentation of the surface or the ability to

resist deformation (160). Its importance also lies in the fact that it is related to other mechanical properties such as tensile strength and abrasion (164). Over time, different hardness testing methods have evolved, categorized according to the forces exerted on the materials, including macro, micro or nano indentation tests. Macroindentation tests use larger forces (1 kgf or more), while microindentation tests use forces between 1 and 1000 gf (gram-force). Nanoindentation tests work at the nanometer level of indentations. Microhardness evaluation proves beneficial in determining the hardness of small samples, complicated geometries, specific phases within a material and surface coatings (165). The actual means of determining microhardness, while method dependent, typically involve creating an indentation on the sample with a diamond indenter by applying a load (166).

1.5.4. Scanning electron microscopy

Scanning electron microscopy (SEM) is highly suitable for investigating the enamel and dentine structure and surface appearance, as it creates high resolution images of hard dental tissues (167, 168). In this testing a concentrated beam of high-energy electrons is used to produce diverse signals at the solid specimen's surface, with the information gathered across a designated surface area of the specimen, creating a two-dimensional image illustrating spatial variances in attributes such as chemical composition, material texture, and orientation (169).

1.5.5. Micro-CT

Micro-focus X-ray computed tomography (micro-CT) is a useful technique which gives insight into mineral density and the structural characteristics of mineral tissues, including teeth (170). Micro-CT allows for the generation of a three-dimensional (3D) map of the interface between the tooth and restoration (171, 172). It is a non-destructive method with the capability to acquire data encompassing volume, depth, surface area, mineral density and imaging (173). MicroCT operates on the principle of x-ray attenuation as it passes through the imaged object or sample. The attenuation follows the equation:

$$Ix = I0e^{-\mu x}$$

where I0 is the initial beam intensity, x represents the distance from the source, Ix is the beam intensity at distance x, and μ is the linear attenuation coefficient (174, 175). The process of reconstructing a 3D image involves rotating either the sample (in desktop systems) or the

emitter and detector (in live animal imaging). This rotation produces a sequence of 2D projections, which are then converted into a 3D representation using a digital technique known as back-projection (176, 177).

1.5.6. Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) is used to obtain high-resolution images of changes in the mineral composition of tooth enamel (178, 179). With this method, the out-of-focus light from the detector can be avoided, eliminating its blurring effect on the collected images, which is particularly effective when examining thick samples, up to one half of the tooth (180). The average grey values are graphed within the lesion to determine the fluorescence values; increased fluorescence corresponds to higher grey values, indicating reduced porosity and dye penetration or increased presence of minerals, while lower values indicate the opposite (179).

1.5.7. Transmission electron microscopy

Transmission electron microscopy (TEM) has been widely used to analyse the crystal structure of dentine (181, 182). The electrons are directed in the form of a beam through an ultra-thin specimen, engaging with the specimen as they pass through, allowing very small details to be observed (183). The special capability of the TEM results from the fact that electrons are charged particles and can be accelerated and precisely focused with the help of electromagnetic fields (184).

1.5.8. Field Emission Gun Electron Probe Micro-Analysis

Field Emission Gun Electron Probe Micro-Analysis (FEG-EPMA) is a method in which a focused electron beam is directed at a sample and the emitted X-rays are analysed. This method typically integrates two closely related analytical techniques — wavelength dispersive spectroscopy and energy dispersive spectroscopy - in one element, the microprobe (185). Its advantages are high spatial resolution, the ability to analyse elements, depth profiling, quantification, and chemical mapping (186).

1.5.9. Polarization-sensitive optical coherence tomography

Polarization-sensitive optical coherence tomography (PS-OCT) proves to be effective for non-destructive quantification of the extent of dentine demineralization and evaluation of the effects of interventions with anticariogenic products (187, 188). Due to the higher scattering coefficient of dentine (187), it is more suitable for the assessment of enamel. It is a three-dimensional optical imaging method comparable to ultrasound but using high-frequency light instead of high-frequency sound waves (189).

2. OBJECTIVES AND HYPOTHESES

2.1. Objective of research

The aim of this study has been to analyse the effect of contemporary ion-releasing materials on remineralization of enamel and dentine. The secondary goals of the research were:

1. Determine the effect of IRMs on the microhardness of enamel and dentine.

2. Determine the effect of IRMs on the appearance of the surface and the chemical composition of enamel and dentine.

3. Quantify the remineralization effect of IRMs in dentine.

2.2. Null hypotheses

Null-hypotheses were:

1. There will be no significant difference in the effect of the tested IRMs on microhardness, surface appearance and chemical composition of demineralized enamel.

2. There will be no significant difference in the effect of the tested IRMs on microhardness, surface appearance and chemical composition of demineralized dentine.

3. There will be no significant difference in dentine density changes obtained by micro-CT analysis.

3. MATERIALS AND METHODS

The Ethics Committee of the School of Dental Medicine, University of Zagreb approved this study (05-PA-30-XXVII-5/2021).

Three parts of research were conducted:

a) Determination of the effect of three IRMs on the microhardness and chemical composition of demineralized enamel.
b) Testing the remineralization effect of restorative IRMs that come in encapsulated form; new glass hybrid cements and alkasites were compared to glass ionomer and calcium silicate materials.
c) Further imaging of ion-releasing materials effect on dentine via Micro-CT.

3.1. MATERIALS USED IN THE STUDY

In the first part of the study, three commercially available materials were evaluated: 3M™ Clinpro™ White Varnish, MI Varnish® and Megasonex® toothpaste (Figure 5). All tested materials were applied topically, directly to the enamel. The materials, along with their respective ingredients and additional information provided by their manufacturers, are shown in Table 2.

Table 2. Materials used for specimen preparation in the first part of the research.

Material	Active ingredient	Additional ingredients	Manufacturer
Clinpro™ White Varnish	22 600 ppm fluoride	Alcohol, resin, water, flavours	3M ESPE, St. Paul, MN, USA
MI Varnish®	CPP-ACP and 5% sodium fluoride	Polyvinyl acetate, ethanol, hydrogenated resin, silicon dioxide, flavours	GC Corporation, Tokyo, Japan
Megasonex®	nano-hydroxiapatite	Sorbitol, glycerin, water, silica, xylitol, tetrasodium pyrophosphate, sodium methyl cocoyl taurate, mica, titanium dioxide, sodium carboxymethylcellulose, citric acid, sodium saccharin, aroma	Panaford B.V., Rotterdam, The Netherlands

Figure 5. Materials used in the first part of the study.

The continuation of the research in the second and third part involved testing the remineralization effect of restorative ion-releasing materials, all of which were in encapsulated form; new glass hybrid cements (EQUIA Forte® HT and Riva Self Cure) and an alkasite (Cention Forte) were compared with glass ionomer (GC Fuji TRIAGE®) and calcium silicate material (Biodentine™) (Figure 6). The tested materials, along with the information provided by the manufacturers, are listed in Table 3.

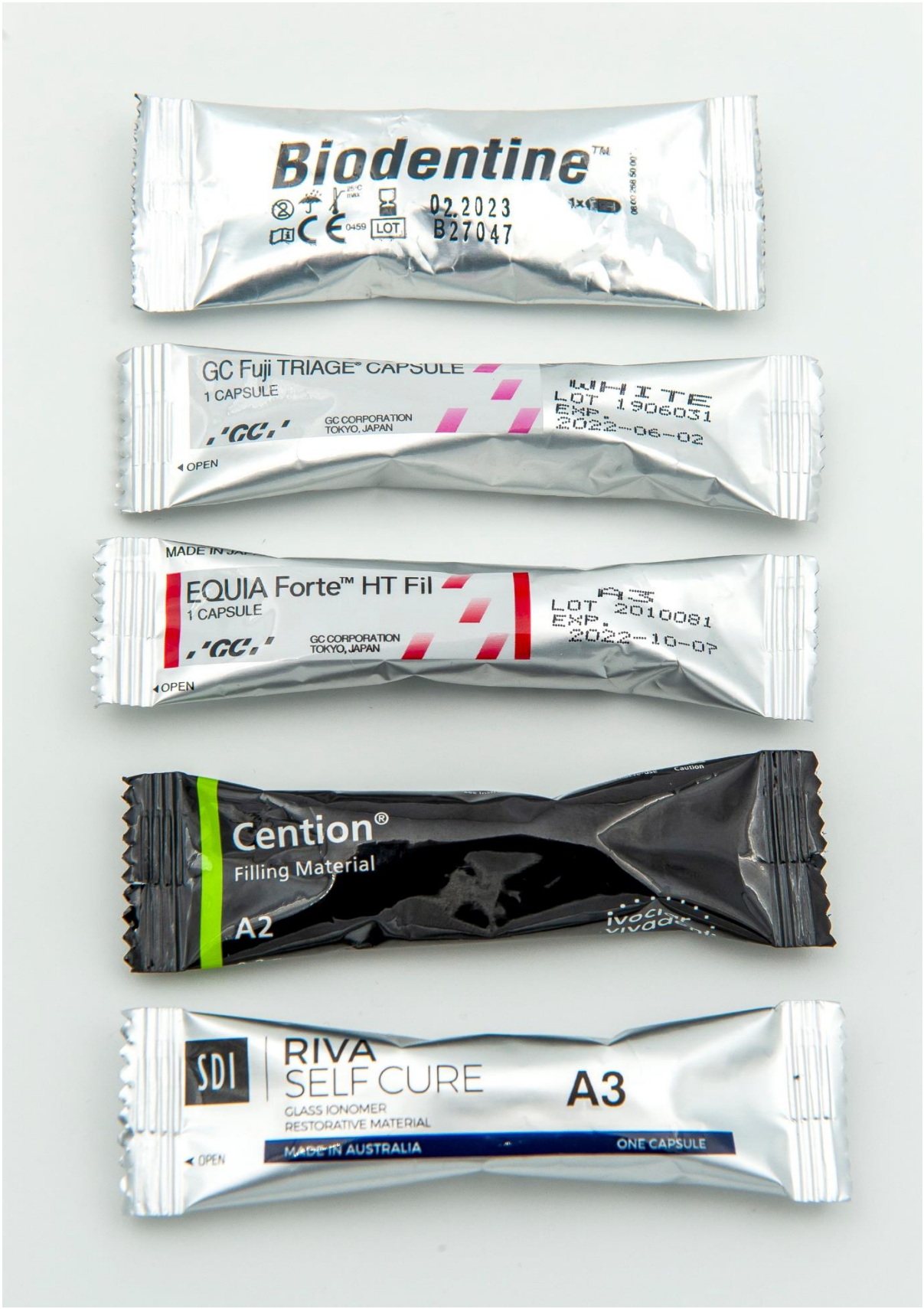


Figure 6. Materials used for specimen preparation in the second part of the study.

Table 3. Materials included in the second and third part of the research.

Material	Type	Composition	Manufacturer
EQUIA Forte® HT	Glass hybrid cement	Powder: fluoroaluminosilicate glass, polyacrylic acid, iron oxide Liquid: polybasic carboxylic acid, wate	GC Corporation, Tokyo, Japan
Riva Self Cure	Glass hybrid cement	Powder: 90-95 % glass powder, 5-10% acrylic acid homopolymer Liquid: water, 20-30% acrylic acid homopolymer, 10-15% tartaric acid	SDI Limited, Bayswater, VI, Australia
Cention Forte	Alkasite	UDMA, DCP, Aromatic aliphatic-UDMA, PEG-400 DMA Barium aluminium silicate glass, Ytterbium trifluoride, isofiller, Calcium barium aluminium fluorosilicate glass, calciumfluorosilicate glass	Ivoclar Vivadent AG, Schaan, Liechtenstein
Biodentine™	Tricalcium silicate-based material	Tricalcium silicate, dicalcium silicate, calcium carbonate and oxide filler, iron	SEPTODONT, Saint-Maur-des-fossés Cedex, France

		oxide shade, and zirconium oxide	
GC Fuji TRIAGE®	Glass ionomer cement	Powder (main components): alumino-silicate glass, polyacrylic acid copolymer, water-soluble polymer, tertiary amine Liquid (main components): Polyacrylic acid, water, tartaric acid, potassium persulfate, sodium hydrogen carbonate	GC Corporation, Tokyo, Japan
3M™ Filtek™ Universal Composite (control group)	Resin-based composite	BIS-GMA, UDMA Bis-EMA, silica/zirconia	3M ESPE, St. Paul, MN, USA

3.2. SAMPLE PREPARATION

3.2.1. Samples for evaluating the microhardness and chemical constitution of demineralized enamel

This segment of the study included 33 healthy extracted human third molars, which had undergone a thorough cleaning process involving scaling and brushing. Following the cleaning procedure, the teeth were preserved in a saline solution at a temperature of 37°C. To create uniform specimens, the teeth were embedded in autopolymerizing acrylic resin (Heraus Kuzler GmbH, Hanau, Germany) within a rubber mold (Figure 7). Subsequently, they were allowed to solidify for 24 hours, resulting in the formation of rectangular blocks. The ensuing polishing procedure was performed using a polishing machine (Presi, Le Loche, Switzerland) with a rotational speed set at 300 rounds per minute (Figure 8). Standard metallographic grinding papers were used to achieve a smooth enamel surface.

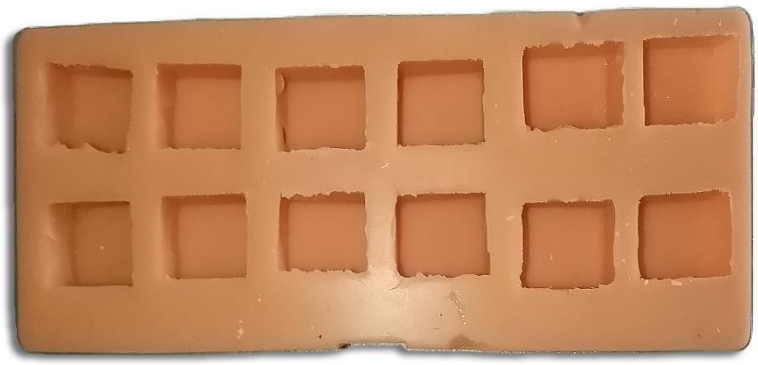


Figure 7. Rubber mold for specimen preparation.

Figure 8. Polishing device used in the study.

To assess microhardness, the samples were divided into three groups, each comprising 10 samples (n=10), through a random allocation process. Additionally, one sample per group was prepared for SEM/EDS analysis (n=1). These samples were sliced to a thickness of approximately 1.5 mm using an IsoMet 1000 Precision Cutter (Buehler, Lake Bluff, IL, USA) and an IsoMet Diamond Wafering Blade with a 12.7-mm shaft and a thickness of 0.5 mm (Buehler, Lake Bluff, IL, USA) at a speed of 250 rpm.

In the demineralization protocol, the buccal enamel surface underwent demineralization through the application of 37% phosphoric acid (DiaDent Group International, Chungcheongbuk-do, Korea) for a duration of three minutes (Figure 9).

Figure 9. Sample demineralization.

After rinsing and drying, remineralization process was initiated by applying the tested remineralizing agents, one in each group. They were applied two times a day at two-minute intervals using a soft brush (3M ESPE, St. Paul, IL, USA). These specimens were stored in saline solution (Croatian Institute of Transfusion Medicine, Zagreb, Croatia) at 37 °C for 14 days. Fresh saline was prepared every 48 hours.

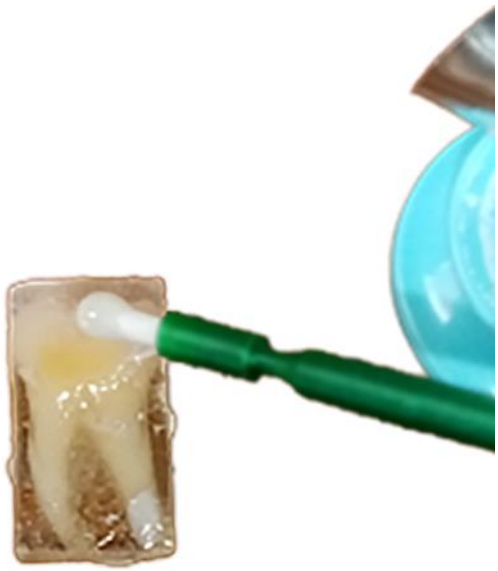


Figure 10. Material application.

3.2.2. Samples for the evaluation of the remineralizing effect of restorative ion-releasing materials on dentine

Seventy-two human molars were categorized into groups for the five materials subjected to testings, alongside a control group. Distinct sets of samples were used for two separate test series, conducted after 14 days and 28 days, respectively. After the removal of the occlusal portion of the crown was removed using the IsoMet 1000 Precision Cutter (Buehler, Lake Bluff, IL, USA), a class I cavity measuring 3 x 1.5 mm in width and 0.5 mm in depth was prepared in each tooth with a diamond bur (Komet, Lemgo, Germany) mounted in a high-speed water-cooled turbine, ensuring that the bottom of the cavity reached the mid-coronal dentine. For direct surface comparison, half of the cavity was coated with acid-resistant nail polish (Markwins Beauty Brands, Inc., Walnut, CA, USA) (see Figure 11).

Figure 11. Prepared samples prior to material application.

In the next step, the samples underwent demineralization though individual immersion in a solution with the following composition: 0.0476 mM sodium fluoride (NaF), 2.2 mM calcium chloride dihydrate ($CaCl_2$•$2H_2O$), 2.2 mM potassium dihydrogen phosphate (KH_2PO_4), 50 mM acetic acid (CH_3COOH) and 10 mM potassium hydroxide (KOH), at pH 5.0 and were kept at 37°C for 14 days in an incubator (NÜVE, Ankara, Turkey) following the protocol established in previous studies (190, 191) (Figure 12).

Figure 12. Ingredients used for demineralization solution preparation.

The cavities were then filled with one of the tested materials. Since the materials were encapsulated, they were prepared following the instructions provided by the manufacturers. These samples were then placed in a saline solution (Croatian Institute of Transfusion Medicine, Zagreb, Croatia) mixed with an equal amount of oral cavity humidifier (Certmedica International GmbH, Aschaffenburg, Germany) for 14 and 28 days in an incubator ES 120 (NÜVE, Ankara, Turkey) at 37 °C, respectively. Every 48 hours, surface was rinsed with a 200-ppm NaF solution. After the incubation period, the teeth were embedded in an autopolymerizing acrylate (Figure 13). All specimens were cut perpendicular to the material line using the IsoMet 1000 Precision Cutter (Buehler, Lake Bluff, IL, USA) (Figure 14).

Figure 13. Sample preparation with autopolymerising acrylate.

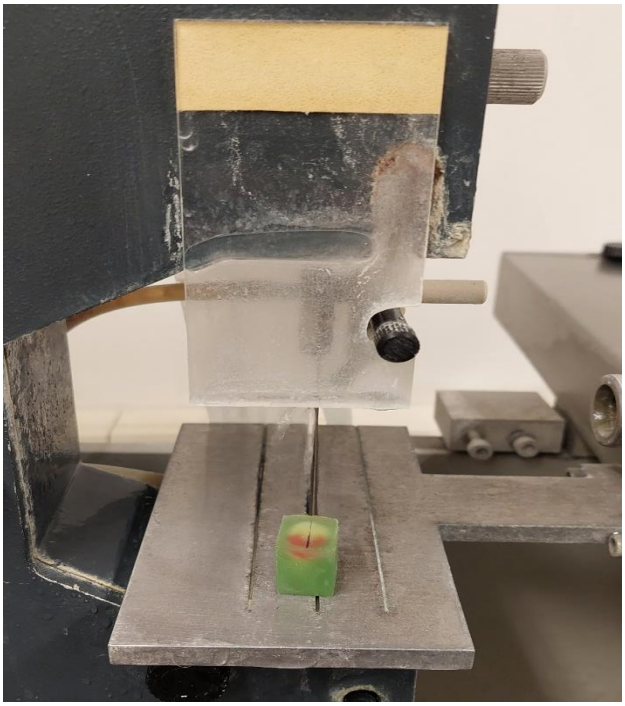


Figure 14. Sample cutting.

In each group (n=10), ten samples (Figure 15) were subjected to microhardness testing, with two more samples prepared for SEM and EDS analysis (n=2). All tests were performed twice, once after 14 days and then again after 28 days of incubation.

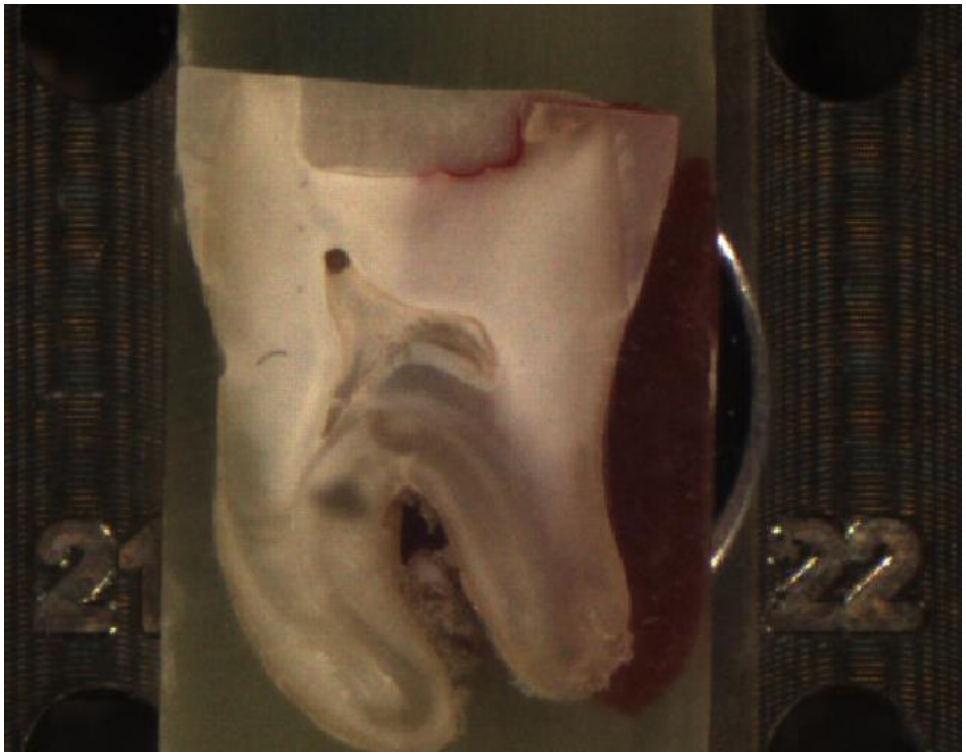


Figure 15. Finished sample for the second part of the study.

3.2.3. Preparation of samples for Micro-CT imaging

Fifteen extracted human third molars were used; the occlusal third of the crown was removed with a diamond saw, exposing the dentine surface. Six 1.5 x 1 mm cavities were prepared on the exposed dentine surface (Figure 16), while the rest of the surface was isolated with nail polish and the prepared cavities were demineralized according to the protocol explained in the previous section. In the next steps, each cavity was filled with the corresponding ion-releasing material. The control group of cavities was filled with a composite

material without bioactive effect. Micro-CT analysis will determine the extent of demineralization and possible remineralization after application of the material; it will be performed before application of the material and after 45 days of incubation in artificial saliva at room temperature.

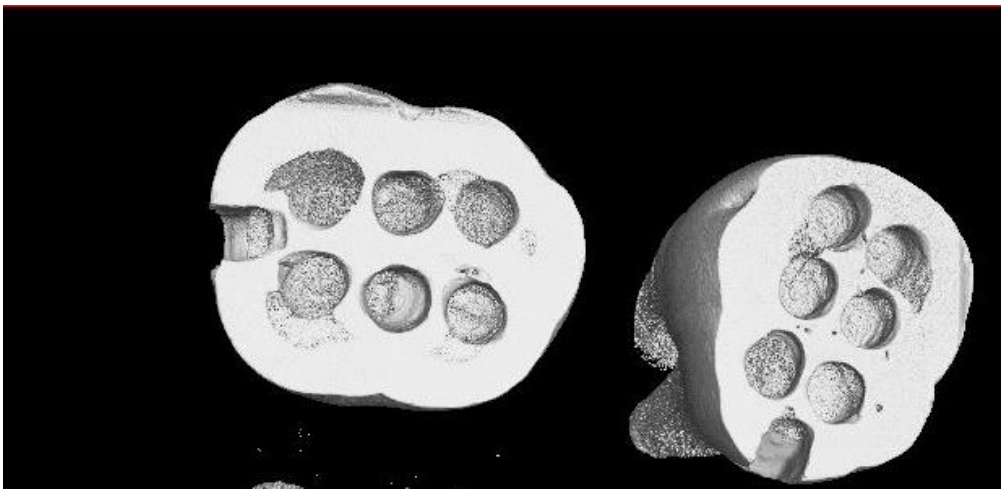


Figure 16. Prepared cavities in the samples for micro-CT analysis.

3.3. ENAMEL PROPERTIES ANALYSIS

3.3.1. Microhardness of the enamel

Vickers microhardness tester KBW 1-V (KB Prueftechnik GmbH, Hochdorf-Assenheim, Germany) (Figure 17) was used for obtaining the data regarding the microhardness of the enamel samples. The microhardness measurements were carried out in three phases: at the initial baseline, after demineralization and after the remineralization phase. A 0.1 kgf load (HV 0.1) was applied for 10 seconds. Each sample was tested three times, and the average result was calculated.

Figure 17. Enamel microhardness evaluation.

3.3.2. Scanning Electron Microscope (SEM) / Energy-Dispersive X-ray Spectroscopy (EDS) Analysis

The SEM/EDS analysis (Figure 18) was performed on one specimen for each tested material. The microscope used was the JSM-7000F (JEOL Ltd., Tokyo, Japan), accelerating voltage of 5 kV, working distance of 10 mm, magnification of 500×. EDS was performed using the Inca 350 EDS system (Oxford Instruments, High Wycombe, UK). Prior to testing, the samples were carefully polished with a brush and then air-dried. This allowed observation of the sample surfaces and determination of specific chemical elements.

Figure 18. Enamel SEM/EDS analysis.

3.4. DENTINE PROPERTIES ANALYSIS

3.4.1. Dentine microhardness analysis

The Qness - Q10 M microhardness tester (ATM Qness GmbH, Golling an der Salzach, Austria) was used to perform the microhardness evaluation of the samples with the Vickers method (Figure 19). After incubation, microhardness measurements were carried out on both sides of the specimen, with two values being recorded for each specimen. The measurement was performed with a load of 0.1 kgf (HV 0.1) for a duration of 10 seconds. Three indentations were made on each specimen and the average result was calculated. The indentations were placed inside the coronal dentine, no more than 200 μm away from the material-dentine junction, with all of them being separated at least three times the size of their diameter.

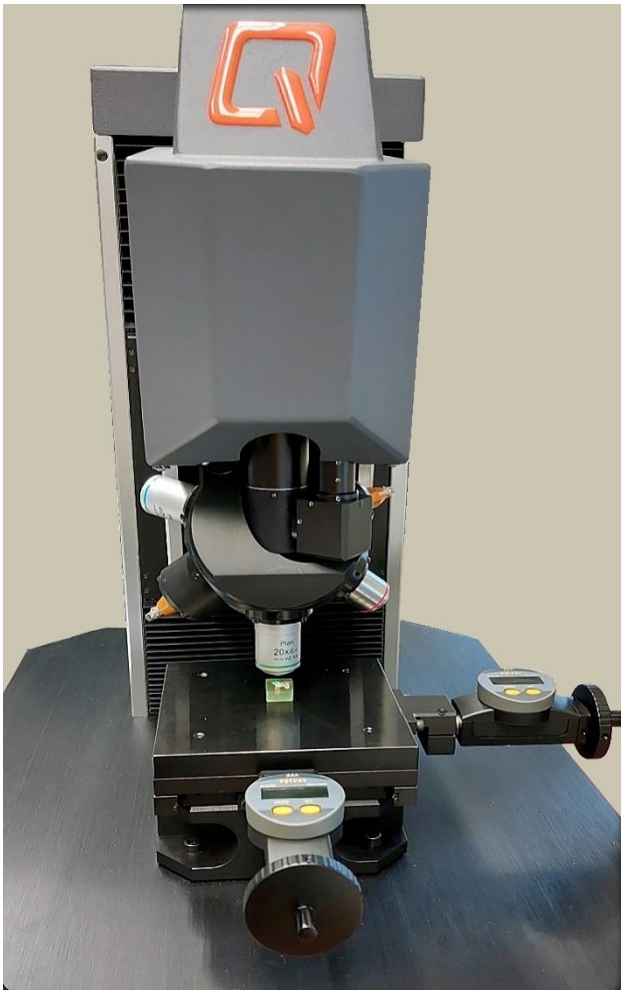


Figure 19. Dentine microhardness analysis.

3.4.2. SEM/EDS analysis

One sample was subjected to the SEM analysis for each group with a Phenom XL scanning electron microscope (Phenom-World BV, Eindhoven, The Netherlands). The imaging parameters used were: 15 kV accelerating voltage, BSD full detector, 60 Pa low vacuum and a scan size of 3840 x 2160. Prior to the analysis, the samples were coated with a 10 mm thick gold layer. This allowed observation of the sample surfaces and the interface between the material and the dentine. Another sample was prepared for EDS evaluation for each tested material at both 14 and 28 days with the Inca 350 EDS system (Oxford Instruments, High Wycombe, UK).

3.4.3. Micro-CT analysis

Firstly, the samples were scanned with micro-CT XT H 225 (Nikon Metrology Europe NV, Leuven, Belgium) before demineralization and after demineralization, to allow post-incubation comparison. The final scan was made after the material placement and an incubation period of 45 days, during which the samples were stored in a mixture of saline and artificial saliva at 37°C in an incubator ES 120 (NÜVE, Ankara, Turkey). During the scanning, the voltage at the source was 110 kV, with a current strength of 240 μA, which corresponds to a power at the radiation source of 26.4 W. The radiation source from this experiment has a ratio of power and focal point size of 1 μm to 1 W, and which resulted in a focal point diameter of about 26 μm. The specified diameter of the focal point also represents the hardware resolution. To ensure the same measurement conditions, the samples were scanned in series of 5 teeth. The scanned samples were reconstructed using VGStudio MAX 3.0.1 64 bit (Volume Graphics GmbH, Heidelberg, Germany). The values of the grey tones were observed in the zones of expected material influence.

Figure 20. Micro-CT device used for remineralization imaging.

3.5. STATISTICAL ANALYSIS

Descriptive statistics (mean, standard deviation) were used for the results analysis, and statistical conclusions are drawn using a mixed ANOVA including repeated measures tests (for microhardness changes due to demineralization and remineralization) and independent samples analysis to compare differences between groups. Post-hoc differences were calculated using the Scheffe post-hoc test for analysis when ANOVA results were significant. The statistical significance level was set at 0.05. Prior to analysis, the distributions were subjected to a Kolmogorov-Smirnov test, which showed that they did not deviate significantly from the normal distribution.

4. RESULTS

4.1. Results of the material influence on enamel surface evaluation

4.1.1. Microhardness testing

The microhardness assessment results are delineated in Table 4. Initial microhardness values showed statistically significant differences among the three groups, as confirmed by the ANOVA test ($p < 0.001$). Notably, all observed variations proved statistically significant, with p-values of 0.024 for the first group versus the second group, 0.008 for the first group versus the third group, and $p<0.001$ for the second group versus the third group (Scheffe's post hoc test). In contrast, no statistically significant correlations were found between these variables, indicating that the initial hardness level does not exert influence on the final hardness level ($r = -0.301$, $p = 0.106$) or the hardness level post-demineralization. This is further backed by the absence of a significant difference among the tested groups after demineralization (ANOVA test, $p = 0.362$).

Table 4. Microhardness values of tested materials in three stages (HV 0.1).

Material		**Initial value**	**Post demineralization**	**Post remineralization**
3M™ Clinpro™ White Varnish	Mean	366.00	190.30	236.57
	Std. Deviation	18.93	23.71	19.42
	Median	367.33	196.72	237.13
MI Varnish®	Mean	343.52	192.73	286.65
	Std. Deviation	26.66	16.37	34.71
	Median	345.20	197.15	293.31
Megasonex®	Mean	393.05	201.89	237.97
	Std. Deviation	16.14	15.30	32.51
	Median	393.87	205.90	231.55

Following the remineralization process, significant inter-group differences emerged, as indicated by the ANOVA test assessing three groups and the mean microhardness values post-demineralization ($p < 0.001$). Notably, the differences were statistically significant between the

samples in the second group (MI Varnish®) in comparison to the other two groups (Scheffe's post hoc test, $p = 0.001$ for both comparisons). In contrast, the first and third groups, represented by 3M™ Clinpro™ White Varnish and Megasonex®, exhibited no significant differences between each other (Scheffe's post hoc test, $p = 0.97$).

4.1.2. SEM/EDS surface evaluation

Representative SEM images of enamel surface before and after remineralization are shown in Figures 21 – 26.

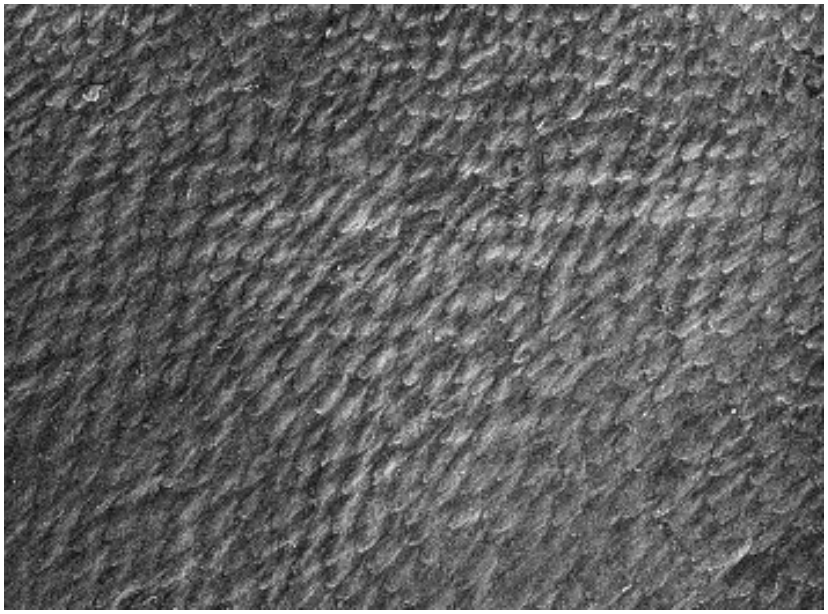


Figure 21. SEM image of the specimen surface before remineralization (3M™ Clinpro™ White Varnish).

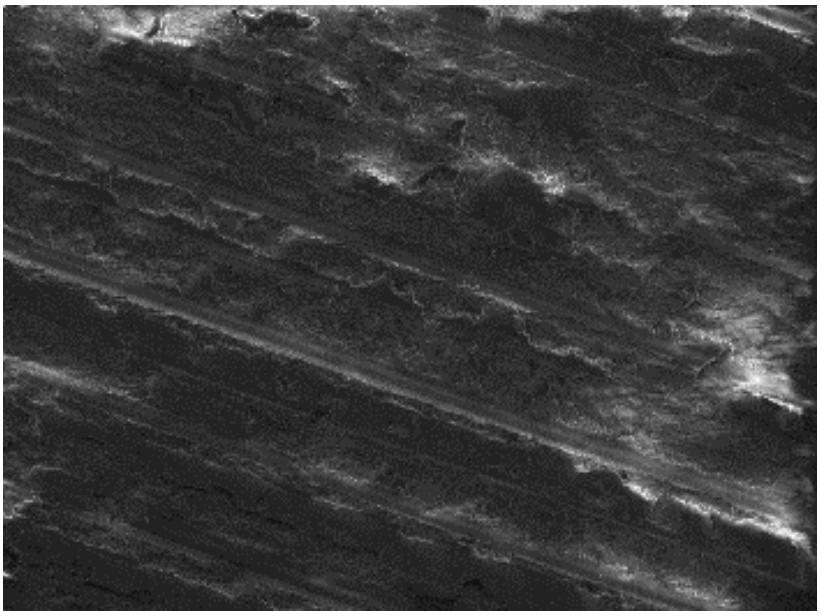


Figure 22. SEM image of the specimen surface before remineralization (MI Varnish®).

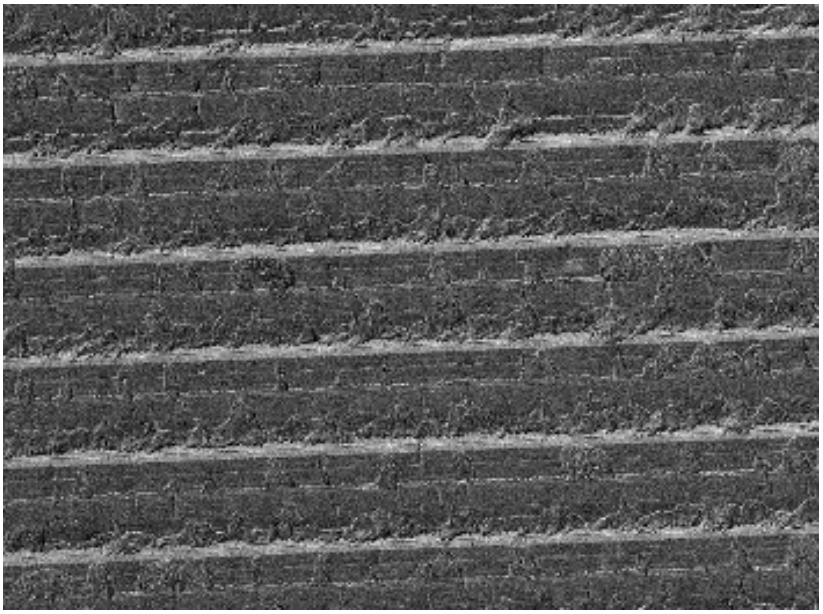


Figure 23. SEM image of the specimen surface before remineralization (Megasonex®).

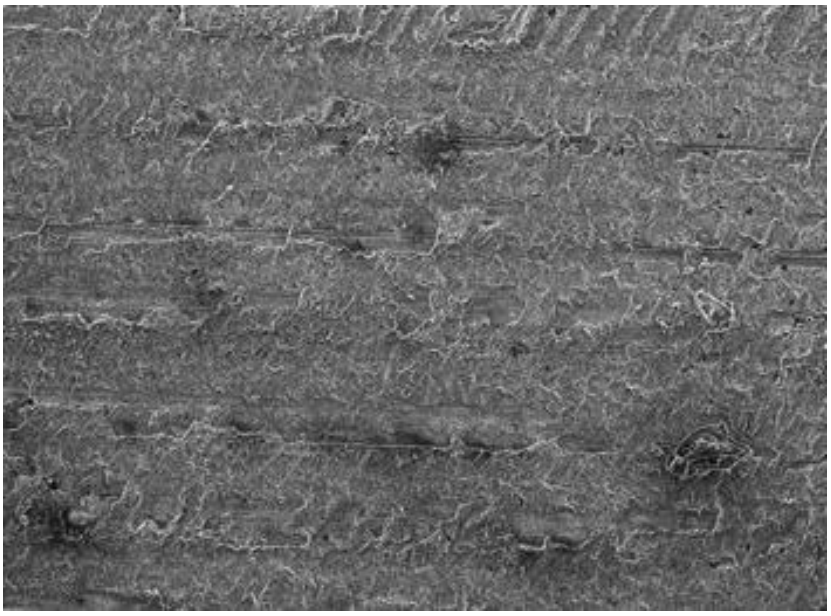


Figure 24. SEM image of the specimen surface after remineralization (3M™ Clinpro™ White Varnish).

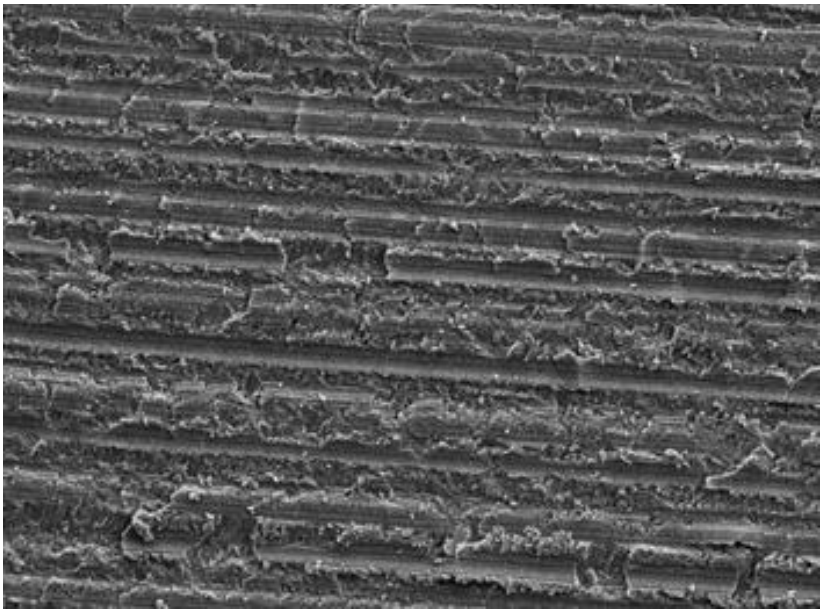


Figure 25. SEM image of the specimen surface after remineralization (MI Varnish®).

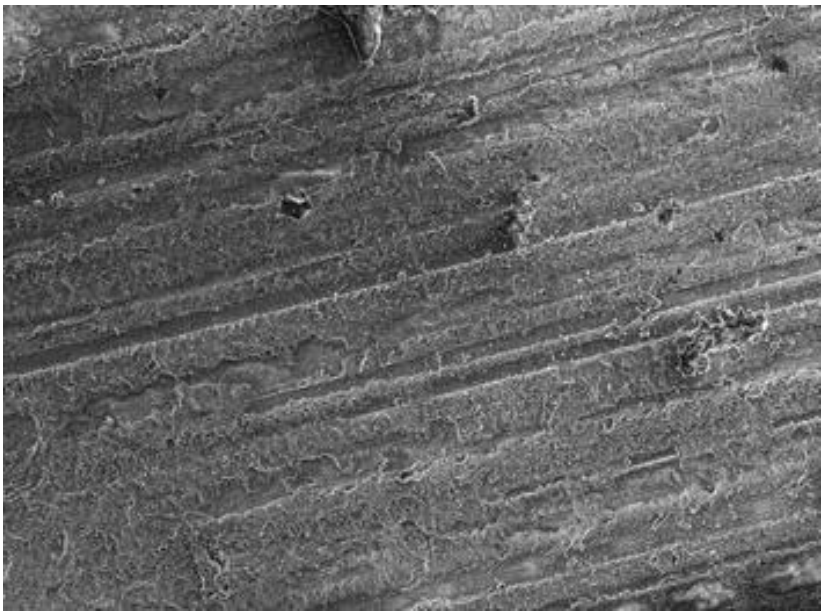
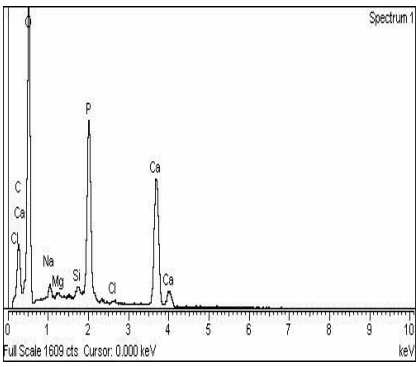


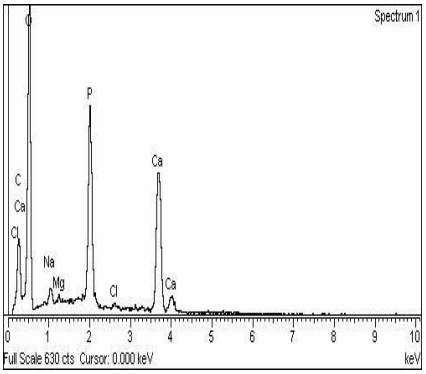
Figure 26. SEM image of the specimen surface after remineralization (Megasonex®).

SEM examination revealed typical enamel surfaces before the demineralization process. Post-remineralization, a more varied array of surfaces was evident, characterized by irregular patterns, porosities, material deposits, and debris. Figure 27 depicts the outcomes of EDS analysis conducted both pre- and post-remineralization, revealing substantial variations in the distribution of specific elements across the tested samples.

3M™ Clinpro™ White Varnish

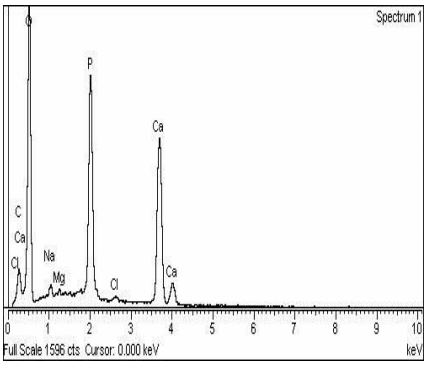


(a)

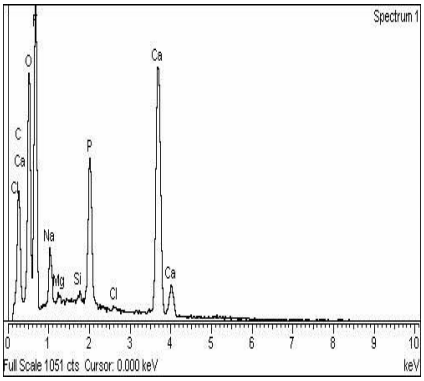


(b)

MI Varnish®

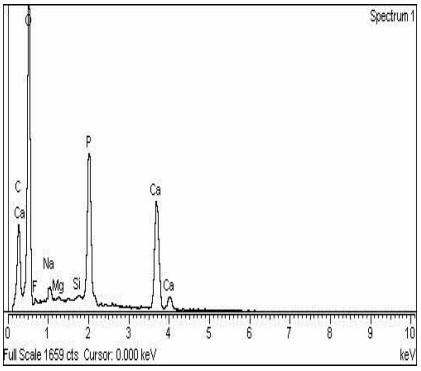


(c)

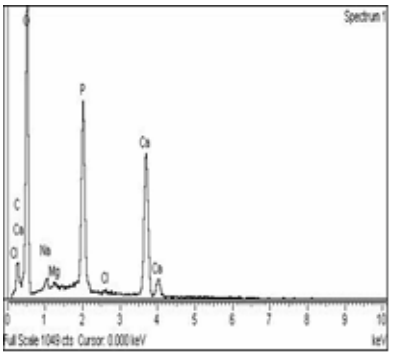


(d)

Megasonex®



(e)



(f)

Figure 27. EDS elemental analysis, showcasing the effects of demineralization and subsequent remineralization on samples treated with 3M™ Clinpro™ White Varnish ((a) and (b)), MI Varnish® ((c) and (d)), and Megasonex® ((e) and (f)).

4.2. Results of the material influence on dentine surface evaluation

4.2.1. Microhardness testing

Microhardness testing results within the area which was coated with nail polish, thus not affected by the protocols, were 66 ± 1.95 (HV 0.1) on average after both examination periods, exhibiting no significant differences among the groups ($p > 0.05$). The mean microhardness values acquired after 14 and 28 days within the zone subjected to the protocols are detailed in Figure 28.

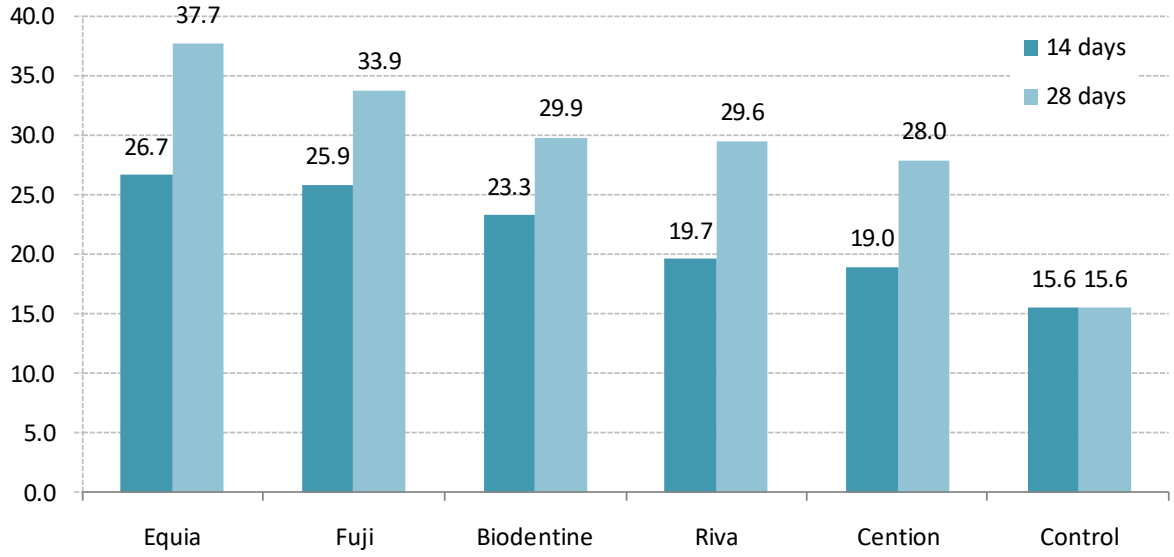


Figure 28. Obtained dentine microhardness and SD values (HV 0.1) after 14 and 28 days.

Significant variations were observed in mean microhardness values after 14 days among most groups ($p < 0.001$), with a few exceptions (Biodentine vs. Cention Forte, $p = 0.08$; Biodentine vs. Riva SC, $p = 0.997$; Riva Self Cure vs. Cention Forte, $p = 0.229$). Likewise, after 28 days, statistically significant differences were present across all groups ($p < 0.001$), except for EQUIA Forte HT and GC Fuji TRIAGE ($p = 0.514$) and Cention Forte and Riva Self Cure ($p = 0.687$).

4.2.2. SEM/EDS analysis in dentine

The results of SEM analysis of the dentine surface are shown in Figures 29 – 30. SEM acquisition parameters are: accelerating voltage of 15 kV, working distance of 8.997 mm, magnification of 2100x, scale bar represents 50 μm.

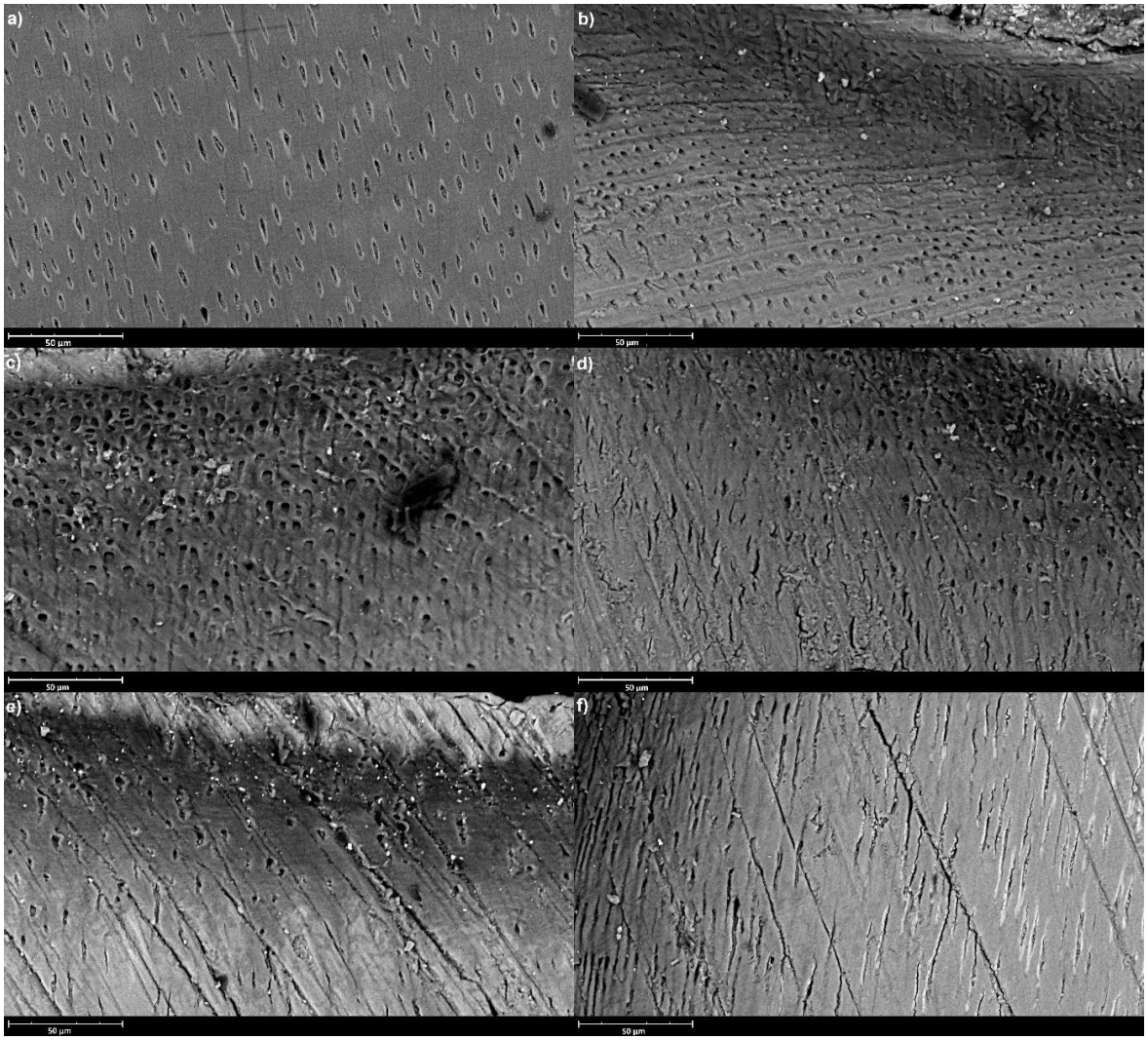


Figure 29. Representative SEM images depicting the surfaces of the samples after 14 days of incubation are presented. The images include: a) Control, b) EQUIA Forte HT, c) GC Fuji TRIAGE, d) Biodentine, e) Riva Self Cure, and f) Cention Forte.

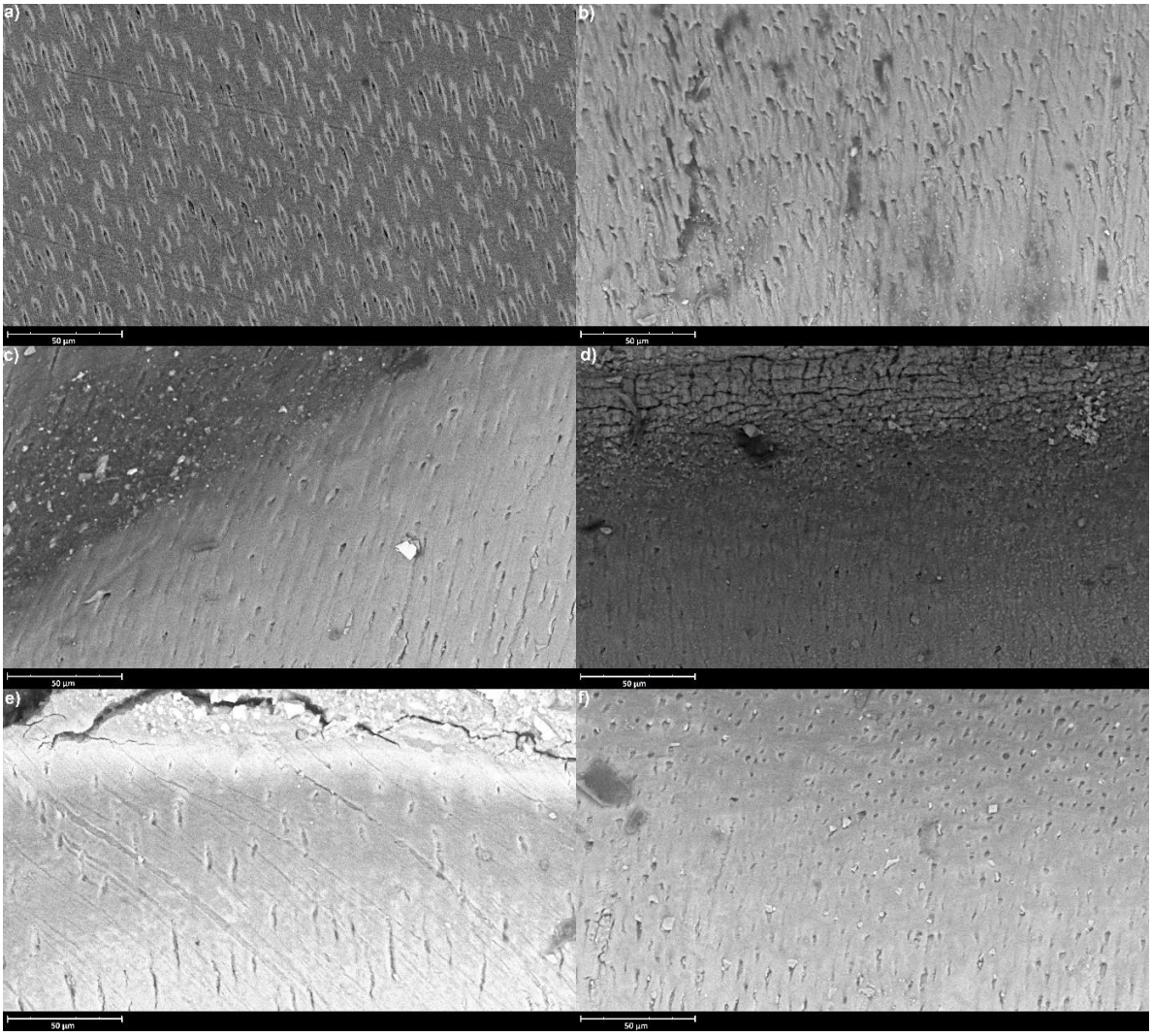


Figure 30. Representative SEM images showcasing the surfaces of the samples following a 28-day incubation period. The images encompass: a) Control, b) EQUIA Forte HT, c) GC Fuji TRIAGE, d) Biodentine, e) Riva Self Cure, and f) Cention Forte.

In Figure 31, results of the EDS analysis are shown for each group following 14 and 28 days of incubation, revealing noticeable disparities in the distribution of elemental shares, with all groups having higher amounts of calcium and phosphates, compared to the control group.

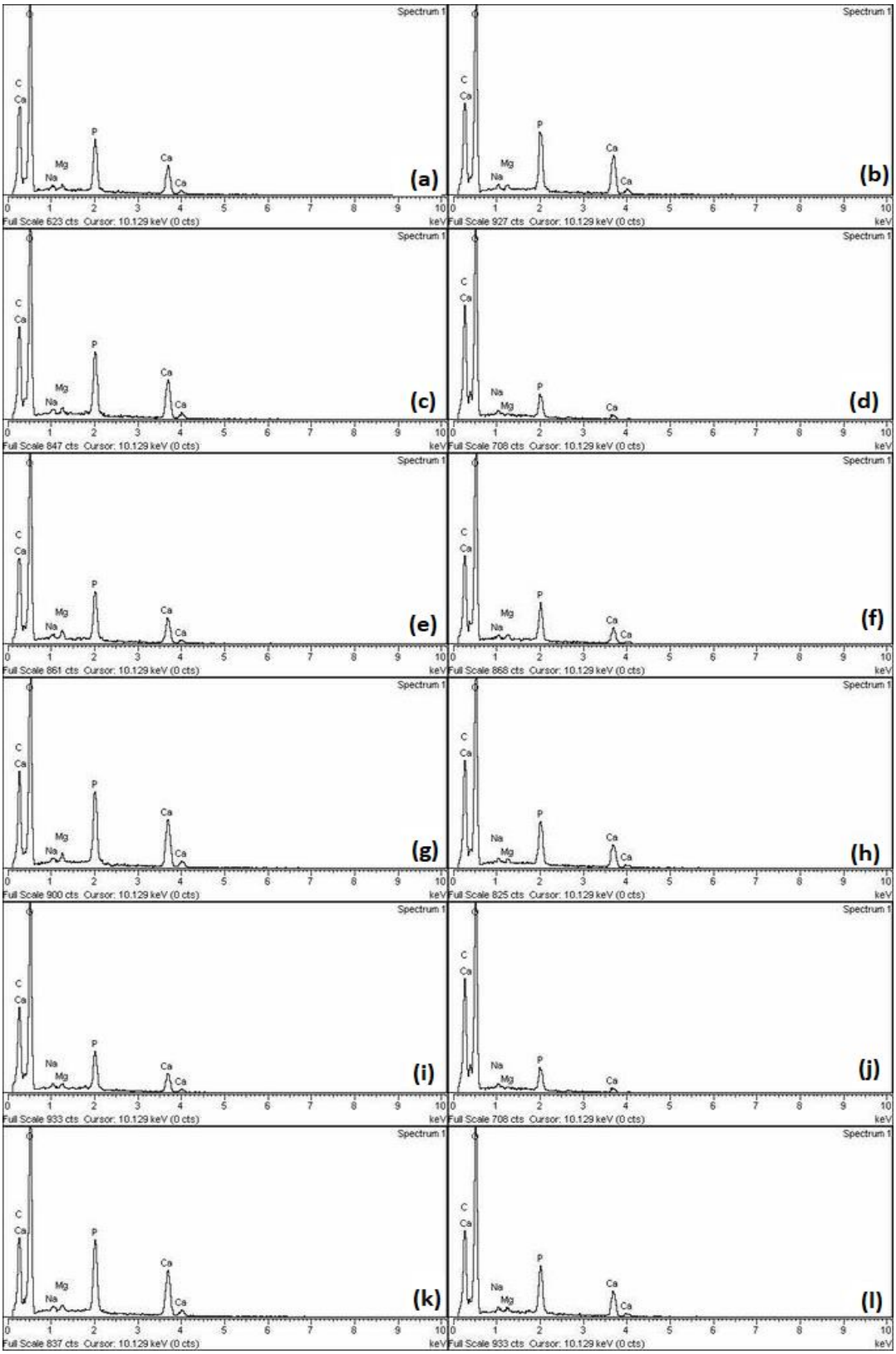


Figure 31. Outcomes from EDS elemental analysis are presented for the 14- and 28-day incubation periods of samples treated with no material ((a) and (b)), EQUIA Forte HT ((c) and (d)), GC Fuji TRIAGE ((e) and (f)), Biodentine ((g) and (h)), Riva Self Cure ((i) and (j)), and Cention Forte ((k) and (l)).

4.2.3. Micro-CT analysis

Figures 32 – 34 were obtained by micro-CT scanning. They show changes in dentine density following de/remineralization protocols.

Figure 32. Baseline micro-CT scan.

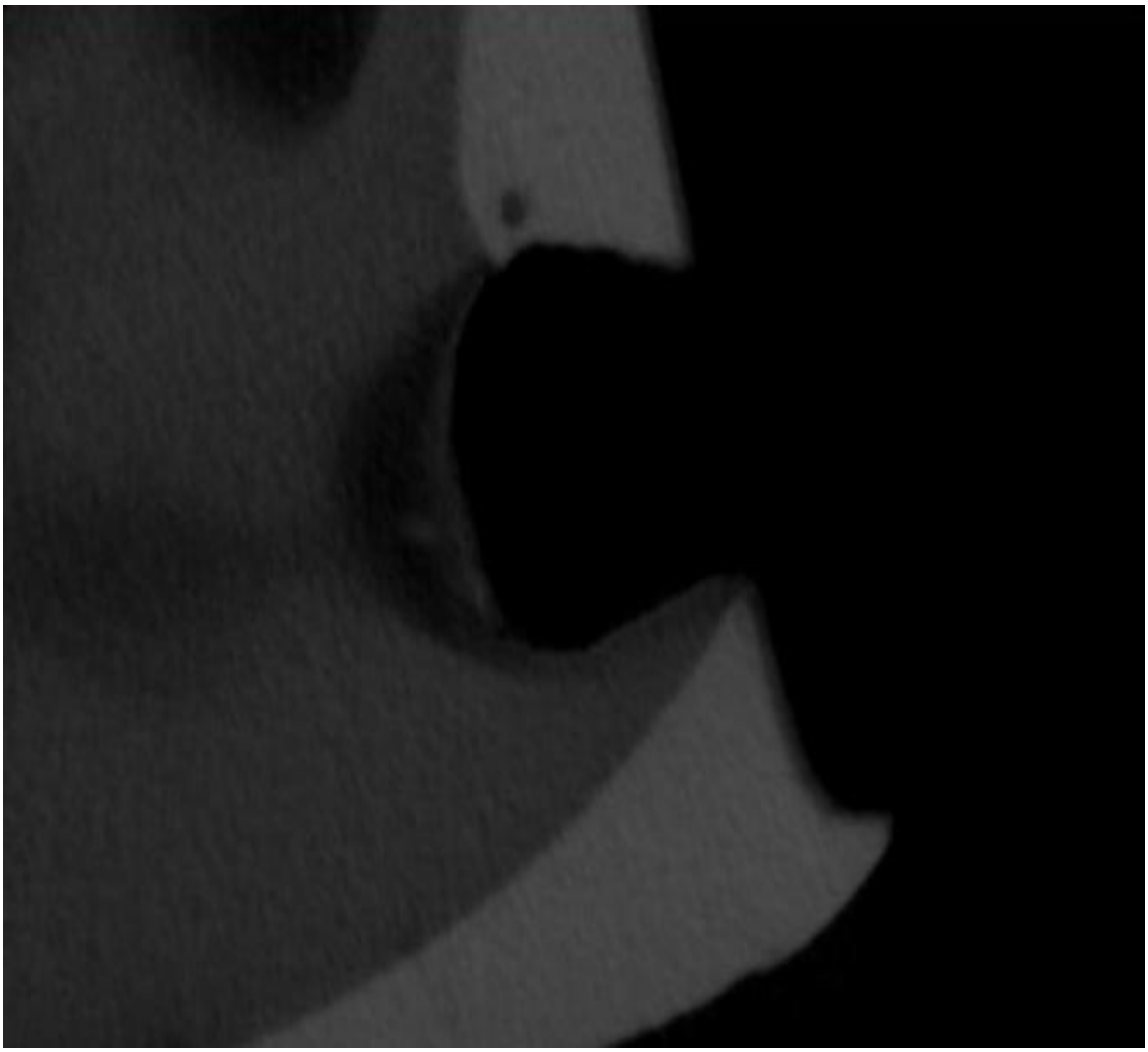


Figure 33. Micro-CT scan taken following demineralization.

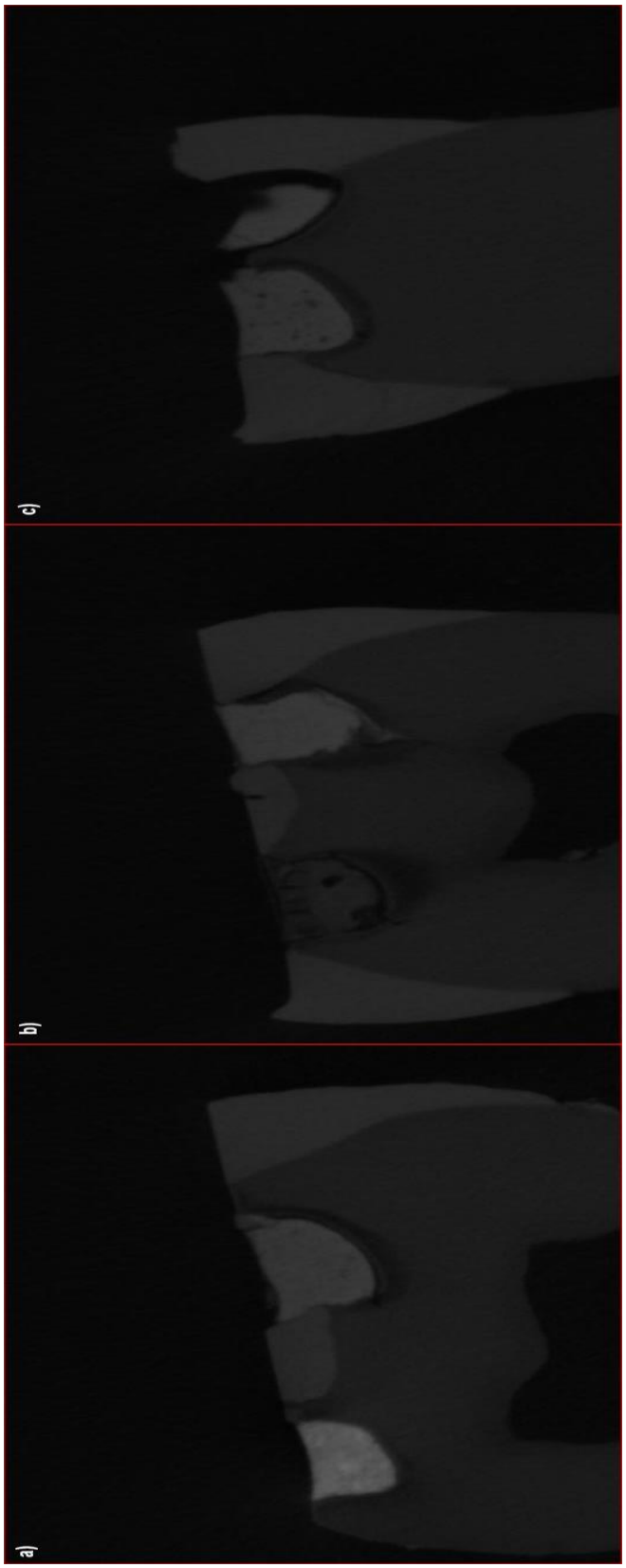


Figure 34. Micro-CT scan taken following the 45-day incubation period a) EQUIA Forte® HT and GC Fuji TRIAGE, b) Riva Self Cure and Biodentine™ c) Cention Forte and composite material.

4.3. Remineralization quantification

Remineralization of enamel and dentine was quantified by calculating the percentage of recovered microhardness following the incubation periods, in relation to demineralized values. Quantification results are shown in Figure 35.

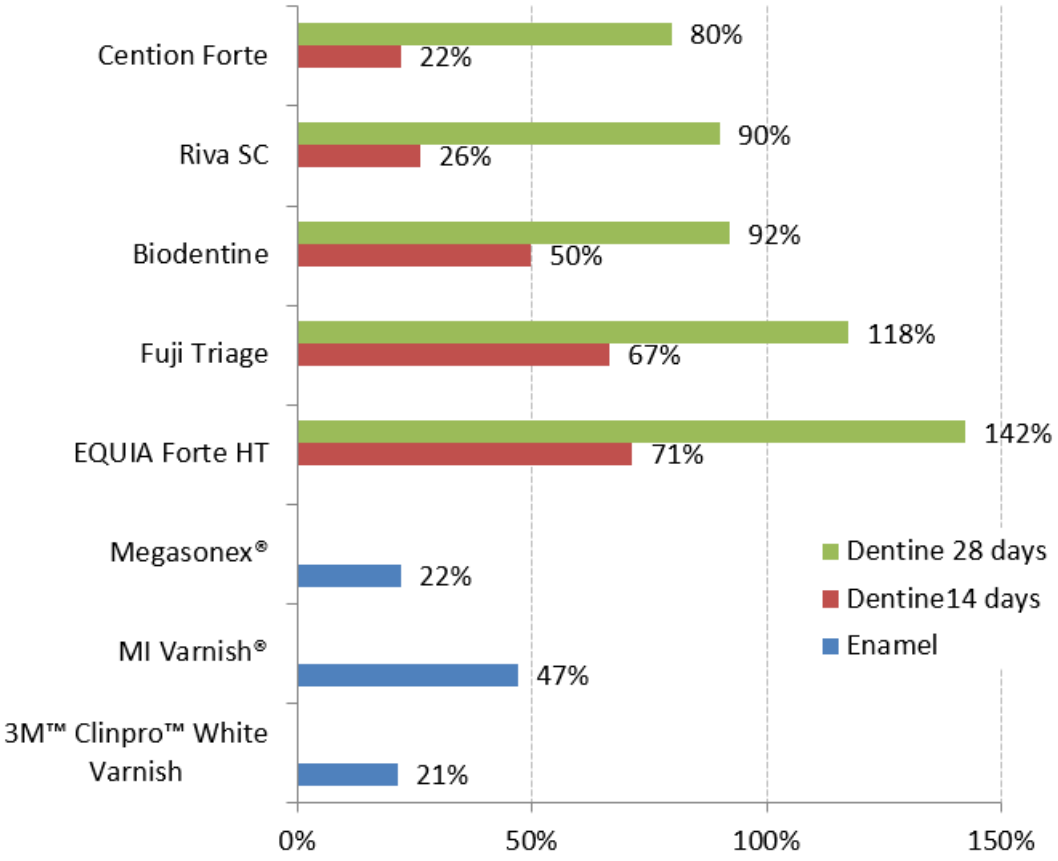


Figure 35. Remineralization quantified by calculating the percentage of recovered microhardness.

5. DISCUSSION

In the present study, the influence of three ion-releasing materials on the microhardness, chemical composition and appearance of demineralized enamel has been investigated. In addition, the effect of five ion-releasing restorative materials on dentine was evaluated by microhardness analysis, evaluation of surface appearance and elemental composition, and micro-CT scans. The decision to start remineralization of an actively progressing initial caries lesion depends on different factors, such as the clinical stage, the radiographic extent of the lesion and the patient's susceptibility to caries, which also serves as an indicator of the probability of the lesion advancing to the cavity (192).

In the first part of the study, the materials 3M™ Clinpro™ White Varnish, MI Varnish® and Megasonex® toothpaste were tested. The choice of materials depended on their chemical properties and composition. Fluoride-based products, such as 3M™ Clinpro™ White Varnish, are most commonly used to remineralize enamel because they increase the pH almost immediately in an acidic environment (193). The initial use of fluorides for caries development control was mainly based on the topical influence on mineral loss and gain at the interface between tooth and oral fluid (194 – 196). When fluorides are present, they can replace the hydroxyl ions in the hydroxyapatite structure, transforming it into fluorapatite (FA). Fluorapatite dissolves at a much lower pH than HA, which stops demineralization (197). In addition, fluorides also affect the metabolism of microorganisms present in the mouth, which can reduce their cariogenic activity (198). However, the use of fluoride has several disadvantages. Firstly, they are by far most effective on the surface of the enamel (199), leaving the deeper areas largely untouched. Consequently, mineralization of the superficial layer can clog the enamel pores, reduce the ion exchange activity of the enamel and prevent complete remineralization of the lesions (200). Furthermore, the efficacy of fluoride-based products is highly dependent on several factors, such as concentration, frequency of exposure and different responses to treatment (201). Water fluoridation, dietary fluoride intake and the presence of fluorides in toothpastes and other oral care products carry a potential risk of causing dental fluorosis (202). All this prompted the evaluation of several possible fluoride alternatives.

The use of CPP-ACP-based products has attracted a lot of attention recently. This is a shift from the earlier view that calcium phosphates were not suitable for enamel remineralization, as calcium and phosphates were deemed not soluble enough and not able to readily localize (203). Stabilizing calcium and phosphate with casein phosphopeptides and preventing calculus formation is a strategy found in several available products, such as MI Varnish®, and has been reported as successful by several researchers (97, 204, 205). However,

as these products usually also contain fluorides, the question of whether CPP-ACP is more effective than fluorides has not yet been fully clarified. A biomimetic approach is also possible as an alternative to fluoride products (206, 207).

Nano-hydroxyapatite (n-HA) is a mineral that resembles the structure of HA present in hard dental tissues and can be incorporated into their structure in the mineral-deprived zones (208). n-HA is commonly found in toothpastes, such as Megasonex®, which was evaluated in this study.

The results of this study refuted the first null hypothesis that there is no significant difference in the effect of the tested ion-releasing materials on microhardness, surface appearance and chemical composition of demineralized enamel. In the current study, the highest microhardness values were obtained for MI Varnish® (286.65 ± 34.07), which was statistically significantly better than the other two materials (p=0.000), possibly due to the combined effect of CPP-ACP and fluoride content. 3M™ Clinpro™ White Varnish (236.57 ± 19.41) and Megasonex® (237.97 ± 32.52) did not differ significantly from each other (p=0.97). CPP-ACP forms a strong bond with the enamel surface, effectively capturing and retaining fluoride ions, thereby prolonging their presence near or on the enamel surface (209), which may benefit the remineralization process. According to Reynolds et al. (210) and Wegehaupt et al. (211), fluorides alone are not sufficient to cause complete remineralization; calcium and phosphate ions, which were not present in 3M™ Clinpro™ White Varnish, are also needed as they contribute to rebuilding the crystal structure of the enamel and facilitate further ion exchange. Similar results were reported by other authors (212, 213). Reports on the formation of a novel amorphous calcium phosphate fluoride phase (ACFP) by CPP-ACP and fluorides reinforced the observations of a synergistic anticariogenic effect of these two compounds (214, 215). Localizing the ACFP at the surface of the tooth may serve as a reservoir for all three ions and simultaneously prevent calculus formation. On the other hand, Vyavhare et al (216) argued that CPP-ACP alone is not suitable for enamel remineralization, as it performed worse compared to fluoride and nano-hydroxyapatite, which achieved similar results. Different results could be explained by the synergistic effect created by the combination of CPP-ACP and fluorides in MI Varnish®.

Apart from the aforementioned disadvantages, the use of fluoride is controversial due to reports of neurotoxicity (217). A 2006 study conducted by the U.S. National Research Council (NRC) evaluated the fluoride standards set by the Environmental Protection Agency

(EPA). The results indicated that fluoride could damage the brain both directly and indirectly and suggested that high levels of fluoride in drinking water could raise concerns about neurotoxic effects, so further research is needed in this area (218). This could be manifested in reduced intelligence, memory impairment, academic difficulties and ADHD, with limited data on the effects of fluoride on depression and anxiety (219). However, the actual risk remains controversial as the actual mechanism of how fluoride might cause such problems is not known and therefore not definitively proven, although there is evidence that the risk may be underestimated (220). Despite this uncertainty, fluoride-free products are becoming increasingly popular with the public.

The synthesis of n-HA can be accomplished by various methods, including coprecipitation and wet chemical precipitation, which is most commonly used due to its high reproducibility, simplicity and cost-effectiveness (221). In addition to the direct depletion of apatite in the demineralized lesion, there are several other mechanisms by which n-HA acts as an anticariogenic agent and promotes remineralization. One of its major advantages is the fact that it serves as a reservoir of calcium and phosphate ions and can act accordingly by keeping the oral environment supersaturated with these ions (106). In addition, its small particles that penetrate the lesion also act as a template, attracting ions that then repair the demineralized structure (106). All this might suggest that n-HA-based products are superior to those containing only fluorides. However, several studies have come to the same conclusion as the present study and have found no significant differences in their remineralizing effect (222–224). One possible explanation for this is that n-HA products work best when they are present in a product at a concentration of around 10% (225), which is explained by the fact that the particles clump together at a higher concentration, effectively closing the pores and preventing further remineralization (222). The actual percentage of n-HA in Megasonex® toothpaste is not known but is likely to be less than 10%.

In the second part of the study, the established glass ionomer cements (GC Fuji TRIAGE®) and tricalcium silicate-based materials (Biodentine™) were compared with novel glass hybrid materials (EQUIA Forte® HT and Riva Self Cure) and an alkasite material (Cention Forte). Glass ionomers were included in the study because, although they are widely used, they are not used for long-term posterior restorations due to their poor mechanical properties, even after modifications such as the addition of resin to their structure (226). Nicholson reported that conventional GICs are still fragile materials with a compressive strength in the range of 150 to 220 MPa (227). RM-GICs were more resistant, but their other

properties remained comparable to those of their conventional counterparts (227). It soon became clear that a major breakthrough and significant changes were needed to make GIC technology applicable for restorations in adult dentition.

They took a step back from conventional GICs and introduced GHs. This time a different approach was taken: instead of simply adding new particles or substances to the structure of the cement, the existing ones were modified. The structure of the material contains two types of fluoroaluminosilicate (FAS) glass fillers; the main fillers (< 25 μm) improve aesthetics and help to regulate setting time, while the smaller and more reactive FAS fillers (< 4 μm) facilitate the release of additional metal ions, which in turn improve the cross-linking of the polyacrylic acid, leading to the improvement in the mechanical properties of the material (228). The most important innovation, however, is the introduction of a resin-based coating that is clinically applied after the material has cured and is light-cured. This coating provides additional material, sealing, reduced wear and improved aesthetics (229, 230). This is probably the explanation for the fact that in this study the highest microhardness values (HV 0.1) were recorded for the samples treated with EQUIA Forte® HT in both test periods (26.7 ± 1.45 37.74 ± 1.56). GC Fuji TRIAGE® followed relatively close behind with results of 25.94 ± 1.35 after 14 days and 33.87 ± 5.57 after 28 days of incubation. The second hybrid glass in the study, Riva Self Cure, performed worse than the two previously mentioned materials with values of 19.66 ± 1.02 and 29.58 ± 1.18 respectively. However, it is noticeable that this material showed the greatest increase in results between the two test periods. These results are partly consistent with the surprisingly few available studies comparing these two types of material. Ghilloti et al (127) included both EQUIA Forte® HT and Riva Self Cure in their study and compared them to a conventional GIC. Their study did not examine microhardness, but concluded that the glass ionomer cement induced more superficial mineral deposition, while the two glass hybrid cements performed better in the deeper area, with all three materials inducing some type of mineral deposition throughout the lesion after 28 days.

While declaratively belonging to the same group of materials, EQUIA Forte® HT and Riva Self Cure generated significantly different results. This may be contributed to a lack of agreement in the sense of terminology that is nowadays present. While the FDI recognizes glass hybrids as a sperate group of materials (231), they are still commonly regarded as high viscosity GICs, often creating confusion (232). This situation opens the door for manufacturers to non-consistently claim their products belonging to the GH group, which is the case for Riva Self Cure, where the presence of new Ionglass™ fillers is emphasized, with the total absence of

resin-based coating, which is an integral part of GH systems, however not commonly used for *in-vitro* studies, such as the present study. In this study, the manufacturers claims were accepted for consistency.

Remineralization ability of calcium silicate-based materials, such as Biodentine™, has already been confirmed in previous studies (233, 234). In this study, microhardness values of 23.35 ± 1.23 and 29.92 ± 1.0 were obtained for this material, which is statistically significantly lower than the results obtained for EQUIA Forte® HT and GC Fuji TRIAGE®. Nevertheless, there is an increase in microhardness; perhaps this is due to the composition with particles of small size (only 2.811 m2/g), which allow a rapid release of calcium and phosphate ions (235). Nevertheless, it is important to mention that this material promotes the secretion of TGF-β1 in cell differentiation and mineralization of the pulp (236), making it particularly suitable for in vivo studies or studies that also replicate the effects on the pulp, as is the case in the present study.

The material for which the lowest microhardness values were obtained was Cention Forte, an alkasite, with a result of 19.01 ± 1.24 and 27.93 ± 1.33, respectively. This material is currently the only alkasite material available on the market (among other variants that differ only in packaging and application method) that contains three main types of fillers: silanized barium aluminium silicate glass, calcium barium aluminium fluorosilicate glass, which is similar to glass ionomers, and calcium fluorosilicate glass (237). Despite the lower initial result, the values obtained increased significantly between the two measurement periods, indicating a possible long-term effectiveness of this material. The positive effects of the alkasite material were reported early on when it was shown to reduce the rate of enamel demineralization compared to conventional composite, confirming its buffering capacity (238, 239). In addition, Banic Vidal et al. found in their study that Cention Forte releases much higher amounts of fluorides than EQUIA Forte® HT, which performed best in the current study (139). These results are in contrast to those of Theerarath et al. (240); in their study, Cention Forte restored a higher percentage of microhardness of demineralized lesions compared to EQUIA Forte® HT, but with no significant difference. One possible explanation for this is the fact that their study used the earlier version of the material where the components were mixed by hand, as opposed to the current study where only encapsulated materials with a fixed powder to liquid ratio were used. Several studies have already reported that even small differences between powder and liquid can significantly affect the ability to release fluoride and the mechanical properties of the material (241, 242). Another explanation for the results obtained with Cention

Forte is the fact that it is a dual-cured material, which was light-cured in this study. Gupta et al (243) reported that self-cured alkasite releases more fluoride than GICs. However, dual-cured materials release fluoride at a slightly slower rate, suggesting that potential remineralization would also occur at a slower rate (239). Nevertheless, studies evaluating the mineralizing properties of this material are still very limited and leave room for further investigation.

The results of this study were contrasted in several studies. Schwendicke et al (244) reported that microhardness does not increase with GICs, whereas it improves significantly when Biodentine™ is used. This is most likely due to the fact that there are significant differences in the study design, as a much simpler demineralization protocol was used in the present study. In addition, the remineralization time was relatively short so that the precipitated ions could not be washed away, which may have influenced the outcome of the microhardness evaluation. Vilela et al (245) in 2023. found no increase in microhardness after the treatment of demineralized dentine with conventional GICs and their modifications even after eight weeks of incubation, which is probably due to the different sample preparation.

The quantification of the remineralization effect of ion-releasing materials refers to the process of measuring and evaluating the extent or degree to which minerals are redeposited in demineralized enamel and dentine. In this study, the percentage of restored microhardness values after remineralization was calculated in comparison to the values after demineralization. This is a new approach to quantifying remineralization, as for this aspect of the study, the materials were compared in the same way for enamel and dentine remineralization. As far as the author is aware, such calculations and comparisons have not been made in previous publications. In this study, the ion-releasing restorative materials restored a higher percentage of dentine microhardness than the materials used for enamel remineralization, although dentine remineralization has already been described as more difficult. However, the most likely reason for this is the fact that during the demineralization protocols, microhardness was reduced much more in dentine than in enamel. For example, the microhardness values in the enamel after demineralization were 53.05% of the initial value, while this percentage was only 23.63% in the dentine, which means that the dentine was more affected by the remineralization protocol.

The study design can significantly influence the outcome of the research as it determines the environment in which the material under investigation behaves in a certain way. In the second and third part of the current study, demineralized lesions were prepared with a solution used in other studies (246). In addition, the remineralization protocol did not include pH

cycling, which is common in other studies. Since simulation of conditions in the oral cavity is not easy to achieve due to the complex influences of salivary flow, unpredictable pH values and individual habits, this type of demineralization protocol was considered sufficient (247, 248) as it reduces the possible errors in performing the examination. Proper demineralization is the crucial step for observing a possible remineralization effect of the material, as the presence of partially demineralized crystals is required to serve as a clean template for mineral deposition (249). Some authors argued against a simplified remineralization protocol claiming that to replicate dynamic oral conditions, it would be beneficial to expose the sample to alternating remineralization and demineralization solutions, reflecting the alternating processes of demineralization and remineralization typical of dental caries (250 – 252). Although this means that it may be difficult to compare the results of studies that were designed differently, it is still possible to draw certain conclusions and observe the behaviour of the materials.

Regarding the duration of the remineralization process, other studies have used different time frames, ranging from a few hours (253) to 28 days (254) to 30 days (255) and more. In this study, the incubation period ranged from 14 to 28 days to allow for easier comparison with other studies. As mentioned earlier, some materials have a more pronounced effect initially, while others work better over time, suggesting that materials should be given time to develop their efficacy.

Indentation testing offers several ways to measure important properties in dentistry, such as microhardness, creep behaviour and modulus of elasticity (256), all of which provide important information about the material or tissue being tested. Microhardness testing is considered a reliable and non-destructive method that allows the observation of de-/remineralization processes in tooth structures and has been used in several studies for both enamel and dentine examinations (257, 258). Although there are several different hardness testing methods, the Vickers and Knopp hardness tests are generally used in dental research. Selecting the appropriate method is important to optimize the accuracy of the results and make them more reliable (256). Various factors play a role in the choice of method, such as the shape, size and thickness of the specimen and the hardness of the material to be tested. Other considerations include the homogeneity of the specimen, the desired statistical confidence limits for the results and the specific purposes for which the hardness data are required (259, 260). The Vickers hardness test is generally considered more user-friendly than other hardness tests because the calculations required do not depend on the size of the indenter and the uniform use of a pyramid-shaped diamond indenter applies to all materials regardless of their hardness

(261). During the measurement, the indenter is pressed into the sample for a certain time. The Vickers hardness number (VHN) is defined as the ratio between the force applied and the surface area of the indentation formed (262). The Knoop hardness test is similar to the Vickers hardness test but has some significant differences. The Knoop hardness test uses an indenter with a rhomboid base (263). In general, the Vickers hardness test is used to assess the hardness of materials within the load range of the microhardness test, whereas the Knoop hardness test is often chosen when assessing the hardness of thin layers such as coatings or investigating cracking in brittle materials (264). Therefore, the Vickers method was chosen for this study.

When discussing possible remineralization, hardness tests alone are not sufficient to draw conclusions; some form of elemental or chemical analysis is desirable. Therefore, SEM/EDS analysis was chosen for this study. SEM analysis captures high-resolution images that provide qualitative information about the morphology of the samples (265), and EDS analysis allows quantification of the major constituents (157). SEM/EDS analysis confirmed the results of the microhardness tests in the assessment of both enamel and dentine remineralization.

In the first part of the study, SEM analysis showed different patterns on the sample surface. After remineralization, irregular mineral clusters and material residues can be observed. The irregularities are most likely due to an uneven restoration of the enamel prism. According to Thimmaiah (266), this restored crystalline structure observed with the SEM is an indicator of healthier enamel. In the images, these minerals appear as whitish, irregular structures, which were observed in all samples, indicating that all materials examined had some degree of restored mineral content. EDS analysis revealed an increase in Ca^{2+} and $[PO_4]_3$ levels after demineralization, with the MI Varnish® group having the highest levels, including the presence of fluoride. CPP-ACP probably caused the presence of fluoride as it binds it tightly to the surface. Prior to remineralization, the demineralization protocol resulted in a significant reduction in calcium and phosphate levels, which is consistent with the mineral loss that occurred. However, Wang et al. observed an even greater mineral loss, which is likely due to a more complex demineralization protocol (267).

The second part of the study showed similar results before and after the remineralization protocols. As the dentine surface is friable, the samples were only lightly polished before examination so that the cut marks remained visible. Here too, mineral clusters were observed to varying degrees in the lesion using the SEM. This is different for each sample due to the

complex dentine properties and differences in composition in different parts of the tooth. The use of EQUIA Forte® HT resulted in the largest number of mineral precipitates observed in the SEM. Although the microhardness increased significantly between the two test periods, the SEM results are comparable for all samples with no significant differences observed. The reason for this is probably a better organized or altered mineral structure after 28 days, which is then less noticeable. In the demineralized area of the control group, a decrease in phosphate content was observed with EDS, which is consistent with the results of Aoba et al (268). After remineralization, calcium and phosphate levels increased again, which corresponds to mineral restoration. Although all tested materials increased the microhardness and the calcium and phosphate values of the demineralized lesion increased, the measured values were still significantly lower compared to healthy dentine. This discrepancy emphasizes the crucial effect that dental pulp, supported by the presence of growth factors, has in facilitating functional mineral deposition (269).

Micro-CT effectively detected the changes in dentine, caused by the presence of ions from the material in the demineralized lesion, possibly forming minerals. Initially, no changes were observed on the dentine surface and normal density was detected. After demineralization, only a slight decrease in density was observed, which was also found in the study by Pires et al (270). This is most likely due to the sensitivity of the method. When X-rays pass through a material, the degree of their absorption or attenuation is influenced by the density and composition of the material. In the context of dentine, a mineralized tissue containing hydroxyapatite crystals, changes in mineral content have a direct effect on X-ray attenuation properties. A higher mineral content in dentine corresponds to greater X-ray attenuation, resulting in darker regions with higher grey values on the micro-CT images. Conversely, a lower mineral content leads to less X-ray attenuation, resulting in lighter regions with lower grey values. The greatest increase in density is observed after the incubation period near the material-dentine interface for all materials examined. On the micro-CT images, this can be recognised as a white accumulation, which corresponds to ion and mineral intercalation. No such findings were observed in the cavities treated with the composite material. A closer look at the images also shows that the grey values in the entire lesion are consistent with the results of the microhardness determination and the SEM/EDS evaluation.

Complete remineralization of the lesion is a hardly achievable goal. This is especially true for dentine, where remineralization attempts often result in heterogeneous crystal formation. The variety of materials that can be used to remineralize enamel and dentine, as well as the range of methods and techniques used to observe remineralization, make it difficult to compare different studies. While this in vitro study does not attempt to replicate oral conditions, it does provide useful information on material and tissue properties and behaviour. In addition, the release of ions and precipitation of minerals are recorded and described, which could ultimately lead to proper remineralization. Nevertheless, it is crucial to interpret the results taking into account the experimental conditions. As researchers continue to explore this area, the results of future studies will likely provide a clearer picture of the potential applications and needed improvements of these innovative remineralization materials.

6. CONCLUSIONS

Considering the limitations of this study, following conclusions can be drawn:

1. There is a significant difference in the effect of ion-releasing materials on tooth enamel. CPP-ACP and fluoride-based MI Varnish® restore a higher percentage of the microhardness of the demineralized enamel lesion compared to 3M™ Clinpro™ White Varnish and Megasonex® toothpaste.

2. All three tested materials for enamel remineralization significantly affect the appearance of enamel.

3. The application of MI Varnish® results in a greater proportion of calcium and phosphate ions in demineralized enamel lesions compared to 3M™ Clinpro™ White Varnish and Megasonex® toothpaste.

4. Products based on nano-hydroxiapatite can be an alternative to products based purely on fluoride.

5. There is a significant difference in the effect of the ion-releasing materials on the dentine. EQUIA Forte HT shows the highest potential to remineralize demineralized dentine lesions compared to the other restorative materials tested by significantly increasing the microhardness values after incubation.

6. All restorative materials tested have a significant effect on the appearance and chemical composition of the dentine surface.

7. Micro-CT is a valuable method to observe the changes in dentine density and to determine the potential remineralization effect of the materials.

8. All the restorative materials tested increased the mineral content of the demineralized lesion.

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8. CURRICULUM VITAE

Ivan Šalinović DMD, was born on August 25th, 1994 in Split, Croatia. He enrolled at the University of Zagreb, School of Dental Medicine in 2013, from which he graduated in 2019. During his college education, he won two Rector's Awards and took part in Erasmus+ Programme, spending one semester at Ludwig-Maximillian University in München, Germany. Since 2020 he has worked as a research and teaching assistant at the Department of Endodontics and Restorative Dentistry, School of Dental Medicine, University of Zagreb, within the Croatian Science Foundation project *Investigation and Development of New Micro- and Nanostructural Bioactive Materials in Dental Medicine, IP-2018-01-1719*.

He regularly attends and actively participates at congresses in Croatia, as well as abroad. Since 2020 he has been a CED-IADR Young Ambassador for Croatia. He is a member of Croatian Medical Association and since 2022 has been a secretary of the Croatian Society for Minimum Intervention Dental Medicine.

8.1. List of publications

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* Publication associated with the doctoral thesis.