

Dye-enhanced laser fluorescence detection on natural caries lesions in primary teeth

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ABSTRACT | *Objective:* This study aimed to investigate the association of two fluorescent dyes to Laser Fluorescence (LF) device in detecting smooth and occlusal natural caries in primary teeth *in vitro*. *Methods:* Measurements were performed with LF only and LF associated to tetrakis (N-methylpyridyl) porphyrin (LF-TMPyP) and protoporphyrin IX (LF-PPIX) in 72 smooth surfaces (from 63 primary molars) and 134 occlusal sites (from 81 primary molars). To validate the suggested technique, surfaces were sectioned and fragments were evaluated under a stereomicroscope. Smooth surfaces were also evaluated by using polarized light microscopy and Knoop microhardness. For both smooth and occlusal surfaces, ROC analyses were performed, and sensitivities, specificities and accuracies were assessed. In smooth surfaces, Pearson's correlation coefficients between LF values and lesions hardness or lesions depth were calculated. *Results:* LF-TMPyP presented higher hardness correlation with lesion depth than other methods in smooth surfaces. Both smooth and occlusal surfaces showed no differences in other parameters among the methods. *Relevance:* The LF-TMPyP might improve performance in quantifying smooth-surface caries lesions in primary teeth. However, sensitivity is improved when caries lesion extends into inner half of the enamel but not to amelodentinal junction when using LF-PPIX in smooth caries lesions. Associating fluorescent dyes does not improve LF performance on occlusal caries.

DESCRIPTORS | Dental Caries; Dyes; Fluorescence; Primary Teeth.

RESUMO | **Fluorescência à laser associada a corantes para detecção de lesões de cárie naturais em dentes decíduos** • *Objetivo:* Este estudo investigou a associação de dois corantes fluorescentes e um dispositivo de fluorescência a laser (FL) na detecção de lesões de cárie naturais nas superfícies lisas e oclusais de dentes decíduos *in vitro*. *Métodos:* Foram realizadas medições com FL e com FL associado à tetrakis(N-metilpiridil)porfirina (FL-TMPyP) e protoporfirina IX (FL-PPIX) em 72 superfícies lisas (de 63 molares decíduos) e 134 superfícies oclusais (de 81 molares decíduos). Para validação, as superfícies foram seccionadas e os cortes obtidos foram avaliados sob estereomicroscópio. As superfícies lisas também foram avaliadas por microscopia de luz polarizada e teste de microdureza Knoop. Para ambas as superfícies a análise ROC foi realizada, e sensibilidade, especificidade e acurácia foram avaliadas. Nas superfícies lisas foram calculados coeficiente de correlação de Pearson entre os valores de FL e dureza ou profundidade das lesões. *Resultados:* FL-TMPyP apresentou maior correlação com dureza e profundidade das lesões do que outros métodos em superfícies lisas. Em ambas as superfícies não houve diferença em outros parâmetros entre os métodos. *Relevância:* A associação FL-TMPyP pode melhorar o desempenho em quantificar as lesões de cárie em superfícies lisas em dentes decíduos. Entretanto, a sensibilidade é melhorada em metade interna do esmalte, mas não na junção amelodentinária quando usado PPIX em lesões de superfícies lisas. A associação de FL com corantes não melhorou o desempenho nas lesões de cárie oclusais.

DESCRITORES | Cárie Dentária; Corantes; Fluorescência; Dentes Decíduos.

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INTRODUCTION

Visual and radiographic methods are currently used for caries detection, however they do not permit quantification of caries lesions.^{1,2} Quantitative methods could provide monitoring of caries lesions in shorter periods,^{3,4} making them conceivable to assess the effectiveness of anticaries agents in abbreviated clinical trials.⁵

Fluorescence has been used to differentiate carious from sound tissue using light/laser with several wavelengths.^{6,7} The laser fluorescence device (LF) chosen in this investigation uses a diode laser that emits a 655-nm red light.⁸ Part of this light is absorbed by chromophores in dental tissues and reemitted at a different wavelength (near-infrared).^{9,10} LF mechanism consists in measuring the fluorescence emitted from existing porphyrins in caries lesions and converting fluorescence values in a numerical scale. Porphyrins that exist in dental caries include protoporphyrin IX (PPIX).¹⁰ This device uses a different principle when compared to other quantitative methods, which are related to mineral loss.¹¹

For initial caries lesions, however, the method has presented inferior performance on occlusal¹²⁻¹⁵ and smooth surfaces^{16,17} since bacterial invasion in these lesions is negligible¹⁸ and the concentration of porphyrins tends to be lower when compared to cavitated lesions. A new approach to improve LF performance in detecting and quantifying early caries lesions was proposed^{19,20} by using the association of LF device with fluorescent dyes. It has presented good results in detecting early demineralization^{19,20} Even if the lesion presents low quantity of porphyrins to be read by the LF device, the dyes can penetrate into lesion porosities and facilitate LF readings.

Despite good results of this caries diagnosis approach,^{19,20} previous studies used artificial caries lesions. As artificial caries lesions are increasingly softened compared to natural ones,²¹⁻²³ they can behave differently when the dyes are used. To the

best of our knowledge, this study pioneered the use of LF associated to fluorescent dyes in natural caries lesions. This initiative is necessary to confirm or reject the good results previously obtained with artificial caries lesions.

Thus, the present study aimed to test the association of LF with two fluorescent dyes in detecting and quantifying natural caries lesions on smooth and occlusal surfaces of primary teeth. Additionally, we intended to check if the dyes could have any residual effect in further LF readings.

MATERIAL AND METHODS

Dyes selection

The Local Ethical Committee approved this study (Protocol 26/04) and teeth were donated by a Bank of Human Teeth. We selected two porphyrins to use as dyes, Protoporphyrin IX (PPIX, Aldrich, Milwaukee, USA) and tetrakis (N-methylpyridyl) porphyrin (TMPyP, Aldrich, Milwaukee, USA). The experiments undertaken to determine the best concentrations and solvents of the dyes were described in earlier studies (1, 2). Both porphyrins were used at 0.2 mM TMPyP dissolved in water, and 4.0 mM PPIX dissolved in water: dimethyl sulfoxide (1:1).

Smooth surfaces – Sample selection

This subsample comprised 72 approximal surfaces of 63 primary molars. The samples were randomly distributed according the type of dye they would receive in order to avoid some selection bias. Thus, 39 surfaces were allocated to the experiment with PPIX and 34 surfaces were used with the TMPyP dye.

The teeth were polished with pumice/water slurry and copiously rinsed with tap water. Then, digital pictures were obtained of each surface. After that, the teeth were stored in saline solution in individual containers at room temperature (ca. 24±1°C).

Smooth surfaces – LF measurements

LF readings were performed by using a DIAGNOdent device (Kavo, Biberach, Germany), following manufacturer's instructions. In this part of the experiment DIAGNOdent was used with B tip (for smooth surface). It was calibrated by using a provided standard made of porcelain prior to the examination and re-calibrated after every ten teeth. We also performed a calibration at a sound surface of each tooth prior to lesion reading. Teeth were taken out from the saline solution, dried with filter paper for 5 s and measured with LF device. The entire surface was evaluated and the highest reading was recorded at each set. One examiner (VML) performed three readings in each site and the mean value was considered for this study.

Part of the teeth sample was used to evaluate LF associated to TMPyP (LF-TMPyP). After initial measurement with LF, samples were immersed in 5 ml of 0.2 mM TMPyP for 60 s, removed and dipped twice into distilled water, dried with filter paper for 5 s and evaluated with LF, the same way as described above. Distilled water was changed for every new specimen. On the other part of the teeth sample, the same procedure was performed using LF readings with 5 ml of 4 mM PPIX (LF-PPIX), maintaining time and methodology described for LF-TMPyP.

At the end of examinations, teeth were washed with water coming from a 3-in-1 syringe, and samples were stored for 30 days. After this period, the same examiner inspected the teeth again following the procedures above mentioned, in order to check the intra-examiner reproducibility and the influence of dyes remainings.

Occlusal surfaces – sample selection

For this part of the study, 81 primary molars were selected. Forty of those molars were used in the experiment with LF-TMPyP and 41 teeth in the experiment with LF-PPIX method. One or two sites

per occlusal surface were selected. Thus, 57 sites were evaluated with LF TMPyP method and other 57 sites with LF PPIX. Teeth were polished as previously described, and digital images of each occlusal surface were registered. Sites were selected and covered by black mask in the digital picture.

Occlusal surfaces were not reexamined since the residual effect of the dye could be resultant of morphology features and not properly of dyes penetration into the caries lesions.

Occlusal surfaces – LF measurements

Measurements performed on occlusal surfaces were similar to those performed on smooth surfaces, except the use of A tip, designed for occlusal surfaces instead of B tip for smooth surfaces, in accordance to manufacturer instructions. Each dye was dipped on the selected site, and after 60 s, visible excess was removed with a drop of water. Subsequently, teeth were dried with compressed air for 3 s just before measurements. Calibration was performed on ceramic standard, and then, on a sound surface of the assessed tooth. One examiner (MMB) performed three readings in each site and the mean value was recorded for each selected site.

Validation

For smooth surfaces, teeth were embedded in resin blocks after the examinations. Histological validation using two hemi-sections was performed as gold standard in assessing the caries lesions depth. Sections were made using a 0.3 mm thick diamond saw mounted in a microtome (Labcut 1010, Extec Co., Enfield, USA). The position in which examiners had registered higher LF value for each surface was used as cutting reference. Therefore, we assumed we analyzed in the following steps, the deepest part of the evaluated caries lesion.

Firstly, two examiners analyzed adjacent sections (halves) in a stereomicroscope (SZPT

Olympus, Tokyo, Japan) by using magnification of 16 to 40× and reflected light in a joint session (VML and FMM). Lesions were classified in a 5-point scale: D0 – no caries; D1 – caries lesion limited to the outer half of the enamel; D2 – caries extending into inner half of the enamel but not to amelodentinal junction; D3 – caries limited to the outer half of the dentine; D4 – caries involving the inner half of the dentine.

After that, while one section (right slice) was examined with a polarized light microscope, the other section (left slice) was assessed by cross-sectional microhardness (CSMH).

For polarized light microscoping, each slice was manually ground and polished with silicon carbide paper (200, 400, 600, 1000 and 1400 grits in sequence) to 100 μm thickness. A light microscope (Axioplan 2, Zeiss, Jena, Germany) coupled to a CCD camera and a computer equipped with image analysis software (Leica Qwin, Leica Microsystems, Heidelberg, Germany) was used to record images, by using transmitted light, a cross polarizer (at 50× magnification) and a quartz plate. Sections were submerged in distilled water at the capturing images. Contrast between sound (negative birefringence) and demineralized enamel (positive birefringence) was detected by the software to determine the maximum lesion depth.

Previous to CSMH analyses, samples were polished with silicon carbide paper (400, 600, 1000 and 1400 grits in sequence) and with 1 and ¼ mm diamond paste on a polishing cloth. The hardness profiles of each lesion were measured across three positions located at the middle of the lesion and at two points located 100 μm to the right and to the left. Indentations were made at 25, 50, 75, 100, 125, 150, 250, and 350 μm from outer enamel surface. Then, we performed 24 indentations in each sample. A Knoop indentations used a 25-gram load for 15 s in a microhardness tester (Shimadzu Micro Hardness Tester HMV-2, Shimadzu Corporation,

Kyoto, Japan) coupled to a computer with a dedicated software (CAMS System, Newage Testing Instruments, Southampton, USA). For each sample, we calculated the integrated area of the curve of the hardness value as function lesion depth (KHN x mm).

For occlusal surfaces, teeth were embedded in resin blocks and serial 250 μm thick sections were obtained by using a 0.3 mm thick diamond saw mounted in a microtome (Labcut 1010, Extec Co., Enfield, USA). Cutting reference was the site marked on the picture previously taken from tooth. Two examiners (FMM and MMB) analyze all sections and both sides of each section in a joint session using the stereomicroscope. Sites were classified in the same 5-point scale described for smooth surfaces.

Statistical analyses

For smooth surfaces, Pearson's correlation coefficients between caries lesions hardness and LF readings with or without dye-enhancing were calculated. Their respective 95% confidence intervals (95% CI) were also found. Pearson's correlation coefficients were also assessed considering caries lesions depth measured by polarized light microscopy.

A receiver operating characteristic (ROC) analysis was conducted to assess the LF performance in detecting smooth-surface caries lesions at three different thresholds obtained by the evaluation in the stereomicroscope: D1 (D0= sound vs. D1 to D4=caries), D2 (D0 to D1=sound and D2 to D4=caries) and D3 (D0 to D2=sound and D2 to D4=caries).

The best cut-off point at each threshold was obtained from ROC analysis. With these cut-off limits, sensitivity, specificity and accuracy were calculated. McNemar change test was applied to check differences between LF and LF associated with

fluorescent dyes. We calculated the intraclass correlation coefficient (ICC) between first and second evaluations for all LF methods.

For occlusal surfaces, ROC analyses were carried out only at two thresholds (D1 and D3, as explained above). Best cut-off points for each method were obtained, and then, sensitivity, specificity, and accuracy were calculated and compared employing McNemar change test. Software was used for all statistical analyses (MedCalc 9.3.0.0, Mariakerke, Belgium), and the level of significance was $p < 0.05$.

RESULTS

Smooth caries lesions

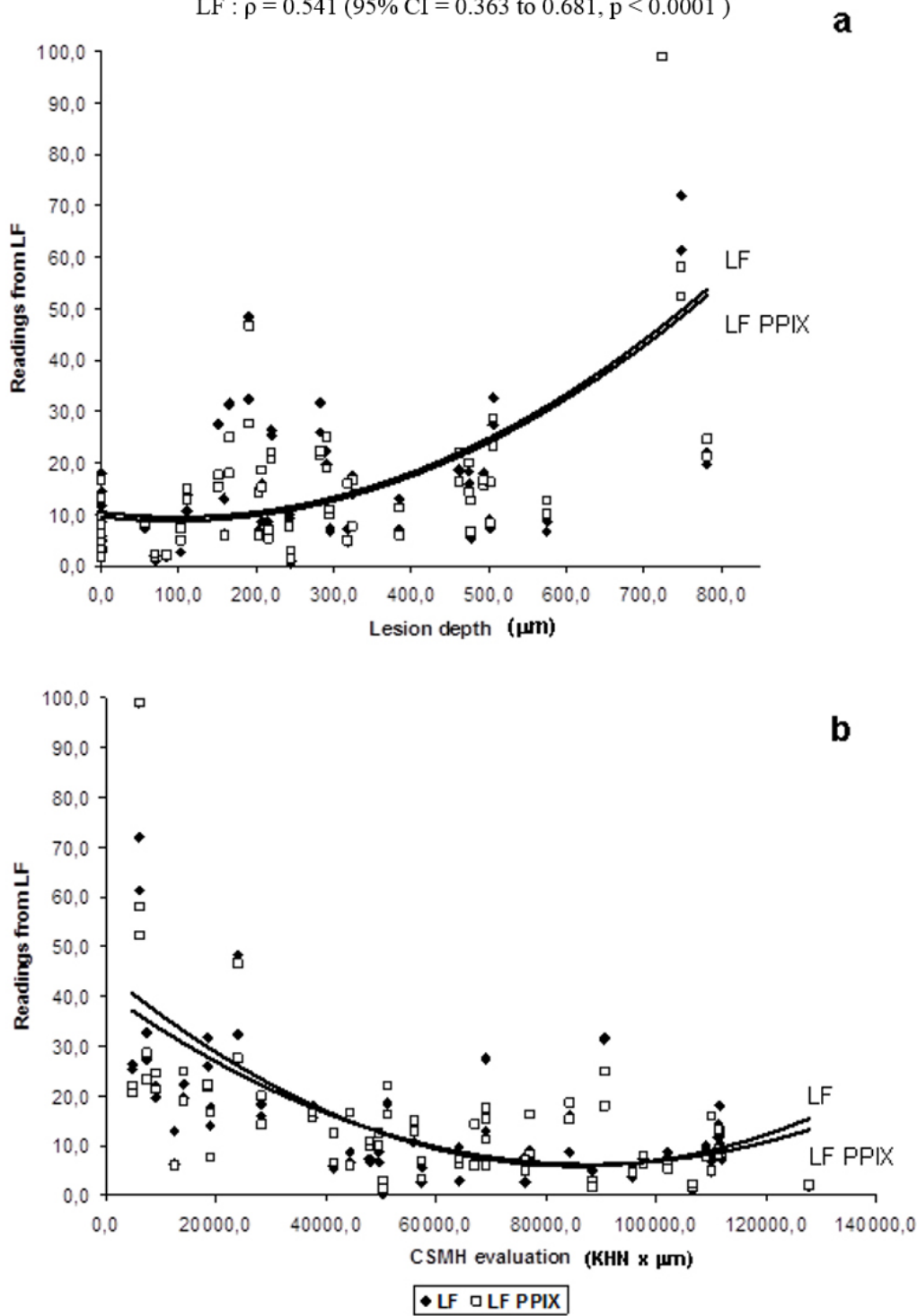
There was significant positive correlation between LF methods associated or not with dyes and lesion depth. With LF PPIX, Pearson's correlation coefficient was 0.583 (95% CI = 0.414 to 0.713, $p < 0.0001$) while the coefficient concerning LF without

dye in this sample was 0.541 (95% CI = 0.363 to 0.681, $p < 0.0001$). Regarding the LF TMPyP, a higher positive Pearson's correlation coefficient was obtained with lesion depth (0.746, 95% CI = 0.615 to 0.837, $p < 0.0001$) compared with the LF with no dye (0.602, 95% CI = 0.421 to 0.736, $p < 0.0001$).

There was negative correlation between the two methods and hardness. The Pearson's correlation coefficients were -0.525 (95% CI = -0.670 to -0.343, $p < 0.0001$) and -0.526 (95% CI = -0.670 to -0.343, $p < 0.0001$) using the LF PPIX and LF alone, respectively. Two-degree polynomial function gave better curve fit with two methods in both lesion depth and mineral loss evaluation (Figure 1). The correlation LF with TMPyP or LF with no dye with hardness was also negative. The Pearson's correlation coefficient using the LF TMPyP method was -0.718 (95% CI = -0.818 to -0.576, $p < 0.0001$) and with the LF alone was -0.645 (95% CI = -0.767 to -0.478, $p < 0.0001$). Two-degree polynomial function also gave better curve fit in these analyses (Figure 2).

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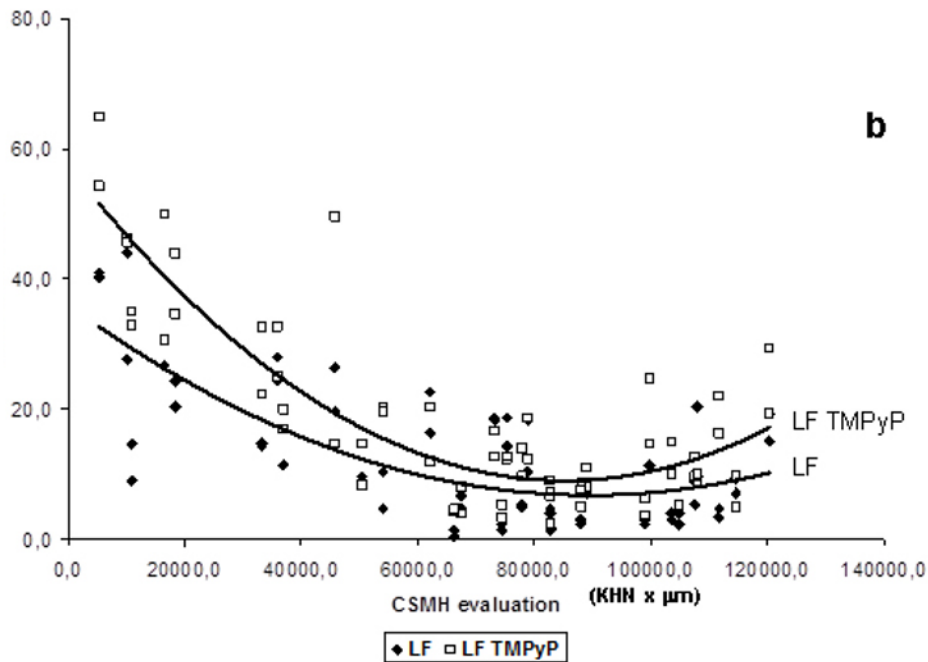
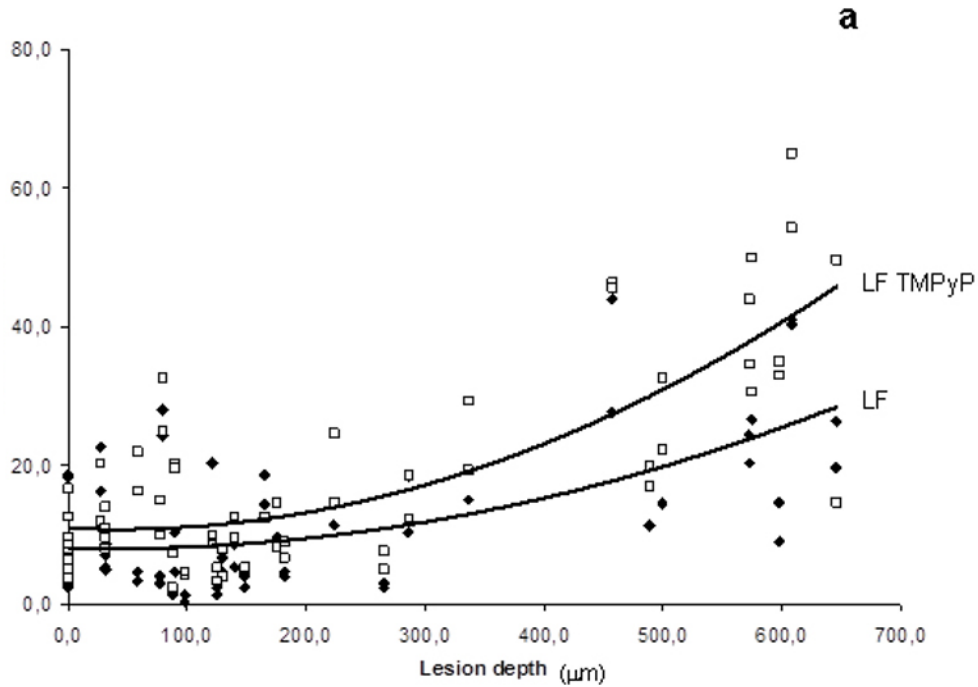
LF PPIX : $\rho = 0.583$ (95% CI = 0.414 to 0.713, $p < 0.0001$)
 LF : $\rho = 0.541$ (95% CI = 0.363 to 0.681, $p < 0.0001$)



LF PPIX : $\rho = -0.525$ (95% CI = -0.670 to -0.343, $p < 0.0001$)
 LF : $\rho = -0.526$ (95% CI = -0.670 to -0.343, $p < 0.0001$)

Figure 1 | Relationship between lesion depth (A) and mineral loss (B) obtained by cross-sectional microhardness analysis (CSMH) of caries lesions and Laser fluorescence (LF) values and with LF associated with Protoporphyrin IX (LF PPIX)

LF TMPyP : $\rho = 0.746$ (95% CI = 0.615 to 0.837, $p < 0.0001$)
 LF $\rho = 0.602$ (95% CI = 0.421 to 0.736, $p < 0.0001$)



LF TMPyP : $\rho = -0.718$ (95% CI = -0.818 to -0.576, $p < 0.0001$)
 LF : $\rho = -0.645$ (95% CI = -0.767 to -0.478, $p < 0.0001$).

Figure 2 | Relationship between lesion depth (A) and mineral loss (B) obtained by cross-sectional microhardness analysis (CSMH) of caries lesions and Laser fluorescence (LF) values and with LF associated with tetrakis(N-methylpyridyl)porphyrin (LF TMPyP)

Concerning the performance in detecting smooth-surface caries lesions, a slightly difference was observed between methods associated or not with the fluorescent dyes. LF PPIX presented higher sensitivity at D2 threshold, and higher specificity and accuracy at D3 threshold (Table 1). Regarding the LF TMPyP, the method achieved higher specificity value

only at D2 threshold. Other parameters did not present statistically significant differences (Table 1).

For all samples, high ICC values were observed (LF PPIX: 0.947, 95% CI = 0.902 to 0.972; LF without PPIX: 0.969; 95% CI = 0.941 to 0.983; LF TMPyP: 0.815 (95% CI = 0.685 to 0.895); LF without TMPyP = 0.900 (95% CI = 0.814 to 0.946).

TABLE 1 | Best cut-off points and performance of Laser Fluorescence (LF) and LF associated with fluorescent dyes (LF TMPyP and LF PPIX), in detecting initial enamel caries lesions (D1), advanced enamel caries lesions (D2) and dentine caries lesions (D3) in smooth surfaces of primary teeth

Methods	Sensitivity			Specificity			Accuracy			Az		
	D1	D2	D3	D1	D2	D3	D1	D2	D3	D1	D2	D3
LF	0.53	0.47	0.82	0.77	0.69	0.72	0.63	0.60	0.73	0.64	0.53	0.80
LF PPIX	0.49	0.67*	0.73	0.87	0.53	0.85*	0.64	0.58	0.83*	0.70	0.58	0.83

D1 cut-off points: LF = 14; LF PPIX = 16; D2 cut-off points: LF = 14; LF PPIX = 10; D3 cut-off points: LF = 16; LF PPIX = 19
 * statistically significant difference between the methods within the same column (p < 0.05). Az = area under ROC curve

Methods	Sensitivity			Specificity			Accuracy			Az		
	D1	D2	D3	D1	D2	D3	D1	D2	D3	D1	D2	D3
LF	0.55	0.73	1.00	0.74	0.75	0.82	0.61	0.74	0.85	0.58	0.74	0.93
LF TMPyP	0.51	0.62	0.80	0.95	0.88*	0.79	0.64	0.77	0.79	0.65	0.73	0.84

D1 cut-off points: LF = 10; LF TMPyP = 17; D2 cut-off points: LF = 10; LF TMPyP = 20; D3 cut-off points: LF = 15; LF TMPyP = 20
 * statistically significant difference between the methods within the same column (p < 0.05). Az = area under ROC curve

Occlusal caries lesions

There were no significant differences between LF methods, associated or not with the fluorescent dyes, in parameters related to performance at both

D1 and D3 thresholds. Generally, the best cut-off points using the fluorescent dyes were higher than the LF alone, but the performance was similar for all methods (Table 2).

TABLE 2 | Best cut-off points and performance of Laser Fluorescence (LF) and LF associated with fluorescent dyes (LF PPIX and LF TMPyP), in detecting initial enamel caries lesions (D1) and dentine caries lesions (D3) in occlusal surfaces of primary teeth

Methods	Cut-off points		Sensitivity		Specificity		Accuracy		Az	
	D1	D3	D1	D3	D1	D3	D1	D3	D1	D3
LF	3	11	0.97	1.00	0.54	0.86	0.78	0.88	0.82	0.94
LF PPIX	5	12	0.91	1.00	0.69	0.88	0.81	0.90	0.85	0.95

Methods	Cut-off points		Sensitivity		Specificity		Accuracy		Az	
	D1	D3	D1	D3	D1	D3	D1	D3	D1	D3
LF	7	13	0.50	0.73	0.86	0.91	0.64	0.88	0.68	0.91
LF TMPyP	13	17	0.59	0.91	0.73	0.87	0.64	0.88	0.68	0.90

There were no significant differences among the methods in any parameters. Az = Area under ROC curve.

DISCUSSION

In this study, we tested the performance of association of LF device with fluorescent dyes using natural caries lesions on occlusal and smooth surfaces. LF-TMPyP association presented better performance than LF-PPIX or LF used alone in quantifying smooth-surface caries lesions. The correlation coefficients between LF-TMPyP readings and microhardness increased considerably when compared to LF readings alone. The same trend was observed for lesion depth assessed by polarized light microscope analyses. Indeed, the association LF-TMPyP had been showed as better in detecting early demineralization in smooth surfaces¹⁹ and artificial caries around brackets.²⁰ On the other hand, the association with PPIX has not been useful, since it has presented similar results than with the device with no dyes.¹⁹ Our findings corroborate these previous results.

The most favorable correlation of LF-TMPyP with lesions hardness and depth could be explained by the chemical properties of this dye. TMPyP is a hydrophilic substance with high affinity for surfaces.²⁴ Enamel caries lesion is usually a subsurface lesion, presenting an apparently intact surface.^{21,25} This surface however, is rough due to the acid attack on it.^{25,26} Thus, the roughness of non-cavitated caries lesions could explain the better interaction with TMPyP, and, consequently, higher performance using this dye.

Since LF device detects organic content of caries lesions instead of mineral loss,^{10,27} it is comprehensible that the incorporation of a fluorescent dye improve the detection of the lesions. Otherwise, in naturally created caries lesions, there are also significant changes in organic content of caries lesions, probably due to the presence of bacterial metabolites.²⁸ Then, the LF by itself presented a satisfactory performance in detecting initial natural caries lesions, and the association with TMPyP did not significantly improve this performance in the

present study, despite its higher correlation with other parameters related to caries lesions.

Differently from observed for detecting very early artificial caries (formed during at maximum 96 hours)(1), PPIX improved significantly LF performance in detecting natural enamel caries (probably formed for a longer period) and made LF more specific and accurate to detect dentine caries lesions. PPIX is an anionic and lipophilic porphyrin produced by some bacteria related to caries lesions.²⁹ The cumulative effect of this kind of porphyrin (both from the lesion and from the dye) could have contributed to improve LF readings at advanced enamel threshold. We can hypothesize that an advanced enamel lesion itself cannot present metabolites enough to be detected by LF whereas the junction of both porphyrins sources does it.

Regarding dentine lesions, they are supposed to have higher concentration of porphyrins themselves, due to their higher level of infection.³⁰ Therefore, no benefits were observed in the sensitivity at this threshold. However, additional porphyrins from the dye contribute more expressively to differentiate sound tissues and enamel lesions from dentine lesions (higher specificity at D3). In conclusion, for smooth surfaces, PPIX can help us in an important task, which is improving the caries detection at D2 threshold, since LF has showed better performance to detect more advanced lesions.^{12,13,15} Nevertheless, despite using dyes, no contribution was observed for initial enamel caries (D1).

On the occlusal surfaces, association with dyes did not improve caries detection compared to the use of LF alone. Since performance of LF device has been satisfactory in occlusal surfaces, although some occurrences of false positive readings,³¹ it is understandable that the association with dyes did not improve the performance. In addition, dyes penetration on occlusal caries can be different to smooth surfaces. This occurrence can be an effect of occlusal morphology.

We did not carry out any method to quantify caries lesions on occlusal surfaces. Actually, quantification of occlusal surfaces is difficult because of the anatomy of these surfaces. Thus, the majority of the studies has performed the validation using a relative scale instead of absolute measurements.³² Considering this limitation, we did not find any advantage in the association of fluorescent dyes and LF device in detecting occlusal caries.

An important prerequisite for a monitoring method is its reproducibility.^{5,33} We evaluated the intra-examiner reproducibility after one month from first measurements. This evaluation had two purposes: checking the reliability itself and evaluating possible residual effect of the dyes. All LF methods presented high values of reliability. LF without dyes presented higher ICC values than LF with the dyes. LF TMPyP showed lower reliability values, which indicates a possible residual effect of this dye. In the oral environment, we suppose dye would be probably removed more efficiently than in laboratory conditions. However, further studies should be conducted to investigate this hypothesis.

Another issue concerning caries diagnosis is caries activity assessment. As active lesions are more porous than inactive lesions, it is probable that the dye might penetrate more significantly in active than in inactive lesions and help to differentiate both statuses of lesions. This premise can explain the lower correlation of LF readings and lesion micro hardness. As we used exfoliated teeth from a bank of teeth, we could not standardize the lesions activity status. This alternative should be tested in further studies and can lead to a possibility to improve caries detection.

In conclusion, LF-TMPyP association might improve performance in quantifying smooth-surface caries lesions in primary teeth. However, LF sensitivity is improved at D2 threshold when using PPIX. The association of the device with fluorescent dyes does not improve the performance in occlusal caries lesions.

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