Acquired pellicle engineering with proteins/peptides: mechanism of action on native human enamel surface

ABSTRACT

Objective: This study investigated the mechanism of action of different proteins/peptides (separately or in combination), focusing on how they act directly on the native enamel surface and on modifying the salivary pellicle. Methods: A total of 170 native human enamel specimens were prepared and submitted to different treatments (2 h; 37 °C): with deionized water, CaneCPI-5, Hemoglobin, Statherin, or a combination of all three proteins/peptides. The groups were subdivided into treatment acting on the enamel surface (NoP – absence of salivary pellicle), and treatment modifying the salivary pellicle (P). Treatment was made (2 h; 37 °C) in all specimens, and later, for P, the specimens were incubated in human saliva (2 h; 37 °C). In both cases, the specimens were immersed in 1% citric acid (pH 3.6; 2 min; 25 °C). Calcium released from enamel (CaR) and its relative surface reflection intensity (%SRI) was measured after 5 cycles. Between-group differences were verified with two-way ANOVA, with "presence of pellicle" and "treatment" as factors ($\alpha=0.05$). Results: The presence of pellicle provided better protection regarding %SRI ($p<0.01$), but not regarding CaR ($p=0.201$). In relation to treatment, when compared to the control group, all proteins/peptides provided significantly better protection ($p<0.01$ for %SRI and Car). The combination of all three proteins/peptides demonstrated the best protective effect ($p<0.01$ for %SRI). Conclusion: Depending on the protein or peptide, its erosion-inhibiting effect derives from their interaction with the enamel surface or from modifying the pellicle, so a combination of proteins and peptides provides the best protection.

Clinical Significance: The present study opens a new direction for a possible treatment with a combination of proteins for native human enamel, which can act directly on the enamel surface as well on the modification of the salivary pellicle, for the prevention of dental erosion.

Keywords: Acquired pellicle; dental erosion; enamel; protein; pellicle modification; saliva.

38 **INTRODUCTION**

39

40 The continuous interaction of dental surfaces with chemical (acid) challenges causes a
41 cumulative loss of dental hard tissue. This process is known as dental erosion [1]. The causative
42 acids can originate from the diet (extrinsic) [2] or from the gastric content of the host (intrinsic)
43 [3]. Several patient-related factors are linked to dental erosion, such as biological, nutritional
44 and behavioral factors [4].

45 Among the biological factors, saliva is considered as one of the most important,
46 constituting a protective role due to its numerous functions [5-8]. It dilutes and buffers the acids
47 and provides ions for remineralization. Furthermore, the presence of saliva in the oral cavity
48 may contribute to the formation of the acquired enamel pellicle (AEP) [9], a thin organic,
49 bacteria-free layer, formed by predominantly salivary proteins that bind to all exposed tooth
50 surfaces [10]. The AEP provides a diffusion barrier to the teeth, consequently reducing the
51 direct contact of acids with the tooth surface, slowing down the dental erosion process [11,12].

52 The incorporation of proteins onto the AEP has been suggested as an alternative
53 measure to further protect the teeth against dental erosion [12-14]. Using proteomic tools, some
54 studies have identified the proteins that remain in the AEP after exposure to acids, suggesting
55 resistance to acid attacks; examples of these acid-resistant proteins are Cystatin B [15,16],
56 Statherin [17] and Hemoglobin [18].

57 These proteins are, therefore, viable options to be used in oral health products to
58 strengthen the AEP and help protect the teeth against dental erosion. Still, when considering the
59 clinical application of these proteins, we must emphasize that one of the most important factors
60 is the cost. In this sense, the use of human Cystatin B is not prohibitive. To overcome this
61 problem, our group cloned a new cystatin derived from sugarcane that was named CaneCPI-5
62 [19].

63 This protein has been shown to be soluble when produced in a bacterial expression
64 system (*Escherichia coli*), which facilitates its production and purification at low costs [19]. An
65 initial study on polished enamel surface assessed by Atomic Force Microscopy showed that the
66 treatment with CaneCPI-5 can modify the AEP and protect teeth against initial erosion, because
67 the protein can strongly bind to enamel [19]. Likewise, the costs of using natural, full length
68 Statherin would run high, so a peptide containing the 15 N-terminal residues of Statherin, with
69 serines 2 and 3 phosphorylated, was recently developed and also showed promising results for
70 protection against initial erosion *in vitro* [20]. In addition, the same protective effect was found
71 in a study using treatment with commercial hemoglobin followed by hydrochloric acid
72 (simulating intrinsic erosion) [21].

73 In this sense, all the above-mentioned proteins have a potential for acquired pellicle
74 engineering, where they can modify the pellicle and protect teeth against acid challenges. To

75 date, all studies involving these proteins/peptides (CaneCPI-5, Hemoglobin and Statherin) were
76 conducted on polished enamel for technical reasons. However, the use of native enamel
77 (unpolished) surfaces is more closely related to the clinical situation, and it can be investigated
78 using a reflection device [22-24], which is what is proposed in this study. Thus, the aim of the
79 present study was to elucidate the mechanism of action of these proteins/peptides (CaneCPI-5,
80 Hemoglobin and Statherin-peptides, separately or in combination) on initial erosion, focusing
81 on how they act directly on the native enamel surface and on salivary pellicle engineering. In
82 this view, we tested the following null hypotheses: 1) there is no difference between the
83 treatments in the absence of the AEP (when they act directly on the enamel surface); 2) there is
84 no difference between the treatments in the presence of the AEP (on modifying the salivary
85 pellicle); 3) the combination of all proteins/peptides does not differ from the single treatments.
86
87 **MATERIALS AND METHODS**
88
89 **Collection of pooled stimulated whole mouth human saliva**
90 Nine adults (average age 33 years) in good general health (the exclusion criteria were:
91 patients with active caries, periodontal disease, smokers, pregnant women and patients with
92 systemic diseases or using long-term medications) with normal salivary flow
93 (unstimulated > 0.3 mL/minute and stimulated > 1.0 mL/minute) donated saliva. For the
94 collection procedure, the volunteers were oriented not to eat or drink, except water, for 2 h
95 before saliva collection. Whole saliva was collected between 9:00 and 10:00 a.m. by chewing
96 Paraffin wax for 10 min and collecting the saliva into cooled vials. The saliva was then pooled
97 (to avoid the effects of idiosyncrasy) and centrifuged (14,000 g for 20 min at 4 °C) [19], the
98 supernatants were separated and the aliquoted saliva was stored at -80 °C until the day of the
99 experiment. As the saliva was pooled, the local ethics committee considers that the saliva
100 cannot be traced for each individual, and they categorize it as “irreversibly anonymized”. In
101 such cases, no previous approval is necessary (Kantonale Ethikkommission: KEK). Still, the
102 volunteers provided their informed oral consent to use the saliva for this study.
103
104 **Preparation of human native enamel specimens**
105 Human third molars were obtained from a pool of teeth. They were visually inspected
106 using a microscope to evaluate the presence of caries, stains and cracks. A total of 170 human
107 enamel specimens were prepared from the buccal surface of the crowns, using two diamond
108 discs (ExtecCorp., Enfield, CT, USA) and a 4 mm spacer attached to a precision cutting
109 machine (ISOMET Low Speed Saw Buehler, Lake Bluff, Illinois, USA) to obtain standard
110 specimens (4 mm × 4 mm). Moreover, the height was standardized to 2 mm by polishing the
111 dentin surface. While the native enamel surface remained unchanged, all other sides were

112 protected with nail polish, leaving only the enamel surface. Lastly, the specimens were cleaned
113 by ultrasonication, using deionized water for 7 min at 25 °C and stored with humidity control
114 (wet gauze) at 4 °C until the time of the experiment. Similarly to saliva, the teeth were acquired
115 from a pooled bio-bank, where the biological material is “irreversibly anonymised” and not
116 traceable to any patient/volunteer. In accordance with the ethical committee, such specimens do
117 not need formal written consent, neither is a previous approval necessary from the ethics
118 committee.

119

120 **Acquisition of proteins and peptides**

121 The sugarcane cystatin (CaneCPI-5) was recombinantly produced in E.coli, as described
122 previously [19]. Briefly, the expressed protein was purified from the soluble fraction of bacterial
123 cultures induced by IPTG (Isopropil-beta-D-Thiogalactosidio), subjected to centrifugation and
124 sonication. Then, the purification was done by affinity chromatography, using columns
125 containing nickel resin Ni-NTA Superflow (Qiagen) [19,25].

126 The peptide derived from statherin containing the 15 N-terminus residues
127 (DpSpSEEKFLRRIGRFG, where “p” means phosphorylation in serine - 2 and 3
128 phosphorylated) was synthesized using the solid phase method [26], according to a standard
129 protocol that used a fluorenylmethoxycarbonyl protecting group (FMOC) as protector of the α-
130 amino groups and t-butyl derivatives to protect the lateral chains of the residues of trifunctional
131 amino acids [27]. Peptides were purified by High-performance liquid chromatography, using
132 C18 column and characterized through the determination of the molecular mass.

133 The human hemoglobin was obtained from Sigma (cat # H7379 - Sigma Aldrich, St.
134 Louis, Missouri, USA) [21].

135

136 **Experimental groups and procedures**

137 The enamel specimens were randomly allocated to 10 groups (n = 17/group), according
138 to the treatment groups and to the experimental procedures: mechanism of action of the
139 proteins/peptides acting directly on the enamel surface without AEP formation (NoP) or
140 mechanism of action on the salivary pellicle (P). Four treatment groups were used for both
141 procedures and the concentrations of each protein/peptide was determined according to previous
142 studies: control (no protein/peptide), CaneCPI-5 (0.1 mg/ml) [19], Hemoglobin (1 mg/ml) [21],
143 Statherin (1.88×10^{-5} M) [20]. All proteins/peptides were diluted in water. Two further groups
144 were tested on specimens with AEP: Statherin solubilized in phosphate buffer, and a
145 combination of the three proteins/peptides (diluted in water).

146 At baseline, the surface reflection intensity (SRI_i) was measured on all specimens, using
147 a hand-held reflectometer [22-24]. The specimens then underwent treatments, according to the

148 test solutions. For that, they were individually immersed in water or protein/peptide solution
149 (250 μL) in microtubes, for 2 h at 37 °C, under agitation of 70 rpm. After that, the specimens
150 were rinsed with deionized water (10 s) and dried with air (5 s).

151 To study the mechanism of action directly on the enamel surface, the specimens were
152 directly submitted to an erosive challenge in microtubes (1 ml, 1% citric acid, pH = 3.6 to
153 simulate the effect of orange juice, 2 min, 25 °C, 70 rpm), then rinsed with deionized water
154 (10 s) and dried with air (5 s) [24]. The citric acid was stored at 4 °C for later analyses of
155 calcium released to the acid (Car). Treatment with the proteins/peptides and acid was repeated a
156 total of 5 times.

157 For the specimens used for the mechanism of action on the salivary pellicle, AEP was
158 formed after the treatment with the protein/peptide solution. For that, the specimens were
159 individually incubated in human saliva in microtubes (250 μL, 2 h, 37 °C, 70 rpm). They were
160 then rinsed with deionized water (10 s) and dried with air (5 s). Afterwards, the specimens were
161 submitted to acid challenge as described above. Likewise, treatment with the proteins/peptides,
162 AEP formation, and acid challenge was repeated for a total of 5 times.

163 At the end of the experiment, all specimens were individually submitted to 1 ml sodium
164 hypochlorite solution (NaOCl) for 5 min, at 25 °C (in order to remove the remaining salivary
165 pellicle layer [28] and hinder its interference on the SRI measurement) and then the final surface
166 reflection intensity (SRI_f) was measured.

167

168 **Surface reflection intensity (SRI)**

169 SRI was measured using a hand-held reflectometer [22-24]. At the beginning (SRI_i) and
170 at the end (SRI_f) of the experiment, each specimen was individually dried (5 s), and the tip of
171 the reflectometer was placed onto the enamel surface, and inclined in different angles until the
172 point highest reflection intensity was registered. For statistical analyses, we used the relative
173 SRI value (%SRI), calculated as follows: $\%SRI = (SRI_f / SRI_i) \times 100$.

174

175 **Analysis of calcium released to the citric acid (Car)**

176 After each erosive challenge, the citric acid was stored. At the end of the experiment,
177 the aliquots used for the 5 cycles for each specimen were pooled (totaling 5 ml of acid per
178 specimen) and were analyzed with an atomic absorption spectrometer (AAS; AAnalyst 400,
179 Perkin Elmer Analytical Instruments, Waltham, MA, USA). Lanthanum nitrate (0.5%,
180 lanthanum nitrate hexahydrate: $La(NO_3)^3 \cdot 6H_2O$) was added to the citric acid to eliminate the
181 interference of other ions [29]. This way, the values of calcium concentrations were used to
182 calculate the total amount of calcium released by each enamel specimen. These calcium values
183 were then normalized to the area of enamel. For more accurate results, the area of the enamel
184 surface had to be more accurately measured. For that, the specimens were measured using a

185 light microscope (Leica, M420) connected to a camera (Leica, DFC495) and a software
186 program IM500 was used to measure the surface area. The total amount of calcium released was
187 expressed in nmol of Ca^{2+} / mm^2 of enamel [23].
188
189 **Scanning electron microscopy (SEM) of the enamel surface**
190 One specimen from each group were used to obtain micrographs under high
191 magnification. For that, the specimens were sputter-coated with gold palladium (100 s, 50 mA)
192 using a sputtering device (Balzers SCD 050, Balzers, Balzers, Liechtenstein) and images were
193 taken at magnification of 500 × at 10 kV (JSM-6010PLUS/LV SEM, JEOL, Tokyo, Japan) [24].
194
195 **Statistical analysis**
196 Initially, the Kolmogorov-Smirnov test was used to assess normality. A first paired t-
197 test was made to compare the treatments of the Statherin solubilized in water or in phosphate
198 buffer. Later, we performed a two-way ANOVA with variables: presence of the AEP (2 factors)
199 and treatment group (4 factors), followed by Tukey's test and Bonferroni´s correction. All
200 statistical analysis considered the significance level of 0.05, and the results are presented as
201 mean ± standard error of the mean.
202
203 **RESULTS**
204 The results were divided into two parts. In the first part, for methodological reasons, we
205 compared the treatment with statherin solubilized in water or in phosphate buffer. In the present
206 study, we used water for all proteins/peptides, and Statherin solubilized in water was able to
207 better protect enamel than when solubilized in phosphate buffer, showing higher %SRI
208 ($p = 0.0158$) and less calcium release ($p = 0.0019$) (Figure 1). So, statherin solubilized in water
209 was used for further analyses.
210 In the second part, we analyzed the effect of the proteins/peptides on the enamel surface
211 (NoP) and on the salivary pellicle (P), considering the control groups and the treatments
212 (CaneCPI-5, Hemoglobin, Statherin, and the combination of all three). In general, all
213 proteins/peptides groups were able to significantly better protect the enamel from erosion when
214 compared to the control group (NoP and P) ($p<0.01$ and $p<0.01$ for %SRI and Car,
215 respectively).
216 Regarding %SRI analysis, the control group presented the least protection (lowest
217 %SRI, $p<0.01$). In the control group, the presence of pellicle was able to protect the enamel
218 (65% ± 2.75) when comparing to the group without pellicle (51% ± 2.75). All proteins and
219 peptide groups protected enamel against erosion, presenting higher %SRI values than the
220 control groups (P and NoP) ($p<0.01$). When acting directly on the enamel surface, there was no
221 significant difference between Hemoglobin (NoP; 75% ± 2.75) and Statherin (73% ± 2.75), but

222 CaneCPI-5 showed better protective effect (106% ± 2.75). On the other hand, when acting on
223 pellicle engineering, there was no difference between the three proteins/peptide groups
224 (85%% ± 2.75, 91% ± 2.75 and 81% ± 2.75, for CaneCPI-5, Hemoglobin, and Statherin,
225 respectively), but the combination of all three proteins/peptides presented the best protective
226 effect (112% ± 2.75) (Figure 2). Observing each protein/peptide group, CaneCPI-5 showed a
227 better protection when acting directly on the enamel surface, Hemoglobin showed better
228 protection when acting on pellicle engineering, and Statherin showed no significant difference
229 in the mode of action (Figure 2).

230 Regarding Car analysis for the NoP study, the control group presented the least
231 protection, showing the greatest calcium release ($p<0.01$). When acting directly on the enamel
232 surface, the control group released most calcium (14.2 ± 0.6), while CaneCPI-5 (8.5 ± 0.6),
233 Hemoglobin (8.9 ± 0.6), and Statherin (9.3 ± 0.6) presented similar protection. When acting on
234 pellicle engineering, there was gradual protection from groups CaneCPI-5 (10.75 ± 0.6),
235 Hemoglobin (9.4 ± 0.6), Statherin (8.3 ± 0.6) and the combination of all three (7.4 ± 0.6). The
236 latter presented the best protection in comparison to the control group (12.5 ± 0.6), but no
237 significant difference between the CaneCPI-5, Hemoglobin and Statherin groups was observed
238 (Figure 3).

239 The images of the enamel surfaces obtained from SEM are observed in Figure 4. A
240 more severe effect of citric acid was observed on the control group, and the least
241 demineralization was more evident for the group treated with the combination of all three
242 proteins/peptides (Figure 4).

243

244 **DISCUSSION**

245 The term "acquired pellicle engineering" has been suggested as an alteration of this
246 organic layer on the tooth surface through the incorporation of proteins and/or peptides that can
247 enhance its protection against demineralization [12,14]. Some proteins and peptides, however,
248 will not only have an effect on engineering of the acquired pellicle, but they may also interact
249 with the tooth surface itself, thereby having an impact on the subsequent deposition of proteins
250 in the acquired pellicle. So, the main objective of this study was to elucidate the mechanism of
251 action of some proteins/peptides (separately or in combination) on the protection against dental
252 erosion, testing how they react directly on the surface of the native human enamel, as well as in
253 the presence of the acquired pellicle.

254 Initially, we compared the statherin peptide, either prepared in water or in phosphate
255 buffer. Statherin is a polypeptide made up of two main regions, a negatively charged N-terminal
256 and a neutral C-terminal. The former has been suggested as the most important region for
257 binding to the tooth surface [30] and protecting the teeth against demineralization [31].

258 Statherin also performs other functions in human saliva, including formation of the AEP,
259 calcium homeostasis and phosphate buffering [32]. Since the latter plays an important role in
260 demineralization, previous experiments have tested the synthesized statherin peptides in
261 phosphate buffers [20,32,33]. In our study, the other proteins/peptides were dissolved in water,
262 and, ideally, all proteins/peptides, including the statherin, should be in the same base solution.
263 This was paramount for the group combining all three proteins/peptides. We now show that
264 there is no difference between statherin prepared in water or in phosphate buffer for the
265 purposes of the present study. In fact, the preparation in water even presented better protective
266 results. Therefore, statherin solubilized in water was used for the main part of the study, and the
267 combination group would also not be affected when water was used.

268 The fundamental aspect of the present study was to assess the behavior of the
269 proteins/peptides when acting directly on the enamel surface (NoP) and on acquired pellicle
270 engineering (P). It is well known that the AEP can protect enamel against demineralization [9]
271 and this was observed in the present study. The SRI analyses show that the presence of the
272 pellicle in the control group demonstrated better protection against dental erosion than control
273 group without pellicle. However, the calcium release analyses were not so clear cut. This may
274 be related to the presence of calcium in the AEP [34]. In the NoP groups, the release of calcium
275 was exclusively from the exposed enamel surface, and it could be expected to be higher since it
276 has no protection from the AEP. However, in the groups with AEP, some of the calcium was
277 also released from the pellicle. More importantly, to form the AEP, the whole enamel specimen
278 was immersed in saliva, and pellicle was formed on all sides of the specimens, even on the
279 surface protected with nail polish. So, when a specimen was immersed in citric acid, calcium
280 was not only released from the exposed dental enamel, but also from the salivary pellicle that
281 covered all sides of the specimen, possibly leading to an overestimation of calcium in the
282 groups containing the AEP. From this perspective, the calcium results must be viewed bearing
283 this restraint in mind.

284 When analyzing each treatment separately, we observed that the CaneCPI-5 group
285 demonstrated a better protective effect when acting directly on the enamel surface (NoP) than
286 when acting on pellicle engineering (P). This may be related to its high binding force to
287 hydroxyapatite, as detected by AFM experiments [19]. This protein is acid-resistant, so we
288 speculate that the CaneCPI-5 has a mechanism of action directly on the enamel surface, where
289 its layer still remains (in parts) after the acid challenge (Figure 5), leading to a protective effect.
290 However, when saliva comes into the equation, other salivary proteins compete with the
291 CaneCPI-5 to occupy the available binding sites on the enamel surface (Figure 5), which
292 justifies the reduced protection in comparison to NoP. Despite this slightly lower protection,
293 CaneCPI-5 still showed an effect on acquired pellicle engineering, since it led to better results
294 than the control group. These results indicate that subsequent studies evaluating the use of

295 caneCPI-5 to protect against dental erosion should apply this protein directly on enamel and not
296 on the acquired pellicle.

297 Regarding hemoglobin, this protein is also a potential candidate to protect against dental
298 erosion. The concentration of this protein is around 3 times higher in the acquired pellicle of
299 reflux patients who do not present erosive tooth wear [18], so it could be one of the protecting
300 factors in these patients. A previous study from our group showed significant protection from
301 hemoglobin when polished enamel was eroded with hydrochloric acid [21], so this protein was
302 also tested here with native human enamel. As expected, hemoglobin also protected enamel
303 against demineralization with citric acid, but it presented a better protective effect in pellicle
304 engineering rather than when acting directly on the enamel surface. This was remarkable, since
305 hemoglobin has strong affinity to adsorb to hydroxyapatite. Its adsorption rate, however,
306 increases as its concentration in the environment increases [35], and as the pH decreases [36].
307 Although our concentration of hemoglobin was relatively high (at 1 mg/ml in comparison to
308 median salivary concentration values in healthy patients of only 0.29 μg/ml) [37], our pH
309 remained neutral. This probably hindered its adsorption onto the enamel surface, but some
310 adsorption still must have occurred, as it presented a protective effect compared to the control
311 group. The exact mechanism of how hemoglobin adheres to enamel is still not well described,
312 and must be further investigated. In any case, our results show that hemoglobin rather had a
313 better mechanism of action on the pellicle engineering, increasing the protective effect of the
314 AEP against dental erosion (Figure 5), corroborating the fact that pellicle of some reflux
315 patients, containing higher amounts of hemoglobin in saliva, can prevent erosive tooth wear
316 [18].

317 Statherin is long known to be one of the precursor proteins forming the basal layer of
318 the AEP because of its high affinity to hydroxyapatite [38,39]. Moreover, similarly to CaneCPI-
319 5, statherin is also an acid-resistant protein, remaining on the enamel surface even after acid
320 attacks [17]. Hence, we expected it to behave in a similar manner to CaneCPI-5. Interestingly,
321 although statherin produced better protective results than the control group, no differences were
322 observed between its action directly on the enamel surface and on pellicle engineering. This
323 could be because there is statherin in saliva, which can already adhere to the enamel surface and
324 compete to occupy the available binding sites on enamel. Once these sites are occupied, no
325 further protection is seen with pellicle engineering with the statherin solution. However, when
326 the statherin peptide solution is used as treatment (without the presence of saliva), the peptide
327 can adsorb onto the enamel binding sites and provide a protective effect [20] (Figure 5). In this
328 case, its binding is related to the 15 N-terminal residues explained earlier. This conformation
329 generates densities of negative charges and phosphate residues that are subsequently attracted
330 by calcium residues within the hydroxyapatite [20,30].

331 The best protective effect observed in our study was from the combination of all three
332 proteins/peptides (Figure 4). This group provided a significantly better protection, when
333 analyzing SRI, in comparison to the other groups. Although the calcium release results were not
334 as clear, as discussed above, there was a tendency for this group to release less calcium (better
335 protection). Based on the results of the other groups with single proteins/peptides, we can
336 speculate the mechanism of action for this combination (Figure 5). We hypothesize that the
337 CaneCPI-5 and statherin will play a more important role in the adsorption onto enamel, while
338 hemoglobin will have a more noteworthy effect on pellicle engineering. Besides, it is also
339 probable that these proteins/peptides have a synergistic effect, which led to the improved SRI
340 results. In fact, the final reflection values were above 100%, and the reasons for this could be
341 twofold: either the enamel was not demineralized, or there was remnant of the salivary pellicle
342 on the enamel surface at the time of final measurements even after immersion in the NaOCl
343 [40]. The former probably did not happen because the acid challenges were able to cause
344 demineralization in all other groups covered with AEP (see other P groups) and it is highly
345 probable it occurred in this group too. So, the latter is more probable, that remnants of the
346 pellicle remained on the enamel even after incubating the specimens in NaOCl [41]. This is
347 plausible, because the synergistic effect of the proteins/peptides probably led to a strong bond to
348 the enamel surface, forming a basal layer strong enough that was not completely removed by
349 the acid or NaOCl (Figure 5). This hypothesis, however, must be further analyzed. Also,
350 additional studies should be carried out combining pairs of proteins/peptides, in order to better
351 understand the mechanism of action of this synergism. For this combination group, however,
352 the NoP procedure was not performed because the solution is aimed for a clinical application
353 where the presence of saliva is indelible.

354 It is important to consider that the incubation time of the enamel specimens is quite long
355 (2 h), but it was performed following previous protocols, allowing comparisons between the
356 studies [19-21]. Moreover, the AEP formation was made *in vitro*, which leads to a different
357 pellicle than that formed *in situ* or *in vivo* [Pela et al. 2020 in press]. Notwithstanding the *in*
358 *vitro* set-up, our results open a path for preventive procedures involving organic components,
359 more specifically using proteins and peptides, which might act on pellicle engineering,
360 hindering enamel demineralization. Additionally, our in vitro set-up not only used human saliva,
361 but also human teeth that were not polished. While the vast majority of other studies involving
362 treatment for dental erosion are conducted on polished dental surfaces (enamel or dentin) [29],
363 we were able to carry out our tests on native surfaces, which is an advantage of the present
364 study, for it is more closely related to the clinic situation. Still, further in situ and in vivo studies
365 might be carried out in the future.

366 Based on our results, all null hypotheses were rejected, and, in conclusion, the treatment
367 with CaneCPI-5 demonstrated a better protective effect directly on the enamel surface,

368 hemoglobin presented a better result on pellicle engineering and the statherin group showed no
369 difference between the two conditions. The combination of all three proteins/peptides provided
370 the best protective effect for native enamel. The probable mechanism of action for the latter is
371 through the adsorption of the proteins/peptides directly on the enamel surface as well as through
372 acquired pellicle engineering.

373

374

375 **Conflict of interest:** The authors declare that they have no conflict of interest.

376

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492 **Figure legend**
493
494 Figure 1. Comparison of the treatment of statherin solubilized in the two forms: water and
495 phosphate buffer. A) Relative surface reflection intensity (%SRI). B) Calcium released to the
496 citric acid (nmol of Ca2+ / mm2 of enamel). Different small letters indicate statistical
497 differences between groups.
498
499 Figure 2. Relative surface reflection intensity (%SRI) according to the different treatments.
500 Light gray columns represent the groups without (NoP) acquired pellicle formation. Dark gray
501 columns represent the groups with (P) acquired pellicle formation. Distinct letters denote
502 significant differences between the treatments.
503
504 Figure 3. Calcium released to the citric acid (nmol of Ca^{2+} / mm^2 of enamel) according to the
505 different treatments. Light gray columns represent the groups without (NoP) acquired pellicle
506 formation. Dark gray columns represent the groups with (P) acquired pellicle formation.
507 Distinct letters denote significant differences between the treatments.
508
509 Figure 4. Scanning electron microscopy images of the enamel surface of the different groups.
510 (14kV - 500x - 50μm). The enamel specimens from the Control (NoP) and Control (P) groups

511 presented clear demineralization pattern, with more porous enamel surface. The other groups
512 also presented some demineralization, with some honey-comb pattern. The Combination (P)
513 group present the faintest demineralization (honey-comb) pattern.
514
515 Figure 5. Model of the mechanism of action of the different proteins/peptides and their
516 combination, directly on the enamel surface and/or on acquired pellicle engineering, with their
517 probable effects before and after erosion. Each row demonstrates the mechanism of a different
518 group, represented by the different colored balls (different proteins/peptides). Left side (two
519 first columns) demonstrates the mechanism directly on the enamel surface (before and after
520 erosion) without the presence of the pellicle. Right side (two last columns) demonstrates the
521 mechanism related to acquired pellicle engineering (before and after erosion).
522