EFFECT OF BISPHOSPHONATES ON THE LEVELS OF RANKL AND OPG IN GINGIVAL CREVICULAR FLUID OF PATIENTS WITH PERIODONTAL DISEASE AND POST-MENOPAUSAL OSTEOPOROSIS

María E. Verde[1], Daniela Bermejo[2], Adriana Gruppi[2], Miriam Grenón[1]

[1] *Periodontology Department, School of Dentistry, National University of Córdoba, Córdoba, Argentina.*

[2] *Center of Biochemistry and Immunology Research. School of Chemistry Sciences. National University of Cordoba, Córdoba, Argentina.*

ABSTRACT

The Receptor activator of nuclear factor-kappa B ligand (RANKL)/RANK/Osteoprotegerine (OPG) system has been proposed as essential for osteoclast biology and identified as key part in regulating the physiology and pathology of the skeletal system. The study of the RANKL/RANK/OPG system has increased the understanding of the mechanisms involved in the bone remodeling process, especially in postmenopausal osteoporosis and periodontal disease.

Bisphosphonates have become the mainstay of the treatment and prevention of post-menopausal osteoporosis. They inhibit the formation and dissolution of calcium phosphate crystals in bone and also osteoclasts, thus reducing bone turnover.Current investigations relate osteoporosis with the appearance and progression of periodontal disease. Although the etiology of both is different, the bone loss present in both shares several characteristics. Thus, therapy used for osteoporosis can be considered of value in the treatment of periodontal disease.

The aim of this study was to evaluate the levels of RANKL, OPG and their relationship in gingival crevicular fluid (GCF) in patients with periodontal disease and postmenopausal osteoporosis/ osteopenia in relation to consumption of bisphosphonates.

We studied 66 periodontal active sites obtained from 17 post-menopausal women patients aged between 45-70 years old with osteoporosis/osteopenia and periodontal disease. GCF samples were collected using sterile filter paper strips. To determine the concentration of RANKL and OPG, a commercial ELISA assay was used. The values of RANKL, OPG and their ratio (RANKL/ OPG) were compared with Mann-Whitney U Test.

The values of RANKL, OPG and their ratio obtained in patients with osteoporosis/osteopenia and periodontal disease with or without bisphosphonates treatment showed no differences. Bisphosphonates do not alter the concentration of RANKL and OPG and their ratio in the GCF of patients with osteoporosis/ osteopenia and periodontal disease, probably because these cytokines may not be the main target of bisphosphonates to inhibit bone resorption in periodontal disease.

Key words: *periodontal disease, RANKL, OPG, bisphosphonates, osteoporosis.*

EFECTO DEL CONSUMO DE BIFOSFONATOS EN LOS NIVELES DEL LIGANDO DEL RECEPTOR ACTIVADOR DEL FACTOR NUCLEAR KAPPA-B Y OSTEOPROTEGERINA EN FLUIDO CREVICULAR DE PACIENTES CON ENFERMEDAD PERIODONTAL Y OSTEOPOROSIS POST-MENOPÁUSICA

RESUMEN

El sistema: Receptor activador del factor nuclear kappa-B ligando (RANKL)/RANK/Osteoprotegerina (OPG) ha sido propuestos como esencial para la biología osteoclástica, ya que ha sido identificado como participante clave en la regulación fisiológica y patológica del sistema óseo. El estudio del sistema RANKL-RANK-OPG ha facilitado la comprensión de los mecanismos intervinientes en el proceso de remodelación ósea, especialmente en la osteoporosis post-menopáusica y la enfermedad periodontal.

Los bisfosfonatos se han convertido en el pilar principal del tratamiento y prevención de la osteoporosis post-menopáusica. Ellos inhiben la formación y disolución de los cristales de fosfato de calcio en el hueso y también inhiben a los osteoclastos reduciendo el recambio óseo.

Actualmente, varios trabajos de investigación asocian la osteoporosis con el inicio y la progresión de la enfermedad periodontal. Aunque la etiología de ambas es diferente, la pérdida de masa ósea comparte varias características y la terapéutica utilizada para la osteoporosis puede ser considera de valor para el tratamiento de la enfermedad periodontal.

El objetivo de este estudio fue evaluar el efecto del consumo de bifosfonatos en fluido crevicular (FC) sobre los niveles de RANKL, OPG y la relación RANKL/OPG en pacientes post-menopáusicas con enfermedad periodontal y osteoporosis/ osteopenia.

Se estudiaron 66 sitios periodontalmente activos obtenidos de pacientes mujeres post-menopáusicas con edades entre 45-70 años de edad con enfermedad periodontal y osteoporosis/ osteopenia. La toma del FC se realizó mediante tiras de papel de filtro estériles. Para determinar la concentración de RANKL y OPG se utilizó el ensayo de ELISA comercial siguiendo las instrucciones del fabricante. Los valores obtenidos de las citoquinas y su relación fueron comparados con el Test U de Mann-Whitney.

No se observaron diferencias en las concentraciones de RANKL y OPG encontradas, ni en su relación, en pacientes con enfermedad periodontal y osteoporosis/osteopenia con y sin tratamiento de bifosfonatos.

Esto sugiere que probablemente estas citoquinas no serian el blanco principal de los bifosfonatos para inhibir la resorción ósea en la enfermedad periodontal.

Palabras clave: *enfermedad periodontal, RANKL, OPG, bifosfonatos, osteoporosis*

INTRODUCTION

Periodontal disease is an inflammatory condition caused by multiple factors where the most significant environmental risk factor is the bacteria that reside in the biofilm. According to the report by Page and Kornman[1], these bacteria are essential but not enough to cause periodontal disease, while factors related to the host, such as heredity and environmental factors like smoking or consumption of certain drugs, are important determinants of the occurrence and severity of this condition.

In the appearance and progression of periodontal disease there is an influence of a wide range of determinants and risk factors, among them osteoporosis/osteopenia and estrogen deficiency are included. Immune and skeletal systems share a variety of regulatory molecules such as cytokines. In the bone marrow, immune cells interact with bone cells. Consequently, the physiology and pathology of one system may affect the other; indeed, abnormal activation of the immune system leads to bone destruction [2].

Members of the tumor necrosis factor alpha superfamily have been proposed as essential for osteoclast biology because they were identified as key parts in the physiological and pathological regulation of the bone system [3]. The members RANKL/RANK/OPG form a system involved in osteoclast formation and activation. The binding of RANKL to its receptor RANK, in the presence of macrophage-colony stimulating factor (M-CSF), provides the fundamental signal to drive the development of osteoclasts from hematopoietic progenitor cells, as well as to activate osteoclasts. OPG binds to RANKL and inhibits bone resorption, preventing both cellular differentiation and function of osteoclasts [4]. Osteoclast recruitment depends on the balance between the RANKL and OPG [5].

The RANK-RANKL-OPG axis is involved in the regulation of bone metabolism in periodontitis and osteoporosis/osteopenia, in which an increase in the relative expression of RANKL and a decrease in OPG can favor the balance to osteoclastogenesis and bone reabsorption [6,7]. Increased RANKL and decreased OPG have been observed in various inflammatory and bone diseases such as osteoporosis, rheumatoid arthritis, periodontal disease, and multiple myeloma [8-10]. Local deregulation of RANKL-OPG levels may lead to alveolar bone reabsorption as has been demonstrated in experimental models of periodontitis [11,12]. Many studies show that, compared to healthy subjects, patients with periodontitis exhibit higher RANKL expression and a reduction of OPG levels in GCF [10,13,14], or gingival tissue [14-17]. Periodontitis and osteoporosis represent main health problems, especially in elderly women. Although the etiology of postmenopausal osteoporosis and periodontal disease is different, the bone loss that occurs in both diseases shares several features [6]. Thus, therapy used for osteoporosis can be considered of value in the treatment of periodontal disease. Among the many drugs for osteoporosis treatment, bisphosphonates are the most commonly prescribed and first-line drugs in most cases [18]. Bisphosphonates have been characterized as modulators of osteoclast function and bone metabolism [19]. Particularly they act on bone tissue, decreasing bone turnover, reducing bone resorption and the number of new bone multicellular units. At cellular level, they reduce the recruitment of osteoblasts and osteoclasts, the adhesion of osteoclasts to the bone as well as the release of cytokines by macrophages. Considering RANKL expression is essential for osteoclast differentiation; the effect of bisphosphonates on osteoclast differentiation could be related to a decrease in the expression of RANKL or by an increase of OPG. On the basis of these properties, several generations of bisphosphonates have been successfully developed in the treatment of post-menopausal osteoporosis, osteopenia and Paget's disease [20].

Considering that RANKL/RANK/OPG system mediates periodontal disease and osteoporosis/osteopenia and that bisphosphonates are established as one of the effective drugs for osteoporosis treatment and that they can act on the RANKL/RANK/OPG mentioned, the aim of this study was to investigate the effect of bisphosphonate treatment on the levels of RANKL and OPG, and their ratio in GFC of post-menopausal women patients with periodontal disease and osteoporosis/osteopenia.

MATERIALS AND METHODS

Study population and clinical examination

A total of 66 samples of GFC were obtained from active periodontal sites of 17 post-menopausal women aged between 45-70 years old with and without bisphosphonate-treatment (risedronate or ibandronate: 150 mg/ 1 tablet a month for at least 3 months, prior to the study), all with chronic

periodontitis and osteoporosis/osteopenia. All women were recruited from the Cimateric Department, University Hospital of Maternity and Neonatology, National University of Cordoba, Argentina. The study protocol was approved by a Bioethical Institutional Committee and explained to all participants who signed the informed consent forms.

Complete medical and dental histories from all patients were considered. None of them had had any systemic illness nor taken therapeutic medication such as antibiotics or anti-inflammatory drugs that could affect the periodontal status for at least 6 months before the study, and also they did not have a history of aggressive periodontitis and they had not received periodontal treatment before they entered the study. The selection of the patients was made according to criteria proposed by the World Health Organization [21]. To determine the clinical periodontal status, all subjects were subject to a clinical periodontal examination including the measurement of probing pocket depth (PPD) and clinical attachment level (CAL) at six sites around each tooth with a manual probe, using a Marquis probe. Dichotomous measurement of supragingival plaque index (PI) and bleeding index (BI) were also recorded.

Diagnosis of osseous and periodontal disease

The presence of 1 or more sites with PPD ≥ 4 mm and ≥ 4 mm CAL [22] in a patient was defined as/considered to be periodontitis. Sites with bleeding on probing were initially defined as active sites [8].

Dual X-ray absorptiometry (DXA) was used to evaluate the bone mineral density, since this method is considered "gold standard" for the diagnosis-prognosis of osteoporosis, monitoring the natural history of the disorder and response to treatment [21]. The diagnosis of osteoporosis/osteopenia was verified from the densitometry reports from proximal femur and lumbar column, following the criteria established by the WHO at the Consensus Development Conference [23].

Osteopenia was defined as a bone mineral density T score (difference between the measured bone mineral density and the mean value for young white women in standard deviations [SDs]) of less than –1 SD or at least –2.5 SD. Osteoporosis was defined by a bone mineral density T score of less than –2.5 SD [23]. The individuals with osteopenia were grouped with those with osteoporosis to form an osteopenia/osteoporosis group.

Sampled sites were then categorized into two groups: a group of 12 post-menopausal women with osteoporosis/osteopenia treated with bisphosphonates (n=55 samples) and a group of 5 post-menopausal women with osteoporosis/osteopenia without bisphosphonates therapy (n = 11 samples).

Collection of Gingival Crevicular Fluid

GCF was obtained using sterile filter paper strips (Periopaper; Oraflow, New York, NY, USA). The selected sites were cleared of supragingival plaque, isolated with cotton rolls and dried with a gentle stream of air to prevent saliva contamination. A sterile Periopaper™ strip (ProFlow Inc., Amityville, NY, USA) was gently inserted into the periodontal pocket until mild resistance was felt and it was left in place for 30 seconds. Mechanical irritation was avoided and strips contaminated with blood were discarded.

After GCF collection, strips were placed in an Eppendorf tube and samples were stored at -80°C before laboratory analysis.

4-5 GCF samples were obtained from post-menopausal women from the group with osteoporosis/osteopenia under bisphosphonate treatment and 2-3 GCF samples were obtained from post-menopausal women from the group with osteoporosis/osteopenia without bisphosphonate therapy.

Paper strips for each patient were pooled, and the GCF was extracted and assayed for the content of RANKL and OPG. GCF was extracted from the paper strips with buffer (50 mM phosphate buffer, pH 7.2, containing protease inhibitors), and collected after centrifugation at 15,000 g and 4ºC for 10 min.

Quantification of RANKL and OPG

Concentrations of RANKL and OPG from GCF samples were determined by ELISA, following manufacturer's instructions (Antigenix America Inc., New York, NY, USA) and the ratio of both cytokines was established.

Briefly, diluted GCF samples were loaded into single wells of a 96-well plate coated with 20 ng/ml of anti-human RANKL antibody or with 4 ng/ml of anti-human-OPG antibody, and incubated overnight at 4°C. Plates were washed four times. After the last wash, 0.2 ug/ml of biotin labelled anti-human

RANKL or OPG were added and incubated for 60 minutes at room temperature.
The reaction was developed with the addition of streptavidin coupled to peroxidase and visualized with the addition of 3.3'-5.5'-tetramethylbenzidine which was transformed into a colored product in the presence of the enzyme. The reaction was stopped by the addition of sulfuric acid and color was measured in a microplate spectrophotometer at 450 nm.
The concentration was measured with reference to standard curves using known amounts of recombinant RANKL and OPG.

Data Analysis

Data were expressed as means ± standard deviation. Taking into account the size of samples, a non-parametric analysis (Mann-Whitney Test U) was used. GraphPad Software (version 5.00 for Windows, San Diego California USA) was used to process the data. P-values ≤0.05 were considered statistically significant.

RESULTS

Clinical parameters of the patients

Fifty five periodontally active sites from twelve patients were analyzed in the study group of post-menopausal women with osteoporosis/osteopenia treated with bisphosphonates (age: 56.21 ± 9.24 years old) and eleven periodontal active sites from five patients were studied in the control group of postmenopausal women without treatment with bisphosphonates (age: 56.55 ± 4.68 years old). Table 1 summarizes the clinical characteristics of subjects included in this study. No statistically significant differences in clinical characteristics (age, PPD, CAL, PI and BI) were observed between groups. In the case of clinical records, PPD and CAL were obtained from an average of measured active sites, while the BI and PI were an average of the entire oral cavity.

Table 1: Baseline characteristics of the study groups (mean ± SD).

	Osteoporosis/ Osteopenia NT	Osteoporosis/ Osteopenia WT	
Clinical parameters	**(n=11)**	**(n=55)**	***P***
Age (years)	**56.55 ± 4.68**	**56.21 ± 9.24**	**0.1898**
PPD (mm site)	3.5 ± 0.7	3.3 ± 1.2	0.493
CAL (mm site)	**3.7 ± 1.19**	**3.2 ± 1.56**	**0.1727**
PI (%)	68.74 ± 25.63	59.14 ± 37.34	0.1815
BI (%)	**54.33 ± 21.88**	**68.12 ± 30.76**	**0.2115**

PPD: Probing pocket depths. CAL: Clinical attachment loss. PI: Plaque index. BI: Bleeding index. NT: Not treated with bisphosphonates. WT: With bisphosphonate treatment.

Levels of RANKL, OPG and RANKL/OPG ratio in GCF from patients treated or not with bisphosphonates

GCF samples obtained from subjects under bisphosphonate treatment (n= 55) showed no significant differences in the levels of RANKL in comparison with the group without bisphosphonates treatment (n = 11), p = 0.88. The values were 26.81 ± 18.66 and 27.78 ± 19.50 pg/ml, respectively (Fig. 1).
OPG showed similar behavior to RANKL. OPG concentrations showed no statistically significant difference (p = 0.62) between both group of patients, whose values were 0.05 ± 0.02 pg/ml in GCF samples from subjects under treatment and 0.04 ± 0.01 pg/ml in GCF samples from patients without treatment (Fig. 2).
As the RANKL/OPG ratio is considered indicative of bone resorption, we investigated if changes in RANKL and OPG levels reflected changes in this ratio. The group under bisphosphonate treatment exhibited a RANKL/OPG ratio of 693.5 ± 561.3 pg/ml, while the group without bisphosphonate treatment showed similar values, 677.7 ± 437.6 pg/ml. There were no significant differences among these groups (Fig. 3).

DISCUSSION

Bisphosphonates have been shown to inhibit the progression of osteoporosis as a result of the reduction of bone loss and they have been characterized as modulators of osteoclast function and bone metabolism [19].

Previous reports suggest that bisphosphonates can reduce not only the maturation and osteoclast function directly, but they can also act indirectly through the synthesis of mediators that interfere with osteoclastogenesis [24-26], further regulating essential signaling molecules involved in osteoclastogenesis such as RANKL [27]. The system RANKL/RANK/OPG acts as a final effector molecular system. RANKL,

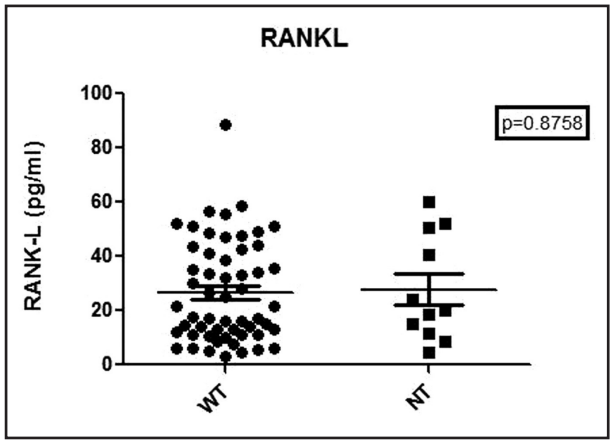

Fig. 1: Distribution levels of RANKL in GCF from active periodontal sites of women with bone disease under bisphosphonate treatment –WT- (n = 55) or without treatment–NT- (n = 11). The individual values represent the concentration of RANKL in GCF [Total RANKL (pg) / volume (ml)] in each patient.

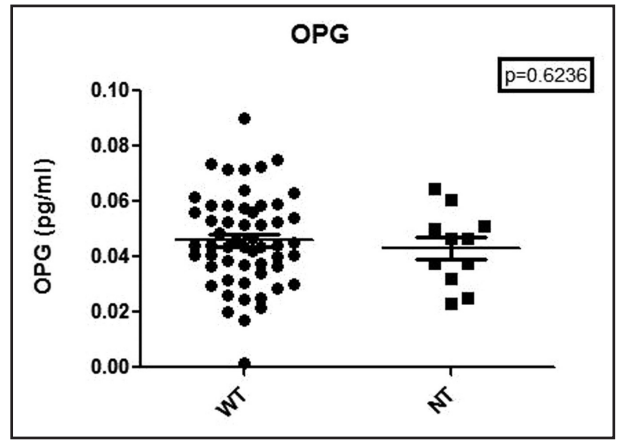

Fig. 2: Distribution levels of OPG in GCF from active periodontal sites of women with bone disease under treatment–WT-(n = 55) or without treatment –NT-(n= 11). The individual values represent the concentration of GCF in OPG [Total OPG (pg) / volume (ml)] in each subject..

RANK, and the decoy receptor OPG, are three key molecules that regulate the recruitment and function of osteoclasts [28]. The binding of RANKL to RANK provides the fundamental signal to drive the development of osteoclasts from hematopoietic progenitor cells, as well as to activate mature osteoclasts. OPG negatively regulates by binding to RANKL, thus inhibits bone reabsorption by preventing both cellular differentiation and function of osteoclasts [4,28]. The recruitment of osteoclasts depends on the balance between RANKL and OPG [5]. To our knowledge, this is the first clinical trial to study the effect of risedronate/ibandronate treatment on RANKL and OPG levels in GCF and their relative ratio in post-menopausal women with osteoporosis/ osteopenia and periodontal disease.

Several studies evaluated the effect of bisphosphonates on the levels of RANKL and OPG; however, the results have been controversial. Tipton et al. [29] demonstrated that the action of alendronate and pamidronate on human gingival fibroblasts, through altering the production of RANKL and OPG, appears to contribute to a microenvironment that favors the inhibition of bone reabsorption due to an increase or no change in the levels of OPG, and a decrease in the production of RANKL. Viereck et al. [30] have demonstrated that bisphosphonates such as pamidronate and zoledronate increase the expression of OPG mRNA in primary human osteoblasts. Pan *et al.* [31] demonstrated that zoledronic acid may inhibit

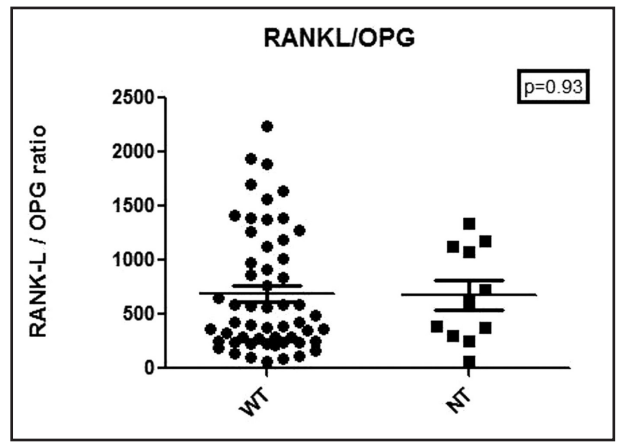

Fig. 3: Distribution levels of RANKL / OPG in GCF from active periodontal sites of women with bone disease under treatment -WT-(n = 55) or without treatment –NT-(n= 11). The individual values represent the concentration in GCF of the RANKL / OPG [Total RANKL (pg) / volume (ml) / total OPG (pg) / volume (ml)] in each subject.

bone reabsorption by reducing the expression of RANKL and increasing transmembrane OPG secretion in cells such as osteoblasts. However, it does not affect gene expression of RANKL and OPG. Dobnig et al. [32] showed a significant increase in serum levels of OPG in patients treated with alendronate and risedronate, whereas serum levels of RANKL were unchanged throughout the treatment period; on the other hand, a positive correlation between changes in serum levels of OPG and BMD was found.

On the contrary, Kim et al. [33] demonstrated that nitrogen-containing bisphosphonates, such as alendronate and pamidronate, do not alter the RANKL and OPG mRNA expression in favor of the inhibition of the osteoclast formation [30]. Alvarez et al. [34] demonstrated that serum OPG decreases after treatment with tiludronate. In contrast, RANKL serum levels and RANKL/OPG ratio are unmodified in patients with Paget's disease. D'Amelio et al. [35] demonstrated that risedronate inhibits the *in vitro* formation of osteoclast by reducing the number and degree of differentiation of osteoclast precursors and by reducing the half-life and the inhibition of the production of pro-osteoclastogenics cytokines. Moreover, risedronate is able to reduce the recruitment of osteoclasts from peripheral blood and this could be the effect of the reduction in cytokine production. Besides, it was observed a reduction in the levels of OPG and RANKL (soluble) in serum during treatment with bisphosphonates. A study by Kwak et al. [36] concluded that risedronate inhibitory effects on osteoclast differentiation are related to decreased expression of RANKL.

According to previous studies, osteotropic agents modulate osteoclast formation through the regulation of RANKL/OPG ratio instead of increasing or decreasing levels of RANKL or OPG alone [37,38]. The increase in the RANKL/OPG ratio results in a favorable condition for osteoclast formation and vice versa.

In this study it was observed that risedronate and ibandronate consumption did not change the GCF RANKL and OPG levels, and RANKL/OPG ratio in patients suffering osteoporosis/osteopenia and periodontal disease. So it could be suggested that RANKL and OPG might not be the main target of bisphosphonates to inhibit bone resorption in periodontal disease, reaffirming the results of Kim et al. [33] and Pan et al. [31].

The discrepancy in the results about the effects of bisphosphonates on the above cytokines can be attributed to several reasons. Firstly, the research design used was different to previous reports. Experimental studies were carried out with mice [33,36] and with human cells *in vitro* [29-31]; however, in this study, the effect of bisphosphonates was evaluated on patients, as in other researches [32,34,35]. Secondly, all drugs analyzed were aminobisphosphonates and they had a different chemical structure. Thirdly, the methods of analysis of the cytokines varied if the gene expression or the presence of proteins was studied.

In conclusion, the present study showed that nitrogen-containing bisphosphonates, ibandronate and risedronate, did not alter the GCF RANKL and OPG levels and their relative ratio in periodontal disease. It could be suggested that RANKL and OPG might not be the main target of bisphosphonates to inhibit bone resorption in periodontal disease.

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CORRESPONDENCE

Dr. María Eugenia Verde
Juan Antonio Barcena 121 – B° Teodoro Felds.
Córdoba Capital. C.P 5009
Argentina
mverde@odo.unc.edu.ar

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