

Salivary and Serum Chromogranin A and α -Amylase in Periodontal Health and Disease

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Background: Salivary stress-related biomarkers in connection with periodontal disease have not been extensively studied. In addition to cortisol as a well-known marker of stress loading, chromogranin A (CgA) and α -amylase (AA) are supposed to link the activity of the neuroendocrine system to local and systemic immune functions and to be related to periodontitis. This study aims to determine CgA and AA in saliva and serum in periodontal health and disease to assess their potential relationship to periodontitis.

Methods: Patients with aggressive (AgP) ($n = 24$) and chronic periodontitis (CP) ($n = 34$) as well as healthy control (CO) ($n = 30$) individuals participated in this study. CgA and AA were determined in saliva and serum with enzyme-linked immunosorbent assay and an adapted clinical amylase test; salivary cortisol was determined using mass spectrometry. Clinical parameters of periodontal disease were evaluated, and their possible correlations with stress-related biomarkers were assessed.

Results: Significantly higher CgA levels were found in the saliva of patients with AgP compared with those in patients with CP and CO individuals ($P < 0.001$). Salivary cortisol levels were higher in the AgP group compared with those in patients with CP ($P < 0.05$). No differences in serum CgA levels and salivary and serum AA activities were found among all groups. A positive correlation was revealed between salivary AA activity or salivary CgA levels and the extent of periodontitis ($P < 0.05$).

Conclusion: The results suggest an association of CgA and cortisol levels as well as AA activity in saliva with periodontitis, especially a significant relationship of salivary CgA and cortisol to AgP. *J Periodontol* 2012;83:1314-1321.

KEY WORDS

α -Amylases; biological markers; chromogranin A; periodontitis; saliva; stress, psychological.

Periodontitis is an inflammatory disease caused by periodontopathic bacteria in the dental biofilm, leading to destruction of the tooth-supporting tissues. Systemic diseases, habits, social factors, and psychologic stress are considered risk factors influencing disease onset and progression.¹ Although psychologic stress was found to be an important risk factor for periodontitis,² the biologic mechanisms of its implication for disease progression remain unclear. Psychologic stress can downregulate the cellular immune response in three different ways: 1) the hypothalamo-pituitary-adrenal (HPA) axis; 2) the peripheral release of neuropeptides; and 3) the sympathetic nervous system (SNS) via the release of adrenaline and noradrenaline.³ The psychoneuroimmunologic model tries to link stress and periodontitis via mentally triggered alterations of immunologic responses. It is assumed that periodontitis is negatively influenced by inappropriate coping behavior under stress, which leads to a centrally mediated immune suppression.⁴ Cortisol, one of the stress-related biomarkers of the HPA axis, has already been shown to be positively associated with the extent and severity of periodontitis.^{5,6}

Saliva testing is a novel diagnostic tool with the advantage of quick, painless, and non-invasive sampling; thus, it may become an important future diagnostic method for various diseases,^{7,8} in

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particular for oral diseases. Many biomarkers have been found in saliva, and some of these were shown to be involved in periodontal disease,⁹ mainly immune-response biomarkers, such as matrix metalloproteinases-8 and -9, osteoprotegerin, and interleukin-1 β .¹⁰ New salivary biomarkers are still required to facilitate the detection of early stages of periodontitis, which may lead to earlier medical intervention.¹¹ In addition to their potential for disease diagnostics, the role of salivary peptides in the pathogenic mechanisms of periodontal disease could be explored.

Cortisol is a well-established stress biomarker and is regulated by adrenocorticotrophic hormone from the pituitary gland. Cortisol levels in saliva are correlated to serum and reliably reflect the activation of HPA, which is considered as an indicator of psychologic stress.¹² Several studies have already evaluated the association of cortisol, stress, and inadequate coping in periodontitis.⁵ It was also reported that salivary cortisol is correlated with alveolar bone loss and with the extent and severity of periodontitis.^{6,13}

Chromogranin A (CgA) is an acidic phosphorylated secretory glycoprotein that is stored and coreleased with catecholamines from the adrenal medulla and secreted by sympathetic nerve endings,^{14,15} as well as by serous and ductal cells of the human submandibular gland.¹⁶

Usually, salivary CgA levels peak at the time of awakening and keep stable during daytime.¹⁷ Higher salivary CgA levels have been observed under various circumstances, such as dry mouth¹⁸ or traffic noise exposure.¹⁹ In contrast, lower salivary CgA levels have been observed under stress-relieving events, such as exposure to negative air ions²⁰ or inhalation of lavender aroma.²¹ In an investigation with children, salivary CgA was shown to be a more sensitive marker of psychologic stress compared with salivary cortisol.²² Because CgA is released during stress and is involved in the innate immunity response to bacteria, fungi, and yeasts by its highly cationic nature,²³ it may play a role in the neuro-immune-protective response to stress accompanied by infectious diseases.²⁴

In addition to CgA, salivary α -amylase (sAA) has been suggested to be a reliable index for reflecting SNS activity, in particular under stress situations.²⁵ So far, only some studies have investigated the relationship of salivary CgA levels and sAA activity to the clinical severity of chronic periodontitis (CP).²⁶⁻²⁸ However, one of these studies included patients after complete periodontal therapy,²⁷ and another study included patients with only three teeth left.²⁶ None of these studies differentiated between CP and aggressive periodontitis (AgP) or assessed CgA and AA in the serum.

Hironaka et al.²⁶ investigated salivary CgA levels in an older population of periodontitis patients and found that higher CgA levels were present in individuals with severe clinical attachment loss (AL). Rai et al.²⁷ concluded that stress and salivary stress markers correlate with periodontal disease. Sanchez et al.²⁸ assumed that stress caused by periodontal inflammation may lead to the release of some salivary proteins, such as sAA. However, it still remains unclear whether such stress markers are related to different types of periodontal disease; therefore, the present study investigates stress-related biomarkers in both CP and AgP patients.

This study aims to reveal CgA levels and AA activity in saliva and serum in AgP and CP patients as well as in healthy individuals to assess their potential association with different types, severity, and extent of periodontitis.

MATERIALS AND METHODS

Study Population

In total, 88 individuals (30 periodontally healthy individuals (CO), 34 patients with CP, and 24 patients with generalized AgP, including smokers and non-smokers) participated in this cross-sectional study, performed from November 2008 to July 2010 in compliance with an approved protocol by the ethics committee of the Medical University of Vienna (EK 623/2007) (Table 1). All patients and CO individuals were recruited at the Department of Periodontology at the Bernhard Gottlieb School of Dentistry after giving written consent. Exclusion criteria for all groups included the following: 1) <20 teeth; 2) any apparent oral infection (i.e., herpes or candida); 3) injuries or bleeding in the oral cavity unrelated to periodontitis; 4) periodontal treatment and antibiotic medication within the past 3 months; 5) salivary gland dysfunction; 6) acute illness (fever, sore throat); 7) systemic disease, mental diseases, immune-suppressive medication, or immunodeficiency; 8) pregnancy or lactation; 9) allergies to benzoic acid; and 10) in particular, intake of sedatives, tranquilizers, or antidepressants.

Clinical Examination

For periodontal diagnostics, probing depth (PD), AL, and bleeding on probing (BOP) were recorded at six sites per tooth by experienced periodontists (HH, KB, ML) at the Department of Periodontology, Medical University of Vienna, Austria, with a periodontal probe.^{||} Every participant underwent a panoramic radiographic examination. Bone loss in the periodontitis groups was additionally evaluated with intraoral radiographs. Only severe forms of periodontitis (supporting bone loss $\geq 30\%$),²⁹ with ≥ 6 teeth with PD ≥ 5 mm or AL ≥ 5 mm, were included. Periodontitis

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Table 1.
Description of the Study Population (N = 88)

Variable	Periodontal Health	AgP	CP
Number of patients	30	24	34
Age (years; mean \pm SD)	33.27 \pm 7.19	31.38 \pm 6.42	43.09 \pm 4.06
Female (n)	18	10	15
Smokers (n)	12	14	17
Number of teeth (mean \pm SD)	28.57 \pm 1.57	28.29 \pm 2.91	27.44 \pm 2.35

was classified according to the American Academy of Periodontology classification of 1999,²⁹ seeing age and pattern of disease as the main criteria for the AgP group. The CO group was determined by the absence of radiographic bone loss, PD \leq 3 mm, and no gingival inflammation.

Saliva and Serum Collection and Biomarker Analysis

Stimulated whole saliva from all groups was collected from 8:00 am to 11:00 am using a rinsing solution of a saliva collection system,[¶] according to the instructions of the manufacturer. Participants were not allowed to eat, drink, smoke, brush their teeth, or put anything else in their mouth from midnight on the day before sampling so as to avoid contamination of the saliva sample. Sampling in the periodontitis groups was exclusively made before a planned conservative periodontal therapy. Peripheral whole blood was collected only after saliva sampling to minimize alterations of salivary biomarkers attributable to possible anxious reactions to the blood collection. After 30 minutes to allow for blood clotting at room temperature, all samples were centrifuged for 10 min at 3,220 rpm at 4°C and immediately aliquoted and frozen at -80°C until analysis.

The percentage of whole saliva per sample was calculated photometrically by means of a saliva quantification kit.[#] CgA levels were analyzed with a CgA enzyme-linked immunosorbent assay (ELISA) kit for saliva (assay range, 0.14 to 33.33 pmol/mL; intra-assay coefficient of variation [CV] <12%; interassay CV <14%)** and a CgA ELISA kit for serum (assay range: 10 to 100 ng/mL; intra-assay CV <9%; interassay CV <10%),^{††} according to the instructions of the manufacturer. AA activity was measured according to the kinetic test for quantitative determination of AA,^{‡‡} following a dilution protocol for saliva (1:50) (assay range: 10 to 4,800 U/L; intra-assay CV <1.4%; interassay CV <3.3%). Cortisol quantification was conducted by ultra performance

liquid chromatography in tandem with mass spectrometry^{§§} (assay range: 0.05 to 8 ng/mL; intra-assay CV <10%; interassay CV <15%). The salivary levels of all parameters were corrected by the actual dilution factor from saliva collection.

Statistical Analysis

All groups were compared with respect to age and the number of teeth by univariate

analyses of variance. Within the periodontitis patients, the differences in periodontal parameters mean PD, mean AL, the number of teeth with PD \geq 5 mm, and the number of teeth with AL \geq 5 mm were analyzed by *t* test, and BOP was compared by the Mann-Whitney *U* test. The dependency among salivary CgA, serum AA, sAA, salivary cortisol, and the variables age, sex, smoking status, or diagnosis (CO versus AgP versus CP) were calculated with univariate linear models. Correlations between different parameters were analyzed using Spearman correlation coefficients. Because salivary CgA, serum AA, sAA, and salivary cortisol were not normally distributed, these variables were log-transformed. All analyses were conducted using statistical software,^{|||} and the significance level was set to 0.05.

RESULTS

Among the 88 participants, 49% were females and 49% smoked cigarettes. In terms of age, the CP group was significantly older than the AgP and CO groups ($P < 0.05$). The number of teeth did not differ between all groups ($P = 0.13$) (Table 1). The results of periodontal examination of periodontitis patients are given in Table 2. BOP, mean PD, mean AL, and the number of teeth with PD \geq 5 mm or clinical AL \geq 5 mm as indicators of disease activity and extent were similar between the AgP and CP groups. Mean CgA levels in saliva were >2 times higher in patients with AgP compared with patients with CP and healthy individuals ($P < 0.001$) (Fig. 1A). There was no significant effect of age, sex, or smoking on salivary CgA levels ($P > 0.31$). No significant difference of sAA activity was observed in all groups

¶ Greiner Bio-One, Kremsmuenster, Austria.

Greiner Bio-One.

** YK070 Human CgA EIA kit, Yanaihara Institute, Shizuoka, Japan.

†† Chromogranin A ELISA kit, Labor Diagnostika Nord, Nordhorn, Germany.

‡‡ Olympus System Reagent 6182, Olympus AU640, Olympus Diagnostic Systems, Center Valley, PA.

§§ Acquity/Xevo UPLC-MS/MS system, Waters, Milford, MA.

||| SAS 9.1, SAS Institute, Cary, NC.

(Fig. 1B). The salivary cortisol levels in the AgP group were significantly higher than those in the CP group ($P < 0.05$) and had a tendency to be higher compared with those in the CO group (Fig. 1C). Concerning serum analysis, CgA levels and AA activity were not significantly different among any groups (Fig. 2).

The correlations between salivary CgA, sAA, or salivary cortisol and clinical parameters within the periodontitis groups were analyzed. Among those parameters, both salivary CgA levels and sAA activities were positively correlated with the number of teeth with PD ≥ 5 mm in periodontitis patients ($P < 0.05$) (Fig. 3). However, this correlation was not identified in the AgP and CP groups separately. Salivary cortisol levels tended to be positively correlated with the number of teeth with PD ≥ 5 mm, but this correlation did not reach a statistical sig-

nificance (Spearman correlation, $\rho = 0.227$; $P = 0.089$) (data not shown). No correlation of CgA and AA in serum with clinical parameters of periodontal disease was found (data not shown). The levels of salivary CgA and serum CgA within all groups did not correlate significantly (Spearman correlation, $\rho = 0.015$; $P > 0.88$) (data not shown), whereas serum AA and sAA significantly correlated (Spearman correlation, $\rho = 0.475$; $P < 0.001$) (data not shown).

DISCUSSION

There are no reliable specific markers that can help to predict periodontal disease because multiple factors trigger disease onset and progression. Inflammatory markers were shown to be useful for evaluating disease progression³⁰ but are lacking in the specific indication for detecting the onset of periodontal disease. Stress-associated factors were suggested to be potential markers for evaluating the etiopathogenesis of periodontitis.²⁶ Among stress-related peptides, CgA and AA in saliva were demonstrated to be related to periodontal disease by some studies.²⁶⁻²⁸ The excessive stress load resulting in the release of these peptides is supposed to be one of the risk factors involved in the pathogenic process of periodontitis.^{2,31} Nevertheless, the biologic function and clinical implication of

Table 2.
Clinical Examination of Patient Groups (N = 58)

Variable	AgP (n = 24)	CP (n = 34)	P value
BOP (%; median interquartile range)	42.05 ± 25.11	31.53 ± 17.91	0.14*
PD (mm; mean ± SD)	3.70 ± 0.90	3.74 ± 1.09	0.87†
AL (mm; mean ± SD)	4.12 ± 0.99	4.19 ± 1.38	0.83†
Number of teeth with PD ≥ 5 mm (mean ± SD)	19.75 ± 8.09	16.35 ± 7.60	0.09†
Number of teeth with AL ≥ 5 mm (mean ± SD)	21.54 ± 7.56	18.68 ± 7.40	0.16†

* Mann-Whitney U test.

† t test.

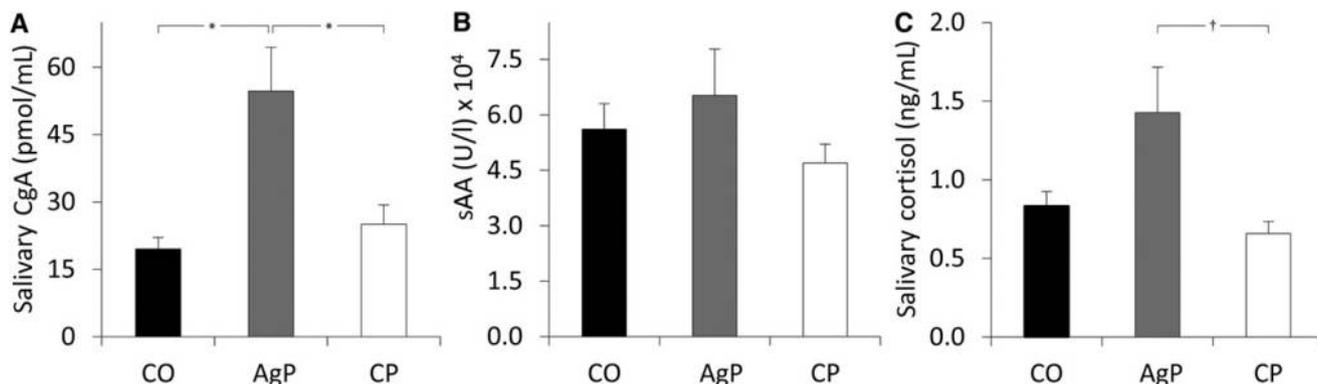
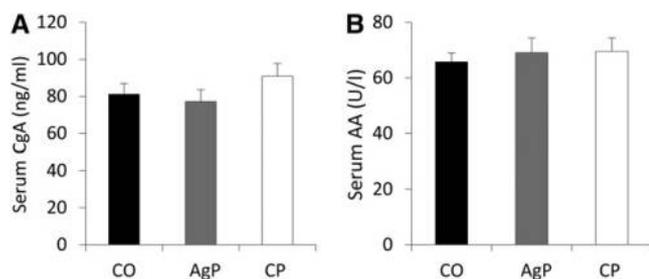
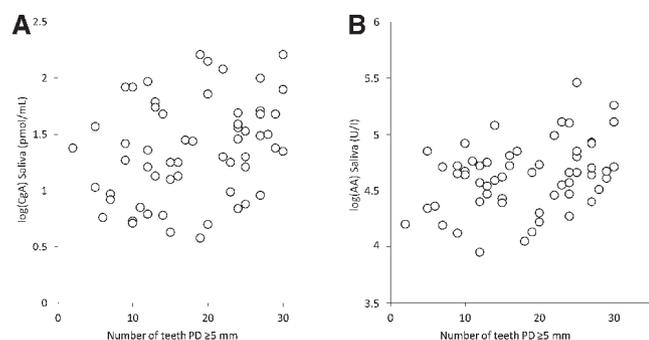


Figure 1. Salivary CgA (A), sAA (B), and salivary cortisol (C) in CO, AgP, and CP groups (mean ± SEM). Salivary CgA levels differed significantly in AgP compared to CO and CP groups (univariate analysis, * $P < 0.001$). Salivary cortisol levels were significantly higher in the AgP group compared to the CP groups (univariate analysis, † $P < 0.05$).

**Figure 2.**

Serum CgA (A) and serum AA (B) in the CO, AgP, and CP groups (mean \pm SEM).

**Figure 3.**

Correlations between CgA in saliva (A) (Spearman correlation, $\rho = 0.290$; $P < 0.05$) or sAA (B) (Spearman correlation, $\rho = 0.302$; $P < 0.05$) and the number of teeth with PD \geq 5 mm in periodontitis (AgP and CP).

such peptides in the pathogenesis of periodontitis still need to be evaluated.

In the present study, salivary CgA levels in the AgP group were revealed as significantly higher compared with the CP and CO groups. Similarly, the levels of salivary cortisol were increased in the AgP group compared with the other groups. Salivary CgA levels were independent of age, sex, or smoking but were significantly associated with the extent of periodontitis. In addition, it was found that salivary CgA levels in all periodontitis patients positively correlated with the number of teeth with deep periodontal pockets. This observation is consistent with that of previous studies,^{26,27} which also demonstrated a positive correlation between salivary CgA and the extent of periodontitis. The increased secretion of these peptides in saliva of AgP patients may be either attributable to the alteration of neuroendocrine immune functions and the reaction of the nervous system interpreting immune activation as a stressor or linked to a higher systemic stress loading.³²

Psychologic stress was shown to be related to periodontitis through changes in behavior and immu-

nologic and inflammatory responses,¹³ and salivary CgA has been suggested to be a biomarker of stress. The activation of the SNS in inflammatory diseases may be expected to alter trafficking of inflammatory cells, the production of inflammatory mediators, tissue injury, and tissue repair.³³ Like catecholamines, CgA or AA might modify the production of pro-inflammatory and anti-inflammatory mediators and thus influence disease activity.

To date, the pathophysiologic functions of CgA in the oral cavity are still not clear. CgA may be not only a stress-related biomarker reflecting the SNS but also a peptide involved in oral bacterial infection. A previous study reported that vasostatin-1, which is the N-terminal fragment of CgA, is released by bovine polymorphonuclear neutrophils (PMNs) during stress.²⁴ Because PMNs form the first line of innate immunity against bacterial attack in periodontal disease, it is also possible that CgA fragments are locally produced by human PMNs in periodontitis. In addition to the submandibular gland and PMNs, Merkel cells may be possible sources of salivary CgA, which was already shown in mammalian oral mucosa.³⁴ Regulatory links exist between the secretion of the stress-related marker CgA and inflammatory conditions. It has been suggested that accumulated PMNs at sites of inflammation, which are stimulated by lipopolysaccharides, provide CgA peptides.³⁵ These peptides have endocrine effects, such as the inhibition of tumor necrosis factor- α -induced extravasation or the stimulation of histamine release. CgA is considered to be a pluripotent prohormone for the modulation of homeostatic processes, such as inflammation and the first phase of microbial invasion.³⁵ The results of this study suggest that CgA may play a role in the communication between the neuroendocrine and immune systems³⁶ and link stress to the local inflammatory reaction in AgP. Higher CgA levels in saliva but not in serum strongly supported the special process of local immune response in patients with AgP.

As reviewed recently,¹ an increasing number of studies focused on the altered inflammatory and immunologic profiles of periodontitis patients caused by stress. In the psychoneuroimmunologic model, the immune system is suppressed and inflammation aggravated by psychologic stress through inadequate coping behavior.⁴ One previous study³⁷ demonstrated that patients with generalized AgP exhibited a significantly increased prevalence of depression compared with individuals in the CP and CO groups. The relationship of psychologic stress to periodontitis was investigated previously with the help of questionnaires, such as the Modified and Perceived Stress Scale³⁸ in an AgP population. Patients with higher stress levels exhibited disease progression over a

5-year observation period.³⁸ Because of the diversity of questionnaires used as psychometric instruments and the lack of a standardized psychologic analysis for the quantification and definition of most psychiatric disturbances,³ biologic markers could be more objective to monitor the psychosocial status. Therefore, salivary cortisol as a biochemical marker for stress was included in this study. In the current study, salivary cortisol levels were higher in the AgP group compared with the CP and CO groups, which indicates an excessive stress loading in this group. Increased salivary CgA levels may also be influenced by cortisol itself because it is supposed that salivary glands are a target for glucocorticoid action,³⁹ but additional investigations are needed to prove a possible interdependence. Increased cortisol levels in periodontitis are assumed to affect immunocompetency through alteration of immunoglobulins and neutrophil function.⁵

It should also be taken into consideration that components of the innate immune system other than neutrophils as well as cytokines and chemokines from the acquired immune system might play a significant and different role in the pathogenesis of AgP and CP.⁴⁰ The susceptibility of the host plays an important role in AgP; for example, unstimulated salivary protein profiles reflecting host immune reactions were found to be different from periodontal health.⁴¹

sAA, another factor released during stress, was reported to be related to periodontitis.⁴¹ In this study, a positive correlation is found between sAA activity and the number of periodontally diseased teeth (PD \geq 5 mm). However, no statistically significant difference in the activity of sAA in stimulated whole saliva was demonstrated among all groups, which is consistent with findings of a recent study.²⁸ So far, significantly higher sAA activities have been reported only in unstimulated whole saliva of periodontitis patients,^{28,41} indicating a possible association of AA to periodontitis. Human salivary glands may be involved in the response to oral inflammatory diseases, including periodontitis.⁴² On the one hand, it has been discussed that the release of AA from the salivary glands was enhanced directly under the stimulation of the inflammatory process of periodontitis.⁴² On the other hand, it was also supposed that the secretion of salivary proteins, including AA, is elevated indirectly through the activation of the SNS under stress loading from the infection process.²⁸ sAA has also been supposed to be a component in saliva involved in the oxidation process in periodontitis⁴³ and to play an inhibitory function against microorganisms.⁴⁴ The correlation between sAA and serum AA may represent an interdependency of serum and salivary activities. However,

this finding seems to have no implication for AA regarding periodontal disease in view of the fact that there were no differences of AA activity among all groups.

The current data indicate higher levels of CgA in the saliva of AgP patients and an association of sAA and salivary CgA with the extent of periodontitis. A higher stress loading in the AgP group needs to be confirmed also by the evaluation of psychologic stress via validated questionnaires. Because of the rather limited sample size in the present study with a cross-sectional design, the present findings need to be proved in the future by longitudinal clinical trials.

CONCLUSIONS

The results of this study suggest that salivary levels of stress markers could be related to the pathogenesis of periodontal disease. Particularly, higher salivary CgA and cortisol levels were found in patients with AgP. Further studies are necessary to assess the association of salivary CgA and sAA with psychologic stress, local and systemic inflammatory mediators, and local bacterial load in periodontal disease.

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