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ORIGINAL INVESTIGATION

Activated matrix metalloproteinase-8 in saliva as diagnostic test for periodontal disease? A case–control study

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Abstract Untreated periodontal disease may influence general health. However, how may a physician, who is not trained in periodontal probing, detect untreated periodontitis? Activated matrix metalloproteinase-8 (aMMP-8) in saliva correlates with periodontal probing parameters. Thus, sensitivity and specificity of a chair-side test for aMMP-8 to detect periodontitis were evaluated. Thirty cases [untreated chronic periodontitis (ChP); 15 generalized moderate and 15 generalized severe] and 30 controls [probing depths (PD) ≤3 mm, vertical probing attachment level (PAL-V) ≤2 mm at <30 % of sites) were examined periodontally (PD, PAL-V, bleeding on probing). Subsequently, the aMMP-8 test was performed. The test kit becomes positive with ≥25 ng/ml aMMP-8 in the sample. The aMMP-8 test was positive in 87 % of ChP and in 40 % of controls. That corresponds to a sensitivity of 87 % and a specificity of 60 %. The sensitivity to detect generalized severe ChP was 93 % (60 % specificity). Backward stepwise logistic regression analysis to explain positive aMMP-8 tests identified exclusively ChP with an odds ratio of 9.8 ($p < 0.001$). Positive results of the aMMP-8 test significantly correlate with generalized ChP. The aMMP-8 test may be used by physicians to detect periodontitis in their patients.

Keywords Activated matrix metalloproteinase-8 · Untreated moderate/severe chronic periodontitis · Saliva · Sensitivity/specificity

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Introduction

Periodontitis, the infectious–inflammatory destruction of tooth-supporting tissues (i.e., connective tissue and bone), is a widespread disease throughout the world [1]. Untreated periodontitis is characterized by periodontal pockets, loss of tooth-supporting tissues (attachment loss), and finally tooth loss. The severity of periodontitis is measured as probing depth (PD) and vertical probing attachment loss (PAL-V) at six sites circularly of each tooth. Therefore, a rigid metal probe that is marked in 1-mm increments is inserted parallel to the tooth axis between tooth and gingiva with a force of 0.2 N. PD is scored from the tip of the probe at the bottom of the pocket to the gingival margin and measures the extend of the inflammatory lesion. PAL-V is scored at the same time from the tip of the probe to the cemento-enamel junction (CEJ; line between tooth root and crown) and measures the severity of periodontal destruction that has already occurred (Fig. 1).

Matrix metalloproteinases (MMP) are enzymes (zinc peptidases) that catalyze the cleavage of tissue proteins. They play important roles in anabolism and catabolism of connective tissue and bone. In health, these enzymes are strictly regulated by inhibitors (tissue inhibitors of

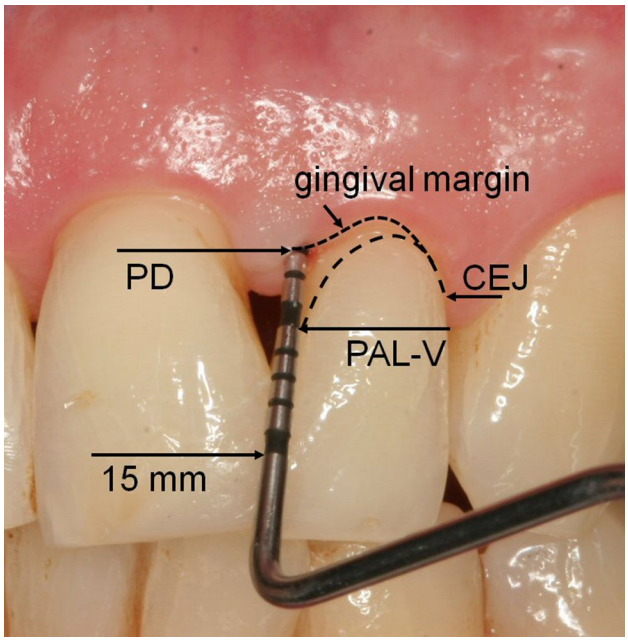

Fig. 1 Periodontal probing: probing depth (PD; 7 mm) is scored from the tip of the probe at the bottom of the pocket to the gingival margin. Vertical probing attachment level (PAL-V; 10 mm) is scored at the same time from the tip of the probe to the cemento-enamel junction (CEJ). The periodontal probe has marking up to 15 mm

metalloproteinases: TIMPs). Only if needed, activated matrix metalloproteinases (aMMPs) are released to degrade connective tissue and bone. In disease, the homeostasis is disrupted and aMMPs are released in abundance. The effects are pathological processes resulting in tissue degradation [2, 3].

Approximately 400 bacterial species are colonizing periodontal pockets, and a further 300 can be found in the rest of the oral cavity [4, 5]. Periodontal pathogens (e.g., *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*) trigger leukocytes, fibroblasts, and macrophages to produce prostaglandins, cytokines (e.g., interleukin-1, tumor necrosis factor-α), and MMPs in the periodontal tissues [6–8]. There is evidence that the level of aMMP-8 in the gingival crevicular fluid (GCF) of periodontal pockets correlates with clinical periodontal parameters [2, 3]. Thus, detection of increased levels of aMMP-8 in the GCF may be used to detect periodontal disease.

There exists a commercially available chair-side test for the qualitative detection of aMMP-8 in crevicular fluid: PerioMarker® test (manufacturer: Dentognostics GmbH, Jena, Germany; distribution: until 2012: Chlorhexamed®, GlaxoSmithKline Consumer Healthcare GmbH & Co. KG, Bühl, Germany; current: Miradent, Hager & Werken GmbH & Co. KG, Duisburg, Germany). The detection limit of the test kit is 25 ng/ml aMMP-8.

There is growing knowledge on interactions between periodontal and general disease (cardiovascular disease [9, 10], diabetes mellitus [11, 12], chronic obstructive pulmonary disease [13], adverse pregnancy outcomes [14]). For a physician, it may therefore be important to know whether her or his patient suffers from periodontal disease or not. This may be clarified easily by periodontal probing. However, physicians are not trained for that. Thus, a simple chair-side test with good sensitivity to detect untreated periodontitis may be used by physicians. The hypotheses behind this study were as follows:

- The aMMP-8 test discriminates between individuals with and without untreated generalized chronic periodontitis (ChP).
- The aMMP-8 test discriminates between individuals with generalized moderate and generalized severe ChP.

Therefore, sensitivity and specificity of a chair-side test for aMMP-8 to distinguish between individuals with and without untreated ChP as well as between patients with generalized moderate and generalized severe ChP were evaluated.

Materials and methods

Patients

A commercially available chair-side test to detect increased levels of aMMP 8 (PerioMarker®, Chlorhexamed®, GlaxoSmithKline Consumer Healthcare GmbH & Co. KG, Bühl, and Miradent, Hager & Werken GmbH & Co. KG, Duisburg) was used at Dr. Matthias Mayer's MMSc. Dental Office (Arndtstr. 14, 60325 Frankfurt am Main) in periodontally healthy individuals and patients with untreated generalized moderate and generalized severe ChP.

Generalized moderate ChP

- Sites with probing depths (PD) ≥3.5 mm [15].
- Attachment loss (PAL-V) 3–4 mm >30 % of sites, PAL-V ≥ 5 mm ≤ 30 % of sites [16].

Generalized severe ChP

- Sites with probing PD ≥ 3.5 mm.
- PAL-V ≥ 5 mm > 30 % of sites [16].

Periodontally healthy

- PD ≤ 3 mm.
- PAL-V ≤ 2 mm at <30 % of sites.

- BOP < 20 %.
- No radiographically detectable bone loss: distance cemento-enamel junction to alveolar crest ≤2 mm [17].

The companies had provided 70 tests for use. During use, the idea arose to retrospectively correlate test results and clinical diagnoses. Thus, a study protocol was submitted to the ethics committee of the Medical Faculty of the Johann Wolfgang Goethe University Frankfurt/Main. All patients who were examined prior to the approval of the ethics committee were evaluated retrospectively. All subsequent patients were recruited prospectively.

All patients were asked about current smoking (yes/no) and education level (basic school, high school, university). The study complied with the rules of the Declaration of Helsinki and was approved by the institutional review board for Human Studies of the Medical Faculty of the Goethe University Frankfurt/Main (application# 144/13).

Inclusion criteria

- At least 18 years of age.
- Clinical diagnosis of generalized moderate or generalized severe ChP or periodontally healthy controls.
- At least five teeth present per quadrant.
- After application to the ethics committee: written informed consent.

Exclusion criteria

- Requirement of systemic antibiotics for measures that may cause transitory bacteraemia (e.g., pocket probing).
- Non-surgical or surgical periodontal treatment within the last 12 months prior to PerioMarker® test.
- Systemic or topical subgingival antibiotics within the last 6 months prior to PerioMarker® test.
- Anti-inflammatory medication (e.g., nonsteroidal anti-inflammatory drugs) during the last 3 months prior to PerioMarker® test.

Clinical examination

The following clinical parameters were assessed at six sites per tooth (mesiobuccal, buccal, distobuccal, mesiolingual, lingual, and distolingual):

- PD and PAL-V to the nearest 1 mm using a manual periodontal probe (PCPUNC 15, Hu-Friedy, Chicago, IL, USA) (Fig. 1).
- BOP 30 s after probing.

At sites where the CEJ was destroyed by restorations, the restoration margin (RM) was used as reference.

Chair-side test

For all periodontitis patients, the aMMP-8 test was performed at least 24 h after clinical examination. In the time interval between aMMP-8 test and clinical examination, no periodontal treatment was rendered. For periodontally healthy controls, the test was done at least 24 h but not more than 7 days after the clinical examination. First of all, patients rinsed with tap water for 30 s. Then, they spat out the water and waited for 1 min. Now, patients rinsed with 5 ml of purified water for 30 s and spat this sample back into the test cup. Approximately 2 ml of the sampled saliva was now sampled with a syringe. After a filter was put onto the syringe, three drops of the saliva were pressed through the filter into the ELISA kit. After 5–10 min, the result was read from the test kit [18]. If both the control and test stripes were visible, the respective test was positive (i.e., ≥25 ng aMMP-8 per ml). The clinical examiner (SIB) judged the results by simple visual inspection. Already a faint test stripe was judged as positive test. All test results were photographed with twofold magnification. All images of the test were then evaluated by a second examiner (PE) who was blinded for the clinical diagnoses.

Statistical analysis

The patient was looked upon as statistical unit. The sensitivity and specificity of the aMMP-8 test were defined as the primary outcome variables. All other parameters were control variables.

For all individuals, cigarette pack-years were calculated. Group frequencies (controls/ChP; controls/moderate ChP/severe ChP) were expressed for sex, current smoking status, education, and aMMP-8 (positive/negative). Group means and standard deviations were calculated for age and BOP. Further, for each individual, the following variables were calculated to describe the periodontal status:

- Mean ± standard deviation of PD and PAL-V.
- Number of pathological PD (≥4 mm) per individual.
- Sum of all PD [19] and sum of all PD with BOP [15], i.e., adding PD and PD plus BOP measurements from all sites (six per tooth) within a patient in an attempt to describe the subgingival wound.
- Periodontal inflamed surface area (PISA) [20, 21].

From these, group means and standard deviations were calculated. Comparisons between groups for dichotomous parameters were made by χ^2 or Fisher's exact test and for all other parameters by Kruskal–Wallis and Mann–Whitney *U* test. Agreement of aMMP-8 test scorings between both examiners (SIB and PE) was estimated as Cohen's kappa.

Table 1 Patient characteristics (chronic periodontitis: ChP) and periodontal variables (probing pocket depth: PD; vertical probing attachment level: PAL-V; bleeding on probing: BOP)

	Without periodontitis N = 30 (1)	ChP N = 30	P^a	Generalized moderate ChP N = 15 (2)	Generalized severe ChP N = 15 (3)	P^b	1 versus 2 *p*	1 versus 3 *p*	2 versus 3 *p*
Female sex (n/%)	20/67 %	14/47 %	0.118	6/40 %	8/53 %	0.225			
Age (years)	28.4 ± 7.9	53.9 ± 10.7	<0.001	54.1 ± 11.9	53.8 ± 9.8	<0.001	<0.001	<0.001	0.819
Current smokers (n/%)	16/53 %	9/30 %	0.067	3/20 %	6/40 %	0.101			
Education			0.090			0.160			
Basic school	5/17 %	11/37 %		4/27 %	7/47 %				
High school	10/33 %	4/13 %		3/20 %	1/6 %				
University	15/50 %	15/50 %		8/53 %	7/47 %				
aMMP-8 positive (n/%)	12/40 %	26/87 %	<0.001	12/80 %	14/93 %	0.001	0.012	0.001	0.291
Mean									
Mean PD (mm)	2.1 ± 0.1	3.8 ± 1.0	<0.001	3.2 ± 0.3	4.4 ± 1.1	<0.001	<0.001	<0.001	<0.001
PAL-V (mm)	0.3 ± 0.0	4.4 ± 1.2	<0.001	3.6 ± 0.4	5.3 ± 1.2	<0.001	<0.001	<0.001	<0.001
BOP (%)	5.9 ± 5.3	39.6 ± 23.3	<0.001	33.0 ± 25.0	46.3 ± 20.2	<0.001	<0.001	<0.001	0.040
PD ≥ 4 (n)	0 ± 0	72.4 ± 32.4	<0.001	50.9 ± 21.3	93.9 ± 27.1	<0.001	<0.001	<0.001	<0.001
Sum PPD (mm)	359.9 ± 23.2	621.5 ± 197.6	<0.001	509.3 ± 79.1	733.6 ± 218.4	<0.001	<0.001	<0.001	<0.001
Sum PD with BOP (mm)	24.2 ± 20.8	310.0 ± 262.3	<0.001	198.1 ± 147.9	421.9 ± 306.2	<0.001	<0.001	<0.001	0.003
PISA (mm^2)	69.7 ± 65.8	1170.0 ± 1048.4	<0.001	644.2 ± 488.9	1695.8 ± 1202.3	<0.001	<0.001	<0.001	0.001

[a] Comparison group 1 with ChP

[b] Comparison group 1, 2, 3

Table 2 Sensitivity and specificity of the aMMP-8 test to distinguish untreated chronic periodontitis (ChP), generalized severe ChP, and generalized moderate ChP, respectively, from individuals without periodontitis

Diagnosis	Without periodontitis	ChP	Total
aMMP-8 test			
Positive	12	26	38
Negative	18	4	22
Total	30	30	60

		95 % confidence interval	
		Lower limit	Upper limit
Sensitivity	0.87	0.68	0.96
Specificity	0.60	0.41	0.77
Positive predictive value	0.68	0.51	0.81
Negative predictive value	0.81	0.59	0.94

Diagnosis	Without periodontitis	Generalized moderate ChP	Total
Positive	12	12	24
Negative	18	3	21
Total	30	15	45

		95 % confidence interval	
		Lower limit	Upper limit
Sensitivity	0.80	0.51	0.95
Specificity	0.60	0.41	0.77
Positive predictive value	0.50	0.30	0.70
Negative predictive value	0.86	0.63	0.96

Diagnosis	Without periodontitis	Generalized severe ChP	Total
Positive	12	14	26
Negative	18	1	19
Total	30	15	45

		95 % confidence interval	
		Lower limit	Upper limit
Sensitivity	0.93	0.66	0.99
Specificity	0.60	0.41	0.77
Positive predictive value	0.54	0.34	0.73
Negative predictive value	0.95	0.72	0.99

Further, for each diagnosis (controls/ChP; controls/moderate ChP; controls/severe ChP), sensitivity, specificity, and positive and negative predictive values were calculated. According to the observed difference, a post hoc sample size calculation is used for the comparison of sensitivity and specificity to detect moderate and severe ChP, respectively (https://www.statstodo.com/SSizSenSpc_Pgm.php).

Using backward stepwise logistic regression analysis, factors should be identified that were associated with positive aMMP-8 tests. The following independent variables were entered into the model: group (control/ChP), sex, age, education, smoking, PD, PAL-V, BOP, PISA. The following parameters were described by dummy variables: group (control = 0, ChP = 1), sex (male = 0, female = 1), smoking status (never and former smoker = 0, current smoker = 1). All factors with $p < 0.05$ were kept in the model. For statistical analysis, a PC program was used (Systat™ for Windows version 12, Systat Inc., Evanston, USA).

Results

From September 21, 2012, to February 24, 2014, thirty cases (untreated ChP; 15 generalized moderate and 15

Table 3 Backward stepwise logistic regression analysis: aMMP-8 test positive in relation to individual and periodontal variables

$N = 60$	Estimate	Odds ratio	Standard error	p	95 % confidence interval	
Constant	−0.405			0.277	Lower	Upper
Chronic periodontitis	2.277	9.750	6.374	<0.001	2.707	35.112

generalized severe) and 30 periodontally healthy controls were examined periodontally and using the aMMP-8 test by SIB at Dr. Matthias Mayer's MMSc. Dental Office (Arndtstr. 14, 60325 Frankfurt am Main). The charts of 33 individuals were evaluated retrospectively. From May 5, 2013, additional 27 individuals were examined prospectively. For periodontitis patients, the mean time interval between clinical examination and aMMP-8 test was 12.8 days (1–41 days). During this interval, no periodontal treatment was rendered. Patient characteristics and frequency of positive aMMP-8 tests of cases and controls are given in Table 1. Periodontally healthy controls were significantly younger and exhibited significantly less positive aMMP-8 test results than ChP patients. Both ChP groups were well balanced according to sex, age, smoking, education, and aMMP-8 test results (Table 1). Due to case definition, ChP exhibited more severe clinical variables than healthy controls and moderate ChP and severe ChP more severe clinical variables than controls (Table 1).

Both examiners (SIB and PE) showed perfect agreement in aMMP-8 test scorings (Cohen's kappa = 1.0). The aMMP-8 test was positive in 87 % of ChP and in 40 % of controls. That corresponds to a sensitivity of 87 % and a specificity of 60 %. The sensitivity to detect generalized severe ChP was 93 % (60 % specificity) (Table 2). Backward stepwise logistic regression analysis to explain positive aMMP-8 tests identified exclusively ChP with an odds ratio of 9.8 ($p < 0.001$) (Table 3). To assess a difference in sensitivity to detect moderate or severe ChP of 16 % (80 %/96 %) with a Type 1 error of 0.05 and 80 % test power, a sample size of 124 (62 in each group) would be required.

Discussion

Up to date, approximately 30 different MMPs have been identified. They can be divided into five major groups: collagenases (MMP-1, MMP-8, MMP-13), gelatinases (MMP-2, MMP-9), stromelysins (MMP-3, MMP-10, MMP-11), membrane-type MMPs (MMP-14, MMP-15, MMP-16, MMP-17), and others [22, 23]. Polymorphonuclear leukocytes and monocyte/macrophages migrate to the sites of inflammatory response to bacterial challenges and release MMP-8 and MMP-9 [22, 24]. An imbalance between MMPs and TIMPs results in periodontal tissue breakdown [22, 23]. Activated MMP-8 levels from GCF have been correlated with periodontal disease and disease progression [25]. A chair-side test for GCF concentrations of aMMP-8 was also roughly associated with periodontal conditions [26]. All these observations demonstrate some correlation of aMMP-8 levels with clinical periodontal parameters. However, what is the additional benefit of the information on aMMP-8 concentrations in GCF? If these concentrations only correlate with clinical parameters (PD, BOP, etc.), there is no clinical use in this information. Clinical information can be collected faster and cheaper.

Periodontitis increases the risk of several general health problems [9, 10, 12–14, 27, 28]. Non-surgical periodontal treatment has been shown to improve metabolic control in type 2 diabetes [29–33]. Thus, physicians should be aware of the periodontal status of their patients. However, physicians are not trained for periodontal probing (e.g., Periodontal Screening Index: PSI) that may easily identify periodontal disease. Simply referring every patient to the dentist for periodontal screening would require additional time and cause additional cost. The aMMP-8 test requires less than 10 min at the physician and identifies most of the patients requiring a dental treatment. A simple questionnaire consisting of eight items to detect periodontitis has been evaluated against different periodontitis case definitions for a US population [34]. The questionnaire alone achieved 59.3 % sensitivity and 57 % specificity to detect periodontitis [at least mild: ≥2 interproximal sites with ≥3 mm PAL-V and ≥2 interproximal sites with ≥4 mm PD (not on the same tooth) or 1 site with ≥5 mm] according to the CDC/AAP case definition. Sensitivity and specificity to detect individuals with PAL-V ≥ 3 mm at least at one site were 86.9 % and 0 % and to detect individuals with PD ≥ 4 mm at least at one site were 71.7 % and 20.8 %. These results are difficult to compare with the results of the aMMP-8 test. For evaluation of the questionnaire, the diagnosis of periodontitis was not limited to ChP and not only untreated periodontitis was included. A case definition for periodontitis based only on PAL-V ≥ 3 mm may also include successfully treated cases that have no effect on systemic disease. It is the parakeratinized and ulcerated pocket epithelium of untreated periodontitis that forms an easy port of entry for oral microorganisms causing systemic effects. If the pocket walls of all periodontally compromised teeth in an untreated patient are combined, the wound surface due to periodontitis is estimated to be as large as 8–20 cm^2 [35].

The size of the wound surface depends primarily on periodontal pocket depths and not attachment loss. After periodontal treatment periodontal pockets may be resolved while attachment loss will persist. Further, in Germany, a survey on the knowledge of the German population on periodontal disease demonstrated massive deficiency of knowledge. Only 29.8 % of 1001 representative Germans answered the question "What is periodontitis?" with "loss of the gums" [36]. It is quite questionable whether in Germany a questionnaire would provide similar sensitivity and specificity. Thus, a simple chair-side test with good sensitivity to detect untreated periodontitis may be used by physicians. However, until now, sensitivity or specificity of a certain aMMP-8 level threshold to distinguish between periodontal health and disease has not been evaluated.

Periodontally healthy controls were quite well balanced to ChP patients regarding sex. They were significantly younger and exhibited significantly less positive aMMP-8 test results than ChP patients. Controls exhibited a tendency to better education and exhibited an insignificantly higher frequency of smokers. It has been shown that the overall GCF MMP-8 levels in smoking patients were lower than in non-smoking patients [37]. Thus, more smokers in the control group may have contributed to the lower frequency of positive aMMP-8 results in the control group. However, multivariate analysis did not identify smoking to be associated with the frequency of positive aMMP-8 tests. Both ChP groups were well balanced according to sex, age, smoking, education, and aMMP-8 test results.

Various variables were assessed to measure the degree of periodontal disease: mean PD and PAL-V, sum of all PD [19] as well as sum of PD with BOP per patient [15] and PISA [20, 21]. The more deep pockets are present, the higher is the probability of BOP [38, 39]. BOP and penetration depth of a periodontal probe are both indicators of subgingival inflammation. However, after entering group (control/ChP), sex, age, education, smoking, PD, PAL-V, BOP, PISA into multivariate logistic regression analysis, only ChP remained in the model to influence frequency of positive aMMP-8 test results. Actually PD and its dependent variables (sum of PD, PISA) and PAL-V are mathematically coupled to the groups. In this study, healthy individuals and ChP patients were distinguished by PD and PAL-V. Thus, the variable with the strongest effect may have thrown the other variables out. This study therefore confirms the correlation of aMMP-8 and clinical periodontal parameters.

This study compares periodontally healthy individuals (BOP = 5.9 %, mean PD = 2.1 mm) with untreated ChP (BOP = 39.6 %, mean PD = 3.8 mm). The aMMP-8 test is quite sensitive to identify diseased (i.e., ChP) individuals from this cohort. However, this study does not provide information how clearly the test may distinguish between gingivitis (e.g., BOP > 20 %, mean PD = 2 mm) and untreated ChP or between successfully treated and untreated ChP. However, in contrast to ChP, BOP was not identified as a factor significantly associated with positive aMMP-8 test. Thus, gingivitis may not be associated with a high frequency of positive aMMP-8 tests. Further research has to be done to clarify these issues.

Within the limitations of the present study, the following conclusion may be drawn:

- Positive results of the aMMP-8 test significantly correlate with generalized ChP and provide substantial sensitivity to distinguish between periodontally healthy individuals and untreated ChP patients.
- The aMMP-8 test may be used by physicians to detect periodontitis in their patients.

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Conflict of interest The authors declare that they have no financial or other relationships that might lead to a conflict of interest.

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