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The role of MMPs inhibitors in the stability of the adhesive interface

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Contents

ABSTRACT

Aims: This thesis aimed to investigate the influence of several protease inhibitors/inactivators, as separate primers or blended within the adhesive resin, on the longevity of dental restorations and on the dentinal enzymatic activity immediately, or after aging *in vitro*. **Methods:** A series of studies was conducted using several different MMPs inhibitors and several adhesive systems. The first three studies investigated the longevity of the hybrid layer by means of microtensile bond strength test, and the enzymatic activity using gelatin and *in situ* zymography, immediately or after 1 year of aging in the artificial saliva. Study 4 tested samples bonded with or without an inhibitor-containing-primer, that were previously aged for 10 years. The degradation of the hybrid layer was observed using transmission electron microscopy, the enzymatic activity in the hybrid layer using *in situ* zymography. Raman spectroscopy was used to investigate whether the active substance was still within the hybrid layer after 10 years. **Results:** The results of the studies showed that the inhibitors/inactivators of endogenous dentinal enzymes were efficient in preserving bond strength after aging *in vitro* when used as separate primers. In the cases when the inhibitory agent was introduced within the adhesive resin, bond preservation was adhesive system- and/or bonding mode-dependent. The enzymatic activity was lower in all experimental groups, at baseline, as well as after aging (for 1 year and 10 years) with a slight influence of the bonding mode. **Conclusions:** The tested protease inhibitors used as separate aqueous primers in etch-and-rinse adhesives seem to be clinically applicable, since the procedure is not overly time-consuming and seems to preserve the hybrid layer over time. As for the inhibitors blended within adhesives, comprehensive studies on the mechanical properties of the adhesives as well as their influence on the longevity of the hybrid layer should be performed before clinical use.

Keywords: Dentin; collagen; enzyme inhibition; hybrid layer; metalloproteinases

INTRODUCTION

"It is said that if you know your enemies and know yourself, you will not be imperilled in a hundred battles; if you do not know your enemies but do know yourself, you will win one and lose one; if you do not know your enemies nor yourself, you will be imperilled in every single battle."

Sun Tzu

Oral diseases and their treatment have high social costs. Tooth decay is in fact the second reason of absence from work after respiratory diseases and affects nearly 100% of the population of developed countries. Considering that the life span is nowadays increasing, there is a large population of the elderly with a poor quality of life due to edentulousness. The estimated worldwide expenditures due to dental diseases amounts to 356.00 billion dollars, with the Western European countries accounting for 28% of this amount. Alarmingly, Italy is among the five countries with the highest expenditures in the World, with 14.92 billion dollars of direct costs. Furthermore, Italy is one of the ten countries with the highest indirect cost of dental diseases due to productivity loss (4.86 billion dollars). Discovering and explaining the mechanisms responsible for these diseases and the possibility of developing better preventive, diagnostics and therapeutic clinical protocols has a huge potential in terms of reducing social costs, decreasing the psychological and financial load and consequently improving patients' life quality.

The adhesion of a direct composite restoration depends on the quality of the micromechanical and, in certain adhesive systems also chemical bond between the adhesive resin and dentin. Adhesion to dentin could be considered as bioengineering and relies on the formation of a layer which contains both dentin and the adhesive resin – the hybrid layer. It is formed when a superficial layer of dentin is demineralized, either by a separate etching step, or in the case of self-etching adhesives, due to the low pH of the primer or the adhesive itself. After demineralization, dentin collagen fibrils are denuded, and ideally, the adhesive resin should penetrate the fibrils and envelope them fully, forming a stable hybrid layer. Aiming to improve the longevity of dental restorations, it is necessary to first unveil the reasons behind their failure, understand in detail all the constituents of the hybrid layer, their biomechanical and chemical properties, possible interactions between them, and the reasons why they degrade. Otherwise, all efforts would be in vain.

Since neither etch-and-rinse (E&R) nor self-etch (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. In the demineralized dentin, endogenous proteases, mainly, matrix metalloproteases (MMPs) and cysteine cathepsins are reactivated, and in the presence of water they can hydrolyze collagen fibers. MMPs are proteases dependent of zinc and calcium ions, which are required to maintain their active state. The true collagenases, MMP-1, -8, -13 and -18, are responsible for collagen degradation. However, they cannot initially bind the collagen triple helix since the binding position is protected by telopeptides. The telopeptidases (MMP-2 and MMP-9) remove the protective telopeptides which facilitates the unwinding of the triple helix and the collagenolytic activity of true collagenases. Therefore, inhibition/inactivation of the MMPs is considered a very important strategy for the preservation of the hybrid layer.

There have been numerous reports on the beneficial effect of protease inhibitors on the longevity of the hybrid layer. Primarily, agents that are able to influence the activity of endogenous dentinal enzymes can be enzyme inhibitors – via a chelating mechanism, and collagen cross-linkers, reported to reinforce the collagen structure and recently also to inactivate the dentinal proteases by changing their molecular mobility.

With the vast array of possibilities investigated in the literature, a confusion could be created in dental practitioners. Hence, there is a necessity for the formation of clear, clinically applicable protocols, that show predictable and favorable results even after long-term intraoral use.

Adhesion to dentin

Adhesion to dentin could be considered as bioengineering *in situ* and relies on the formation of a layer which contains both dentin and the adhesive resin intertwined – the hybrid layer (Breschi et al., 2008; D. H. Pashley et al., 2011). Adhesive systems laid ground for contemporary dentistry and shifted the development of materials and therapeutic concepts in a new direction. Due to rapid technological development, nowadays there is a vast array of adhesive systems available on the market. The developments have aimed to resolve the most important weakness of the adhesive-dentin interface – susceptibility to hydrolytic degradation, as well as to simplify the adhesive procedure. There are three main adhesive concepts: the etch-and-rinse (E&R), the self-etch (SE), and the universal adhesive systems. They differ in terms of composition, acidity, their interaction with dentin, and the clinical protocols. They can be applied in one, two or three clinical steps (D. H. Pashley et al., 2011; Van Meerbeek et al., 2011).

Etch-and-rinse adhesive systems

The first requirement of this system is the demineralization of the superficial layer of dentin which removes the smear layer, exposes the organic matrix and makes it permeable for the adhesive resin. The dental substrate is etched with 35-37% phosphoric acid and thoroughly rinsed with water (Pashley et al., 1993). After dentin demineralization, collagen fibril network is exposed, surrounded by water and fragile. The specific requirement of handling demineralized dentin is not to overly dry it. In case this happens, the collagen fibrils collapse, and the adhesive resin cannot penetrate the organic network. Hence, etched dentin should be only blot dried and water-wet bonding should be applied, as suggested for the first time by Kanca et al. (Kanca, 1992). Since this layer is hydrophilic and the adhesive monomers are hydrophobic, the primers are used to increase the wettability of collagen fibrils with hydrophobic resin. The primers are amphiphilic, meaning that they contain both hydrophilic and hydrophobic monomers. In 3-step E&R systems, the primer is

in a separate bottle and facilitates the penetration of the third part of the system – the hydrophobic adhesive resin (Swift et al., 1995). The 3-step E&R system is considered as the gold standard in this group, since it showed the best mechanical properties and bond strength to dentin if used in a proper way (De Munck et al., 2003). Another advantage of the 3-step system is that therapeutics could be implemented into etchant, primer and/or adhesive, which could render the system antimicrobial or anti-enzymatic, or both (D. H. Pashley et al., 2011). The downside of the 3-step system is that it is very technique-sensitive. Whenever a system contains several steps that need to be performed in a specific way, the possibility of error increases. Therefore, simpler E&R systems, 2-step, have been implemented, combining the primer and adhesive into one bottle. These adhesives are applied in two layers, with the first one serving as the primer (D. H. Pashley et al., 2011). However, the monomers in these blends are not miscible with water and are therefore dissolved in acetone or ethanol, but since demineralized collagen is comprised of approx. 70% water, the interdiffusion of these two different materials is not adequate. Hence, it was necessary to add a certain percentage of hydrophilic monomers, such as two-hydroxyethyl methacrylate (HEMA), into the adhesive blends in order to improve the interdiffusion with dentin (Swift et al., 1995; Van Meerbeek et al., 2005). However, the incorporation of the more hydrophilic monomers into these systems has rendered them more permeable and prone to hydrolytic degradation. Enamel bonds with E&R systems are more durable compared to dentin bonds (Reis et al., 2009). If *in vitro* bond strength test was applied to teeth that were restored and aged as intact resin-bonded crowns, and cut into sticks only before testing, the bond strength was preserved possibly due to the protective effect of peripheral enamel seal. If, on the other hand, the teeth were restored, immediately cut and aged as sticks, the bond strength deteriorated significantly (De Munck et al., 2003; Gamborgi et al., 2007). The protective properties of the peripheral enamel seal however, were shown to be less important *in vivo*, since clinical studies showed deterioration of the adhesive layer in class I

restorations, probably due to functional and thermal stresses (Carrilho et al., 2007c; Hebling et al., 2005).

Self-etch adhesive systems

In the SE systems on the other hand, there is no separate etching step, since the primers and/or adhesives are acidic, and they simultaneously etch dentin and penetrate the collagen network which should provide better resin infiltration compared to the E&R systems (Breschi et al., 2004, 2003b; Spencer et al., 2000). Further, there is significantly less post-operative sensitivity after bonding with SE compared to E&R adhesives (Franklin R Tay et al., 2002a). Depending on the aggressiveness of the system and the method of dentin preparation, SE hybrid layers contain parts of the smear layer including the smear plugs that prevent dentinal fluid from wetting the surface during bonding procedures (Koibuchi et al., 2001; Oliveira et al., 2003). With regard to the chemical composition and acidity, SE adhesives interact differently with dentin and create hybrid layer thickness of a few hundred nanometers (ultra-mild SE approach, pH > 2.5), around 1 µm (mild SE approach, pH ~2), 1-2 µm (intermediately strong SE approach, pH between 1 and 2), and several micrometers deep (strong SE approach, pH≤1) (Koshiro et al., 2006). There are 2-step systems, which contain a primer and an adhesive resin, and there are one-bottle systems. As in the case of E&R adhesives, the golden standard is the more "complex" system, since it prepares the dentinal tissue for adhesion in a more adequate way. The SE adhesives also contain functional monomers which can bind to dentin chemically, and their adhesive performance depends on the affinity of these monomers towards hydroxyapatite (Van Meerbeek et al., 2011). For instance, a functional monomer 10-methacryloyloxydecanethylene phosphoric acid (MDP), incorporated in the Clearfil SE (Kuraray) has the ability to bind to hydroxyapatite chemically, reinforcing the adhesive bond. The Adhesion-Decalcification concept explains that in the reaction of SE adhesives with dentin, the acidic component of the adhesive initially always binds to calcium, causing the release of phosphate

($PO4^{3-}$) and hydroxide (OH^-) ions from hydroxyapatite. The strength of the bond between the acid and the calcium will determine if demineralization, or chemical bonding to hydroxyapatite will occur. In mild SE adhesives with MDP, chemical bonding will take place. On the other hand, when the strong ones are used, the surface will demineralize (Yoshida et al., 2001; Yoshioka et al., 2002). In general, SE bonds show adequate durability, but are still prone to loss of bond strength over time (Tjäderhane et al., 2013b).

Universal adhesive systems

The universal adhesive systems are more similar by composition to the SE systems and can be used both in the E&R and SE mode, providing more versatility to clinicians. They also contain functional monomers as the SE systems and are more acidic compared to the E&R adhesives (Hanabusa et al., 2012; Inoue et al., 2005; Toledano et al., 2001; Yoshida et al., 2000). Depending on the system, they can contain a separate primer, or be one-bottle-systems. There is still scarce evidence on bond strength of universal adhesives compared to the standard systems. So far, it seems that if used in SE mode, they present better bond strength after aging, than in the E&R mode (Marchesi et al., 2014). Further, in comparison to SE or E&R systems, the results were material-dependent. Some systems performed similarly as the controls, while others performed worse (Muñoz et al., 2013).

Different adhesive systems interact with dentin in specific ways, creating hybrid layers whish are microstructurally distinct, as observed using scanning electron microscopy (SEM) or transmission electron microscopy (TEM) (**Figure 1**). These differences were also investigated by means of the gold immunolabeling technique (Breschi et al., 2004). Resin-infiltrated dentin specimens, both in E&R and SE mode, were incubated with a primary anti-collagen type I antibody and a secondary gold-conjugated antibody, to examine gold labelling under SEM. The E&R adhesive showed weak gold labeling in the first half of the hybrid layers, while the bottom half showed intense gold labeling. This indicates that the deepest parts of the hybrid

layer in the E&R adhesives are not fully infiltrated by the adhesive resin. On the other hand, the hybrid layer created with a SE adhesive system showed a uniform weak labeling throughout the resin-dentin interface, signaling better homogeneity. Interestingly, the distribution of the areas of interfacial nanoleakage in E&R and SE system correlated with the presented immunolabeling, demonstrating that there are more nanoporosities in the bottom of the hybrid layer, especially in the E&R adhesives, marking the initial points of hybrid layer degradation (Suppa et al., 2005).

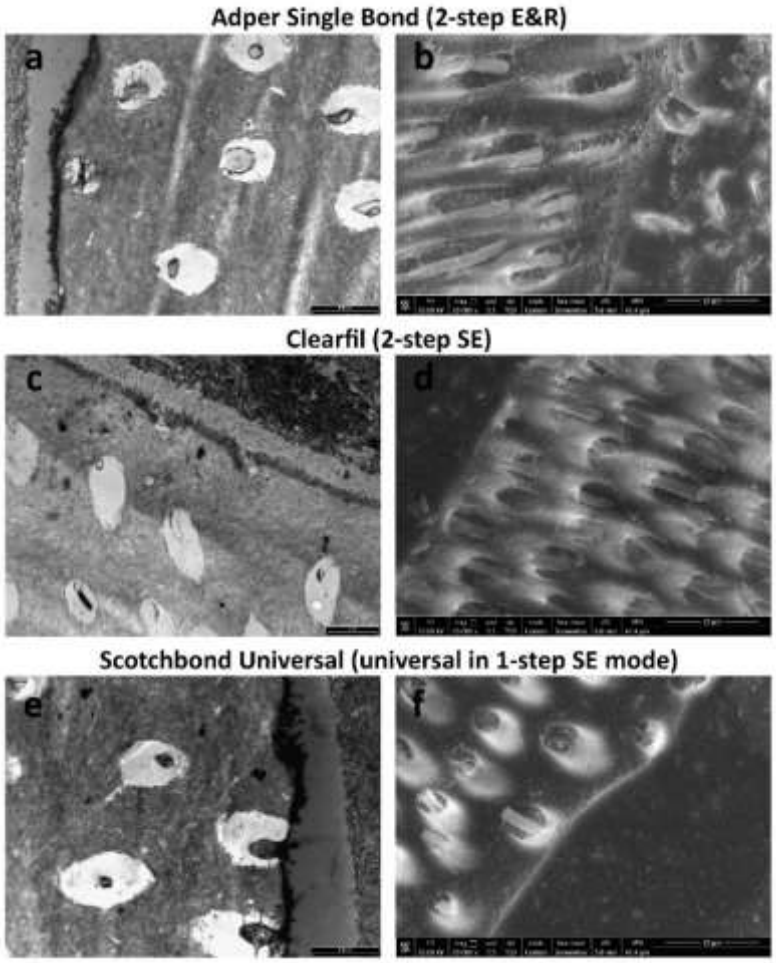

Figure 1. Hybrid layer formed with different adhesive systems as presented on TEM (a, c, e) or FEI-SEM (b, d, f): (a, b) Adper Single Bond, 3M Espe (2-step E&R); (c, d) Clearfil SE, Kuraray (2-step SE); (e, f) Scotchbond Universal (universal adhesive in 1-step SE mode).

Dentin structure in relation to bonding

The composition and structure of dentin varies in different parts of the tooth due to its tubular structure. Overall, it is composed of approximately 70 weight % or 45 volume % of mineral tissue, and 20 weight % or 33 volume % of the organic tissue, with water as the remaining fraction (Nanci, 2008; Tjäderhane et al., 2009).

The presence of tubules is the main characteristic of dentin. This characteristic of the dental tissue influences its mechanical behavior, permeability and bonding properties (Carvalho et al., 2012; Tjäderhane et al., 2009). Since dentinal tubules are in a radial disposition, and of a conical shape with the wider part facing the pulp, the deeper the dentin – the larger is the surface occupied by dentinal tubules. Hence, superficial dentin presents a higher surface of intertubular dentin, that is mainly organic matrix constituted of collagen fibrils, unlike peritubular dentin, which is high in mineral content. Both intertubular dentin and the tubules are equally important for the adhesion to dentin due to the interdiffusion with the adhesive resin (Gwinnett, 1993; Pashley et al., 1995; Tay and Pashley, 2004). Hence, regional variations in dentin structure could influence the quality of adhesion. Further, the surface water content is higher in deeper dentin, leading to lower bond strengths (Prati and Pashley, 1992). Different factors, such as aging, carious and reparatory processes, preparation of dentin, proximity to the pulp tissue, use of diverse chemical cleaning or whitening agents, can all influence bonding to dentin significantly (Carvalho et al., 2012).

Given that in clinical settings, the dentinal substrate available for bonding is rarely completely sound, it is important to stress out the differences in the bonding quality in these cases. Depending on the clinical situation, caries removal method, the extent of the removal of carious dentin, often governed by preferences and personal experience of the operator, the dentinal substrate available for bonding could be partially caries-infected and/or -affected (de Almeida Neves et al., 2011). The caries process causes structural changes in the dentin. The mineral content and

crystallinity of the hydroxyapatite are reduced, and are followed by the changes in the structure of collagen (Wang et al., 2007). Hence, the mechanical properties of dentin are hampered, which influences adhesion to carious dentin. The hybrid layers in caries-infected and -affected dentin have been reported to be thicker and poorly infiltrated by the bonding agent (Hsu et al., 2008). Furthermore, carious dentin contains a higher percentage of water (Ito et al., 2005), making the hybrid layer more prone to hydrolytic degradation. Polymerization of dental adhesives on caries-affected dentin has also been shown to be less efficient compared to sound dentin (Spencer et al., 2005). The majority of the studies investigating bonding to dentin *in vitro* have been performed on sound dentin, which might be less relevant form the clinical standpoint, since an operator is most often faced with cavities which include an array of different substrates, from enamel, to sound dentin, caries-infected or -affected dentin, or sclerotic dentin. The available *in vitro* research on bonding to caries-affected dentin showed a 20-50% lower bond strength of this substrate compared to sound dentin (Ceballos et al., 2003; de Almeida Neves et al., 2011; Yoshiyama et al., 2002). Further, the immediate bond strength in caries-affected dentin seems to be higher when E&R adhesives are used compared to SE systems (Erhardt et al., 2008; Yoshiyama et al., 2000). However, these differences seem to disappear after short-term aging (Erhardt et al., 2008). Long-term aging studies are necessary to further investigate this matter. On the other hand, long-term clinical retrospective studies have demonstrated a high level of success of posterior composite restorations over time (Da Rosa Rodolpho et al., 2011; Opdam et al., 2010), regardless of the fact that the majority of the posterior cavities contain a portion caries-affected dentin. This disparity could be due to the fact that the cavities also contain, as previously mentioned, enamel and sound dentin, superior bonding substrates, which are usually the structures directly exposed to the oral cavity, with the caries-infected/-affected dentin being in the deeper portions of the cavity. Altogether, this could enhance the clinical durability of the restorations.

Sclerotic dentin is another specific dentinal substrate to be considered in terms of adhesive bonding. It is characterized by a superficial layer of hypermineralized dentin, often containing trapped microorganisms, under which is a layer of dentin with denatured collagen fibrils. The tubules are filled with mineral crystallites (Tay and Pashley, 2004). All these features influence bonding properties of sclerotic dentin. Bond strength measured on this substrate is lower compared to sound dentin (Kwong et al., 2002), probably due to the fact that the standardized etching procedures are inadequate in this case and should be modified. It has been shown that resin tags and intertubular dentin hybridization contribute to bond strength about 20% each (Gwinnett, 1993; Pashley et al., 1995; Tay and Pashley, 2004). Due to the specific structure of sclerotic dentin this hybridization is significantly reduced, which could be the cause of poor bond strength. A mere prolongation of etching time showed an improvement in some but not in all the tested adhesives (Lopes et al., 2004), indicating that this solution time might not be sufficient to resolve issues such as bacterial infiltration or denatured collagen fibrils.

Hence, it is clear that the dentin is a highly variable dynamic tissue, and it is therefore difficult to control bonding on dentin and to predict the longevity of the hybrid layer in each individual case. To better understand the mechanisms of adhesion to dentin, and its limitations, it is necessary to understand its molecular structure and organization.

Dentin collagen structure

The majority of the dentin extracellular matrix (90%) is formed of collagen type I. Collagen molecule is formed by three strands of amino acids – two α1 and one α2 strand. Each of these strands forms a left-handed helix around its own axis. Further, these three strands intertwine into a right-handed triple helix, forming the collagen molecule (300 kDa, 300 nm of length, 1.5 nm diameter, containing around 1000 amino acids) (Gelse et al., 2003; Shoulders and Raines, 2010). The central triple-helical region comprises the largest part of the chain, over 95%, while the non-

helical regions – carboxyterminal (C-terminal telopeptide) and aminoterminal (N-terminal telopeptide) comprise the remaining 5% of the chain (Yamauchi and Shiiba, 2008). The collagen molecules form fibrils by joining in bundles along their long axes. Between the neighboring collagen chains, there is a gap of 67 nm (Veis, 2003). This structure is connected by inter and intramolecular cross-links in such a way that the C-terminal region of one collagen molecule reacts with the N-terminal region of the adjacent molecule. The cross-links render dentin collagen extremely resilient. For instance, it does not denature after 15 s of acid etching in dental procedures (35-37% phosphoric acid), while dermal collagen would not withstand this treatment (Breschi et al., 2003b, 2003a; Oyarzún et al., 2000). Over-etching could, however, induce structural changes in the collagen molecules (Breschi et al., 2003b) as well as proteoglycans (PGs) (Hedbom and Heinegård, 1993; Scott, 1988) and should therefore be avoided. Dentin collagen is not degraded easily, but once it is, it cannot be replaced, since it does not turn over (Schlueter and Veis, 1964).

Collagen fibrils represent the pillar of the extracellular matrix and are perpendicularly connected with non-collagenous proteins, most importantly the PGs (Breschi et al., 1999; Lin et al., 1993). The PGs consist of a core protein, glycosaminoglycans (GAGs) and linkage proteins (Goldberg and Takagi, 1993) and help maintain the three-dimensional integrity of the collagen fibril bundles (Cheng et al., 1999; Goldberg and Takagi, 1993). Furthermore, the PGs are involved in the process of dentin mineralization and can regulate the affinity of collagen to water by binding and organizing water molecules. This can affect the substitution of water during bonding procedures (Hedbom and Heinegård, 1993; Oyarzún et al., 2000; Scott, 1988; Vogel et al., 1984). The three-dimensional relationships between the different parts of the ECM have been investigated and revealed using precise selective immunolabeling protocols (Breschi et al., 2003a, 2003b, 2002; Oyarzún et al., 2000). **Figure 2** shows partially demineralized dentin with a distinct collagen network in the intertubular and peritubular dentin as presented in SEM.

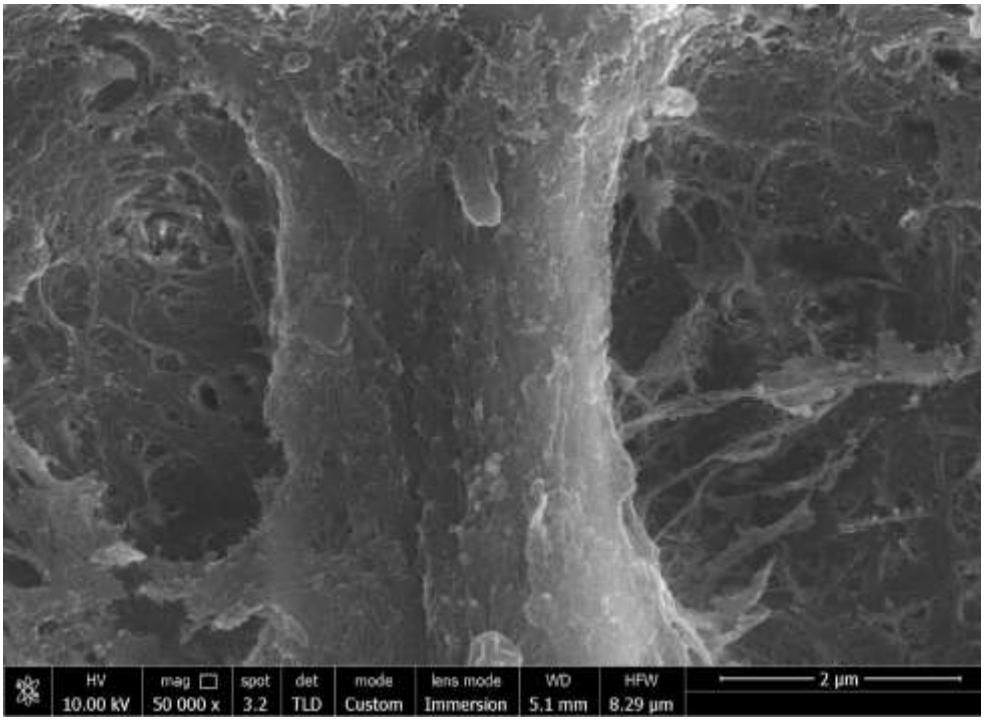

Figure 2. Partially demineralized dentin peri- and intertubular collagen – FEISEM

Degradation of the hybrid layer

Since the hybrid layer consists of different components, deterioration of each of these components can lead to the degradation of the whole system. Primarily, the hybrid layer degrades due to hydrolysis and disorganization of the collagen fibrils and of the adhesive resin (Breschi et al., 2018).

Degradation of the adhesive resin

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., 2008; Tay and Pashley, 2003). 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 (Franklin R Tay et al., 2002b; Franklin R. 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., 2011b). 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 hybrid layer. 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). It has been reported that the time indicated by the manufacturers for the evaporation of the solvent are insufficient and should be prolonged (Milena Cadenaro et al., 2009; Ikeda et al.,

2008, 2005). 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 the collagen fibrils

Since neither E&R 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, matrix metalloproteases (MMPs) and cysteine cathepsins are reactivated (Hannas et al., 2007; Y. Liu et al., 2011; Nascimento et al., 2011; Vidal et al., 2014), and in the presence of water they can hydrolyze collagen fibrils (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” that maintains the enzyme in the inactive form. MMPs also contain other domains which determine substrate specificity and other interactions (Hannas et al., 2007). The MMPs are secreted in the 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-form into the 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; Sulkala et al., 2007), stromelysin MMP-3 (Mazzoni et al., 2011a), enamelysin MMP-20 (Shimada et al., 2009; Sulkala et al., 2007) and most recently MMP-7 (Mazzoni et al., 2018b). 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 (Tjäderhane and Haapasalo, 2009). After the demineralization of dentin, these protases 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 hybrid layer (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; Mazzoni et al., 2015, 2012).

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 ¼ and ¾ 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, collagenases cannot initially cleave the collagen triple helix since the binding position is sterically blocked by C-telopeptides, making the 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 gelatin zymography in dentin powder (Mazzoni et al., 2007). The same research team further demonstrated that the MMPs can be activated by acid etching, E&R 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 hybrid layer (Breschi et al., 2018; Tjäderhane et al., 2013b).

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 and B in dentin have recently been described using immunolabeling technique (Scaffa et al., 2017). Cathepsins K and B 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 CTs in active and chronic carious lesions (Nascimento et al., 2011). The activity of cathepsins is more pronounced as the depth of the cavity increases. Hence, it is reasonable to believe that the odontoblast-secreted cathepsins have implications in the active caries lesions. The increase of the activity of cysteine cathepsins 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. 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 (Brömme and Wilson, 2011). "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 (5 Å) to accommodate the whole collagen molecule (15 Å), 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, inhibit it. 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 ¼ and ¾ 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).

Strategies to reduce hybrid layer degradation

As explained previously, on the bottom of the hybrid layer the denuded collagen fibrils surrounded by water are not fully enveloped in resin, enabling collagen degradation by endogenous proteases, and leading to plasticization of the adhesive resin, and mechanical strain on the exposed fibrils. Sharing a common foe, loss of collagen and resin plasticization deteriorate in the presence of water, with collagen degradation possibly preceding the loss of resin. After the initiation, the

process enters a vicious cycle, since the degradation of collagen may accelerate resin plasticization, causing in turn, due to leaching of the resin, further collagen degradation and the increase in water flow. Hence, there are several bonding strategies designed to reduce adhesive interface degradation, aiming either to eliminate water between the exposed collagen fibrils, thereby preventing their degradation, or prevent the loss of collagen either by collagenolytic enzyme inhibition or increasing collagen resistance to degradation.

Removal of the unbound/residual water within the HL

It is clear that water is the most notorious enemy of the longevity of the hybrid layer. In a biological setting such as dentin and the oral environment, it is impossible to fully eliminate the influence of water. However, it is possible to influence the presence and distribution of water in several key moments during the adhesive procedure, that could significantly reduce the negative influence of water on the long run, or at least postpone it.

The adhesive formulations containing dimethacrylates capable of creating strong, highly crosslinked resin polymers, are solvated in ethanol, since they are not miscible with water, and a water solution could lead to phase changes in the adhesive. This could further interfere with the monomer infiltration of the acid-etched dentin (Spencer and Wang, 2002). Hence, manufacturers produce their adhesive formulations in ethanol solvents to ensure the mixture is in a single phase (Spencer and Wang, 2002). However, placing an ethanol-rich hydrophobic adhesive resin onto the water-rich demineralized dentin leads to poor interdiffusion between these two components of the hybrid layer (D. H. Pashley et al., 2011). Over-drying acid-etched dentin is however highly unadvisable due to the collapse of the collagen fibrils, among other reasons explained previously. Hence, a change in the wet-bonding technique was proposed by Pashley et al. (Pashley et al., 2007) replacing rinse-water with ethanol prior to bonding. This could preserve the three-dimensional conformation of the collagen fibrils, while enabling infiltration of the

hydrophobic resin. Further, Tay et al. (Tay et al., 2007) reported excellent resin-dentin bonding by applying bis-GMA, the least soluble dimethacrylate, to ethanol-saturated dentin. This procedure prevents phase separations and reduces the presence of residual water in the resin-dentin bonds (Ayar, 2016). In the absence of water, matrix proteases cannot cleave collagen, the hydrophobic resins are less prone to hydrolytic degradation and this technique enables infiltration of more hydrophobic resins into the hybrid layer (Hiraishi et al., 2005; Shin et al., 2009). Although ethanol can remove and replace unbound water from demineralized dentin (Agee et al., 2015), there is still a certain percentage of bound water within the collagen fibrils. Molecular dynamic computer simulations were used to recreate the three layers of bound water in collagen matrices (Jee et al., 2016). It was confirmed that the first and second layers of tightly-bound water in collagen could not be replaced by ethanol, but 50% of the bound water in the outermost layer of bound water could. This could bring ethanol-solvated resin closer to the gap regions of collagen, possibly contributing to the inactivation of collagen-bound proteases.

Generally, there is approximately 70 vol% (D. H. Pashley et al., 2011) water in acid-etched dentin just prior to adhesive application. Another approach trying to reduce residual water in hybrid layers is the use of SE primers. As described previously, exposed collagen fibrils are better embedded into SE than the E&R adhesives (Breschi et al., 2004, 2003b; Spencer et al., 2000; Stape et al., 2015). The SE adhesive systems could be considered “dry bonding systems” since the primed dentin is only dried, not rinsed after “self-etching”, and is further sealed with a solvent-free adhesive. These primers contain acidic monomers such as 10-methacryloyloxydecanethylene phosphoric acid (MDP) in higher concentrations than used before for wet-bonding adhesives. They water is added in these primers just in a sufficient amount to allow the acidic monomers to become ionized, and to solubilize the mineral phase of dentin. These water concentrations are proprietary, but are generally in the range of 20-25 vol% (Hiraishi et al., 2005).

Another approach to control rinse-water or dentinal fluid flow involves the use of dimethyl sulfoxide (DMSO) as a primer or a solvent and due to low surface energy DMSO can be used as a solvent to facilitate radical polymerization reactions. Interestingly, it possesses the polarity needed to break down water's self-association and forms stable complexes with water to create "hydrophobic water" (Catalán et al., 2001; Vishnyakov et al., 2001). Recent studies have shown increase both in immediate and long-term bond strength (Guo et al., 2017; Stape et al., 2016b, 2016a, 2015; Tjäderhane et al., 2013a). Perhaps the increase in immediate bond strength is due to DMSO-induced improvement in dentin wettability (Mehtälä et al., 2017) and consequential better adhesive penetration (Stape et al., 2015). Better infiltration of the monomers could lead to longer preservation of resin-dentin bonds. Furthermore, DMSO might also have MMP-inhibitory effects (Tjäderhane et al., 2013a).

Calcium-chelation dry bonding

Can we use the three-dimensional conformation of the collagen fibril to preserve the fibrils mineralized while removing only the interfibrillar minerals? Recent research has revealed that collagen fibrils behave as if they were size-exclusion beads of Sephadex in column chromatography. While molecules with molecular weights smaller than 600 Daltons can enter collagen, molecules larger than 40 KDa are excluded (Chiba et al., 2016; Takahashi et al., 2013; Toroian et al., 2007). Phosphoric acid, with a molecular weight of 100 Da, is small enough to permeate throughout collagen fibrils, solubilizing both extra- and intrafibrillar mineral, leaving dentin completely demineralized and soft.

If the standard etchant is exchanged with a calcium chelator of a large molecular weight, (i.e. 15 wt% 225,000 Da), like sodium polyacrylate, then the chelator would be too large to permeate collagen. Only apatite mineral from the extrafibrillar space would be removed, creating interfibrillar spaces for inward monomer diffusion and uptake into the hybrid layer. Mai et al. tested this agent and after 30 sec of chelation,

the reaction was stopped by water rinsing and air-drying (Mai et al., 2017). This technique enables dry bonding since the completely mineralized fibrils are too stiff to shrink or collapse during the evaporation of residual water with strong air blasts (Mai et al., 2017). Furthermore, in this novel approach, the MMPs remain inactive within the mineralized fibrils.

Remineralization of hybrid layers

Biomimetic remineralization is a novel approach for the preservation of the hybrid layer which entails ion-releasing materials, intended to mimic the natural mineralization process. The water-filled regions in the incompletely infiltrated hybrid layers that can slowly solubilize the insoluble collagen fibrils. The infiltration of apatite could remineralize the collagen fibrils, and simultaneously fossilize the MMPs. Tay and Pashley (Tay and Pashley, 2008) showed that Portland cement in a phosphoprotein analog-containing fluid can form a meta-stable amorphous calcium-phosphate, which can deposit inside the collagen organic network in the form of apatite crystallites. Further studies confirmed this concept (Gu et al., 2011; J. Kim et al., 2010a; Y. K. Kim et al., 2010) and highlighted the importance of the presence of biomimetic analogs, since the mineralization achieved without the analogs was not in the form of apatite crystallites, but of extrafibrillar mineral deposits. As a step forward towards a clinically applicable system, the authors performed a follow-up study, where the phosphoprotein analog was bonded directly to collagen, instead of having it in the solution (Abuna et al., 2016; Sauro et al., 2015). Dentin collagen was completely remineralized after 3 to 4 months. It should be possible to cover resin-bonded dentin with “therapeutic” resin composite containing slow-release remineralization reagents that can diffuse through the adhesive layer, into water-filled regions of the hybrid layers to remineralize naked collagen fibrils. The demineralized regions can be “back-filled” with nanoscopic apatite crystallites. This results in physical displacement of free water, thereby preventing protease hydrolysis of collagen. Recently, certain studies investigated

the efficiency of E&R (Sauro et al., 2015) and SE (Abuna et al., 2016) experimental adhesives and primers doped with phosphoprotein biomimetic analogs to preserve the HL longevity. Microtensile bond strength was preserved in the experimental groups after 3- and 6-month storage in the dentin specimens bonded with E&R and SE adhesive systems, respectively. Although very interesting, this strategy has not yet become applicable in everyday clinical practice. Furthermore, since remineralization requires several months to occur, there is a risk that by that time the degradation of the collagen fibrils will have already started. Hence, it is important to combine the remineralization strategy with inhibition/inactivation of host derived proteases, and possibly also with the reinforcement of the collagen structure. Therefore, the focus of this thesis will be on the inhibition of the enzymatic activity as a strategy to prevent the degradation of the hybrid layer.

Inhibition of the enzymatic activity

The inhibition of endogenous collagenolytic activity can occur by a chelating mechanism, since the activity of the MMPs is dependent on the metal ions that can be chelated. Further, many of the inhibitors are cationic agents that can bind to negatively charged parts of the targeted molecules.

Of the numerous MMPs inhibitors, *chlorhexidine (CHX)* has been studied the most. It has been used in dentistry as an antimicrobial agent and is therefore suitable for use as a primer. Apart from its antimicrobial properties, CHX was shown to inhibit the activity of the MMPs (Gendron et al., 1999) and cysteine cathepsins (Scaffa et al., 2012). As low as 0.2% solutions of CHX have been shown to increase the longevity of the hybrid layer (Brackett et al., 2009; Breschi et al., 2010c; Campos et al., 2009; Carrilho et al., 2007c, 2007a; Leitune et al., 2011; Loguercio et al., 2016; Mobarak, 2011; Zheng et al., 2014). However, CHX binding was shown to be a reversible mechanism, and probably due to leaching, the resin-dentin interfaces treated with CHX were shown to be unstable after 18 months (Ricci et al., 2010; Sadek et al., 2010). *In vitro* studies demonstrated that cationic CHX binds to anionic

sites in mineralized and demineralized dentin (Carrilho et al., 2010; J. Kim et al., 2010b). However, sodium chloride was able to displace CHX from both mineralized and demineralized dentin, indicating that CHX binding to dentin is only electrostatic (J. Kim et al., 2010b). CHX is a strong base and has a cationic structure at physiological pH. The cationic part of CHX molecule could bind to the negatively charged part of the organic, as well as mineral substrates. The reaction with the organic part is presumed to be with the carboxylic and hydroxyl groups of collagen and noncollagenous phosphoproteins, while in the hydroxyapatite CHX is presumed to bind to the mineral phosphates (J. Kim et al., 2010b). Moreover, CHX not only has the possibility to bind to, but also showed excellent substantivity to oral tissues (Carrilho et al., 2010). Despite possible leaching, the use of CHX as additional therapeutic primer is still the most common *in vitro* and *in vivo* tested method to stabilize the adhesive interface with clinical applicability due to the large availability of CHX in dental offices and the limited clinical application time (as low as 30 s showed efficacy in inhibiting the enzymatic activity of dentin) (J. Kim et al., 2010b).

Quaternary ammonium compounds, positively charged at physiological pH, can inhibit the endogenous enzymatic activity of dentin using a cationic mechanism, similar to CHX. One of the quaternary ammonium compounds already tested as an MMPs inhibitor is *benzalkonium chloride (BAC)*, which is a mixture of alkylbenzyl-dimethylammonium chlorides of various alkyl chains that strongly binds to demineralized dentin and that was shown to have an immediate enzyme inhibiting effect comparable to that of CHX (Sabatini et al., 2015; Sabatini and Patel, 2013; Arzu Tezvergil-Mutluay et al., 2011), with promising results in bond strength preservation over time (Sabatini et al., 2015; Sabatini and Patel, 2013). Integrating methacrylates into these compounds (quaternary ammonium methacrylates - QAMs) could improve their efficiency. The BAC-containing etchant has been available on the market and has been originally incorporated due to its antimicrobial

properties, but has recently gained more interest also for its possible anti-enzymatic properties.

Ethylenediaminetetraacetic acid (EDTA) has been used for decades in the endodontic therapy for the enlargement of the root canals due to its chelating properties. EDTA removes the Ca^{2+} from the collagen matrices, and binds the Zn^{2+} ions from the catalytic site of the MMPs (Carvalho et al., 2000; Thompson et al., 2012). However, several downsides of this agent have been reported, such as long application time and reversibility caused by water solubility (Carrilho et al., 2009).

Different pharmaceutical agents unrelated to oral pathologies have also been shown to have an inhibiting effect on the MMPs through a chelating mechanism. For instance, *bisphosphonates*, in particular, polyvinylphosphonic acid, has shown good immediate results, but with questionable longevity (Tezvergil-Mutluay et al., 2010). Further, *tetracycline* and its analogs (doxycycline and minocycline) have shown collagenases- and gelatinases-inhibitory properties (Lauhio et al., 1995; Sorsa et al., 2006; Sulkala et al., 2001). The evidence on the protective effect of these antibiotics on the resin-dentin bond is scarce, and special caution in use of these agents in dentistry should be taken due to the dark photo-oxidation stains it can cause on dental tissue.

Further, specific inhibitors of the MMPs and cysteine cathepsins have been produced and tested in terms of bond strength preservation. *Galardin* incorporated within a primer of the E&R adhesive has shown a reduction in the degradation of the HL after one year (Breschi et al., 2010a). Similarly, *SB-3CT*, a specific MMP-2 and -9 inhibitor showed a positive influence on HL preservation (De Munck et al., 2010b). Specific inhibitors for cysteine cathepsins, such as *E-64* and *odanacatib* express their inhibiting effect through binding to the enzyme at the cleavage site, mimicking the substrate (Turk et al., 2012), but the data on effect on dentin bond durability is very limited (Yang et al., 2013).

Cross-linking agents

Researchers have attempted to enhance the naturally occurring cross-linking mechanism using chemical substances with cross-linking properties. The ability of cross-linkers to preserve the hybrid layer can be attributed to several mechanisms. Firstly, they are reported to reinforce the collagen structure by making additional cross-links between the collagen molecules, making them less prone to hydrolysis. Secondly, they can also cross-link MMPs and inactivate them by changing their molecular mobility, further disabling the degeneration process (Bedran-Russo et al., 2014, 2008; Xu and Wang, 2011).

Aldehydes, such as *glutaraldehyde (GD)*, are used as tissue fixatives, but have been proposed as potential cross-linking agents for the use in dentistry, since they form covalent bonds between the amino groups of proteins and the two aldehyde groups of glutaraldehyde. In particular, GD binds the ε-amino groups of peptidyl lysine and hydroxylysine residues within the collagen scaffold. GD has been extensively studied as a cross-linking agent in dentistry and has yielded positive results (Bedran-Russo et al., 2008, 2007; Chen et al., 2016; Han et al., 2003; Hass et al., 2015; Hiraishi et al., 2013; R. Liu et al., 2011; Seseogullari-Dirihan et al., 2016; Xu and Wang, 2011; Zhou et al., 2016), confirming the validity of the cross-linking approach (Nimni, 1988; Sung et al., 1999). Although aldehydes are very efficient in the preservation of the hybrid layer, these primers could be cytotoxic. Therefore, less toxic agents, such as *carbodiimides* and *plant-derived cross-linkers* have been recently studied (Bedran-Russo et al., 2014) and are perhaps more suitable for clinical practice.

EDC (*1-ethyl-3-(3-dimethylamino-propyl) carbodiimide)*, was demonstrated to achieve a similar bond strength preservation to that of GD and has been extensively investigated (Bedran-Russo et al., 2010; Annalisa Mazzoni et al., 2013; Mazzoni et al., 2018a) with the advantage of a much lower cytotoxicity. EDC contains a functional group with the formula RN=C=NR and can react with ionized carboxyl groups in proteins to form an O-acylisourea intermediate that can react with a non-

proteinated amino group and an adjacent protein chain to form a stable covalent amide bond between the two proteins. Its ability to increase the stiffness of demineralized dentin matrix (Bedran-Russo et al., 2010; Ryou et al., 2016) and the hybrid layer (Zhang et al., 2017) has been demonstrated. Moreover, EDC has a two-fold activity since it can cross-link both helical and telopeptide domains in collagen and also preventing telopeptidase activity that would normally remove telopeptides (Tezvergil-Mutluay et al., 2012). Recent zymographic assays on dentin powder (Annalisa Mazzoni et al., 2013; Mazzoni et al., 2018a) and *in situ* zymographic three-dimensional images obtained by confocal microscopy (Mazzoni et al., 2017, 2014), clearly demonstrated that when EDC is used as additional therapeutic primer on acid-etched dentin, it also has the ability to silence dentinal MMPs, even after a 1-year storage in artificial saliva (Mazzoni et al., 2017).

Plant-derived cross-linking agents are another prominent group, gaining much interest in the scientific community, suitable for clinical use due to their high potency and low cytotoxicity. The most studied of these agents are *genipin*, *polyphenolic compounds*, such as *tannins*, *proanthocyanidins* and *curcumin* (Al-Ammar et al., 2009; Bedran-Russo et al., 2007; Epasinghe et al., 2012; Hiraishi et al., 2013; Liu et al., 2013; R Seseogullari-Dirihan et al., 2015; Seseogullari-Dirihan et al., 2016).

Apart from chemical compounds, the cross-linking effect can also be achieved through physical and photochemical treatment, such as ultraviolet A (UVA) (Roda Seseogullari-Dirihan et al., 2015) or gamma irradiation, heating and drying (Hayashi et al., 2010).

Another important feature of cross-linking agents is their ability to react with the collagen-degrading enzymes (Mazzoni et al., 2014; Scheffel et al., 2014; Tezvergil-Mutluay et al., 2012). To date, GD (Camila Sabatini et al., 2014), EDC (Mazzoni et al., 2014, 2012; Tezvergil-Mutluay et al., 2012) and plant-derived cross-linking agents (Khaddam et al., 2014; Zhang et al., 2012) have shown this ability.

MMPs inhibitors and cross-linkers blended in adhesive resins

The described inhibitors and cross-linkers have been initially tested as separate primers in aqueous solutions and have shown promising results (Breschi et al., 2010c; Maravic et al., 2017; Annalisa Mazzoni et al., 2013; Sabatini et al., 2015; R Seseogullari-Dirihan et al., 2015). However, in accordance with the current tendency to simplify dental procedures, there have been efforts to incorporate these agents into one of the components of the adhesive system. Further, while cross-linkers are thought to permanently inactivate the MMPS, the effect of inhibitors could be reversible, since they could leach out of the hybrid layer, given their solubility in water (CHX) (Ricci et al., 2010; Sadek et al., 2010). Hence, another reason behind this tendency is that a slow release of an inhibitor from the adhesive could prolong its activity and preserve the hybrid layer over a longer period of time.

Inhibitors and cross-linkers have been blended into experimental and commercially available etchants, primers, or adhesive resins, yielding mixed results. There are various risks related to adding a new component into a stable system, since there could be repercussions on the quality of polymerization and mechanical properties of the adhesive. A recent study (Moharam et al., 2018) found no adverse effects of the incorporation of CHX digluconate (0.5, 2 and 4 wt%) into an experimental E&R adhesive on degree of conversion (DC). Cadenaro et al. (M. Cadenaro et al., 2009) also found that the incorporation of 1% or 5% CHX within resin blends of increasing hydrophilicity did not affect DC, but it decreased the elastic modulus significantly, which stresses the need for comprehensive investigation of different mechanical properties of experimental adhesives before consideration for clinical use. CHX was further reported not to adversely affect DC, water sorption, ultimate tensile strength or solubility of an E&R adhesive, while preserving bond strength for 12 months (Stanislawczuk et al., 2014). Da Silva et al. (da Silva et al., 2015) tested experimental E&R adhesives doped with several inhibitors: Galardin, Batimastat, GM1489 and CHX diacetate and presented similar results with CHX and GM1489.

Moreover, although five different QAM-containing adhesive blends showed an increase in DC, a decrease in Young's modulus and ultimate tensile strength was observed (Hoshika et al., 2014). Methacryloyloxydodecylpyridinium bromide (MDPB) is an example of a QAM that has already been incorporated into a commercial adhesive system (Clearfil Protect Bond, Kuraray Noritake Dental Inc., Osaka, Japan) (Imazato et al., 2007, 2003). The advantage of MDBP is that it can copolymerize with methacrylates in the adhesive resin (D. Pashley et al., 2011; Tezvergil-Mutluay et al., 2015) and can also inhibit the MMPs activity (D. Pashley et al., 2011; Tezvergil-Mutluay et al., 2015). Following the good results obtained with MDPB, other QAMs were investigated as MMPs inhibitors with encouraging results, comparable to CHX (A Tezvergil-Mutluay et al., 2011).

Another approach to be considered is the incorporation of the cross-linkers into adhesive blends, since they might have a multiple role in resin-dentin preservation. For instance, quercetin is a flavonoid, an antioxidant with cross-linking properties, known to down-regulate expression of MMP2 and -9 in prostate cancer cells. Gotti et al. (Gotti et al., 2015) and Yang et al. both investigated the incorporation of quercetin into AdperTM Single Bond 2 (3 M ESPE, St. Paul, MN, USA) (Gotti et al., 2015; Yang et al., 2017), and the former reported lower DC and bond strength in the experimental adhesive blend, while the latter presented a decrease in DC only in the group containing higher concentration of quercetin, with the preservation of the hybrid layer over time. This discrepancy could be due to the differences in the concentration of the active substance (5 wt% vs 100/500/1000 μg/mL). Gotti et al. also investigated two other adhesive systems and different antioxidants, and only the Clearfil SE (Kuraray Medical; Tokyo, Japan) presented no impairment of DC in the experimental groups. The high variability in the achieved results leads to the conclusion that the mechanical properties of a certain adhesive blend could be impaired or improved depending on the physicochemical interactions of the components of the adhesive system with a therapeutic compound added in a certain

concentration. Hence, efforts should be made towards matching appropriate inhibitors in adequate concentrations with appropriate adhesive resin blends (Pallan et al., 2012).

Adhesive systems doped with different therapeutic agents could be less dependent on the composition of the adhesive and concentration of the therapeutic agent if certain fillers, that are also carriers of the active substance, could be incorporated within the adhesive. This could have a double impact, since fillers have been shown to improve mechanical properties of adhesives (Moharam et al., 2018), and the addition of an inhibitor/cross-linker would not disturb the balance in the liquid part of the adhesive. Hence, there would be less potential impact on polymerization quality. Moreover, some of these carriers could be "programmed" to release inhibitors gradually, ensuring a prolonged inhibitory effect. There have been several reports on such experimental adhesives recently. Bottino et al. (Bottino et al., 2013) first investigated whether incorporation of Halloysite® nanotubes into an adhesive would influence its mechanical properties. They found no differences in DC, microhardness and bond strength compared to the control when 20 wt % of nanotubes were added. Further, the same group investigated whether encapsulation of doxycycline into the nanotubes would cause changes in DC and microtensile bond strength, but the system remained stable (Feitosa et al., 2014). Most recently, the authors loaded doxycycline into Halloysite® nanotubes and performed a comprehensive investigation of physicochemical properties of this adhesive, as well as its anti-MMP potential (Palasuk et al., 2018). DC and Knoop's microhardness were not impaired. The adhesive showed dose-dependent antimicrobial and anti-MMP properties, while preserving cell viability. Further, Yan et al. (Yan et al., 2018) reported that CHX-encapsulated mesoporous silica-modified dentin adhesive can inhibit *S. Mutans* while preserving DC and bond strength after collagenase aging of 1 month in lower concentrations of CHX-mesoporous silica particles (1 and 5%). In the group containing the highest concentration of the

particles (10%), DC as well as bond strength were impaired. This was probably due to the tendency of nanoparticles to form agglomerates when they are present in a higher concentration, which was confirmed using FEISEM. The release of CHX was measured for 30 days and reached a plateau after day 10. Other mechanical properties of this adhesive should be investigated, as well as its anti-enzymatic potential. Furthermore, the ability of the nanocarriers of therapeutic agents to penetrate between the demineralized collagen fibrils should be considered and investigated due to their size and the small intermolecular spaces between collagen fibrils. In the case that nanoparticles could not infiltrate resin tags, therapeutic agents could not be delivered to the most critical parts of the hybrid layer. Although all these approaches are valid, the most optimal way to singlehandedly reduce collagen degradation would be biomimetic remineralization, which would protect the collagen fibrils, cover and inactivate proteases, remove the remaining water, and enhance the mechanical properties of the hybrid layer. Recently, there have been reports that the use of primers doped with analogs of phosphoproteins and Ca/P-doped SE adhesives leads to biomimetic remineralization, with the preservation of bond strength and better mechanical properties of the hybrid layer (Abuna et al., 2016; Sauro et al., 2015). Analogs of phosphoproteins play a very important role in the mineralization, since without them, the mineralization occurs in the form of extrafibrillar mineral deposits (Breschi et al., 2018; Gu et al., 2011; J. Kim et al., 2010a; Y. K. Kim et al., 2010). Before transiting from this exciting *in vitro* to clinical concept, there is a long path to be crossed, but the future seems to carry innovative solutions, which could lead us to the next era in adhesive dentistry.

Aims and hypotheses

Four separate studies were performed as a part of the present thesis, and each study was executed in accordance with their own specific aims. However, all of them share the same general objectives:

1) To investigate whether protease inhibitors/inactivators influence the preservation of bond strength, immediately or after aging in artificial saliva at 37°C.
2) To investigate whether protease inhibitors/inactivators influence enzymatic activity within dentin powder or the hybrid layer, immediately or after aging in artificial saliva at 37°C.

The null hypotheses were as follows:

1) The protease inhibitors/inactivators do not influence bond strength, immediately or after aging in artificial saliva at 37°C.
2) The protease inhibitors/inactivators do not influence the enzymatic activity within dentin powder or the hybrid layer, immediately or after aging in artificial saliva at 37°C.

STUDY 1

Chlorhexidine incorporated within a commercially available adhesive influences long-term bond strength and endogenous enzymatic activity

The aim of this study was to investigate, by the means of micro tensile bond strength test, gelatin and *in situ* zymography, the influence of 0.2% CHX incorporated within a commercially available adhesive on long-term bond strength and endogenous enzymatic activity.

The null hypotheses were the following:

1) presence of CHX within the adhesive system does not affect bond strength immediately or after 12-month-aging in artificial saliva at 37°C;

2) activation of endogenous MMPs is not related to the presence of CHX within the adhesive system or the bonding strategy.

Materials and Methods

Microtensile bond strength test

Non-carious molars (N=15 for each group) stored in 0.5% chloramine in water at 4ºC were used within 1 month after extraction. The occlusal enamel was removed using a low speed diamond saw under water irrigation (Micromet, Remet, Bologna, Italy) and dentin was exposed to create a flat surface for conventional bonding. Specimens were assigned to treatment groups and bonded according to manufacturer's instructions (**Table 1**):

1. Dentin surfaces were etched for 15 s with 35% phosphoric-acid gel (3 M ESPE, St. Paul, MN, USA) and rinsed with water. Peak Universal Bond adhesive system in E&R mode (containing 0.2% CHX) was applied;
2. Dentin surfaces were etched and rinsed as in group 1. Scotchbond Universal (SBU) adhesive system in the E&R mode was applied – control;
3. Peak Universal Bond adhesive system in SE mode (containing 0.2% CHX) was applied to unetched dentin;
4. SBU adhesive system in SE mode was applied to unetched dentin – control.

Further, 4-mm thick resin composite build-ups were created (Filtek Z250, 3M ESPE, St. Paul, MN, USA) and polymerized for 40 s using a light-emitting diode curing unit (Curing Light 2500; 3M ESPE). Non-trimming technique (Carrilho et al., 2007b) was used to obtain resin-dentin sticks with cross-sectional area of approximately 0.9 mm^2. Each stick was measured, and the dimensions recorded for bond strength calculation. Within each of the groups, the sticks were equally divided into two parts and stored at 37ºC in artificial saliva prepared in accordance with Pashley et al. (Pashley et al., 2004), for 24h (T0) or for 12 months (T12). The sticks were stressed until failure using a simplified universal testing machine at a crosshead speed of 1 mm/min (Bisco Inc., Schaumburg, IL, USA). The evaluation of failure modes and classification as cohesive (C), adhesive (A), or mixed (M) failures was done according to Breschi et al. (Breschi et al., 2009). Although recorded, the number of prematurely de-bonded sticks per each group was not included in the statistical

analysis, since all premature failures occurred during the cutting procedure, which was performed at time zero and did not exceed 3% of the total number of tested specimens.

Table 1. Components, compositions, and application procedure of the tested adhesives (information supplied by the manufacturer).

Adhesive system	Composition	Manufacturer's instructions
Peak Universal Bond (Ultradent Products Inc., South Jordan, UT)	1. Etchant: 35% phosphoric acid 2. ethyl alcohol, 2-hydroxyethyl Methacrylate, Methacrylic Acid, Chlorhexidine di(acetate) 0.2%	ER: 1. Apply etchant for 20 s 2. Rinse for 5 s 3. Air dry 2 s 4. Apply a puddle coat of the adhesive and gently agitate for 10 s (Peak Universal Bond) or 20 s (SBU) 5. Dry 10 s using ¼ to ½ air pressure (Peak Universal Bond) or 5 s (SBU) 6. Light polymerize for 10 s at 1200 mW/cm2 SE: 1. Initial use of Peak SE Primer requires activation of the two components separated in the syringe 2. Application of the Peak SE Primer with microbrush for 20 s using continuous scrubbing on dentin. Do not scrub enamel. 3. Thin/dry for 3 s using air/water syringe or high-volume suction directly over preparation 4. Apply a puddle coat of the adhesive and gently agitate for 10 s (Peak Universal Bond) or 20 s (SBU) 5. Dry 10 s using ¼ to ½ air pressure (Peak Universal Bond) or 5 s (SBU) 6. Light polymerize for 10 s at 1200 mW/cm2
*Scotchbond Universal (3M ESPE, Neuss, Germany)	1. Etchant: 35% phosphoric acid 2. 2-hydroxyethyl methacrylate, bisphenol a diglycidyl ether dimethacrylate (BISGMA), decamethylene dimethacrylate, ethanol, water, silane treated silica, 2-propenoic acid, 2-methyl-, reaction	

*In the SE mode, the procedure starts from step 4

Gelatin zymography

The zymographic essay on dentin extracts was performed according to the protocol of Mazzoni et al. (A Mazzoni et al., 2013) to investigate the expression of MMP-2 and -9. Enamel, cement and the pulp were removed from 7 sound human molars and the dentin was reduced to powder using a Retsch mill (Model MM400, Retsch GmbH, Haan, Germany). The powder from all the teeth was mixed together and a pool of dentin powder was created. Aliquots of dentin powder were divided into 6 groups: G1 – mineralized dentin powder (control); G2 – demineralized dentin powder treated with 10 wt% phosphoric acid for 10 min (control); G3 – demineralized as G2 and treated with Peak Universal Bond for 30 min; G4 – demineralized as G2 and treated with SBU for 30 min; G5 – mineralized dentin powder treated with Peak Primer for 30 min followed by Peak Universal Bond application for 30 min; G6 – mineralized dentin powder treated with SBU for 30 min. The groups treated with the adhesives or primer were subjected to a series of three suspensions in 1mL of acetone followed by centrifuges (20.800xg for 20 min), in order to properly remove the adhesive resin (Mazzoni et al., 2012). Further, the specimens were suspended in the extraction buffer (50 mM Tris–HCl pH 6, containing 5 mM $CaCl_2$, 100 mM NaCl, 0.1% Triton X-100, 0.1% nonionic detergent P-40, 0.1 mM $ZnCl_2$, 0.02% NaN_3) and kept for 24h at 4°C under constant agitation, after which they were sonicated for 10 min (at ≈ 30 pulses) and centrifuged (20.800g; 20 min; 4°C). The supernatant was separated and re-centrifuged two times in order to remove traces of the powder. The protein content was concentrated by centrifugation in Vivaspin concentrators (10,000 kDa cut-off; Vivaspin Sartorius Stedim Biotech, Goettingen, Germany) for 30 min at 25°C (15,000g, 3 times) and the protein concentration was determined using Bradford assay (Bio-Rad, Hercules, CA, USA). The extracted protein aliquots were diluted in Laemmli sample buffer in a 4:1 ratio. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 10%) was performed under non-reducing conditions. The gel contained 1 mg/mL of fluorescently labelled (2-methoxy-2,4-diphenyl-3(2H)-furanone) gelatin. Pre-

stained low-range molecular weight SDS-PAGE standards (Bio-Rad) were used as molecular-weight markers. After electrophoresis, the gels were washed for 1 h in 2% Triton X-100, incubated in activation solution (50 mmol/L Tris–HCl, 5 mmol/L $CaCl_2$, pH 7.4) for 48 h and photographed under long wavelength ultraviolet light illumination (Gel Doc XR System, Bio-Rad). The zymographic assay was performed and analyzed in triplicate.

In-situ zymography of resin-dentin interfaces

One-millimeter-thick slabs of middle/deep dentin were obtained from extracted human third molars (N=3) using the low-speed Micromet saw with water-cooling. Each slab was further divided into four parts as to test all the investigated groups on the same substrate. Silicon-carbide paper (600-grit) was used to create a standardized smear layer on each dentin surface. One surface of each quarter of a slab was treated with adhesive systems as described for µTBS test. This was followed by a 1-mm build-up with flowable composite (Filtek 250 flow; 3M ESPE); the composite was polymerized for 40 s using a light-emitting diode light-curing unit (Curing Light 2500; 3M ESPE). After completion of those procedures, the bonded assemblies were sectioned vertically into 1-mm-thick specimens to expose the adhesive-dentin interface. Each specimen was glued to a microscope slide, ground down approximately to the thickness of 50 µm and polished. *In-situ* zymography was performed following the protocol reported by Mazzoni *et al*. (Mazzoni et al., 2014). Self-quenched fluorescein-conjugated gelatin mixture (E- 12055; Molecular Probes, Eugene, OR, USA) was placed on the specimen covering the polished resin-dentin surfaces and then protected with a coverslip. The specimens were incubated for 12 h at 37°C in a humid chamber avoiding direct contact with water, or exposure to light. Confocal laser scanning microscope was used to examine the specimens after incubation (excitation wavelength, 488 nm; emission wavelength, 530 nm; Model A1-R; Nikon, Tokyo, Japan). For each assembly, a series of images were made (one image per each 1 µm into the depth of the sample) to show the hydrolysis of the

quenched fluorescein-conjugated gelatin substrate, presented as green fluorescence. ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to quantify integrated density of the fluorescence signals, which correspond to the endogenous enzymatic activity.

The statistical analysis

μTBS test results were analyzed using the two-way ANOVA test followed by pair-wise comparisons using the Tukey Test. Since the *in situ* zymography data failed to comply with normality requirements (Shapiro-Wilk test), Kruskal Wallis test was used to compare the density of the fluorescence signal within the different investigated groups. The significance threshold was set at $p<0.05$. All the analyses were performed in Sigma plot v. 12.0 (Systat Software, Inc).

Results

Microtensile bond strength test

The mean values and standard deviations of the μTBS are listed in **Table 2**, and graphically presented in **Figure 3**. Bond strength was significantly higher in the experimental compared to the control groups, immediately, as well as after aging ($p<0.05$). The bond strength after 12-month aging was significantly lower in all tested groups compared to immediate bond strength ($p<0.05$), apart from SBU SE group where the values were lower after 12 months, but not significantly.

Failure mode distribution of the de-bonded specimens is shown in **Table 3**. The predominant failure mode was the mixed, followed by the adhesive failure mode in all the tested groups.

Table 2. μTBS obtained by applying Scotchbond Universal or Peak Universal Bond adhesive system on the dentin surface. Values are mean ± standard deviation [number of premature failed sticks/number of intact sticks tested]. T0 and T12 indicate specimens that were tested after storage of 24 h and 12 months, respectively.

	T_0	T_{12}
Peak Universal E&R (0.2%CHX)	50.1 ± 11.0^{aB} MPa [7/102]	40.7 ± 8.6^{bB} MPa [6/98]
SBU E&R (control)	38.9 ± 12.8^{aA} MPa [5/93]	21.6 ± 7.7^{bA} MPa [4/109]
Peak Universal SE (0.2%CHX)	53.0 ± 11.3^{aB} MPa [7/92]	43.9 ± 11.7^{bB} MPa [5/108]
SBU SE (control)	35.8 ± 13.0^{aA} MPa [4/103]	33.6 ± 11.1^{aC} MPa [8/96]

†Premature failures due to preparation procedures were not included in the statistical analysis (p>0.05). Different superscript lower-case letters (in rows) indicate statistical difference in storage time. Different superscript upper-case letters (in columns) indicate statistical difference in different adhesive protocols.

Table 3. Percentages of failure modes after microtensile test.

Adhesive system	T_0	T_{12}
Peak Universal E&R (0.2%CHX)	35% A 65% M	45% A 55% M
SBU E&R (control)	50% A 50% M	50% A 50% M
Peak Universal SE (0.2%CHX)	35% A 5%CD 65% M	60% A 40% M
SBU SE (control)	30% A 10%CD 60% M	45% A 55% M

Fractures were classified as: A, adhesive; CD, cohesive failure in dentin; CC, cohesive failure in resin composite; M, mixed failure.

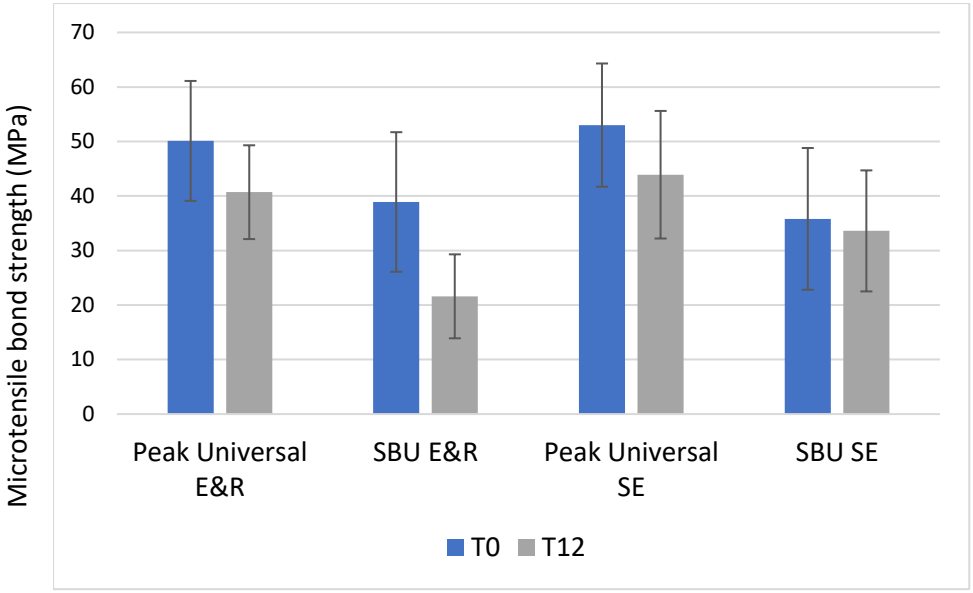

Figure 3. Graph summarizing the bond strength values (MPa) reported in Table 1.

Gelatin zymography

Gelatinolytic activity of the investigated groups is shown in **Figure 4**. Mineralized dentin powder (G1; Lane 1) showed week enzymatic activity in the corresponding to the molecular weights of MMP-2 and -9. Demineralized dentin extract (G2; Lane 2) showed multiple forms of gelatinolytic enzymatic activity, with the weak MMP-2 active and pro-form at 66 kDa and 72 kDa, as well as a stronger signal of MMP-9 active and pro-forms at 86 kDa and 92 kDa, respectively. The active enzyme forms were more pronounced. In both E&R and SE Peak groups (Lanes 3 and 5, respectively), there is an absence of the activity of MMP-2, while the activity of MMP-9 seems to be reduced. On the other hand, enzymatic activity in the E&R and SE SBU groups is more pronounced compared to the controls (Lane 4 and 6, respectively). Quantification of enzymatic activity is shown in **Figure 5** and corresponds to the qualitative results.

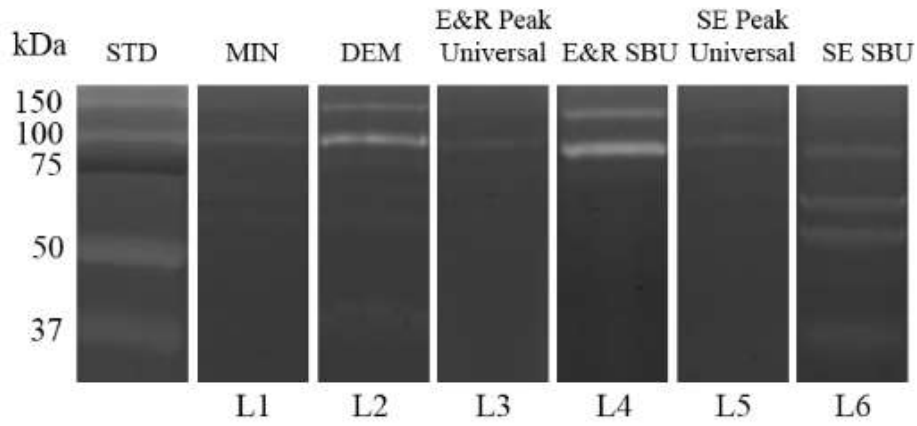

Figure 4. (a) Gelatin zymography: Figure showing the differences in the enzymatic activity between the investigated groups presented as light bands in the area of the molecular weights of pro- and active MMP-2 and MMP-9. *STD* = Standard; *MIN* = Mineralized dentin powder; *DEM* = Demineralized dentin powder; *E&R* = Etch-and-Rinse Mode; *SE* = Self-Etch Mode; *SBU* = Scotchbond Universal.

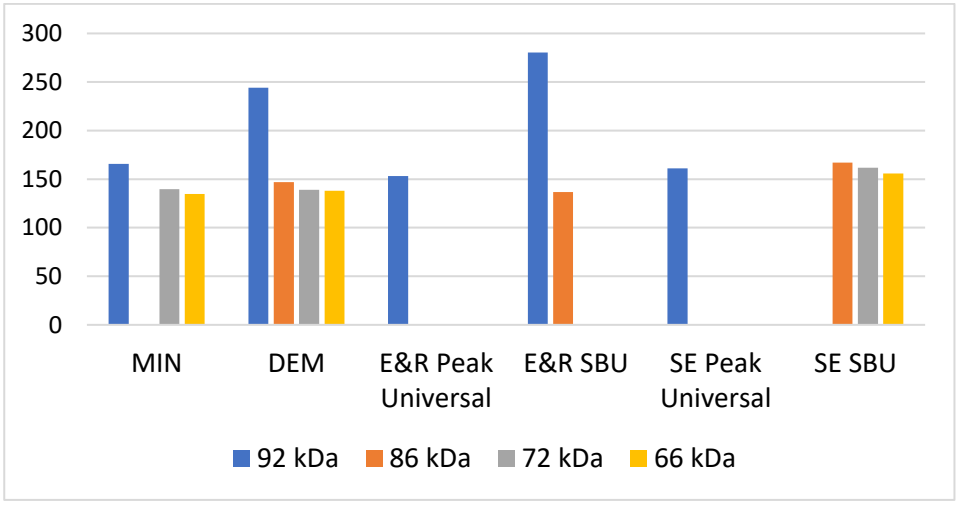

Figure 5. Graph illustrating the densitometric evaluation of bands obtained from the zymographic analysis of proteins extracted from dentin powder.

In situ zymography results

The results obtained on the confocal microscope revealed differences in the fluorescence signal exhibited by different tested groups. The level of the enzymatic activity corresponds to the density of the green fluorescence signal (**Figures 6,7**). Quantification of the density of the fluorescence (**Figure 6**) demonstrated a reduction in enzymatic activity in the hybrid layers created with Peak Universal adhesive used in the E&R mode, compared to the control SBU used in the same mode ($p<0.05$). Both adhesives used in the SE mode showed a similar level of fluorescence ($p>0.05$), while they exhibited lower enzymatic activity compared to the same adhesives used in E&R mode. Interestingly, Peak used in the E&R mode showed a similar activity as SBU used in SE mode.

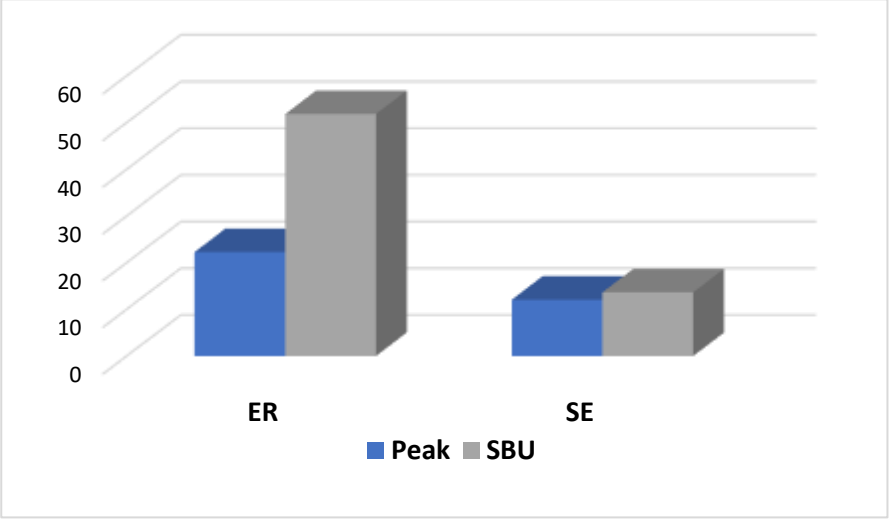

Figure 6. Graph illustrating the quantification of the enzymatic activity of the tested groups**.**

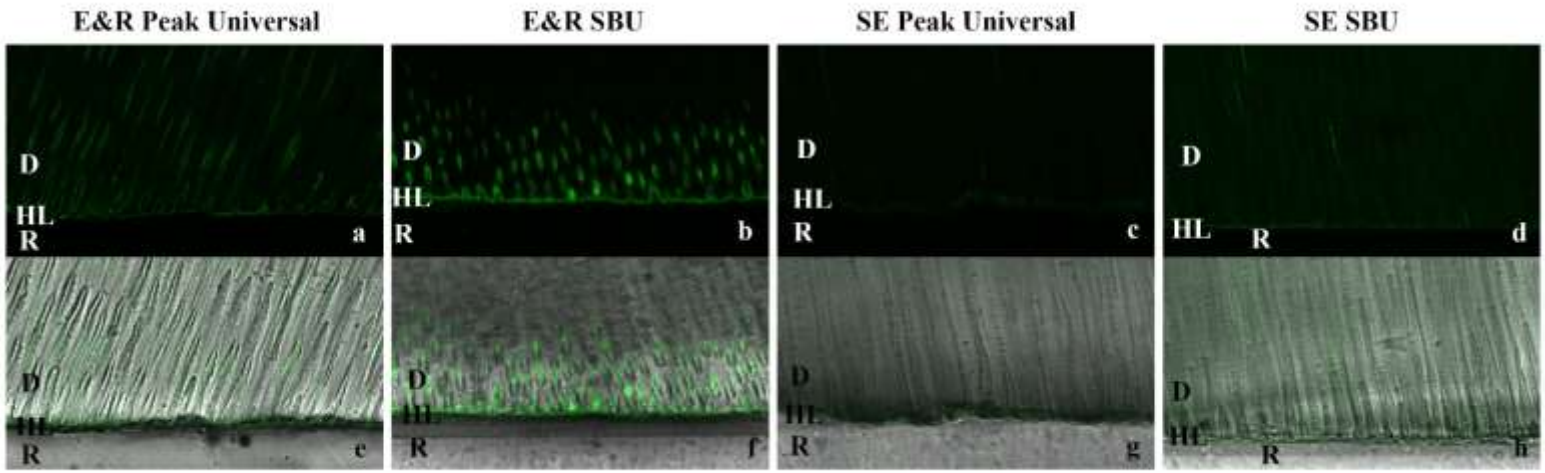

Figure 7. Resin-dentin interfaces bonded with Peak Universal or SBU, in E&R or SE mode, incubated with quenched fluorescein-labeled gelatin; (a,b,c,d) Images acquired in green channel, showing fluorescence (identifying intense endogenous enzymatic activity) in dentinal tubules and within the HL created using different protocols; (e,f,g,h) Images of resin-dentin interfaces created using different protocols, obtained by merging differential interference contrast image (showing the optical density of the resin-dentin interface) and image acquired in green channel (showing enzymatic activity). *E&R* = Etch-and-Rinse mode; *SE* = Self-Etch mode; *SBU* = Scotchbond Universal; *D* = Dentin; *HL* = Hybrid Layer; *R* = Resin Composite

STUDY 2

Effect of Benzalkonium Chloride on dentin bond strength and endogenous enzymatic activity

The aim of this in vitro study was to evaluate, at baseline (T0) and over time (T12 months), the effect of a multi-mode universal adhesive compared with two experimental formulations blended with different concentration of benzalkonium chloride (BAC) on bond strength and endogenous enzymatic activity.

The null hypotheses were as follows:

1) BAC blended within the adhesive formulation does not affect bond strength immediately or after aging in the artificial saliva at 37°C;

2) has no effect on inactivation of endogenous dentine MMPs immediately or after aging in the artificial saliva at 37°C.

Materials and Methods

Microtensile bond strength test

Microtensile bond strength test was performed according to the protocol described in Study 1 with the following investigated groups bonded according to manufacturer's instructions (**Table 4**):

1. Dentin surfaces were etched for 15 s with 35% phosphoric-acid gel (3 M ESPE, St. Paul, MN, USA) and rinsed with water. All-Bond Universal was generously applied according to the manufacturer's instructions.
2. All-Bond Universal was directly applied on untreated dentin according to the manufacturer's instructions for the SE mode.
3. Dentin surfaces were etched for 15 s with 35% phosphoric-acid gel (3 M ESPE, St. Paul, MN, USA) and rinsed with water. All-Bond Universal & 0.5% BAC was generously applied according to the manufacturer's instructions.
4. All-Bond Universal & 0.5% BAC was directly applied on untreated dentin according to the manufacturer's instructions for the SE mode.
5. Dentin surfaces were etched for 15 s with 35% phosphoric-acid gel (3 M ESPE, St. Paul, MN, USA) and rinsed with water. All-Bond Universal & 1% polymerizable BAC was generously applied according to the manufacturer's instructions.
6. All-Bond Universal & 1% polymerizable BAC was directly applied on untreated dentin according to the manufacturer's instructions for the self-etch mode.

Table 4. Components, compositions, and application procedure of the tested adhesives (information supplied by the manufacturer).

Adhesive system	Composition	Manufacturer's instructions
All-Bond Universal (BISCO, Inc., Schaumburg, IL, U.S.A.)	1. Etchant: 35% phosphoric acid 2. Bisphenol A Diglycidylmethacrylate, ethanol, 2-Hydroxyethyl Methacrylate, MDP	E&R: 1. Etch enamel and dentin using an etchant for 15 seconds. 2. Rinse thoroughly. 3. Remove excess water by blotting the surface with an absorbent pellet or high volume evacuation (HVE) for 1-2 seconds, leaving the preparation visibly moist. 4. Apply two separate coats of adhesive, scrubbing the preparation with a microbrush for 10-15 seconds per coat. Do not light cure between coats. 5. Evaporate excess solvent by thoroughly air-drying with an air syringe for at least 10 seconds, there should be no visible movement of the adhesive. 6. Light cure for 10 seconds. SE: Follow the procedure for E&R starting from step 2.
All-Bond Universal + 0.5% benzalkonium chloride	1. Etchant: 35% phosphoric acid 2. Bisphenol A Diglycidylmethacrylate, ethanol, 2-Hydroxyethyl Methacrylate, MDP, 0.5% BAC	
All-Bond Universal + 1% methacrylate benzalkonium chloride	1. Etchant: 35% phosphoric acid 2. Bisphenol A Diglycidylmethacrylate, ethanol, 2-Hydroxyethyl Methacrylate, MDP, 1% BAC methacrylate	

Gelatin zymography

Gelatin zymography was performed according to the protocol described in Study 1 with the following investigated groups:

- **G1:** dentin left mineralized (DP), as control;
- **G2**: demineralized with 10 wt% phosphoric acid for 10 min to simulate the first step of the etch-and-rinse approach;
- **G3**: All-Bond Universal was applied on untreated dentin powder;
- **G4:** All-Bond Universal + 0.5% BAC applied on untreated dentin for 30 min to simulate the self-etch approach;
- **G5:** All-Bond Universal + 1% polymerizable BAC applied on untreated dentin for 30 min to simulate the self-etch approach;
- **G6:** DDP as for L2 treated with All-Bond Universal for 30 min;
- **G7:** DDP as for L2 treated with All-Bond Universal + 0.5% BAC for 30 min;
- **G8:** DDP as for L2 treated with All-Bond Universal + 1% polymerizable BAC for 30 min.

In situ zymography of resin-dentin interfaces

In situ zymography was performed as described in Study 1, and the tested groups correspond to the groups listed in the *Microtensile bond strength* chapter of Study 2. The samples were bonded using identical protocols.

Results

Microtensile bond strength test

Means and standard deviations for microtensile bond strength (in MPa) at T0 and T12 are reported in **Table 5**. Statistical analyses of variance (ANOVA) showed that only the variables "adhesive" and "aging" significantly affected the results, but not the variable "application mode". Tukey *post-hoc* test underlined that ABU+1%BAC methacrylate performed significantly worse than ABU and ABU+0.5%BAC. Furthermore, all groups showed a detectable and significant decrease in microtensile bond strength values over time (**Figure 8**).

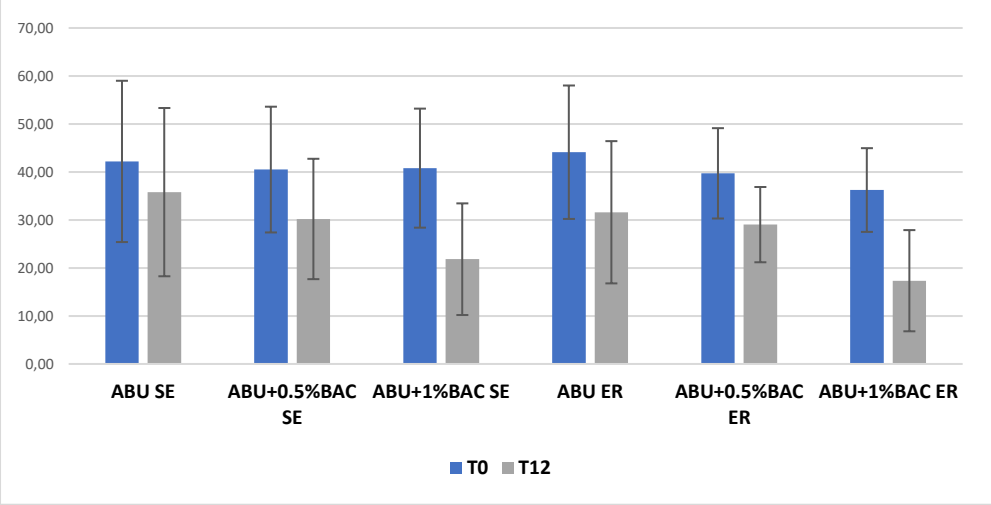

Figure 8. Microtensile bond strength graph.

Table 5. Microtensile bond strengths (mean ± SD, in MPa) of different groups immediately after bonding (T0) and after 12 months of aging (T12) in artificial saliva.

Mode	**SE**			**ER**		
Adhesive	ABU	ABU+0.5%BAC	ABU+1%BAC	ABU	ABU+0.5%BAC	ABU+1%BAC
T0	42.2^{aA}±16.8	40.5^{aA}±13.1	40.8^{aA}±12.4	44.1^{aA}±13.9	39.7^{aA}±9.4	36.2^{bA}±8.7
T12	35.8^{aB}±17.5	30.2^{aB}±12.5	21.8^{bB}±11.6	31.6^{aB}±14.8	29.0^{aB}±7.8	17.4^{bB}±10.5

Different lower-case letters indicate significant differences ($p<0.05$) within the same row, different upper-case letters indicate significant differences ($p<0.05$) within the same column.

Gelatine Zymography

Zymography results are shown in **Figure 9**. Zymographyc assay revealed that all the BAC-containing formulations tested in the E&R mode decreased MMPs expression. When employed in the SE mode, All-Bond Universal increased the activity of MMP-2 and -9, while BAC-containing adhesive formulations showed reduced expression of MMP-2, and almost complete inhibition for MMP-9, qualitatively and quantitatively (**Figure 9, 10)**.

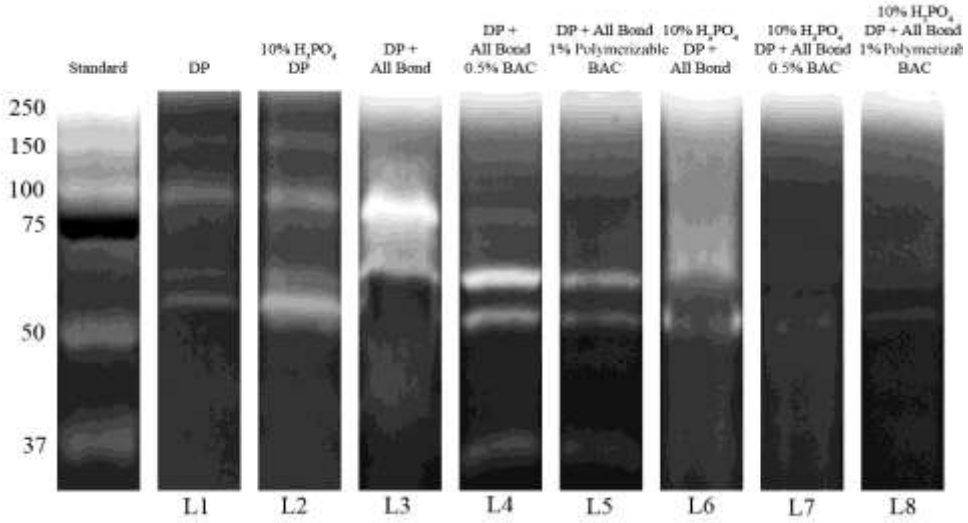

Figure 9. Zymogram of all tested groups

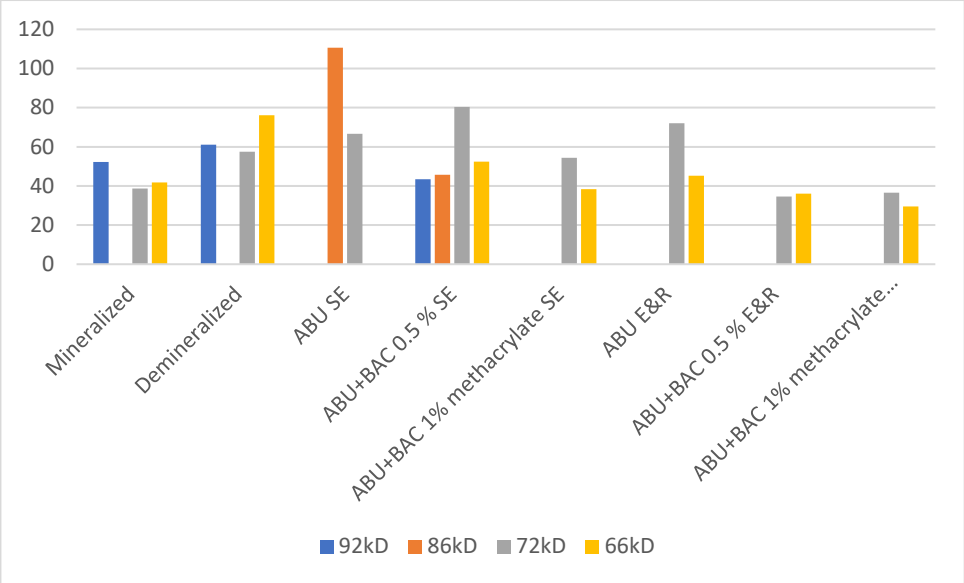

Figure 10. Quantification graph of enzymatic activity.

In situ Zymography

In situ zymography qualitative and quantitative results are shown in **Figures 11-15**. *In situ* zymography demonstrated a decrease in enzymatic activity at T0 in all the experimental groups. All the groups showed a general trend of enzymatic activity increase after aging, except for the ABU SE + 1% methacrylate group, which showed further decrease. Altogether, the experimental groups bonded with ABU + 1% methacrylate showed the lowest level of fluorescence over time, regardless of the bonding strategy employed, which was not in accordance with the μTBS results.

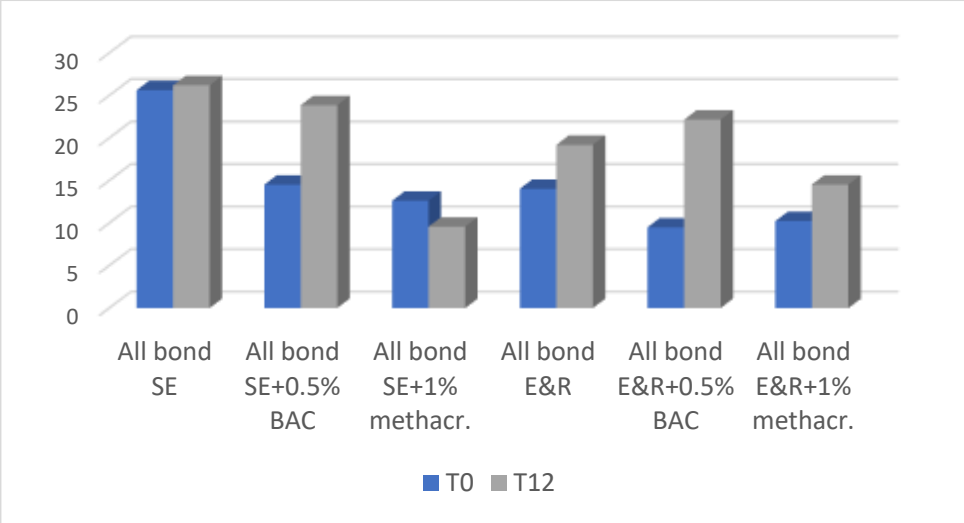

Figure 11. In situ zymography quantification graph.

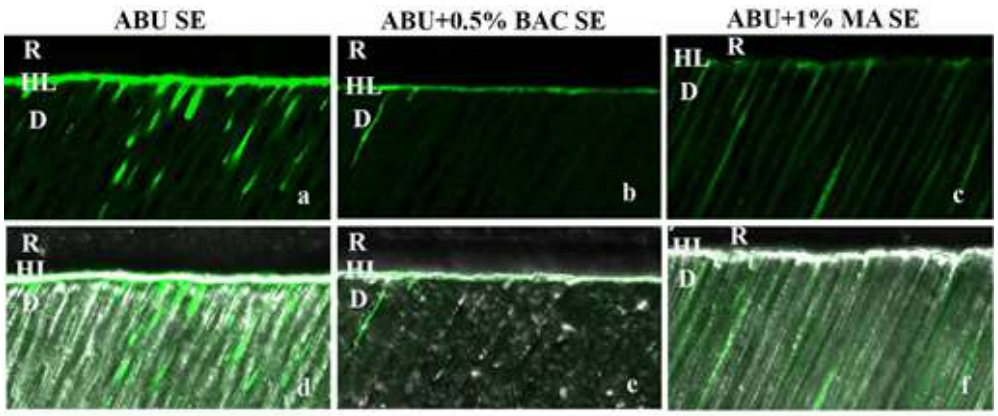

Figure 12. *In situ* zymography analysis of ABU SE, ABU+0.5%BAC SE and ABU+1%BAC SE at T0. (a,b,c,) Images acquired in green channel, showing fluorescence (identifying intense endogenous enzymatic activity) in dentinal tubules and within the HL created using different protocols; (d,e,f) Images of resin-dentin interfaces created using different protocols, obtained by merging differential interference contrast image and image acquired in green channel. D = Dentin; HL = Hybrid Layer; R = Resin Composite.

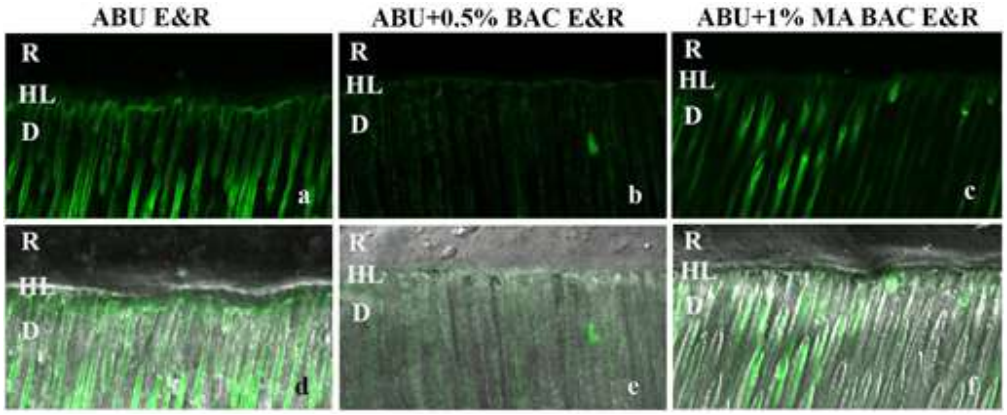

Figure 13. *In situ* zymography analysis of ABU ER, ABU+0.5%BAC ER and ABU+1%BAC ER at T0. (a,b,c) Images acquired in green channel, showing fluorescence in dentinal tubules and within the HL created using different protocols; (d,e,f) Images of resin-dentin interfaces created using different protocols, obtained by merging differential interference contrast image and image acquired in green channel. D = Dentin; HL = Hybrid Layer; R = Resin Composite.

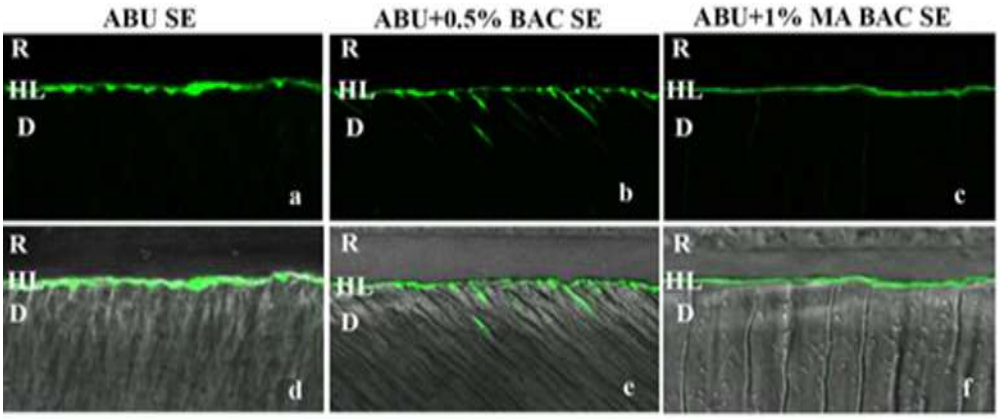

Figure 14. *In situ* zymography analysis of ABU SE, ABU+0.5%BAC SE and ABU+1%BAC SE at T12. (a,b,c) Images acquired in green channel, showing fluorescence in dentinal tubules and within the HL created using different protocols; (d,e,f) Images of resin-dentin interfaces created using different protocols, obtained by merging differential interference contrast image and image acquired in green channel. D = Dentin; HL = Hybrid Layer; R = Resin Composite.

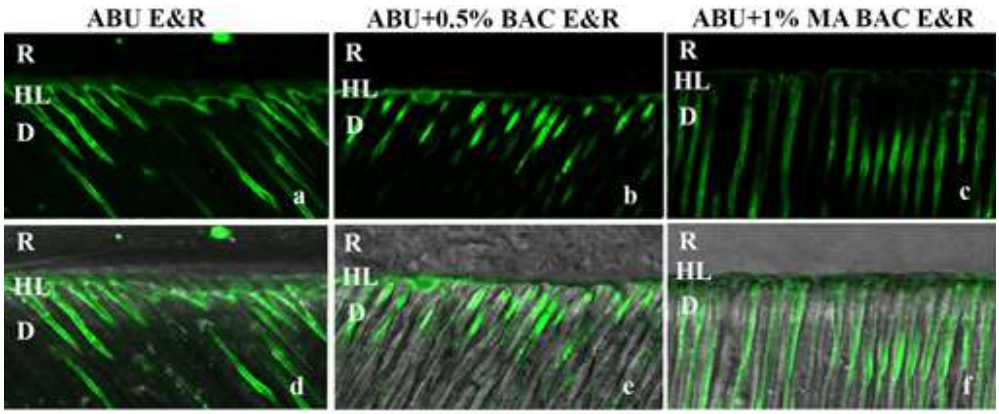

Figure 15. *In situ* zymography analysis of ABU ER, ABU+0.5%BAC ER and ABU+1%BAC ER at T12. (a,b,c) Images acquired in green channel, showing fluorescence in dentinal tubules and within the HL created using different protocols; (d,e,f) Images of resin-dentin interfaces created using different protocols, obtained by merging differential interference contrast image and image acquired in green channel. D = Dentin; HL = Hybrid Layer; R = Resin Composite.

STUDY 3

Experimental use of an acrolein-based primer as collagen cross-linker for dentine bonding

The objective of the present study was to investigate the long-term effect of the use of 0.01 wt% acrolein (ACR) aqueous solution as an additional primer, on the mechanical durability of resin-bonded dentine created with a simplified etch-and-rinse adhesive. Zymography of dentine extracts and *in-situ* zymography of resin-dentine interfaces were additionally performed to analyze the potential inhibition effect of ACR on dentinal MMPs.

The null hypotheses tested were that the use of ACR as a collagen cross-linker:

1) has no effect on bond strength deterioration over time and

2) has no effect on inactivation of endogenous dentine MMPs.

Materials and Methods

Microtensile bond strength test

Microtensile bond strength test was performed according to the protocol described in Study 1 with the following investigated groups:

The teeth were divided and randomly assigned to 2 treatment groups (n = 18).

1. Group 1 (experimental): the acid-etched dentine was pre-treated with 0.01 ACR wt% aqueous solution (MilliporeSigma, St, Louis, MO, USA) for 1 min, gently air-dried and bonded with Adper Scotchbond 1XT (3M ESPE) in accordance with the manufacturer's instructions. The composition of the adhesive and instructions for use are shown in **Table 6**.
2. Group 2 (control): Adper Scotchbond 1XT was applied directly on the etched dentine in accordance with manufacturer's instructions.

In both groups, the adhesive was light-cured (Curing Light 2500; 3M ESPE) for 20 sec after solvent evaporation. For time 0 (T0), the sticks were stored in artificial saliva (Pashley et al., 2004) for 24 h at 37 °C; for time 1 year (T12), the sticks were stored for 1 year in artificial saliva at 37 °C.

Table 6. Components, compositions, and application procedure of the tested adhesives (information supplied by the manufacturer).

Adhesive system	Composition	Manufacturer's instructions
Adper™ Scotchbond™ 1 XT Adhesive (3M ESPE)	1. Etchant: 35% phosphoric acid 2. Ethanol, 2-hydroxyethyl-methacrylate, bisphenol-A-diglycidyl-ether-dimethacylat, urethandimethacylate, water	1. Etch dentin using for 15 s. 2. Rinse thoroughly for 10 s. 3. Blot excess water using acotton pellet or mini sponge. 4. Immediately apply 2-3 consecutive coats of adhesive for 15 s. Gently airdry for 5 s to evaporate solvents. 5. polymerize for 10 s.

Gelatin zymography

Gelatin zymography was performed in dentin powder according to the protocol described in Study 1. For group 1, the dentine powder was demineralized with 10% phosphoric acid to simulate the etching procedure used in the application of an etch-and-rinse adhesive. For group 2, the dentine powder was demineralized in the same manner as group 1 and then treated with 0.01 wt% ACR solution at 4°C for 30 min. For the control, after demineralization with 10% phosphoric acid, the dentine powder was incubated with 2 mM of 1,10-phenanthroline at 4°C for 30 min.

In situ zymography of resin-dentin interfaces

In situ zymography was performed as described in Study 1, and the tested groups correspond to the groups listed in the *Microtensile bond strength* chapter of Study 3. The samples were bonded using identical protocols and tested at T0 (after aging in the artificial saliva for 24h at 37°C).

Statistical analysis

Analysis of μTBS data was performed using the tooth as the statistical unit; bond strength data from each tooth were averaged to obtain the mean bond strength for that tooth. The acquired data (n = 18) were evaluated for compliance with the normality (Shapiro-Wilk test) and equality of variance (modified Levine test) assumptions required for parametric statistical analysis. Because these assumptions were not violated, the data were analysed with a two-factor analysis of variance, to examine the effects of “with/without ACR” and “storage time” on μTBS. Post-hoc pairwise comparisons were conducted using the Holm-Sidak method. For all analyses, statistical significance were set at $\alpha = 0.05$.

As for the *in situ* zymography data, the integrated density of the fluorescence signals was quantified as indicator of the dentine enzymatic activities of the tested groups. Because the data were not normally distributed even after nonlinear transformation, they were analysed using the Mann-Whitney U-test, with $\alpha = 0.05$.

Results

Microtensile bond strength

The results of μTBS are shown in **Table 7** and **Figure 16**. The table presents the means and standard deviations of μTBS (in MPa) of groups 1 and 2 at T0 and T12, respectively. Two-factor analysis of variance revealed significant difference for the factor "with/without ACR" ($p < 0.001$) and the factor "storage time" ($p < 0.001$). The interaction of these two factors was also statistically significant ($p < 0.001$). Within the factor "with/without ACR", pairwise comparisons indicated no significant difference in μTBS between the two bonding methods at T0 ($p > 0.05$), and significant difference between the two bonding methods at T12 ($p < 0.05$). Within the factor "storage time" (T0 *vs* T12), pairwise comparisons indicated significant decline in μTBS for the subgroups "adhesive only" ($p < 0.05$) and "0.01 wt% ACR + adhesive" ($p < 0.05$).

Table 7. Means and standard deviations (mean ± SD) of μTBS of Group 1 (0.01% acrolein pre-treatment) and Group 2 (control) immediately after bonding (T0) and 12 (T12) of aging in artificial saliva at 37°C.

Treatment groups (MPa)	Storage Time	
	T0	T12
SB1XT+0.01%Acr	44.6±14.2[a] (43A/30CC/5CD/22M)	46.4±6.1[a] (30A/10CC/0CD/60M)
SB1XT	40.1±8.2[a] (20A/8CC/25CD/47M)	24.6±6.0[b] (25A/20CC/8CD/57M)

Differences were considered significant at $p<0.05$. The level of significance was adjusted according to the Bonferroni's correction. Groups with the same superscripts are not statistically significant ($p>0.05$). Percentages of the failure modes (reported in square rounds) after microtensile test analyzed by stereomicroscopy were classified as: A, adhesive; CC, cohesive in composite; CD, cohesive in dentin; and M, mixed failure. Bond reduction after storage report the percentage of mean bond reduction after 1 year of storage.

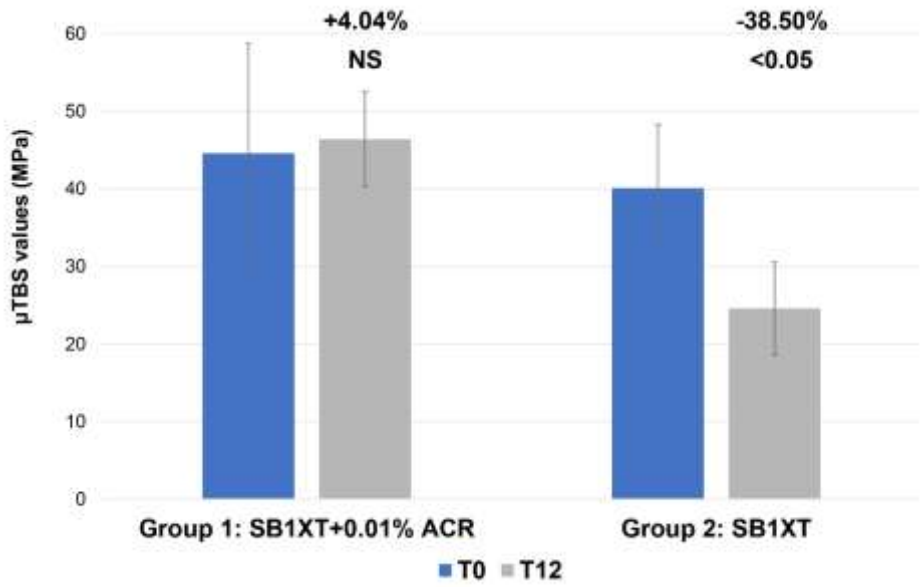

Figure 16. Graph summarizing the bond strength values (MPa) reported in Table 1.

Gelatin Zymography

The pro-form and active form of recombinant human MMP-2 and -9 for the positive control are shown in lane 1 of **Figure 17a**. Phosphoric acid-demineralized dentine extracts contained multiple forms of gelatinolytic enzymes, including a 72 kDa MMP-2 pro-form, a fainter 86 kDa band corresponding to the active form of MMP-9, and other minor gelatinolytic bands (**Figure 17a**, lane 2). Pre-treatment of demineralized dentine powder with 0.01 wt% ACR resulted in almost complete inactivation of the pro-form and active form of MMP-2 and the active form of MMP-9 (**Figure 17a**, lane 3). A band around 100 kDa was still detectable, which could be attributed to a complex pro-form of MMP-9. Densitometric evaluation of the zymography bands (**Figure 17b**) confirmed that ACR inactivated the pro-form and active form of MMP-2. A band around 100 KDa was still present, although it had a lower intensity compared to the MMP-9 active form. Control zymograms produced by incubating the phosphoric acid-demineralized dentine powder with 2 mM of 1,10-phenanthroline showed no enzymatic activity (data not shown).

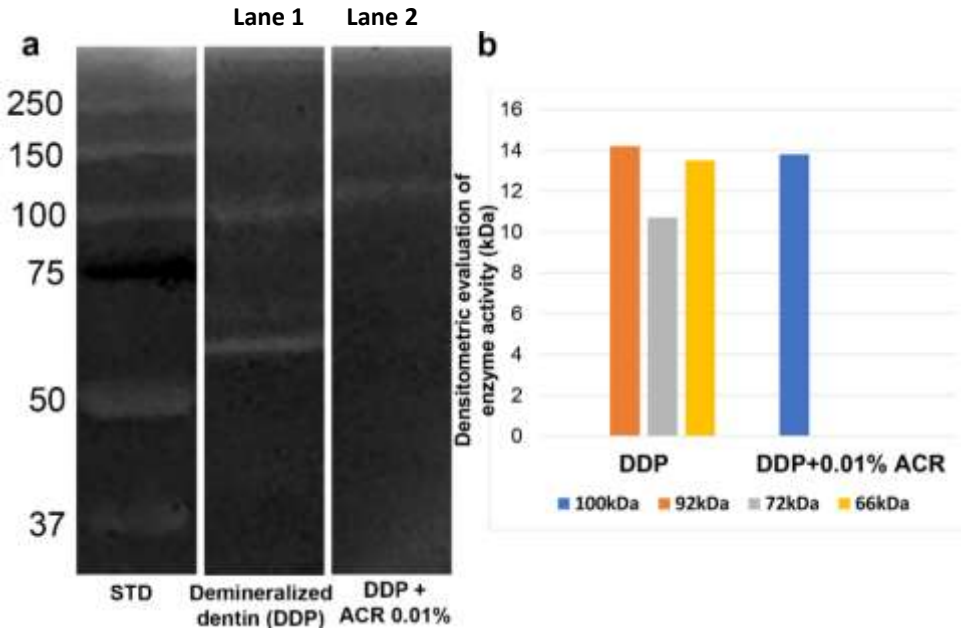

Figure 17. (a) Acrolein zymographic analysis. Lane 1: demineralized dentin powder showing activity of pro- form of MMP-9 (92 kDa) and active form of MMP-2 (66 kDa). Lane 2: demineralized dentin powder after incubation with 0.01% ACR showing complete inactivation of MMP-2, and reduced MMP-9 activity, although a complex form of MMP-9 around 100 kDa is still detectable; (b) Graph illustrating the densitometric evaluation of bands obtained from the zymographic analysis of proteins extracted from dentin powder**.**

In-situ zymography of resin-dentine interfaces

Confocal laser scanning microscopy images of *in-situ* zymography and superimposition of the fluorescence with light microscopy of the resin-dentine interfaces are shown in **Figure 18**. Specimens that were pre-treated with 0.01 wt% ACR prior to adhesive application exhibited minimal green fluorescence within the hybrid layer (**Figures 18a,c**). In contrast, specimens that were bonded using the dentine adhesive alone (control) exhibited intense green fluorescence within the hybrid layer (**Figures 18b,d).** Quantification of the fluorescence of the specimens (**Figure 19**) indicates a 42.76% reduction in enzymatic activity when the ACR-based primer was used before the bonding procedure. The difference between the control and experimental group is statistically significant ($p < 0.05$).

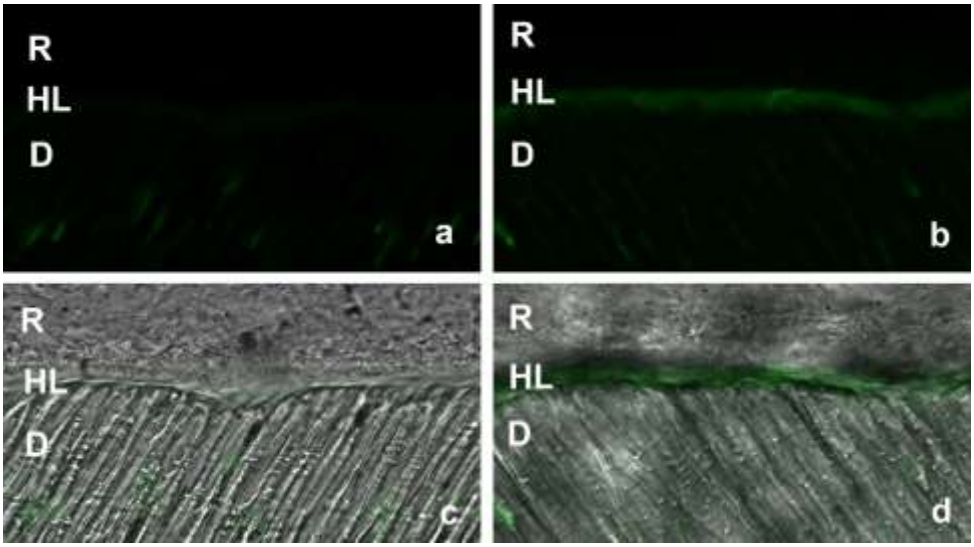

Figure 8. Resin-bonded dentin interfaces prepared with SB1XT with or without ACR pre-treatment, incubated with quenched fluorescein-labeled gelatin; (a) Image acquired in green channel, showing fluorescence (identifying intense endogenous enzymatic activity) in dentinal tubules and within the HL created with SB1XT with ACR pre-treatment; (b) Image acquired in green channel of the HL created by the application of SB1XT to acid-etched dentin without ACR pre-treatment showing higher fluorescence; (c) Image of SB1XT with ACR pre-treatment, obtained by merging differential interference contrast image (showing the optical density of the resin-dentin interface) and image acquired in green channel (showing enzymatic activity); (d) Image of HL created with SB1XT without ACR pre-treatment obtained by merging differential interference contrast image and image acquired in green channel; *SB1XT* = Adper Scotchbond 1 XT; *ACR* = Acrolein *D* = Dentin; *HL* = Hybrid Layer; *R* = Resin Composite.

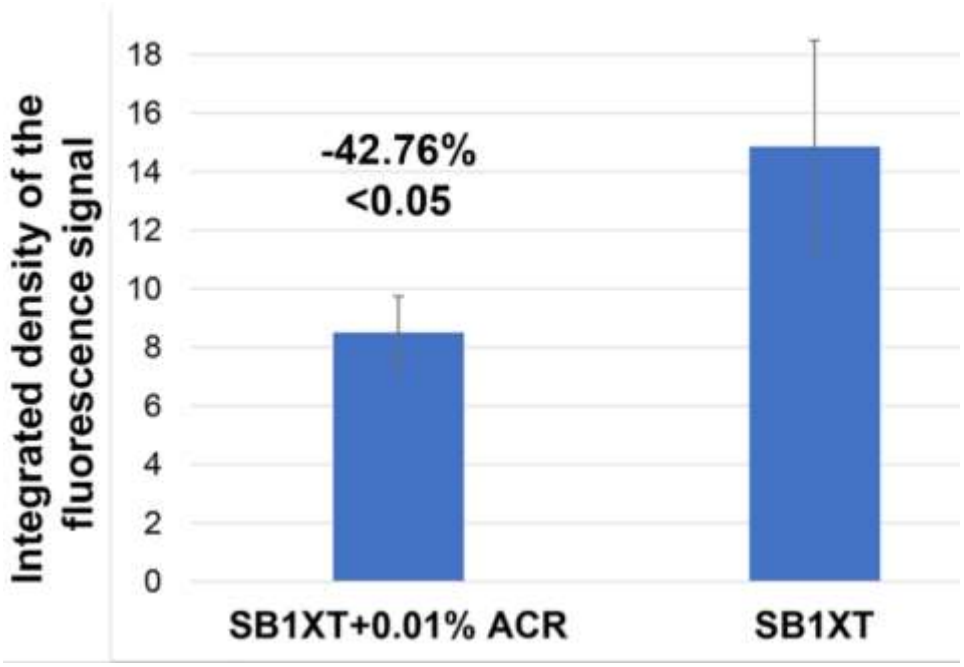

Figure 19. Graph illustrating the quantification of the enzymatic activity of the tested groups**.**

STUDY 4

Does chlorhexidine preserve the hybrid layer *in vitro* after 10-years aging?

Depending on the MMP-s inhibiting substance that was used, and whether it was used as a separate primer or incorporated within the adhesive blends, the experimental groups tested in the first 3 studies showed to the most part potent inhibitory activity towards endogenous enzymes, and preserved bond strength after 1 year of aging *in vitro*. However, since the inhibitors, such as CHX are largely soluble in water, their effect could be reversible, since they could leach out of the hybrid layer (Ricci et al., 2010; Sadek et al., 2010). Hence, it is of utmost importance to investigate whether the effect of enzyme inhibitors can be preserved within the hybrid layer over a longer period of time. Therefore, the research team that I am a part of started a comprehensive study 10 years ago, storing aged samples treated with CHX 0.2% as a separate primer, and as a part of the present thesis, we investigated whether the anti-enzymatic properties and the preservation of the hybrid layer remain in the experimental groups after this 10-year aging period in artificial saliva. Further aim was to investigate whether CHX was still present in the hybrid layer after 10 years of aging.

The null hypotheses of the study were:

1) CHX does not retain its MMPs inhibitory properties after 10-year aging in artificial saliva;

2) CHX does not influence the preservation of the hybrid layer after 10-year aging in artificial saliva;

3) CHX is no longer present in the hybrid layer after 10 years of aging in artificial saliva.

Materials and methods

In situ zymographic assay

In situ zymography was performed as described in Study 1, and the tested groups are the following:

1. Group 1 (G1) was etched for 15 s using a with 35% phosphoric acid for 15 s (etching gel, 3M ESPE; St Paul, MN, USA), air dried gently, and left moist for the wet-bonding procedure with Adper Scotchbond 1XT (SB1XT, 3M ESPE). The adhesive was applied according to the manufacturer's instructions;
2. In group 2 (G2), the samples were etched as in G1, then the dentin was treated with a 0.2% CHX containing additional primer for 60 s, gently air dried and left moist, and the wet-bonding procedures with SB1XT were performed as in G1.

The samples were tested at T0 (after aging in the artificial saliva for 24h at 37°C) and T10yr (after aging in the artificial saliva for 10 years at 37°C).

Transmission electron microscopy of resin-dentine interfaces

Bonding of the samples was performed in the same way as described previously for the *in situ* zymography test. The specimens were aged for 10 years in artificial saliva at 37°C. Further, they were decalcified in 0.1 M formic acid/sodium formate (pH 2.5) followed by fixation in Karnovsky's fixative (2.5 wt% glutaraldehyde and 2% paraformaldehyde in 0.1 mol/L cacodylate buffer; pH, 7.3) for 8 h. The samples were post-fixed in 1% osmium tetroxide for 1 h. The decalcified specimens were dehydrated in ascending concentrations of ethanol (50%-100%), immersed in propylene oxide as a transition medium, and embedded in pure epoxy resin. Ultrathin sections (90 nm) were cut with an and mounted on nickel grids. Grids were stained with 4% uranyl acetate (15 min) and Reynold's lead citrate (15 min) for examination with a JEM-1230 TEM (JEOL, Tokyo, Japan) at 110 kV.

Identification of CHX by Micro-Raman Spectroscopy

Bonded sticks prepared as for *in situ* zymography were aged for 10 years in artificial saliva at 37°C and tested for identification of CHX in the hybrid layer. Micro-Raman spectroscopy was performed using Senterra spectroscopy (Bruker Optik; Ettlingen, Germany). The micro- Raman spectrometer was first calibrated for zero and then for coefficient values using a silicon sample. Samples were analyzed using the following micro-Raman parameters: 20 mW neon laser with 532 nm wavelength, spatial resolution of ca 3 µm, spectral resolution of ca 5 cm^{-1} and 100X magnification (Olympus UK; London, UK) to a ca 1-µm beam diameter. The bonding area was visualized and focused at 100X magnification. The spectra were taken in intertubular dentin, in the middle of the hybrid layer, selected arbitrarily. Accumulation time per spectrum was 30 s and 6 co-additions were taken per point. The data were post-processed using the dedicated Opus Spectroscopy Software version 6.5 and consisted of analysis with modeling, which allowed distinguishing spectral components of the adhesive and dentin.

Results

In situ zymographic assay

Results of the *in situ* zymographic assay are shown in **Figure 20 and 21** and were assessed qualitatively and quantitatively. At T0, there is a pronounced fluorescence in the hybrid layer of the control group, while 28% less pronounced signal was demonstrated in the CHX-pretreated groups ($p < 0.05$). A 40% lower level of fluorescence in the experimental group compared to the control group was sustained even after 10-years aging ($p < 0.05$), maintaining the fluorescence on a similar level as at baseline ($p > 0.05$).

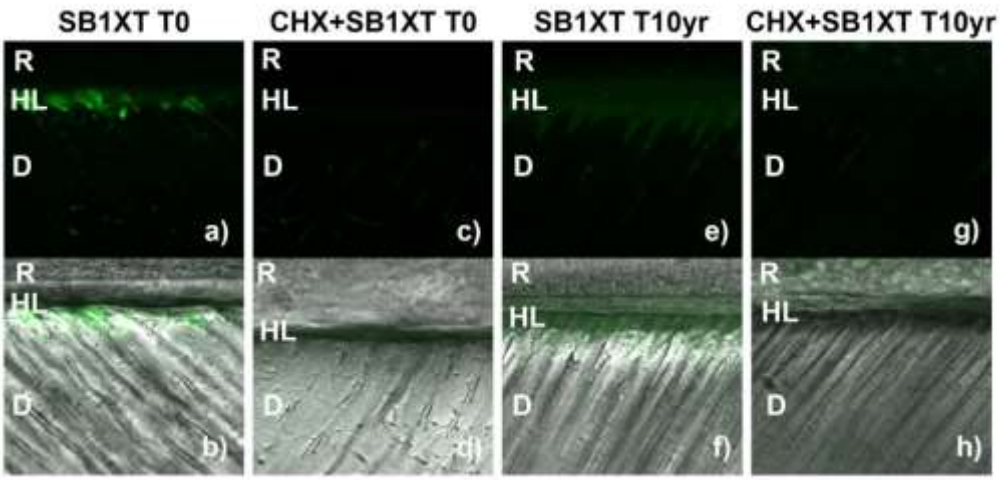

Figure 20. Resin-dentin interfaces incubated with quenched fluorescein-labeled gelatin; (a) Image acquired in green channel, showing fluorescence (identifying intense endogenous enzymatic activity) in dentinal tubules and within the HL created with SB1XT without CHX pre-treatment at T0; (b) Image of SB1XT without CHX pre-treatment, obtained by merging differential interference contrast image (showing the optical density of the resin-dentin interface) and image acquired in green channel; (c) Image acquired in green channel of the HL created by the application of SB1XT to acid-etched dentin with CHX pre-treatment at T0 showing lower level of fluorescence; (d) Image of HL created with SB1XT without CHX pre-treatment obtained by merging differential interference contrast image and image acquired in green channel at T0; (e) Image acquired in green channel, showing fluorescence in the HL created with SB1XT without CHX pre-treatment at T10-yr; (f) Image of SB1XT with CHX pre-treatment at T10-yr, obtained by merging differential interference contrast image and image acquired in green channel; (g) Image acquired in green channel of the HL created by the application of SB1XT to acid-etched dentin with CHX pre-treatment at T10-yr showing lower level of fluorescence; (h) Image of HL created with SB1XT with CHX pre-treatment obtained by merging differential interference contrast image and image acquired in green channel at T10-yr; SB1XT = Adper Scotchbond 1 XT; CHX = Chlorhexidine D = Dentin; HL = Hybrid Layer; R = Resin Composite.

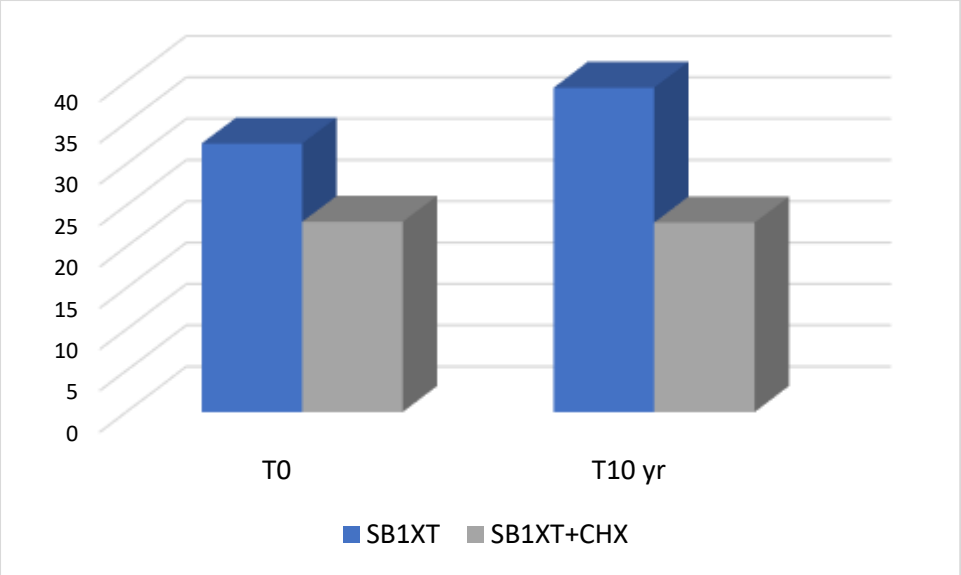

Figure 21. Graph illustrating the quantification of the enzymatic activity of the tested groups.

Transmission electron microscopy of resin-dentine interfaces

The results depicting the level of preservation of the hybrid layer as assessed under TEM (*data not shown – unpublished results*) showed that in G1 (SB1XT E&R – 10-year aging), 98% of the hybrid layer had been degraded. Only the resin tags were linking the adhesive and composite with the underlying intertubular dentin. In G2 (SB1XT E&R + CHX – 10-year aging), 95 % of the hybrid layer is intact. In the 5% of the hybrid layer that had degraded, the collagen fibrils were sparse and had partially degraded microfibrillar strands and short chains. The hybrid layer was approximately 3µm thick.

Identification of CHX by Micro-Raman Spectroscopy

All the registered Raman spectra of the investigated groups were within the region of 400-2200 cm^{-1}, and are associated with adhesive, dentin mineral, collagen and CHX bands. The main active peaks that characterize the adhesive system are at 1113 cm^{-1} (C-O-C), 1454 cm^{-1} (CH_2), 1608 cm^{-1} and 1638 cm^{-1} (C=C) and 1720 cm^{-1} (C=O).

The characteristic peaks that can be attributed to mineral dentin are at 960 (PO_4^{-3}) and 1070 cm^{-1} (CO_3^{-2}), and to the collagen at 1242/1280 cm^{-1} (amide III), 1452 cm^{-1} (CH_2) and 1667 cm^{-1} (amide I). These peaks can be observed in all the experimental groups.

However, the peaks that are assigned to CHX, at 670 cm^{-1} (C-Cl), 1268 and 1564/1608 cm^{-1} (unprotonated/protoneted bands associated to CHX, respectively) (Jones et al., 2000) could be identified only in SB-CHX group.

Apart from the presence of the described bands, the chemical profile by Raman revealed interesting differences between the groups. In the SB1XT group, the first 5μm into the depth of the resin-dentin interface show properties of the hybrid layer, since signals of hybridization with adhesive penetration (C-O-C, CH_2, C=C, C=O), very slight PO_4^{-3} peaks as well collagen peaks (amide III) can be identified, indicating that the mineral has been removed from this region during acid etching. The amide I peaks were absent, possibly due to the damage of the collagen fibrils, since both amide peak III and I are sensitive to the molecular conformation of polypeptide chains (Toledano et al., 2015; Xu and Wang, 2012, 2011). Starting from 6th μm, there is an increase of intensity of PO_4^{-3} and CO_3^{-2} peaks, as well as slight collagen vibrations of CH_2 and amide I, demonstrating the characteristics of dentin.

In the SB-CHX group, on the other hand, characteristic spectra of the hybrid layer were well defined from the 1st to the 8th μm. More intense vibrations on region at 670 cm^{-1} (black hand) were identified in comparison to the control group. These vibrations could probably be attributed to C-Cl bands of CHX. This peak was present in the entire mapping, while the characteristic peak attributed to CHX (1564 cm^{-1}; green hand) was identified only the top spectrum on mapping (Jones et al., 2000; Loguercio et al., 2016). Furthermore, other atypical vibrations in the region of 1268 cm^1 (red hand) throughout the entire spectra, and exclusively in the SB-CHX group.

Starting from the 9th µm signals of dentin substrate can be detected due to the increase of intensity of mineral peaks (PO_4^{-3} and CO_3^{-2}). Nevertheless, signals of interaction of adhesive system remained in these spectra.

However, the CHX contains two basic -CNH-NH-CNH- groups both of which readily accept a proton to form a protonated species with several tautomeric forms (Jones et al., 2000). Thus, during the interaction of CHX with the demineralized dentin, protonation of amide groups of CHX with the carboxylic acid/hydroxyl from dentin occurs. Certainly, the tautomeric forms resulted by the protonation can be identified by the additional peak at 1268 cm^{-1}, and as previously demonstrated at 1608 cm^{-1} (Jones et al., 2000). However, the 1608 cm^{-1} could have been overlapped, since it is a common peak within adhesive system. Thus, it could not be confirmed in this analysis. The graphical data are not presented – *unpublished results*.

DISCUSSION

The presented thesis demonstrated that the inhibitors/inactivators of endogenous dentinal enzymes were efficient in preserving bond strength after 12 months of aging, and hybrid layer integrity after 10 years of *in vitro* aging in artificial saliva at 37°C when used as a separate primer. In the cases when the inhibitory agent was introduced within the adhesive resin, the bond preservation was adhesive system- and/or bonding mode-dependent. Hence, the first hypothesis can be partially rejected. The enzymatic activity was lower in all experimental groups, at baseline, as well as after aging (for 1 year and 10 years) with a slight influence of the bonding mode. Hence, the second hypothesis of the thesis can be rejected.

Discussion of the results

Pashley et al. were the first to show that endogenous dentinal enzymes can degrade exposed collagen fibrils over time in the absence of bacteria, and that this effect can be diminished by the use of CHX (Pashley et al., 2004). Since then, there have been numerous reports on the beneficial effect of protease inhibitors on bond strength preservation over the course of time (Bedran-Russo et al., 2007; Breschi et al., 2010a; Carrilho et al., 2007c, 2007a; Hebling et al., 2005; Mazzoni et al., 2017; R Seseogullari-Dirihan et al., 2015). In particular, the inhibitors chosen to be used in the present study are CHX and BAC. CHX is an antimicrobial agent used in dentistry for years, available in every dental office, and therefore very convenient and widely accepted as an additional primer aiming to inhibit MMPs. BAC, on the other hand, contains a QAM-group and inhibits MMPs in a similar way as CHX, but is also dissolvable in ethanol and acetone.

It is well known that low concentrations of CHX can inhibit MMPs and cysteine cathepsins (Gendron et al., 1999; Scaffa et al., 2012). It is hypothesized that there are several mechanisms responsible for the affinity of CHX towards dentin as a substrate (J. Kim et al., 2010b). CHX is a strong base and has a cationic structure at physiological pH. The cationic part of CHX molecule binds to the negatively charged part of the target molecule. It has not been completely explored how CHX reacts with the oral tissues, but it is assumed that there is a cationic-anionic reaction of CHX with organic, as well as mineral substrates. The reaction with the organic part is presumed to be with the carboxylic and hydroxyl groups of collagen and noncollagenous phosphoproteins, while in the hydroxyapatite, CHX is presumed to bind to the mineral phosphates (J. Kim et al., 2010b). Moreover, CHX not only has the possibility to bind to, but also showed excellent substantivity to oral tissues (Carrilho et al., 2010).

Several authors established the beneficial effect of CHX water solution used as a primer in the preservation of the μTBS over time (Breschi et al., 2010b, 2009;

Campos et al., 2009; Loguercio et al., 2016; Zheng et al., 2014). On the other hand, certain authors found no influence of CHX water solution on bonding performance (Manso et al., 2014), or a negative influence on mechanical properties of the hybrid layer after the use of this inhibitor (Hiraishi et al., 2009). Nevertheless, the majority of the available research is in favor of the use of CHX.

In every-day dental practice, it is of great importance for patients, as well as for dental practitioners, to make chair-time as short as possible. Therefore, there is a tendency to avoid additional steps in the restorative procedure. With that in mind, efforts have been made to incorporate protease inhibitors/cross-linkers within one of the components of bonding systems. Another reason behind this tendency is that some of the inhibitors are soluble in water and are thus thought to leach out of the hybrid layer after a certain period of time (De Munck et al., 2010a; Hashimoto et al., 2000; Komori et al., 2009). Since adhesives act like semipermeable membranes (Breschi et al., 2009), it is assumed that the adhesive layer could serve as a reservoir of protease inhibitors, released into the hybrid layer over time, which could contribute to the durability of the adhesive bond. Therefore, in the presented thesis, we tested a commercially available adhesive system with 0.2% CHX included within the adhesive (Peak Universal), and an experimental adhesive system with BAC blended within a commercially available adhesive (0.5% BAC or 1% methacrylate BAC in AllBond Universal).

The CHX-doped adhesive system revealed significant improvement in bond stability, immediately, as well as after aging compared to the control adhesive system. After a 12-month storage in artificial saliva, a decrease in bond strength was noted in all the tested groups. However, the CHX-containing groups showed higher bond strength values, in fact, higher than the T0 values in the control group. These results differ from the results of a similar recent study (Sabatini, 2013) in which the same commercial adhesive system was used as the experimental group. Sabatini et al. found no differences between the control and experimental group in shear bond

stress, regardless of aging (6 months), or the bonding mode (E&R/SE). Further, Sabatini et al. confirmed the efficacy of CHX in inhibiting the dentinal MMPs using a zymographic assay, testing only the effect of CHX aqueous solution. This finding is in accordance with our results. However, in our study, gelatin zymography was for the first time performed following all the steps required by the manufacturers for the clinical use of Peak Universal and SBU systems in E&R and SE mode. Hence, the results of Sabatini et al. are not fully comparable to our study, due to the different methodology, control group and aging time. Moreover, perhaps the aging of 6 months was not long enough to reveal differences between the tested groups. Furthermore, the control group in the study of Sabatini et al. was Peak LC, which is a material very similar to Peak Universal, since they are produced by the same company, with the difference that Peak LC does not contain 0.2% CHX. Since we used a different system as the control group (SBU), we cannot exclude the possibility that Peak Universal is superior to SBU as an adhesive system and that the differences are not driven by the presence of CHX. However, since the present study did not only test the microtensile bond strength, but also showed, using two different zymographic assays, that the Peak Universal adhesive system presents lower dentinal enzymatic activity compared to SBU, and having in mind previous studies that have shown preservation of the hybrid layer with the use of CHX, we could hypothesize that there is a correlation between the CHX within Peak Universal and better bond strength.

The results of other studies with CHX incorporated into one of the parts of the adhesive system varied, especially in SE mode. Certain studies showed that the incorporation of CHX within adhesive blends, used in the E&R mode could contribute to the preservation of bond strength after a 12-month water storage (da Silva et al., 2015; Stanislawczuk et al., 2014). Yiu et al. (Yiu et al., 2012) found mixed results, where the effect of CHX was in correlation with the composition of the adhesive used in the E&R mode. Zhou et al. found that the incorporation of 0.1%-

1% CHX into a SE primer could preserve dentin bond strength after a 12-month aging (Zhou et al., 2009). Conversely, a study incorporating 2% CHX into a SE adhesive found no influence of CHX on bonding performance after 6 months (Pomacóndor-Hernández et al., 2013).

On the other hand, BAC incorporated into one of the parts of the adhesive system demonstrated mixed results in the available literature. There is an etchant on the market containing 1% BAC (Bisco), initially produced due to antimicrobial properties of BAC, but recently studied also for its anti-collagenolytic potential. However, the groups treated with 1% BAC etchant showed deterioration of the hybrid layer after 6 months (El Gezawi et al., 2018; C Sabatini et al., 2014), 12 months (Sabatini et al., 2015; Sabatini and Pashley, 2015; Tekçe et al., 2016) and 18 months of aging (Sabatini and Patel, 2013). This could be due to the fact that BAC bonds to dentin electrostatically and around 50% of BAC could be washed off along with the etchant (Arzu Tezvergil-Mutluay et al., 2011). Hence, blending BAC in several concentrations in different adhesives has been tested in terms of bond strength preservation and anti-MMP properties. Since BAC can be dissolved in ethanol and acetone, and its activity is rather consistent in different pH, it was assumed that it could be incorporated into a large variety of adhesive blends maintaining its anti-enzymatic properties (Sabatini et al., 2015).

In the majority of the available studies, BAC incorporated in different concentrations (0.25-2%) into predominantly E&R adhesives yielded preservation of bond strength. BAC in the concentrations of 0.25%, 0.5%, 1% and 2% incorporated into two different E&R adhesives (Optibond Solo Plus and All-Bond 3, Bisco) demonstrated preservation of bond strength (microshear bond strength test) after 18 months of aging, and in the lower concentrations, even an increase of bond strength was noted (Sabatini and Patel, 2013). Adper Single Bond blended with 0.5% and 1% of BAC showed preservation of bond strength after 6 months in both tested concentrations (C Sabatini et al., 2014), or 1 year of aging with 0.5% BAC (Sabatini et al., 2015).

However, the 1% BAC group demonstrated bond strength comparable to the control group after 1-year aging. When a certain percent of a new component is incorporated into an adhesive, especially in a form of a liquid, there is a risk of jeopardizing the balance between the components of the system. This could have a detrimental effect on degree of conversion, elastic modulus, tensile stresses and other mechanical properties of the adhesive (M. Cadenaro et al., 2009). Hence, perhaps the polymerization and material properties have been affected by the incorporation of a higher concentration of BAC into the adhesive blend in this case. In fact, this is what we believe happened also in the present study, since we demonstrated loss of bond strength after aging, comparable to the control group in the case of 0.5% BAC, or even more pronounced in the case of 1% methacrylate BAC, both in the E&R and SE mode. Moreover, the 1% methacrylate BAC showed lower bond strength already at the baseline compared to other investigated groups. On the other hand, Sabatini et al. (Sabatini and Pashley, 2015) investigated the same adhesive system that was considered in the present thesis – All Bond Universal (Bisco) with 0.5%, 1 and 2% BAC or methacrylate BAC blended within the adhesive, used in E&R mode, and bond strength was preserved in all the experimental groups after 6 and 12 months of aging. A possible reason for these discrepancies in the results could be due to the technology of the incorporation of the active substance in the adhesive or perhaps to the fact that different light-curing units have been used in the two studies.

Methacrylate BAC added to ABU in this investigation has yielded the worst results regarding bond strength after aging. However, it demonstrated the lowest level of enzymatic activity compared to other tested groups, especially after aging, at T12. We hypothesize that both these results can be attributed to poor degree of conversion in this new system where additional methacrylates were added and did not polymerize properly. Hence, this caused leaching out of the non-polymerized monomer, facilitating the breach of water within the hybrid layer that became more

permeable, leading to hydrolysis of the organic and resin component of the hybrid layer. On the other hand, leaching of the BAC methacrylate monomer could have induced better MMP-inhibitory properties. These effects were particularly apparent after aging.

Also, generally, there are certain differences in the results of studies incorporating inhibitors in E&R and SE adhesives. It is thought that acid etching during E&R procedures can denaturize enzymes within the etched dentin, due to the low pH values of the phosphoric acid. However, Mazzoni et al. (Mazzoni et al., 2006) showed that enzymatic activity in dentin could be reactivated after acid etching. The authors hypothesize that acid-etching inactivates the superficial layer of the enzymes, while activating the residual latent enzymes within the underlying demineralized dentin. The SE adhesives, on the other hand, have a pH 1.5 – 2.7, which means that the more acidic adhesives could denaturize the enzymes, while the ones with a higher pH value could contribute to the activation of enzymatic activity (Nishitani et al., 2006). Moreover, the protease inhibitors which act via chelation bind to calcium chloride released from dentinal tissue by the influence of acids (especially the primers of SE adhesives) (Frassetto et al., 2016; Gendron et al., 1999), which could diminish the inhibitory effect of CHX. Furthermore, in the SE systems, the hybrid layer is created simultaneously as the acidic monomer etches into the dentin. Therefore, it is assumed that there are less denuded collagen fibrils at the bottom of the hybrid layer compared to the E&R system, and consequently less amount of activated MMPs (Breschi et al., 2004). Perhaps this is why CHX might have less influence on dentinal enzymatic activity in SE adhesives. This corresponds to the *in situ* zymography results of the present Study 1, since the activity in the SE groups was much lower compared to the E&R groups, regardless of the presence of CHX in the bonding system. Further, what can be clearly observed on *in situ* zymography figures in both in Study 1 and 2 is that there is a different distribution of the fluorescent signal in E&R compared to SE groups, regardless of the adhesive system

used. In the SE systems, the enzymatic activity is limited to the first several µm of the hybrid layer and the signal rarely penetrates the dentinal tubules, while in the E&R groups the signal clearly goes more deeply into the dentin and the enzymes within the dentinal tubules seem to be more activated. Hence, the overall activity in the E&R groups could be higher compared to SE groups, although the results might not always demonstrate it since we quantify the enzymatic activity only in the first 15 µm of the hybrid layer, since we believe this is the most critical area. All the aforementioned differences could be responsible for the discrepancies in the results found in different studies investigating E&R and SE dentin bonding strategies.

Since the inhibitors demonstrated discrepancies in the results, another strategy has been widely considered and investigated. Based on the premises that native cross-links improve the tensile properties of collagen fibrils and increase the resistance of the collagen matrix against enzymatic degradation (Bedran-Russo et al., 2014), different natural and chemical cross-linking agents have been used experimentally prior to adhesive application (Al-Ammar et al., 2009; Bedran-Russo et al., 2007; Cova et al., 2011; Macedo et al., 2009; Mazzoni et al., 2014). Collagen cross-linking agents have the ability to reinforce the collagen fibrils network by inducing intra- and intermolecular cross-links. This may prevent unwinding of the triple helix of collagen molecules, which is necessary for the true collagenases to reach the site on the collagen which they can cleave. This results in the increase in resistance of the collagen matrix to MMP activities (Chung et al., 2004). Unlike cross-linkers, the inhibitors are electrostatically bonded with the polymerised resin network and may therefore leach out of the hybrid layer after 18-24 months (De Munck et al., 2010a; Hashimoto et al., 2000; Komori et al., 2009). Moreover, recently published studies showed that some of the cross-linking agents are also capable of inactivating dentinal MMPs (Cova et al., 2011; Mazzoni et al., 2014). As a part of the present thesis, we considered acrolein (2-propenal) as a cross-linker from the group of aldehydes, since other members of the same family, such as glutaraldehyde were

shown to positively affect the mechanical properties of dentine and collagen fibrils in several connective tissues (Al-Ammar et al., 2009; Bedran-Russo et al., 2007; Macedo et al., 2009). Application of ACR before bonding to acid-etched dentine resulted in significantly better preservation of μTBS after one year of *in vitro* aging (the bond strength was even slightly higher) and in a decreased enzymatic activity of the MMPs in the exposed collagen matrix. Hence, both null hypotheses of Study 3 can be rejected.

The influence of crosslinkers, such as glutaraldehyde, on the mechanical properties of the dentine organic matrix, as well as on the preservation of the hybrid layer has been widely investigated (Bedran-Russo et al., 2008, 2007; Chen et al., 2016; Han et al., 2003; Hass et al., 2015; Hiraishi et al., 2013; R. Liu et al., 2011; Seseogullari-Dirihan et al., 2016; Xu and Wang, 2011; Zhou et al., 2016). Glutaraldehyde improves the mechanical properties and reduces the degradation rate of biological tissues (Nimni, 1988; Sung et al., 1999) by bridging the amino groups of lysine and hydroxylysine residues of different collagen polypeptide chains with monomeric or oligomeric cross-links (Adkins, 1970; Al-Ammar et al., 2009; Bedran-Russo et al., 2007; Dos Santos et al., 2011; Macedo et al., 2009). Similarly to glutaraldehyde, ACR is a well-known cross-linking agent and is used as a tissue fixative (Cheung et al., 1990, 1985; Macedo et al., 2009). Hence, ACR was included in the present experiments to investigate the influence of a the most reactive α,β-unsaturated aldehyde molecule on cross-linking of demineralized dentin. Therefore, it can easily bind to glutathione, as well as react with cysteine, histidine and lysine protein residues. The ACR cross-linking reaction starts with two molecules of ACR that react with the free amino terminal groups of lysine (or hydroxylysine), forming Nε-(3-formyl-3,4-dehydropiperidino)lysine (FDP-lysine). The electrophilic α,β-unsaturated carbonyl moiety is retained in FDP-lysine, allowing it to react further with sulfhydryl compounds of glutathione, creating new intra- and inter-molecular cross-links (Stevens and Maier, 2008).

The advantage of our study is that ACR was applied on dentin for 1 min, a clinically acceptable time, and significantly shorter compared to the one reported in the studies with other cross-linking agents (Al-Ammar et al., 2009; Bedran-Russo et al., 2007; Castellan et al., 2010; Dos Santos et al., 2011). Even when ACR was applied for only 1 min, the bond strength of the tested adhesive was preserved after one year. Hence, the results of the present study may be considered more clinically relevant when compared to glutaraldehyde, which requires 1 h of application on the dentin surface to be effective (Castellan et al., 2010; Dos Santos et al., 2011).

Apart from creating cross-links within the dentin collagen matrix, bond preservation in acid-etched dentine that is treated with cross-linking agents may also be related to their ability to inactivate endogenous MMPs in the exposed dentin collagen matrix (Mazzoni et al., 2018a, 2017, 2014, 2006; Nishitani et al., 2006). It has been hypothesized that cross-linking agents also cross-link MMPs, changing their molecular mobility, altering the three-dimensional conformation of the catalytic domain of MMPs, or changing their negatively-charged ionised carboxyl groups into positively-charged amide groups, which consequently inactivates the MMPs. Unlike in the case of protease inhibitors, inactivation of the proteases by cross-linkers is non-specific (Nascimento et al., 2011; Tersariol et al., 2010), and the created bonds are covalent, and not electrostatic, which should render the anti-enzymatic activity of cross-likers long-lasting and more efficient. The results of gelatin and *in situ* zymography of the present study support the hypothesis that stabilization of hybrid layers created after priming with ACR is related to the inactivation of dentin gelatinolytic activities, since the reduction in the enzymatic activity was noted in dentin powder, as well as in the hybrid layer, respectively. Further, hypothetically, the inactivation capability of ACR may be associated with the modification of Cys residues in the MMPs, which, in turn, results in the inactivation of these proteolytic enzymes (Aldini et al., 2011; Gugliucci et al., 2007), but no definitive structure characterization or molecular dynamics simulation have been provided.

Similar to glutaraldehyde, a disadvantage of ACR compared to the other cross-linking agents is the cytotoxicity that may arise from residues of unreacted or degraded cross-linking agents (Han et al., 2003). However, ACR is widely present in cooked foods as well as in the environment (Stevens and Maier, 2008). Moreover, a very low concentration of ACR was employed (0.01%) in the present study. The U.S. Environmental Protection Agency (U.S. Environmental Protection Agency, 2003) published general recommendations for the safety levels regarding the ingestion of ACR based on studies performed on rats. The studies tested the consequences of chronic ACR ingestion and reported that the no-observed-adverse-effect-level (NOAEL) of ACR was 0.05 mg/kg per day. Hence, after the application of a total uncertainty factor of 100, the recommended reference dose (RfD) is 0.0005 mg/kg per day. The World Health Organisation suggests a tolerable ACR intake of 0.0075 mg/kg per day (Gomes et al., 2002). One drop of the solution that was used in the present study contains 0.05 mg of ACR. This means that the ACR primer may be safely used on adults, especially considering that the intake of ACR during dental procedures would be rare, or completely prevented, due to the use of a rubber dam during clinical restorative procedures. Nevertheless, since there are other sources of the ACR in the environment, the cytotoxicity of ACR should be further investigated by testing serial dilutions of ACR on dental pulp stem cells prior to its recommendation for clinical use.

The aging time used in the first three presented studies could be considered short, and *in vitro* and clinical studies with a longer monitoring time should be conducted to assess the efficacy of this adhesive system. However, the clinical studies conducted thus far have shown mixed results on the protective effect of inhibitors, such as CHX, on the hybrid layer. The study by Araújo et al. (Araújo et al., 2015) investigated whether there was a clinical advantage of the use of 1% CHX primer over the course of 2 years, while Sartori et al. (Sartori et al., 2013) conducted a 5-year clinical follow up, using a 2% CHX primer. Both studies investigated non-carious

cervical lesions and neither found a difference in survival in the CHX group as compared to the control group. Non-carious lesions of the V class are quite specific due to the presence of sclerotic dentin, structurally different compared to sound or caries-affected dentin, which might have influenced the results. Carrilho et al. (Carrilho et al., 2007b), on the other hand, found that the use of a CHX primer preserves bond strength in restorations of the I class, after 14 months of intra-oral use. Thus far, the longest *in vitro* aging tested in samples treated with CHX primer, was reported by Loguercio et al. (Loguercio et al., 2016), demonstrating by means of Raman spectroscopy that there is in fact CHX in the hybrid layer of the tested samples, and by means of μTBS test that the bond strength is higher in the CHX-treated samples after 5 years of *in vitro* aging. As to our knowledge, the presented Study 4 is so far with the longest reported *in vitro* aging time regarding the effect of inhibitors on the preservation of the hybrid layer.

The Study 4 investigated the effect of CHX used as an additional aqueous primer during bonding procedures on endogenous enzymatic activity and the preservation of the hybrid layer after 10-year aging in artificial saliva at 37°C. CHX seemed to retain its MMPs inhibitory properties after 10-year aging in artificial saliva. Further, preservation of the hybrid layer was shown in the CHX-pretreated specimens, while the hybrid layers in specimens of the control group were almost completely degraded. Also, as shown in the Raman spectroscopy, peaks belonging to CHX were present in the hybrid layer only of the experimental samples after 10 years of aging in artificial saliva. Hence, all the null hypotheses of Study 4 can be rejected.

We have already discussed the inhibitory potential of CHX shown in numerous studies, and we can safely say that CHX does improve bond strength over a certain period of time. But, these studies left us with a big question – does this effect last? As demonstrated in the present research, the inhibitory activity of CHX seems to persist even after 10-year *in vitro* storage in artificial saliva at 37°C. As for evaluating this fact in terms of hybrid layer preservation, we demonstrated for the

first time using TEM analysis that 95 % of the hybrid layer in the CHX-treated group was intact, while 98 % of the hybrid layer in the control group has been degraded. In the control group, there were only resin tags linking the adhesive and composite with the underlying intertubular dentin. In the 5 % of the hybrid layer in CHX-containing group that had degraded, the collagen fibrils were sparse and showed partially degraded microfibrillar strands and short chains. Furthermore, the peaks that were assigned to CHX could be identified only in SB-CHX group by Raman spectroscopy, and not in the control group. Therefore, we could hypothesize that the CHX has influenced significantly the preservation of the hybrid layer after such a long period of time. These results add substantially to the knowledge on the long-term effects of CHX and further reinforce the importance of MMPs inhibition in the preservation of the hybrid layer.

As presented in the results of this thesis, as well as in the available literature, there are several factors to be considered when engaging testing of different MMP-inhibitory agents into any clinical protocol, or in a part of an adhesive system. Firstly, the possibility of clinical application and convenience of the clinician and patient should always be considered. If a certain procedure is efficient but prolongs the chair time for more than 5 min, it is not applicable in every day practice. Also, if the procedure is overly practitioner-dependent, there could be a lot of variability in the efficiency of the treatment. Further, if an active component is added within the adhesive, it can influence the material properties of the adhesive in a detrimental way. Moreover, there have been a lot of discrepancies in the results of studies performed in E&R and SE modes. This could be attributed both to the composition of the materials, which differ from each other, as well as the chemical interactions that the active substance has with the individual components of the adhesive blend. Also, importantly, the inhibitors themselves have different interactions with the substrate depending on whether the substrate has been previously etched or not. For instance, exploring the substantivity of CHX to dentin, Carrilho et al. compared

the substantivity in three substrates, the mineralized, partially and totally demineralized dentin, and found that CHX has the best substantivity to partially demineralized dentinal substrate (Carrilho et al., 2010). This could probably be due to the fact that in such a substrate, CHX can exert its affinities towards the extracellular organic matrix, as well as towards hydroxyapatite in the underlying mineralized dentin. In the present studies, the dentin was partially demineralized, possibly contributing to the prolonged effects of CHX.

Nevertheless, it could be recommended to clinicians in the every-day practice to use CHX as a separate primer or incorporated within an adhesive system, since it exhibited beneficial effects on the longevity of the hybrid layer and prolonged anti-enzymatic activity. Further research involving other adhesive systems with a long aging time should be performed in order to distinguish between the effects of CHX versus the effects of the bonding material, both being important factors in the degradation of the hybrid layer.

Discussion of the methods

In order to explain the reasoning behind the choice of methods used in the present thesis, it is necessary to better understand their advantages and limitations.

The Guidelines of the Academy of Dental Materials on *in vitro* testing of dental composite bonding effectiveness to dental substrate have recommended μTBS test as the optimal measure of the strength between the composite material and dental tissue (Armstrong et al., 2017). This technique is relatively simple, and it should be reproduced easily in different laboratories, facilitating the comparison between the results of different published studies and also repetition if deemed necessary. However, there has been a lot of variability between the studies, even though they were using the same testing method and the same adhesive material, probably due to differences in specimen geometries, loading configurations and modulus of elasticity of the restorative composites (Roeder et al., 2011). Hence, the present

study has been conducted in compliance with the guidelines proposed by the Academy of Dental Materials.

The influence of etching and adhesive procedures on endogenous dentinal enzymatic activity has been successfully tested using zymography and *in situ* zymography, with each of the experimental methods carrying their own advantages and disadvantages. Gelatin zymography demonstrates the presence of endogenous proteases, as well as their enzymatic activity in powdered dentin and precisely identifies between the pro- and active forms of both MMP-2 and -9. The activity can be quantified and compared. The drawback of this method could be that it is performed using dentin powder and the clinical procedures cannot be followed exactly as recommended in the manufacturers' instructions due to the requirements and the specificity of the test. This method is not particularly simple to perform using dental adhesives. Care must be taken that the adhesives do not polymerize during the procedure, and the samples should be protected from the light as much as possible during all the steps involving adhesives. Unlike gelatin zymography, the *in situ* zymography cannot differentiate between gelatinolytic enzymes and we cannot be certain which of the gelatinolytic enzymes we can attribute the activity to. However, the strength of this method and an advantage of *in situ* zymography compared to gelatin zymography is that it visually and spatially maps the dentinal endogenous enzymatic activity. Hence, we can note in which part of the sample and of the hybrid layer there is the highest amount of activity, and we can also recognize the morphological differences in the surface treated with different adhesive procedures. The activity shown by *in situ* zymography can also be quantified. The limitation of both zymographic assays is that gelatin is used as the substrate, and it is not the preferred substrate of true collagenases. However, gelatinases, MMP-2 and -9 were shown to be pivotal in the initiation of the collagen hydrolysis, and since they are abundant in dentin, we believe they are valid indicators of the overall proteolytic potential of MMPs.

TEM is a very powerful microscopical tool that offers the highest magnification, information on element and compound structure and surface features. Images produced using TEM are detailed and of high-quality. However, their downside is that the preparation of the samples is quite laborious, since in the case of our study, the samples first need to be cut, bonded, aged, and then demineralized and embedded into a resin. Further, the samples need to be electron transparent, or in other words, cut in extremely thin sections so that the electrons could pass through. The equipment for the preparation of the samples, as well as the TEM itself are costly.

Raman spectroscopy represents a powerful tool which is more than suitable for testing the structure, spatial arrangement of molecules and structural analyses of nearly all materials. It is a non-destructive technique which avoids contamination of the sample. The samples can be in solid (if transparent), liquid or gaseous, both organic and inorganic, and no preconditioning of the sample is needed before the testing. Moreover, it is highly sensitive, specific, fast and the intensity of the measured peaks corresponds to the concentration of that molecule. The main drawback of this method could be the high cost of the very sensitive instrument. Also, metals cannot be tested using this method, but this limitation is not applicable in the present study. Further, fluorescence can appear as a competing process along with Raman scattering, but this effect can be controlled by choosing a suitable laser wavelength (Vašková, 2011).

Hence, all the methods used as a part of the thesis are well established in the field of the present research area, precise, repeatable, and their advantages heavily outweigh their limitations.

Future perspectives

Efforts should be made towards matching appropriate inhibitors in adequate concentrations with appropriate adhesive resin blends as to achieve the best possible material properties and the least detrimental influence of dentinal proteases. All the investigated new systems should be tested comprehensively, including their adhesion to dentin, anti-enzymatic activity, degree of conversion and material properties, immediately and after aging. Perhaps the future lies in the novel nano-carriers of the active substances, which could potentially provide better mechanical properties of the adhesive itself, as well as slow release of the active substance over a long period. Also, biomimetic remineralization should be further investigated and a clinically applicable protocols should be achieved. However, even the remineralization protocols should include protease inhibitors, since remineralization takes time, and if the dentinal enzymes are not silenced during this period, degradation could precede remineralization.

Conclusions

The results of the present thesis lead to several conclusions:

- Protease inhibitors/inactivators used as separate primers still provide more predictable results regarding preservation of the hybrid layer over time (even after 10 years) compared to inhibitors blended into adhesive resins, possibly since they do not impair the mechanical properties of the adhesive resin itself;
- When the inhibitory agent was introduced within the adhesive resin, the bond preservation was adhesive system- and/or bonding mode-dependent; hence, the preservation of the hybrid layer is influenced by several different factors, and is not only inhibitor-dependent;
- All the tested inhibitors, whether as separate primers, or blended within the adhesive, inhibited to some point the dentinal enzymatic activity. This effect was present at the baseline, after 1 year or 10 years of aging.
- Moreover, CHX was present within a hybrid layer after 10 years of aging, even though it was “only” electrostatically bonded with dentin. The theory about leaching out of the inhibitor could hence be reconsidered. Further, we could expect that the cross-linkers would be even more likely to remain within the hybrid layer.

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