

# Preventing and Arresting Primary Tooth Enamel Lesions Using Self-Assembling Peptide P<sub>11</sub>-4 *In Vitro*

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**ABSTRACT** **Objectives:** To evaluate self-assembling peptides (SAP) for caries prevention and arrest in primary tooth enamel *in vitro*. **Materials and Methods:** Overall, 180 extracted primary teeth were used. In the prevention experiment ( $n = 20$  samples per group), self-assembling peptide for prevention (SAPP), fluoride varnish/mouthwash (FV/FMW), casein-phosphopeptide amorphous-calcium phosphate (CPP-ACP), and nanohydroxyapatite (nHA) were applied. Samples were subjected to a demineralizing pH cycling for 14 days. In the arrest experiment ( $n = 15$ /group), 60 samples were pre-demineralized; induced lesions were treated using self-assembling peptide for repair (SAPR), FV, CPP-ACP plus fluoride, and resin infiltration (RI) and submitted to pH cycling. Mineral loss and its differences as well as lesion depth were determined using transversal microradiography. Numerical data were tested for normality using Shapiro–Wilk’s test and were compared using Kruskal–Wallis test followed by pairwise comparisons utilizing multiple Mann–Whitney U tests with Bonferroni correction. The significance level was set at  $P < 0.05$  within all tests. **Results:** FV (median:  $-46.3$  [interquartile range:  $175.52$ ] vol%  $\times \mu\text{m}$ ) and FMW ( $-33.35$  [ $124.65$ ] vol%  $\times \mu\text{m}$ ) prevented caries significantly more effectively than all other groups ( $P < 0.001$ ), which did not show significant preventive effects. RI (median:  $4949.70$  [ $1637.20$ ] vol%  $\times \mu\text{m}$ ) and FV (median =  $6076.05$  [ $5190.08$ ] vol%  $\times \mu\text{m}$ ) arrested lesions, whereas SAPR and CPP-ACPF did not show such arrest. **Conclusions:** FV and FMW showed the largest caries-preventive effect, whereas RI and FV arrested lesion progression in primary tooth enamel *in vitro*.

**KEYWORDS:** Biomimetic remineralization, caries, microradiography, prevention, primary teeth, self-assembling peptides

## INTRODUCTION

Caries in primary dentition is one of the most prevalent conditions of humankind,<sup>[1]</sup> with more than 500 million untreated cases and more than 120 million incident cases each year. Conventional treatment of caries lesions in primary teeth using restorative approaches is challenging due to a combination of behavioral and micro- and macro-anatomic factors, and failure rates of plastic restorations in the primary dentition being high specifically due to secondary caries.<sup>[2]</sup> Caries in the primary dentition is a major

reason for hospitalization for both routine treatments and emergencies.<sup>[3]</sup>

Hence, there is a great need for both preventing and arresting carious lesions in the primary dentition. The most accepted strategies for prevention are the delivery of fluoride, mainly via toothpaste or, in high-risk

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individuals, varnishes, gels, or mouthwashes, as well as routine oral hygiene care and dietary control. The use of fluoridated toothpaste for caries prevention is supported by a large body of evidence.<sup>[4]</sup> Similarly, fluoride varnish (FV) and fluoride mouthwash (FMW) have been found to be highly efficacious for caries prevention,<sup>[5,6]</sup> and if applied risk-adjusted are cost-effective.<sup>[7]</sup>

Fluoride has been well known for its inhibitory effect on demineralization and enhancing effect of remineralization. During demineralization phases *in vivo*, sugars are converted into acids and once the critical pH of enamel (pH = 5.5) is reached, mineral dissolution occurs. The presence of fluoride in the dental biofilm can inhibit the demineralization process by the formation of fluoroapatite because of hydroxyapatite dissolution. Since the critical pH of fluoroapatite is pH = 4.5, it allows the precipitation of minerals back to the tooth structure and prevents the net demineralization. In addition, fluoride can enhance the remineralization process. When sugar ingestion has stopped, the pH rises above 5.5, and the salivary remineralizing effect is enhanced by the presence of fluoride in the biofilm. At high pH levels, biofilm fluid is supersaturated with respect to hydroxyapatite as well as fluoroapatite and hence the lost ions are efficiently recovered by the tooth.<sup>[8]</sup>

Alternative interventions to control the balance between de- and remineralization on the surface of primary teeth, specifically enamel, have been sought; among them are (a) casein-phosphopeptide amorphous calcium phosphate (CPP-ACP) with or without the addition of fluoride for home and in-office use, (b) nanohydroxyapatite (nHA), or (c) self-assembling peptides P<sub>11-4</sub> (SAP).<sup>[9]</sup>

- (a) After its application, CPP-ACP accumulates in the supragingival plaque and, under low pH conditions, releases Ca<sup>2+</sup> and PO<sub>4</sub><sup>3-</sup> ions, which precipitate and are thought to prevent net demineralization and enhance remineralization of incipient lesions.<sup>[10]</sup>
- (b) Synthetic nHA is known for being a bioactive and biocompatible material that resembles enamel apatite crystals in their morphology, structure, and crystallinity. The nano-sized nHA particles are believed to fill enamel defects on the enamel surface and create a new layer of synthetic surface enamel, preventing demineralization and enhancing remineralization.<sup>[11-13]</sup>
- (c) SAP are oligomer  $\beta$ -sheet-forming peptides (Ace-Gln-Gln-Arg-Phe-Glu-Trp-Glu-Phe-Glu-Gln-Gln-Nh<sub>2</sub>), which when subjected to specific environmental conditions have the ability to self-assemble into fibrillar scaffolds, thereby creating

a  $\beta$ -sheet called “nanotapes.” The process of self-assembly continues while the nanotapes connect by pairing and transform into ribbons, which further self-assemble to form fibrils and fibers,<sup>[14]</sup> leading to scaffold-like structures attracting calcium and phosphate deposition.<sup>[14-16]</sup> SAP is thereby supposed to facilitate biomimetic remineralization of hard dental tissue<sup>[15]</sup> and has been found to be efficacious clinically as well.<sup>[17]</sup>

Similarly, for lesion arrest, the in-office application of FV or CPP-ACP, for example in higher concentrations,<sup>[18]</sup> or SAP has been suggested.<sup>[19]</sup> Alternatively, resin infiltration (RI), where the lesion is infiltrated with lowly filled resins, which are light-cured and subsequently block any acid diffusion into the lesion body and hence mineral loss from it, can be applied to inhibit caries lesion progression.<sup>[20]</sup> There is a robust body of clinical data supporting RI, for example to arrest proximal carious lesions, mainly in the permanent dentition.<sup>[21]</sup>

Overall, most studies on preventing and/or arresting carious lesions using the described measures were conducted in the permanent, not the primary dentition. The body of evidence comparing fluoride applications, other mineral suppliers, RI or SAP is extremely limited. Therefore, we aimed at comparing caries prevention and inhibition of lesion progression using SAP against those of other established measures *in vitro*, hypothesizing SAP to have no superior caries-preventive and arresting properties compared with the other materials under investigation.

## MATERIALS AND METHODS

### STUDY DESIGN

This study followed the CRIS (Checklist for Reporting *In vitro* Studies) guidelines<sup>[22]</sup> and was based on the fundamentals of ethical research practice. Informed consent was obtained from all patients' legal guardians to include children's teeth in the experiments. This study assessed the caries-preventive and -arresting effect of SAP and various alternatives, namely FV, FMW, CPP-ACP, CPP-ACP with fluoride (CPP-ACPF), and nHA for prevention, and FV, CPP-ACPF, and RI for arrest in primary tooth enamel *in vitro* [Table 1].

A combined study design was chosen, with samples being either prepared, the preventive strategies applied (Experiment 1, Preventive Study), and then challenged with demineralization (using pH cycling), or with the samples being prepared, pre-demineralized using acetic acid for 21 days (to induce an artificial carious lesion), the application of arresting interventions (Experiment 2, Arrest Study), and then challenged with demineralization (using pH cycling). For both study

parts, mineral loss and lesion depth were assessed using transverse microradiography (TMR). The study flow is summarized in Figure 1.

**SAMPLE SIZE ESTIMATION**

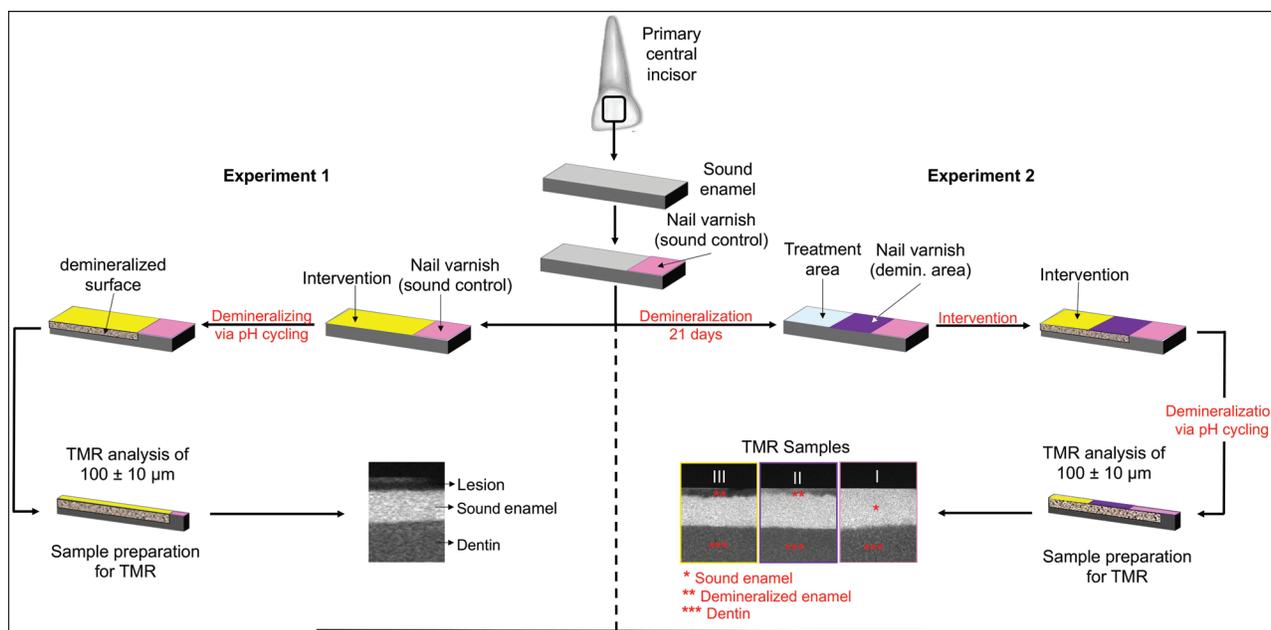
A sample size estimation was performed to have adequate power to apply a two-sided statistical test of the research hypothesis (null hypothesis) that there is no difference SAP and FV in preventing and

inhibiting carious lesions progression of primary teeth. The estimation was built on the results of Sindhura, Vemulapalli *et al.*, in which the mean difference was 0.26 and the standard deviation was 0.21.<sup>[23]</sup> Assuming an alpha ( $\alpha$ ) level of 0.05 (5%), a beta ( $\beta$ ) level of 0.20 (20%), that is, a power = 80%, and an effect size ( $d$ ) of (1.24), the required sample size ( $n$ ) was 12 samples per group. Sample size calculation was performed using G\*Power version 3.1.9.2.<sup>[24]</sup>

**Table 1: The caries-preventive and caries-arresting products used**

Intervention	Form	Effective component	Manufacturer
SAPP (Curodont Protect)	Gel	Self-assembling peptide P <sub>11-4</sub>	Credentis, Windisch, Switzerland
SAPR (Curodont Repair)	Solution	Self-assembling peptide P <sub>11-4</sub>	Credentis, Windisch, Switzerland
FV (Profluorid varnish)	5% NaF Varnish	22.600 ppm fluoride	Voco, Coxhaven, Germany
CPP-ACP (Tooth Mousse)	Paste	Casein phosphopeptide amorphous calcium phosphate	GC, Tokyo, Japan
CPP-ACPF (MI Paste)	Paste	Casein phosphopeptide amorphous calcium phosphate fluoride	GC, Tokyo, Japan
FMW (NaF Solution)	Mouthwash	500 ppm NaF	Charité pharmacy, Berlin
nHA (Biorepair)	Mouthwash	Nanohydroxyapatite	Dr. Kurt Wolff, Bielefeld, Germany
RI (ICON)	Solution	Resin Infiltration	DMG, Hamburg Germany

SAPP = self-assembling peptide for prevention, SAPR = self-assembling peptide for repair, FV = fluoride varnish, CPP-ACP = casein phosphopeptide amorphous calcium phosphate, CPP-ACPF= casein phosphopeptide amorphous calcium phosphate fluoride, FMW = fluoride mouthwash, nHA = nanohydroxyapatite, RI = resin infiltration



**Figure 1: Study flow.** Sound enamel was ground, polished, and sectioned to enamel-dentin samples. Parts of the sound polished enamel were protected against demineralization using nail varnish to serve as sound controls. The remaining exposed enamel was either submitted to preventive strategy application (left side) or pre-demineralized to induce artificial caries lesions (right side), to be arrested using arresting strategies. The treated surfaces were exposed to pH cycling for 14 days. Samples of 100 ± 10 µm thickness were prepared for transverse microradiography and microradiographically analyzed (left side: the lesion induced by pH cycling, sound enamel, and dentin beneath it; right side: I sound enamel and dentin, II pre-demineralized enamel, sound enamel, and dentin beneath it, III the lesion after further demineralization using pH cycling and sound enamel and dentin beneath it)

Several studies done on remineralization comparing SAP with other remineralizing agents used a range of 10–22 samples per group, with an average of 16 samples/group. Therefore, in Experiment 1 (Prevention Study), a sample size of  $n = 20$  and in Experiment 2 (Arrest Study),  $n = 15$  was chosen, to have a minimum of 25% extra samples to compensate for any loss of samples during preparation expected due to the limited thickness of enamel in primary anterior teeth.

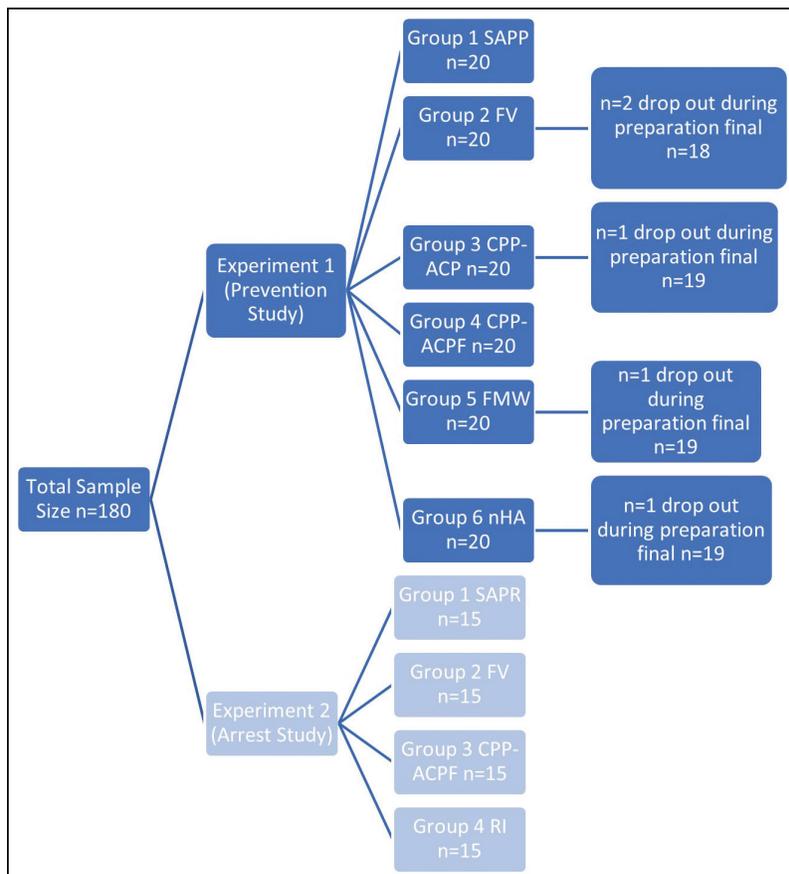
**SPECIMEN PREPARATION**

One hundred eighty sound primary anterior teeth (incisors and canines) obtained from Egyptian patients after exfoliation under an ethically approved protocol (ethical committee of the Ain Shams University, FDASURcIR022024) were collected and stored in 0.5% Chloramine T solution for a maximum of two months. There are more than 15 different storage solutions suggested in the literature for the storage of extracted teeth or enamel specimens, Chloramine T was chosen for being a well-known storage medium with antibacterial properties that can prevent bacterial growth during the storage period.<sup>[25]</sup>

Teeth were cleaned and those with stains, cracks, and carious or developmental defects were excluded. The root was separated from the crowns at the cemento-enamel junction using a water-cooled diamond coated band saw (Band Saw Exakt 300cl; Exakt Apparatebau, Norderstedt, Germany). The samples were embedded in epoxy resin (Technovit 4071, Heraeus Kulzer, Hanau, Germany), with the labial surfaces of incisors and the lingual surfaces of canines facing upward, ground flat, and polished sequentially (Mikroschleifsystem; Abrasive Paper WS flex 18C, SiC 1200–4000, Exakt Apparatebau, Norderstedt, Germany) until a surface of approximately 2mm × 2mm enamel was exposed. All samples were checked under light microscopy (Durchlichtmikroskop “Axioskop 2,” Fa. Zeiss, Oberkochen, Deutschland) to make sure that an enamel surface was still present, and that dentine was not exposed.

**INTERVENTIONS**

The 180 samples were divided into the two experimental arms: In Experiment 1 (Prevention Study), 120 samples were used to assess the caries-preventive effect, whereas in Experiment 2 (Arrest Study), 60 samples were used



**Figure 2:** Flow chart, showing the sample sizes for all groups in both Experiments 1 and 2. Experiment 1: SAPP (self-assembling peptide for prevention); FV (fluoride varnish); CPP-ACP/CPP-ACPF (casein-phosphopeptide amorphous-calcium-phosphate without/with fluoride); FMW (fluoride mouthwash); nHA (nanohydroxyapatite). Experiment 2: SAPR (self-assembling peptide for repair); FV (fluoride varnish); CPP-APF (casein-phosphopeptide amorphous-calcium-phosphate fluoride); RI (resin infiltration)

to assess the lesion arrest [Figure 2]. One-third of the exposed surfaces of all samples was protected against the subsequent demineralization challenge using a nail varnish (Maybelline New York Express Finish 40, New York, USA), serving as baseline (sound control).

### Experiment 1 (Prevention Study)

To test the caries-preventive effect, the remaining two-thirds of the exposed surfaces of the 120 samples were treated using one of six interventions ( $n = 20$ /group, Table 1) before being challenged for demineralization:<sup>[1]</sup> SAPP (Curodont Protect, Credentis, Windisch, Switzerland),<sup>[2]</sup> FV (5% NaF Profluorid, Voco, Cuxhaven, Germany),<sup>[3]</sup> CPP-ACP (Tooth Mousse, GC, Tokyo, Japan),<sup>[4]</sup> CPP-ACP plus fluoride (CPP-ACPF, MI Paste Plus, GC, Tokyo, Japan),<sup>[5]</sup> 500 ppm sodium FMW (pharmacy of the Charité – Universitätsmedizin Berlin),<sup>[6]</sup> and nHA mouthwash (Biorepair Mouth Wash, Dr. Kurt Wolff, Bielefeld, Germany). Curodont Protect gel was applied on a semidry surface with a microbrush, rubbed in, and left for a couple of minutes to dry. It was then washed away as instructed by the manufacturer. FV, CPP-ACP, and CPP-ACPF were applied on a dry surface with a microbrush and left 30 min to set; then, they were rinsed off with water to mimic the conditions of the oral cavity. Both mouthwashes were utilized once daily after the demineralization cycle for 15 min by storing the samples in them, whereas the other samples were stored in distilled water during that time.

### Experiment 2 (Arrest Study)

To test lesion arrest, the remaining two-thirds of the exposed surfaces of the 60 samples were pre-demineralized using 3 mM  $\text{CaCl}_2$ , 3 mM  $\text{KH}_2\text{PO}_4$ , 0.006 mM methylhydroxydiphosphanate (MHDP), 50 mM  $\text{CH}_3\text{COOH}$ , and 10 M KOH. The pH was adjusted to 4.95 using KOH for 21 days (Carl Roth, Karlsruhe, Germany).<sup>[26]</sup>

Half of the demineralized surface was covered with a nail varnish to allow the assessment of the mineral loss of the lesions after the first demineralization challenge and prior to the second demineralization challenge. The remaining one-third of the exposed surface of the sample received one of four interventions ( $n = 15$ /group, Table 1) prior to being challenged again for demineralization:<sup>[1]</sup> SAPR (Curodont Repair, Credentis, Windisch, Switzerland),<sup>[2]</sup> 5% FV (5% NaF Profluorid, Voco, Cuxhaven, Germany),<sup>[3]</sup> CPP-ACPF (MI Paste Plus, GC),<sup>[4]</sup> and RI (Icon DMG, Hamburg, Germany). For SAPR, samples were etched with 37% phosphoric acid (Fine Etch 37, Spident, Korea) for 5 s and rinsed with tap water. After drying the surface, Curodont Repair (In Vitro Vial, Credentis, Windisch,

Switzerland) was dissolved without any further purification in 50  $\mu\text{L}$  distilled water applied on each sample and left 5 min for setting. FV and CPP-ACPF were applied as described in experiment 1. Before RI, samples were etched using 37% phosphoric acid (FineEtch 37) for 5 s. The specimens were thereafter washed and dried using Icon Dry for 30 s and infiltrated using Icon Infiltrant for 3 min. After removing the excess material, light-curing was performed using an LED curing light (Valo, Ultradent, Salt Lake City, USA) with an intensity of 1400  $\text{mW}/\text{cm}^2$  for 40 s from < 1 mm distance. The procedure was repeated, with the infiltrant being applied for only 1 min, as recommended by the manufacturer.

### DEMINERALIZATION CHALLENGE USING pH CYCLING

All samples were subsequently subjected to a demineralizing pH cycling using a demineralization solution containing 2.2 mM  $\text{CaCl}_2$ , 2.2 mM  $\text{NaH}_2\text{PO}_4$ , and 50 mM acetic acid adjusted to a pH of 4.8 by NaOH (Carl Roth, Karlsruhe, Germany). The remineralizing solution contained 1.5 mM  $\text{CaCl}_2$ , 0.9 mM  $\text{NaH}_2\text{PO}_4$ , and 0.15 M KCl adjusted to a pH of 7.0 by KOH (Carl Roth, Karlsruhe, Germany). Each group was cycled separately for 8 h in 100 mL demineralizing solution and 16 h in 100 mL remineralizing solution for 14 days at room temperature without agitation. Between the de- and remineralizing cycles, the samples were washed with distilled water. The mouthwashes in the prevention groups were renewed daily.<sup>[27]</sup>

### TRANSVERSAL MICRORADIOGRAPHY

Samples were cut along their longitudinal axes (Band Saw Exakt, Exakt Apparatebau, Norderstedt, Germany) and thereafter, thin plano-parallel slices with a thickness of  $100 \pm 10 \mu\text{m}$  were prepared (Mikroschleifsystem, Exakt Apparatebau, Norderstedt, Germany). In Experiment 1, a total of five samples were lost during their preparation for the TMR analysis, since the enamel of human primary anterior teeth was very thin. Among the five samples, two were from FV group, one from CPP-ACP, one from FMV, and one from nHA. Despite this loss, the estimated sample size was reached.

The samples were placed on film holders and exposed to a nickel-filtered copper radiation source operating at 20 kV and 20 mA with an exposure time of 10 s. Films (Fine 71337, Fujifilm, Tokyo, Japan) were developed according to the manufacturer's instructions under standardized conditions. The microradiographs were analyzed with a digital image-analyzing system (XC 77 CE, Sony, Tokyo, Japan) interfaced with a universal microscope (Axioskop 60318, Zeiss, Oberkochen,

Germany) and a personal computer (TMR for Windows 2.0.27.2, Inspector, Research, Amsterdam, Netherlands). Calibration standardization was done using an aluminum step-wedge with different aluminum thicknesses, and a calibration curve between aluminum thickness and gray levels was constructed.

### STATISTICAL ANALYSIS

Our primary outcome was mineral loss (vol%/µm), and the secondary outcome was the lesion depth (µm). Numerical data were tested for normality by checking their distribution and by using Shapiro–Wilk’s test. Data were found to be nonparametric, so they were presented as median and interquartile range values and were analyzed for intergroup comparisons using Kruskal–Wallis test followed by pairwise comparisons utilizing multiple Mann–Whitney U tests with bonferroni correction. The significance level was set at  $P < 0.05$  within all tests. Statistical analysis was performed with R statistical analysis software version 4.1.0 for Windows.<sup>[28]</sup>

## RESULTS

### EXPERIMENT 1 (PREVENTION STUDY)

Descriptive statistics for mineral loss and lesion depth change are presented in Tables 2 and 3, respectively.

Results of intergroup comparisons for mineral loss change presented in Table 4 showed that there was

a significant difference between different groups ( $P < 0.001$ ). The highest median value was found in CPP-ACP (2,099.4 [2,021.75]) followed by SAPP (2,089.85 [1,014.45]) and then CPP-ACPF (1,606.00 [899.82]), nHA (1,074.6 [893.8]), and FMW (−33.35 [124.65]) whereas the lowest value was found in FV (−46.3 [175.52]). Post hoc pairwise comparisons showed values of SAPP, CPP-ACP, and CPP-ACPF to be significantly higher than values of FMW and FV ( $P < 0.001$ ). In addition, they showed the value of nHA to be significantly higher than FV ( $P < 0.001$ ). Median values for change in mineral loss in different groups are presented in Figure 3.

Results of intergroup comparisons for lesion depth change presented in Table 4 showed that there was a significant difference between different groups ( $P < 0.001$ ). The highest median value was found in CPP-ACPF (69.40 [27.95]) followed by SAPP (55.30 [17.12]) and then CPP-ACPF (54.30 [12.67]), nHA (44.20 [22.85]), and FMW (−0.55 [2.88]) whereas the lowest value was found in FV (−1.40 [3.90]). Post hoc pairwise comparisons showed values of SAPP, CPP-ACP, CPP-ACPF, and nHA to be significantly higher than values of FMW and FV ( $P < 0.001$ ). Median values for change in lesion depth in different groups are presented in Figure 4.

**Table 2: Experiment 1 (Prevention Study) descriptive statistics for mineral loss difference (baseline-intervention)**

Treatment	Mean	95% CI		SD	Median	IQR
		Lower	Upper			
SAPP	2,163.30	1,723.29	2,603.32	1,003.99	2,089.85	1,014.45
FV	−61.84	−146.13	22.44	182.45	−46.30	175.52
CPP-ACP	2,383.42	1,668.24	3,098.60	1,590.54	2,099.40	2,021.75
CPP-ACPF	1,563.30	1,175.03	1,951.56	885.91	1,606.00	899.82
FMW	82.07	−134.75	298.88	469.32	−33.35	124.65
nHA	954.93	625.54	1,284.32	732.55	1,074.60	893.80

95% CI = 95% confidence interval for the mean, SD = standard deviation, IQR = interquartile range, SAPP = self-assembling peptide for prevention, FV = fluoride varnish, CPP-ACP = casein phosphopeptide amorphous calcium phosphate, CPP-ACPF = casein phosphopeptide amorphous calcium phosphate fluoride, FMW = fluoride mouthwash, nHA = nanohydroxyapatite

**Table 3: Experiment 1 (Prevention Study) descriptive statistics for lesion depth difference (baseline-intervention)**

Treatment	Mean	95% CI		SD	Median	IQR
		Lower	Upper			
SAPP	58.78	52.72	64.84	13.84	55.30	17.12
FV	−0.84	−5.53	3.84	10.14	−1.40	3.90
CPP-ACP	58.25	45.61	70.89	28.11	69.40	27.95
CPP-ACPF	48.86	38.98	58.74	22.53	54.30	12.67
FMW	1.21	−8.05	10.48	20.05	−0.55	2.88
nHA	40.82	28.73	52.91	26.89	44.20	22.85

95% CI = 95% confidence interval for the mean, SD = standard deviation, IQR = interquartile range, SAPP = self-assembling peptide for prevention, FV = fluoride varnish, CPP-ACP = casein phosphopeptide amorphous calcium phosphate, CPP-ACPF = casein phosphopeptide amorphous calcium phosphate fluoride, FMW = fluoride mouthwash, nHA = nanohydroxyapatite

**EXPERIMENT 2 (ARREST STUDY)**

Descriptive statistics for mineral loss and lesion depth change are presented in Tables 5 and 6, respectively. Results of intergroup comparisons for mineral loss change presented in Table 7 showed that there was no significant difference between different groups regarding the change of mineral loss values from baseline to intervention application ( $P = 0.152$ ) and from demineralized samples to intervention application ( $P = 0.328$ ). For (baseline-intervention) difference, the highest median value was found in SAPR (9,799.00 [8,828.80]) followed by CPP-ACPF (8,071.90 [6,801.03]) and then FV (6,076.05 [5,190.08]) whereas the lowest value was found in RI (4,949.70 [1,637.20]). For demineralized-intervention difference, the highest median value was found in SAPR (5,202.40 [7,279.80]) followed by CPP-ACPF (2,249.60 [6,503.62]) and then

RI (1,271.60 [903.10]) whereas the lowest value was found in FV (1,177.50 [2,632.43]). Median values for change in mineral loss in different groups are presented in Figure 5.

Results of intergroup comparisons for lesion depth change presented in Table 7 showed that there was no significant difference between different groups regarding the change of lesion depth values from baseline to intervention application ( $P = 0.343$ ) and from demineralized samples to intervention application ( $P = 0.247$ ). For (baseline-intervention) difference, the highest median value was found in CPP-ACPF (164.65 [52.17]) followed by FV (161.15 [90.97]) and then SAPR (159.10 [91.00]) whereas the lowest value was found in RI (114.80 [48.40]). For (demineralized-intervention) difference, the highest median value was found in SAPR (33.30 [55.10]) followed by CPP-ACPF (17.70

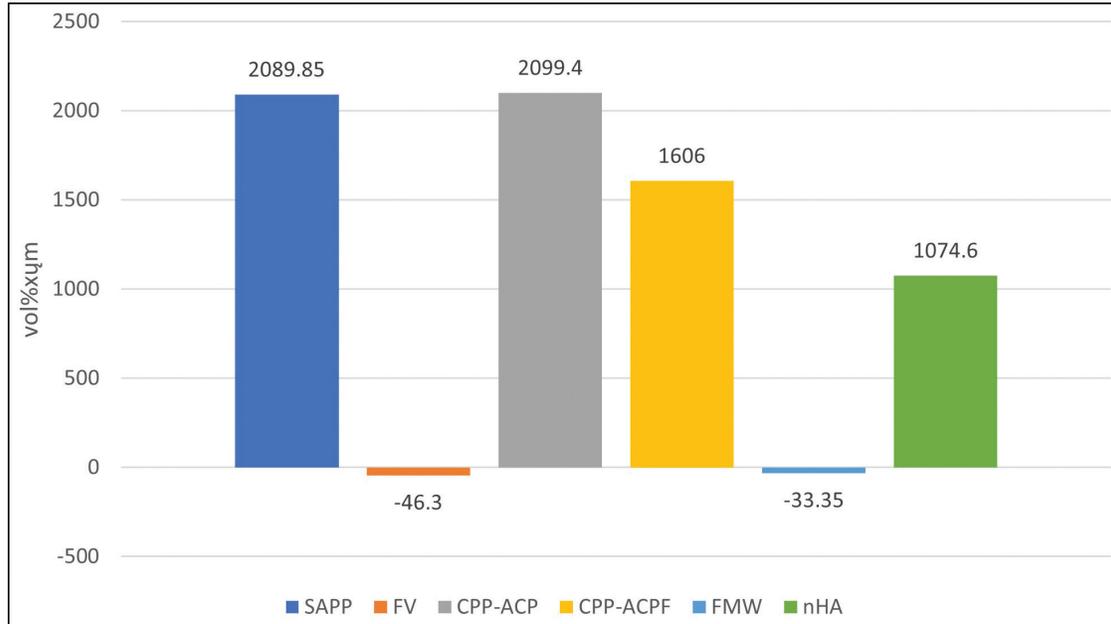


Figure 3: Experiment 1 (Prevention Study) median values for mineral loss change

**Table 4: Experiment 1 (Prevention Study) intergroup comparisons for mineral loss difference and lesion depth difference (baseline-intervention)**

Mineral loss [Median (IQR)]						P value
SAPP	FV	CPP-ACP	CPP-ACPF	FMW	nHA	
2,089.85 (1,014.45) <sup>A</sup>	-46.3 (175.52) <sup>C</sup>	2,099.4 (2,021.75) <sup>A</sup>	1,606.00 (899.82) <sup>A</sup>	-33.35 (124.65) <sup>BC</sup>	1,074.6 (893.8) <sup>AB</sup>	<b>&lt;0.001*</b>
Lesion depth [Median (IQR)]						P value
SAPP	FV	CPP-ACP	CPP-ACPF	FMW	nHA	
55.30 (17.12) <sup>A</sup>	-1.40 (3.90) <sup>B</sup>	69.40 (27.95) <sup>A</sup>	54.30 (12.67) <sup>A</sup>	-0.55 (2.88) <sup>B</sup>	44.20 (22.85) <sup>A</sup>	<b>&lt;0.001*</b>

Medians with different superscript letters within the same horizontal row are statistically significantly different\*; significant ( $P \leq 0.05$ ); IQR=interquartile range, SAPP = self-assembling peptide for prevention, FV = fluoride varnish, CPP-ACP = casein phosphopeptide amorphous calcium phosphate, CPP-ACPF= casein phosphopeptide amorphous calcium phosphate fluoride, FMW = fluoride mouthwash, nHA = nanohydroxyapatite

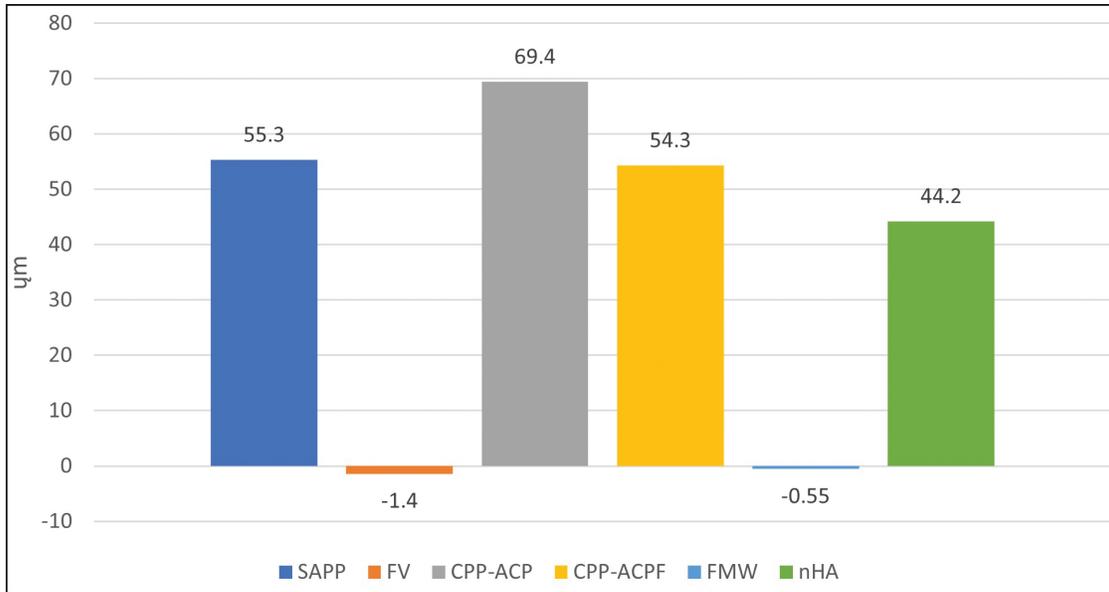


Figure 4: Experiment 1 (Prevention Study) median values for lesion depth change

Table 5: Experiment 2 (Arrest Study) descriptive statistics for mineral loss difference

Difference	Treatment	Mean	95% CI		SD	Median	IQR
			Lower	Upper			
Baseline-intervention	SAPR	8,841.05	5,856.55	11,825.54	5,490.28	9,799.00	8,828.80
	FV	6,423.55	3,831.49	9,015.61	4,581.29	6,076.05	5,190.08
	CPP-ACPF	7,876.33	5,423.65	10,329.01	4,682.27	8,071.90	6,801.03
	RI	4,442.78	3,580.10	5,305.47	1,587.00	4,949.70	1,637.20
Demineralized-intervention	SAPR	4,392.39	1,739.18	7,045.60	4,880.84	5,202.40	7,279.80
	FV	950.45	-689.53	2,590.43	2,898.55	1,177.50	2,632.43
	CPP-ACPF	3,199.59	1,200.68	5,198.51	3,816.02	2,249.60	6,503.62
	RI	1,314.48	715.32	1,913.65	1,102.22	1,271.60	903.10

95% CI = 95% confidence interval for the mean, SD = standard deviation, IQR = interquartile range, SAPR = self-assembling peptide for repair, FV = fluoride varnish, CPP-ACPF= casein phosphopeptide amorphous calcium phosphate fluoride, RI = resin infiltration

Table 6: Experiment 2 (Arrest Study) descriptive statistics for lesion depth difference

Difference	Treatment	Mean	95% CI		SD	Median	IQR
			Lower	Upper			
Baseline intervention	SAPR	163.76	123.61	203.91	73.86	159.10	91.00
	FV	166.52	91.93	241.10	131.83	161.15	90.97
	CPP-ACPF	150.71	120.17	181.25	58.30	164.65	52.17
	RI	122.47	90.66	154.28	58.52	114.80	48.40
Demineralized intervention	SAPR	46.39	3.55	89.24	78.82	33.30	55.10
	FV	35.56	-34.42	105.54	123.69	14.90	37.65
	CPP-ACPF	20.00	5.76	34.24	27.19	17.70	40.68
	RI	3.78	-19.48	27.05	42.80	1.00	31.00

95% CI = 95% confidence interval for the mean, SD = standard deviation, IQR = interquartile range, SAPR = self-assembling peptide for repair, FV = fluoride varnish, CPP-ACPF= casein phosphopeptide amorphous calcium phosphate fluoride, RI = resin infiltration

[40.68]) and then FV (14.90 [37.65]) whereas the lowest value was found in RI (1.00 [31.00]). Median values for change in lesion depth in different groups are presented in Figure 6.

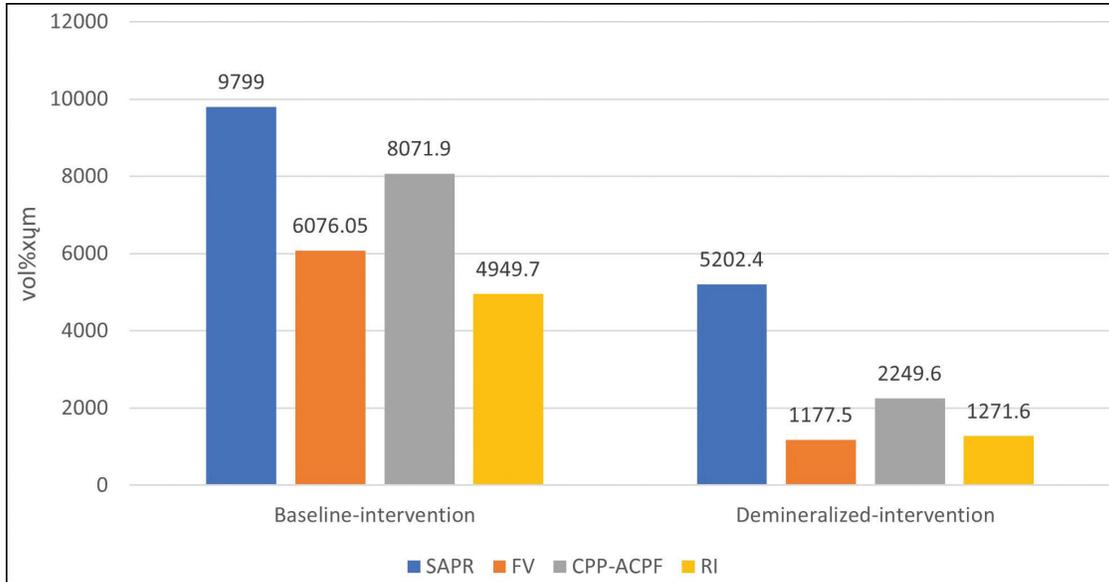
## DISCUSSION

A range of novel caries-preventive and -arresting strategies based on both inhibition of demineralization and facilitation of remineralization are nowadays

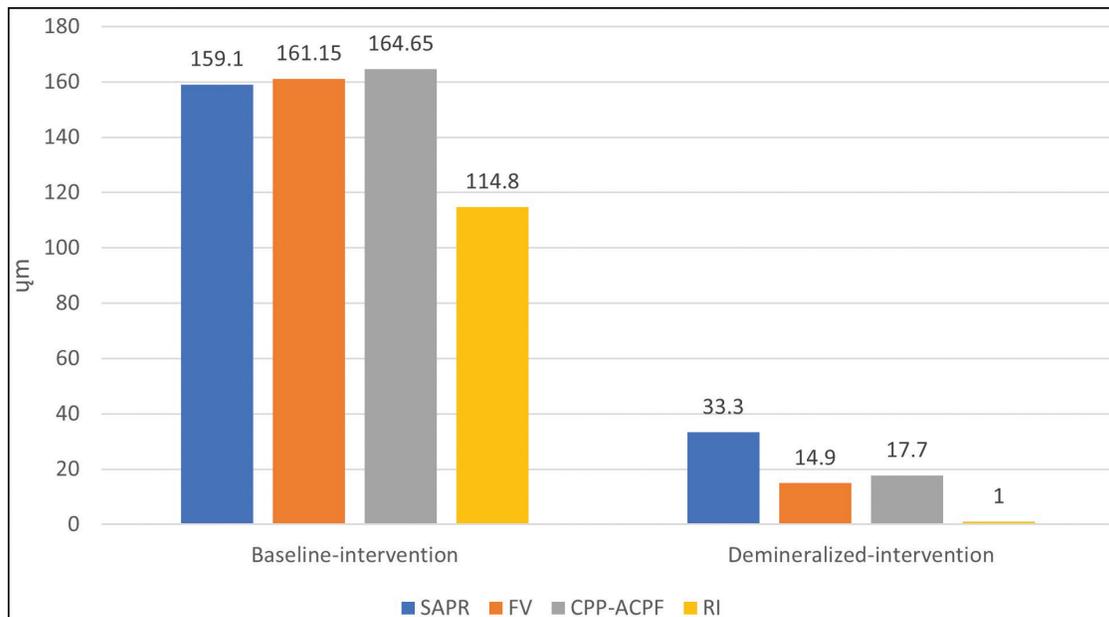
**Table 7: Experiment 2 (Arrest Study) intergroup comparisons for mineral loss difference and lesion depth difference**

Difference	Mineral loss [median (IQR)]				P value
	SAPR	FV	CPP-ACPF	RI	
Baseline intervention	9,799.00 (8,828.80)	6,076.05 (5,190.08)	8,071.90 (6,801.03)	4,949.70 (1,637.20)	0.152
Demineralized intervention	5,202.40 (7,279.80)	1,177.50 (2,632.43)	2,249.60 (6,503.62)	1,271.60 (903.10)	0.328
Difference	<i>Lesion depth [median (IQR)]</i>				<i>P value</i>
	<i>SAPR</i>	<i>FV</i>	<i>CPP-ACPF</i>	<i>RI</i>	
Baseline intervention	159.10 (91.00)	161.15 (90.97)	164.65 (52.17)	114.80 (48.40)	0.343
Demineralized intervention	33.30 (55.10)	14.90 (37.65)	17.70 (40.68)	1.00 (31.00)	0.247

IQR = interquartile range, SAPR = self-assembling peptide for repair, FV = fluoride varnish, CPP-ACPF= casein phosphopeptide amorphous calcium phosphate fluoride, RI = resin infiltration



**Figure 5: Experiment 2 (Arrest Study) bar chart showing median values for mineral loss change**



**Figure 6: Experiment 2 (Arrest Study) median values for lesion depth change**

available. Especially in younger children and primary teeth, the availability of efficacious alternatives to fluoride are of relevance given concerns of acute and chronic toxicity of fluoride and the associated negative public sense.<sup>[29,30]</sup>

SAP have been proposed as one such alternative. In the present study, a range of novel strategies that are suggested to prevent caries and inhibit lesion progression against each other were compared with primary tooth enamel *in vitro*.

In Experiment 1 (Prevention Study), six different agents were compared for their caries-preventive effect, namely SAPP, FV, CPP-ACP, CPP-ACPF, FMW, and nHA. Regarding the prevention of caries lesions, established means such as FV and FMW were found to reduce mineral loss, having the least mineral loss difference, whereas novel strategies such as SAPP, CPP-ACP, CPP-ACPF, and nHA did not have any significant preventive effect *in vitro*. Lesion depth findings were similar to those of mineral loss, where FV and FMW had the least lesion depth difference compared with SAPP, CPP-ACP, CPP-ACPF, and nHA.

In Experiment 2 (Arrest Study), four agents were tested for their remineralizing effect, namely SAPR, FV, CPP-ACPF, and RI. SAPR was the main material under investigation, that is, claimed to provide subsurface remineralization compared with FV, the gold standard remineralizing agent. We also compared against CPP-ACPF and RI. There was no statistically significant difference regarding the four groups in their ability to inhibit lesion progression. Nevertheless, when comparing the four groups, RI was found to inhibit the lesions most effectively from progressing, followed by FV, whereas again novel strategies such as CPP-ACPF and SAPR did not have any significant inhibition of lesion progression effect *in vitro*. We accepted our null hypotheses.

This study has several strengths and limitations. First, data on novel strategies, especially SAP, are scarce, and so far, no study has tested SAP to prevent caries or inhibit lesion progression in primary teeth. Previous studies on SAP focused on human enamel from permanent teeth or bovine enamel. Both show different mineralization and maturation potential than human primary tooth enamel. Second, SAP have largely been tested for lesion arrest (i.e., facilitation of remineralization), not prevention,<sup>[31]</sup> and this study is one of the few testing this novel strategy for preventive applications as well as caries arrest. Third, the assessment of mineral loss using TMR is highly sensitive and a valid method that was not employed in previous studies on SAP.

Instead, scanning electron microscopy (SEM), surface microhardness, or laser fluorescence<sup>[32,33]</sup> had been used. Notably, the chosen assessment method has not evaluated the specific mineral volume based on the thickness of each histological site of measurement, but on the mean whole ground section thickness. As sound enamel naturally has variations in density, this will result in a heterogeneous thickness along specimens, which may have introduced some distortion. However, this distortion will be identical across groups with some chance, limiting the risk of bias. Fourth, and as a limitation, the employed *in vitro* protocol may have biased our findings to some degree. Notably, grinding and polishing the enamel surface has removed the aprismatic enamel surface layer, which is hypothesized to be required for SAP and SAPR action: On prismatic enamel, columnar calcium of the hydroxyapatite crystal is not available any longer; it is assumed that peptide matrix development may be impeded to some degree in prismatic compared with aprismatic enamel. Nevertheless, the described setup was used as only then valid mineral loss measurements in TMR are possible. Moreover, and notable, the aprismatic outermost layer of enamel is usually gradually worn off in a clinical setting, too, at least occlusally, and any clinical efficacy of self-assembling peptides might be reduced if strongly relying on this aprismatic enamel being available. In addition, previous studies had, by large, employed polished specimens, which is why it was aimed at retaining this concept for reasons of comparability.<sup>[31-33]</sup> Fifth, SAP was applied only once, that is, before the 14-day pH cycling period for reasons for standardization (other materials, e.g., FV, were also only applied once). The manufacturer recommends applying it once or twice per week. Sixth, the fluoride products tested have a relatively high F concentration (> 100 m of F), and thus the formation of CaF<sub>2</sub> deposits is their main mechanism of action. Notably, the calcium source for such formation is saliva, whereas in our study neither human nor artificial saliva was employed. Also, for SAP calcium and phosphate from saliva support, the formation of *de novo* hydroxyapatite crystals occurred within the three-dimensional peptide matrix.<sup>[14-16]</sup>

Application frequency and the availability of saliva might explain the differences between our findings and those yielded *in situ* and *in vivo*.<sup>[6]</sup> Notably, the lack of saliva applies to all groups, and the superiority of FV, for instance, may not necessarily be explained with it. Furthermore, an etching step was employed when testing lesion arrest using SAP to mimic its clinical application. Etching of our polished samples might

have removed the remaining pseudo-intact surface layer of the lesion claimed to be needed for SAP to effectively remineralize the enamel. The etching step was suggested *in vivo* to clean the pseudo-intact surface of enamel from pellicle and remove mineral debris; both are not present in artificially induced enamel lesions.

A range of findings needs to be discussed. The SAP were not effective in preventing caries or inhibiting lesion progression. A limited body of evidence on SAP is available, as discussed, and our findings align with some, but not all of the reported studies. This might be partially due to the mentioned methodological reasons (application time and frequency, absence of aprismatic enamel, lack of saliva, and pellicle formation). However, especially for SAP, there remain a number of questions toward its hypothesized preventive mechanism: SAP are designed to assemble in the acidic environment of an active caries lesion and then attract minerals present in human saliva. It is unclear how this mechanism should apply to prevent lesions. In our study, it cannot be excluded that the material was washed away during the first demineralization cycles, or that only very thin peptide layers formed on the sound enamel surfaces, possibly insufficient to protect the enamel from subsequent demineralization.

Topical fluoride is considered the gold standard for caries prevention and lesion arrest.<sup>[8]</sup> This was confirmed by our study. There is evidence suggesting that SAP should be combined with fluoride to harness their complementary mechanism and location of action when it comes to inhibiting lesion progression (fluoride mainly acts on the pseudo-intact surface of initial lesions,<sup>[8]</sup> whereas SAP is suggested to diffuse into the subsurface body of the lesion<sup>[29]</sup>). In our study, a possible advantage of the used FV (5% NaF) was its consistency and stickiness. It is possible that FV not only had a chemical but also mechanical effect by “sealing” the surface, thereby protecting it from demineralization. Such a sealing effect has been described for FV in studies on root caries prevention.<sup>[34]</sup> Given that we also found FMW (containing 500 ppm NaF) to be efficacious, this explanation may not fully apply, though (notably, however, FMW was provided daily in contrast to most other alternatives).

RI is well known for its lesion progression inhibition in non-cavitated lesions by infiltrating carious enamel porosities and thereby occluding the diffusion pathways, leading to caries arrest.<sup>[21,35,36]</sup>

RI has been demonstrated to be superior to fluoride-based alternatives for lesion arrest by a range of clinical

studies,<sup>[21]</sup> also in the primary dentition,<sup>[37]</sup> and our data also point in this direction. Notably, the application of RI is technique-sensitive, something that may be relevant, especially in the primary dentition and in children. As we removed the excess infiltrant material, a pure sealing effect of RI (instead of truly infiltrating the lesion) can be excluded.

CPP-ACP and CPP-ACPF did not show any caries-preventive or lesion progression inhibiting effect in our study, which may be attributed to the fact that their effect is believed to be enhanced by the presence of a biofilm, which acts as a reservoir for the delivered calcium and phosphate ions and hence prevents mineral loss in intermittent periods of demineralization. Moreover, and as discussed for SAP, the application frequency of CPP-ACP and CPP-ACPF might have been insufficient.

Based on our results, a range of future directions can be derived. First, future *in vitro* studies assessing SAP should aim at mimicking the aprismatic enamel layer even when using ground specimens. Second, the application of saliva prior to remineralization may be recommended in an *in vitro* setting to allow pellicle formation and simulate clinical conditions as far as possible. Alternative, *in situ* designs may be employed. Third, mineral loss measurement should not focus on artifact-prone methods (such as laser-fluorescence) or unsuitable proxies (such as SEM evaluation), but they should strive to truly determine the mineralization effects, for example using TMR, transverse wavelength-independent microradiography, or micro-CT. Last, clinical studies should be employed before translating our findings into any clinical recommendations; so far, clinical data have largely focused on lesion remineralization, as described.

In conclusion, and within the described limitations, FV (5% NaF) and FMW (500 ppm NaF) showed consistent and significant caries-preventive effects on human primary teeth enamel *in vitro*, whereas RI and FV were shown to be effective to inhibit caries lesion progression in this setup. SAP, CPP-ACP, CPP-ACPF, and nHA did not show any significant caries-preventive or progression inhibition effects.

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**CONFLICTS OF INTEREST**

The authors declare no conflict of interest, and the study has received no funding.

**AUTHORS CONTRIBUTIONS**

Development of protocol: FS, NW, NK, GA, laboratory investigations: NW, KE, analysis and interpretation of data: FS, KE, MK, manuscript: NW, KE, FS, NK, final approval of manuscript: FS, NW, NK, GA, KE, MK.

**ETHICAL POLICY AND INSTITUTIONAL REVIEW BOARD STATEMENT**

This study followed the CRIS (Checklist for Reporting *In-vitro* Studies) guidelines<sup>[19]</sup> and was based on the fundamentals of ethical research practice. Informed consent was obtained from all patients' legal guardians to include children's teeth in the experiments.

**PATIENT DECLARATION OF CONSENT**

Not applicable.

**DATA AVAILABILITY STATEMENT**

Data are available on request from the corresponding author, Nour Wahba, e-mail: [nourwahba@gmail.com](mailto:nourwahba@gmail.com).

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