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ORIGINAL ARTICLE

Quantification of matrix metalloproteinases MMP-8 and MMP-9 in gingival overgrowth

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KEYWORDS

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Abstract *Background:* Matrix metalloproteinases (MMPs) are proteolytic enzymes involved in extracellular matrix remodeling of all body tissues, including oral tissues such as gingival tissue. Expression levels of MMPs are widely studied as important biomarkers for explaining the biochemical mechanisms and evolution of many oral diseases.

Objective: Demonstrate the sensitivity, reproducibility, repeatability, and robustness of the dot blot assay for the relative quantification of MMP-8 and MMP-9 expression levels in patients with GO associated with orthodontic treatment.

Methods: A validated dot blot assay was used to compare the relative expression levels of MMP-8 and MMP-9 in gingival samples. Methodological variability, reproducibility, sensitivity and robustness were determined with the use of control samples from healthy donors (G1). Next, expression levels were measured in gingival tissue from patients with mild and moderate gingival overgrowth associated with orthodontic treatment (G3 and G4) and patients without gingival overgrowth but with a history of using orthodontic appliances (G2).

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Results: Dot blot assay demonstrated that MMP-8 and MMP-9 expression levels were higher in patients with gingival overgrowth and distinguished those with moderate clinical grade (G4) from those with mild overgrowth (G3). In addition, patients with a history of orthodontic treatment showed similar expression levels to the control group two years after removing orthodontic appliances.

Conclusions: With the assay used, we were able to detect differences in MMP-8 and MMP-9 expression in patients with different levels of severity of gingival overgrowth. Dot blot could be used to measure MMPs during the onset and progression of gingival overgrowth.

1. Introduction

Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteolytic enzymes. An important function is to regulate the extracellular matrix (ECM) remodeling process in all body tissues. Such turnover is regularly accomplished through direct degradation of proteins such as collagen, fibronectin, and proteoglycans. Therefore, their expression results in a variety of physiological and pathological events (Cui et al., 2017).

Under normal physiological conditions, Tissue Inhibitors of Metalloproteinases (TIMPs) locally regulate the catalytic activity of MMPs, and during pathological circumstances, both MMPs and TIMPs may vary. However, in dental diseases such as dental caries, gingivitis, pulpitis, periodontitis, oral cancer and gingival overgrowth (GO), an alteration in the MMP expression levels is frequently found (Liu and Khalil, 2017). In particular, significantly increased expression levels of MMP-8 and MMP-9 in gingival crevicular fluid and gingival tissue of patients with orthodontic appliances have previously been described, linked to collagen remodeling processes (Kapoor et al., 2019). This likely occurs in response to the use of adapted fixed devices during treatment. Likewise, these patients frequently develop gingival overgrowth, a pathology that is considered as a generalized or localized increase in volume of the gingival tissues (Beaumont et al., 2017).

Thus, MMP expression levels can be affected by genetic background, i.e., gene polymorphisms (Perunovic et al., 2015), as a result of biological causes, such as oral infectious diseases, or due to physical trauma such as that caused by the presence of orthodontic appliances (Boelen et al., 2019). Therefore, studies aimed to measure changes in MMP expression levels are still necessary to deepen in the biochemical foundations of many oral pathological processes in dentistry (Sambandam and Neelakantan, 2014). Furthermore, the measurement of any qualitative and quantitative changes in MMP expression under different physiological conditions is important for the advancement of biomedical sciences (Maciejczyk et al., 2016).

Currently, enzyme-linked immunosorbent assay (ELISA), zymography and Western blot immunoassay are the most common methods used for these purposes and have advantages and some limitations; for instance, zymography is a relatively low-cost test in comparison with the others. In addition, by zymography levels of specific enzyme forms or active forms of gelatinases MMP-2 and MMP9 can be detected with high sensitivity when its substrate is included in polyacrylamide gels and their TIMPs are absent or are present at low levels (Vandooren et al., 2013). However, it is not an assay of choice for quantitative analysis due to the demanding nature of standardization and the requirements of long-term processing, among other reasons.

For an efficient identification of MMPs, ELISA and Western blot assays are recommended. Although these methods require effort and a significant amount of time, both immunoassays are characterized by high sensitivity and specificity, depending on the quality of the antibodies used. In particular, the ELISA quantitative method is faster and more sensitive than the others, but it does not discriminate between active and inactive forms of MMP and multimers and protein complexes that often compose these proteins (Bencsik et al., 2017).

Regarding Western blot assay, is important to mention that it is known as a useful method for detecting specific proteins in complex biological samples. Its efficiency may be improved when implementing chemiluminescence technique, allowing the sensitivity and stability of detected signals to be increased. It is also worth mentioning that this method allows a particular protein to be detected, but it does not make it possible to quantify its amount unless a known concentration of a pure protein sample is available for study to construct a calibration curve and perform an absolute quantification, similar to ELISA assays. Another important difficulty reported for this method is its limited capacity for transferring high-molecular-weight proteins, a situation that researchers try to overcome through modifications of transfer methods (Ghosh et al., 2014).

A third type of immunoassay, called dot blot, has been shown to be versatile, sensitive and helpful for measuring expression levels as well as the presence of posttranslational modifications of proteins (Wang et al., 2013; Putra et al., 2014). However, despite having been developed in 1982, it has not been widely considered to study MMP expression in oral pathologies (Surendran et al., 2015).

The dot blot method is characterized by the simplicity of its procedure and its high sensitivity. It became a promising assay to quantify changes in MMP expression levels in clinical cases that involve remodeling of gingival tissue. In our study, we aimed to demonstrate the sensitivity, reproducibility, repeatability, and robustness of the dot blot assay for relative quantification of MMP-8 and MMP-9 expression levels in patients with GO associated with orthodontic treatment.

2. Materials and methods

A pilot study was designed to compare relative MMP-8 and MMP-9 expression levels in gingival tissue between a control group and samples obtained from patients with GO. The following methodological steps were established:

2.1. Sample selection and experimental groups

A total of twenty gingival tissue biopsies were obtained from systemically healthy patients (between 13 and 35 years old) who underwent gingival surgery in a dental clinic at the University of Cartagena. Subjects were selected using non-probability sampling for convenience, taking into account the inclusion and exclusion criteria previously established. Patients were divided into four study groups, each one including 5 individuals: **Group 1**, a control group of healthy patients without clinical evidence of GO and no record of using orthodontic appliances. **Group 2**, patients without clinical evidence of GO but with a history of using orthodontic appliances. **Groups 3** and **4**, patients diagnosed with mild and moderate GO, respectively, due to orthodontic treatment (Fig. 1). Donors were informed about the study purpose, the surgical procedure, and its possible risks. All of them signed an informed consent form, approving donation of gingival tissue. In case of minors, a responsible adult signed the authorization. MMP-8 and MMP-9 expression levels were determined in all collected samples.

2.2. Sampling

The gingival tissue samples were obtained through gingivectomy. The same surgical protocol was performed in both gingival overgrowth patients and healthy patients. Local anesthesia (lidocaine 2%) was applied on the area to intervene, and a n°15 scalpel blade was used to cut and remove the gingival margin. The tissues were washed with isotonic saline to eliminate blood residues. Then, samples were stored at −80 °C until processing.

This research was approved by the institutional ethics committee according to scientific and technical standards for health research established in Resolution No. 008,430 1993 of the Health Ministry of the Colombian republic, as well as the Helsinki Declaration by the World Medical Association. The authors acknowledge complete autonomy during development of this work and declare the absence of any conflict of interest.

2.3. Protein extraction

Twenty to 40 mg of tissue were treated two times with PBS-Triton X-100 (1%) protein extraction buffer supplemented with a protease inhibitor (Protease Inhibitor Cocktail. AMRESCO. Code. M221-1 ml). First, 80 µL of solution was added to macerate the tissue on ice using a manual homogenizer for 10 min, and lysates were then sonicated 3 times for 60 s and centrifuged at 12,000 rpm, at 4 °C for 15 min. The supernatants were recovered and placed in new tubes. A second extraction from the remaining sediment tissue was performed, with 50 µL of extraction buffer added following the same conditions as previously described. Supernatants with protein extracts were pooled, and their concentration was determined by Bradford Protein Assay using a calibration curve with a BSA standard (Bradford 1976).

2.4. Dot blot assay

Samples from the control group (G1) were used to optimize and validate the dot blot assay. During validation, PVDF membranes (Immun-Blot® PVDF Membrane for Protein Blotting. Cat. 162-0177) were selected and activated with methanol and water (1 and 5 min, respectively). Next, each protein extract was manually placed in a range of 0.5–10 µg. Extraction buffer was used as a blank, and the applied volume was adjusted to 4 µL (Fig. 2). Dried membranes were activated again and then blocked at 25 °C for one hour with skim milk in phosphate-buffered saline (PBS), 10% w/v. Then, membranes were incubated for 2 h at room temperature with primary antibodies using blocking reagent as diluent. The mouse monoclonal antibodies anti-MMP-8 (Antibody - MyBiosurce MBS833512; Dilution 1: 2000) and mouse Anti-Human MMP-9 (AbD Serotec MCA2735; Dilution 1: 4000) were used for determinations of MMPs 8 and 9, respectively.

Next, excess of antibody was eliminated with two washes of 5 min each using blocking solution, and membranes were then incubated for 1 h with secondary Goat anti-mouse IgG-HRP Conjugate-Antibody (MyBiosurce MBS330074; Dilution 1: 5000). Membranes were treated with three sequential washes of 10 min each, the first one in Tween 20 (0.05%v/v in blocking solution), the next in blocking solution and the final one in PBS solution.

Membranes were incubated for two minutes in a solution of chemiluminescent substrates A and B (Novex ECL, HRP Chemiluminescent substrate reagent kit, Invitrogen), after which excess solution was removed, and then uptake of chemiluminescent signal was measured in a transilluminator (Chemi-Doc MP Imaging System, Bio-Rad). Capture of images was performed every 24 s.

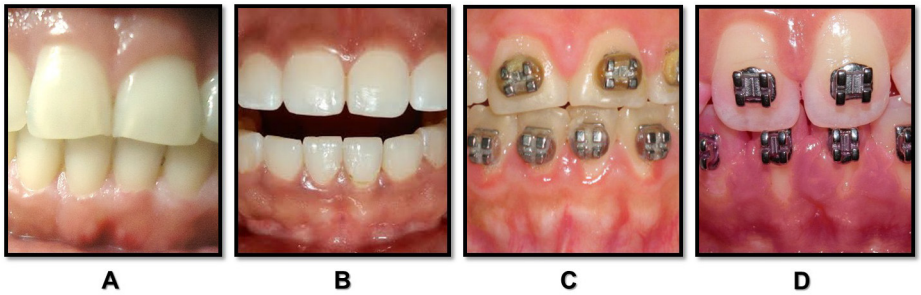

Fig. 1 Clinical features of gingival tissue from donors. Periodontally healthy patients without historical (Group 1) and with historical (Group 2) use of orthodontic appliances, and patients diagnosed with mild (Group 3) and moderate (Group 4) gingival overgrowth associated with orthodontic treatment, respectively. A = Group 1; B = Group 2; C = Group 3 and D = Group 4.

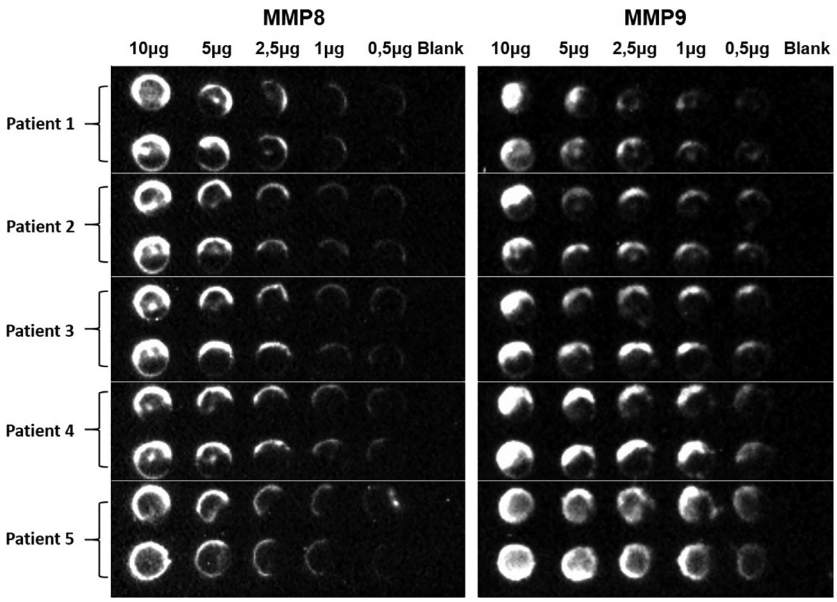

Fig. 2 A representative image of the dot blot Group 1. The five points of each sample were spotted in duplicate. Exposure time: 617.1 s.

Intensities of signals of MMP-8 and MMP-9 were determined by optical densitometry using Image Lab software (Bio-Rad). The circular volume tool was used to delimit the shape of each point, including the blank. To assure a uniform analysis, a reading area of 52.8 mm^2 was established for all the points, and the general background was subtracted. The signal intensity values (Volumes) of each point were exported to an Excel sheet, and the signal intensity was graphed against the protein mass to obtain the calibration curves. The dot blot assay was repeated for validation, and the linearity, repeatability, intralaboratory reproducibility and robustness were evaluated. Data were analyzed using Excel 2013 and ANOVA test with Bonferroni correction to evaluate significance among groups.

2.5. Application of dot blot in clinical samples

The validated dot blot assay was applied to clinical samples from patients of G2, G3 and G4 following the previously mentioned procedure. Assays were performed on independent membranes, using one membrane for each study group. On all membranes, five samples corresponding to each group were placed in duplicate.

3. Results

A dot blot assay was optimized to quantify MMP-8 and MMP-9 in gingival tissue using selective monoclonal antibodies. Dot volume, amount of protein and best timing for capturing chemiluminescence signal were initially adjusted to establish the assay conditions. As a result, the following characteristics were determined: dots of 4 µL of gingival protein extracts containing MMPs in a range of 0.5–10 µg produced a strong chemiluminescent signal with a low background at 10 min. To demonstrate the reliability of the application, the optimized method was validated according to analytical criteria such as linearity, precision (based on repeatability and intralaboratory reproducibility), and robustness. In this sense, it was necessary to have a good protein extraction method. The protein extraction protocol performed for all study samples showed yields between 43 and 54.8 µg of protein per mg of processed tissue, achieving enough protein sample for the all proposed analyzes.

Taking into account the intrinsic variability in biological replicates from patients with inflammatory processes (G2 to G4), validation of the established methodology was conducted from samples obtained from G1. In this manner, to verify linearity, six-point matrix-matched calibration curves were traced in a range from 0 to 10 µg of gingival tissue proteins to compare with an instrumental response (dot volume intensity). Regression analysis showed good linearity for both MMPs within the studied range. The mean values of the linear correlation coefficients obtained were 0.989 (±0.004) and 0.992 (±0.002) for purified gingival tissue samples for MMP-8 and MMP-9, respectively. Instrumental responses from each patient were averaged and are presented as a function of applied protein mass and MMP type. The mean intensity of dot volume as a function of protein mass ranged between 13.5 and 270.6 (SD < 4.1) for MMP-8, while that for MMP-9 ranged between 10.4 and 246.6 (SD < 24.8). Good linear correlation coefficients were obtained in both cases, with 0.9936 for MMP8 and 0.9906 for MMP9. Table 1 shows the calibrated curve equations obtained for each MMP type.

To check the intralaboratory reproducibility, the samples were extracted and analyzed. Duplicate samples were evaluated on three different days, and a total of thirty experiments were performed to evaluate this characteristic. The results are included in Table 1. Linear correlation coefficients with values of 0.9890 for MMP8 and 0.9920 for MMP9 (SD < 0.004) were obtained when comparing instrumental responses collected as a function of applied protein mass in both cases. Data analysis demonstrated good repeatability and reproducibility of the designed method in the evaluated range.

Table 1 Method repeatability and reproducibility in the analysis of MMP-8 and MMP-9 in five patients of the Control Group. The reproducibility analysis was performed on 3 different days.

	MMP8			MMP9		
	m	b	R^2	m	B	R^2
Day 1	26,243	−0,1439	0,9873	26,302	8,438	0,992
Day 2	25,721	6,1151	0,9936	23,544	3,7742	0,9906
Day 3	23,711	−0,1597	0,9855	23,798	11,336	0,9943
Mean	25,225	1,937	0,989	24,548	7,849	0,992
SD	1,337	3,618	0,004	1,524	3,815	0,002
Repeatability (intraday)[+]	$y = 25{,}721X + 6{,}1151$ $R^2 = 0{,}9936$			$y = 23{,}544X + 3{,}7742$ $R^2 = 0{,}9906$		
Reproducibility (interday)[+]	$y = (25{,}23 \pm 1{,}34)X + (1{,}937 \pm 3{,}62)$ $R^2 = 0{,}989 \pm 0{,}004$			$y = (24{,}548 \pm 1{,}52)X + (7{,}849 \pm 3{,}82)$ $R^2 = 0{,}992 + 0{,}002$		

[+] The mass of protein extract spotted for patients was in the range of 0.5–10 μg (n = 5). Average calibration equation. Uncertainty values calculated at a 95% confidence limit.

Finally, the method robustness for each MMP was determined through a comparison of slopes presented on calibration curves obtained from various analyses performed on different days using an ANOVA test. Referred analysis did not show a significant difference between slopes, proving the robustness of the method.

3.1. Application

Our validated methodology was applied to a quantitative analysis of MMP-8 and MMP-9 in patients without clinical evidence of GO but with a history of using orthodontic appliances (G2) and in patients diagnosed with mild (G3) and moderate GO (G4) associated with orthodontic treatment. The results were compared against those obtained from healthy donors (G1). It was observed that the lowest levels of MMP expression corresponded to individuals in group 2, while patients in group 4 showed the highest expression levels, preceded by individuals in group 3, as expected. The samples from group 4 showed an MMP-8 expression level 9.9 ± 5.3 times higher than the control group, with 12 ± 6.5 times increased MMP-9 expression compared to G1. For group 3, the MMP-8 and MMP-9 expression levels were 3.4 ± 1.2 and 3.3 ± 0.8 times higher, respectively, when compared to the control group. Finally, in healthy donors with a record of using orthodontic appliances (G2) similar expression levels to G1 were found, with values of 1.2 ± 0.4 and 1.4 ± 0.4 for MMP-8 and MMP-9, respectively (Figs. 3 and 4).

An ANOVA test with Bonferroni correction was performed to compare slopes between study groups (1–4). For MMP-8, a statistically significant difference ($P < 0.005$) was found, but only when comparing groups 1–3 with group 4. Such a difference was observed for MMP-9 expression level when comparing groups 1 and 2 with groups 3 and 4.

4. Discussion

In this study, a dot blot method based on employing monoclonal antibodies for relative quantification of MMP-8 and MMP-9 in human gingival tissue was proposed. The assay was optimized and validated, demonstrating its linearity, repeatability, reproducibility, and robustness.

Application of a previously referred methodology represented an efficient, practical, fast and relatively economical alternative when compared to other methods such as Western blot assay and zymography. With reference to sample preparation, a good performance was obtained with the chosen protein extraction process. Mechanical methods and a soft

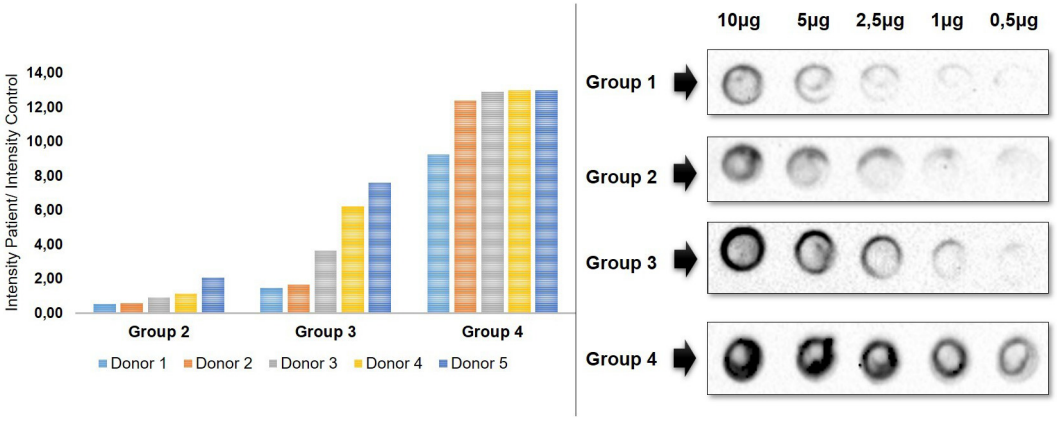

Fig. 3 Expression of MMP-8. Left: Graph represents the relationship between the signal strength of the replicates of each group and the mean intensity of the control. The point chosen for comparison was 2.5 μg. Right: Representative image of a dot blot from each study group for MMP-8.

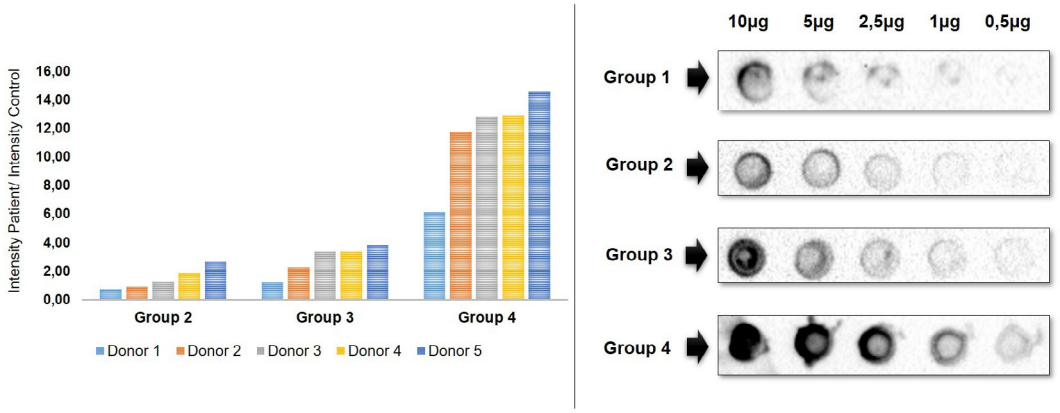

Fig. 4 Expression of MMP-9. Left: Graph represents the relationship between the signal strength of the replicates of each group and the mean intensity of the control. The point chosen for comparison was 2.5 µg. Right: Representative image of a dot blot from each study group for MMP9.

extraction buffer (PBS-Triton X-100 1%) were employed in our procedure; separation processes or aggressive treatments that could include chemical agents were avoided to prevent protein degradation. Since the samples did not require any separation procedure, the processing time was limited to the number of samples to analyze, and considering that they were placed directly on the PVDF matrix, loss of proteins was null, and the processing time was optimized, achieving a good procedure performance. Therefore, it can be said that this method is useful and fast when working with a large series of samples without compromising the precision of the results. Some authors, such as Putra et al, validated a dot blot method through calibration curves for protein quantification employing two different signal processing systems (Putra et al., 2014). From curves, they obtained a linear correlation coefficient (R2) from 0.965 to 0.988, while Guillermin also validated a dot blot quantitative method for measuring protein expression levels, reporting R2 values of 0.97–0.99 (Guillemin et al., 2009), both cases in agreement with the results presented in this study.

It is important to note that our method is reproducible, and it allowed the comparison of various groups, each one with different clinical characteristics. It should be emphasized that although MMP standardization was executed from protein extracts, specific monoclonal antibodies with the ability to recognize only MMP-8 or MMP-9 in a complex protein mixture were used, demonstrating the reliability of the obtained data. Recently, monoclonal antibodies have been used to selectively measure changes in the expression levels of MMP-8 during the progress of periodontal diseases and their treatment with immunofluorometric assays (Rathnayake et al., 2017, Alassiri et al., 2018).

Additionally, the protein dot blot method was also demonstrated to be useful when evaluating samples from patients with a history of using orthodontic appliances, allowing us to measure overexpression of MMP-8 and MMP-9 in the gingiva of patients with GO due to orthodontics. Development of said pathology implies the occurrence of inflammatory and remodeling processes in gingival tissue, which requires MMP-mediated mechanisms for extracellular matrix remodeling (Zanatta et al., 2014, Li et al., 2018). Similar results were reported by Surlin et al., who found that patients with the absence of GO exhibited a significantly increased level of MMP-8 in gingival crevicular fluid the first 4–8 h after installing orthodontic appliances, and MMP-8 and MMP-9 expression levels were more notable in cases when GO showed clinical inflammation (Surlin et al., 2010, 2012). Healthy donors (G1) showed the lowest expression levels among all groups. As there are no standard reference values to measure MMP-8 and MMP-9 expression levels in gingival tissue, the results obtained from this group represent basal expression levels of MMP-8 and MMP-9 in healthy gingiva, associated with physiological functions of remodeling and regeneration.

Even though there was not a significant difference between study groups 2 and 3, the MMP levels for group 2 were higher in all points of curves. These results were interesting, given that samples from these patients were obtained at least one year after orthodontic appliances were removed.

The presence of MMPs that persist in gingival tissue after appliance removal can be associated with other local factors that may result from orthodontic treatment, such as metal release. Nickel, for example as a transition metal, has the ability to generate reactive oxygen species that could be linked to the activation of MMPs (Golz et al., 2014; Hussain et al., 2016; Pazzini et al., 2016).

As evidenced by the present results, a validated protein dot blot assay allowed differentiation between mild and moderate GO. MMP-8 and MMP-9 expression levels were higher in patients with moderate GO in comparison to mild GO, suggesting that MMP expression is related to the clinical condition of gingival tissue and can be considered as a molecular marker for severity classification of this pathology.

However, despite natural biological variability among patients, even in those belonging to the same study groups and with atypical expression levels of MMPs 8 and 9 in healthy donors, validated methodologies have proved to be reproducible and sensitive when measuring these metalloproteinases in gingival clinical samples. Therefore, the importance of the dot blot assay is its compliance of use and its utility to identify variations of expression levels of mentioned proteins, even when study groups present different clinical characteristics.

It is necessary to emphasize that the amounts of tissue required to perform the referred assay was obtained under ethical approval and was compliant with the clinical needs of each

patient. For this reason, execution of this method will not represent a risk for any donor and will guarantee obtaining reliable data for an adequate quantification of MMPs.

5. Conclusions

- Implementation of the protein dot blot methodology for relative quantification of MMPs proved to be fast, highly sensitive, repeatable and economical.
- Through protein dot blot, it was possible to detect differences in MMP-8 and MMP-9 expression between study groups.
- Finally, MMP-8 and MMP-9 measurements with a protein dot blot assay and the associations of detected MMP levels with periodontal status may be considered as a biochemical indicator of onset and progression of GO associated with orthodontics.

6. Ethical statement

Donors were informed about study purpose, surgical procedure, and its possible risks. All of them signed an informed consent form, approving donation of gingival tissue. In case of minors, a responsible adult signed authorization.

This research was approved by institutional ethics committee according to scientific and technical standards for health research established in Resolution No. 008430 1993 of Health Ministry of Colombian republic, as well as Helsinki Declaration by World Medical Association. Authors acknowledged complete autonomy during development of this work and declared absence of any conflict of interest.

7. Contribution of authors

All authors have made substantial contributions to conception and design of the study. JO, ER, AD, DM, were involved in data collection and its analysis. ER, DM, AD, and JO were involved in data interpretation, drafting of manuscript and revising it critically, in the same way, all participants have given final approval of the present version to be published.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sdentj.2020.07.001.

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