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Inflammatory biomarkers in dentinal fluid as an approach to molecular diagnostics in pulpitis

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Abstract

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Aim To explore a set of inflammatory biomarkers obtained from dentinal fluid (DF) from patients with symptomatic irreversible pulpitis (IP), reversible pulpitis (RP) and normal pulp (NP).

Methodology A cross-sectional exploratory study was performed, recruiting 64 patients on the basis of their respective pulp condition. DF samples were obtained from all patients (23, from IP patients; 20, from RP patients; and 21, from NP patients). Quantification of biomarkers was performed using a Luminex® MAGPIX platform system and multiplex assay kits. The Kruskal–Wallis test was used for comparisons with regard to pulp state. A simple logistic regression model and the odds ratio (OR) with a 95% level of confidence ($P = 0.05$) were used to evaluate associations between biomarker levels and pulpal diagnosis. The performance discrimination of the biomarkers was evaluated through the construction of a receiver operating characteristic (ROC) curve by calculating the area under the curve (AUC) for IP versus RP after logistic regression modelling. Youden criteria were used to establish cut-off points for biomarkers alone with AUC > 70 and P-value < 0.05, or estimated probabilities from the multivariable logistic model.

Results The biomarkers that had significantly higher values in participants with IP versus RP were IL-1α, VEGF-α and FGF acid ($P < 0.05$). FGF acid (OR: 12.62; $P = 0.0085$; CI 95% 1.91–83.29) and VEGF-α (OR: 2.61; $P = 0.0252$; CI 95% 1.13–6.03) were associated with pulp diagnoses of IP versus RP. The AUC-ROC curve for FGF acid was 0.79. The model containing FGF acid, IL-1α, IL-6 and TIMP-1 had an AUC-ROC of 0.92 for IP versus RP with a significant difference from the FGF acid ROC curve ($P = 0.0231$).

Conclusions Dentinal fluid could be used to assay pulpal mediators in the molecular diagnosis of pulpitis. Despite the limitation of the clinical diagnostics used in the present study, it was possible to detect a difference between irreversible symptomatic pulpitis and reversible pulpitis associated with the following combined biomarkers: FGF acid + IL-6 + IL-1α, +TIMP-1.

Keywords: biomarkers, diagnosis, Endodontics, pulpitis.

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Introduction

Decision-making during the clinical management of deep caries lesions and the diagnosis of pulpitis is highly complex. It is critical when the decision is one of whether to perform vital pulp therapy or root canal treatment, which mainly depends on the ability of the clinician to distinguish amongst the different stages of pulp inflammation (Ricucci *et al.* 2014). Therefore, current pulpal diagnostic procedures aimed at determining the state of inflammation of the pulp involve obtaining a symptomatic history of the patient's case, thermal and electrical pulp tests, and radiographic examinations (Mejare *et al.* 2012). However, these do not provide accurate information about the true histological pulpal status (Seltzer *et al.* 1963). Endodontic molecular diagnosis has received significant attention, with a focus on the inflammatory molecules involved in the pulpal inflammatory reaction in response to bacteria (Rechenberg & Zehnder 2014). Inflamed pulp is seen in an increasing number of inflammatory cells with synthesis of pro-inflammatory mediators, such as proteases, growth factors, chemokines and cytokines, all of which exacerbate the immune–inflammatory response (Hahn & Liewehr 2007, Cooper *et al.* 2010). These mediators could also play a role as biological markers, reflecting the inflammatory state of the pulp as a more accurate and biologically sound diagnostic tool in endodontics (Zanini *et al.* 2017). One example is the fibroblast growth factors (FGFs) related to angiogenesis, tissue healing and embryological development (Beenken & Mohammadi 2009). Other reports have indicated that interleukin-1 beta (IL-1b), interleukin-1 alpha (IL-1α), interleukin 4 (IL-4), interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 12 (IL-12 subunit p40), C-X-C motif chemokine 10 (CXCL 10), tissue inhibitor of metalloproteinase-1 (TIMP-1), tumour necrosis factor α (TNFα) and matrix metalloproteinases (MMPs) are also involved in inflammatory mechanisms in the pulp (Zehnder *et al.* 2003, Elsalhy *et al.* 2013, Abd-Elmeguid *et al.* 2013).

Dentinal fluid is an extracellular fluid contained within dentinal tubules and corresponds to a blood ultrafiltrate from the capillaries of the pulp, and it has a very similar composition to plasma (Coffey *et al.* 1970). It may also represent a noninvasive source of inflammatory markers related to inflammatory pulp status. Dentinal fluid could represent a surrogate source of biomarkers with potential diagnostic utility, reflecting the pulp status and more specifically discriminating between symptomatic irreversible and reversible pulpitis. The aim of this study was to explore a set of inflammatory biomarkers in the dentinal fluid of patients with a diagnosis of symptomatic irreversible pulpitis (IP), reversible pulpitis (RP) or normal pulp (NP).'

The null hypothesis was that it is not possible to detect inflammatory biomarkers in the dental fluid of patients diagnosed with symptomatic irreversible pulpitis (IP), reversible pulpitis (RP) or normal pulp (NP).

Materials and methods

Study design, participants and enrolment

A cross-sectional exploratory study was conducted, designed and reported, in accordance with the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines. It was approved prior to patient recruitment by a Scientific Ethics Committee of Servicio de Salud Metropolitano Oriente (SSMO06_12_2016) and conducted following Good Clinical Practice guidelines and the Declaration of Helsinki. The study protocol was clearly explained to all the participants of the study, who signed an informed consent form. From March 2016 to August 2017, consecutive patients referred for endodontic treatment to the Universidad de los Andes Health Center, located in San Bernardo (Santiago, Chile), were screened for enrolment. After comprehensive clinical and radiological examinations, 64 patients aged 12–55 years were recruited. The inclusion criteria were as follows: incisor, premolar or molar teeth with a clinical diagnosis of normal pulp, reversible pulpitis or symptomatic irreversible pulpitis, with no previous pulp exposure and normal apical periodontal space on radiographic examination. The exclusion criteria were as follows: the presence of periodontal disease (periodontal pockets deeper than 3 mm); systemic disorders or conditions such as diabetes mellitus, osteoporosis, pregnancy and breastfeeding; intake of medications that could influence pulp tissues 3 months prior to the beginning of the study; endodontically treated teeth; internal or external root resorption on clinical or radiographic examination; teeth with a history of avulsion; teeth with clinical and/or radiographic evidence of root fracture; teeth that could not be completely isolated with a rubber dam; teeth with radiographic evidence of canal calcification; and teeth with open apices.

Clinical procedures

All the clinical examinations, diagnostics and recordings were performed by two trained endodontists on the basis of a standard operating procedure (SOP). The samples for the study were dentinal fluid obtained from the layer of dentine affected by caries in teeth with IP and RP diagnosis. In the case of teeth with NP, the sample was taken in the cavity 2 mm before pulp communication, calculated from the previous radiograph.

Patients recruited according to the inclusion criteria were subjected to a clinical procedure performed with a head-mounted lens with a magnification of 3.5× and a working distance of 400 mm (EyeMag Model Pro F; Carl Zeiss AG, Oberkochen, Germany). Anaesthesia (2% lidocaine hydrochloride with 1 : 80 000 epinephrine; Septodont, Saint-Maur-des-Fosses, France) was used with infiltration or inferior alveolar nerve block techniques. Caries and infected dentine were removed, starting with an excavator (No 47/48; Maillefer, Ballaigues, Switzerland), and followed, if necessary, with low-speed burs of different sizes (Dentsply Sirona, Ballaigues, Switzerland), leaving exposed hard or leather-type dentine (Nyvad *et al.* 2013). Isolation using a rubber dam (Hygenic; Coltène/Whaledent AG, Altstätten, Switzerland) disinfected with 70% alcohol solution (Merck KGaA, Darmstadt, Germany) was performed in all cases. A gingival protection barrier (OpalDam® SDS; Ultradent, UT, USA) was used to avoid contamination of the sample by achieving a better marginal seal.

Pulpitis status was categorized as normal pulp (referred for extraction for orthodontic purposes), reversible pulpitis (referred for prosthetic purposes) and irreversible symptomatic pulpitis (referred for root canal treatment) (Fig. 1a). Diagnostic criteria were applied according to the Glossary of Endodontic Terms (AAE 2016). Reversible pulpitis was defined as a clinical diagnosis based on subjective and objective findings indicating that the inflammation should resolve and the pulp return to normal. Symptomatic irreversible pulpitis was defined as a clinical diagnosis based on subjective and objective findings, meaning that the vital inflamed pulp was incapable of healing. Additional descriptors were lingering thermal pain, spontaneous pain and referred pain (for further details see Table S1). Subsequently, for the evaluation of the data, a code was assigned to each patient allowing him/her to be identified confidentially, and the data were entered into a specific database, thereby ensuring privacy and confidentiality. The information recorded included age, gender, tooth number, sensitivity test and pulpal diagnosis. The sample size was established arbitrarily for convenience, owing to the exploratory nature of the study.

Dentinal fluid sampling

Dentinal fluid was obtained using a Polyvinylidene difluoride membrane (PVDF) of 0.45 μm pore size, 13 mm diameter (Durapore®; Millipore, Bedford, MA, USA), handled with sterile tweezers (Straumann, Basel, Switzerland). The clinical procedure of taking each sample was performed meticulously to avoid contact of the PVDF membrane with the rubber dam. The PVDF membrane was kept inside the cavity for 2 min and then inserted in a 1.5 mL sterile microcentrifuge tube (Eppendorf®; Merck, Darmstadt, Germany; Fig. 1b). All samples were transported in an IsoFreeze® Flipper (Model 5152G75; Thomas Scientific, Swedesboro, NJ, USA) from the Clinic to the Center for Research in Biology and Oral Regeneration (CIBRO) of the University of Los Andes, Santiago, Chile (Fig. 1c) and stored at –80 °C for protein analyses.

Sample preparation

The PVDF membranes were thawed at room temperature. Each PVDF membrane was transferred to a 2 mL Eppendorf® tube with a standard volume of 350 μL phosphate-buffered saline (PBS 1X; Gibco PB, Thermo Fisher, CA, USA) supplemented with 0.1% Tween 20 (Sigma, Germany) for 30 min at room temperature. Subsequently, samples were stirred and then centrifuged for 5 min at 10 000 × ***g*** in a benchtop centrifuge (PrismR Labnet, Edison, NJ, USA) refrigerated at 4°C (Fig. 1d).

Selected biomarkers

Fourteen biomarkers were selected based on a previous literature search and were assembled in three high sensitivity kits (R&D Systems® Inc, Minneapolis, MN, USA), expressed in pg mL^{-1} as follows:

- Human Magnetic Luminex Screening Assay 13 plex. For 3 biomarkers: PDGF-bb, IL12p40 and TIMP-1 (LXSAHM-03; R&D Systems® Inc).
- Human Magnetic Luminex Screening Assay 3 plex. For six biomarkers: CXCL-10, IL-1α, MMP-9,

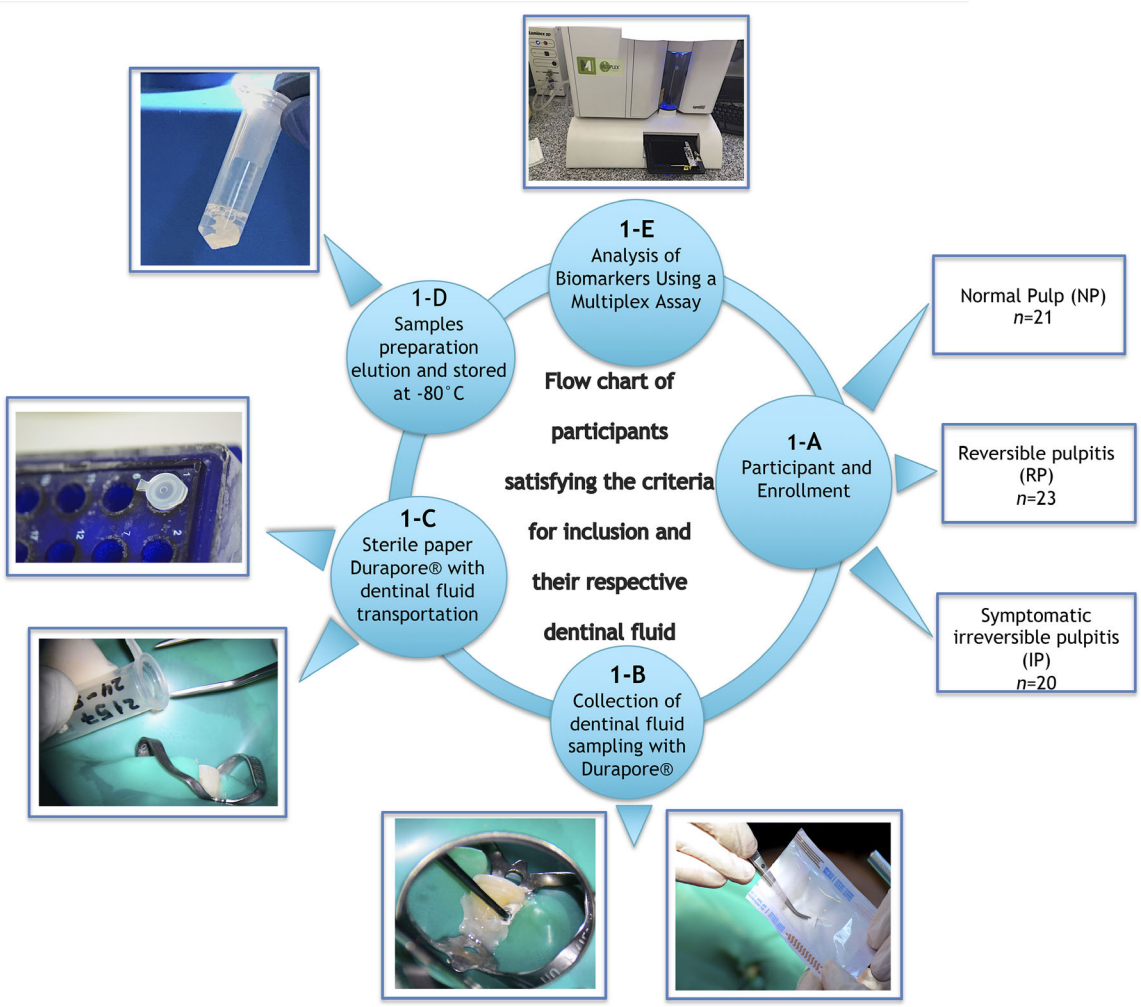

Figure 1 Flow chart of the methodology used in the study. (a) three experimental groups were evaluated: teeth with reversible pulpitis (RP) (n = 23), teeth with symptomatic irreversible pulpitis (IP) (n = 20) and caries-free teeth with normal pulp (NP) (n = 21). The criteria for this diagnostic categorization were in accordance with the classification of the American Association of Endodontists (AAE). (b) hydrophilic PVDF paper (Durapore® 0.45 μm, 13 mm diameter) was held with sterile tweezers to obtain the dentinal fluid. The paper was kept in the cavity for 2 minutes. (c) Each sample was stored in a 1.5 mL Sterile Eppendorf ®, labelled with a code assigned to the patient with the diagnosis and date. All samples were transported in an Iso-Freeze® Flipper from the Clinic to the Center for Research in Biology and Oral Regeneration (CIBRO) of the University of Los Andes. (d) Samples were stored in a freezer at –80°C to avoid denaturation until subsequent analysis. (e) Protein analysis of dentinal fluid was performed by a Luminex® MAGPIX platform system using a multiplex assay based on colour-coded polystyrene beads (LXSAHM, Milliplex multi-analyte profiling (MAP) kits, R&D Systems Europe, Abingdon, UK).

MMP-3, VEGF-α and FGF acid (LXSAHM-06; R&D Systems® Inc).

- Human High Sensitivity Cytokine Premixed kit. For five biomarkers: IL-1b, IL-4, IL-6, IL-8 and TNFα (FCSST-09-05; R&D Systems® Inc).

A lower limit of detection (LOD) was considered in the data analysis.

Analysis of biomarkers in dentinal fluid using a multiplex assay

Protein analysis of dentinal fluid was performed by a blinded operator through use of a Luminex® MAGPIX (Thermo Scientific) platform system, with a multiplex assay based on colour-coded polystyrene

beads (LXSAHM, Milliplex multi-analyte profiling (MAP) kits (R&D Systems Europe) (Fig. 1e). The protocol and panel, including the 14 analytes measured, are listed on the company's website (www.rndsystems.com). All assays were carried out according to the manufacturer's instructions. Standard curves were constructed by making a serial dilution according to the manufacturer's recommendations. Median fluorescence intensity (MFI) values for each analyte were converted into absolute concentration values via serial dilution of standards with known concentrations, using a five-parameter logistic (5-PL) curve-fit generated by the MILLIPLEX® Analyst 5.1 software (Millipore; Merck KGaA, Darmstadt, Germany). Biomarker concentration results were expressed in pg mL^{-1}. Sterile PVDF membranes were used as an internal control for each analyte (Ballal *et al.* 2017).

Statistical analysis

Statistical analysis was performed by considering the affected tooth as the unit of analysis. All data were tested for normality using the Shapiro–Wilk test by pulp status. All variables had an asymmetric distribution; therefore, they were described by median and interquartile range. For comparisons according to pulp state, the Kruskal–Wallis test with multiple comparisons was used. Also, analysis of the association between standardized biomarker levels and the outcome as a dichotomous variable (IP versus RP) was carried out using a simple logistic regression model, reporting the odds ratio (OR), 95% confidence interval and p-values. The statistical value of $P < 0.05$ was considered significant. An exploratory biomarker screening was performed through the construction of receiver operating characteristic (ROC) curves by calculating the area under the curve (AUC) for IP versus RP after logistic regression modelling. Multivariable logistic regression with backward stepwise selection with a *P*-value higher than 0.25 for the removal of variables was used. Transparent Reporting of a multivariable prediction model for Individual Prognosis or Diagnosis (TRIPOD) was undertaken, and internal validity was assessed with a bootstrapping procedure for a realistic estimate of the performance of the model (100 samples with replacement from the original sample). The optimism and shrinkage correction for coefficients and AUC ROC were estimated. The frequency of biomarkers selected in 100 samples was calculated, and the equality of two ROC areas was assessed. Calibration was performed by plotting observed proportions and predicted probabilities, and the results of the Hosmer and Lemeshow test were then reported. Youden criteria were used to establish cut-off points for biomarkers alone with AUC > 70 and a *P*-value < 0.05, or estimated probabilities from the multivariable logistic regression model. Sensitivity, specificity, predictive values and likelihood ratios were presented. The results obtained were analysed using STATA (version 15.1; StataCorp, College Station, TX, USA).

Results

Demographic data on the participants and tooth type by pulp status are presented in Table 1. In the analysis of the results, the limit of detection (LOD) described for each analyte by the manufacturer was considered and registered. See Table S1.

The biomarkers detected over the LOD by the Luminex® MAGPIX platform in all the study groups were as follows: IL-1β, TNF-α, IL-4, IL-1α, VEGF-α, FGF acid, IL-8, TIMP-1, MMP-9 and IL-6. However, some sample data results were below the LOD, for the following analytes: 3 samples under LOD for IL-8, 1 sample for IL6, 15 samples for TIMP-1, 1 sample for FGF acid and 1 sample for MMP9. PDGF-BB, IL12p40, CXCL10 and MMP-3 were not assigned a concentration value because the values were beneath the LOD according to the specifications and details of each kit for each analyte delivered by the manufacturer (https://www.rndsystems.com/products/performance-assay_luhm200#product-reviews), as described in Table S2.

Concentrations of the selected biomarkers by pulp status are presented in Table 2. IL-1α, VEGF-α and FGF acid had significantly higher values in participants with IP than in those with RP (15.21 vs. 13.59, 17.18 vs. 14.09, and 12.76 vs. 10.61 pg mL^{-1} median values, respectively, $P < 0.05$; Table 2).

The biomarkers that demonstrated an association with a diagnosis of IP versus RP were FGF acid (OR: 12.62; $P = 0.0085$; CI 95% 1.91–83.29) and VEGF-α (OR: 2.61; $P = 0.0252$; CI 95% 1.13–6.03). The relationship between standardized biomarker levels and IP versus RP is shown in Table 3.

Biomarker ROC curves for the detection of IP versus RP are presented in Fig. 2. Biomarkers that showed acceptable discrimination (AUC >=0.70) by themselves for IP versus RP were FGF acid

Table 1 Description of data of the study participants: age, sex and tooth type of enrolled individuals by pulp status

Variable	Normal pulp (n = 21)	Reversible pulpitis(n = 23)	Symptomatic irreversible pulpitis (n = 20)	Total
Age (years) [p50 (IQR)]	14 (3)	30 (25)	37.5 (25.5)	25 (24.5)
Sex [n (%)]				
Female	15 (71.43%)	16 (69.57%)	18 (90%)	49 (76.56%)
Male	6 (28.57%)	7 (30.43%)	2 (10%)	15 (23.44%)
Tooth type [n (%)]				
Incisors	0 (0%)	7 (30.43%)	4 (20%)	11 (17.19%)
Canines	0 (0%)	0 (0%)	4 (20%)	4 (6.25%)
Premolars	21 (100%)	9 (39.13%)	10 (50%)	40 (62.5%)
Molars	0 (0%)	7 (30.43%)	2 (10%)	9 (14.06%)

P50: Median; IQR, interquartile range.

Table 2 Biomarkers description in pg mL^{-1} by pulp status

Biomarker (pg mL^{-1})	Normal pulp (n = 21) P50 (IQR)	Reversible pulpitis (n = 23) P50 (IQR)	Symptomatic irreversible pulpitis (n = 20) P50 (IQR)
IL-1b	0.34 (0)†	0.34 (1.48)	1.23 (3.63)†
TNFα	0.93 (0.14)†	0.93 (0.14)	1.07 (0.14)†
IL-4	17.11 (1.16)	16.72 (1.54)	17.3 (1.52)
IL1-α	11.15 (2.45)†	13.59 (7.31)‡	15.21 (6.78)†,‡
VEGF-α	14.09 (2.05)	14.09 (8.09)‡	17.18 (3.87)‡
FGF-Acid	10.91 (3.02)†	10.61 (5.32)‡	12.76 (4.39)†,‡
IL8	0.13 (0.13)*,†	0.36 (1.09)*	1.125 (4.39)†
TIMP-1	6.07 (10.24)†	19.59 (77.8)	20.57 (167.25)†
MMP9	50.5 (122.86)*,†	283 (2959.59)*	1271 (13,956)†
IL-6	0.56 (0.12)†	0.62 (0.13)	0.62 (0.62)†

P50: median IQR, interquartile range; SD, standard deviation.
*,†,‡Significant differences in a row by Kruskal–Wallis test.

(AUC = 0.79; 95% CI 0.65–0.99) and IL-1α (AUC = 0.70; 95% CI 0.54–0.86). Combining FGF acid, IL-1α, IL-6 and TIMP-1, the AUC-ROC curve for RP versus IP diagnosis was 0.92 (95% CI 0.84–0.99), with a significant difference from the FGF acid curve (P = 0.0231).

An optimism score was calculated (0.048), and the adjusted AUC ROC was 0.87 (95% CI 0.79–0.95). A score for a combination of the four biomarkers mentioned above was estimated using the constant and coefficient of a logistic regression model. The formulation of the score proposed is –10.45 + 0.74*FGF acid + 4.64*IL6 – 0.06*IL1α – 0.01*TIMP1. The shrinkage factor was 0.66, and the intercept calibration was –0.064; thus, the corrected model was –10.51 + 0.49*FGF acid + 3.06*IL6 – 0.04*IL1α – 0.007*TIMP1. Calibration plots for the proposed score (FGFacid + IL6+IL1α + TIMP1), the distribution of individuals by the predicted probability of IP by pulp status group and the observed versus predicted probability are all presented in Fig. 3. The cut-off points, found via the estimation of Youden's index for FGF acid and FGFacid + IL6+IL1α + TIMP1 for IP versus RP, are shown in Table 4.

Table 3 Relationship between standardized biomarkers level concentration and symptomatic irreversible pulpitis versus reversible pulpitis (n = 43, univariate logistic regression model)

Biomarker	Odds ratio	95% CI	P-value	% selected*
IL-1b	1.32	[0.67–2.59]	0.4164	
TNFα	1.89	[0.77-4.64]	0.164	
IL-4	1.57	[0.79–3.10]	0.1948	
IL1-α	1.06	[0.58–1.94]	0.8525	58%
VEGF-α	2.61	[1.13–6.03]	0.0252*	
FGF Acid	12.62	[1.91–83.29]	0.0085*	99%
IL8	1.57	[0.65–3.77]	0.3159	
TIMP-1	1.70	[0.79–3.68]	0.1759	64%
MMP9	1.89	[0.66–5.44]	0.2359	
IL-6	1.70	[0.77–3.72]	0.1865	85%

CI, confidence interval.
*100 bootstrap stepwise selection.

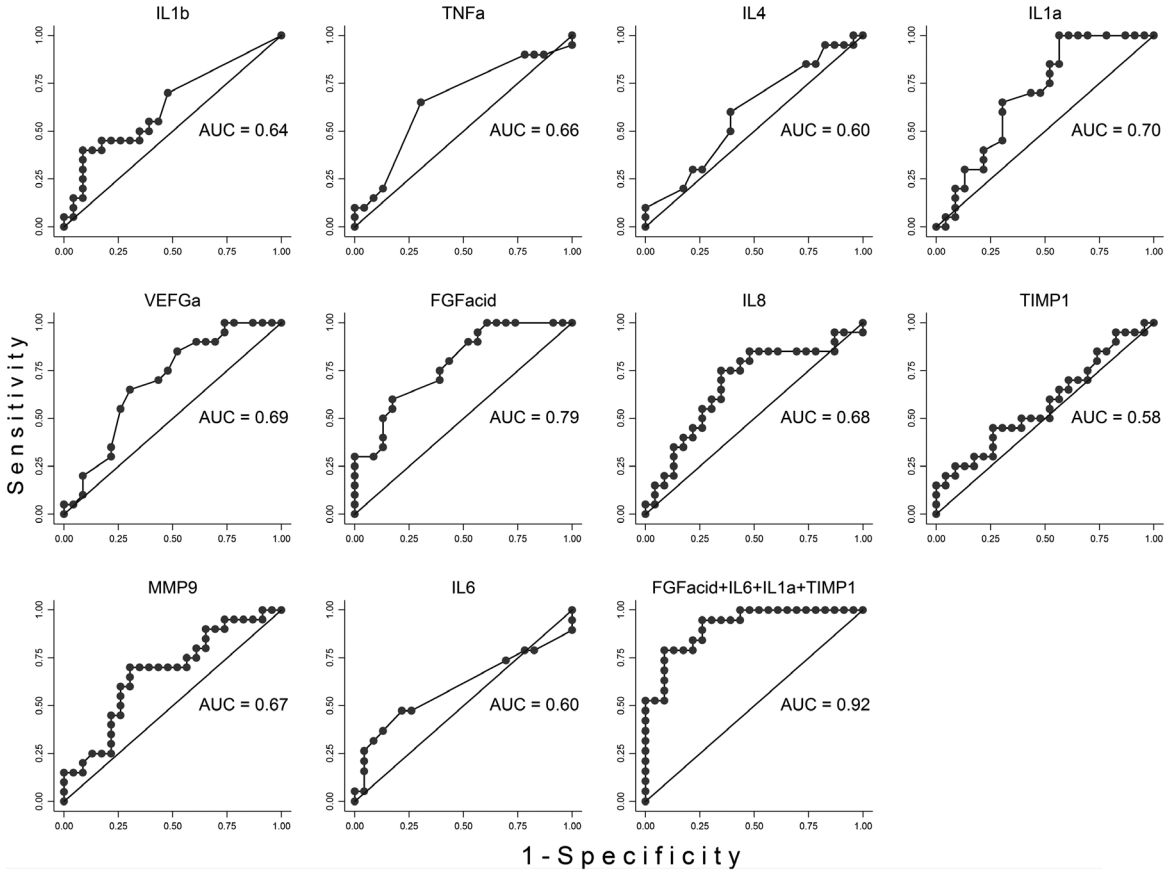

Figure 2 Discriminatory performance of biomarkers and score proposed (FGF acid + IL6+IL1α + TIMP1) for symptomatic irreversible pulpitis versus reversible pulpitis.

Discussion

The most significant contribution of this work is the fact that this is the first study to report on the use of dentinal fluid, in which the analysis of multiple analytes is considered simultaneously and by means of a highly sensitive test. These results could lay the groundwork for developing a biomarker panel for a molecular diagnostic kit.

The results of this study suggest that dentinal fluid composition has the potential to be used as a noninvasive tool for pulpal diagnosis. Specifically, a significant capacity of discrimination in the detection of IP versus RP diagnosis when FGF acid, IL-1α, IL-6 and TIMP-1 biomarkers are combined, and the precision of their diagnostic performance was encouraging.

Currently, the diagnosis of pulp pathosis tends to occur quite late, and the available tools used in clinical practice are imprecise and subjective, because they only measure the pulp nerve response and not the blood flow of the pulp, which gives a much better definition of pulp status (Mejàre *et al.* 2012). Thus, the development of minimally invasive approaches is a crucial element in endodontics, requiring the most accurate diagnostic tools, especially for cases of irreversible versus reversible pulpitis (Bjørndal *et al.* 2019).

Dentinal fluid (DF) is an extracellular fluid, and its composition is controlled by odontoblasts under normal conditions. However, any disturbance, such as dentinal exposure or dental caries, may lead to a change in DF composition, which is then more likely to be formed as a transudate from pulpal capillaries (Ozok *et al.* 2004). It has been reported that DF may be used for a 'patient-specific diagnostic test for pulp disease' and may also serve as a liquid biopsy medium to evaluate the concentrations of the constituents of pulpal tissue fluid (Maita *et al.* 1991).

In the current study, DF was obtained with the Durapore® PVDF membrane, with promising results.

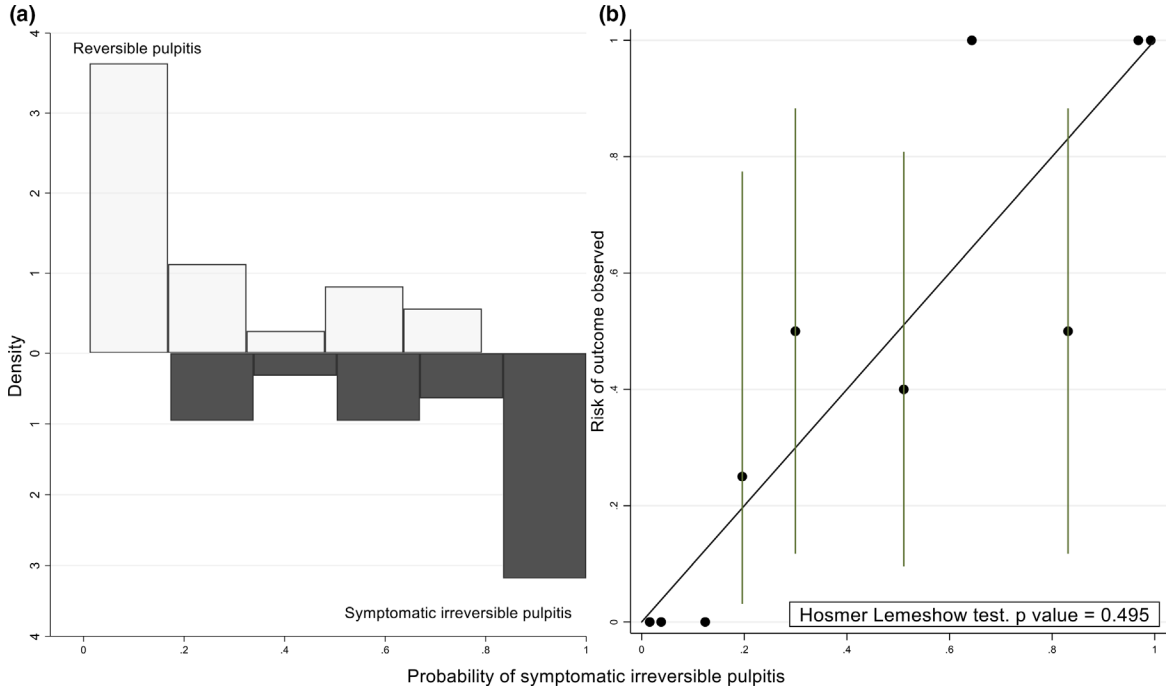

Figure 3 Calibration plots for score proposed (FGF acid + IL6+IL1a + TIMP1). (a) Distribution of individuals by predicted probability of symptomatic irreversible pulpitis and by pulp status group. (b) Probability of observed versus predicted score.

Table 4 Performance of FGF acid and irreversible status score cut-off for detection of irreversible symptomatic pulpitis versus reversible pulpitis

	FGF Acid		FGF Acid + IL-1α + IL-6 + TIMP-1	
	Value	95% CI	Value	95% CI
Cut-off	11.99	–	−3.39	–
Sensitivity	60.00%	36.10–80.90%	78.90%	54.40–93.90%
Specificity	82.60%	61.20–95.00%	91.30%	72.00–98.90%
Positive predictive value	75.00%	47.60–92.70%	88.20%	63.60–98.50%
Negative predictive value	70.40%	49.80–86.20%	84.00%	63.90–95.50%
Likelihood ratio (+)	3.45	1.32–9.01	9.08	2.37–34.83
Likelihood (−)	0.48	0.27–0.85	0.23	0.1–0.56
Youden's index	0.43		0.70	

CI, confidence interval.

However, these results do not accord with findings reported by Zehnder *et al.* (2014), who compared the effectiveness of several cellulose membranes versus a PVDF membrane, and concluded that cellulose membranes with a larger pore size could be used to collect measurable amounts of MMP-2 from the dentine of healthy teeth in 9 out of 13 cases, compared with PVDF membranes, which only afforded success in 1 out of 13 cases ($P < 0.05$) (Zehnder *et al.* 2014). A likely explanation of this difference lies in the method used for the acquisition of the sample. In the present study, a standardized sampling protocol was established, which included a 2-min sample collection time, as well as appropriate transport and storage of the samples at –80 °C. In addition, a different sample processing method was performed, in which PBS 0.1% Tween 20 elution buffer was used, which probably allowed better elution of biomarkers.

The results of the current study support a differential profile of inflammatory biomarkers amongst patients with symptomatic IP in comparison with RP. IL-1α, VEGF-α and FGF acid had significantly higher

values in individuals with IP than in those with RP (15.21 vs. 13.59, 17.18 vs. 14.09 and 12.76 vs. 10.61 pg mL^{-1} median values, respectively). In addition, the biomarkers that demonstrated an association with the pulp diagnoses of IP versus RP were FGF acid (OR: 12.62; $P = 0.0085$; CI 95% 1.91–83.29) and VEGF-α (OR: 2.61; $P = 0.0252$; CI 95% 1.13–6.03).

These results may be explained by the fact that FGF acid and VEGF are two growth factors and are consequently essential components for dental pulp repair in response to damage (Mullane *et al.* 2008). FGF acid is secreted by fibroblasts and has marked mitogenic potential, because it allows differentiation of pulp stem cells in fibroblasts and proliferation of endothelial cells for neoangiogenesis in affected areas (Boyle *et al.* 2014). VEGF-α is a glycoprotein with the ability to increase vascular proliferation and blood vessel permeability, which initially helps repair by encouraging the chemotaxis of cells to the inflamed site (Hahn & Liewehr 2007). In addition, FGF acid has a marked action on endothelial cells, in close relationship with the function of VEGF (Jeanneau *et al.* 2017). Jeanneau *et al.* (2017) reported the importance of these two molecules in the pulp repair process, describing how, 6 h after damage, the expression of both growth factors could already be seen in the first inflammation–repair reactions.

Although FGF acid and VEGF-α have been reported only a few times in the literature on pulp inflammation, the present results are in agreement with those reported in another study where there was a significant difference ($P < 0.01$) in the expression of FGF acid and VEGF-α in the irreversible-versus-reversible pulpitis group (Abd-Elmeguid *et al.* 2013). However, the samples in the reported study were obtained from pulp tissue and not from noninvasive sampling of the DF.

A rather exploratory analysis was proposed that will provide inputs to future studies, allowing efforts to be concentrated on the detection of biomarkers that turn out to have a better performance and, from this, calculate a sample size to carry out a more confirmatory study in the future.

The discriminatory performance of the biomarkers was evaluated through the construction of receiver operating characteristic (ROC) curves by calculating the area under the curve (AUC) for IP versus RP after the development of a logistic regression model. The biomarkers that showed acceptable discrimination (AUC >= 0.70) by themselves for IP versus RP were FGF acid (AUC = 0.79; 95% CI 0.65–0.99) and IL-1α (AUC = 0.70; 95% CI 0.54–0.86). The third type of molecule found in our investigation corresponded to IL-1α. It is well known that IL-1α is the initiator of the cellular immune response because it is secreted by activated monocytes and macrophages. It also induces release of prostaglandin and stimulation of fibroblasts (D'Souza *et al.* 1989). When comparing literature reports with the results obtained in the present study, fibroblasts are again seen to be important precursors of the immune–inflammatory response at the pulp level. On the other hand, Wisithphrom *et al.* (2006) reported in their study that IL-1α can induce pulp destruction by differential regulation of MMPs and TIMPs.

The results also demonstrate that, when combining FGF acid, IL-1α, IL-6 and TIMP-1, the AUC-ROC curve for IP versus RP diagnosis was 0.92 (95% CI 0.84–0.99) with a significant difference from the FGF acid curve ($P = 0.0231$). It has been reported that the immuno-inflammatory response does not depend on a single analyte, but rather on a combination, because the inflammatory and immune responses are inherently complex processes (Zehnder *et al.* 2003). The expression of inflammatory mediators in pulp inflammation, measured by multiplex assays, has been described previously in the literature (Abd-Elmeguid & Yu 2009, Cooper *et al.* 2010). For this reason, 14 biomarkers were studied simultaneously in this experiment, rather than considering each one separately.

One of the drawbacks of the present study was the limited sample size, which was selected arbitrarily for convenience, owing to the exploratory nature of the study. Large-scale validation of the current results is essential. As in all other previous studies using molecular analysis of dentinal fluid, in this study there was a limitation regarding the true diagnosis of the teeth under investigation. Future studies should assess the real predictive value of the markers under investigation in order to establish a correlation between biomarkers in DF with the result of the treatment outcome. Another limitation is the low volume of sample material obtained from DF, which makes it a very delicate technique, dependent on the operator and on the type of quantitative/qualitative analysis performed. To overcome this last limitation, standardization of the acquisition of the sample, storage, and conservation of the sample, as well as its reading, were carried out to validate the method. In addition, the Luminex® High-Performance Assays

used are the most accurate and precise bead-based multianalyte profiling kits. Luminex technology is a highly sensitive method that is critical for immunoassays, allowing analytes present at low levels to be measured accurately. Without adequate sensitivity, analytes on low abundance can be missed entirely, even if they are present in a sample. In addition, it can be difficult to compare healthy samples with diseased samples, since overexpressed analytes in diseased states are often present at very low levels in healthy individuals.

Due to the small sample volume, it was not possible to quantify the total protein concentration. However, this information is relevant and should be considered in future studies. Future studies are planned to perform this quantification with Micro BCA and to normalize the concentrations of the analytes under investigation.

Innovative and precise strategies based on molecular diagnosis offer promise and may be relevant for determining the appropriate clinical indications for vital pulp therapies and optimization of prognosis. The present study shows that optimization of sampling using DF allows use of a less invasive technique. With this pilot study and innovative research, it was hoped to establish the basic internal validity of a diagnostic test to establish a differential profile of specific biomarkers and associate them with pulpal diagnoses. Future work will aim to develop an accurate diagnostic kit based on a molecular approach, which could help to make the clinical distinction between reversible and irreversible pulpitis. One example of this is the immunochromatographic tests based on the immunological capture of a coloured colloid during its passage through a membrane, in which an antibody or an antigen is immobilized. This process is fast, simple, reliable and easy to interpret.

Conclusions

Dentinal fluid has potential as a medium for molecular diagnosis of pulp status. Despite the limitations of the true clinical diagnostic criteria used in the present study, it was possible to detect a difference between irreversible symptomatic pulpitis and reversible pulpitis, which was associated with the following combined biomarkers: FGF acid + IL-6 + IL-1α, + TIMP-1. These molecules, obtained from dentinal fluid, could be useful potential biomarkers for chair-side diagnostic tests of irreversible versus reversible pulpitis.

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Conflict of interest

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Criteria for this diagnostic categorization were applied according to the classification proposed by the American Association of Endodontists (AAE).

Table S2. Analyte specifications with LOD and Assay Range described for the manufacturer. Also, the negative controls C- experimental values.