

# Effects of fluoride and calcium phosphate-based varnishes on pH, lactic acid, and trace elements in saliva: a randomized clinical trial.

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## Research article

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## Abstract

**Background:** When dental plaque is not regularly removed, bacteria break down sugars in the diet forming acids as by-products. Lactic acid is the main acid involved in caries. As acids accumulate minerals are lost from the surface layer of the tooth. The imbalance in demineralization/remineralization favors the loss of calcium and phosphate from the teeth. Saliva contains the most important microelements for the remineralization and maturation of dental tissue and plays a crucial role in maintaining the oral environment. Fluoride is the agent par excellence in preventing and detaining cavities. However, remineralization may be hampered by limited levels of calcium and phosphate, and new products have been developed to ensure a constant supply. The aim of this study was to investigate the effect of the application of two varnishes – MI Varnish (5% sodium fluoride with CPP-ACP) and Clinpro White Varnish (5% sodium fluoride with *f*TCP) – applied every three months in children with high caries risk for 12 months on salivary pH, lactic acid concentrations and trace elements.

**Methods:** We conducted a controlled, randomized clinical trial where MI Varnish and Clinpro White Varnish were applied quarterly in children with a high risk of caries, for 12 months. We included 58 children aged 4-12 years, assigned to control (placebo), Clinpro and MI groups. Baseline and three-monthly saliva samples were taken. We assessed changes in pH, lactic acid concentrations and trace elements in saliva.

**Results:** At 12 months, all groups showed a nonsignificant increase in pH levels and a reduction in lactic acid, which was greatest in the placebo group. There was a significant reduction in  $^{24}\text{Mg}$  ( $p < 0.001$ ),  $^{31}\text{P}$  ( $p = 0.033$ ) and  $^{66}\text{Zn}$  ( $p = 0.005$ ) levels in the placebo group ( $p \leq 0.05$ ), but not in the other elements studied:  $^{23}\text{Na}$ ,  $^{27}\text{Al}$ ,  $^{39}\text{K}$ ,  $^{44}\text{Ca}$ ,  $^{52}\text{Cr}$ ,  $^{55}\text{Mn}$ ,  $^{57}\text{Fe}$ ,  $^{59}\text{Co}$ ,  $^{63}\text{Cu}$ ,  $^{75}\text{As}$ ,  $^{111}\text{Cd}$ ,  $^{137}\text{Ba}$ ,  $^{208}\text{Pb}$  and  $^{19}\text{F}$ .

**Conclusions:** Neither pH, lactic acid concentrations or most salivary trace elements were useful in defining patients at high risk of caries or in monitoring the effect of MI Varnish and Clinpro White Varnish after three-monthly application for 12 months.

**Trial registration:** ISRCTN registry, ISRCTN13681286. Registered 26 May 2020.

## Background

According to the 2015 Global Burden of Disease, untreated caries in permanent teeth were the most prevalent condition worldwide, affecting 2.4 billion people. Furthermore, untreated caries in deciduous teeth were the tenth most prevalent condition, affecting 621 million children worldwide [1]. Tooth decay is a multifactorial, sugar-dependent disease, mediated by cariogenic biofilm [2]. When dental plaque is not regularly removed, bacteria metabolize dietary saccharides [3] leading to an imbalance in demineralization/remineralization dynamics that favors the loss of calcium ions and phosphate from the teeth [4].

Lactic acid, the predominant end product of sugar metabolism, is the main acid involved in caries formation. As acids accumulate in the fluid phase of the biofilm, the pH falls, and the surface layer of the tooth is partially demineralized. Once sugars are removed from the mouth by salivary dilution and swallowing, biofilm acids can be neutralized by the buffering action of saliva [2]. Saliva, the main vehicle in enamel remineralization, contains the most important microelements for the remineralization and maturation of dental tissue (calcium, sodium, magnesium, zinc, fluoride) [5], proteins and glycoproteins, and plays a crucial role in maintaining the oral environment, preserving a neutral pH that enables remineralization [2].

Fluoride is the agent par excellence in preventing and detaining cavities [6]. Fluoride varnishes were developed to prolong the contact time between fluoride and tooth enamel [7]. Systematic reviews, meta-analyses and clinical guidelines worldwide recommend fluoride varnishes as caries inhibitors and repairers of initial caries lesions in temporary and permanent teeth [8,9,10]. As remineralization may be hampered by the limited bioavailability of calcium and phosphate, new products have been developed to ensure their constant supply [11]. Two of the most used products are amorphous calcium phosphate stabilized with casein phosphopeptide (CPP-ACP) and tricalcium phosphate modified by fumaric acid (*f*TCP) [12,13].

CPP-ACP forms aggregates that prevent calcium phosphate precipitation, demineralization and promotes remineralization [14]. CPP-ACP has been shown to have anti-anticariogenic activity in *in vitro* experiments, in animals and *in situ* in humans [15,16,17]. MI Varnish (GC Tooth Mousse; GC Corporation, Tokyo, Japan) also contains sodium fluoride 5%. Functionalized TCP acts as a low-dose calcium and phosphate delivery system [18] and has been shown to remineralize *in vitro* [16]. Clinpro White Varnish (3M ESPE, Saint Paul, MN, USA) is an *f*TCP varnish that also contains 5% sodium fluoride.

Saliva has been widely studied as a possible indicator of susceptibility to caries, including searching for salivary physiology parameters, antioxidant levels, proteins, microelements and trace elements that may indicate the risk of caries [5]. The application of varnishes *in vivo*

modifies the composition of the biofilm and saliva in the short term – 30 minutes [19] or 50 hours [20]. However, as far as we know, the effect of varnish application on the composition of saliva in the long term (one year) has not been studied. Neither have controlled, randomized clinical trials monitored the influence of 5% sodium fluoride with calcium phosphate varnishes on pH, lactic acid concentration and salivary trace elements [15,21,22].

The objective of this study was to carry out a controlled, randomized clinical trial to study the effects of the coating with MI Varnish and Clinpro White Varnish, applied quarterly to children at high risk of cavities, on the evolution of pH, lactic acid concentrations and salivary trace elements for 12 months. The null hypothesis was that the application of the two varnishes applied quarterly for 12 months does not change either pH, lactic acid concentrations or levels of trace metals in the saliva of children at high risk of caries.

## Materials And Methods

### Trial design

We conducted a controlled randomized trial with a parallel group design between June 2017 and December 2018. The study was double blinded for patients and the statistical analysis. Dental professionals could not be blinded as the commercial presentation and characteristics of the two varnishes were distinguishable. The study was approved by the Ethics Research Committee and the Research Biosecurity Committee of the University of Murcia, Spain (CIS: 1499/2017; CBE 50/2017). The trial was retrospectively registered on May 26, 2020: ISRCTN13681286, <http://www.isrctn.com/ISRCTN13681286>.

### Participants

The study was carried out at the University of Murcia Dental Clinic, Hospital General Universitario Morales Meseguer, Murcia. Inclusion criteria were children aged 4-12 years attending the Integrated Child Dentistry Clinic of the University of Murcia for checkups or dental treatment who presented a high or extreme risk of caries according to the CAMBRA protocol. A high or extreme risk of caries was defined as a patient who presented  $\geq 3$  or more cavitated or noncavitated (incipient) carious lesions or restorations (visually or radiographically evident) or teeth missing due to caries in last 36 months [23].

Exclusion criteria were: 1) children who had received fluoride varnish or other permanent surface treatment containing fluoride in the previous 6 months, e.g. children who had pits and fissures sealant; 2) children fitted with orthodontic apparatus 3) children living in an area with fluoridated drinking water; 4) children with moderate or severe fluorosis or other morphological or anatomical abnormalities of dental development 5) children with systemic diseases causing physical limitations; 6) children with allergy or proven/suspected sensitivity to milk proteins.

Before the study, an informative leaflet was provided to all parents/guardians, who were shown the expected benefits and risks of the study, after which written informed consent was obtained. A questionnaire was administered in parents containing questions on demographic factors, dietary habits, history of disease, fluoride therapy, and use of medication and vitamin supplements (Supplementary file 1).

The sample size (n=13 patients/group) was calculated using the evolution data of lactic acid and the existing index of loss of children treated by our clinic. An alpha risk of 0.05 and a beta risk of 0.20 (power 0.8) in a bilateral contrast was accepted to detect a minimum difference of 2.0 between two groups, assuming that there were 3 groups, and a standard deviation of 3.0. A loss to follow up of 45% was estimated. We randomly allocated participants to intervention groups using a computer-generated randomization list. AJOR generated and concealed the allocation sequence to intervention groups until assignment. APP enrolled and assigned participants to intervention groups.

## Clinical evaluation and interventions (Interventions, compliance measure, and clinical visual evaluation time points)

### Patient selection

The history and examination were carried out by an experienced pediatric dentist, who underwent three training sessions on written and visual instructions, standardization, and calibration with participants *in vivo*. Caries lesions were recorded using a mirror and a WHO probe according to International Caries Detection and Assessment System criteria (ICDAS II) [24]. The ICDAS II system has two-digit coding for

the detection criteria of primary coronal caries. The first refers to tooth restoration and is coded from 0 to 9. The second digit is used for coding caries, from 0 to 6.

Verbal and written oral hygiene instructions and dietary advice were given to participants and their responsible adults to facilitate and strengthen preventive measures (Supplementary file 2). Each participant received a fluoridated toothpaste with 1450 ppm of fluoride (Lacer Junior, Lacer SA, Barcelona, Spain) and a manual toothbrush (Lacer Junior, Lacer SA, Barcelona, Spain) which was changed every 3 months. Instructions were given to avoid other sources of fluoride during the study period (environmental products, supplements, professional or other dental products). At each check-up we verified that study hygiene guidelines and the use of the study toothpaste were being complied with. There were five check-ups: baseline and 3, 6, 9 and 12 months. Fluoride and the prevalence of caries were recorded at baseline and 12 months.

## Saliva samples

At the beginning of each check-up, 3.5 ml of unstimulated saliva was collected for 5 minutes in a sterile polyethylene tube. Children had not ingested water or food for 1 hour before the examination. Saliva samples were stored at  $-20^{\circ}\text{C}$  until measurement. The schedule at which appointments were made was restricted to 15:00 to 18:00 hours for maximum avoidance of circadian fluctuations in the variables studied [25]. Saliva collection was always performed in the waiting room to avoid the effect of anxiety on the amount and composition of saliva.

## Experimental groups, application of varnishes.

Patients were assigned randomly to the control group (placebo), the Clinpro White Varnish<sup>®</sup> group, and the MI Varnish<sup>®</sup> group. The composition of the study materials is shown in table 1.

Table 1. Product composition according to material safety data sheets (MSDS)

Product	Manufacturer	Composition
MI Varnish	GC, Leuven, Belgium	30-50% polyvinyl acetate, 10-30% hydrogenated MSDS rosin, 20-30% ethanol, 5% sodium fluoride, 1-5% CPP-ACP, 1-5% silicon dioxide
Clinpro White Varnish	3 M ESPE, Saint Paul, MN, US	30-75% pentaerythritol glycerol ester of colophony resin, 10-15% n-hexane, 1-15% ethyl alcohol, 5% sodium fluoride, 1-5% flavour enhancer, 1-5% thickener, 1-5% food grade flavour, <5% fTCP.

CPP-ACP: protein casein-phosphopeptide with amorphous calcium phosphate; fTCP: functionalized tricalcium phosphate.

After obtaining saliva samples and before application of the varnishes, the teeth were dried with dry compressed air and isolated with cotton rolls and saliva ejector. Then, 0.25 ml of varnish was applied to the surface of the teeth and allowed to dry for 30 seconds. In the placebo group, distilled water was applied with a brush identical to that used to apply the varnishes. Patients were instructed not to rinse their mouths, not to eat or drink for an hour and not to brush until 4-6 hours after application, in accordance with the manufacturers' instructions. The procedure was repeated every three months for one year (Figure 1 [see Supplementary file 3]). During the 12-month study period, patients received conventional dental treatment (extractions, seals, pulp treatments, etc.). To avoid uncontrolled sources of fluoride, the materials used were always fluoride-free.

## Outcome measures

### Caries index

The dmfs/DMFS indexes were calculated from the ICDAS II scores (the second digit ranged from 3 to 6) [24], transforming them into the decayed, missing and filling values of the ICDAS.

### pH and Lactic acid

Saliva samples were thawed and shaken for 10 seconds at  $20^{\circ}\text{C}$  (ClassicVortex Mixer, Velp Scientifica, Italy) and 15  $\mu\text{L}$  of the saliva sample was poured onto a pH test strip (range 4.0–9.0; Code. 1.16996.0001; Reflectoquant<sup>™</sup> Merck, Darmstadt, Germany) which was introduced in a RQflex<sup>®</sup>10 reflectometer (Merck Millipore, Darmstadt, Germany) to provide the pH value. 30  $\mu\text{L}$  of the saliva sample was

poured onto a lactic acid test strip (range 1.0-60.0 mg/L; Code. 1.16127.0001; Reflectoquant™ Merck, Darmstadt, Germany) which was introduced in a RQflex® 10 reflectometer (Merck Millipore, Darmstadt, Germany) to provide the lactic acid value.

## Fluoride

Fluoride concentrations were measured using an ion-specific fluoride electrode (Orion 9609 BNWP, Thermo Fisher Scientific Inc. Waltham, USA) coupled to an ion analyzer (Orion EA-940 Thermo Fisher Scientific Inc. Waltham, USA). Before each reading, samples were shaken with a vibrator (Classic Vortex Mixer, Velp Scientifica, Italy) to homogenize the sample. The electrode was calibrated beforehand with standard solutions from 0.125 to 2.0 ppm F, mixing 1mL of each standard solution with 1mL of TISAB II (1.0 M pH acetate buffer 5.0; 1.0 M NaCl and 4% CDTA). Once calibrated, the samples were read, for which we mixed 1mL of each saliva sample with 1mL of TISAB II (Hanna Instruments, Woonsocket, Rhode Island, USA). The results in mV were converted into fluoride concentrations (ppm) using the standard calibration curves measured immediately before the analysis.

## Trace elements

We analyzed 2 ml of the homogenized sample using mass spectrometry with inductively coupled argon plasma (ICP-MS Agilent 7900; Agilent Technologies Inc.; CA; USA). Ultrapure water (18.2 M  $\Omega$ ) from a water purification system (Milli-Q® Reference A+, Merck Millipore) was used to prepare the standard reagents and solutions: 100  $\mu$ L of the saliva sample was diluted up to 1 mL with a 2% HNO<sub>3</sub> Suprapur solution in ultrapure water. The samples were introduced in a self-sampling spectrometer by the impulse of a peristaltic pump, and were atomized, ionized and the ions generated detected and quantified subsequently by mass spectrometry. The isotopes selected for each element studied were: <sup>23</sup>Na, <sup>24</sup>Mg, <sup>27</sup>Al, <sup>31</sup>P, <sup>39</sup>K, <sup>44</sup>Ca, <sup>52</sup>Cr, <sup>55</sup>Mn, <sup>57</sup>Fe, <sup>59</sup>Co, <sup>63</sup>Cu, <sup>66</sup>Zn, <sup>75</sup>As, <sup>111</sup>Cd, <sup>137</sup>Ba and <sup>208</sup>Pb.

## Statistical analysis

Values are expressed as mean  $\pm$  standard deviation. The Kolmogorov-Smirnov test was used to determine sample normality and the Levene test for equality of variance. Pearson's chi-square test was used to determine between-group differences in sex and a one-way ANOVA test for differences in age. To determine within-group differences in age distribution according to sex we used the Mann-Whitney test. To detect between-group differences in DMFS and dmfs values a Kruskal-Wallis test was performed and one-way analysis of variance was used to detect differences in the Turesky et al Modified Quigley Hein Plaque Index.

Differences in concentrations of trace elements, pH and lactic acid between baseline and 3, 6, 9 and 12 months were determined by simple variance analysis of repeated measures. When there were differences between the times, two-by-two comparisons were made using the Holm-Sidak test.

A paired t-test was used to analyze the within-group evolution of fluoride concentrations between baseline and 12 months when there was normality and a Wilcoxon test when there was no normality. One-way ANOVA was used to detect between-group differences in the same period. A value of  $p < 0.05$  was considered significant. The analysis was made using the SigmaStat 3.5 statistical software package (Systat Software Inc., Point Richmond, CA, USA).

## Results

Of the 80 patients initially reviewed, only 58 met the inclusion criteria (Figure 2 [see Supplementary file 4]). After randomization, 18 were assigned to the control group, 19 to the Clinpro group and 21 to the MI group. Of these, 25 children were lost throughout the study. Of the 33 patients who finally completed the study, 12 were in the placebo group, 10 in the Clinpro group and 11 in the MI group.

There were 16 females and 17 males with a mean age of  $7.09 \pm 2.55$  [4–12 years]. 12 were aged  $< 6$  years and 21  $> 6$  years. The baseline characteristics of sex, age, age distribution according to sex, the Turesky et al Modified Quigley Hein Plaque Index and the dmfs/DMFS of the three groups were similar (Table 2). The DMFS was significantly higher in the Clinpro group ( $p = 0.039$ ).

Characteristic	Control group	Clinpro group	MI group	P value
<b>Demographic</b>				
Age – years	6.91 ± 2.59	7.6 ± 2.36	6.81 ± 2.63	P = 0.766 One-way analysis of variance
Sex – Age	8 (6.12±2.36)	4 (6.25±1.26)	4 (6.00±2.16)	(P = 0.283)
Female	4 (8.50±3.51)	6 (8.50±2.59)	7 (7.29±2.93)	(χ <sup>2</sup> test)
Male	P=0.214 Mann-Whitney test	P= 0.171 Mann-Whitney test	P=0.527 Mann-Whitney test	
<b>Clinical</b>				
Turesky et al Modified Quigley Hein Plaque Index	1.99 ± 0.54	2.76 ± 0.89	2.23 ± 0.74	P=0.057 One- way analysis of variance
dmfs (baseline)	18.33 ± 10.07	32.34 ± 19.93	23.66 ± 18.51	P = 0.353 (K-W)
DMFS (baseline)	0.57 ± 1.32	3.70 ± 3.38	1.36 ± 2.25	P = 0.039 (K-W)

Table 2. Baseline characteristics (mean±SD)

Sex – Age: Age distribution according to sex. The DMFS index counts the total number of decayed (D), missing (M) and filled (F) tooth surfaces of permanent dentition. The dmfs index counts the total number of decayed (d), missing (m) and filled (f) tooth surfaces of primary dentition.

## pH

Baseline pH was similar in all groups (placebo 7.69±0.27; Clinpro 7.68±0.29; MI 7.62±0.32. At 12 months, all groups showed slight non-significant increases in pH (Table 3 [see Supplementary file 5]).

## Lactic acid

All groups showed a similar concentration of lactic acid in time 0. Lactic acid concentrations decreased throughout the study and the decrease was non-significantly higher in the control group than in the two varnish groups. (Table 3 [Supplementary file 5]).

## Trace elements

There was a significant reduction in <sup>24</sup>Mg concentrations in the placebo group between baseline, 3 and 6 months versus 9 months, and 3 months versus 12 months (p= <0.001); <sup>31</sup>P in the placebo group between baseline versus 3, 6, 9 and 12 months (p = 0.033); and <sup>66</sup>Zn between baseline versus 9 and 12 months in the placebo group (p= 0.005) (Table 4 [see Supplementary file 6]). Concentrations of the other trace elements studied appear in table 3 [see Supplementary file 5].

## Discussion

The null hypothesis was partially proven, since the application of the varnishes did not change either the pH, the concentration of lactic acid or most trace metals studied.

The age of the children included ranged from 4 to 12 years, a population in which caries prevention is common [23]. The varnishes used contained 5% sodium fluoride, equivalent to 22,600 ppm or 1.19 M of fluoride, and calcium phosphate in two chemical forms: CPP-ACP and fTCP. *In vitro* studies have demonstrated their preventive and remineralizing capacity as they generate supersaturated calcium and phosphate solutions in biofilm and saliva [16, 26, 27].

In vulnerable populations, such as infants and children, saliva is the perfect diagnostic medium due to its non-invasive collection, and easy handling and storage [28]. A wide range of saliva biomolecules are related to the physiological state and provide useful data on oral and systemic diseases. We selected pH, lactic acid and some trace elements in total unstimulated saliva as biomarkers of the predisposition to caries and the ability of varnishes to modify the risk [2, 10, 27]. Reports [29] have found differences in saliva composition depending on whether there is caries or not, but found no difference between stimulated and unstimulated saliva in children, or according to sex or the dental status.

The baseline pH value was between 7.6 and 7.7, although the patients were at high or extreme risk of cavities, and the value did not change significantly throughout the study in any group. Although some reports [30, 31] have recorded lower pH values and observed significant differences in pH in unstimulated saliva in children without versus children with early childhood caries (7.20 vs. 6.07) [32], other studies recorded a wider range than ours in salivary pH values in children with active caries (6.20 - 7.90) and found no correlation between pH and caries activity [32,33]. Therefore, the static measurement of salivary pH is of little use in assessing the risk of caries [34] because, within 20-40 minutes, saliva neutralizes pH variations caused by sugary foods and the activity of microorganisms [2, 35], while it is the acidification of the pH of the biofilm in the area of the lesion that determines the generation of tooth decay and correlated with a high risk of caries [2, 16, 35, 36].

There are few studies of lactic acid concentrations in children's total saliva. Fidalgo et al. 2013 [37] and Pereira et al. 2019 [29] detected a higher concentration of lactate and other organic acids (acetate and n-butyrate) in the saliva of children with caries. We found higher baseline lactic acid values than did other studies [29], probably because our patients were at are high and extreme risk of caries, lactic acid concentrations were not significantly reduced in any group throughout the study.

The role of trace elements present in saliva on caries remains unclear [38]. Of the trace elements measured in our study, Ca, P, F, Mg, Zn and Cu are, in some way, related to tooth mineralization. However, their saliva concentrations do not always reflect the degree of tooth demineralization/remineralization, caries or its risk [31, 39]. It might be thought that concentrations of Ca and P, the main components of tooth hydroxyapatite, would be increased in saliva during tooth demineralization of the tooth, although some reports have found an inverse relationship between caries and salivary calcium [33, 40, 41] and phosphate [40] levels, while other studies found no relationship [41].

The application of calcium phosphate varnishes should reflect an increase in the concentrations of these two ions in children's saliva, as has been observed *in vitro*. Cochrane et al. 2014 [42] and Shen P et al. 2011 [21], determined *in vitro* release of calcium, phosphate and fluoride ions in five varnishes (MI Varnish Clinpro White Varnish, Esmal Pro, Bifluorid 5, and Duraphat) and found a greater cumulative release of the ions by MI Varnish. The calcium concentrations observed in our study did not increase significantly after varnish application because saliva was collected 3 months after each application and there is no cumulative *in vivo* effect of *in vitro* studies, since the varnish only remains *in situ* for up to 24 hours as it is eliminated by chewing, salivary flow, rubbing by the cheeks and tongue and oral hygiene [20]. Phosphorus was significantly reduced at 12 months in the control group but not in the varnish groups.

Baseline fluoride levels in all groups were around 0.05 ppm and there was a non-significant increase, which was higher in the MI group than in the other groups, reaching  $0.0920 \pm 0.0402$  ppm. Initial concentrations were similar to those described by Sekhri et al. 2018 [38] in caries-free groups and higher than those described by Dehailan et al. 2017 [19] and Rechman et al. 2018 [43]. As one of the exclusion criteria was consumption of fluoridated running water, the higher levels of baseline salivary fluoride of study children might be due to consumption of external sources of fluoride, such as bottled waters. In fact, a study that analyzed the fluoride content of 20 bottled water brands marketed in the area of origin of the study children found fluoride concentrations between 0.05 ppm and 0.95 ppm [44]. Increases in salivary fluoride seem to predict increases in fluoride content of dental plaque fluid, implying that both would be good indicators of intraoral levels of fluoride [45].

There is no consensus on the significance of Cu, Zn and Mg levels in relation to caries. Brookes et al. 2003 [46] suggested  $\text{Cu}^{2+}$  might have a direct protective effect on enamel dissolution, although other studies [34, 39,47,48,49] suggest a high Cu level is observed in patients with caries and comes from destroyed hydroxyapatite crystals. We found high salivary Cu levels in our sample with a baseline dmfs between  $18.33 \pm 10.07$  and  $32.34 \pm 19.93$ , and the levels did not fall in any group throughout the study. We also found high zinc levels. Elevated Cu and Zn values could reflect the action of saliva's antioxidant systems, as both ions act as co-enzymes of superoxide dismutase [49]. Sejdini et al. 2017 [50] suggested Mg promotes caries resistance, so children with low Mg concentrations would have a high caries index. This might explain the low levels of Mg recorded in our children, which are similar to those recorded by Rajesh et al. 2015 [51]. While the application of varnishes kept Mg levels stable during the follow-up, there was a fall in levels in the control group.

Saliva is the main remineralizing agent and generally protects the teeth. Knowledge of its composition may help detect deficiencies in patients at high risk of caries and thus provide individualized treatments that reverse the risk [47]. The parameters studied in our work did not serve to define a risk situation or monitor the treatment with fluoride varnishes and calcium phosphate. Continuous salivary flow and the influence of the diet, hormonal status, hydration status and anxiety levels have on salivary composition, and the short half-life of varnishes in the oral environment may have been influencing factors. It is estimated that the half-life of CPP in plaque is 124.8 minutes and that casein is hydrolyzed by salivary bacteria in a similar time [11]. Therefore, varnishes must release their ions in a relatively short time and the initial high ion load is diluted over time.

One limitation of the study could be the measurement of variables in saliva. Although there is no difference in the composition of some elements between plaque and saliva (sodium, ammonium, potassium, magnesium and chlorine), the metabolic activity of bacterial plaque can vary the inorganic composition between plaque and saliva [50]. It may be useful to measure the composition of bacterial plaque and not the composition of saliva to determine the risk of caries and their monitoring. Additionally, possible loss to-follow up may be another limitation. Controlled, randomized clinical trials often incur problems due to patients lost throughout the study. Likewise, children at high or extreme risk of caries often miss appointments due to socioeconomic or other factors.

In conclusion, the quarterly application of two calcium phosphate varnishes, MI Varnish and Clinpro White Varnish, for 12 months, did not change the pH, lactic acid concentrations or most trace metals in the saliva of children at high and extreme risk of caries, and therefore these parameters were not useful in monitoring the follow up of varnish application.

## Declarations

### Ethics approval and consent to participate

- Ethics approval

The statement detailing Ethics approval is shown in lines 117-119.

Approval was obtained on 19/05/2017 by Ethics Research Committee and the Research Biosecurity Committee of the University of Murcia (University of Murcia: Merced Campus, Calle San Cristo 1, 30001, Murcia, Spain; +34 (0)868 88 3614; [comision.etica.investigacion@um.es](mailto:comision.etica.investigacion@um.es)), ref: CIS: 1499/2017; CBE 50/2017.

- Consent to participate

Written informed consent to participate in the study was obtained from participant's parents or legal guardians and the statement to this effect appears in lines 138-139 of the manuscript.

- Consent for publication

The written informed consent contains a paragraph detailing the consent form used by our institution. According to the consent form, the details of the study are to be done in

accordance with the Law on the protection of personal data. Also, the data obtained will be communicated to the scientific community in the framework of public conferences and via publications freely available to the general public on the Internet.

## Availability of data and materials

All data generated or analyzed during this study are available within the article and its supplementary materials or from the corresponding author, APP, upon reasonable request.

## Competing interests

The authors declare that they have no competing interests.

## Funding

This study was financed solely by the University of Murcia.

## Authors' contributions

A.P.P. established and validated quantitative pH and Lactic acid measurement methods and performed data acquisition, statistical analysis and data interpretation. C.S.M., A.P.S. and A.J.O.R. contributed to quantitative data acquisition, analysis and interpretation. A.P.P. and A.J.O.R. wrote the initial manuscript. Y.M.B., C.S.M, and A.P.S. contributed to data interpretation and the final version of the manuscript. A.P.P. conceptualized and realized the human study. A.J.O.R. and Y.M.B. conceptualized the project and supervised the works. All authors reviewed the manuscript.

## Acknowledgements

Not applicable.

## Additional Files

- **File name: Supplementary file 1**

Title of data: Clinical History

Description of data: A questionnaire was administered in parents containing questions on demographic factors, dietary habits, history of disease, fluoride therapy, and use of medication and vitamin supplements.

- **File name: Supplementary file 2**

Title of data: Instructions given to participants.

Description of data: oral hygiene instructions and dietary advice were given to participants and their responsible adults to facilitate and strengthen preventive measures.

- **File name: Supplementary file 3**

Title of data: Figure 1.

Description of data: Clinical Trial Design.

- **File name: Supplementary file 4**

Title of data: Figure 2.

Description of data: Flow diagram of the progress through the phases of the study. CONTROL(Placebo), CLINPRO (Clinpro White Varnish) and MI (MI Varnish).

- **File name: Supplementary file 5**

Title of data: Table 3.

Description of data: Trace elements with no significant changes.

- **File name: Supplementary file 6**

Title of data: Table 4.

Description of data: Trace elements with significant changes

- Supplementary file 7: CONSORT checklist

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