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LABORATORY AND CLINICAL PERSPECTIVES OF UNIVERSAL ADHESIVES

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ABSTRACT

Aim: The continuous evolution of adhesive dentistry has led to the development of universal adhesives (UAs) designed to simplify clinical procedures while maintaining optimal bonding efficacy under various conditions. However, degradation of the resin-dentin interface remains a critical issue, primarily due to enzymatic activity and the intrinsic complexity of the dentin substrate. This doctoral research aimed to investigate strategies to enhance the long-term stability of the hybrid layer and bond performance through four complementary approaches: (1) the use of glutaraldehyde-based desensitizing agents as functional dentin primers; (2) the application of the Immediate Dentin Sealing (IDS) technique for indirect restorations; (3) the evaluation of the effects of endodontic irrigants on adhesive performance; and (4) the incorporation of bioactive materials into adhesive systems to promote remineralization and enzymatic protection.

Materials and Methods: Extracted human teeth were sectioned to obtain standardized dentin specimens. Different universal adhesive systems were applied following various clinical protocols, including etch-and-rinse (ER), self-etch (SE), and selective enamel etching strategies (SEE). In specific groups, Gluma Desensitizer (glutaraldehyde + HEMA) was applied as a pre-treatment prior to adhesive application. For IDS, freshly cut dentin was immediately sealed with a universal adhesive and stored under simulated clinical conditions before testing. Additional groups were pre-treated with endodontic irrigants (NaOCl, EDTA, CHX) to evaluate their influence on bonding performance. Adhesive interfaces were tested using microtensile bond strength (μ TBS) and shear bond strength (SBS) tests, both immediately and after thermocycling or water aging. Scanning Electron Microscopy (SEM) and confocal laser scanning microscopy (CLSM) were employed to assess hybrid layer (HL) morphology, nanoleakage, and dentinal tubule occlusion. *In situ* and gelatin zymography were performed to visualize matrix metalloproteinase (MMP) activity within the adhesive-dentin interface. In the final experimental phase, bioactive formulations containing ion-releasing components (Ca , PO_4^{3-} , F^-) were evaluated for their remineralization potential and ability to reduce enzymatic degradation. Statistical analysis was conducted using ANOVA and post-hoc tests with a significance level set at $p < 0.05$.

Results: The application of Gluma Desensitizer significantly increased bond strength values ($p < 0.05$) and reduced nanoleakage compared with untreated controls. SEM observations revealed partial occlusion of dentinal tubules and cross-linking effects on the collagen matrix, which corresponded to reduced MMP and cathepsin activity, as confirmed by *in situ* zymography.

The IDS technique produced significantly higher μ TBS values and improved marginal adaptation compared with delayed dentin sealing, demonstrating a more uniform HL with reduced micropermeability.

In contrast, the use of NaOCl as an irrigant negatively affected bond strength and resin infiltration due to collagen denaturation and oxygen residue formation, while EDTA-treated dentin exhibited a more favourable substrate for SE adhesives, maintaining fibrillar integrity. CHX pre-treatment showed intermediate results, partially preserving collagen structure and enzymatic stability.

The incorporation of bioactive components into adhesive or restorative formulations resulted in ion exchange at the adhesive-tooth interface, promoting the precipitation of apatite-like deposits and reducing enzymatic fluorescence signals over time. These effects suggest enhanced remineralization capacity and potential protective behaviour against hybrid layer degradation.

Conclusion: The results from this doctoral research underline the multifactorial nature of adhesive durability and the importance of biological and chemical control at the resin-dentin interface. Desensitizing agents such as Gluma not only reduce hypersensitivity but also act as collagen-stabilizing primers, while IDS effectively protects freshly cut dentin and strengthens the adhesive interface. The influence of endodontic irrigants on dentin substrate highlights the need for optimized irrigation protocols to prevent adverse chemical effects on bonding. Furthermore, bioactive adhesive systems capable of releasing therapeutic ions offer a promising avenue for developing restorative materials that combine adhesion, protection, and regeneration. Overall, this research supports a shift in adhesive dentistry towards biofunctional and integrative strategies, where chemical bonding, enzymatic inhibition, and biomimetic remineralization converge to ensure more stable, durable, and biologically compatible adhesive interfaces.

INTRODUCTION

Among the most impactful developments were the introduction of composite resins and acid-etching techniques. The pioneering work of Michael Buonocore and Rafael Bowen laid the foundation for the concept of dental adhesion, marking a transition from the traditional principle of “extension for prevention” (Black, 1891) to the modern philosophy of minimally invasive dentistry. Buonocore’s demonstration of adhesion to dental hard tissues in 1955 initiated the continuous evolution of bonding materials and adhesive strategies, profoundly influencing restorative clinical practice. Despite significant technological advancements, achieving and maintaining a stable and durable bond to dental tissues remains a major clinical challenge (Sano et al., 1994).

Bonding to enamel relies predominantly on micromechanical retention, which is highly stable (Buonocore, 1955). This process involves partial demineralization of enamel hydroxyapatite by acid etching, followed by resin infiltration into the microporosities and subsequent polymerization, resulting in a resin-enamel complex (Nakabayashi et al. 1982).

Similarly, dentin bonding depends on the infiltration of resin monomers into the collagen matrix after partial removal of the inorganic content. Upon polymerization, a collagen-reinforced interdiffusion zone known as the “hybrid layer” (HL) is formed (Pashley et al. 2011; Breschi et al. 2008). The interface between the cured adhesive and the remaining mineralized dentin is mediated by collagen fibrils that extend from the mineralized base into the HL. These fibrils are rich in non-collagenous proteins, including growth factors and matrix-bound proteolytic enzymes (Mazzoni et al. 2009). Although these enzymes, such as matrix metalloproteinases (MMPs) and cysteine cathepsins (CCs), are physiologically involved in dentin development, they become reactivated during bonding procedures, regardless of whether an etch-and-rinse (ER) or self-etch (SE) approach is used. This enzymatic activity initiates the progressive breakdown of collagen fibrils that serve as the anchorage between resin and dentin, ultimately compromising the integrity of the adhesive interface (Tjäderhane et al., 2013). MMPs and CCs are the primary enzymatic agents implicated in the time-dependent degradation of the dentin collagen matrix. Consequently, strategies aimed at inhibiting or deactivating these enzymes have become essential to ensure the long-term durability of the resin-dentin bond.

Furthermore, although significant strides have been made in improving the effectiveness and longevity of dental materials, post-operative sensitivity remains a frequent complication following adhesive procedures, particularly in deep dentin cavities where tubules are more numerous and wider (Brännström, 1966; Pashley, 1990; Orchardson , 2006).

In summary, contemporary adhesive dentistry is increasingly focused on two main objectives: reinforcing the tooth–adhesive interface through strategies aimed at preventing or limiting enzymatic degradation of the HL, and reducing post-operative sensitivity, with the overarching aim of improving the long-term clinical performance of adhesive restorations (Tjäderhane et al., 2013; Breschi et al., 2018).

One effective strategy to mitigate these issues is the application of desensitizing agents. Their mechanism is based on the precipitation of proteins and the obliteration of dentinal tubules, which not only reduces sensitivity but may also contribute to improved resin infiltration and bond stability. Additionally, they exert an inhibitory effect on MMPs, supporting the durability of adhesive interfaces (Hebling et al., 2005; Stawarczyk et al., 2011; Pashley et al. 2011; Breschi et al., 2018).

A further approach to enhance bonding efficacy and minimize dentinal sensitivity is the Immediate Dentin Sealing (IDS) technique. This method involves the immediate application of adhesive resin to freshly cut dentin before impression-taking or provisionalization. IDS has been shown to enhance bond strength, reduce microleakage, and protect the dentin-pulp complex from bacterial contamination during the temporization phase (Magne, 2005; Magne et al., 2007; Bertschinger et al., 1996).

Another important aspect pertains to adhesive procedures in endodontically treated teeth. While post-operative sensitivity is not a concern in this context, maintaining high bonding efficacy is still crucial, especially considering the possible effects of root canal irrigants on the adhesive interface. This could have resonance in case of composite resin pre-endodontic restoration, which adhesive interface can be mostly in contact with the different irrigant solutions during the devitalization procedures. Indeed, sodium hypochlorite (NaOCl), EDTA, and chlorhexidine (CHX), while essential for disinfection, may chemically and structurally alter the dentin substrate and influence both bond strength and HL stability. Understanding how these irrigants interact with dentin and adhesive systems is therefore crucial for the long-term success of bonded restorations in endodontically treated teeth (Menezes et al. 2004; Ari et al. 2003; Prado et al. 2011).

Alongside these developments, a new class of restorative materials is gaining increasing relevance in daily clinical practice: bioactive materials. These not only demonstrate excellent adhesive and mechanical properties, but also possess the unique ability to release therapeutic ions that promote remineralization and repair of dental tissues. Their capacity to interact dynamically with the surrounding biological environment positions them as a promising option for enhancing the longevity and biological performance of adhesive restorations. Specifically, bioactive materials

capable of releasing calcium, phosphate, and fluoride ions offer a valuable alternative to traditional composites and adhesives. These materials can support the remineralization of adjacent dentin while enhancing the integrity of the adhesive interface through modulation of enzymatic activity and the formation of more stable chemical bonds (Hench, 2015; Sauro et al., 2025; FDI, 2022; Mazzoni et al., 2015).

Altogether, these innovations are reshaping not only restorative dentistry but the entire field of dental medicine. A comprehensive evaluation of the influence of these different materials and techniques on dentin bonding properties and enzymatic activity is still lacking. Their investigation could unveil the underlying factors determining dentin adhesion longevity and possibly offer new recommendations for improving bonding procedures (Pashley et al., 2011; Tjäderhane et al., 2013; Breschi et al., 2018).

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ADHESION SUBSTRATES

Adhesion (from Latin “Adhaerere”, that means “be attached”) is defined as the sticking of two surfaces to one another. The advent of adhesion to hard tissues of the tooth defined a turning point and featured a new era in dentistry. (Buonocore, 1955; Nakabayashi et al., 1982; Van Meerbeek et al., 2020). The substrates more frequently available for adhesion in restorative dentistry are enamel and dentin, but also ceramic, composite resin and other indirect restorative materials. Dental adhesion depends on the properties of components: assembly materials (such as cement and adhesive), the tooth, and prosthesis (Van Meerbeek et al., 2011; Pashley et al., 2011). The mode of adhesion can be divided into five main categories: mechanical, chemical, by absorption, electrostatic and by diffusion. Mechanical adhesion relies on mechanical interlocking which provides retention and a durable form of bonding (Buonocore, 1955). Chemical adhesion involves the modification of the surface etch, and two dissimilar surfaces are connected by an active monomer (Nakabayashi et al., 1982; Yoshida et al., 2000). The absorption theory suggests that adhesion is due to Van der Waals forces (each molecule has a negative pole or a positive pole) or to London’s forces (random movement of atoms) (Kinloch, 1987). The electrostatic theorizes a double electric layer on the interface between a metal and a polymer (Mittal, 1976). Finally, the diffusion theory proposes that molecules cross each other on the interface.

Moreover, there are various requirements for a correct and effective adhesion: surface cleaning (Nassar et al. 1995); contact angle and wettability (Paddy et al. 1992, Bouquet et al. 1984); viscosity and flowability of the adhesive (Fan et al. 1975); surface roughness (Bussher et al. 1984); polymerization of the adhesive (Spoto et al. 2013); geometry of the adhesive interface (Marshall et al. 2016).

Achieving a strong and long-lasting adhesive bond to dentin requires an optimal interaction between the adhesive system and the underlying substrate. One of the key factors influencing this process is the presence of the smear layer, a thin film formed on the dentin surface during cavity preparation. The smear layer is a 1-2 μm thick layer of cutting debris created during instrumentation. It includes dentin particles and organic remnants, occluding dentinal tubules as smear plugs. While it reduces dentin permeability and sensitivity, it may interfere with adhesion. Total-etch systems remove it completely; SE systems partially dissolve it, promoting better interaction with demineralized dentin (Pashley et al. 2011).

Even the absence of any one of the previous factors may irreversibly compromise the adhesive bond between the dental substrate and the restorative material.

Enamel

Tooth enamel, the most mineralized tissue in the human body, is composed of over 95% (by weight) carbonated apatite. Its mechanical properties derive from a hierarchical structure of enamel rods (or prisms), each 4-8 μm in diameter, composed of tightly packed hydroxyapatite crystallites. The rods exhibit a keyhole-like cross-section, with the “head” facing the crown and the “tail” toward the root. Crystallites are parallel in the head but diverge ($\sim 65^\circ$) in the tail. Rods are arranged perpendicularly to dentin and tilt near the CEJ. Interrod enamel surrounds the rods and has the same composition but different crystal orientation. The boundary between rod and interrod enamel is the rod sheath. Organic matter ($\sim 1\text{-}2\%$) surrounds each rod and plays a role in guiding crystal growth, mediated by amelogenin, enamelin, and ameloblastin proteins during the secretory stage of enamel formation. In the maturation stage, matrix proteins are degraded by KLK4 protease.

Due to its high mineral content and low organic composition, enamel represents the most favourable dental substrate for achieving durable adhesion. The abundance of exposed hydroxyapatite crystals provides a chemically and mechanically stable surface that readily interacts with adhesive monomers following acid etching. This etching process creates a micro-porous surface that allows deep resin infiltration and the formation of a strong micromechanical interlocking, the so-called *resin tags*. Resin tags are microscopic extensions of adhesive resin that penetrate into the microporosities of acid-etched enamel or dentin. They create micromechanical interlocking between the resin and the tooth substrate, contributing significantly to the overall bond strength and durability of the adhesive interface.

As a result, bonding to enamel is generally more predictable, durable, and less affected by ageing phenomena compared with dentin, where the hybrid layer is more prone to hydrolytic and enzymatic degradation.

Enamel can be classified as prismatic (or rod) and aprismatic (or rodless) according to the presence or absence of enamel prisms. Prismatic enamel is the predominant structure and consists of tightly packed hydroxyapatite crystallites organized into enamel rods, which provide mechanical strength and fracture resistance (Ten Cate, 1994; Simmer & Hu, 2021). In contrast, aprismatic enamel, typically found in the outermost and innermost layers, lacks the rod organization and exhibits crystallites arranged parallel to the surface. This structural homogeneity makes aprismatic enamel less permeable and less responsive to acid etching, influencing adhesive procedures (Gwinnett, 1992; Perdigão, 2010)

Principles of adhesion on enamel

Etching: Acid etching is the use of an acidic substance to prepare the tooth's natural enamel for the application of an adhesive. The acid roughens the surface microscopically, increasing retention of resin sealant. Etching of dental enamel with acid removes the smear layer and opens enamel tubules. The application of weak acid, usually 30% phosphoric acid roughens the surface before bonding a resin filling or veneer. In the early 1950s, Dr. Michael Buonocore first introduced the idea of acid etching enamel to increase bonding of resin to tooth structure. Retention of dental resin materials is increased significantly by prior treatment of the enamel surfaces with few inorganic acids or chelators. Acid etch creates microscopic spaces in enamel (increasing surface roughness) into which the bonding agent/adhesive can flow, aiding the bonding process (micromechanical retention). When desiccated, a surface that has been etched appears chalky white or frosted. The use of phosphoric acid gels in enamel etching offers several advantages. These materials significantly enhance the resin-enamel bond, thereby strengthening the adhesive properties of restorations and contributing to an effective marginal seal that helps prevent microleakage. Moreover, the thixotropic behavior of etching gels provides excellent control during application, allowing precise placement on the intended area without excessive spreading. However, some drawbacks are also associated with their use. Etching gels require a completely dry working field to achieve optimal results, as moisture contamination can compromise adhesion. Additionally, the higher viscosity of gels may reduce their ability to uniformly wet the enamel surface, potentially affecting the consistency of the etching pattern. In certain cases, self-etch adhesives may not remove the smear layer as effectively, which can result in a less retentive surface and decreased bond strength.

Many studies have shown that enamel should be etched for no more than 30 s.

Primer: Although the application of a primer on enamel does not negatively affect adhesion, its use is unnecessary when the enamel surface has been properly etched and dried. In such conditions, micromechanical retention predominates, and the adhesive can effectively penetrate the etched micro-porosities without the need for additional hydrophilic components. Therefore, the primer, essential for bonding to moist dentin, offers no significant benefit on etched enamel and may even introduce unnecessary moisture into the interface.

Bonding: The adhesive agents used on the enamel are liquid, unfilled, low viscosity acrylic resins that wet the surface micropores. They consist mainly of BIS-GMA (bisphenol A-glycidyl methacrylate) or UDMA (urethane dimethacrylate). Both monomers are viscous and hydrophobic,

for this reason they are often diluted with other more hydrophilic monomers of lower viscosity, such as TEG-DMA (triethylene glycol dimethacrylate) or EG-DMA (ethylene glycol dimethacrylate) (Grandini et al. 1999).

Dentin

Dentin, which makes up the bulk of tooth structure, is less mineralized than enamel (~72% mineral, 20% organic, 8% water). It is formed by odontoblasts during dentinogenesis and features microscopic tubules (diameter: 0.9–2.5 μm), which extend from the pulp to the dentin-enamel junction (DEJ) or dentin-cement junction (DCJ). These tubules carry odontoblastic processes and dentinal fluid and contribute to dentin's permeability and sensitivity. Furthermore, its tubular structure influences mechanical properties, permeability, and bonding behavior. Superficial dentin is rich in intertubular collagen, while deeper dentin has higher tubule density and moisture, which weakens adhesion.

Several factors (aging, caries, sclerosis, chemical treatments) modify its bonding performance. Caries-affected dentin exhibits reduced mineral content, altered collagen, and increased water, leading to weaker and thicker hybrid layers. Bond strength in caries-affected dentin is 20-50% lower than in sound dentin. Sclerotic dentin, with hypermineralized layers and occluded tubules, also shows poor adhesion, often requiring modified etching times.

Dentin types include:

- *Primary dentin*: The main structural component, including mantle dentin (outer layer, less mineralized) and circumpulpal dentin.
- *Secondary dentin*: Formed after root development, growing slowly and asymmetrically, contributing to pulp chamber shrinkage with age.
- *Tertiary dentin*: Formed in response to stimuli. It may be reactionary (by existing odontoblasts) or reparative (by new cells), with structure depending on stimulus intensity.

Newly secreted predentin (10-47 μm) is unmineralized and stains lighter than mature dentin.

Dentin collagen structure

The extracellular organic matrix (ECM) consists mainly of type I collagen fibrils, stabilized by inter- and intramolecular cross-links and supported by non-collagenous proteins such as

proteoglycans and phosphoproteins. Collagen molecules form triple helices ($\alpha 1$, $\alpha 2$ chains) that organize into fibrils and bundles.

Cross-linking and non-collagenous components preserve mechanical integrity and regulate water interaction, key to HL formation. Dentin collagen has minimal turnover, accumulating stable cross-links that resist denaturation during acid etching.

Principles of adhesion on dentin

Etching: Adhesion to dentin is more complex, due to its chemical composition and its structural heterogeneity. The ability of adhesives to bond to dentin depends on two main factors:

- 1) Demineralization of the substrate, which partially removes the mineral phase and increases the receptivity of the substrate;
- 2) Passive infiltration of monomers in the demineralized layer.

The total etching technique, in which enamel and dentin are etched simultaneously, was introduced by Fusayama in 1980 (Fusayama, 1980). Orthophosphoric acid removes the smear layer, demineralizes the dentinal surface by 5 μm , so that collagen fibers are exposed and the dentinal tubules are freed, forming tubular-shaped micro cavities (Pashley, 1992). The resinous monomers, in contact with the etched dentin, form a matrix of collagen fibers reinforced with resins forming the “hybrid layer” or “interdiffusion zone” and it is composed of collagen, resinous monomer and components of the smear layer (Nakabayashi, 1992). Dentin should only be demineralized to the depth required for the desired infiltration. Many studies have shown that dentin should be etched for no more than 15 seconds. For this reason, the etchant must be applied first on the enamel and then on the dentin, so as not to cause an “overetching” of the substrate.

Primer: Following etching, the collagen fibers collapse as they are deprived of hydroxyapatite crystals. Moreover, the air-drying of dentin after etching eliminates the water from the matrix, causes the fibrils to collapse and therefore reduces the interfibrillar spaces necessary for the penetration of the resin to have a correct mechanical interlocking. To avoid this problem, a surfactant agent (primer) must be deposited on the dentinal surface, in order to determine an expansion and separation of the collagen fibers and matrix. Furthermore, the primer transforms the hydrophilic surface of dentin into hydrophobic, favoring adhesion. Thanks to the primer, the adhesive is able to optimally wet all the surface dentin and create a lasting bond over time with the composite resin (Nordenvall et al. 1980; Nakabayashi et al 1992; Eliades 1994). All this is allowed by the peculiar structure of the primer: it is an amphiphilic composite, i.e. with a hydrophilic part, which

binds to the dentinal substrate, and a hydrophobic part with a methacrylate group, capable of binding to the composite resin (Grandini et al. 1999). Essential for adhesion is 2-hydroxyethyl methacrylate (HEMA), due to its excellent wettability characteristics. However, the primer may also contain any other hydrophilic monomers (NTG-GMA, PMDM, PENTA), diluted in solvents such as acetone or ethanol, which favor the disappearance of water in moist dentin (Jacobsen et al. 1994). For this reason, the wet bonding technique is used: it is therefore essential to reach the correct degree of dentinal humidity, without exceeding in drying or “over wetting”, to have an adequately hydrated collagen network (Cerutti et al. 2007). Afterwards, the solvent contained in the primer should be evaporated with a light flow of air for at least 10 seconds, before bonding (Haws, 1997).

Bonding: It involves applying a thin layer of adhesive (60 to 120 μm) on the dental substrate with a brush, to allow the formation of the support structure of the adhesive interface, between the tooth and the composite resin. After the removal of smear layer and smear plug from the dentine surface, the liquid resin penetrates into the spaces forming “resin tags”, which interdigitate with the dentinal structure. Resin tags increase the available surface and increase the bond strength by 10-30% (Gwinnet, 1993). In this case, the use of the air-water syringe to thin the adhesive layer is done carefully, to avoid the formation of an uneven surface. The use of a microbrush is therefore indicated, so as to directly remove the excess resin through the bristles of the brush.

Hybrid Layer (HL)

The hybrid layer (HL) is a micromechanical interdiffusion zone formed at the interface between demineralized dentin and adhesive resin during bonding procedures. It results from the infiltration of adhesive monomers into the exposed collagen fibril network after removal or modification of hydroxyapatite by acid etching or self-etching approaches. Once polymerized, the adhesive resin interlocks with the collagen matrix, creating a resin-collagen composite structure that provides the fundamental basis for resin-dentin adhesion. The quality and durability of this interface largely depend on the extent of resin infiltration and on the preservation of the collagen scaffold during bonding procedures.

The formation of the HL is strongly influenced by the condition of the dentin substrate, the presence and modification of the smear layer, and the balance between dentin demineralization and resin infiltration. Ideally, adhesive monomers should completely infiltrate the depth of the demineralized dentin matrix. However, incomplete resin penetration frequently occurs, leaving zones of exposed collagen fibrils that are vulnerable to degradation. These poorly infiltrated regions

are associated with nanoleakage phenomena, characterized by the penetration of fluids and ions within the HL, which may accelerate interfacial degradation over time (Breschi et al., 2008).

The HL is considered the most critical and degradation-prone component of the adhesive interface. Its deterioration is mainly related to hydrolytic degradation of resin components and enzymatic breakdown of collagen fibrils. Endogenous dentinal enzymes, particularly MMPs and CCs, can be activated during acid demineralization and contribute to the progressive degradation of the collagen network. In addition, the hydrophilic nature of contemporary adhesive systems facilitates water sorption and resin plasticization, further compromising the mechanical properties of the interface.

Since the HL consists of multiple interacting components, degradation affecting either the collagen matrix or the polymerized resin may lead to progressive failure of the entire adhesive system. For this reason, several strategies have been proposed to enhance hybrid layer stability, including the use of MMP inhibitors, collagen cross-linking agents, ethanol-wet bonding techniques, and bioactive or remineralizing materials aimed at preserving collagen integrity and promoting long-term interfacial stability (Breschi et al., 2008; Breschi et al., 2018).

Adhesive Resin Degradation

Hydrolytic degradation is a chemical reaction during which the covalent bonds between polymers are disrupted in the presence of water, leading to loss of resin mass (Breschi et al., 2018; Tay and Pashley, 2004). The well-known discrepancy between dentin and the adhesive resins in the affinity towards water, along with the tendency to simplify dental procedures, caused changes in the composition of the simplified adhesives. The hydrophilic monomers that are added into simplified adhesive blends are essential for the infiltration of the resin into the collagen network (Swift et al., 1995; Van Meerbeek et al., 2005), but render the adhesive more prone to water sorption and more permeable (Tay et al., 2002). Moreover, the amphiphilic resin blends are susceptible to nano-phase separation phenomenon, especially in the more simplified systems. Hence, heterogenous resin layers are created, with the separation of the more hydrophilic and the more hydrophobic phase (Wang et al., 2006; Ye et al., 2008, 2007). The restorations are intraorally constantly exposed to occlusal forces and temperature changes, which eventually hampers the interfacial stability allowing oral fluids and water to penetrate the resin, especially in the more hydrophilic adhesives, or the hydrophilic phase of the heterogenous blends (Gale and Darvell, 1999).

The degradation starts with water diffusion through the more hydrophilic part of the adhesive layer, leading to the creation of water-filled channels that degrade the hydrophilic phase of the adhesive (Jacobsen and Söderholm, 1995; Mazzoni et al., 2011a). As the solubilized resin leaches out, the underlying collagen fibrils become denuded and are more exposed to hydrolytic degradation by endogenous proteases (Cadenaro et al., 2005). One of the key factors in the stability of the resin-dentin interface is the quality of the polymerization of the adhesive. All the adhesive systems, irrespective of the number of the components in the system, display a certain degree of incomplete polymerization, which could facilitate hydrolytic degradation (Breschi et al., 2008; Cadenaro et al., 2005). Hence, any factor that can further adversely affect degree of conversion can facilitate the degradation of the HL. With regard to this, it is important to mention that the adhesive resins contain up to 50% of solvents, that can be water, ethanol or acetone, depending on the system.

These solvents need to be evaporated by air stream before polymerization or they may remain trapped within the matrix during photopolymerization (Reis et al., 2010; Yoshida et al., 2007). Further, the standard photo-initiators, such as camphorquinone, that were adequate for the hydrophobic monomers, might not yield the optimal degree of cure of hydrophilic resins (Wang et al., 2006). The addition of alternative hydrophilic photo-initiators such as TPO (ethyl 4-dimethylaminobenzoate and diphenyl(2,4,6-trimethylbenzoyl)-phosphine oxide), in addition to conventional camphorquinone, has been proposed to improve the DC of hydrophilic adhesive systems (Ilie and Hickel, 2008; Wang et al., 2006).

Degradation of collagen fibrils

Since neither ER nor SE adhesives can fully penetrate the dentin collagen network, denuded collagen fibrils surrounded by water are always present on the bottom of the hybrid layer after the bonding procedures, and this is the weak area of any resin-dentin interface (Frankenberger et al., 2005; Sano et al., 1995; Wang and Spencer, 2002). In the demineralized dentin, endogenous proteases, mainly, MMPs and CCs are reactivated (Hannas et al., 2007; Nascimento et al., 2011; Vidal et al., 2014), and in the presence of water they can hydrolyze collagen fibers (Pashley et al., 2004). MMPs are a group of Zn^{2+} and Ca^{2+} dependent endogenous proteases, with implications in various physiological and pathological processes in dentin (Mazzoni et al., 2009a). The MMPs contain several prodomains, two of which are in common for all: propeptide domain which contains the key cysteine residue and the catalytic domain, which contains the Zn-binding site. These two

domains form the “cysteine switch” which maintains the enzyme in an inactive form. MMPs also contain other domains which determine substrate specificity and other interactions (Hannas et al., 2007). The MMPs are secreted in pro-form and due to several different factors, they can turn to the active form. In the pro-form, the cysteine residue from the propeptide domain is bonded to the zinc atom on the active site. Irrespective of the activation mode, the underlying mechanism is always the same: the cysteine is dissociated from the zinc atom in the active site, and exchanged by water molecules, which transforms the pro- into active form of MMP (Van Wart and Birkedal-Hansen, 1990). So far, several MMPs have been identified in dentin: gelatinases MMP-2 and MMP-9 (Mazzoni et al., 2009b, 2007), collagenase MMP-8 (Shimada et al., 2009), stromelysin MMP-3 (Mazzoni et al., 2011b), enamelysin MMP-20 (Shimada et al., 2009) and most recently MMP-7 (Mazzoni et al., 2018). They are thought to play part in the mineralization of collagen matrix during tooth development, after which they remain trapped and inactive in the mineralized dentin. After the demineralization of dentin, these proteases are reactivated. The MMPs are believed to be involved in a vast range of dental hard tissue pathologies, including dental caries (Mazzoni et al., 2015; Tjäderhane et al., 2015), degradation of the HL (Mazzoni et al., 2014) and dentin erosion (Buzalaf et al., 2015). In particular, MMP-2 and MMP-9 are important in caries progression (Tjäderhane et al., 1998; Vidal et al., 2014) and degradation of the hybrid layer (Apolonio et al., 2017; A. Mazzoni et al., 2013;). The MMPs can degrade practically all components of the extracellular matrix (ECM). However, only true collagenases, MMP-1, -8, -13 and -18, are responsible for collagen degradation (Visse and Nagase, 2003). The cleavage occurs on specific sites, creating $\frac{1}{4}$ and $\frac{3}{4}$ fragments of the collagen molecule. These fragments are thermally unstable and can easily be degraded by other members of the MMP family, or a wide range of proteases (Perumal et al., 2008). Nevertheless, they cannot initially cleave the collagen triple helix since the binding position is sterically blocked by C-telopeptides, making collagen triple helix positioned in such a way that the large collagenase molecule cannot fit (Perumal et al., 2008). Hence, removing the protective telopeptides is the key factor which facilitates the unwinding of the triple helix and the collagenolytic collagen cleavage by true collagenases (Mirigian et al., 2013). The telopeptidases which enable this process are MMP-2 and -9 (Perumal et al., 2008). The activity of these gelatinases, the most abundant in dentin, and pivotal in the collagen degradation process, has been extensively investigated by Mazzoni et al. (Mazzoni et al., 2009b, 2007). Apart from demonstrating MMP-2 and -9 as the intrinsic constituents of dentin organic matrix using immunolabeling technics (Mazzoni et al., 2009b), Mazzoni et al. also investigated the activity of these MMPs with precise identification of

the pro- and active forms of both MMP- 2 and -9 using gelatine zymography in dentin powder (Mazzoni et al., 2007). The same research team further demonstrated that the MMPs can be activated by acid etching, ER as well as SE adhesive systems (A. Mazzoni et al., 2013; Mazzoni et al., 2012, 2006). Therefore, inhibition/inactivation of the MMPs is considered a very important strategy for the preservation of the HL (Breschi et al., 2018; Tjäderhane et al., 2013). CTs are another important intrinsic constituent of dentinal organic matrix with implications in physiological and pathological processes in dentin. They can contribute to the degradation of the ECM and foreign proteins (Mazzoni et al., 2009a). All human cathepsins have the same active site, formed by cysteine and histidine residues, with three well-defined substrate binding subsites (Turk et al., 1998). The presence and distribution of cathepsins K (CTK) and B (CTB) in dentin have recently been described using immunolabeling technique (Scaffa et al., 2017). CTK and CTB may play an important role in bone remodeling or collagen degradation during orthodontic tooth movement (Mazzoni et al., 2009a).

Furthermore, there is high increase in the activity of cysteine CTs in active and chronic carious lesions (Nascimento et al., 2011). The activity of cathepsins is more pronounced with the increased depth of the cavity. Hence, it is reasonable to believe that the odontoblast-secreted CTs have implications in the active caries lesions. The increase of the activity of CTs in the chronic caries lesions could indicate their role in the activation of growth factors and consequently in the stimulation of defensive pulpal reactions (Nascimento et al., 2011; Tersariol et al., 2010). Interestingly, almost all collagenolytic activity of the CTs can be attributed to CTK which is specific in several ways. CTK, a papain-like cysteine protease, is predominantly expressed in osteoclasts and held responsible for the degradation of bone collagen (Bromme and Okamoto, 1995). It can cleave collagen in multiple positions, in the helical as well as non-helical parts (Garnero et al., 1999), while other CTs can cleave only the non-helical parts of the collagen molecule. It is capable of disintegrating compact collagen fibers into fragments and further solubilizing them into soluble peptides (Panwar et al., 2013). "Productive" GAGs, such as chondroitin 4-sulfate, or chondroitin 6-sulfate are necessary for the activation of CTK. Since the active site of the CTK is too small to accommodate the whole collagen molecule, it is hypothesized that the CTK-GAG complex conformationally changes the active site of the proteases, enlarges it, enabling the cleavage of collagen (Li et al., 2002). While some GAGs activate CTK, others, such as heparin, act in its inhibition. Hence, the activity of CTK is fully regulated by GAGs (Li et al., 2004). The mechanisms in which the MMPs and CTs degrade collagen is different, but they can also work synergistically, and create a

cascade of events leading to the degradation of the hybrid layer (Nascimento et al., 2011; Tersariol et al., 2010). In summary, there are two main pathways of collagen degradation. One involves the MMPs, where in the joint activity of telopeptidases and true collagenases, the collagen molecule is fragmented into $\frac{1}{4}$ and $\frac{3}{4}$ fragments which are thermally unstable and can further be degraded by a wide range of proteases and non-collagenolytic MMPs. The other pathway is mediated by CTK-GAG complexes which can degrade collagen without the cooperation with other proteases (Li et al., 2004).

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ADHESIVE SYSTEMS

Adhesive systems can be classified according to different characteristics: generation, type of solvent, mechanism of action against the dentine substrate, number of adhesive steps.

Classification of adhesive systems

Classification by generation: traditionally, adhesive systems have been classified into “generations,” a term used to describe the evolution of bonding strategies over time, with each generation reflecting distinct compositional and procedural features introduced during a specific period.

I Generation

In first generation adhesive systems (early 1950s), the main constituent was NPG-GMA (N-phenylglycine glycidyl methacrylate), which facilitated the adhesion between enamel, dentin and composite resins, through the calcium ions present on the surface. They had a high poly shrinkage and thermal expansion, but low bond strength (2-3 MPa).

II Generation

Second generation adhesives (late 1960s-early 1970s) comprised several calcium-chelating acrylic resins, with phosphoric groups within. The smear layer was preserved to have more calcium ions available, but the adhesion forces were still low (5-6 MPa) (Smith et al. 1994)

III Generation

The third generation of adhesives brought significant innovations. Nakabayashi and Fusayama showed that dentin etching did not cause pulp necrosis or inflammatory reactions. The concept of hybrid layer began to be defined in order to have a good bond between dentin and resin (Nakabayashi et al. 1982). The primers were mainly composed of monomers such as 4-META or HEMA, the bondings instead consisted of uncharged resin, enriched with monomers, such as BIS-GMA and HEMA.

IV Generation

The fourth generation dentinal adhesives entered the market in the 90s and, unlike the previous ones, are still widely used. They consist of etchant, primer and bonding agent and they are applied in three sequential steps. They have obtained clearly valid clinical results and for this reason they are still present on the market. This generation was introduced by Kanca and Gwinnett in 1992 (Kanca, 1992; Gwinnet, 1993).

V Generation

Fifth generation adhesives are the first to introduce 2-step systems, i.e. etching applied in a single step, then primer and bonding in a single package (Van Meerbeek et al. 2003). This generation was introduced to simplify the classic 3-step systems, reducing times and margins of error, while maintaining the same adhesive performances.

VI Generation

Adhesive systems of the sixth generation were introduced with the aim of further facilitating compared to those of the fifth generation and solving the problem of wet bonding. They are 2-step adhesive systems, defined as self-conditioning, in which etching and primer are in a single solution: we are talking about acid primers, whose application involves the use of bonding directly, skipping the rinsing phase. This generation is closely related to the second generation, because there is not the complete dissolution of the smear layer, but it becomes part of the adhesive substrate (Tay et al. 2003).

VII Generation

An attempt was made to reduce operator-dependent errors and application time with 1-step systems. Etchant and primer are in a single solution, to which the bonding is joined before application on the dental tissue. Despite being more recent, these adhesives have a significantly lower bond strength to dentin than fourth, fifth and sixth generation adhesives (Van Meerbeek et al. 2003).

VIII Generation

These are the latest generation ones, also called “universal adhesives”. These are 1-step adhesive systems, consisting of etchant, primer and bonding in a single solution (*all in one, one bottle*).

Classification by type of solvent: taking into consideration the important role of their solvent (Perdigao et al. 2000), adhesives are also classified according to the type and concentration of this constituent. Accordingly, the following adhesive can be distinguished: acetone-based, water-based and alcohol-based.

- Acetone-based adhesives: acetone is a very volatile solvent, which has the role of displacing the water as it evaporates. These adhesives are often contained in ER systems, where they reduce viscosity and increase the wettability of the system. Kanca studies have shown acetone to be a better solvent than water, especially when mixed in a primer that contains HEMA (Kanca, 1992; Jacobsen et al. 1995). Acetone is considered a better solvent than water because it efficiently displaces residual moisture from the dentin surface, enhances monomer infiltration into the collagen network, and evaporates rapidly, leaving a more uniform and durable hybrid layer” (Jacobsen et al. 1995). These solvents have a very high vapor pressure following the application on dentin. Evaporating very easily, they must be dried gently. This leaves the dentine substrate moist to create an optimal hybrid layer.
- Water-based adhesives: they allow the dissociation of weak acids for the demineralization of enamel and dentin, for this reason they are used in self-etching systems. Their purpose is to leave the cavity well hydrated. Post-etching drying must be greater than acetone or alcoholic bases.
- Alcohol-based adhesives: they evaporate faster than water-based ones, but more slowly than acetone-based ones. Their drying must be “intermediate” compared to the two previous types (Hilton et al. 2013).

Classification by mechanic action against dentin substrate: the classification that best characterizes adhesive systems was proposed by Van Meerbeek et al. in 2003, as it considers the interaction mode with the substrate and the number of clinical application steps. Nowadays, 2 main adhesion strategies are identified: ER adhesive systems, which remove the smear layer and SE adhesive systems (also called etch and dry), which dissolve and incorporate the smear layer. A new group of

materials called universal or multi-mode adhesive systems has recently been marketed. The main feature of these systems is that they can be applied according to different adhesion strategies.

Classification by number of steps: finally, adhesives can be classified according to the number of clinical steps present in the adhesive procedure. This classification is the most used (Van Meerbeek et al. 2003) and divides adhesive systems into: 3-steps, 2-steps and 1-step.

3-steps: also referred to as etch and rinse (ER) adhesives, they require sequential application of etchant, primer and bonding. Today they are considered the gold standard, thanks to excellent clinical results and their ease of use even in complex cases. The only disadvantage is due to the time and methods of application which, to obtain satisfactory results, must be rigorous and accurate.

2-steps: these adhesives have two distinct phases of application: ER formulation and SE formulation. ER in two steps involves, as the first step, the application of the single etchant (as in the three steps), while primer and bonding are in a single solution. It is easier to use and takes less time when using them. Despite these advantages, 2-steps systems have a lower bond strength than 3-step systems. Furthermore, they are not suitable systems for all clinical cases (they are not compatible with dual or self-curing resins). In the 2-steps SE as the first step there is the application of etchant and primer in a single solution followed by drying without rinsing, in order not to remove the primer and finally the bonding is applied.

1-step: Etchant, primer and bonding are in one single component. They are “all in one”. They can also be in separate packs and then be mixed at the time of use. Very often it is etched anyway before the application, in this case it is not a pure 1-step technique.

Etch-and-rinse (ER) adhesive systems

ER adhesive systems can be applied in 3- or 2- steps, always using an initial acid-etching phase on both enamel and dentin. Dental substrate etching is traditionally carried out with a gel of phosphoric acid at concentrations that vary between 30% and 40% (pH = 0.1-0.4), which is first applied to enamel and then to dentin, for a period of no longer than 15 seconds on dentin and no less than 15 seconds on enamel.

When dealing with these multi-step adhesive systems, one of the more delicate steps relates precisely to the acid etching, including rinsing and the drying phase. After the acid is rinsed off, the procedures to dry the tooth should allow a low level of humidity at enamel while keeping the dentin hydrated to optimize the adhesion process. However, this is a difficult clinical task to achieve.

Dehydration of the demineralized dentin leads to the collapse of the exposed collagen mesh, reducing or nullifying the spaces between the fibrils and preventing them from acting as channels through which monomers may penetrate and spread within.

For ER adhesives, the wet bonding technique is recommended as a routine procedure to achieve effective dentin bonding (Kanca, 1992). This is particularly important when using acetone-based primers, as acetone cannot form hydrogen bonds and therefore promotes collagen re-expansion (Pashley et al., 2007). This technique consists of leaving the dentin moist in the post-conditioning phase, preventing the collapse of the collagen network, which is crucial in ensuring a suitable infiltration of the adhesive monomers throughout the nano-sized voids in the organic mesh. For this, excess water in the cavity must be removed with a dry sponge or a short air blast. However, the level of moisture that must be maintained on dentin to allow adequate solvent-based water displacement remains unclear and subject to ambiguity. If dentin presents an over-wet condition, excess water determines phase separation of the hydrophobic and hydrophilic components, resulting in the formation of blister and resin globules at the resin-dentin interface. This potentially weakens the resin-dentin bonds and is particularly more concerning when simplified systems are applied. On the other hand, when the dentin surface is excessively desiccated, the collapsed and altered collagen structure determines an incomplete inter-tubular resin infiltration. Interfaces are characterized by infiltrated areas along the dentinal tubules and superficially, forming a very fine HL delimiting an incompletely infiltrated sub-superficial hybridoid region, rich in collagen and susceptible to premature breakdown.

Self-etching (SE) or etch-and-dry adhesive systems

The mechanism underlying these adhesive systems does not involve complete removal of the smear layer; instead, it modifies the smear layer to increase its permeability, allowing better interaction with and penetration of adhesive monomers. Contrary to their ER counterpart, SE adhesives are not rinsed with water after application, but only air-dried, causing the evaporation of the solvents present. SE systems can be divided into “mild” or “strong” depending on the pH (Van Meerbeek et al. 2001). Strong adhesive systems have a very acid pH ($\text{pH} < 1$), while mild type adhesives have a higher pH. In order for the resin tags to form in the dentinal tubules, it is necessary that the SE systems have a pH lower than 1, therefore strong SE are the choice. This low pH is able to dissolve the calcium and phosphate ions of dentine, which are not rinsed, unlike the ER systems. These ions are unstable in an aqueous environment and for this reason make the adhesive interface

unstable (Hilton et al. 2013). SE adhesives, unlike ER, not only have micromechanical adhesion but also have a chemical type (Van Meerbeek et al. 2003; Yoshida et al. 2004). This is due to the “descaling adhesion” concept: in the first phase all acids bind to the calcium of hydroxyapatite (chemical bonds). At the same time, phosphate ions (PO_4^{3-}) and hydroxide ions (OH^-), coming from hydroxyapatite, are released. In this way the surface remains electrically neutral. The molecules of the adhesive will remain adhered thanks to the formation of a bond with calcium salts will be formed.

Universal Adhesives systems

Universal adhesives (UAs), also known as multi-mode adhesives, were developed in response to the clinical demand for simplified and less technique-sensitive bonding procedures (De Munck et al., 2005). These adhesive systems are designed to be applied using different strategies, including etch-and-rinse (ER), self-etch (SE), or selective enamel etching (SEE), allowing clinicians to adapt the bonding approach according to the clinical situation and substrate characteristics (Hanabusa et al., 2010). Their versatility has contributed to widespread clinical adoption, as a single adhesive system can be used for multiple indications and restorative procedures.

A distinctive feature of UAs is the incorporation of functional acidic monomers, most notably 10-methacryloyloxydecyl dihydrogen phosphate (10-MDP), which is capable of forming stable chemical bonds with hydroxyapatite through ionic interaction with calcium. This chemical interaction has been associated with improved bonding stability and the formation of nano-layered structures at the adhesive interface, potentially contributing to enhanced resistance to hydrolytic degradation.

Despite these advantages, UAs present some intrinsic limitations. Their relatively mild acidity results in a reduced etching effect on enamel compared with phosphoric acid, which explains why SEE with 35% orthophosphoric acid is frequently recommended to improve enamel bond strength and marginal sealing. Furthermore, the simplified formulation of UAs often requires a balance between hydrophilic and hydrophobic components, which may increase water sorption and permeability after polymerization. This characteristic may contribute to long-term degradation of the adhesive interface through hydrolysis and plasticization of the polymer matrix.

Although UAs represent the most recent evolution of adhesive systems, current evidence suggests that they have not introduced substantial improvements in long-term bonding performance compared with well-established multi-step adhesive systems. Their main advantage

lies in clinical versatility and procedural simplification rather than in a significant increase in bond durability. Ongoing research is therefore focused on improving their chemical stability, reducing water permeability, and enhancing the long-term preservation of the hybrid layer.

Longevity of adhesives

Dental adhesives are subjected to the oral environment, which is inherently complex and chemically aggressive (Teixeira et al. 2021). Over time, this can lead to issues such as marginal discoloration and secondary caries. Numerous studies have confirmed that bond strength tends to diminish as restorations age. The combined effect of occlusal loads and thermal fluctuations causes expansion and contraction of the materials, resulting in microleakage (Frassetto et al. 2016). For this reason, identifying adhesives with long-term stability remains a primary goal in restorative dentistry.

Biocompatibility

During dental practices, biocompatible materials and techniques must always be used. For example, the use of an etching agent for prolonged times or on insufficient thickness of dentin (less than 1 mm from the pulp tissue) can cause a pulp reaction. Even some composite resins if erroneously applied can cause damage or even minimal pulp irritation (Stanley et al. 1975).

Bioactive materials and adhesive systems

The term *bioactivity* is commonly used to describe a specific category of biomaterials capable of inducing apatite formation and triggering beneficial biological responses, such as targeted cellular or tissue reactions, under suitable conditions. The concept of bioactive materials was first introduced in biomedical science by Larry Hench, the creator of bioactive glass (Hench, 2015). His original formulation, *Bioglass 45S5*, was initially developed for orthopedic use because it produced a specific biological interaction at the tissue-material interface, creating a bond through apatite deposition. Later studies demonstrated its ability to promote remineralization and adhesion to dental hard tissues, leading to its adoption in dentistry. Similarly, Kokubo et al. proposed that any material capable of forming an apatite-like layer when immersed in simulated body fluid should be considered *bioactive* (Kokubo et al. 2006). This definition was extended to include materials that promote apatite nucleation or growth when exposed to saliva or biological fluids. In dentistry, the

concept gained further importance with the introduction of Mineral Trioxide Aggregate (MTA) by Dr. Mahmoud Torabinejad in the early 1990s (Torabinejad et al. 1995). This calcium silicate-based cement demonstrated excellent biocompatibility and the ability to stimulate apatite-like deposition and tissue regeneration, particularly in endodontics and pulp protection.

Over the past two decades, the understanding of *bioactivity* has evolved significantly. Modern bioactive materials are designed to engage in complex interactions with biological environments-through mechanisms such as the release of therapeutic ions, modulation of immune responses, or even targeted drug delivery, broadening their potential applications in oral and medical contexts. (Chan et al. 2023). Consequently, the concept of bioactivity has gained substantial attention in dental materials research, and the term is now frequently used in the marketing of restorative products (Sauro et al. 2021).

However, despite its popularity, the definition of *bioactivity* in dental materials remains ambiguous, leading to possible misuse. To address this, the FDI World Dental Federation issued a policy statement clarifying the term and providing guidelines for its responsible use (Schmalz et al. 2023). According to the FDI, bioactive restorative materials must elicit deliberate, localized, and beneficial biological effects without toxicity, while maintaining their fundamental role of replacing lost dental tissues for a durable period. Based on these principles, the concept of Advanced Bioactive Restorative Adhesive Materials (ABRAM) has been proposed (Sauro et al. 2025). Such materials should exhibit several key functional properties:

- (i) the capacity to chemically induce and deposit apatite-like minerals, promoting remineralization of demineralized dental tissues and sealing interfacial gaps to prevent secondary caries;
- (ii) controlled antibacterial activity against oral biofilms, reducing caries and inflammation without contributing to antimicrobial resistance;
- (iii) high biocompatibility, allowing interaction with pulpal cells and stimulation of odontoblast activity or stem cell differentiation, thus maintaining pulp vitality or aiding tissue repair.

It is crucial, however, that the designation “bioactive” be supported by solid experimental evidence. Therefore, both scientific publications and commercial communications should reserve this term exclusively for dental adhesive materials that demonstrably meet these biological and functional criteria (Ferracane et al. 2022; Sauro et al. 2025).

In recent years, the concept of *bioactivity* has been increasingly extended to adhesive systems, particularly to universal adhesives, which represent the most versatile and advanced generation of bonding agents. These materials are designed not only to promote micromechanical

retention through the formation of a hybrid layer but also to establish chemical and biological interactions with the dentin substrate. The inclusion of functional monomers such as 10-MDP (10-Methacryloyloxydecyl dihydrogen phosphate) allows the formation of stable calcium-phosphate salts, providing a chemical bond to hydroxyapatite and improving the long-term stability of the adhesive interface (Yoshida et al., 2004; Van Meerbeek et al., 2011). Recent research has explored the incorporation of bioactive fillers or ion-releasing compounds within universal adhesives to enhance remineralization potential and reduce degradation over time. For instance, the addition of calcium-phosphate nanoparticles, bioactive glasses, or zinc-doped formulations has demonstrated the ability to promote mineral deposition and inhibit MMPs activity, which is crucial for preserving the integrity of the HL (Sauro et al., 2018; Mazzoni et al., 2013; Tjäderhane et al., 2013). These findings align with the evolving concept of biofunctional adhesives, in which bonding agents are no longer regarded as passive intermediaries but as active components capable of modulating the dentin microenvironment and supporting tissue preservation (Breschi et al., 2018).

Some universal adhesives, therefore, may be considered bioactive systems, as they combine chemical bonding, enzyme inhibition, and remineralization mechanisms within a single formulation. Their ability to stabilize the HL and counteract hydrolytic and enzymatic degradation addresses one of the most persistent challenges in adhesive dentistry, the long-term durability of the resin-dentin bond. Consequently, the integration of bioactive principles into UAs represents a promising direction toward achieving enhanced longevity, biological compatibility, and functional stability of adhesive restorations, bridging the gap between traditional restorative materials and the new generation of ABRAM (Sauro et al. 2025; Breschi et al., 2021).

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EMERGING CLINICAL AND BIOLOGICAL PERSPECTIVES IN THE USE OF UNIVERSAL ADHESIVES

UAs represent the latest evolution in adhesive dentistry, combining versatility, chemical interaction, and simplified protocols. Their “multi-mode” capability, allowing both *ER*, *SE* and *SEE* approaches, has broadened their clinical applications beyond conventional restorative procedures. Contemporary research is now exploring novel and biologically oriented perspectives that exploit the functional and chemical potential of these systems to enhance both clinical outcomes and biological compatibility.

Dentin Hypersensitivity (DH)

One significant application of UAs involves the management of DH. UAs can effectively reduce DH by occluding dentinal tubules through the infiltration and polymerization of hydrophilic monomers that penetrate into the tubules and form resin tags after curing. This micromechanical sealing limits dentinal fluid movement, thereby reducing the hydrodynamic stimulus responsible for hypersensitivity (Pashley et al., 2011). Moreover, functional monomers such as 10-MDP can chemically interact with the calcium ions of hydroxyapatite, forming stable calcium-phosphate nanolayers that enhance the durability of the seal over time (Yoshida et al., 2004). To reduce DH, several approaches have been proposed, including the use of desensitizing agents capable of occluding the dentinal tubules. This mechanism complements that of Gluma Desensitizer (Heraeus Kulzer, Germany), composed of 5% glutaraldehyde and 35% HEMA, which acts by coagulating plasma proteins inside the dentinal tubules and cross-linking exposed collagen fibrils. The resulting protein–collagen network decreases dentinal permeability and reinforces the structural stability of the substrate (Cova et al., 2011; Maravic et al., 2020). When used in combination, Gluma provides immediate desensitization, while UAs ensure long-term tubule sealing through micromechanical interlocking and chemical bonding mechanisms. Thus, the DH condition, resulting from the exposure of dentinal tubules and fluid movement within them, can be mitigated by agents that occlude or cross-link dentinal collagen. *Gluma Desensitizer* has been shown to precipitate plasma proteins inside dentinal tubules and to stabilize collagen fibrils through cross-linking mechanisms (Pashley et al., 2011). When used in combination with universal adhesives, Gluma may enhance tubular sealing and reduce postoperative sensitivity without adversely affecting bond strength (Cova et al., 2011; Maravic et al., 2020). This integration highlights the dual clinical and biological benefits achievable when desensitizing and adhesive strategies are combined.

Immediate Dentin Sealing (IDS)

A second important frontier is represented by the IDS technique, described by Magne (Magne et al. 2007) IDS involves the application of an adhesive system directly after tooth preparation and prior to the impression or provisionalization phase. By sealing freshly exposed dentin immediately, the technique preserves bond integrity, reduces fluid contamination, and protects the pulp from bacterial and thermal insults. The use of universal adhesives for IDS has gained increasing popularity due to their low film thickness, compatibility with resin cements, and chemical adhesion potential through functional monomers such as 10-MDP. Clinical and laboratory studies have demonstrated improved long-term bond durability, enhanced marginal adaptation, and reduced postoperative sensitivity when IDS is performed with UAs (Tian et al., 2016; Polesso Patias et al 2025).

New Bioactive materials

In parallel, universal adhesives are being reinterpreted as bioactive or biofunctional interfaces rather than passive bonding agents. The concept of *bioactive adhesives* has been developed notably by Sauro and co-workers (Sauro 2024; Melo et al. 2022; Ferracane et al. 2023), who demonstrated the potential of incorporating calcium-silicate, bioactive glass, or nanofilled calcium-phosphate particles into adhesive formulations. These materials can release remineralizing ions (Ca^{2+} , PO_4^{3-} , Si^{4+}), buffer the local pH, and promote mineral precipitation within the HL. Moreover, certain bioactive formulations can inhibit enzymatic degradation by reducing MMPs activity and preserving the integrity of the collagen network (Breschi et al., 2018; Mazzoni et al., 2015). In this context, UAs represent a promising platform for integrating bioactivity and remineralization strategies, bridging the gap between restorative adhesion and biomimetic tissue preservation.

Influence of endodontic irrigants on adhesive system performance

Another emerging field concerns the interaction between UAs and dentin came in contact with endodontic irrigants. Irrigation solutions such as sodium hypochlorite (NaOCl), ethylenediaminetetraacetic acid (EDTA), and chlorhexidine (CHX) profoundly alter the chemical and morphological properties of dentin, affecting its wettability and mineral-organic balance (García-Varela et al., 2024). These changes can compromise adhesion by removing or denaturing the collagen scaffold. However, UAs, thanks to their mild acidity and the presence of functional monomers like 10-MDP, are capable of establishing stable calcium-phosphate complexes even on

chemically altered dentin (Stevens et al., 2024). Recent in vitro studies have demonstrated that UAs can maintain satisfactory bond strength and interfacial adaptation when applied after NaOCl/EDTA irrigation, suggesting an improved chemical tolerance and adhesion reliability under endodontic conditions (Yuan et al., 2022).

Collectively, these applications illustrate how UAs are evolving beyond simple bonding agents into multifunctional materials capable of addressing a range of clinical challenges. Their adaptability allows integration into various treatment protocols, such as desensitization, immediate dentin sealing, and endodontic restorative sequences, while their compositional flexibility opens the door to bioactive and enzyme-inhibiting modifications. The clinical and biological versatility of UAs thus represents one of the most promising frontiers in modern adhesive dentistry. Future research should aim to refine the chemical formulations of UAs to enhance their biofunctional performance, improve long-term HL stability, and expand their clinical reliability across different substrates and treatment conditions. By combining chemical adhesion, biological compatibility, and therapeutic functionality, universal adhesives are poised to become central components of minimally invasive and biologically driven restorative dentistry.

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EXPERIMENTAL APPROACHES FOR THE EVALUATION OF ADHESIVE INTERFACES

MICROTENSILE BOND STRENGTH TEST: Background, Principles, and Applications

The microtensile bond strength (μ TBS) test, first introduced by Sano in 1994 (Sano et al. 1994), represented a major advancement in the evaluation of the adhesive interface between restorative materials and dental hard tissues. Traditional macrotensile and shear bond strength (SBS) tests demonstrated limitations due to non-uniform stress distribution and the large bonded area, which often led to cohesive failures within the substrate rather than at the true interface. The μ TBS method was developed to overcome these issues by testing small bonded areas, thereby improving stress distribution and increasing the sensitivity of the method (Sano et al., 2019).

The rationale behind the development of the μ TBS test was to create a more precise and reproducible method for assessing the integrity of the resin-dentin interface. By using smaller specimens, typically beams or sticks with a cross-sectional area of approximately 1 mm² (Fig 1-3), researchers could obtain multiple specimens from a single tooth, significantly improving the statistical reliability of the results. Traditionally, two techniques have been described: the trimming and non-trimming methods. In the former, hourglass-shaped specimens are prepared from resin-dentin beams, whereas in the latter, the beams are simply sectioned into multiple sticks (Goracci et al, 2004). Owing to its time efficiency and the ability to obtain a larger number of test specimens, the non-trimming method has become increasingly popular over time. Moreover, because each specimen represents a localized area of bonding, the test allows for the detection of regional variations within the same tooth, such as differences between coronal, middle, and apical dentin. The test's ability to reveal subtle variations in adhesion makes it particularly useful for evaluating new adhesive systems, including UAs, which are designed to perform effectively under multiple bonding strategies (SE, ER, or SEE).

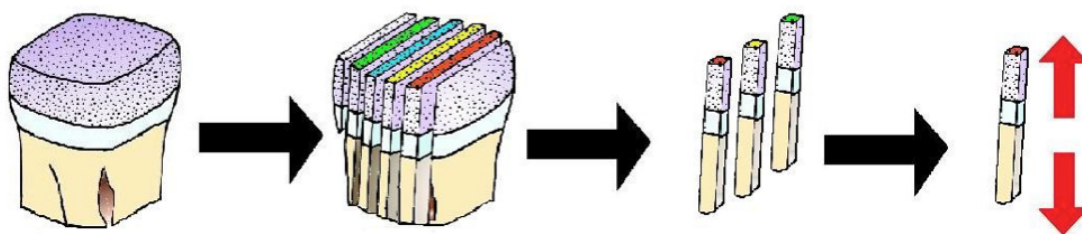


Fig. 1. Graphic diagram illustrating the sectioning procedure and the subsequent preparation of the sticks that will later be subjected to the microtensile bond strength test.

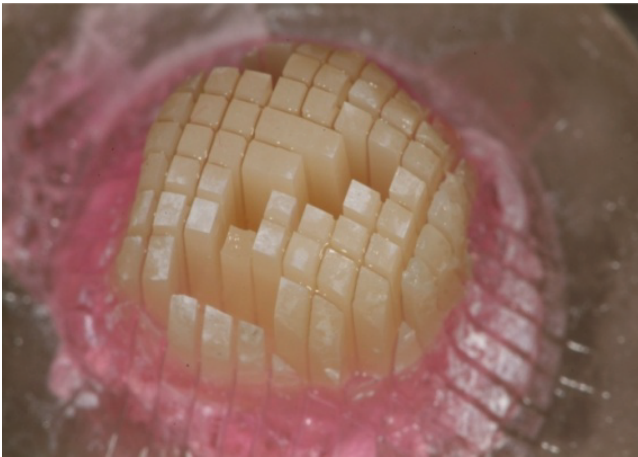


Fig. 2. Representation of a specimens after 2 sagittal cuts to obtain the sticks.



Fig. 3. The sticks are collected and, in the case of artificial aging, divided into baseline and aged groups. Each stick consists of one part dentin and one part resin composite, separated by the adhesive interface, which is subjected to tensile stress during testing.

Methodological Principles

In the μ TBS test, flat dentin surfaces are prepared, and the adhesive and composite resin are applied following the manufacturer's instructions. After light-curing, the restored tooth is sectioned, usually with a low-speed diamond saw under water cooling, into small sticks or beams with defined dimensions. Each beam is fixed in a testing jig and subjected to a tensile load until failure, using a universal testing machine at a crosshead speed typically between 0.5 and 1.0 mm/min (Fig. 4). The bond strength is then calculated by dividing the maximum load (in Newtons) by the bonded area (in mm^2), yielding a value expressed in Megapascals (MPa) (Fig.5). Sano et al. emphasized several methodological improvements over the years, such as the "non-trimming" technique, which avoids mechanical damage to the specimen during preparation. They also discussed the influence of variables such as dentin depth, tubule orientation, and residual dentin thickness on measured bond

strengths. The μ TBS test has since been adapted for use on different substrates, including sound, caries-affected, and sclerotic dentin, as well as root canal dentin.

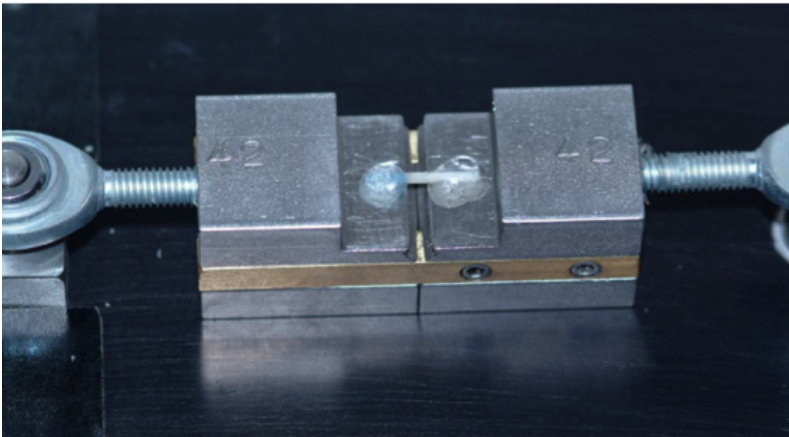


Fig. 4. The sticks are glued to the metallic support, taking care to not touch the adhesive interface that is located in the center as to be stressed with the test.



Fig. 5. Measurement of the stick dimensions using a digital caliper.

The μ TBS test offers several important advantages over traditional macrotensile or shear methods:

- High discriminative power: it can detect subtle differences in adhesive performance that may be masked in larger-area tests.
- Efficient sample use: multiple micro-specimens can be prepared from a single tooth, enhancing statistical significance and reducing biological variability.
- More uniform stress distribution: the small bonding area minimizes stress concentration and reduces cohesive failures within the substrate.

- Correlative potential: the test can be combined with morphological or chemical analyses (e.g., SEM, TEM, nano-leakage studies) to better understand failure mechanisms at the interface.

Despite its advantages, the μ TBS test also presents several technical challenges. The preparation of micro-specimens is labor-intensive and requires precise cutting to avoid premature failures. Specimen handling and fixation in the testing device can also introduce variability. Furthermore, environmental conditions such as humidity and temperature, as well as differences in loading rate, can influence the results. For these reasons, Sano et al. recommend meticulous standardization of experimental conditions and cross-validation with complementary analytical techniques (Sano et al. 1994).

Application and Clinical Relevance

The μ TBS test has been widely used to evaluate the performance of new generations of UAs, which aim to simplify bonding protocols without compromising durability. It allows researchers to assess the bond strength after various pre-treatments (such as exposure to endodontic irrigants or IDS) and after aging procedures like thermocycling or prolonged water storage. The test is also essential for evaluating how bioactive materials or desensitizing agents (e.g., Gluma or HEMA-based primers) interact with dentin collagen and influence hybrid layer integrity. Long-term studies using μ TBS testing have shown that bond strength decreases over time, mainly due to hydrolytic degradation and enzymatic activity within the HL. Therefore, μ TBS testing remains a cornerstone for investigating the durability of adhesive interfaces and the potential of innovative strategies, such as MMP inhibitors, chelating agents, or bioactive monomers, in order to stabilize the resin-dentin bond.

The μ TBS test is a precise and reliable method for evaluating adhesive performance at the microscopic level. Its historical evolution, methodological flexibility, and sensitivity make it one of the most widely adopted techniques in dental materials research. As highlighted by Sano et al. (2019), μ TBS testing continues to provide valuable insights into the behavior of adhesives under different clinical and laboratory conditions, contributing significantly to the development of more durable and effective universal adhesive systems.

SHEAR BOND STRENGTH TEST: Principles, Applications, and Relevance in Adhesive Dentistry

The Shear Bond Strength (SBS) test is one of the most conventional and extensively used mechanical methods for evaluating the adhesive performance between restorative materials and dental hard tissues. The test measures the maximum shear stress that an adhesive interface can withstand before failure when subjected to a force applied parallel to the bonded surface. Although it has been gradually complemented by more refined techniques such as the μ TBS test, the SBS test remains a useful and practical tool for preliminary screening of adhesive systems and bonding protocols.

The SBS test has been used for decades as a standard approach to evaluate the bond strength of dental adhesives, cements, and composite materials to enamel and dentin. Its popularity is due to its simplicity, relatively fast execution, and minimal equipment requirements. The method was initially developed to provide a straightforward measurement of the adhesive interface's mechanical integrity; however, subsequent studies have demonstrated that the test is strongly influenced by factors such as specimen geometry, loading configuration, and stress distribution within the bonded area (Sirisha et al., 2014). Despite these inherent limitations, the SBS test remains valuable for comparing materials under standardized conditions. In adhesive dentistry, it has been widely used to evaluate the initial bond strength of new universal adhesive systems, before and after aging, and under different bonding strategies (SE, ER, or SEE).

In a typical SBS test, the flat surface of enamel or dentin is prepared and treated with the adhesive system according to the manufacturer's instructions. A resin composite is then applied onto the bonded surface through a cylindrical mold, defining a bonding area typically ranging from 2 to 3 mm in diameter. After polymerization and storage (or aging, if applicable), the specimen is mounted in a universal testing machine. A chisel-shaped or knife-edge blade applies a load parallel to the bonding interface at a controlled crosshead speed (usually 0.5–1.0 mm/min) until failure occurs (Fig.6). The maximum load at failure (N) is recorded and divided by the bonded area (mm²), yielding the bond strength value expressed in megapascals (MPa). The fractured surface is often examined under stereomicroscopy or scanning electron microscopy (SEM) to determine the failure mode (adhesive, cohesive, or mixed).

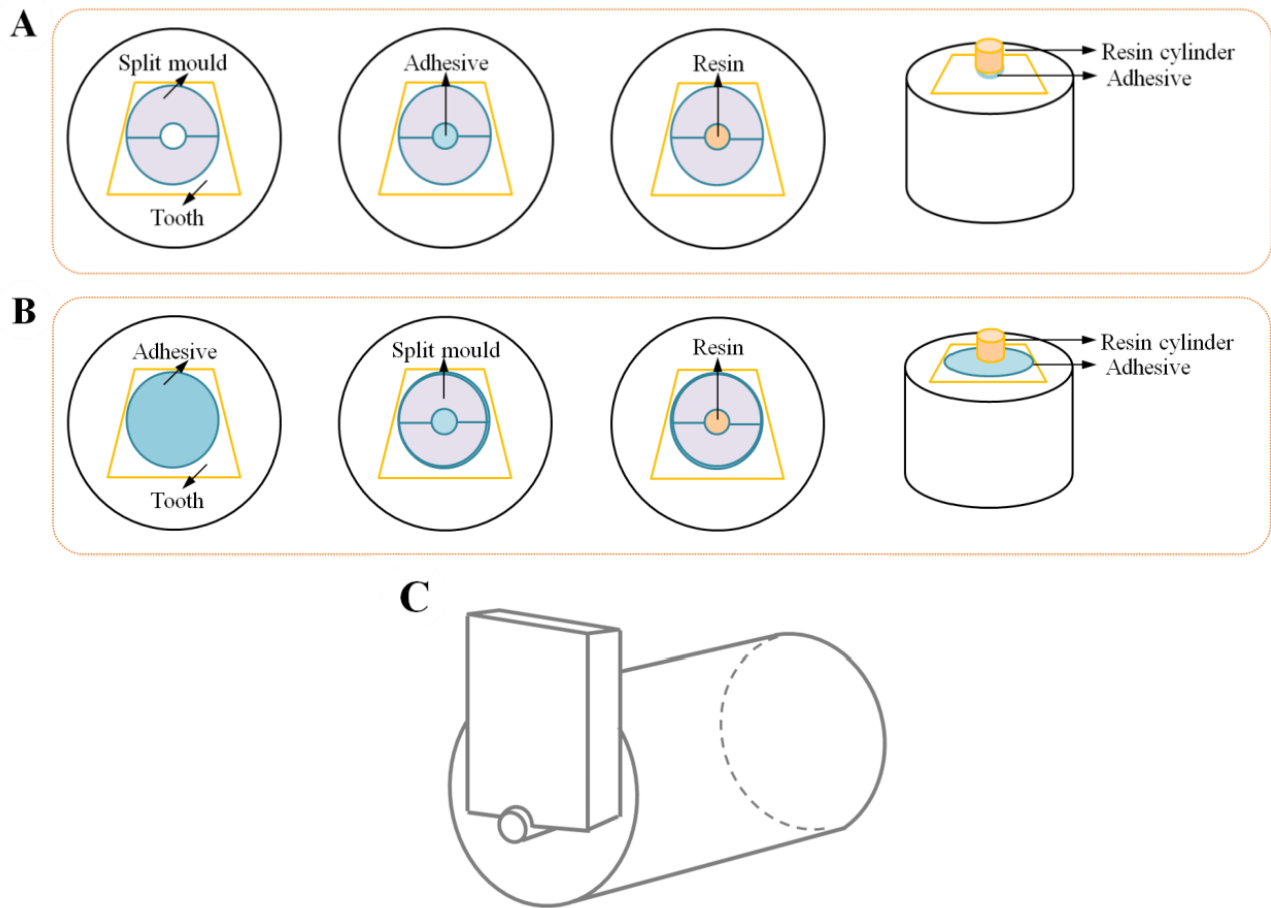


Fig. 6. Schematic representation of the shear bond strength test.

A more refined variation, the micro-shear bond strength (μ SBS) test, was later introduced to improve stress distribution and reduce premature substrate failures by using smaller bonded areas (often less than 1 mm²). This modification allows testing multiple specimens from a single tooth and increases the method's sensitivity to detect small differences between adhesive systems. The SBS and μ SBS test offer several important advantages:

- Simplicity and practicality: The test is easy to perform, requires limited specimen preparation, and can be conducted with basic mechanical testing equipment.
- High throughput: Multiple samples can be tested efficiently, allowing direct comparison among different adhesives or surface treatments.
- Adaptability: The SBS test can be applied to both enamel and dentin, and under various conditions (e.g., before and after thermocycling, on sound or sclerotic dentin, after exposure to endodontic irrigants).

- Educational and screening value: It remains an excellent screening tool for identifying promising adhesive systems before performing more complex evaluations like μ TBS or fatigue testing.

Despite its wide use, the SBS test has important methodological limitations. The primary issue lies in the non-uniform stress distribution at the adhesive interface. Finite element analyses have shown that stresses are concentrated near the loading edge rather than being evenly distributed, resulting in mixed stress patterns (shear, tensile, and compressive components). Consequently, fractures may occur within the substrate or composite rather than at the true adhesive interface, leading to over- or underestimation of bond strength values (El Mourad, 2018). Other sources of variability include specimen alignment, blade geometry, crosshead speed, and the mechanical properties of the substrate itself. Moreover, the SBS test is not ideal for evaluating the interfacial integrity of small or irregular bonding areas, such as root dentin or cervical lesions. For these cases, the μ TBS test provides a more accurate assessment.

Applications in Universal Adhesive Research

In recent years, the SBS test has been employed to evaluate the performance of universal adhesives, particularly in studies assessing the influence of pre-treatments or environmental factors on immediate bond strength. It has been used to compare the effects of endodontic irrigants, chlorhexidine application, and bioactive agents on adhesive performance, as well as to test the effectiveness of different bonding strategies (ER vs. SE). Furthermore, SBS testing is often conducted both before and after aging procedures to evaluate the short-term stability of the adhesive interface. These tests provide useful preliminary insights into how universal adhesives behave under clinical-like conditions, even though they do not fully simulate the complex degradation mechanisms that occur in the oral environment.

While the SBS test remains an important method for screening adhesive systems, it is generally considered less precise and less discriminative than the μ TBS test. The μ TBS method allows a more uniform stress distribution and provides data on regional variations in adhesion, making it more suitable for mechanistic and durability studies. However, due to its simplicity and reproducibility, the SBS test is still widely accepted for initial comparative analyses of adhesives, including those based on novel chemistries or universal formulations. The SBS test continues to play a significant role in dental adhesive research as a fast, practical, and informative method for assessing interfacial adhesion. Although it has limitations in terms of stress distribution and

accuracy, its ease of use and versatility make it a valuable preliminary tool in the development and evaluation of modern universal adhesives. For comprehensive understanding, SBS results should be interpreted alongside complementary methods such as μ TBS testing, nanoleakage evaluation, and aging simulations, to better predict long-term clinical performance.

IN SITU ZYMOGRAPHY: Principles, Application, and Significance in dentin-adhesive interface studies

The *in situ* zymography technique is a highly sensitive microscopic method used to visualize and quantify the activity of endogenous proteolytic enzymes, mainly MMP and CTs, within dental tissues. In adhesive dentistry, this method is particularly valuable for assessing the enzymatic degradation of the HL over time, a key factor affecting the long-term durability of the resin-dentin bond (Mazzoni et al., 2013; Breschi et al., 2018).

In situ zymography is based on the hydrolysis of a fluorescently labeled gelatin substrate, such as fluorescein-conjugated DQ™ gelatin. This substrate is initially non-fluorescent; however, when cleaved by active proteases, it releases fluorescent fragments that emit a measurable signal. The distribution and intensity of this fluorescence reflect the location and relative activity of MMPs and cathepsins within the sample. The analysis is typically performed using confocal laser scanning microscopy (CLSM), which enables high-resolution visualization of enzyme activity within the adhesive-tooth interface. This approach allows researchers to directly observe the degradation patterns in the HL, dentin tubules, and peritubular regions (Mazzoni et al., 2009; Gu et al., 2018).

After preparation of dentin-adhesive specimens, the bonded interface is sectioned into thin slabs (usually 1-2 mm thick) (Fig.7). The samples are incubated in a humid chamber with the DQ™ gelatin solution for a defined period, typically ranging from 12 to 24 hours at 37°C. After incubation, the specimens are rinsed and examined under CLSM using excitation/emission wavelengths of approximately 488/530 nm. Fluorescent areas indicate active enzymatic degradation. The fluorescence intensity is often quantified using image analysis software, providing a semi-quantitative assessment of proteolytic activity. Comparisons between experimental groups (e.g., different adhesive systems, dentin pre-treatments, or aging conditions) allow for the evaluation of how specific variables influence enzyme activation and HL stability.

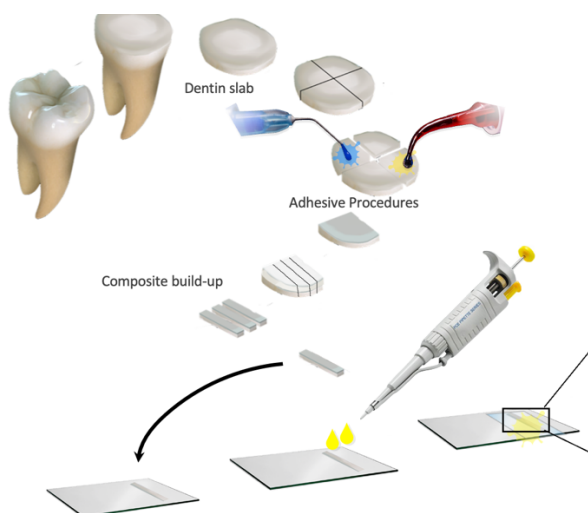


Fig. 7. Representative scheme of sample preparation for in situ zymography.

Applications in Adhesive Dentistry

The activation of endogenous proteases, particularly MMP-2 and MMP-9, has been identified as one of the main mechanisms responsible for the degradation of the hybrid layer in adhesive restorations (Tjäderhane et al., 2013; Mazzoni et al., 2015). *In situ* zymography provides a direct means of visualizing this process, allowing researchers to correlate enzymatic activity with bond strength loss, nanoleakage formation, and hybrid layer disorganization. This method has been extensively used to evaluate:

- The effectiveness of MMP inhibitors, such as chlorhexidine or quaternary ammonium methacrylates, in preserving collagen integrity.
- The influence of dentin conditioning protocols (e.g., phosphoric acid vs. chelating agents like EDTA or PAANs) on enzymatic activation.
- The impact of thermocycling and artificial aging on the reactivation of dentin proteases.
- The performance of bioactive and universal adhesives designed to remineralize or protect the HL from enzymatic degradation.

For instance, Gu et al. (2018) used *in situ* zymography to demonstrate that extrafibrillar demineralization using high-molecular-weight chelating agents significantly reduced enzymatic activity within the HL, correlating with enhanced bond durability. Similarly, Mazzoni et al. (2009, 2011) reported that both ER and SE adhesives can reactivate latent MMPs, highlighting the

importance of enzyme inhibition strategies for long-term adhesion. The *in situ* zymography offers several important advantages:

- Direct visualization of enzymatic activity at the microscopic level.
- High sensitivity and spatial resolution, allowing localization of protease activity within specific dentin regions.
- Non-destructive approach, preserving the structural integrity of the HL for complementary imaging (e.g., SEM or CLSM).
- Correlative potential with mechanical and chemical analyses, such as bond strength testing or nanoleakage studies.

Although *in situ* zymography provides valuable qualitative and semi-quantitative data, it does not measure absolute enzyme concentrations or kinetics. The fluorescence signal reflects the net enzymatic activity but can be influenced by experimental variables such as substrate penetration, incubation time, and sample thickness. Additionally, distinguishing between MMP and cathepsin activity may require the use of specific inhibitors during incubation (e.g., EDTA for MMPs, E-64 for cathepsins). Another limitation is that aging procedures, such as thermocycling or prolonged water storage, may affect enzyme activity by altering the collagen microenvironment. Therefore, careful experimental design and standardized protocols are essential for reproducibility (Mazzoni et al., 2013).

Relevance to Universal Adhesives

In the context of UAs, *in situ* zymography has become a powerful tool to study the biological stability of the resin-dentin interface. Universal adhesives contain acidic functional monomers (e.g., 10-MDP) that promote chemical bonding with hydroxyapatite but can also induce mild demineralization, potentially exposing collagen fibrils to enzymatic attack. By combining *in situ* zymography with mechanical testing, researchers can assess how new adhesive formulations, desensitizing agents (e.g., Gluma), or bioactive components influence enzyme suppression and HL preservation. The technique thus bridges morphological, biochemical, and mechanical perspectives in adhesive research, offering a comprehensive understanding of HL degradation mechanisms.

In situ zymography represents a crucial analytical method for evaluating the enzymatic stability of the adhesive interface. It enables direct visualization of MMP and cathepsin activity and

provides meaningful insights into the degradation dynamics of the HL. When used alongside bond strength testing and microscopic imaging, it contributes to a holistic understanding of resin-dentin bond durability, particularly in the development and optimization of next-generation UAs and bioactive adhesives.

OTHER ANALYTICAL METHODOLOGIES: GELATIN ZYMOGRAPHY AND NANOLEAKAGE ANALYSIS

Gelatin zymography is an electrophoretic technique used to assess the enzymatic activity of MMPs, particularly MMP-2 and MMP-9, within demineralized dentin or protein extracts. This method allows for the qualitative and semi-quantitative evaluation of both the pro- and active forms of these enzymes, which play a pivotal role in collagen degradation at the resin-dentine interface. The procedure involves extracting total proteins from powdered dentine previously treated with experimental or control materials. Protein extracts are separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions using gels containing fluorescently labelled gelatin as the substrate. Following electrophoresis, gels are incubated in an activation buffer (typically Tris-HCl with CaCl_2) to allow enzymatic digestion of the embedded gelatin. The resulting lytic bands, corresponding to areas of substrate degradation, indicate the presence and relative activity of gelatinolytic enzymes. The bands are visualized under ultraviolet light and quantified using digital image analysis software (e.g., ImageJ). This technique enables the differentiation between the latent (pro-) and active forms of MMPs, providing insights into the inhibitory or activating effects of experimental treatments on endogenous dentinal proteases. In adhesive dentistry, gelatin zymography has been widely employed to evaluate how bioactive or remineralizing systems can reduce enzymatic degradation of the HL and prolong the longevity of adhesive bonds (Mazzoni et al., 2013; Chou et al., 2025).

Nanoleakage analysis is an optical or electron microscopic method used to assess the ultrastructural integrity and permeability of the resin-dentin interface. It visualizes fluid penetration through nanometric voids or channels within the HL and adhesive layer, which are associated with hydrolytic degradation and reduced bond durability. Specimens are typically prepared by restoring dentin surfaces with adhesives labelled with a fluorescent dye such as Rhodamine B and subsequently immersing them in a Lucifer Yellow solution. This tracer penetrates nanoscale defects or water-filled pathways along the adhesive interface. Confocal laser scanning microscopy (CLSM) is then employed to generate optical sections and three-dimensional reconstructions of the interface. The fluorescence patterns allow differentiation between sound,

gap-free hybrid layers and degraded, dye-permeable regions. The extent of dye infiltration provides a semi-quantitative indication of the interface's permeability and structural stability. This method is often used in combination with aging protocols such as thermocycling to simulate clinical degradation and to compare the protective effects of different adhesive systems or bioactive treatments (Pashley et al., 2011; Chou et al., 2025).

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LABORATORY STUDIES ON ADHESION, ENZYMATIC DEGRADATION, AND HYBRID LAYER PRESERVATION

Objectives

This chapter presents four *in vitro* studies, designed to investigate different strategies aimed at improving the durability and stability of the resin-dentin bond. Despite the continuous evolution of adhesive systems, the degradation of the HL remains a major limitation for long-term restoration success. Therefore, the experimental work focused on the analysis of mechanical performance, morphological characteristics, and enzymatic activity at the adhesive interface under different clinical and material-related conditions.

The first study evaluated the effect of various dentin treatments and adhesive strategies on and enzymatic activity, combining mechanical testing (μ TBS), scanning electron microscopy (SEM), and *in situ* zymography to correlate structural integrity with enzymatic inhibition.

The second study further explored the influence of adhesive application protocols and surface treatments on the morphology and stability of the HL. Using similar methodologies (μ TBS, SEM, and *in situ* zymography), it aimed to identify the optimal conditions for preserving collagen integrity and limiting matrix metalloproteinase (MMP) activation.

The third study assessed the immediate and aged bonding performance of different adhesives through micro-shear bond strength tests (μ SBS) and *in situ* zymographic analysis. The focus was placed on quantifying the enzymatic degradation occurring at the bonded interface, providing insight into the mechanisms underlying bond failure over time.

Finally, the fourth study investigated the performance of experimental bioactive materials and their potential to inhibit enzymatic activity while promoting interface remineralization. Through a multimodal approach - including μ TBS, field emission SEM, confocal microscopy, *in situ* and gelatine zymography - this research provided a comprehensive understanding of how biofunctional materials can interact with dentin to reinforce the hybrid layer and prolong adhesive longevity.

Together, these four studies form a cohesive body of research that bridges the gap between fundamental mechanisms of dentin bonding and the development of innovative, bioactive adhesive systems. Their combined findings contribute to the understanding of how chemical composition,

substrate condition, and biological activity determine the long-term success of adhesive restorations

STUDY 1

Glutaraldehyde-based desensitizers' influence on bonding performances and dentin enzymatic activity of universal adhesives

Objectives

The aim of this study was to evaluate the influence of two glutaraldehyde-based desensitizers differing in consistency and delivery form (L: GLUMA Desensitizer, Heraeus Kulzer and G: GLUMA Desensitizer PowerGel) prior to universal adhesives application on microtensile bond strength (μ TBS) to dentin and endogenous enzymatic activity (MMPs). The null hypotheses tested were that:

1) dentin pre-treatment with glutaraldehyde-based primers prior to adhesive procedures with two universal adhesives does not influence microtensile bond strength immediately or after aging;

2) dentin pre-treatment with glutaraldehyde-based primers prior to adhesive procedures with two universal adhesives does not influence endogenous dentinal enzymatic activity immediately or after aging;

3) there are no differences between two tested universal adhesives in terms of bond strength and enzymatic activity.

Materials and methods

Microtensile bond strength test (μ TBS)

Microtensile bond strength test (μ TBS): Forty-eight freshly extracted non-carious human molars were obtained from anonymous individuals following their informed consent under a protocol approved by the Ethical Committee of the University of Bologna, Italy (protocol N°: 71/2019/OSS/AUSLBO). Tooth crowns were removed with a low-speed diamond saw under water cooling (Microremet, Remet, Casalecchio di Reno, Italy) to expose enamel-free middle/deep coronal dentin. Cut dentin surfaces were examined with a stereoscopic microscope to ensure that they were devoid of defects and enamel remnants. A standardized smear layer was created on each dentin surface using #320- grit wet silicon carbide paper and water lubrication. In the experimental groups, prior to adhesive application, the dentin surfaces were pre-treated for 30 s with one of the following desensitizers available in 2 consistencies (Kulzer GmG, Hanau, Germany): the liquid

GLUMA Desensitizer (L) and the gel GLUMA Desensitizer Powergel (G). After application, the specimens were thoroughly water-rinsed as indicated by the manufacturer. In the control groups, no dentin pretreatment was performed. Then, two universal adhesives were employed for the bonding procedures in the SE mode: IBU - iBond universal (Kulzer), or AU - Adhese Universal (Ivoclar Vivadent, Schaan, Liechtenstein). In the end, the following groups were formed according to the desensitizer/adhesive combination (n = 8): 1) L/IBU; 2) G/IBU; 3) IBU (CTRL); 4) L/AU; 5) G/AU; 6 AU (CTRL). Complete details and instructions of the materials used in the study are presented in Table 1. After polymerization of the respective adhesive, a build-up was created (two 2 mm-thick layers) with a nanohybrid resin composite (Venus Pearl, Kulzer). Light curing of the adhesive resin and each layer of composite resin was performed for 20 s with a light-emitting diode (LED) curing light (ELIPAR™ DeepCure-S, 3 M, St Paul, MN, USA; light output > 1000 mW/cm² and wavelength 430–480 nm). The bonded specimens were serially sectioned to obtain sticks with ~0.9 mm × ~0.9 mm cross-sectional area, following the non-trimming technique of the microtensile bond strength test (μTBS). The exact dimension of the sticks was measured using a pair of digital calipers. Bond testing was performed after the sticks were aged in artificial saliva at 37 °C for 24 h (T0) or 12 months (T12). Each beam was stressed under tension to failure using a simplified bond testing machine (Shear Bond Tester; Bisco, Schaumburg, IL, USA) at a crosshead speed of 1 mm/min. The number of prematurely debonded specimens in each experimental group was recorded. Null bond strength values were not included in the statistical analysis because the number of prematurely debonded sticks did not exceed 3% of the total number of tested specimens and were similarly distributed within the groups (Maciel Pires et al. 2022). A single observer evaluated each side of the fractured sticks with a stereomicroscope at 50× magnification to determine the mode of failure. Failure was classified as adhesive at the dentin interface (A), cohesive in dentin (CD), cohesive in composite (CC) or mixed failure (M; adhesive and cohesive fractures occurred simultaneously).

Material	Composition	pH	Mode of use
Adhese Universal <i>Ivoclar Vivadent, Schaan, Liechtenstein</i>	MDP, MCAP, HEMA, Bis-GMA, D3MA, water, ethanol, highly dispersed silicon dioxide, initiators and stabilizers	2.5- 3	1. The adhesive is scrub on dentin for 20s; 2. Air-spray with oil- and moisture-free compressed air until a glossy, immobile film layer results; 3. Light-cure using a LED light-curing unit for 20s.
iBOND Universal <i>Kulzer GmgH, Hanau, Germany</i>	Phosphonic acid acrylate, HEMA, D3MA, highly dispersed silica, ethanol, catalysts, stabilizers, fluoride	1.6–1.8	1. The adhesive is scrub on dentin for 20s; 2. Disperse the adhesive with an oil- and moisture-free compressed air until a glossy, immobile film layer result; 3. Light-cure using a LED light-curing unit for 10s.

GLUMA Desensitizer <i>Kulzer GmgH, Hanau, Germany</i>	Purified water, (HEMA), glutardialdehyde,	3.66	1. Apply the desensitizer for 30 - 60s; 2. Thoroughly rinse off the desensitizer with water spray and dry with oil-free air; 3. Continue with the bonding procedures.
GLUMA Desensitizer Powergel <i>Kulzer GmgH, Hanau, Germany</i>	Purified water, (HEMA), glutardialdehyde, pyrogenic silicic	3.66	1. Apply the desensitizer for 30 - 60s; 2. Thoroughly rinse off the desensitizer with water spray and dry with oil-free air;
VENUS Pearl <i>Kulzer GmgH, Hanau, Germany</i>	Amorphous silica, triethylen glycol dimethacrylate, trimethoxysilylpropyl methacrylate, Phenyl 1,2-propandion, methyl methacrylate, acetic acid	Not defined	

Table 1. Chemical composition of the materials used in the study and bonding procedures.

Scanning Electron Microscope (SEM) examination

After μ TBS, two representative debonded sticks per group were selected (with a bond strength value close to the mean value of the group) and prepared for scanning electron microscopy (SEM) evaluation. Each specimen was fixated in a 2.5% glutaraldehyde 0.1 M cacodylate buffer (pH

7.4), dehydrated in ascending ethanol solutions (50%, 70%, 80%, 90%, 95% and 100%), and dried using hexamethyldisilazane. Then, they were mounted on aluminum specimen stubs, coated with 8÷10 nm gold particles and observed under a field-emission gun scanning electron microscope (FEG-SEM; Nova NanoSEM 450; FEI, Eindhoven, NL).

In situ zymography of resin-dentin interfaces

One-millimeter-thick slabs of middle/deep coronal dentin were obtained from four extracted human third molars using a low-speed saw (Micromet) under water-cooling. Two dentin slabs were obtained from each tooth. Each slab was further divided into 4 pieces so that testing of the six experimental groups was performed using the same dentin substrate. A standardized smear layer was created on each dentin surface using #600-grit silicon carbide paper under water cooling. Identical bonding procedures were performed as previously described for the μ TBS forming the same 6 groups ($n = 4$). Resin-dentin interfaces were exposed by cutting the bonded specimens vertically into 1 mm-thick sticks using the slow-speed saw under water cooling. The sticks were fixed to glass slides with cyanoacrylate glue ground down and polished to obtain $\sim 50 \mu\text{m}$ thick slabs using a series of wet silicon carbide papers. Self-quenched fluorescein-conjugated gelatin was used as the MMP substrate (E-12,055, Molecular Probes, Eugene, OR, USA) for in situ zymography at T0 and T12 (Mazzoni et al. 2013). The fluorescent gelatin mixture was placed on top of each slab and covered with a glass coverslip. The slides were incubated in a humidified chamber at 37 °C overnight. During incubation, the assemblies were prevented from direct contact with water and were protected from exposure to light. After incubation, the microscopic slides were examined using a confocal laser scanning microscope (laser excitation wavelength 488 nm; emission wavelength 530 nm; Model A1-R; Nikon, Tokyo, Japan). To visualize the hydrolysis of the quenched fluorescein-conjugated gelatin substrate as an indicator of endogenous gelatinolytic activity, 3 z-stack images ($\sim 15 \mu\text{m}$ thick) were made per specimen. The steps between the optical sections were of $1 \mu\text{m}$ and the z-stacks were made from the top of the specimen and into the depth of $\sim 15 \mu\text{m}$. The images were made in the center and towards the two ends of the specimen, always making sure that the distance from the enamel (where present) was at least 1 mm. Enzymatic activity was quantified as the integrated density of the fluorescence signals by means of the ImageJ software (National Institutes of Health, Bethesda, MD, USA) using a rectangular selection ($100 \times 20 \mu\text{m}$) placed over the hybrid layer and dentin (3 measurements per image). The quantification was performed after discarding several initial slices as to exclude parts of the specimen with the background noise in the signal attributed to the activated gelatin substrate on top of the specimen. After the removal of the initial slices also

the differential interference contrast (DIC) image reached maximum focus and this point was considered repeatable and ideally suited for the execution of the measurements in all the specimens.

Statistical analysis

Data sets obtained from bond strength testing and in situ zymography were first validated individually for their normality (Shapiro-Wilk test) and equality of variance (Brown-Forsythe test). For the μ TBS test, as data were normally and equally distributed, the three-way analysis of variance (ANOVA) was performed to identify the effects of the 3 independent variables involved in the testing: adhesive systems, dentin desensitizer and aging time. Post-hoc pairwise comparisons were conducted using the Tukey test. Regarding the in situ zymography results, since data were not normally distributed (Shapiro-Wilk test, $P < 0.05$), they were analyzed using the Kruskal-Wallis test and a Pairwise Multiple Comparison Procedures (Dunn's Method). All analyses were performed using a statistical software (Sigmaplot v.14.0.; StataCorp LLC, College Station, TX, USA) and the statistical significance was preset at $\alpha = 0.05$.

Results

Microtensile bond strength test (μ TBS)

Mean μ TBS results and standard deviations of the experimental groups tested at baseline and after 1 year of artificial storage are presented in Table 2. Statistical analysis revealed that dentin pre-treatment ($F = 20.131$; $P < 0.001$), the type of adhesive ($F = 100.587$; $P < 0.001$) and aging ($F = 25.905$; $P < 0.001$) significantly influenced the results. The interaction between the adhesive and aging was also significant ($F = 3.931$; $P = 0.048$). AU showed higher bond strength compared with IBU ($P < 0.001$). At baseline, specimens pre-treated with L resulted in higher bond strength compared to G ($P < 0.001$) and control group ($P < 0.001$). No statistically significant differences were found between G and the control groups ($P = 0.556$). Laboratory aging statistically decreased bonding values, irrespective of the experimental group ($P < 0.001$). After 1 year of storage in artificial saliva no differences in bond strength were observed between L and G regardless of the adhesive ($P = 0.001$). However, pre-treating dentin with the two desensitizers resulted in higher bond strength when compared to the controls, independent of the adhesive product used for bonding procedures ($P < 0.001$). Table 3 summarizes the percentage distribution of failure modes identified after μ TBS test at T0 and T12. A predominance of CC failures, followed by the A ones was observed in all groups, irrespective of the adhesive and dentin pre-treatment. After storage, the percentage of the A failure mode decreased and the CC failures further increased for pre-treated groups (irrespective of

whether it was L or G), while the control groups did not change tendency after aging (independent of the type of adhesive). A certain percentage of CD and M fractures were observed among groups, with an equal distribution independent of the adhesive, dentin pretreatment and aging.

Densensitizer/Adhesive	T ₀ (MPa) [†]	T ₁₂ (MPa) [†]
L/IBU	24.03 ± 7.43 ^{c,A}	24.37 ± 10.70 ^{a,b,A}
G/IBU	23.05 ± 14.26 ^{c,A}	18.57 ± 10.67 ^{b,A}
IBUCTRL	20.59 ± 6.86 ^{c,A}	16.59 ± 7.76 ^{b,A}
L/AU	43.16 ± 10.51 ^{a,A}	30.41 ± 9.55 ^{a,B}
G/AU	32.86 ± 1.63 ^{b,A}	27.54 ± 14.95 ^{a,A}
AUCTRL	31.50 ± 11.27 ^{b,A}	24.51 ± 11.72 ^{a,b,A}

Table 2. Summary of microtensile bond strength results obtained from the six experimental groups immediately (T₀) and after 12 months of laboratory aging (T₁₂). Abbreviations: L - liquid desensitizing agent GLUMA Desensitizer; G - desensitizing agent GLUMA Desensitizer Powergel; IBU - iBond Universal; AU - Adhese Universal; CTRL - Control: no dentin pretreatment. Different lower-case letters indicate significant differences within the same column (p < 0.05). Different upper-case letters indicate significant differences within the same row (p < 0.05). [†] Values are means ± standard deviations, in megaPascals (MPa).

	Failure mode (%)							
	T ₀				T ₁₂			
	A	CC	CD	M	A	CC	CD	M
L/IBU	44%	49%	5%	2%	32%	61%	2%	5%
G/IBU	38%	51%	8%	3%	32%	57%	0%	11%
IBUCTRL	21%	64%	8%	8%	22%	63%	9%	6%
L/AU	34%	32%	19%	15%	18%	42%	22%	18%
G/AU	28%	51%	6%	15%	26%	60%	9%	5%
AUCTRL	25%	68%	3%	5%	19%	67%	10%	5%

Table 3. Failure mode distributions and their percentages identified among the experimental groups after bond strength testing at baseline (T₀) and 1 yr of laboratory storage in artificial saliva (T₁₂). Failure modes: A - adhesive at the dentin interface; CC - cohesive in composite; CD - cohesive in dentin; M - mixed.

Scanning Electron Microscope (SEM) examinations

Representative SEM images at T₀ and T₁₂ are shown in Fig. 1 and 2 respectively (magnifications 200x, 2000x and 5000x). SEM analysis showed the presence of smear layer when no dentin pretreatment was performed (control groups in Figs 1 C and G and Figs 2 C and G), especially when compared to the experimental groups where the tubules orifices were mostly visible and wide

(Figs 1 A, B, D, E and Figs 2 A, B, D, E). These situations were observed both at baseline (Fig. 1) and after laboratory storage (Fig. 2). Remnants of resin composite bulk were observed in all the experimental groups at baseline showing, however, rough and untight surfaces cautiously indicating mixed failures (Fig. 1). Higher concentration of dentinal tubules with larger amount of impregnated resin tags were observed at T12 for AU when compared to IBU, irrespective of the desensitizer used (Fig 2 D and E). At the same timepoint, IBU revealed a softer and plastic appearance with sparsely distributed bubble formations when it was used after application of the liquid desensitizer or alone (CTRL group) (Fig. 1 A and C, respectively). This conformation was only occasionally present when IBU was used after the application of G (Fig. 2 B).

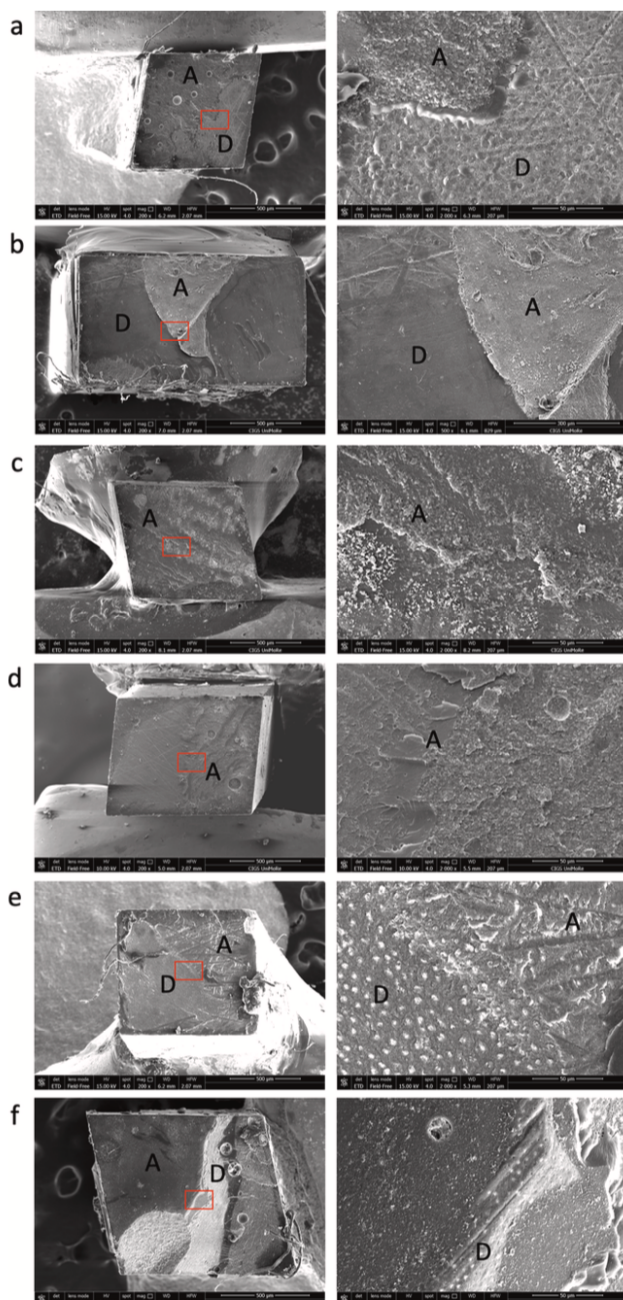


Fig. 1. Representative field emission scanning electron microscope micrographs of the adhesive interfaces of fractured microtensile bond strength sticks (the dentin side) at baseline (T0): left - view of the whole adhesive surface; right - enlarged view of the area marked with the red selection. Magnification: 200 x, left; 2000 x, right. (a) L/IBU: mixed failure with the presence of remnants of resin composite. (b) G/IBU: mixed debondings with the present of resin composite still attached on the dentin surface. (c) IBUCTRL: mixed failure, the presence of soft-appearance material was noted over the dentin surface. (d) L/AU: this group showed the highest percentage of mixed failures among groups at baseline. (e) G/AU: mixed failure, with the presence of sparsely distributed resin tags occluding dentinal tubules. (f) AUCTRL: mixed failures. A = adhesive resin; D = dentin.

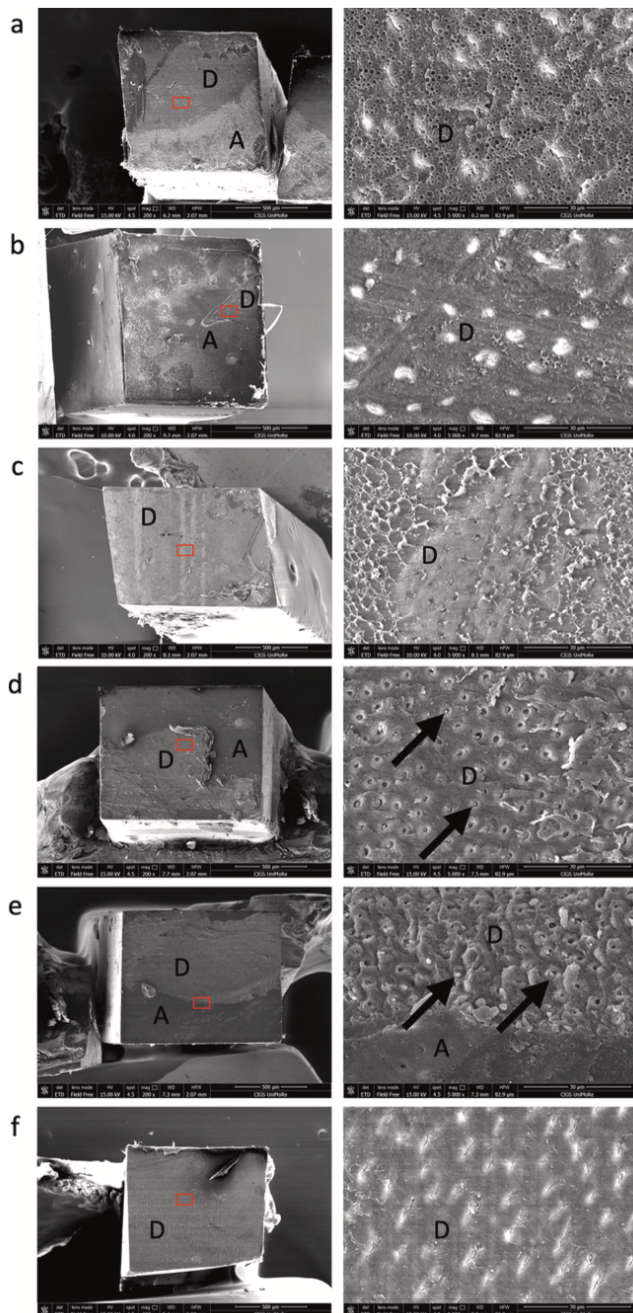


Fig. 2. Representative field emission scanning electron microscope micrographs of the adhesive interfaces of aged (T12) fractured microtensile bond strength sticks (the dentin side): left, view of the whole adhesive surface; right, enlarged view of the area marked with the red selection. Magnification: 200 x, left; 5000 x, right. (a) L/IBU: mixed fracture, with the presence of a porous with intermittent bubbles cross overing the resin. (b) G/IBU: mixed failure with some resin tags and sparsely distributed soft resin remnants. (c) IBUCTRL: mixed fracture with some opened dentinal tubules with no evident resin penetration. The surface was also characterized by the presence of plastic filaments, rendering the surface rough and irregular. (d) L/AU: mixed failure showing exposed dentinal tubules with thin extruding resin tags formations. (e) G/AU: mixed failure with the presence of opened dentinal tubules intermittently occluded by thin and sparse resin impregnation. (f) AUCTRL: mixed failure with the presence of smear layer on the dentin surface occluding the underneath tubule orifices. A = adhesive resin; D = dentin; Black arrow = resin tags in the dentinal tubule.

In situ zymography

Representative confocal images of the tested groups are shown in Figs. 3 and 4 for time T0 and T12, respectively. The percentages of HLs exhibiting hydrolysis of the quenched fluorescein-conjugated gelatin at T0 and T12 are shown in Fig. 5. The green fluorescence signals at T0 identified from the AU control group were statistically lower compared to those exhibited by the L/AU group ($P < 0,001$), whilst G/IBU showed statistically higher levels of fluorescence than IBU (CTRL) group ($P = 0,022$). In all the tested groups, the fluorescence density level was lower after aging. At T12 all the groups tested showed comparable endogenous enzymatic activity irrespective of the adhesive and the desensitizing agent used ($P > 0.05$).

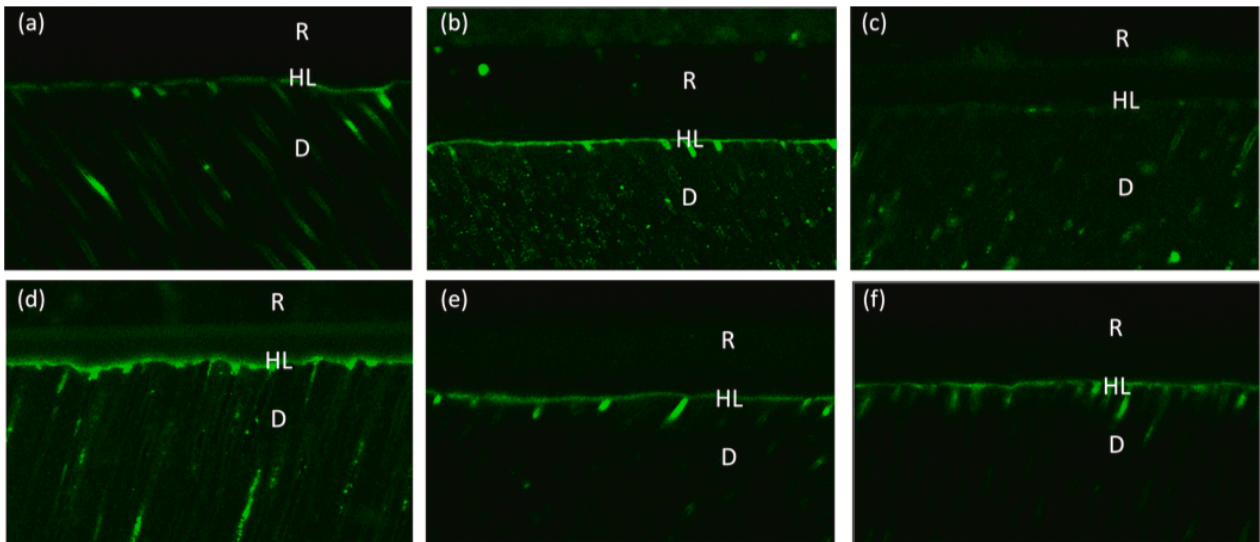


Fig. 3. Resin-bonded mid-coronal dentin interfaces prepared with L/IBU (a) G/IBU (b), IBUCTRL (c), L/AU (d), G/AU (e) and AUCTRL (f) at T0, incubated with quenched fluorescein-labeled gelatin. All the images were acquired in green channel, showing fluorescence (identifying intense endogenous enzymatic activity) in dentinal tubules and within the HL. D = Dentin; HL = Hybrid Layer; R = Resin Composite.

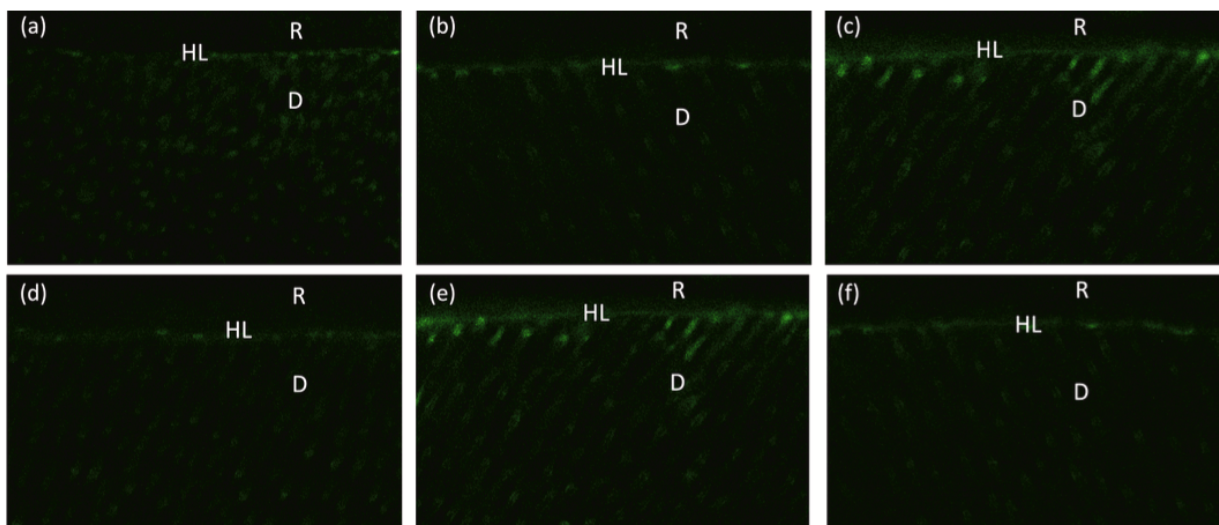


Fig. 4. Resin-bonded mid-coronal dentin interfaces prepared with L/IBU (a) G/IBU (b), IBUCTRL (c), L/AU (d), G/AU (e) and AUCTRL (f) at T12, incubated with quenched fluorescein-labeled gelatin. All the images are acquired in green channel, showing fluorescence (identifying intense endogenous enzymatic activity) in dentinal tubules and within the HL. D = Dentin; HL = Hybrid Layer; R = Resin Composite.

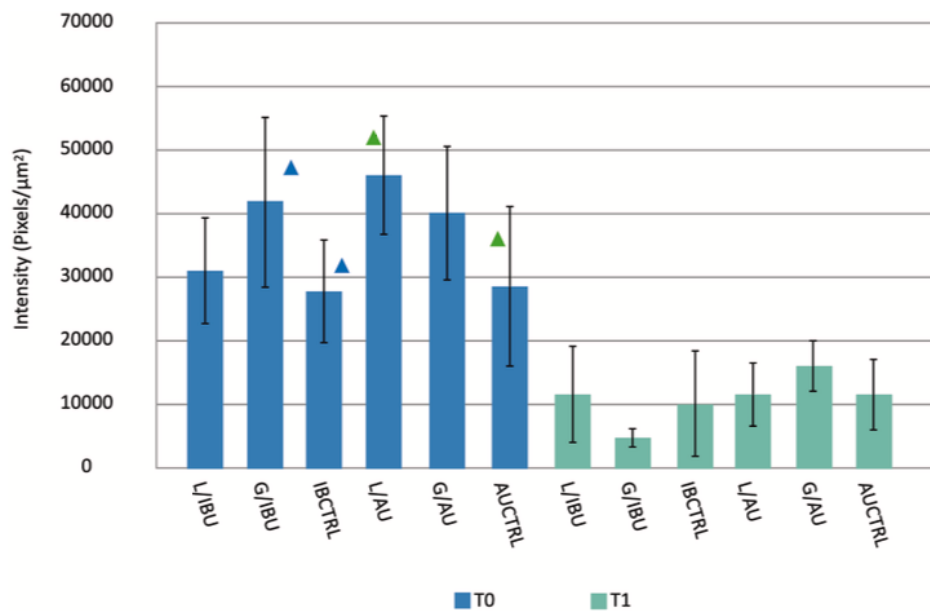


Fig. 5. Quantification (Pixels/μm²) of the fluorescence obtained in the different groups at T0 and T12 by means of in situ zymography showing reduced enzymatic activity in all T12 groups irrespective from the adhesive and desensitizing agent used.

STUDY 2

The effect of Immediate Dentin Sealing on enzymatic activity and bond-strength of hybrid ceramic CAD/CAM onlays: a comparative study of two universal adhesives

Objective

To elucidate the effect of immediate dentin sealing (IDS) with two universal adhesive systems on microtensile bond-strength (μ TBS) and enzymatic activity (MMPs) of CAD/CAM hybrid ceramic onlays. This laboratory study tested the subsequent research hypotheses:

- 1) different universal adhesives used for IDS technique would improve bonding performance of CAD/CAM hybrid ceramic onlays luted with a silane-containing universal cement immediately and after artificial aging;
- 2) IDS performed with universal adhesives would have a different influence on MMPs activity immediately and after artificial aging.

Materials and methods

Specimen preparation

The specimen preparation for bond-strength testing was carried out by the same operator according to the guidelines of the Academy of Dental Materials (ADM) (Armstrong et al. 2017). Freshly extracted intact human molars obtained from healthy adult donors after their informed consent and University Ethical Committee Approval (protocol N°: 71/2019/OSS/AUSLBO) were used in the study. The middle-deep dentin was exposed by cutting the teeth perpendicularly to their long axis using a low-speed diamond saw (Microremet, Remet, Casalecchio di Reno, Italy) under water cooling. Subsequently, a standardized smear layer was created on the exposed dentin surface with #600-grit wet silicon-carbide (SiC) paper (Sebold et al. 2022). The specimens were water rinsed and gently dried with oil-free air flow for 5 s being careful not to desiccate the dentin surface. Specimens were then randomly assigned to the following groups (n=8 per group, as suggested by the ADM guidelines (Armstrong et al. 2017)), according to the adhesive used for IDS:

Group IDS QB: Clearfil Universal Bond Quick (Kuraray, Noritake, Tokyo, Japan);

Group IDS SB: Scotchbond Universal Plus (3M, Seefeld, Germany);

Group CTR: Control group (no IDS was performed).

In Groups IDS QB and IDS SB, the adhesives were applied on dentin surfaces in SE mode and polymerized with a light emitting diode lamp (LED; 1,470 mW/cm², wavelength: 430-480 nm; Elipar Deep cure, 3M, St. Paul, MN, USA), according to manufacturers' instructions. Next, the sealed dentin surface was covered with a glycerin gel and light-cured for additional 10 s. The gel was then removed using a cotton pellet soaked with 70% ethyl alcohol (Pheerarangsikul et al. 2022). A 3 mm thick temporary restoration (Cavition, GC, Tokyo, Japan) was then placed on the dentin surfaces in all groups, and the specimens were stored in artificial saliva at 37°C for 1 week. The detailed composition of the materials used, and their application mode can be seen in Table 1. Following the provisional phase, the material was mechanically removed using a dental explorer and the dentin surfaces were cleaned with pumice-water slurry for 5 s at 500 rpm under 3.5x magnifying loupes. To simulate definitive cementation, 3 mm thick CAD/CAM hybrid ceramic onlays (Katana AVENCIA Block, Kuraray, Tokyo, Japan) were air-abraded (0.2 MPa, 30 µm Al₂O₃) and cleaned in an ultrasonic bath (ethanol and water, 1:1 ratio) for 1 min. Next, the onlays were cemented using a silane-containing universal composite cement (PANAVIA SA Cement Universal, Kuraray, Tokyo, Japan) in the self-adhesive mode under standardized seating pressure (Mancuso et al. 2022). The cemented onlays were tack-cured for 2 s and the excess cement was removed using a dental explorer. After the final resin cement light-curing for additional 40 s (Elipar Deep cure, 3M), the specimens were placed in artificial saliva and stored in an incubator at 37 °C for 24 h.

Microtensile bond-strength (µTBS) testing

After storage, the specimens were serially sectioned to obtain approximately 1-mm-thick sticks in accordance with the non-trimming technique of the microtensile bond-strength (µTBS) test and stored in artificial saliva at 37 °C for 24 h. Afterwards, half of the specimens were subjected to µTBS testing after 24 h (T₀), while the other half was subjected to artificial aging in thermocycling machine (T_t: 10.000 cycles, 5-55 °C, dwell time 30 s; SD-Mechatronik, Westerham, Germany). The dimension of each stick (ca. 0.9 mm × 0.9 mm × 8 mm) was measured using a digital caliper (± 0.01mm) and the bonded area was calculated for subsequent conversion of µTBS values in Newtons (N) into units of stress (MPa). The sticks were stressed to failure using a testing machine (Bisco, Schaumburg, IL, USA) at a crosshead speed of 1 mm/min. Failure mode analysis was performed using stereomicroscope at 40x magnification (Stemi 2000-C; Carl Zeiss Jena GmbH, Jena, Germany). The failures were classified as: Adhesive at the dentine/cement interface – AD, adhesive at the

CAD/CAM ceramic restoration/cement interface – AC, Cohesive - C (within dentin – CD, cement – CA or restoration – CC), Mixed (adhesive and cohesive failures occurred simultaneously – M).

Scanning electron microscope (SEM) analysis of the fractured interfaces

Representative fractured sticks from the μ TBS test (n=2 per group, with bond-strength values close to the group mean) were used for the scanning electron microscope (SEM) analysis. Briefly, the sticks were fixed in a 2.5% glutaraldehyde in 0.1% cacodylate buffer solution and subsequently dehydrated in ascending ethanol concentrations (50%, 70%, 80%, 90%, 95%, 100%) and HMDS. The fractured sticks were then mounted on metal stubs and gold-palladium sputter-coated before evaluation at different magnifications (50 x, 200 x, 1,000 x at accelerating voltage of 15.00 kV) (D'Alessandro et al. 2024).

In situ zymography analysis

The *in situ* zymography analysis was performed in accordance with a previous study (Mazzoni et al. 2012). The middle deep dentin of non-carious human molars (n= 3), based on a previous study (Maravic et al. 2021), was exposed using a low-speed diamond saw under water cooling. Next, the cementation procedures were performed according to the previously defined groups and the specimens were cut vertically into 1mm-thick slabs to expose the resin-dentin interface or the hybrid layer. Half of the specimens were processed immediately for *in situ* zymography analysis, while the other half was subjected to thermocycling procedures as described above. Each bonded slab with the exposed resin-dentin interface was glued to a microscope slide and ground down to obtain approximately 50 μ m-thick specimens. The *in situ* zymography was performed using quenched fluorescein-conjugated gelatin as the MMP substrate. The gelatin stock solution (diluted 1:8 with the dilution buffer and an anti-fading agent) was placed on top of each slab and covered with a coverslip. Slides were light-protected and incubated in a humidified chamber at 37°C. Hydrolysis of quenched fluorescein-conjugated gelatin substrate, indicative of endogenous gelatinolytic enzyme activity, was assessed by examination under a confocal microscope (Leica SP8, Leica Microsystems GmbH, Wetzlar, Germany; ex:488nm and em:lp530nm). The hydrolysis of the quenched fluorescein-conjugated gelatin substrate, indicative of MMPs' activity, was quantified as the integrated density of the fluorescence signals for each image using ImageJ software (National Institutes of Health, Bethesda, MD, USA) by one operator who was unaware of the group belonging.

Statistical analysis

Since the data from μ TBS test and *in situ* zymography analyses were not normally and homogeneously distributed (failed Kolmogorov–Smirnov and modified Levene’s tests, respectively, $p < 0.05$), the non-parametric Kruskal-Wallis and Dunn’s post-hoc tests were run. All the analyses were performed by a statistician blinded to the groups using SigmaPlot 14.0 (Systat Software, Chicago, IL, USA). The significance level was set at $\alpha = 0.05$.

Results

The results from the μ TBS test are presented in Table 2. The IDS QB group, which used a universal adhesive and resin cement from the same manufacture, showed higher strength at T_0 compared to control and IDS SB groups ($p < 0.001$). No statistically significant difference ($p = 0.138$) was observed between control group and IDS SB groups at T_0 . After thermocycling, the bond-strength significantly decreased ($p < 0.05$) in IDS QB and CTR groups. Both IDS groups demonstrated higher bond-strength values compared to the CTR group ($p < 0.05$) after thermocycling.

Representative SEM images (magnification 50x, 200x and 1000x, respectively) of the fractured specimens at T_0 and T_t are shown in Figures 1 and 2. In the CTR group, open dentinal tubules were clearly visible at T_0 , with very limited presence of resin composite cement. On the other hand, the dentinal tubules were partially (QB) or mostly occluded (SB) in the IDS groups, with some air bubbles present in the IDS QB group. After thermocycling, the morphology of the fractured specimens in the CTR group remained largely unchanged. Rough surface with some microcracks were visible in the IDS QB group, while no exposed tubules could be seen in either of the IDS groups.

Regarding the data retrieved from *in situ* zymography analysis, at T_0 , significant differences in the levels of enzymatic dentinal activity between the groups ($p < 0.05$) were observed in the following order: IDS QB > IDS SB > CTR. After thermocycling, IDS QB showed a higher level of enzymatic activity compared to the CTR ($p = 0.022$), while there were no other differences between the groups (Figure 3). Representative images of confocal laser scanning microscopy are presented in Figure 4. A more prominent fluorescence, alongside the hybrid layer and spreading towards dentinal tubules, was observed in the IDS QB group, while low signal was observed in the CTR group. In general, there was a tendency in reduction of fluorescence signal in all groups after thermocycling.

Material	Composition	Application protocol	Batch number
Clearfil Universal Bond Quick (Kuraray Noritake, Tokyo, Japan)	Bis-GMA ethanol HEMA 10-MDP Hydrophilic amide monomers Colloidal silica Silane coupling agent Sodium fluoride Camphorquinone Water	Apply in rubbing motion, no waiting, mildly air-dry (≥ 5 s) until the adhesive no longer moves. No light curing.	LOT 220289
Scotchbond Universal Plus Adhesive (3M, Seefeld, Germany)	Brominated dimethacrylate HEMA 10-MDP 2-Propenoic acid, 2-methyl-, 3-(triethoxysilyl) propylester, reaction products with silica and 3-(triethoxysilyl)- 1-propanamine Ethanol Water Synthetic amorphous silica, fumed, crystalline-free γMPTES Camphorquinone Copolymer of acrylic and itaconic acid N,n- dimethylbenzocaine APTES DEGDMA Acetic acid, copper(2+) salt, monohydrate	Apply a thin layer to the adhesive substrate with micro-tip applicator and rub for 20 s. Air-blow gently for 5 s. No light curing.	LOT 8101151
PANAVIA SA Cement Universal (Kuraray Noritake, Tokyo, Japan)	Paste A: 10-MDP, bis-GMA, TEG-DMA, hydrophobic aromatic dimethacrylate, HEMA, silanated barium glass filler, silanated colloidal silica, camphorquinone, peroxide, catalysts, pigments Paste B: hydrophobic aromatic dimethacrylate, silane coupling agent, silanated barium glass filler, aluminum oxide filler, surface treated sodium fluoride, camphorquinone, accelerators, pigments	Before the first use, let a small amount bleed out and discard it. Attach a mixing tip, extrude a small amount, then apply the material on the surfaces to be luted. Light-cure for 2 to 5 seconds or let self-cure for 2 to 4 minutes, then remove the excess cement.	LOT 830018

Table 1. Brand, dental manufacturer, chemical composition, application protocol and batch number of the materials used in this study.

Groups	T ₀	T _t
IDS QB	12.7 ± 6.0 ^{A, a} (88M/8AD/4AC/0C)	8.0 ± 3.6 ^{A, b} (96M/4AD/0AC/0C)
IDS SB	5.7 ± 0.8 ^{B, a} (88M/3AD/9AC/0C)	6.6 ± 0.6 ^{A, a} (94M/6AD/0AC/0C)
CTR	8.3 ± 4.5 ^{B, a} (73M/19AD/8AC/0C)	3.4 ± 1.6 ^{B, b} (83M/17AD/0AC/0C)

Table 2. Microtensile bond-strength values (MPa) with standard deviations after 24h of artificial saliva storage (T₀) and after 10,000 thermocycles (T_t). Different superscript lower letters indicate differences within the rows, different superscript upper letters indicate differences within the columns. The percentages of different failure modes are presented in parentheses under each respective bond-strength value. AD – adhesive at the dentin interface; AC – adhesive at the CAD/CAM ceramic restoration interface; cohesive – C (within dentin – CD, cement – CA or restoration – CC); M - mixed failure.

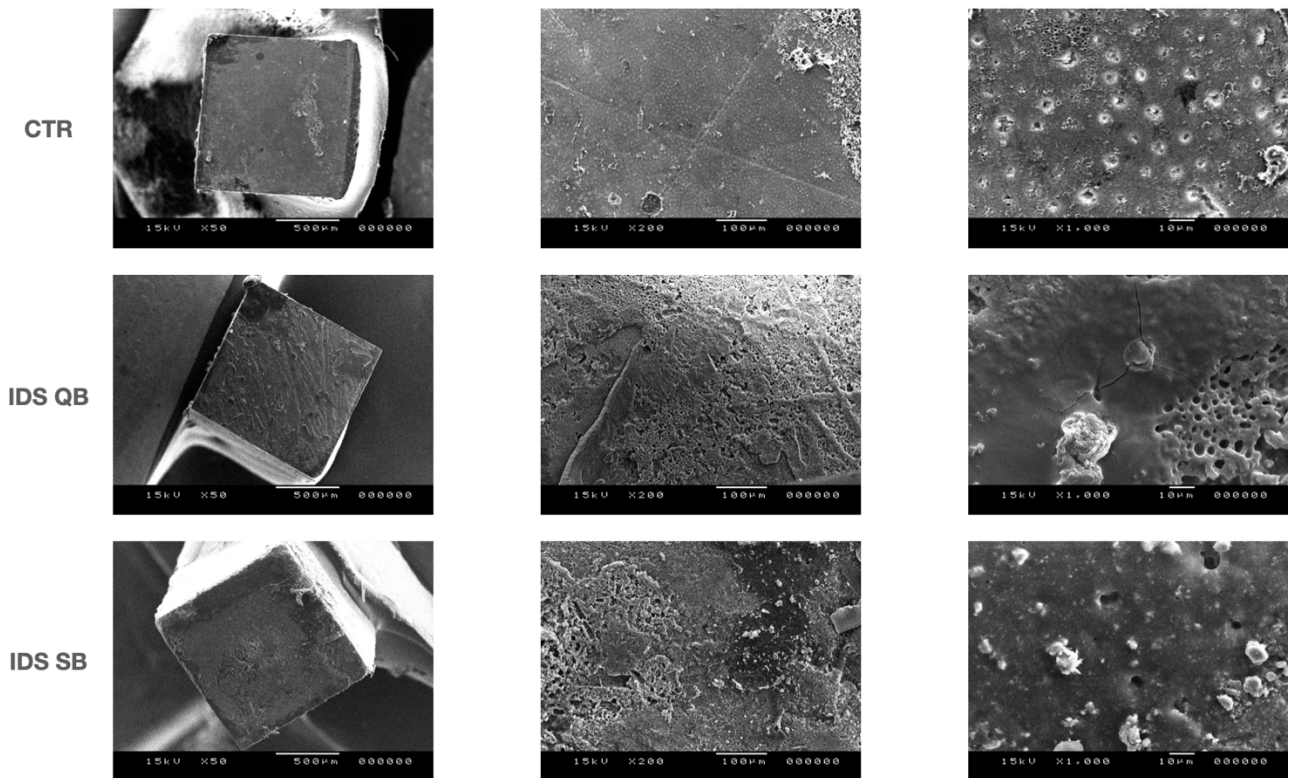


Fig. 1. Scanning electron microscopy microphotographs (50x, 200x and 1000x magnifications) of the fractured specimens at baseline.

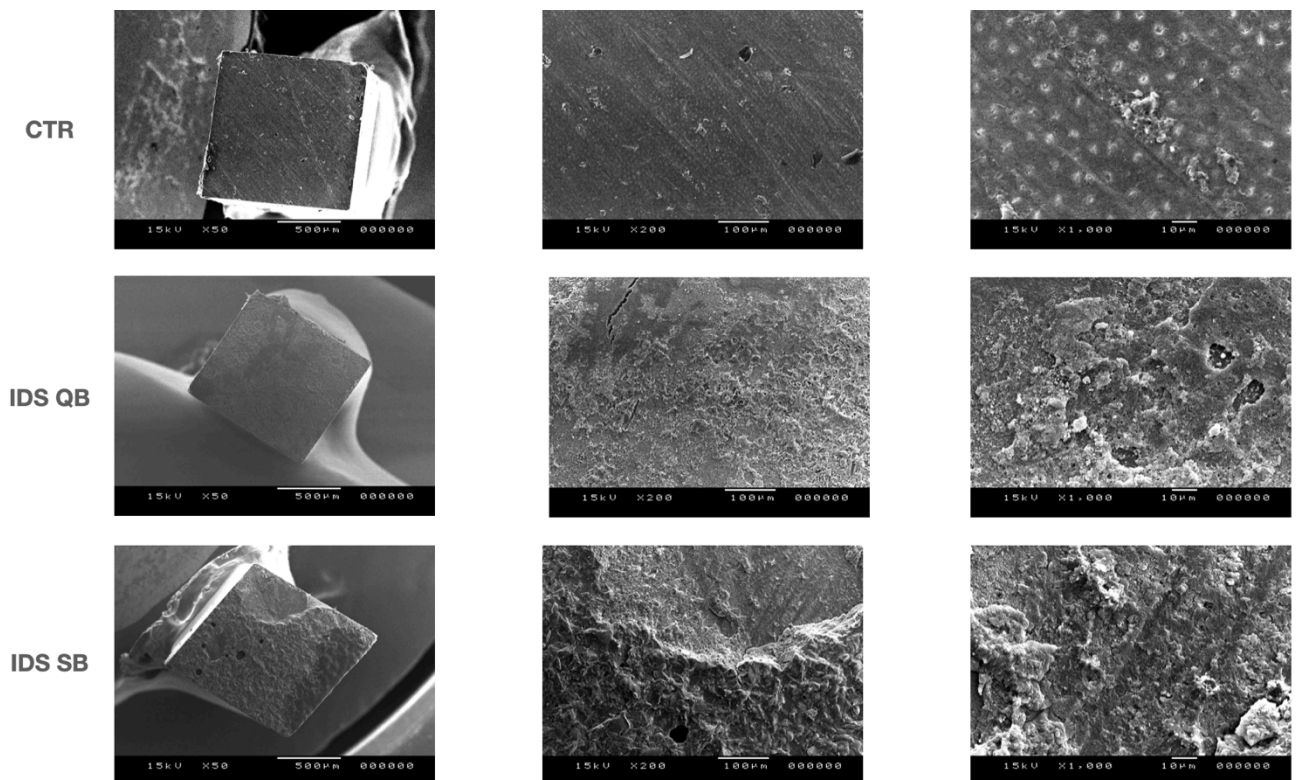


Fig. 2. Scanning electron microscopy microphotographs (50x, 200x and 1000x magnifications) of the fractured specimens after 10.000 thermocycles.

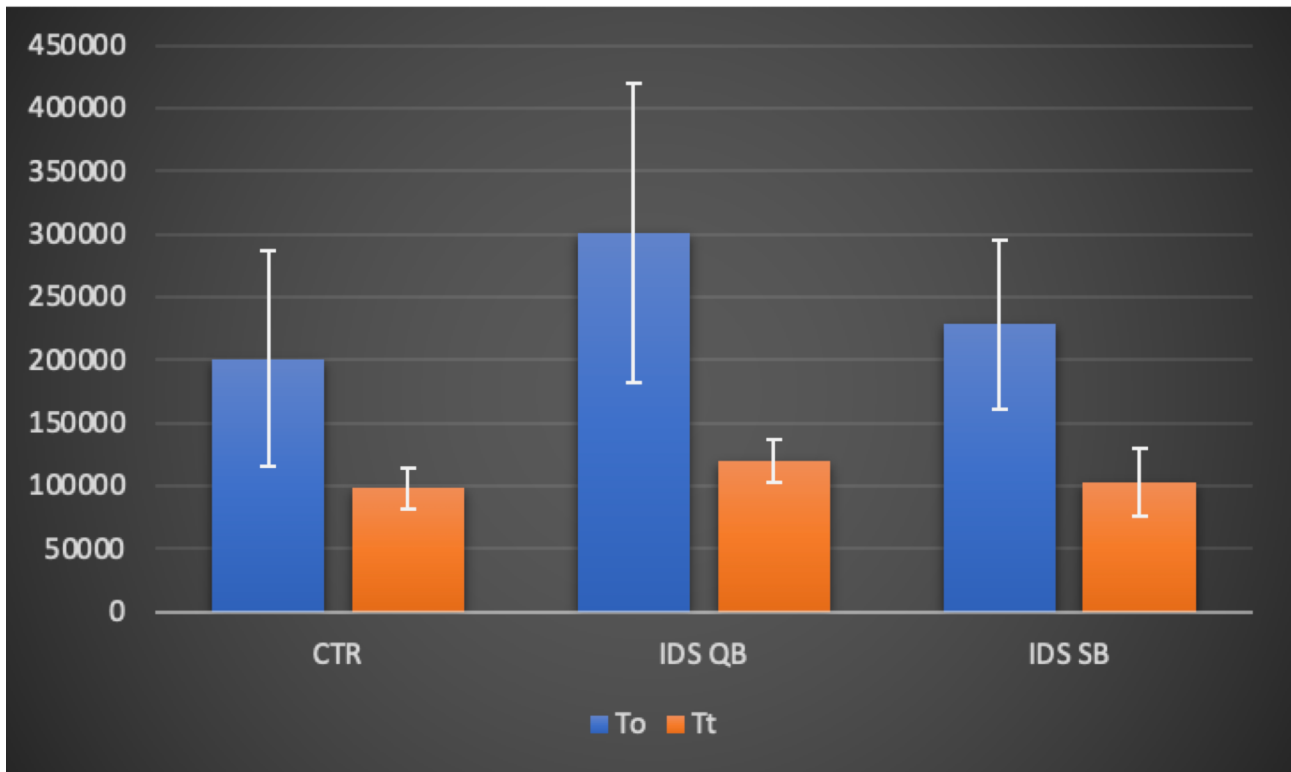


Fig. 3. Gelatinolytic activity, expressed as the percentage of the green fluorescence within resin/dentin interfaces created in experimental groups at baseline (T_0) and after thermocycling procedure (T_t). Values are means and standard deviations. For comparison of the factor “group”, columns that are labelled with the different upper-case letters (T_0) or lower-case letters (T_t) are significantly different ($p < 0.05$).

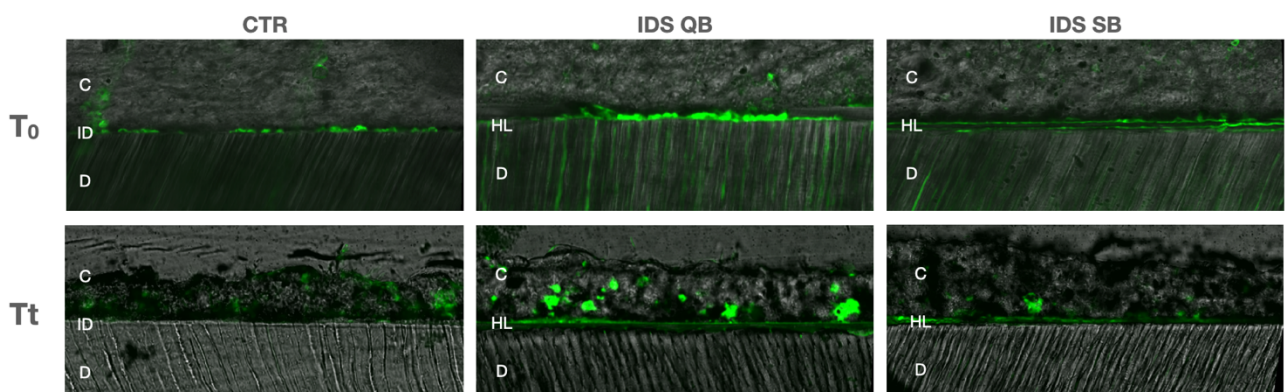


Fig. 4. Confocal laser scanning microscopy images of resin-dentin interfaces that were incubated with quenched fluorescein-labelled gelatin in experimental groups at baseline (T_0) and after thermocycling procedure (T_t). Abbreviations: D-dentin; HL-hybrid layer; ID-interdiffusion zone.

STUDY 3

Root canal irrigants and their role in the adhesion of pre-endodontic restorations

Objectives

Therefore, this study aimed to evaluate the influence of different irrigating solutions used during endodontic treatment on the immediate (T0) and artificially aged (TC) micro-shear bond strength (μ SBS) and endogenous dentinal enzymatic activity of pre-endodontic adhesive resin composite restorations. The null hypotheses tested were that:

- 1) intracanal irrigants produced no effect on bond strength between the dentin and the pre-endodontic resin composite restoration immediately and after TC;
- 2) intracanal irrigants produced no effect on endogenous enzymatic activity immediately and after TC.

Materials and Methods

Samples preparation

Sixteen freshly extracted non-carious human molars were obtained from anonymous individuals following their informed consent under a protocol approved by the Ethical Committee of the University of Bologna, Italy (protocol N°: 71/2019/OSS/AUSLBO). Teeth were collected and stored in a physiological solution at 4 °C until use, however no more than 1 month. Teeth were carefully observed under a stereomicroscope and those with signs of caries, posts, crowns, previous endodontic treatments and restorations, demineralization, abrasion, fractures and cracks were not included. Tooth crowns were removed using a low-speed diamond saw under water cooling (Microremet, Remet, Casalecchio di Reno, Italy) to expose deep coronal dentin, proximal to the pulp chamber. The cut was made 1 mm above the cemento-enamel junction (CEJ). Subsequently, the roots of the molars were removed with a second cut at a more apical level. Thus, the resulting tooth samples had an enamel portion very close to the CEJ and deep dentin, simulating clinical conditions (Fig. 1A). Afterwards, the specimens were examined under a stereomicroscope to ensure they were free from defects and embedded in acrylic resin (ProBase Cold resin, Ivoclar vivadent technical, Schaan Liechtenstein) using a cylindrical mold taking care to not touch the exposed dentin-enamel

surface (Fig. 1B). Finally, the specimens were ground with a #180-grit wet silicon-carbide (SiC) abrasive paper until a flat enamel and dentin surfaces were obtained. Consequently, a standardized smear layer was created by #240-grit wet SiC paper. To closely replicate the clinical scenario of a pre-endodontic resin composite restoration, the adhesive procedures were performed at the periphery of the teeth, both on the enamel and dentin (Fig. 1C). The materials used for restorative procedures are represented in Table 1 and handled strictly following manufacturer's instructions. A universal bonding system used in SE mode (IBOND Universal, Kulzer, Hanau, Germany) was applied with a micro brush, rubbed for 20 s, air-dried for 5 s and light-cured for 10 s with a light-emitting diode (LED) lamp (Curing Light Elipar™ DeepCure-L, 3 M™, St Paul, MN, USA; wavelength range 430–480 nm; light intensity 1.47 mW/cm²). A teflon ring mold (1.84 mm x 3 mm) (Josic et al. 2021) was placed on the periphery of the teeth and used to produce resin composite build-ups. The mold was filled with two 1.5-mm thick increments of a nanohybrid resin composite (shade A3; Venus Pearl, Kulzer, Hanau, Germany), and each layer was light-cured for 20 s. Excess of composite was meticulously removed with a dental explorer before final curing. Given that a sample size of $n = 10$ per group ensures at least 80% statistically significant differences with a standard error value of 0.05, a total of 20 resin composite build-ups per group were used. At the completion of the restorative procedures, the specimens were stored in distilled water in an incubator at 37 °C until use. The following groups were formed based on the irrigation protocol performed ($n = 20$): **C**: No treatment; **SH**: 5.25% sodium hypochlorite for 10 min + final water rinse; **CHX**: SH + final 2% chlorhexidine rinse for 2 min; **EDTA**: SH + final EDTA rinse for 2 min. The irrigating solutions were then placed in specific containers, and the previously prepared samples were positioned upside down inside the containers, ensuring that they remained in constant and complete contact with the irrigants for the required duration. After each irrigating protocol, the specimens were washed with water. Then, half of the specimens ($n = 10$ /group) were stored in artificial saliva and incubated in a humidified chamber at 37 °C overnight before being tested with the μ SBS test (T0). The second half was subjected to thermal cycles (TC; THE100, Mechatronik, Feldkirchen-Westerham, Germany) with 10,000 cycles, dwell time of 30s at 5 to 55 °C and 5 s interval between each bath.

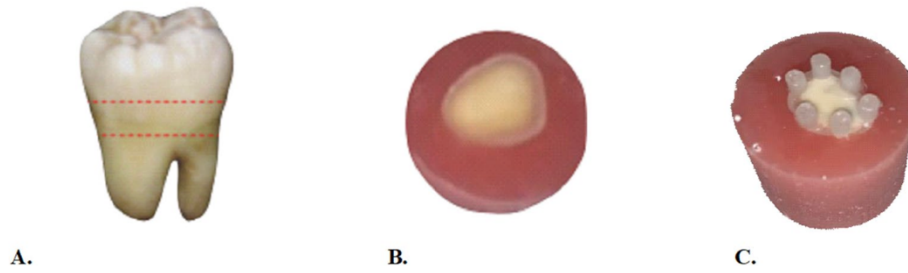


Fig. 1. Representation of specimens' preparation. **A:** discs of deep dentin were exposed (**A**); The dentin discs were embedded in self-curing acrylic resin (**B**); resin composite cylinders were bonded at the periphery of the deep dentin discs, to simulate clinical procedures of pre-endodontic restorations (**C**). In these conditions, the specimens came in contact with the irrigating solutions for the time estimated according to the testing group.

Product	Component	Description
iBond Universal	Methacrylate Monomer	DUDMA, 10-MDP, 4-META
	Solvents	Acetone, Water
	Additional Components	Stabilizers, Camphorquinone, Ethanol, Amine Accelerator
Venus Pearl	Resin Matrix	TCD-Urethane Acrylate, UDMA, TEGDMA
	Filler	Barium-Aluminum-Boro-Fluoro Silicate Glass, Silica, Titanium Dioxide
	Pigments	Fluorescent Pigments, Metal Oxide Pigments, Organic Pigments
	Initiators and Additives	Aminobenzoic Acidifying Agent, BHT, Camphorquinone

Table 1. Composition of iBond universal and Venus Pearl (Kulzer). DUDMA: polyurethane dimethacrylate; 10-MDP: 10-Methacryloyloxydecyl dihydrogen phosphate; 4-META: 4 methacryloxyethyl trimellitate anhydride; TCD-Urethane Acrylate: Toluene-2,4-Diamine-Urethane acrylate; UDMA: urethane dimethacrylate; TEGDMA: triethylene glycol dimethacrylate; BHT: butylated hydroxytoluene.

Micro-shear bond strength test (μ SBS)

At the completion of each storage period, the specimens were mounted in a testing machine (Ultra Tester™, Ultradent, Cologne, Germany) and the μ SBS test was executed. The machine's chisel shaped blade was positioned at the adhesive interface to measure the bond strength at a crosshead speed of 0.5 mm/min. The μ SBS was calculated as megapascals [MPa] using the following formula: stress [MPa] = force [N]/bonding area [mm²] (Josic et al. 2021).

In situ zymography

Additional 3 teeth were used for the in situ zymography and MMPs activity quantified at baseline and after TC. The teeth used in this case were immediately frozen after extraction and thawed at the time of sample preparation. Two 1-millimeter-thick slabs of middle/deep coronal

dentin were obtained from extracted human third molars using a low-speed saw (Micromet) under water cooling. Two dentin slabs were obtained from each molar. Each slab was further divided into 4 pieces so that testing of the four experimental groups was performed using the same dentin substrate (Breschi et al. 2023). A standardized smear layer was created on each dentin surface using #240-grit silicon carbide paper under water cooling. Identical bonding procedures were performed as previously described for the μ SBS test, forming the same 4 groups according to the different irrigating protocols. Resin-dentin interfaces were exposed by cutting the bonded specimens vertically into 1 mm-thick sticks using the slow-speed saw under water cooling. Half of the specimens were tested after 24 h (T0) and the other half after 10,000 TC. After aging, the sticks were fixed to glass slides with cyano-acrylate glue ground down and polished to obtain $\sim 50 \mu\text{m}$ thick slabs using a series of wet silicon carbide papers. Self-quenched fluorescein-conjugated gelatin was used as the MMPs substrate (E-12,055, Molecular Probes, Eugene, OR, USA) for in situ zymography at T0 and TC (Breschi et al. 2023). The fluorescent gelatin mixture was placed on top of each slab and covered with a glass coverslip. The slides were incubated in a humidified chamber at 37 °C overnight or subjected to thermocycling. During incubation, the assemblies were prevented from direct contact with water and were protected from exposure to light. After incubation, the microscopic slides were examined using a confocal laser scanning microscope (laser excitation wavelength 488 nm; emission wavelength 530 nm; Leica SP8, Leica Microsystems GmbH, Wetzlar, Germany). To visualize the hydrolysis of the quenched fluorescein-conjugated gelatin substrate as an indicator of endogenous gelatinolytic activity, 3 z-stack images ($\sim 15 \mu\text{m}$ thick) were made per specimen. The steps between the optical sections were of $1 \mu\text{m}$ and the z-stacks were made from the top of the specimen and into the depth of $\sim 15 \mu\text{m}$. Enzymatic activity was quantified as the integrated density of the fluorescence signals by means of the ImageJ software (National Institutes of Health, Bethesda, MD, USA) using a rectangular selection ($100 \times 20 \mu\text{m}$) placed over the HL and dentin (3 measurements per image). The quantification was performed after discarding several initial slices as to exclude parts of the specimen with the background noise in the signal attributed to the activated gelatin substrate on top of the specimen. After the removal of the initial slices also the differential interference contrast (DIC) image reached maximum focus and this point was considered repeatable and ideally suited for the execution of the measurements in all the specimens.

Statistical analysis

The data from the μ SBS test (calculated in MPa) and in situ zymography were normally (Shapiro Wilk test) and homogeneously (Brown-Forsythe test) distributed ($p > 0.05$). Consequently, two-way analysis of variance (2-way ANOVA) and pairwise multiple comparison procedures (Holm-Sidak method) were used to evaluate the effects of factors: “irrigating protocol” and “aging”. Statistical significance was set at $\alpha = 0.05$ (SigmaPlot 14.0; Systat Software Inc., Berkshire, UK).

Results

The bond strength results are illustrated in Table 2. The two-way ANOVA demonstrated that μ SBS values were statistically influenced by both the irrigation protocol ($p = 0.011$) and aging ($p < 0.001$), with their interactions being also statistically significant ($p = 0.016$). The pairwise multiple comparison procedures (Holm-Sidak method) demonstrated that at T0, irrigation with EDTA achieved the highest bond strength values compared to all the other groups (EDTA > SH, $p = 0.001$; EDTA > CHX, $p = 0.002$; EDTA > CTR, $p = 0.032$), while no differences were observed among C, SH, and CHX groups ($p > 0.05$). TC significantly reduced bond strength values in all groups ($p < 0.001$), with no differences observed between the various irrigating protocols ($p > 0.05$). The results of gelatinolytic activity, expressed as green fluorescence intensity within the HL, are illustrated in Figs. 2 and 3. The enzymatic activity was significantly influenced by the irrigation protocol ($p < 0.001$) and aging ($p < 0.001$), with their interactions being statistically significant ($p < 0.001$), as demonstrated by the two-way ANOVA test. At T0, the SH group exhibited the highest enzymatic activity (SH > C, $p = 0.009$; SH > CHX, $p < 0.001$; SH > EDTA, $p = 0.033$), while the EDTA group showed similar values to the C group ($p = 0.525$). In contrast, the CHX group demonstrated the lowest fluorescence values (EDTA > CHX, $p < 0.001$; CTR > CHX, $p = 0.004$). After TC, the enzymatic activity increased across all groups ($p < 0.001$). Specifically, the post-hoc test demonstrated that the SH group recorded the highest overall values, followed by the C group (SH > C, $p < 0.001$; SH > CHX, $p < 0.001$; SH > EDTA, $p < 0.001$; C > CHX, $p < 0.001$; C > EDTA, $p < 0.001$). The CHX and EDTA groups exhibited lower and comparable enzymatic activity ($p = 0.079$).

	T0	T1
C	18.1±6.4 b, A	10.3±5.3 a, B
SH	16.1±5.2 b, A	7.1±3.2 a, B
CHX	16.7±6.4 b, A	8.5±2.5 a, B
EDTA	22.7±7.8 a, A	8.0±3.3 a, B

Table 2. μ SBS test values in MPa (mean $\pm$ standard deviation). Different uppercase letters indicate statistically significant differences ($p < 0.05$) within the same row; different lowercase letters indicate statistically significant differences ($p < 0.05$) within the same column. C: control group; SH: sodium hypochlorite; CHX: chlorhexidine; EDTA: Ethylenediaminetetraacetic acid.

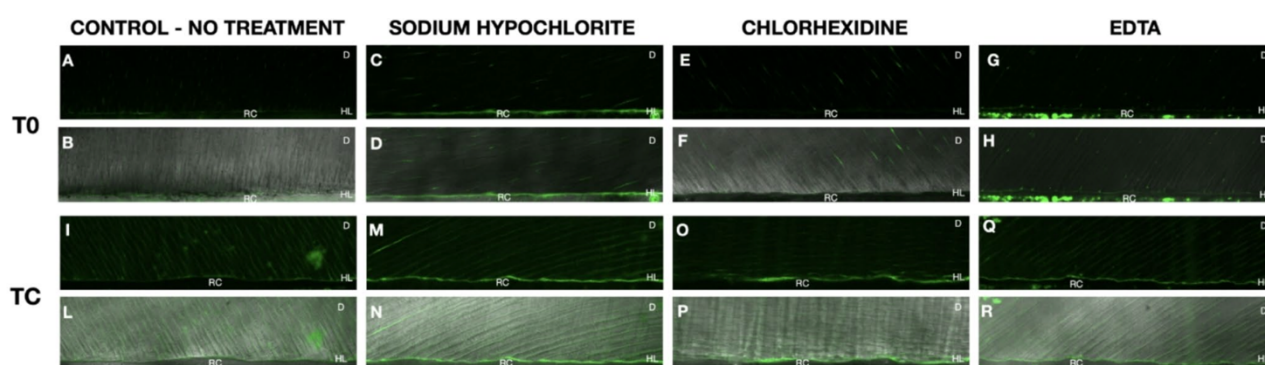


Fig. 2. In situ zymography analysis of the resin/dentin interfaces. RC: resin composite; HL: hybrid layer; D: dentin. **A, C, E, G, I, M, O, Q:** images acquired in green channel, showing fluorescence (the greater the green fluorescence, the higher the enzymatic activity) in dentinal tubules and within the HL. **B, D, F, H, L, N, P, R:** images obtained by merging differential interference contrast image (showing the optical density of the resin-dentin interface).

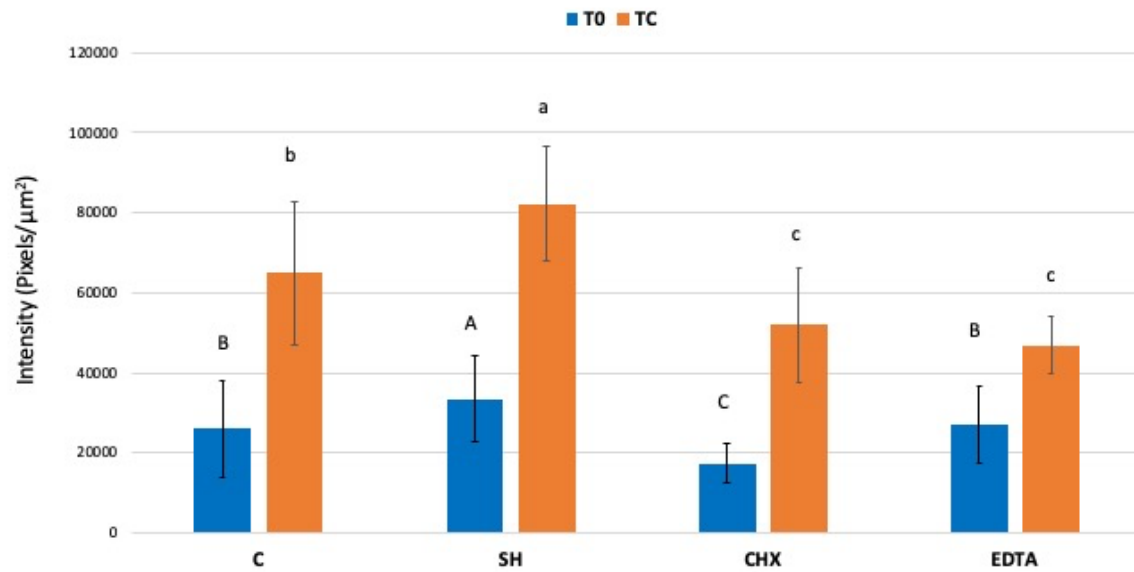


Fig. 3. Graphical representation of gelatinolytic activity values, expressed as the percentage of green fluorescence in the resin/dentin interfaces within the radicular dentin created in the experimental groups. The values are represented as means $\pm$ SD. Groups significantly different from each other ($p < 0.05$) were marked in different uppercase letters at T0 and different lowercase letters at TC. There was a significant increase in enzymatic activity in all the groups after aging ($p < 0.05$).

STUDY 4

Effects of a biomimetic dual-analogue primer on the bonding performance of an experimental ion-releasing adhesive system – An in vitro study

Objectives

This study aimed at assessing the bonding performance of an experimental two-component Dual-Analogue Primer (DAS) containing Fluoride-Doped Calcium Phosphate (FDCP) applied on acid-etched dentine in combination with a biomimetic dual-analogue dentine conditioner (PRM), and evaluate their effects on endogenous dentinal enzymatic activity. This aim was accomplished by testing the microtensile bond strength (μ TBS), fractographic, and resin-dentine interface ultramorphology/nanoleakage analyses at baseline (T0) and after thermocycling (TC). Moreover, the dentine enzymatic activity was also assessed through in situ zymography analysis, as well as gelatine zymography on protein extracts obtained from dentine powder and subsequently treated with the tested materials. This in vitro study hypothesized that the use of the experimental adhesive system containing FDCP used with or without the biomimetic primer would demonstrate both at T0 and after TC:

- 1) superior bonding performance compared to the control materials (a glass-ionomer cement, and a conventional universal adhesive);
- 2) improved hybrid layer integrity and nanoleakage expression;
- 3) reduced endogenous dentinal enzymatic activity.

Materials and Methods

Preparation of the experimental materials

An experimental FDCP was synthesised as described in earlier studies (Alambiaga-Caravaca et al. 2024; Sauro et al. 2023). This was achieved using a 1:1 molar ratio of beta-tricalcium phosphate and monocalcium phosphate monohydrate, combined with calcium hydroxide (10 wt.%) and calcium and sodium fluoride salts in a 1:1 molar ratio at a concentration of 10 wt.% (designated as VS10; particle size ω 50 μ m). The FDCP was then added (20 vol%) to a resin-based material, resulting

in an experimental resin bond (VS10-R20%), which consisted of urethane dimethacrylate (UDMA, 55 wt%), ethoxylated bisphenol-A dimethacrylate (Bis-EMA, 20 wt%), and 1,6-hexanediol ethoxylate diacrylate (HEDA, 25 wt%). Additionally, camphoroquinone (CQ) and ethyl 4-dimethylaminobenzoate (DMAB) were included (1 mol%), along with 0.5 mol% of trimethylbenzoyl-diphenylphosphine oxide (TPO) and 2,4-dihydroxybenzophenone (DHBP). Butylated hydroxytoluene (BHT) was added as a stabiliser (0.01 wt%). A silica nanofiller with an average diameter of 16 µm (AEROSIL® R972, Evonik Industries AG, Essen, Germany) was also incorporated (26 wt%) to adjust the viscosity of this latter resin. Furthermore, the FDCP was included (10 vol%) in an experimental primer (AD/VS10-R10 %; pH 3.1), which was formulated using UDMA (26 wt%), Bis-EMA (5 wt%), HEDA (10 wt%), N-acrylamide (NACA, 16 wt%), and 10-Methacryloyloxydecyl dihydrogen phosphate (MDP, 15 wt%). The solvents comprised bi distilled water, absolute butanol, and absolute ethanol (total: 23 wt%). CQ and DMAB were incorporated at 1 mol%, with TPO and DHBP at 0.5 mol%. BHT was added at a concentration of 0.01 wt%. Additionally, a water-based biomimetic dual-analogue dentine conditioner was prepared by dissolving 5 wt% polyacrylic acids (PAA, Mw 1800) and 5 wt% sodium tripolyphosphate (TPP) (Chou et al. 2025). The pH of the primer was adjusted to 7.0 using NaOH. All reagents were sourced from Merck Life Science SLU (Madrid, Spain), except for the MDP, which was provided by Pulpdent Corporation (Watertown, MA, USA).

Preparation of dentine specimens

Sixty-four sound human molars extracted for surgical and/or periodontal aims under a protocol reviewed and approved by the Ethical Committee (ethical approval number: CEEI23/401, University CEU Cardenal Herrera, Valencia, Spain) were used in this study. Preceding extraction, patients were fully informed with a written consent form on the use of the teeth for scientific purposes. The teeth were stored in distilled water at 4 °C for no longer than 3 months. The roots were removed 1 mm beneath the cemento–enamel junction using a diamond-embedded blade (#12,205 – EXTEC diamond wafering blade) mounted on a low-speed cutting machine (Isomet 1000, Buehler Co. Chicago, Illinois, USA) under continuous water cooling. A second parallel cut was made to remove the occlusal enamel, and mid-coronal dentine was exposed using #320-grit SiC papers. Four main groups (n = 16 teeth/group) were formed based on the materials tested in this study:

CTR – control: the dentine surface of the specimens was acid-etched for 10 s (37 % H₃PO₄, SDI Ltd. Bayswater, Australia) and subsequently rinsed copiously with distilled water (10 s). A universal

adhesive (SCH: Scotchbond Universal, 3 M Oral Care, Seefeld, Germany) was brushed for 10 s, air-blown for 5 s to evaporate the solvent, and then light-cured for 10 s using a LED (1200 mW/cm²) light source (Radii Plus. SDI, Ltd.). Subsequently, the specimens were restored by applying 3 layers (2 mm each) of Filtek™ Supreme XTE (3 M Oral Care), individually light cured for 30 s.

GIC – control: the dentine surface of the specimens was conditioned with a 10 % polyacrylic acid for 10 s (SDI Ltd.) and subsequently rinsed copiously with distilled water. Two capsules of glass ionomer cement (GIC; RIVA SC, SDI Ltd.) were mixed (10 s) using an amalgam mixing device and finally applied. The GIC was left undisturbed for 10 min to allow a correct setting reaction.

EXP: The experimental two-component DAS constituted of an FDCP-containing primer (AD/VS10-R10 %) and bond resin (VS10-R20 %) was employed. Firstly, the dentine surface of the specimens was acid-etched for 10 s (37 % H₃PO₄, SDI Ltd.) and subsequently rinsed as described above. The resin primer AD/VS10-R10 % was applied using a micro-brush for 10 s, air-blown for 5 s to evaporate the solvent, followed by the application of a layer of bond resin (VS10-R20 %). Finally, both components were light-cured together for 20 s (Radii plus, SDI Ltd.). Finally, 2 layers (1 mm each) of Filtek™ Supreme XTE (3 M Oral Care) were applied and light cured as described previously.

EXP-PR: The acid-etched (10 s of 37 % H₃PO₄) dentine specimens were first treated with the biomimetic PRM, which was brushed (0.5 ml) on the surface for 60 s. The dentine surface was then gently air-blown for 5 s, bonded, and light-cured as described above using the AD/VS10-R10 %, and VS10-R20 %. Subsequently, 2 layers (1 mm each) of Filtek™ Supreme XTE (3 M Oral Care) were applied and light cured as described previously.

Micro-tensile bond strength (μTBS), failure modes, and Fe-SEM fractographic analysis

All the bonded/restored specimens created as described in chapter 2.2 were sectioned across the resin-dentine interface in both X and Y directions (Isomet 1000, Buehler Co) to obtain approximately 20 composite-tooth matchsticks from each tooth, with a cross-sectional area of 1 mm². Half of such matchsticks from each group were stored in distilled water for 24 h (T0; baseline), while the remaining half part of the matchsticks was aged in a thermocycling system containing deionised water (TC; 10,000 cycles; 5 – 55 °C, 30 s of exposure time, 5 s dwell time) before microtensile testing. This latter procedure was performed using a microtensile bond strength device with a stroke length of 50 mm, a peak force of 500 N, and a displacement resolution of 1 mm. To

analyse the modes of failure of each specimen, stereoscopic microscopy operating at 50x magnification was employed, and the fractures were categorized as a percentage of adhesive (A), mixed (M), or cohesive (C) failures. Five representative fractured specimens from each sub-group were first desiccated overnight and then mounted on aluminum stubs with carbon glue. The specimens were gold-sputter coated and analyzed with an ultra-high-resolution analytical focused ion beam scanning electron microscope (FIB-SEM, Thermo Scientific Scios 2 DualBeam, Waltham, MA, USA) in secondary electron mode at 10 kV. The normality of μ TBS data was evaluated using the Shapiro-Wilk test ($p \leq 0.05$). The homogeneity of variance was calculated using the Brown-Forsythe test. For all tests, the variances were homoscedastic ($p \leq 0.05$). Data were then analysed using a two-way Analysis of Variance (ANOVA Factors: material and aging protocol) and Holm-Sidak multiple comparison test ($\alpha \rightarrow 0.05$). SigmaPlot v. 14.0 (Systat Software GmbH, Düsseldorf, Germany) was used.

Bonded-Dentine interfaces – ultramorphology/nanoleakage confocal microscopy

Further extracted human molars ($n=3/\text{group}$) were prepared and restored as described above in session “*Preparation of dentine specimens*”, but with the tested materials doped with 0.05 % Rhodamine B (RoB: Sigma Chemicals) (Sauro et al. 2021). After storage in water at 4 °C for 24 h, the restored teeth were sectioned longitudinally into 1 mm-thick slabs to expose the resin-dentine interfaces using the same low-speed water-cooled diamond saw and aged as previously described above (Baseline-T0; Thermocycling-TC). Subsequently, the specimens were coated with a fast-setting nail varnish, applied 1 mm from the bonded interface, and left undisturbed for 30 min. They were subsequently immersed in a Lucifer Yellow (LY; Sigma Chemicals) water solution (0.1 wt.%) for 24 h. Afterwards, the specimens were ultrasonicated in distilled water for 3 min and then polished for 30 s each side with a #600-grit, followed by #2400-grit SiC abrasive papers. The specimens were ultrasonicated again in distilled water for 3 min and immediately submitted to confocal microscopy ultra-morphology analysis (CLSM - Olympus FV1000, Olympus Corp., Tokyo, Japan), using a 63X/1.4 NA oil immersion lens with simultaneous LED illumination at 488 and 543 nm. Reflection and fluorescence images were obtained using specific bandpass filters for RoB and LY and a 1- μ m z-step to section the specimens optically to a depth of up to 20 μ m below the surface (Profeta et al. 2013). The z-axis scan of the interface surface was arbitrarily pseudo-coloured for improved visualization and compiled into single and topographic projections using the CLSM image-processing software

(Fluoview Viewer, Olympus). The configuration of the system was standardized and used in constant settings for the entire investigation. Each dentine interface was completely investigated and finally, 5 representative images were randomly captured and recorded; these represented the most common morphological features observed along with the bonded interfaces (Sauro et al. 2021).

In situ zymography analysis - Confocal microscopy

An in situ zymography was performed using additional extracted molars (n=3/group) prepared and restored as described above in session 2.2. After storage in water at 4 °C for 24 h, the restored teeth were sectioned longitudinally into 0.7-mm-thick slabs to expose the resin-dentine interfaces using the same low-speed water-cooled diamond saw as described above. Within each group, the resin-dentine slabs were equally divided into 2 groups based on the aging protocol (Baseline (T0) or Thermocycling (TC)). The slabs were progressively polished with wet SiC papers with increasingly fine grit sizes (#600-, #1.200-, #4.000-grit) to obtain approximately 0.4 ($\leftrightarrow$ 0.1) mm-thick sections and ultra-sonicated in distilled water for 1 min to remove the excess smear layer created during the polishing procedures. To produce the fluorescent substrate, 1.0 mg/mL of a stock solution containing self-quenched fluorescein-conjugated gelatine (E-12,055; Molecular Probes, Eugene, OR, USA) was prepared by adding 1.0 mL deionised water to the vial containing the lyophilised gelatine. The substrate was stored at 20 °C until use. The gelatine stock solution was diluted 10 times with dilution buffer (NaCl 150 mmol/L, CaCl 25 mmol/L, Tris-HCl 50 mmol/L; pH 8.0). Then, the slabs were immersed in a 2 ml quantity of gelatine mixture and incubated for 24 h at 37 °C under continuous agitation (D'Alessandro et al. 2024). After incubation, the specimens were ultra-sonicated in distilled water for 1 min to remove the excess of gelatine/fluorescein and immediately analysed using a confocal laser scanning microscope (CLSM, Olympus FV1000) and a 40X/1.4 NA oil immersion lens with excitation/emission wavelength of 488/530 nm. Several z-stack images were obtained at the resin dentine interface (~15 μ m thick, one image per each 1 μ m into the depth of the specimens) of each slice. The settings configuration of the system was standardized throughout the entire investigation. Three to five z-stack images with the most significant morphological features observed along the dentine-material interfaces were randomly captured to qualitatively analyse the hydrolysis of the quenched fluorescein-conjugated gelatine substrate, indicative of endogenous gelatinolytic enzyme activity.

Gelatin zymography

Gelatin zymography was performed as described by Mazzoni et al. (Mazzoni et al. 2012) Ten human freshly extracted sound permanent teeth were devoid of enamel, cementum, and pulp tissue and immediately frozen. The teeth were triturated into powder (MM400, Retsch GmbH, Haan, Germany). Aliquots of dentine powder (100 mg) were divided into 4 groups:

G1—demineralised dentine (control) powder demineralised with 10 wt.% H_3PO_4 for 10 min at 4 °C;

G2—demineralized dentine (as G1) treated only with the biomimetic dual-analogue primer for 30 min at 4 °C;;

G3 – demineralised as G1 and treated with a water solution of FDGP (10 vol %) for 30 min at 4 °C,, and

G4 – demineralised as G1, treated with the biomimetic dual-analogue primer as G2, and then with the FDGP-containing solution as G3.

All the groups were tested in duplicate. Due to technical reasons, it was not possible to process the specimens with the GIC. After the etching procedure performed in all groups, H_3PO_4 was neutralised using 5 M sodium hypochlorite (NaOH), centrifuged for 20 min at 4 °C; (20,800↓ g), rinsed twice with water, and re-centrifuged under the same conditions. After the treatment with experimental solutions (G2–4), the powder was centrifuged to remove the experimental solution and then rinsed with distilled water and centrifuged again (20 min, 4 °C; C, 20,800↓ g). To extract protein, all dentine powder aliquots were further suspended in extraction buffer (50 mM Tris–HCl containing 5 mM CaCl_2 , 100 mM NaCl, 0.1 % Triton X-100, 0.1 % non-ionic detergent P-40, 0.1 mM ZnCl_2 , 0.02 % NaN_3 ; pH → 6) for 24 h at 4 °C; , sonicated for 10 min, and centrifuged for 20 min at 4 °C; . The supernatant was removed and re-centrifuged to remove any powder remnants. The protein content was concentrated in a centrifugal concentrator with 10,000 KDa cut-off (Vivaspin Sartorius Stedim Biotech, Goettingen, Germany) for 30 min at 25 °C; (15,000 g, until concentrating all the liquid up to 75 µl). The total protein concentration of the dentine ex- tracts was determined using Bradford assay (Bio-Rad, Hercules, CA, USA). Dentine protein aliquots were diluted in Laemmli sample buffer at a 4:1 ratio and subjected to electrophoresis under non reducing conditions in 10 % sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS PAGE) containing 1 mg/mL of fluorescently labelled gelatine. Pre-stained, low-molecular-weight SDS-PAGE standards (Bio-Rad)

were used as molecular-weight markers. The zymography was performed 3 times. After electrophoresis, the gels were washed in 2 % Triton X-100 (1 h) and incubated in the zymography activation buffer (50 mmol/L Tris-HCl, 5 mmol/L CaCl₂, pH 7.4) for 48 h. The gel was scanned with an ultraviolet light scanner (ChemiDoc Universal Hood, Bio-Rad, Hercules, CA, USA). Zymographic bands were identified and quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Results

Micro-tensile bond strength (μ TBS), failure modes, and Fe-SEM fractographic analysis

The two-way ANOVA demonstrated that both the tested materials ($P \leq 0.001$) and aging protocol ($P \rightarrow 0.002$), as well as their interaction ($P \leq 0.001$), influenced μ TBS. Overall, GIC demonstrated inferior bond strength compared to all the other groups ($P \leq 0.001$), the CTR group obtained higher bond strength results compared to EXP and EXP-PR at T0 ($P \leq 0.001$), while there were no significant differences between EXP and EXP-PR ($P \rightarrow 0.835$). The specimens of the CTR group (commercial adhesive/composite system) had the highest ($P \leq 0.001$) bond strength values (45.8 $\leftrightarrow$ 9.9 MPa) at baseline and failed mainly in cohesive and mixed modes (56.4 % and 30.8 %, respectively) with no bonding pre-fail (Table 1). The specimens failed in adhesive and mixed mode that were analysed through SEM, showed fractures beneath the hybrid layer (Fig. 1A), leading to a dentine surface characterised by a lack of resin-infiltrated collagen fibrils (Fig. 1A1 and A2). After TC, CTR specimens showed a significant drop ($P \leq 0.001$) in bond strength (31.8 $\leftrightarrow$ 12 MPa) compared to the T0 values. The SEM fractographic analysis (Fig. 2A) showed that the interface fractured within the hybrid layer, and clear signs of collagen degradation were observed particularly near dentinal tubules (Fig. 2A1, Fig. 2A2). GIC specimens failed mainly in cohesive modes (80 %) and 27.1 % of the matchsticks pre-failed before the bonding test. This group showed the lowest ($p \leq 0.001$) bond strength values (6.1 $\leftrightarrow$ 4.8 MPa) at baseline (T0; Table 1). The specimens observed under SEM showed a significant amount of remaining cement on the dentine surface (Fig. 1B), indicating a degree of adhesion preservation despite the failure (Fig. 2B1 and B2). After TC, GIC demonstrated no significant drop ($P \rightarrow 0.864$) in bond strength (6.5 $\leftrightarrow$ 5.4 MPa) compared to the T0 values. However, these specimens showed a well-mineralised dentine surface (Fig. 2B and 2B1), and in many cases, these surfaces remained partially covered by the GIC (Fig. 2B2). The specimens created using the experimental DAS (EXP) failed mainly in adhesive and mixed modes (45 % and 42.5 %, respectively).

respectively), but with no bonding pre-fail. These specimens had bond strength values of $26.2 \leftrightarrow 10$ MPa (T0; Table 1). The SEM analysis showed that fracture occurrences were located within the hybrid layer (Fig. 1C) with the exposed dentine characterised by the presence of resin tags and few uninfiltreated collagen fibrils (Fig. 1C1); such exposed collagen fibrils appeared to be often covered by resin (Fig. 1C2). After TC, the EXP specimens showed no significant drop ($P \rightarrow 0.694$) in bond strength ($24.4 \leftrightarrow 13.6$ MPa) compared to the T0 values. These specimens failed again mainly in adhesive modes and mixed modes and 12.5 % of them pre-failed before the bond strength test (Table 1). The EXP specimens submitted to TC (Fig. 2C) revealed during the SEM analysis a dentine characterised by few exposed collagen fibrils mode (Fig. 2C1), often covered by mineral deposition mode (Fig. 2C2). The specimens created using the experimental DAS in combination with the biomimetic dual-analogue PRM (EXP PR) failed mainly in adhesive and mixed modes (51.1 % and 46.3/%, respectively), and there was no bonding pre-fail. These specimens had bond strength values of $27.1 \leftrightarrow 8.4$ MPa (T0; Table 1), which were similar to those of EXP ($P \rightarrow 0.810$). When the specimens that failed in adhesive and mixed mode were analyzed through SEM (Fig. 1D), the exposed dentine appeared mostly protected by resin (Fig. 1D1). At increased magnification (Fig. 1D2), few exposed collagen fibrils were often observed. After TC, the EXP-PR specimens showed no significant drop ($P \rightarrow 0.582$) in bond strength ($26.7 \leftrightarrow 13.7$ MPa) compared to the baseline values; these values showed no significant difference compared to the CTR and EXP specimens ($P \in 0.05$). These specimens failed again mainly in adhesive modes and mixed modes and only 9.8 % of specimens pre-failed before the bond strength test (Table 1). The SEM analysis revealed, the exposed dentine was primarily shielded by a mineral layer covering both the dentinal tubules and collagen fibrils (Fig. 2D and 2D1); these latter were seldom visible even at higher magnifications (Fig. 2D2).

GROUP	CTR	GIC	EXP	EXP-PR
T0	45.8 ± 9.9^{a1} (12.8/30.8/ 56.4) [pre-fail 0]	6.1 ± 4.8^{b1} (6.5/13.5/80) [pre-fail 27.1]	26.2 ± 10.0^{c1} (45.0/42.5/ 12.5) [pre-fail 0]	27.1 ± 8.4^{c1} (51.1/46.3/ 2.6) [pre-fail 0]
TC	31.8 ± 12.1^{a2} (32.5/51.2/ 16.3) [pre-fail 9.3]	6.5 ± 5.4^{b1} (20.4/54.6/ 25.0) [pre-fail 5.5]	24.4 ± 13.6^{c1} (42.5/40.0/ 17.5) [pre-fail 12.5]	26.7 ± 13.7^{ac1} (44.1/48.8/ 7.1) [pre-fail 9.8]

Table 1 Microtensile bond strength test values both at baseline after 24 h (T0) and after thermocycling (TC), along with the respective standard deviation. In parentheses are the percentages of fractures determined by fractographic analysis (A/M/C). The values in square brackets indicate the percentages of pre-failures. Same lowercase letters indicate no significant difference in rows. Same numbers indicate no significant difference in columns.

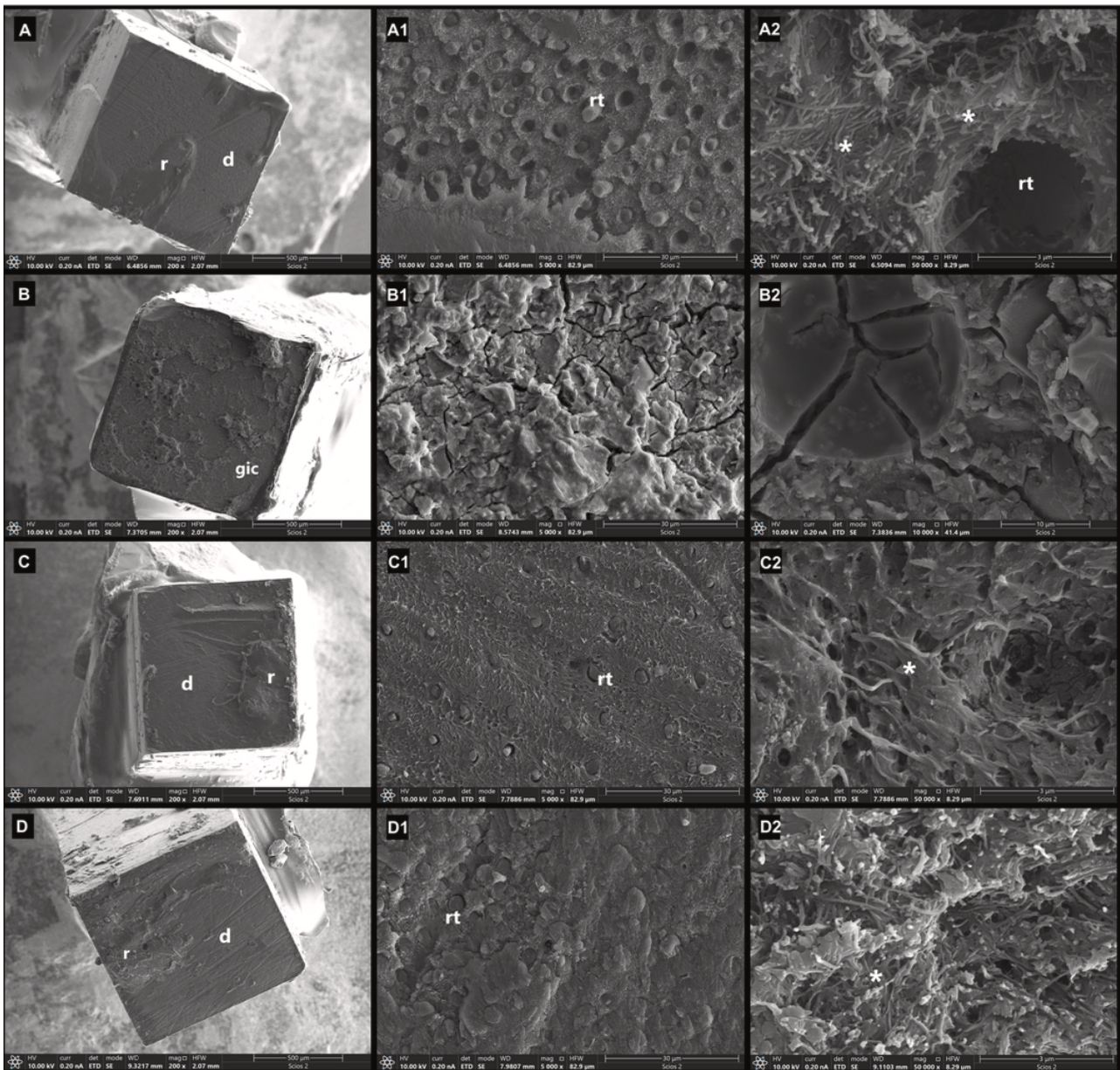


Fig. 1. Scanning electron microscopy – fractography specimens at baseline (T0). **A:** Representative SEM fractography of a specimen created by the application of the CTR (adhesive/composite) that failed in mixed mode showing both dentine (d) and remaining composite (r). At higher magnification, it is possible to see (A1) that the fracture occurred underneath the hybrid layer, and the dentine surface is characterised by the presence of few poorly resin-infiltrated collagen fibrils (*) and resin tags (rt) inside dentine tubules (A2). **B:** The specimens created with GIC that failed in mixed mode show a clear presence of remaining cement (gic) on the dentine. At higher magnifications (B1, B2), it is possible to note also the dentine part of the mixed failure remains still protected by the presence of GIC. **C:** Representative SEM fractography of a specimen created by applying the experimental restorative system (EXP) that failed in mixed mode. At higher magnification. (C1), it is possible to see that the fracture occurred within the hybrid layer and that the dentine is characterised by resin tags (rt) inside the dentinal tubules and a few exposed dentine collagen fibrils. At higher magnifications (C2), such as exposed collagen fibrils seem partially covered by the resin (*). **D:**

Representative SEM fractography of a specimen created by applying the experimental restorative system in combination with the biomimetic primer (EXP-PR) that failed in mixed mode. In this case, the exposed dentine observed at higher magnification (D1) appears mainly protected by the resin, since the fracture occurred within the adhesive leaving the tubules occluded by resin tags (rt). Only at higher magnification (D2), it is possible to see a few collagen fibrils still partially exposed (*).

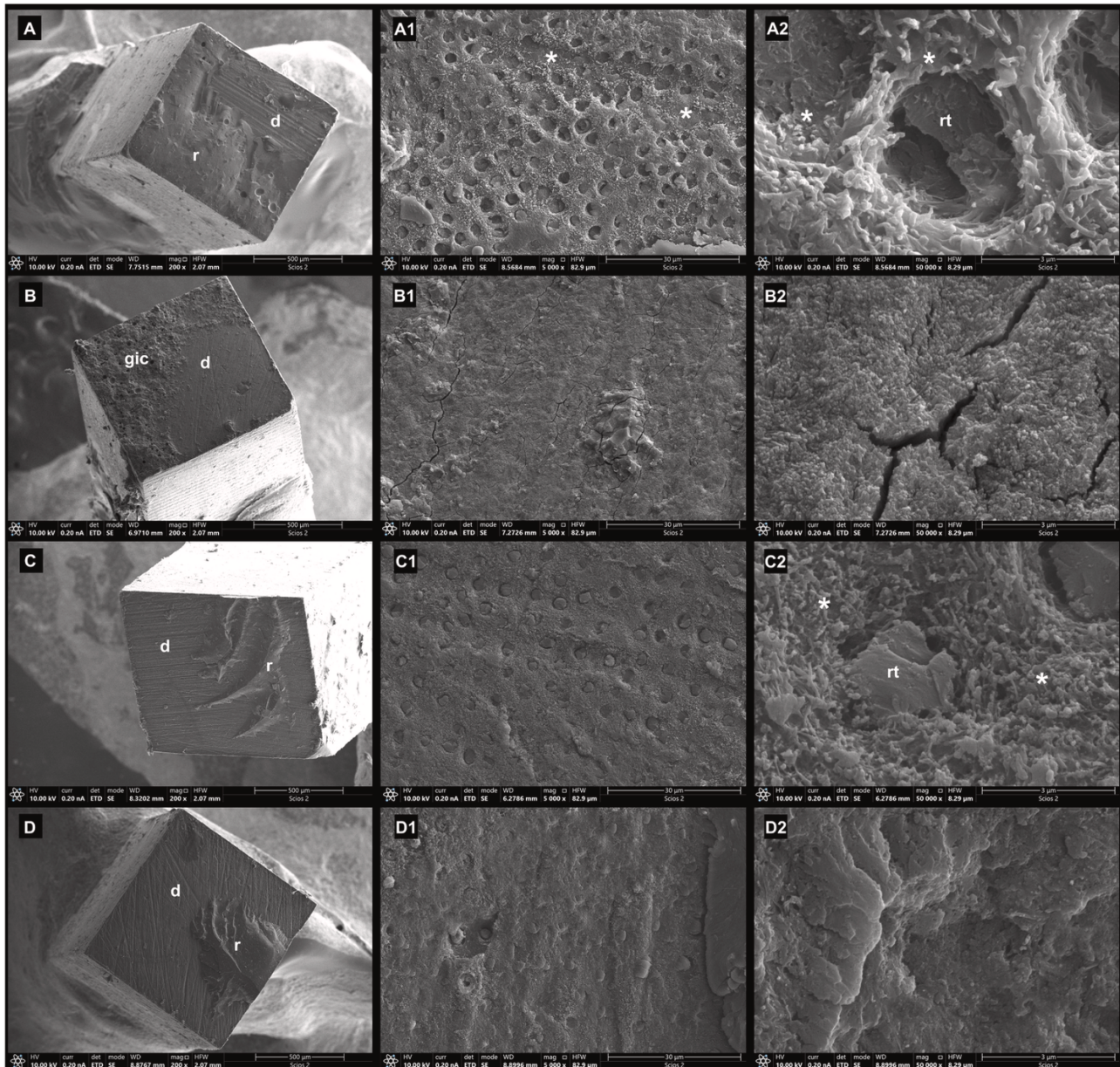


Fig.2 Scanning electron microscopy – fractography specimens after thermocycling. **A:** Representative SEM fractography of a specimen created by the application of the CTR (adhesive/composite) that failed in mixed mode showing both dentine (d) and remaining composite (r). At higher magnification, it is possible to see (A1) that the fracture occurred within the hybrid layer, and the dentine surface is characterised by zones devoid of collagen fibrils (*). It is also possible to note (A2) clear signs of collagen degradation tags (*), especially in the proximity of dentinal tubules; many of them still present the presence of fractured resin tags

(rt). **B:** The specimens created with GIC that failed in mixed mode show remaining cement (gic) and exposed dentine (d). At higher magnifications (B1, B2), it is possible to note an exposed mineralised dentine surface with no clear presence of remaining GIC. **C:** Representative SEM fractography of a specimen created by applying the experimental restorative system (EXP) that failed in mixed mode. At higher magnification (C1), it is possible to see that dentine is characterised by resin tags (rt) still inside the dentinal tubules and a few exposed dentine collagen fibrils. Moreover, (C2), such as exposed collagen fibrils seem to be partially covered by mineral deposition (*). **D:** Representative SEM fractography of a specimen created by applying the experimental restorative system in combination with the biomimetic primer (EXP-PR) that failed in mixed mode. The exposed dentine observed at higher magnification (D1) appears mainly protected by a mineral layer that covers both dentinal tubules and collagen fibrils; these latter are rarely found at even higher magnification (D2).

Bonded-Dentine Interfaces – ultramorphology/nanoleakage confocal microscopy

This chapter details the confocal single projection imaging results obtained in both transmission and fluorescence modes (using dyes RoB and LY) for resin-dentine specimens created with the various materials tested in this study. The analyses were conducted at two time points: T0 and TC, providing insights into the structural characteristics and integrity of the resin-dentine interface. The specimens of the CTR group revealed at T0 the presence of short resin tags (Fig. 3A) and a clear presence dye penetration within the hybrid layer (Fig. 3A1). When the specimens were observed in transmission/fluorescence mode (Fig. 3A2), it was possible to see a hybrid layer with a thickness of 9–12 μm devoid of gaps. After TC, the CTR specimens revealed the absence of resin tags (Fig. 4A). When the specimens were observed in double-fluorescence (Fig. 4A1) and transmission fluorescence image (Fig. 4A2) mode, clear gaps were observed at the bonding interface, with a hybrid layer notably infiltrated by the LY dye. At T0, GIC showed an interface that was often free of gaps (Fig. 3B), along with a very thin ($\sim 1 \mu\text{m}$) bonding interdiffusion layer. However, the double fluorescence (Fig. 3B1) and transmission-fluorescence images (Fig. 3B2) revealed that the interdiffusion layer was highly porous and/or permeable to the LY dye, indicating a potential for fluid movement throughout. After TC, the presence of several gaps in the material residues was observed (Fig. 4B). The interdiffusion layer was found to be still highly porous and/or permeable to the LY dye (Figs. 4B1 and B2). The images of the experimental DAS (EXP) highlighted the presence of resin tags doped with FDCP fillers penetrating the dentine tubules for several microns (Fig. 3C1). When the specimens were imaged in double-fluorescence (Fig. 3C1) and transmission/fluorescence

mode (Fig. 3C2), it was possible to confirm a gap-free resin-dentine interface, characterised by a highly porous hybrid layer permeable to the LY dye. After aging, the presence of long resin tags penetrating the dentine tubules for several microns (Fig. 4C) was detected, while in the double fluorescence (Fig. 4C1) and transmission/fluorescence images (Fig. 4C2), the resin-dentine interfaces were characterised by tiny gaps above a hybrid layers still infiltrated by the LY dye. The resin-dentine specimens in group EXP-PR exhibited long resin tags and a well-defined hybrid layer (Fig. 3D). In double-fluorescence (Fig. 3D1) and transmission/fluorescence images (Fig. 3D2) a slight diffusion of the dye within the hybrid layer was observed. Conversely, after TC, it was still possible to appreciate the presence of long resin tags and a well-defined hybrid layer without gaps or any signs of degradation (Fig. 4D). Although the double-fluorescence images (Fig. 4D1) presented no evidence of dye diffusion into the hybrid layer, LY traces were observed within the dentinal tubules. Additionally, the transmission/fluorescence image (Fig. 3D2) demonstrated the presence of an intense reflective layer beneath the hybrid layer, which was devoid of any dye infiltration.

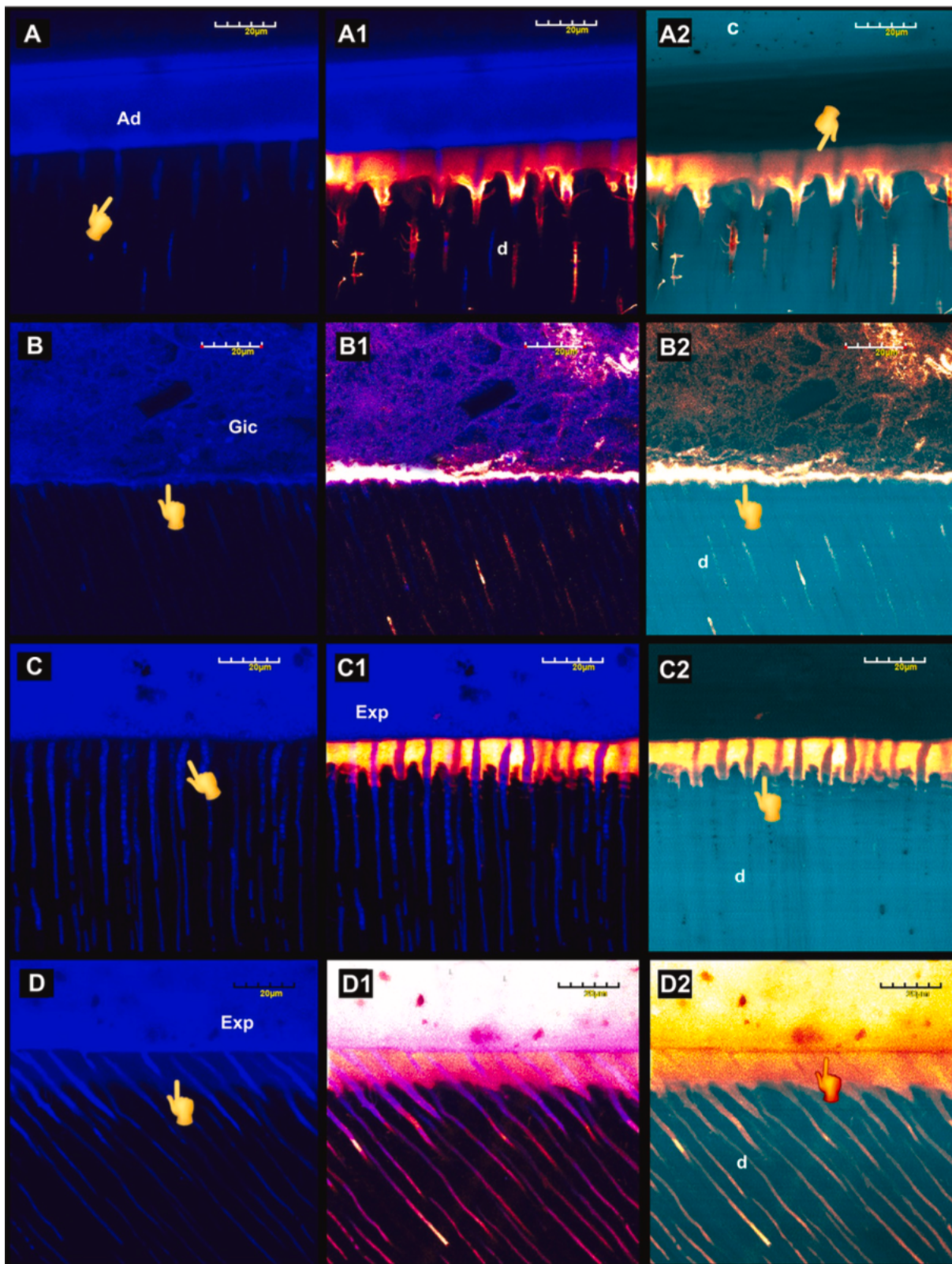


Fig. 3. Interface Ultramorphology/Nanoleakage - Confocal Microscopy T0 (Baseline). Confocal single projection images obtained in transmission and fluorescence (dyes: RoB and LY) mode of the resin-dentine specimens created with the different materials tested in this study and analysed at T0. **A:** This picture shows the control (CTR) Scotchbond Universal adhesive (ad) labelled with RoB applied in acid-etched dentine (d); note the presence of short resin tags (pointer). **A1:** in this double-fluorescence image it is possible to observe

a clear presence of nanolakege within the hybrid layer. This latter transmission/fluorescence image shows that the hybrid layer has a thickness of 9–12 μm and the interface is devoid of gaps (pointer) (A2). **B:** The RoB-labelled GIC created an interface often free of gaps, and a very thin interdiffusion layer ($\sim 1\mu\text{m}$). B1 and B2 are respectively double-fluorescence and transmission/fluorescence images, which show that the interdiffusion layer is highly porous and/or permeable to the LY dye. **C:** This picture shows the experimental adhesive and composite (EXP) labelled with RoB. Please note the presence of resin tags doped with the FDCP fillers penetrating the dentine tubules for several microns (pointer). C1 and C2 are respectively double-fluorescence and transmission/fluorescence images, which show a gap-free resin-dentine interface, characterised by the presence of a hybrid layer highly porous and/or permeable to the LY dye. **D:** The RoB-labelled experimental adhesive and composite applied in etched dentine primed with the dual-analogue primer (EXP-PR) created long resin tags and a well-defined hybrid layer (Pointer). D1 and B2 are respectively double-fluorescence and transmission/fluorescence images, which show a slight diffusion of the LY dye in the hybrid layer, but an evident presence of the LY within the adhesive and composite (pointer).

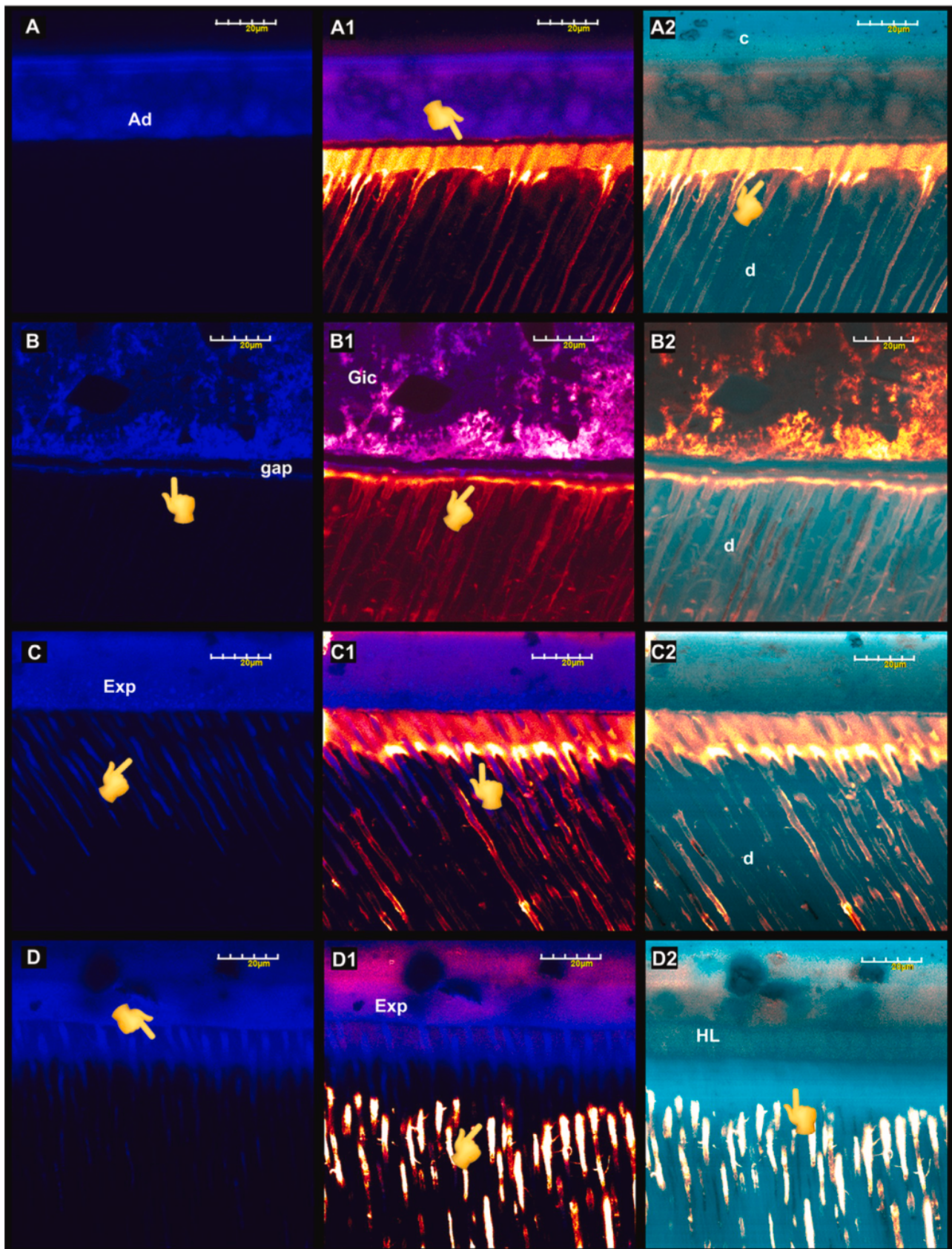


Fig. 4. Interface Ultramorphology/Nanoleakage - Confocal Microscopy TC (Thermocycling). Confocal single projection images obtained in transmission and fluorescence (dyes: RoB and LY) mode of the resin-dentine specimens created with the different materials tested in this study and analysed after TC. **A:** This picture

shows the control group (CTR) with the Scotchbond Universal adhesive (ad) labelled with RoB applied in acid-etched dentine (d); note the absence of resin tags (pointer), but several water droplets that exudate from the adhesive during the imaging process. **A1:** in this double fluorescence image it is possible to observe the presence of a gap between the adhesive; a hybrid layer notably infiltrated by the LY dye (pointer) can be seen also in the transmission-fluorescence image (**A2**). **B:** The GIC is characterised by a gap (pointer) situated above a very thin remaining Gic layer and an interdiffusion layer highly porous and/or permeable to the LY dye (**B1** and **B2**). **C:** This picture shows the experimental adhesive and composite (EXP) labelled with RoB, where it is possible to note the presence of long resin penetrating the dentine tubules for several microns (pointer). **C1** and **C2** are respectively double-fluorescence and transmission/fluorescence images, which show a resin dentine interface, characterised by the presence of a tiny gap and a hybrid layer highly porous and permeable to the LY dye (pointer). **D:** This is a representative image of the resin-dentine interface created with the experimental adhesive and composite applied in etched-dentine pre-treated with the dual-analogue primer (EXP PR). It is possible to see several long resin tags and a well-defined hybrid layer without gaps or signs of degradation (Pointer). **D1.** Double-fluorescence image that shows no diffusion of the LY dye in the hybrid layer, but the LY only penetrates the dentinal tubules several microns away from the hybrid layer (pointer). **D2** A transmission/fluorescence image showing the presence of an intense reflective layer just underneath the hybrid layer (pointer), which is devoid of any LY penetration; again, LY only penetrates the dentinal tubules several microns away from the hybrid layer.

In situ zymographic analysis - Confocal microscopy

This chapter presents the findings from the fluorescence analysis of resin-bonded mid-coronal dentine interfaces prepared with different materials: CTR, GIC, EXP, and EXP-PR. The interfaces were evaluated at baseline (1) and after TC (2) to assess endogenous enzymatic activity. At baseline, a slight fluorescence signal was observed at the resin- dentine bonding interface (Fig. 5A1) and along the dentine several microns (~100 µm) beneath that bonding interface of the specimens in the CTR adhesive/composite group. Notably, a strong fluorescence signal indicating some level of enzymatic activity was detected from the dentinal tubules. After TC, an increased fluorescence was noted at the hybrid layer and within the dentinal tubules just below the bonding interface. The specimens of the GIC group showed at T0 an evident fluorescence signal along the entire GIC-dentine interface and inside the dentinal tubules, indicating significant enzymatic activity (Fig. 5B1). After TC, the interface maintained its integrity, although the enzymatic activity remained consistent (Fig. 5B2). The specimens in the EXP group showed enzymatic activity in the dentinal tubules several microns (~50–80 µm) beneath the bonded interface (Fig. 5C1). Interestingly, no

increase in endogenous enzymatic activity was observed after TC, and the resin-bonding interface and hybrid layer remained free from fluorescence signal (Fig. 5C1). The hybrid layer exhibited only a weak fluorescence signal from the dentinal tubules in the EXP-PR group specimens. This indicates limited enzymatic activity at the bonding interface created with EXP-PR (Fig. 5D1). Following TC, there was almost no endogenous enzymatic activity along the entire resin-bonding interface (Fig. 5D2).

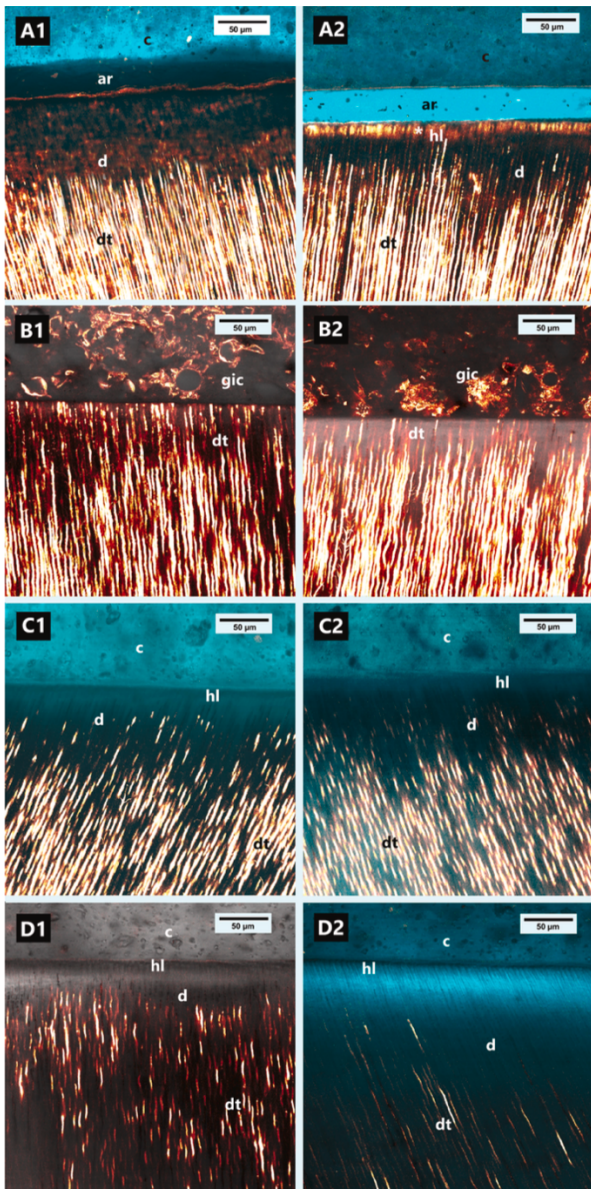


Fig. 5. Confocal microscopy - transmission/fluorescence images in situ zymography. Resin-bonded mid-coronal dentine interfaces prepared with CTR (A) GIC (B), EXP (C), PR-EXP (D), at baseline (1) and after thermocycling (2) showing fluorescence (identifying intense endogenous enzymatic activity). (d) → Dentine; (dt)→ Dentinal tubules; (hl) → Hybrid Layer; (c) → composite; (ad)→ Adhesive; (c)→ Composite; (gic)→ Glass Ionomer Cement.

A1: A slight fluorescence can be observed (Baseline) at the resin-dentine bonding interface (*) and along the dentine several microns (~ 100 µm) underneath that interface; at this latest distance from the interface, it is possible to see a strong fluorescence signal from the dentinal tubules.

A2: After thermocycling aging, an increased fluorescence can be observed at the hybrid layer and inside the dentinal tubules just under the bonding interface.

B1: An evident fluorescence signal can be seen along all the GIC-dentine interface and inside the dentinal tubules.

B2: Specimens after thermocycling - a similar fluorescence signal can be seen along all the GIC-dentine interfaces and inside the dentinal tubules.

C1: This resin-bonding interface and the hybrid layer appear free from any fluorescence signal; the enzymatic activity can be seen only in the dentinal tubules several microns (~ 50–80 µm) underneath that interface.

C2: Specimens after thermocycling - no increase in endogenous enzymatic activity is visible, and the resin-bonding interface, as well as the hybrid layer are free from any fluorescence signal. Also, in this case, the dentinal tubules several microns (~ 50–80 µm) underneath that interface present a clear fluorescence.

D1: The resin-bonding interface and the hybrid layer also in this case are free from any fluorescence signal, while the fluorescence signal from the dentinal tubules is quite weak.

D2: Specimens after thermocycling - it is possible to note almost no endogenous enzymatic activity along the entire resin-bonding interface, with only very few dentinal tubules showing a slight fluorescence signal.

Gelatin zymography

Gelatin zymography demonstrated a modest activity of pro- and active forms of MMP-2 and MMP-9 in the control – demineralized dentine group. There was a slight increase in the activity of all the enzymes in the dentine powder treated with the biomimetic primer (PR), while the FDCP-containing water solution silenced all the endogenous enzymatic activity. On the other hand, the dentine treated with the combination of PR and FDCP restored the level of activity previously measured in the control group. In general, the active form of MMP-2 presented the strongest bands in all the tested groups (Fig. 6).

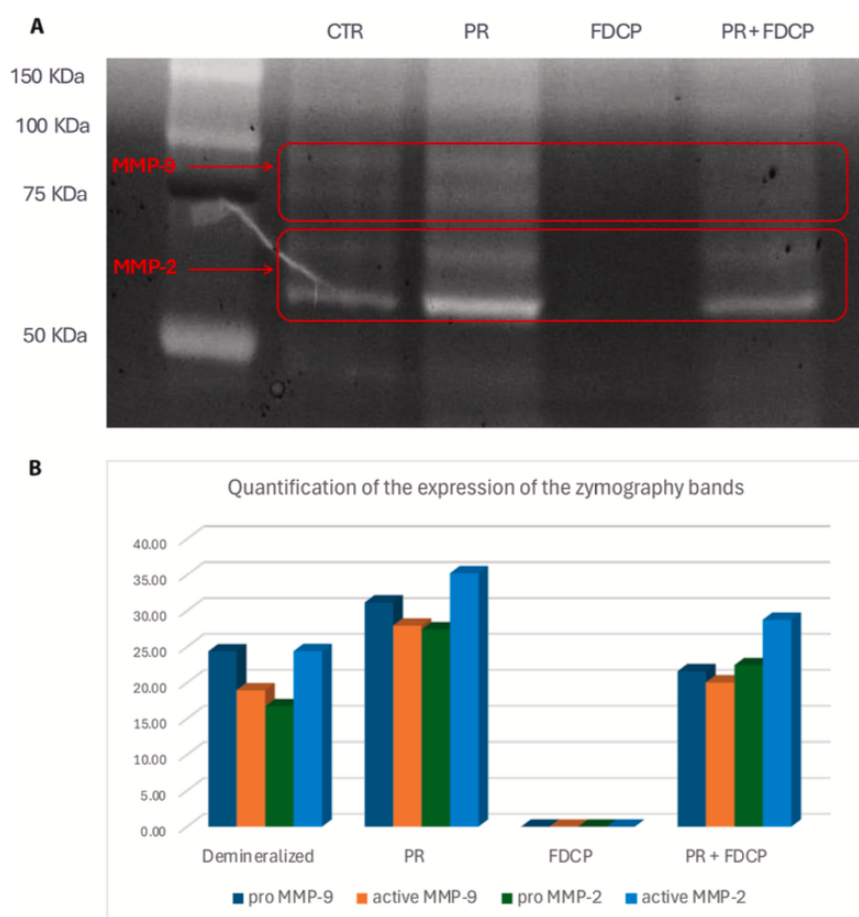


Fig. 6. Gelatin zymography. A) Gel electrophoresis; B) Quantification of the expression of pro- and active forms of MMP-2 and MMP-9. CTR – control group (demineralised dentine); PR – biomimetic primer; FDCP – fluoride-doped calcium phosphates water solution.

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DISCUSSION

The continuous evolution of adhesive dentistry has led to the development of UAs designed to simplify clinical procedures while ensuring reliable performance under a wide range of operative conditions. Despite these advances, achieving a durable and stable HL remains one of the major challenges in restorative dentistry, especially when the dentinal substrate has been modified by pre-treatments, endodontic procedures, or physiological aging. The four studies presented in this thesis explored different but complementary aspects that affect the long-term integrity of the adhesive interface, namely: the desensitizing and cross-linking effect of *Gluma* on DH, the clinical and structural benefits of the IDS technique, the influence of endodontic irrigants on adhesive behaviour, and the promising role of bioactive materials in enhancing remineralization and biological stability.

The first investigation focused on the mechanism of *Gluma Desensitizer*, a formulation based on glutaraldehyde and hydroxyethyl methacrylate (HEMA). The findings confirmed its ability to effectively occlude dentinal tubules through protein coagulation induced by glutaraldehyde reacting with plasma proteins and collagen fibrils within the dentinal tubules. This mechanism reduces dentinal fluid movement, an essential factor in controlling DH, while also promoting cross-linking between glutaraldehyde and exposed collagen fibrils. Such cross-links can stabilize the collagen matrix, decrease its permeability, and enhance its resistance to enzymatic degradation mediated by MMPs and cysteine cathepsins CTs (Breschi et al., 2018; Tjäderhane et al., 2013). When used prior to universal adhesives, *Gluma* therefore acts not only as a desensitizing agent but as a functional dentin primer, improving HL quality and contributing to long-term bond preservation (Stawarczyk et al. 2011). These biochemical effects may explain the reduced nanoleakage and improved mechanical stability observed when *Gluma* pre-treatment is combined with both ER and SE universal systems (Pashley et al. 2011).

Parallel to these findings, the IDS technique has emerged as an evidence-based clinical strategy to protect freshly cut dentin and maintain bond integrity during indirect restorative procedures. The concept, introduced by Magne and Belser (2002) and later substantiated by Magne et al. (2007, 2014), involves applying an adhesive resin immediately after tooth preparation to achieve optimal hybridization before bacterial contamination or dentinal dehydration occur. Numerous laboratory and clinical studies have demonstrated that IDS results in significantly higher bond strength, marginal adaptation, and reduced microleakage compared with delayed

dentin sealing (Magne, 2014; Van den Breemer et al., 2019; Hardan et al., 2021). These improvements are primarily attributed to the polymerization of the adhesive on a pristine dentinal surface, which allows for deeper monomer infiltration and the formation of a more homogeneous HL, thus limiting nanoleakage and hydrolytic degradation (Breschi et al., 2018). Additionally, IDS minimizes post-operative sensitivity by sealing the dentinal tubules immediately after preparation, stabilizing dentinal fluid dynamics, and preventing bacterial penetration. When integrated with UAs containing functional monomers such as 10-MDP, IDS further enhances chemical bonding to hydroxyapatite and promotes the formation of stable calcium-phosphate nanolayers, reinforcing the adhesive interface at both micromechanical and chemical levels (Van den Breemer et al., 2019; Maravic et al., 2021). This synergy highlights IDS as a highly effective and clinically relevant approach to optimize the performance and durability of universal adhesive systems in modern indirect restorative workflows.

A third component of this thesis addressed the complex interaction between endodontic irrigants and the adhesive performance of dentin bonding agents. Irrigants such as sodium hypochlorite (NaOCl), ethylenediaminetetraacetic acid (EDTA), and chlorhexidine (CHX) are indispensable for root canal decontamination, yet they can significantly alter the chemical composition and surface energy of dentin. NaOCl, while an effective organic tissue solvent, can denature collagen and leave an oxygen-rich layer that inhibits adhesive polymerization. EDTA, by contrast, provides gentle chelation of the mineral phase while preserving collagen fibrils, facilitating better interaction with SE adhesives (Breschi et al., 2020). The data obtained confirmed that post-irrigation residues or alterations in dentin wettability may impair monomer diffusion and polymerization kinetics, leading to decreased bond strength and greater interfacial degradation (Santos et al. 2006; Toledano et al. 2002). Consequently, understanding the interaction between irrigation protocols and adhesive systems is essential for achieving predictable adhesion in endodontically treated teeth, particularly when universal adhesives are used as pre-endodontic sealing or coronal sealing materials.

The final part of this research explored the emerging field of bioactive adhesive materials, a rapidly expanding area within restorative dentistry. Bioactive materials are defined as those capable of eliciting specific biological responses at the interface with tissues, typically through the release of therapeutic ions such as calcium, phosphate, and fluoride, which stimulate the precipitation of apatite-like crystals (Hench, 2015; Cadenaro et al., 2023). In adhesive dentistry, such materials may remineralize demineralized dentin, restore ionic equilibrium, and counteract the progressive

hydrolytic and enzymatic degradation of the HL. The incorporation of bioactive components into adhesives or resin cements thus marks a conceptual transition, from passive bonding systems to interactive materials capable of promoting tissue regeneration and long-term stability. Moreover, ion-releasing adhesives exhibit antibacterial activity, reduce acidogenic biofilm formation, and may assist in maintaining pulpal vitality in deep cavities or near pulp exposures. However, despite their promising potential, a clear consensus regarding the standard definition and testing of bioactivity in dental materials remains elusive (FDI, 2022). The scientific community continues to emphasize the need for robust in vitro and clinical data before bioactive claims are accepted as evidence-based (Breschi 2018 ; Braga et al. 2020).

Collectively, the results from these four studies underscore the multifactorial nature of adhesion to dentin. The durability of the HL is not determined exclusively by the chemistry of the adhesive but rather by a delicate interplay of substrate conditions, biological activity, and environmental factors. Pretreatment with glutaraldehyde-based agents enhances matrix stability by creating additional covalent cross-links within the collagen fibrils; the IDS technique ensures immediate hybridization and protection of the freshly cut dentin surface from contamination; endodontic irrigation protocols critically influence monomer infiltration and polymerization; and bioactive systems contribute to remineralization, enzymatic inhibition, and overall interface stability.

UAs, by combining hydrophilic and hydrophobic monomers, functional phosphate groups such as 10-MDP, and compatibility with multiple bonding strategies, represent a versatile platform capable of integrating all these complementary mechanisms. Their multifunctional role as bonding agents, sealers, and bio-interactive mediators positions them at the forefront of restorative material innovation, bridging the gap between traditional adhesion science and regenerative dentistry.

From a translational perspective, these findings emphasize that the longevity of adhesive restorations cannot rely solely on the intrinsic properties of the adhesive resin but must also consider the dynamic biological environment of dentin. Strategies that target enzymatic inhibition, collagen stabilization, and remineralization represent a paradigm shift from purely mechanical retention toward biologically integrated adhesion. This new perspective supports the concept of a “*biofunctional HL*,” capable not only of resisting degradation but also of interacting beneficially with surrounding dental tissues.

CONCLUSIONS

The collective evidence presented in this thesis highlights that the long-term success of adhesive restorations depends on both the chemical performance of the adhesive system and the biological characteristics of the dentinal substrate. Glutaraldehyde-based desensitizers such as *Gluma* not only reduce dentinal hypersensitivity but enhance collagen cross-linking and hybrid layer resistance. The IDS technique achieved with different universal adhesives offers superior bonding by allowing early and controlled hybridization. Optimizing endodontic irrigation protocols prevents adverse chemical interactions and supports adhesive polymerization, ensuring consistent dentin sealing. Meanwhile, the advent of bioactive materials introduces a regenerative dimension, fostering remineralization, antibacterial protection, and pulpal biocompatibility. Taken together, these findings point towards a future where adhesive dentistry evolves from being purely restorative to becoming bio-functional, integrating adhesion, protection, and regeneration within a single clinical workflow. UAs, when combined with evidence-based pretreatments and bioactive innovations, have the potential to achieve more predictable, durable, and biologically stable bonding outcomes.

Future research should continue to focus on optimizing the chemical composition and nanostructure of UAs to promote ion release and bioactivity without compromising polymerization or mechanical properties. Moreover, clinical studies with long-term follow-up are needed to validate *in vitro* results and to establish standardized protocols for evaluating bioactive performance. The integration of nanotechnology, smart delivery systems, and biomimetic principles may further advance the development of next-generation adhesives that are not merely inert bonding agents, but active participants in maintaining the health and integrity of the dentin-resin interface.

Ultimately, this line of investigation contributes to a broader vision of adhesive dentistry, one that moves beyond the pursuit of immediate bond strength toward the creation of stable, self-protective, and biologically interactive adhesive interfaces. Such progress represents a crucial step toward the long-term success and predictability of adhesive restorations, aligning restorative materials more closely with the natural resilience and regenerative potential of dental tissues.

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