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To etch or not to etch, Part II: On the hydrophobic-rich content and fatigue strength of universal adhesives

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ABSTRACT

Objective: To determine whether smear layer management, *via* conservative etching protocols, and the hydrophobic-rich content of hybrid layers would affect the fatigue strength of resin-dentin interfaces.

Methods: Bar-shaped dentin beams obtained from sound third molars were wet-polished for 30 s. Dentin was etched with 32 % ortho-phosphoric acid for 3 or 15 s, 10 % meta-phosphoric acid for 15 s or by a prime-and-rinse application using a mild universal adhesive (Scotchbond Universal, 3M ESPE). Self-etch application served as control. Coating was performed with a solvent-free bisGMA-based resin. Composite buildups were made with a nanofilled composite. Resin-dentin beams with twin-bonded interfaces were sectioned and stored in deionized water for 24 h at 37 °C before 4-point flexural quasi-static monotonic testing (n = 16). Stress-life fatigue behavior was evaluated under cyclic loading (n = 35) by the staircase method at 4 Hz. The tension side of cyclic-loaded unfractured beams were evaluated under SEM, along with the micro-morphology of etched dentin surfaces. Monotonic data was analyzed by two-way ANOVA followed by the Tukey Test and cyclic-loaded data by Kruskal-Wallis on Ranks ($\alpha = 0.05$).

Results: Etching protocols and higher hydrophobic-rich content produced significantly higher fatigue life distributions ($p < 0.05$). Dentin demineralization was ranked as OPA 15 s > MPA 15 s > OPA 3 s > P + R > SE. Less aggressive etching and coating reduced crack formation at hybrid layers.

Significance: Current oversimplification trends in resin-dentin bonding constitute a trade-off between hybridization quality and easier adhesive handling. Controlled dentin etching

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and increasing the hydrophobic-rich content of hybrid layers may be necessary to extend the longevity of mild universal adhesives.

1. Introduction

Adhesive dentistry focusses not only on improving the longevity of tooth-bonded interfaces, but it also aims to simplify restorative procedures [1]. Universal adhesives have been introduced as an alternative to allow a personal choice regarding the use of a single bonding agent in etch-and-rinse or self-etch mode [1]. The promise of bonding to glass-rich (*i.e.*, *via* silane) and glass-poor (*i.e.*, *via* MDP) ceramics without the need of additional steps (*e.g.*, use of separate silane coupling agents) further contributed to the great acceptability by clinicians. Undoubtedly, the multiuse possibilities of such simplified "all-in-one" adhesives certainly characterized one of the greatest advancements in resin-dentin bonding introduced by manufacturers during the last decade. Furthermore, specific clinical scenarios may benefit from a particular application mode over another (*i.e.*, etch-and-rinse *vs.* self-etch), which confers more individualized bonding procedures with just a single product.

Although quite appealing from a clinical standpoint, universal adhesives have generated debate over their optimum form of application, especially considering the dentin substrate [2–6]. There is growing evidence that the performance of universal adhesives varies according to the application mode [2]. Not only *in vitro* findings point towards their underperformance in self-etch mode [3], but *in vivo* studies also begin to show reductions in retention rates, poor marginal adaptation and higher marginal staining when dentin is not etched [2,4,5,7]. There are indications that the bonding effectiveness of mild universal adhesives may be impaired by more clinically relevant smear layers [3], suggesting insufficient smear layer dissolution when used following manufacturer instructions [3,8]. Similar issues attributed to insufficient smear layer encapsulation have been long reported to one-step self-etch adhesives [9], which coincidently present quite similar monomer-solvent contents. Universal adhesives rely on improved monomer-solvent blending and not necessarily on specially designed acidic monomers to be used in etch-and-rinse or self-etch mode [10]. The notion that resin-dentin bonding may be negatively affected by the inability of adhesives to effectively etch through weakly-attached smear layers is not new [11]. *In vivo* findingsof universal adhesives [2,4,5,7] show that the etch-and-rinse approach produces improved clinical outcomes in terms of retention rates. Confusion arises with conflicting microtensile studies reporting that acid etching does not improve *in vitro* dentin-bonding performance of universal adhesives [6,12]. Microtensile bond strength testing is indeed considered the standard for current resin-dentin bonding assessment [13]; however, it may fail or only timidly signalize material dependency regarding the application mode of universal adhesives [12]. Commonly employed monotonic tests generally do not differentiate between resin-dentin bonding protocols as effectively as cyclic loading approaches tested under fatigue [3]. It is evident that determining the most effective mode of application of universal adhesives remains highly controversial requiring further investigations. To elucidate such inconsistencies, more discriminative mechanical testing approaches could be employed to better understand alternative techniques or additional strategies proposed to enhance the dentin bonding performance of universal
adhesives [14].

The turning point for effective self-etching bonding lies on adequate acidic-monomer etching through the smear layer with minimal demineralization of the underlying dentin [15]. Hence, a separate demineralizing step, especially using stronger acids (*e.g.*, H_3PO_4), seems to go against the emerging trend in adhesive dentistry of avoiding dentin etching [1]. Nonetheless, removal of thicker/denser smear layers may be necessary to optimize the bonding performance of ultra-mild or even mild universal adhesives [2–5,7]. The high hydrophilicity of such simplified bonding resins further challenges their long-term durability [4,16,17]. Increasing the hydrophobic-rich content of universal adhesives has been an effective approach to improve *in vitro* [17] and *in vivo* [4] bonding performance of universal adhesives. It is of crucial interest to determine the extent in which hybrid layers with higher hydrophobic content, etched at different depths, would affect the dentin bonding performance of mild universal adhesives. In this regard, the recently proposed prime-and-rinse approach [18,19] or less aggressive etching agents could potentially contribute to improve the interaction of mild universal adhesives to dentin without excessive collagen exposure. Therefore, the primary aim of this study was to determine whether different etching strategies and/or the hydrophobic-rich content of hybrid layers would affect the fatigue strength of dentin interfaces bonded with a mild universal adhesive. The tested null hypotheses were that (i) different approaches used for controlled dentin etching and (ii) increasing the hydrophobic-rich content of hybrid layers would have no effect on the bonding performance of a mild universal adhesive.

2. Materials and methods

Sixty-six extracted sound third molars were obtained with informed consent from patients (age 18–34 years) under a protocol (#23-2003) approved by the University of Oulu, Finland. Teeth were stored at 4 °C in 0.9 % NaCl containing 0.02 % NaN_3 to prevent microbial growth and were used within 1 month after extraction.

Table 1 – Adhesive system compositions, etching agents and application modes.

	Composition	~ pH	Application mode
Scotchbond Universal Adhesive (3M-ESPE)	MDP phosphate monomer, dimethacrylate resins, HEMA, methacrylate modified polyalkenoic acid copolymer, filler, ethanol, water, initiators and silane	2.7	Self-etch * Etch-and-rinse ** Prime-and-rinse ***
Scotchbond Multi-Purpose Adhesive (3M-ESPE)	bisGMA, HEMA, dimethacrylates and photoinitiators	–	Coating# 3 s (OPA 3 s) 15 s (OPA 15 s)
Scotchbond Universal Etchant (3M-ESPE)	32 % ortho-phosphoric acid, water, synthetic amorphous silica, polyethylene glycol and aluminum oxide	0.1	
Meta-phosphoric acid (Sigma-Aldrich)	10 % meta-phosphoric acid, distilled water	1.3	15 s (MPA 15 s)

Abbreviations: bisGMA = bisphenol glycidyl methacrylate; TEGDMA = tryethylene glycol dimethacrylate; MDP = methacryloyloxydecyl dihydrogen phosphate; HEMA = 2-hydroxyethyl methacrylate; 3 s = dentin etching for 3 s; 15 s = dentin etching for 15 s; OPA = ortho-phosphoric acid, MPA = meta-phosphoric acid. * Self-etch: active universal adhesive application for 20 s; gentle air-drying for 10 s; light curing for 10 s. ** Etch-and-rise: dentin etching according to the experimental group (OPA 3 s, OPA 15 s or MPA 15 s); water rinse for 15 s; blot-drying; active universal adhesive application for 20 s; gentle air-drying for 10 s; and light curing for 10 s. *** Prime-and-rinse: active universal adhesive application for 20 s; water rinse for 15 s; gentle air-drying for 5 s; active universal adhesive application for 20 s; gentle air-drying for 10 s; and light curing for 10 s. # Coating: universal adhesive application according to the experimental group without light curing; active Scotchbond Multi-Purpose Adhesive application for 20 s; gentle air-drying for 5 s; and light curing for 10 s.

2.1. *Experimental design and bonding protocols*

The bonding performance of resin-dentin interfaces produced by a mild universal adhesive (Scotchbond Universal Adhesive: SU, 3M ESPE; pH 2.7) [20,21] applied in self-etch mode and after different etching protocols were assessed. Dentin etching was performed with 32 % ortho-phosphoric acid (Scotchbond Universal Etchant, 3M ESPE, St. Paul, MN, USA) for 3 s (OPA 3 s), 15 s (OPA 15 s) or 10 % meta-phosphoric acid etching for 15 s (MPA 15 s). A prime-and-rinse approach (P + R) was also evaluated [18,19]. Bonded interfaces were assessed in terms of 4-point flexural strength (n = 16/group) and stress life fatigue (n = 35/group). Table 1 shows adhesive system compositions, etching agents and application modes. The resultant dentin etching morphology was evaluated by SEM. The experimental design was composed of two study factors. Dentin etching, in five levels composed by (i) self-etch application, (ii) 15 s and (iii) 3 s etching with 32 % ortho-phosphoric acid, (iv) 10 % meta-phosphoric etching for 15 s and the (v) prime-and-rinse approach (P + R). Hydrophobic-rich content of the hybrid layer in two levels composed by (i) the hydrophobic content offered by the tested mild universal adhesive or (ii) presence of an additional solvent-free bisGMA-based resin coating (Scotchbond Multi-Purpose Adhesive, 3M ESPE) mixed with the uncured universal adhesive layer on the dentin surface. The additional coating essentially transformed the mild universal adhesive, originally classified as one-step, into a two-step universal adhesive, which was applied in self-etch or etch-and-rinse mode according to the studied etching protocols. Ten groups were obtained for flexural strength and stress-life fatigue behavior testing following the twin-bonded interface (TBI) approach [22]. The exception was that the cross-sectional area of specimens was reduced to roughly 0.9 mm^2. Smaller cross-sectional areas were selected for the monotonic and cyclic loading to be in line with a previous study [3], thereby allowing more precise comparisons. Standardized smear layers were produced by wet-polishing mid-coronal dentin surfaces with 320-grit SiC paper (Buehler-MET II, Buehler; grit size ≈ 36 μm), for 30 s. The self-etch application mode was performed according to the manufacturer's instructions. After smear layer standardization, dentin surfaces were rinsed with air-water blasts for 10 s, air-dried for 5 s, followed by active application of one coat of the universal adhesive for 20 s under manual pressure equivalent to approximately 40 g for 20 s, gentle air-drying for 10 s and light curing for 10 s using a LED unit (Elipar Deepcure, 3M ESPE) at 1400 mW/cm^2. For the experimental groups, dentin surfaces were etched with 32 % ortho-phosphoric acid (Scotchbond Universal Etchant, 3M ESPE, St. Paul, MN, USA) for 3 or 15 s or with an aqueous 10 % meta-phosphoric acid solution (meta-Phosphoric acid, Merck Group, Darmstadt, Germany) for 15 s. Etched dentin surfaces were then rinsed for 15 s and blot-dried with absorbent paper following the wet-bonding technique [23]. One coat of the universal adhesive was actively applied on the dentin surface under similar manual pressure, air-drying was gently performed for 10 s and light curing for 10 s. The prime-and-rinse (P + R) protocol [18,19] consisted of active application of one coat of the universal adhesive, as in the self-etch group, followed by water rinsing for 15 s, gentle air-drying for 5 s, active application of a second coat of the universal adhesive for 20 s, gentle air-drying for 10 s and light curing for 10 s. In groups composed by the additional coating, after the active application of the universal adhesive for 20 s and solvent evaporation, a bisGMA-based bonding resin (Scotchbond Multipurpose Adhesive, 3M ESPE) was actively applied on the dentin surface for 20 s and mixed with the uncured universal adhesive. Film thinning was performed by air-drying for 5 s and light curing for 10 s. A nanofilled composite (Filtek Supreme XTE, 3M ESPE) was used as the

restorative material to produce the bar-shaped specimens for monotonic and cyclic loading. All bonding procedures were carried out by the same pair of calibrated operators.

2.2. Characterization of the fatigue behavior

Bar-shaped dentin beams, roughly 1 × 1 × 8 mm, were obtained from 46 sound third molars. Roots were removed 1 mm below the cervical line and discarded. Crown segments were longitudinally sectioned occluso-cervically to produce mesio-distal slabs which were wet-polished with 320-grit SiC paper (Buehler-MET II, Buehler) for 30 s, for dentin and smear layer standardization, and perpendicularly sectioned at the mid-coronal region with a slow-speed diamond saw (Isomet, Buehler Ltd, Lake Bluff, IL, USA) under water cooling. Only one dentin beam was obtained from each slab. Dentin beams were randomly selected to produce twin-bonded interface specimens (TBI) in a specially designed aluminum mold as described by Mutluay et al. [22]. Briefly, dentin beams were placed inside the mold with the tubules oriented nominally parallel to the bonding interface. Bonding was concomitantly performed on both opposing dentin surfaces according to each specific group. After dentin hybridization, a nanofilled composite (Filtek Supreme XTE, 3M ESPE) was applied in a single increment to fill the mold cavities on both sides of the dentin beam and light-cured for 20 s on both sides using a LED unit (Elipar Deepcure, 3M ESPE) with output intensity of 1400 mW/cm^2. The bonded sections were carefully released from the mold, inspected for voids and flaws, lightly wet-polished with 600- and 1200-grit SiC paper and longitudinally sectioned with a slow-speed diamond saw (Isomet, Buehler Ltd) to obtain twin-bonded interface samples roughly 0.9 × 0.9 × 12 mm as shown in Part I [3]. A minimum of 51 TBI samples were prepared for each group with average cross-sectional area of 0.87 mm^2 (± 0.11). Specimens were re-inspected for flaws at the bonded interface using a stereomicroscope (Leica M60, Leica Microsystems) with 40 × magnification and stored in artificial saliva [24] at 37 °C for a minimum of 48 h prior to testing. Samples presenting flaws were discarded and replaced. TBI specimens were tested under quasi-static and cyclic flexure using a universal testing system (Electropuls E1000, Instron) with load capacity of 250 N and sensitivity of 0.025 %. All experiments were performed with specimens fully immersed in artificial saliva at room temperature. TBI specimens were placed on a four-point flexural fixture so that the load was applied on the occlusal surface. Quasi-static loading was applied at a rate of 0.05 mm/min. The flexural strength (*FS*) of the beams was calculated using conventional beam theory [25] in terms of the maximum measured load (*P*) in N and beam geometry (width *b*, thickness *h* in mm) according to $FS = 3Pl/bh^2$, where *l* is the distance from interior and exterior supports (*l* = 2 mm). Sixteen specimens (n = 16) were evaluated per group. Cyclic loading of the TBI specimens was conducted using the same flexure configuration under load control with frequency of 4 Hz and stress ratio (R = ratio of minimum to maximum cyclic load) of 0.1. A minimum of thirty-five specimens (n = 35) were evaluated per group. The cyclic loading experiments followed the staircase fatigue method beginning at approximately 80 % of the determined 4-point flexural strength. Such values were identified from the quasi-static loading and followed sequential reductions in the order of 10 % until failure. The process continued until reaching a stress amplitude (MPa) at which specimens did not fail within a minimum of 1.2 × 10^6 cycles. The cyclic stress amplitude was plotted in terms of the number of cycles to failure in log-base format. The data was fit through a non-linear regression with a Basquin-type model, according to equation $\sigma = A(N)^B$, where A and B are the fatigue-life coefficient and fatigue-life exponent, respectively. The apparent endurance limit was estimated from the models for a fatigue limit defined at 1 × 10^7 cycles [22,26].

2.3. Detection of crack propagation sites

The unfractured sides of TBI specimens, which withstood a minimum of 10^4 loading cycles, were evaluated by scanning electron microscopy (SEM) to identify the origins of failure and potential weak links at resin-dentin bonded interfaces. Specimens were lightly wet-polished with SiC papers 600-, 1200-, 2000- and 4000-grit SiC paper and ultrasonically cleaned in water for 2 min. Specimens were dehydrated in a series of ascending ethanol series (50, 70, 80, 90 and 3 × 100 %), fixed in hexamethyldisilazane, mounted on aluminum stubs, sputtered with gold/palladium and analyzed on backscattering mode at 15 kV (Phenom ProX, Phenom-World). SEM micrographs (1000–12,000 × magnification) were taken to analyze the entire extension of the bonded interfaces locate at the tensile side of specimens. Representative micrographs of the most common crack propagation sites were obtained by a blinded-calibrated operator.

2.4. Dentin etching patterns

Twenty mid-coronal dentin disks measuring roughly 1 mm in thickness were sectioned from sound third molars (n = 2/group) using a slow speed diamond saw (Isomet, Buehler Ltd). The absence of enamel remnants was verified with a stereomicroscope (Leica M60, Leica Microsystems) at 40 × magnification by an experienced and blinded operator. Standardized smear layers were created on the occlusal surface by wet polishing the exposed dentin surfaces for 30 s with 320-grit SiC paper (Buehler-MET II, Buehler). Bonding protocols were performed on the occlusal dentin surfaces according to each specific group with the exception that bonding resins were left unpolymerized. Resin monomers were then copiously rinsed away with water spray for 15 s followed by ultrasonic cleaning in distilled water for 5 min. Coated samples were initially rinsed with 50 % and 100 % ethanol, to remove high-molecular weight monomers, and then with water for 60 s, to re-expand collagen. Specimen were immediately dehydrated in ascending ethanol series (50, 70, 80, 90 and 3 × 100 %) avoiding direct exposure of etched surfaces with air (*i.e.*, surfaces were always kept moist when changing solutions) and fixed in hexamethyldisilazane. Half of the specimens were longitudinally fractured in liquid nitrogen to expose a cross-sectional view of the etched surface, while the remaining specimens exposed an occlusal view of the dentin etching patterns. Samples were mounted on stubs, sputtered with gold/palladium and analyzed on

backscattering mode at 15 kV (Phenom ProX, Phenom-World) at 1000–12,000 × magnification.

2.5. Statistical analyses

Data normality and equality of variance were confirmed by Shapiro-Wilk and Levene tests, respectively. Four-point flexural strengths obtained after quasi-static loading measurements (Shapiro-Wilk test = 0.421; Levene test = 0.595) were analyzed with two-way ANOVA and Tukey test. Fatigue life distributions were compared using Kruskal-Wallis on Ranks. Significance levels were set at 5 % ($\alpha = 0.05$). Statistical analyzes were performed on IBM SPSS Statistics for Windows, version 23 (IBM Corp., Armonk, NY, USA).

3. Results

3.1. Quasi-static 4-point flexural strength

The cross-sectional area of TBI specimens were not significantly different between groups ($p = 0.42$). Two-way ANOVA revealed that "dentin etching" ($p < 0.001$; $\eta^2 = 0.374$) and "hydrophobic coating" ($p = 0.022$; $\eta^2 = 0.35$) had significant effects ($p < 0.05$) on the 4-point flexural strength of the tested universal adhesive. No interactions were observed between both study factors (*i.e.*, "dentin etching" and "hydrophobic-rich content"). Bar-shaped TBI specimens presented no significant differences regarding resin-dentin cross-sectional areas 0.87 mm^2 (± 0.11) between groups ($p = 0.62$). 4-point flexural bond strengths are reported in Table 2. No significant differences were detected between the self-etch and conventional 15 s etching with ortho-phosphoric acid (OPA 15 s) for the tested mild universal adhesive ($p > 0.05$). Meta-phosphoric acid etching for 15 s (MPA 15 s) and ortho-phosphoric acid etching for 3 s (OPA 3 s) significantly increased flexural strengths in the order of 35 % and 45 % compared to non-etched dentin, respectively ($p < 0.05$). The prime-and-rinse approach (P + R) was not statistically different from the self-etch application ($p > 0.05$). Coating produced only marginal increases in flexural strength. Pairwise comparisons were unable to identify significant differences in flexural strength between corresponding groups with or without coating. Fractures involved the bonded interfaces without exclusive cohesive fractures in either dentin or composite. Self-etching produced adhesive failures involving the hybrid layer with substantial exposure of underlying dentin. Application modes involving the different dentin etching approaches and hydrophobic coating presented remnants of hybridized dentin depicting failures above the hybrid layer. Areas presenting failures below the hybrid layer were characterized by sparse collagen exposure in fractured prime-and-rinsed interfaces, whereas collagen fibrils were frequently found after the remaining etching approaches, especially for 15 s etching with ortho-phosphoric acid (OPA 15 s).

3.2. Fatigue response

The cross-sectional area of TBI specimens were not significantly different between groups ($p = 0.693$) for cyclic loading. Fatigue life diagrams (S-N curves) are shown in Fig. 1. Basquin-type power law models are listed for each group describing the mean fatigue strength distribution for each bonding protocol. Pairwise comparisons for the fatigue life distributions according to Kruskal-Wallis on Ranks are shown in Table 2. The self-etch mode produced the lowest stress amplitudes ($p < 0.05$). Ortho-phosphoric acid etching for 15 s (OPA 15 s) and the prime-and-rinse approach (P + R) produced significantly higher stress amplitudes than the self-etch mode ($p < 0.05$). Ortho-phosphoric acid etching for 3 s (OPA 3 s) and meta-phosphoric acid etching for 15 s (MPA 15 s) were not statistically different producing the highest stress amplitudes for groups without coating. In general, increasing the hydrophobic-rich content significantly increased stress

Table 2 – Stress-life fatigue response, power law constants and estimated endurance limits for TBI resin-dentin interfaces produced by a mild universal adhesive (Scotchbond Universal, 3M ESPE) under different etching conditions followed by hydrophobic coating.

		A (MPa)	*B*	R^2	*4-point flexural strength (MPa)**	*Endurance limit (MPa)*	*Fatigue life distributions pairwise comparison***
Control	Self-etch	35.11	-0.074	0.65	45.07 ± 8.52^{c}	10.65	D
	OPA 15 s	34.89	-0.038	0.55	49.69 ± 8.26^{bc}	18.91	C
	OPA 3 s	28.69	-0.014	0.50	65.79 ± 11.7^{a}	22.89	AB
	MPA 15 s	42.47	-0.046	0.61	60.59 ± 10.31^{ab}	20.23	B
	Prime-and-rinse	30.61	-0.029	0.68	48.94 ± 10.17^{bc}	19.61	C
Coating	Self-etch	33.97	-0.033	0.57	48.6 ± 9.22^{bc}	19.96	B
	OPA 15 s	37.88	-0.025	0.61	57.21 ± 12.54^{bc}	25.31	A
	OPA 3 s	33.47	-0.019	0.51	69.81 ± 12.02^{a}	24.64	A
	MPA 15 s	43.08	-0.039	0.50	64.92 ± 11.93^{a}	22.98	A
	Prime-and-rinse	31.37	-0.022	0.51	49.23 ± 9.41^{bc}	22.01	AB

R^2 values represent the coefficient of determination for each model. ± represent standard deviations.

* Different lower-case letters indicate significant differences for 4-point flexural strength according to the Tukey Test. Endurance limits were calculated at 1×10^7 cycles. *Abbreviations:* OPA = 32 % ortho-phosphoric acid; MPA = 10 % meta-phosphoric acid.

** Different capital letters indicate significant differences in fatigue life distributions according to Kruskal-Wallis on Ranks.

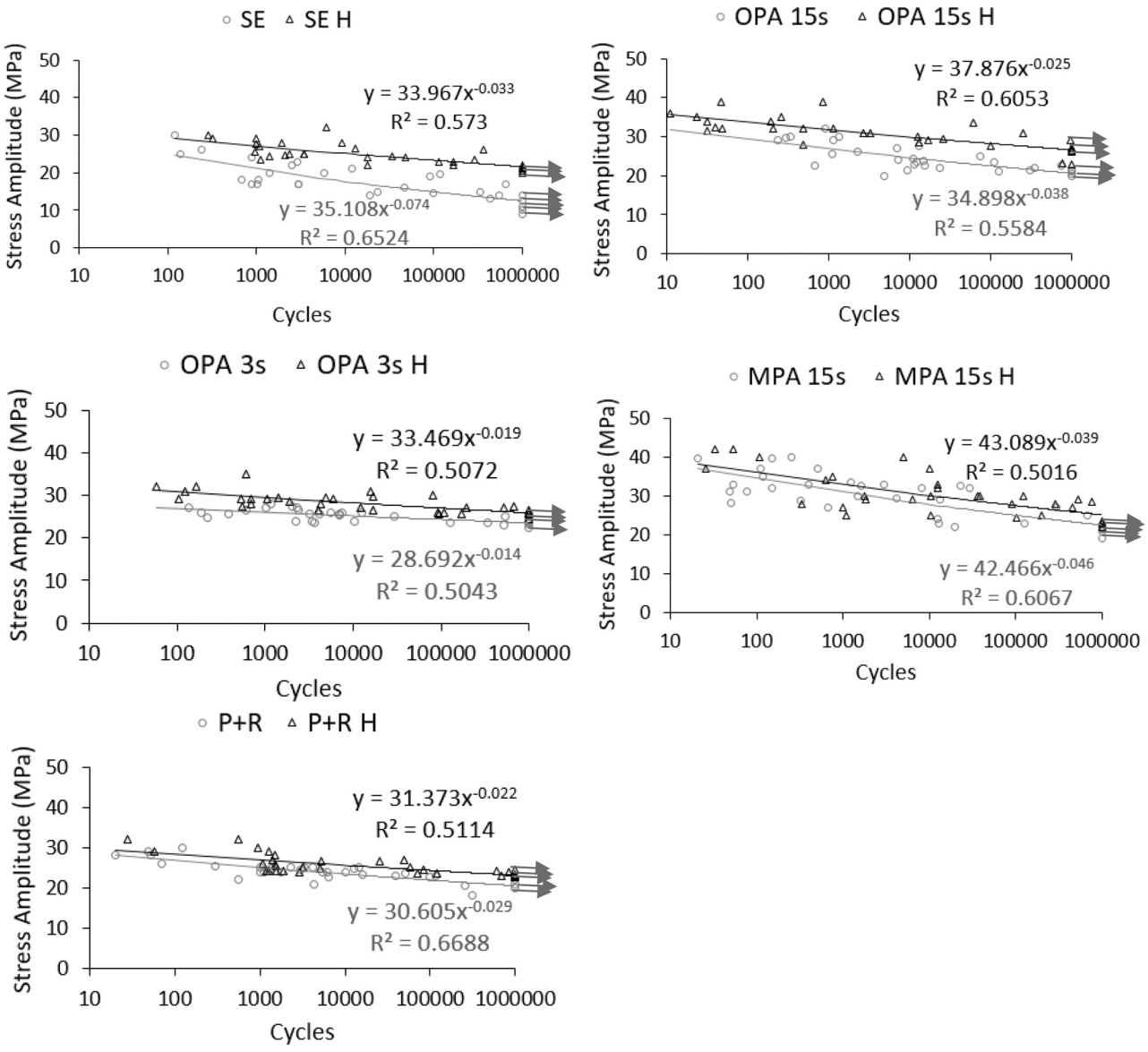

Fig. 1 – Fatigue life diagrams (S-N curves) of a mild universal adhesive (Scotchbond Universal, 3M ESPE) bonded to dentin in self-etch mode (SE) and after etching for 3 and 15 s using OPA, MPA for 15 s or after the prime-and-rinse approach (P + R). Hydrophobic coating (H) was performed by a adding a solvent-free hydrophobic resin over the uncured universal adhesive. Note that data points with arrows represent those specimens that reached 1.2×10^6 cycles and the test was discontinued. R^2 values represent the coefficient of determination.

amplitudes and thus fatigue resistance. The biggest impact was observed for coated interfaces bonded in self-etch mode (SE), which presented an 87 % increase in the endurance limit compared to applying the universal adhesive in the self-etch mode alone. Coating and dentin etching for 15 s with ortho-phosphoric acid (OPA 15 s) with meta-phosphoric acid for 15 s (MPA 15 s) and the prime-and-rinse approach (P + R) produced significantly higher stress amplitudes than their corresponding groups without coating ($p < 0.05$), resulting in 33.8 %, 13.6 % and 16.1 % increase in endurance limit, respectively. No significant differences in stress amplitudes were observed for coating or not dentin interfaces etched with ortho-phosphoric acid for 3 s (OPA 3 s). Increasing the hydrophobic-rich content of OPA 3 s samples marginally increase the endurance limit (7.6 %).

3.3. *Crack formation sites*

Etching protocols and coating affected crack formation in resin-dentin interfaces. Cohesive cracks in composite and in the bulk of the adhesive layer were identified in all samples regardless of bonding protocols. In general, more aggressive etching approaches increased crack formation at the hybrid layer surrounding areas. Coating displaced cracks away from

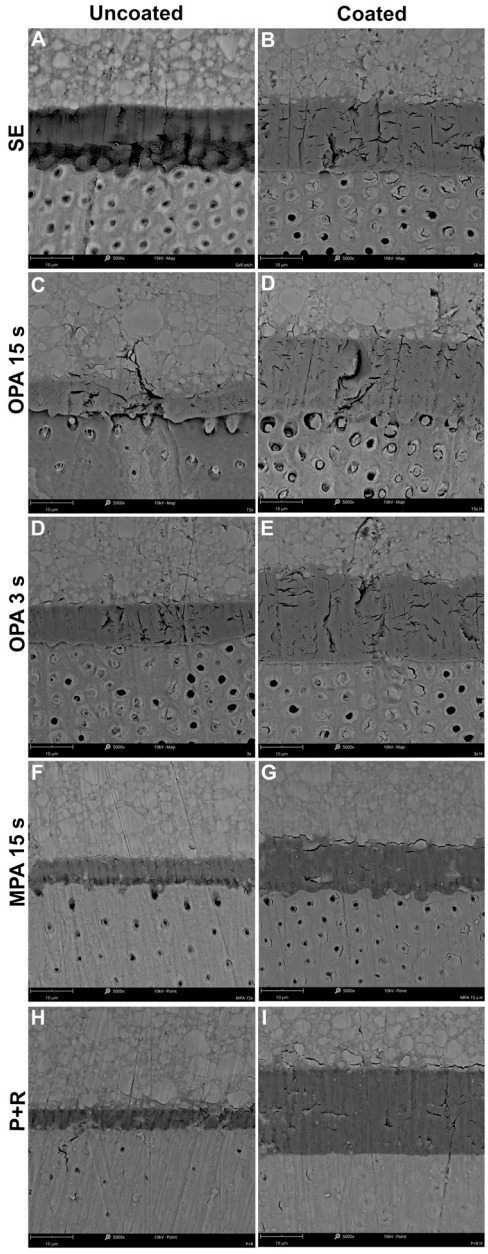

Fig. 2 – Representative SEM micrographs showing the profile view of the tensile side of unfractured TBI resin-dentin specimens subjected to a minimum of 10^4 cycles. Bonding protocols consisted of a mild universal adhesive (Scotchbond Universal, 3M ESPE) used in self-etch mode, after OPA etching for 3 or 15 s, MPA etching for 15 s, following the prime-and-rinse approach and with or without the proposed coating using a bisGMA-based resin (Scotchbond Multipurpose Adhesive, 3M ESPE).

the hybrid layer towards the composite-adhesive boundaries. Fig. 2 shows representative SEM micrographs for all groups. Self-etch (SE) samples (Fig. 2 **A)** presented cracks mostly located at areas adjacent to the hybrid layer, forming deep-continuous depressions (8–11 µm wide) at the bottom of the adhesive layer, which were more evident with longer cycles. These areas contained "microcracks" covering most of the surface. Increasing the hydrophobic content of self-etched interfaces (Fig. 2 **B)** resulted in fewer cracks involving the hybrid layer with a slight increase in cohesive cracks at the adhesive layer. For 15 s ortho-phosphoric etching (OPA 15 s; Fig. 2 **C**), cracks were commonly located at the bottom of hybrid layer extending towards the bulk of the adhesive layer. "Microcracks" could be identified in collagen-containing areas (4–6 µm wide), but to a lesser extent than SE. Increasing the hydrophobic-rich content of OPA 15 s (Fig. 2 **D)** produced lower incidence of cracks at the hybrid layer and surrounding areas with an increase in cohesive crack formation in the adhesive-composite boundaries. Ortho-phosphoric etching for 3 s (OPA 3 s; Fig. 2 **D**) produced cracks with smaller dimensions at the hybrid layer and surrounding areas compared to the OPA 15 s, which were further reduced with higher hydrophobic content (Fig. 2 **E)**. Prime-and-rinse samples (P + R; Fig. 2 **H**) exhibited similar crack formation as in OPA 3 s with lower incidence of cracks at the hybrid layer boundaries, which were also reduced by higher hydrophobic content (Fig. 2 **I)**. Meta-phosphoric acid etching for 15 s (MPA 15 s; Fig. 2 **F**) produced small cracks at hybrid layer boundaries with a shallow 2–3 µm wide depression zone. Increasing the hydrophobic-rich content MPA15 s (Fig. 2 **G)** reduced overall crack formation at hybrid layer boundaries with increased cracks at the adhesive-composite surrounding areas.

3.4. *Dentin etching patterns*

Bonding protocols resulted in different etching patterns with contrasting smear layer impregnation, dissolution of peritubular dentin, smear plug removal and collagen exposure. Representative SEM micrographs of etching patterns for all bonding protocols are shown in Fig. 3. Dense 3–4 µm thick smear layer was produced by the 320-grit SiC paper (Fig. 3 **A–B)** covering the entire dentin extension. The etching protocols produced different extensions of dentin/smear layer dissolution and collagen exposure. The tested universal adhesive presented low capacity to etch trough the smear layer when actively applied for 20 s following manufacture's recommendations (Fig. 3 **D–E)**. Self-etch application resulted in well-adapted smear plugs fully blocking dentinal tubules. Cross-sectional views (Fig. 3 **E)** revealed smear plugs reaching a depth of roughly 5–9 µm into dentinal tubules. Smear layer remnants were identified covering most of the underlying mineralized dentin with low collagen exposure in sporadic areas. Bonding protocols incorporating a separate etching step before universal adhesive application improved resin-dentin interaction (Fig. 3 **G–U)**. Collagen exposure varied according to etching agents and application times. The extension of dentin demineralization regarding the etching protocols could be ranked as OPA 15 s > MPA 15 s > OPA 3 s > P + R > SE. Ortho-phosphoric acid application for 15 s (OPA 15 s; Fig. 3 **G–H**) completely removed the smear layer resulting in the largest demineralization extension. Intertubular dentin was demineralized to approximately 5–11 µm in depth resulting in fully dissolution of peritubular cuffs. Peritubular dentin demineralization could be identified up to 28 µm deep inside dentinal tubules. Meta-phosphoric acid etching for 15 s (MPA 15 s; Fig. 3 **O–P**) produced lower demineralization with complete smear layer removal. Peritubular

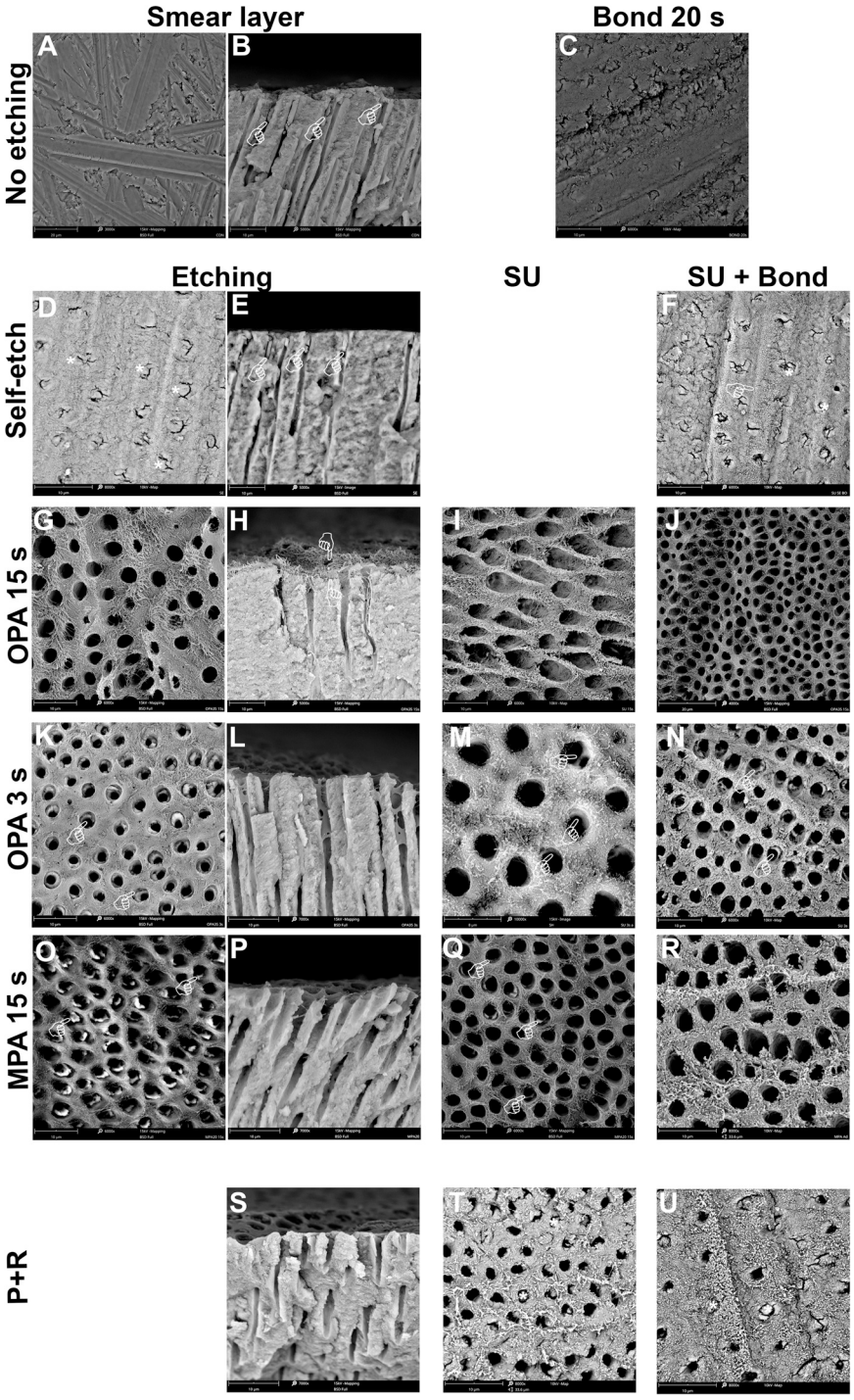

Fig. 3 – Representative SEM micrographs showing dentin-etching patterns of less aggressive etching agents combined or not with a mild universal adhesive. Loosely attached smear layer to the underlying dentin produced by 320-grit SiC paper (A and B) and after the application of the solvent-free hydrophobic resin (C). Occlusal and cross-section views of dentin etching patterns produced by Scotchbond Universal (3M ESPE; SU) in self-etch mode (D and E), ortho-phosphoric acid etching for 15 s (G and H; OPA 15 s) and 3 s (K and L; OPA 3 s), meta-phosphoric acid for 15 s (O and P; MPA 15 s) and following the prime-and-rinse approach (S and T; P + R). Note the inability of the mild adhesive to properly etch through the entire extension of the smear layer in self-etch mode and the different collagen exposures after the etching protocols. Cumulative dentin etching produced by SU combined with OPA 15 s (I), OPA 3 s (M) and MPA 15 s (Q). Cumulative dentin etching produced by SU and the hydrophobic coating (Bond) in self-etch mode (F) and combined with OPA 15 s (J), OPA 3 s (N), MPA 15 s (R) and following the P + R approach (U).

cuffs were superficially demineralized reaching roughly 2–3 µm in depth inside dentinal tubules. Demineralized collagen was not found in areas below the demineralized peritubular cuffs. Ortho-phosphoric acid etching for 3 s (OPA 3 s; Fig. 3 **K–L**) was considerably less aggressive, albeit complete smear layer dissolution also occurred. Smear plugs were removed producing superficial dissolution of peritubular cuffs reaching roughly 1–2 µm in depth inside dentinal tubules. Collagen exposure was slightly lower than MPA 15 s. Intertubular dentin was only superficially demineralized, exposing a thin collagen layer (approximately thickness 1–1.5 µm). Collagen exposure was not identified inside dentinal tubules in areas below peritubular cuffs. The prime-and-rinse approach (P + R) was the least aggressive etching protocol. The smear layer was mostly removed with few areas presenting thin smear layer remnants **(**Fig. 3 **S–T)**. Peritubular cuffs were not dissolved and smear plug partial removal resulted in tubular disocclusion. Smear plug occluded tubules were frequently identified **(**Fig. 3 **T)**. Intertubular dentin was superficially etched exposing a thin layer of collagen. Exposed collagen could not be identified inside dentinal tubules **(**Fig. 3**S)**. Active application of the bisGMA-based bonding resin for 20 s (Bond 20 s) did not produce substantial effects on smear layer thickness **(**Fig. 3 **C)**. The underlying mineralized dentin remained covered by a thick smear layer and collagen demineralization was not identified. Coating (SU + Bond) did not substantially modify the originally obtained etching patterns, except for a slight general increase in the extension of demineralization. More diffused collagen exposure, albeit still considered superficial, occurred for the self-etch application mode and the prime-and-rinse approach (P + R). A discrete increase in peritubular cuff demineralization was observed for OPA 3 s and MPA 15 s. The additional coating step produced no noticeable modifications for OPA 15 s.

4. Discussion

The approaches used for dentin demineralization had a profound effect on the fatigue strength of the tested mild universal adhesive. Therefore, the first null hypothesis was rejected. Since fatigue testing is a laborious method, the first step in the study design was to determine etching agents that could potentially remove smear layer without excessive collagen exposure. A series of SEM micrographs of different acids applied for different etching times (data not shown) were analyzed to determine the most promising options to be associated with the tested mild universal adhesive. Ortho-phosphoric etching for 3 s (OPA 3 s) was included in the present study due to the previously reported higher fatigue strengths compared to self-etch and OPA 15 s applications [3]. The present study corroborated the latter. Although quite effective to improve bonding of the tested mild universal adhesive to dentin *in vitro*, 3 s etching may be too short of a period for cavities with larger dimensions. Due to 32 % ortho-phosphoric acid's high dentin demineralization, extending the etching time for as little as a couple of seconds may invariably overexpose collagen resulting in lower calcium content at the interface [8]. Complete dentin demineralization technically counteracts the beneficial MDP-calcium chemical bonding, which contributes to improved bonding performance of universal adhesives [27]. To prevent this, bonding protocols with milder acidity were assessed aiming for better clinical applications allowing more convenient etching times without excessive dentin demineralization. All bonding protocols composed by an additional etching step improved fatigue strengths compared to the simplified self-etch application.

The emerging general assumption that mild universal adhesives can perform equally well in both self-etch or etch-and-rinse modes remains a matter of debate [2,4–8]. Controversies [2,4–8] regarding the best-performing application mode (*i.e.*, self-etch *vs* etch-and-rinse) for universal adhesives may be related to sample preparation and test setups normally used for resin-dentin bonding assessment. Fatigue testing of resin-dentin interfaces has been shown to be more discriminative than the standard microtensile test [3]. In this study, fatigue testing detected improvements in bonding performance showing significant differences between etching protocols [3]. Such differences were not detected in previous studies [6]. Failure of previous studies to simulate more clinically relevant smear layers (*e.g.*, use of 600-grit SiC paper for direct bonding [13]) prior to dentin hybridization with universal adhesives is a key factor for such inconsistencies. Smear layer standardization with 600-grit SiC paper (grit size ≈ 14.5 µm), employed in the vast majority of studies, does not reflect denser/thicker smear layers produced on bur-cut dentin [28]. Less dense smear layers with reduced thickness invariably facilitate bonding of self-etch resins to the underlying dentin [28–30]. Failure to properly simulate smear layers invariably contribute to unrealistic overestimations of the bonding performance of some universal adhesives *in vitro*. A recently performed meta-analysis [6], including resin-dentin microtensile studies, claimed that "prior acid etching does not improve the bond performance of universal adhesives" and "self-etch mode can achieve a perfect bond strength". Such type of statements must be analyzed with extreme caution, especially if meant to be extrapolated to the clinical scenario. This is reinforced by randomized clinical studies showing that universal adhesives may indeed perform better in etch-and-rinse mode [2,4,5,7].

Dealing with the smear layer before the use of mild universal adhesives may be necessary to improve the bonding performance of such resin-dentin interfaces. The presence of the smear layer creates a barrier against monomer diffusion [31], which imposed a detrimental effect on the fatigue resistance of the tested universal adhesive. H_3PO_4-etching for 15 s has been shown to improve immediate bond strengths of some universal adhesives; however, no effect was observed for the same mild universal adhesive used in this study [32]. H_3PO_4-etching for 15 s increased the endurance limit (roughly 77 %) producing significantly higher fatigue life distributions compared to the self-etch application. This confirms the discriminative power of the fatigue testing approach [3] showing that more clinically relevant smear layers can interfere in bonding of universal adhesives to dentin. This could go unnoticed if only monotonic testing setups were employed (*i.e.*, microtensile and microshear testing).

Choosing an effective approach to remove the smear layer without excessive collagen exposure becomes a critical step for successful resin-dentin bonding. Meta-phosphoric acid [HPO_3] is an interesting chemical with lower etching capacity compared to the conventionally used ortho-phosphoric acid [H_3PO_4]. It produces metaphosphate anions [PO_3^-] in aqueous solutions through the ionization of the single hydroxyl group [-OH] to produce a hydrogen ion [H^+]. In this study, meta-phosphoric acid concentration (*i.e.*, 10 %) was lower than those previously reported (*i.e.*, 40 %) [33,34]. Considering the etching capacity of universal adhesives, the rationale for selecting a lower meta-phosphoric acid concentration was to reduce collagen exposure (2–7 µm, depending on application times) produced by higher concentrations (*i.e.* 40 %) [34]. Although higher meta-phosphoric acid concentrations seems necessary to bond etch-and-rinse resins to dentin [33,34], the more conservative demineralization approach employed in this study produced significant improvements in fatigue resistance. Under the tested conditions, smear layer was fully removed, which improved the interaction of the mild universal dentin with the underlying dentin. This was an important aspect in improving the bonding performance of the tested mild universal adhesive considering that the self-etch application was unable to etch through the smear layer resulting in the lowest fatigue resistance. MPA 15 s endurance limit was comparable to OPA 3 s, roughly 90 % higher than the self-etch application, standing out as the most effective etching protocols. Improvements in endurance limits produced by MPA 15 s occurred with a substantial reduction in the overall collagen exposure compared to OPA 15 s. No significant differences were observed between the fatigue life distributions of MPA 15 s and OPA 3 s. Nonetheless, the possibility to safely etch dentin without the risk of excessive collagen overexposure, in case of accidental short extensions in etching times (*i.e.*, 1–2 s), constitutes an interesting advantage to MPA 15 s over OPA 3 s. This becomes more critical during long-term aging of the bonded interfaces considering the ability of endogenous enzymes to hydrolyze collagen and impair resin-dentin bonding [23,35]. Curiously, metaphosphate anions produced by meta-phosphoric acid may present a cross-linking effect on dentin collagen, which could further contribute to more durable resin-dentin bonded interfaces [33]. More studies are necessary to evaluate the long-term bonding performance of universal adhesives to HPO_3-etched dentin.

Although quite promising, meta-phosphoric acid is not readily available for clinical use yet. To bypass this limitation, a prime-and-rinse approach (P + R) was tested [18,19]. This procedure consisted of active application of the universal adhesive for 20 s, followed by water rinsing and reapplication of a second layer of the same adhesive for 20 s. The universal adhesive acted as a separate etching agent, similarly to conventional ortho-phosphoric acid etching, but with lower demineralizing potential. Lower collagen exposure was detected for the prime-and-rinse approach compared to the remaining etching protocols. A single application of the mild universal adhesive, following the conventional self-etch mode (SE), was unable to etch through the smear layer consistently. However, the double application of the mild universal adhesive interposed by water rinsing, albeit not fully, increased smear layer removal. Dentin can buffer the acidity of simplified self-etch resins limiting their etching depth [31]. Extending the total application time benefits smear layer removal. As a result, the endurance limit produced by the prime-and-rinse approach was roughly 83 % higher than the self-etch mode. Fatigue life distributions were significantly higher as well. Hence, improvements in resin-dentin bonding by the prime-and-rinse approach were evident, but with significant lower fatigue life distributions and marginally lower endurance limits (5–16 %) than MPA 15 s and OPA 3 s protocols. This was likely due to the presence of residual smear layer in some areas, which albeit thin, might have prevented optimal resin-dentin interaction. It is important to notice that the prime-and-rinse approach produced comparable bonding effectiveness to the OPA 15 s etching with substantial lower collagen exposure, which may retard interface degradation on the long run. The prime-and-rinse approach offers a simple and fast procedure to reduce the negative impact of smear layers on mild universal adhesives. Future studies should assess the prime-and-rinse approach on bur-produced smear layers.

Another key factor on resin-dentin bonding durability is the quality of the methacrylate-based polymer structure forming the hybrid layer. Dentin etching has a two-fold mechanism in improving the bonded interfaces of universal adhesives. Etching improves resin-dentin interaction by removing the smear layer, which may also act as residual contaminants, preventing polymer chains from getting closer during polymerization [32]. As a result, higher monomer conversions have been reported at the bonded interface of universal adhesives after H_3PO_4 etching [32]. Although material dependent, etching-associated improvements in monomer conversion contributes to higher bond strengths of universal adhesives [32]. Likewise, improved monomer conversion produced by dentin etching [32] certainly contributed to the substantially higher fatigue strengths. Simplified adhesives possess a more hydrophilic character compared to multi-step systems, which affects polymerization [36]. Both monomer type and the presence of organic solvents contribute to the hydrophilicity character of bonding resins. This is a requirement for adequate dentin bonding using current bonding protocols [23]. Nonetheless, the possibility of free radicals to initiate polymerization within the hydrophilic-rich phase of bonding resins is low compared to a more hydrophobic-rich phase [36]. Hence, the use of hydrophilic-rich monomer blends invariably affects the final polymer structure and downgrade its mechanical properties [37]. The high hydrophilic monomer content [36] and residual solvents [38] hinder the formation of cross-linked polymers reducing the overall polymer quality. Differently from previous studies, where the application of the bisGMA-based resin was performed over a pre-cured universal adhesive layer, forming a distinct-protective layer [4,16,17,32], in the present study the solvent-free resin with higher hydrophobic-rich content was actively mixed *in situ* with the universal adhesive before curing. The rationale for using this application approach was to allow higher inward diffusion of hydrophobic-crosslinking monomers into the hybrid layer. Determination of whether coating, as suggested in previous studies [4,16,17,32], is more or less effective than increasing the hydrophobic-rich content

of hybrid layers is beyond the scope of this study. Noteworthy, adding a distinct hydrophobic coat over pre-cured universal adhesives contributes to higher monomer conversion [32,39], higher dentin bond strengths [17,32] and lower nanoleakage [17]. It is reasonable to speculate, however, that increasing the hydrophobic-rich content of hybrid layers, and not exclusively that of the adhesive layer, may further benefit the bonded interface. Higher resin-dentin bond strengths, albeit not significant, have been reported after the application of a hydrophobic resin onto uncured one-step self-etch adhesives [40]. In this study, increasing the hydrophobic-rich content of hybrid layers produced higher endurance limits and significantly higher fatigue life distributions than the hydrophilic-rich counterparts, so the second null-hypothesis was rejected.

The extent in which coating improved the fatigue resistance (14–88 % higher endurance limits) varied according to the etching protocol. Curiously, the highest impact occurred for the self-etch application with an 88 % higher endurance limit. Active application of the solvent-free hydrophobic resin had no substantial effects on smear layer removal. The application of the additional hydrophobic resin for 20 s, over the uncured universal adhesive, invariably extended the etching period. This improved smear layer encapsulation, as shown by the SEM etching pattern characterization, allowing better interaction between the universal adhesive and the underlying dentin surface with minimal collagen exposure. When a separate etching step was performed, the effect of increasing the hydrophobic-rich content of hybrid layers on fatigue strengths showed a positive correlation with demineralization depth. OPA 15 s, with the highest collagen exposure, presented the highest increase in endurance limits (roughly 34 %) after the application of the hydrophobic resin. Furthermore, turning simplified universal adhesives into hydrophobic-rich multi-step systems mitigated the effects of residual smear layer on dentin bonding of universal adhesives. Additional coating using a bisGMA-based resin not only produced higher fatigue resistances, but also reduced etching-associated bonding variabilities. No significant differences were detected between hybrid layers with a hydrophobic-richer phase when a separate etching step was performed. Nonetheless, the relative larger increase in the endurance limit of OPA 15 s (88 % higher) compared to the remaining less aggressive etching protocols (13.6–16.1 % higher) may be related to the limited diffusion of high molecular weight cross-linking monomers within collagen [41,42]. Hybridization is a demanding and non-homogenous process [41,42]. Lower extensions of demineralized collagen may be more easily infiltrated by the hydrophobic content offered by the universal adhesive. Higher demineralization depths invariably limit the uptake of cross-linking monomers. The additional bisGMA-resin coating increased the availability of cross-linking monomers, likely facilitating their uptake by collagen. Clearly, such improvements in fatigue resistance raise questions about the extent in which current trends of oversimplifying resin-dentin adhesive procedures may be contributing to sub-optimal bonding performance. Future studies should evaluate the effect of dentin etching and hydrophobic-rich content on the fatigue resistance of universal adhesives after long-term aging.

5. Conclusion

Determination of fatigue strengths proved to be a valuable approach for assessing resin-dentin bonded interfaces. Additional measures to improve resin-dentin interactions were necessary to optimize the bonding performance of the tested mild universal adhesive. While self-etch bonding underperformed, modification of the smear layer *via* controlled etching protocols greatly enhanced resin-dentin interaction resulting in higher apparent endurance limits. Although depth-of-etching-dependent, increasing the hydrophobic-rich content of hybrid layers produced higher fatigue strengths contributing to lower variability in bonding performance. Therefore, the association of adequate smear layer management and the increase of hydrophobic-rich content of hybrid layers, albeit more laborious, may be required to truly extend the durability of universal adhesives submitted to oral challenges.

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