Endo180 modulation by bisphosphonates and diagnostic accuracy in metastatic breast cancer

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Background: Endo180 (CD280; MRC2; uPARAP)-dependent collagen remodelling is dysregulated in primary tumours and bone metastasis. Here, we confirm the release and diagnostic accuracy of soluble Endo180 for diagnosing metastasis in breast cancer (BCa).

Methods: Endo180 was quantified in BCa cell conditioned medium and plasma from BCa patients stratified according to disease status and bisphosphonate treatment (n = 88). All P-values are from two-sided tests.

Results: Endo180 is released by ectodomain shedding from the surface of MCF-7 and MDA-MB-231 BCa cell lines. Plasma Endo180 was significantly higher in recurrent/metastatic (1.71 ± 0.87; n = 59) vs early/localised (0.92 ± 0.37; n = 29) BCa (P < 0.0001). True/false-positive rates for metastasis classification were: 85%/50% for the reference standard, CA 15-3 antigen (28 U ml⁻¹), ≤ 97%/≥ 36% for Endo180, and ≤ 97%/≥ 32% for CA 15-3 antigen + Endo180. Bisphosphonate treatment was associated with reduced Endo180 levels in BCa patients with bone metastasis (P = 0.011; n = 42). True/false-positive rates in bisphosphonate-naive patients (n = 57) were: 68%/45% for CA 15-3 antigen; ≤ 95%/≥ 20% for Endo180, and ≤ 92%/≥ 21% for CA 15-3 antigen + Endo180.

Conclusion: Endo180 is a potential marker modulated by bisphosphonates in metastatic BCa.
receptor that interacts with extracellular and intracellular collagens (Wienke et al., 2003). Endo180 expression is correlated with tumour grade, disease progression and poor prognosis in BCa (Wienke et al., 2007), head and neck cancer (Sulek et al., 2007), prostate cancer (Kogianni et al., 2009) and glioblastoma multiforme (Huijbers et al., 2010). Endo180-dependent collagen remodelling has mechanistic roles in the promotion of BCa progression (Curino et al., 2003, 2006, 2007; Wienke et al., 2007; Kogianni et al., 2009; Huijbers et al., 2010) and metastatic bone lesion pathology (Caley et al., 2011). In this study, we report that Endo180 ectodomain is released from BCa cells and measurement of soluble Endo180 in plasma, alone or in combination with CA 15-3 antigen, provides an accurate method for the classification of metastatic disease. The results also indicate that Endo180 levels are modulated following treatment with bisphosphonates.

**PATIENTS AND METHODS**

**Preparation of conditioned medium and cell lysates.** MCF-7-Endo180 and MDA-MB-231 cells were cultured as previously described (Sturge et al., 2003, 2006; Wienke et al., 2007). Protein was precipitated from conditioned medium and cell lysates prepared as described in Supplementary Materials and methods.

**Immunoblot analysis.** Conditioned medium pellets and corresponding cell lysates were resolved using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE). Immunoblot analysis was carried out using A5/158 or 39.10 mouse anti-human Endo180 monoclonal antibodies (1 μg ml⁻¹) or CAT-2 rabbit anti-mouse Endo180 polyclonal antibody (1.5 μg ml⁻¹) (Sturge et al., 2003, 2006, 2007; Wienke et al., 2007; Kogianni et al., 2009; Huijbers et al., 2010) and streptavidin–alkaline phosphatase conjugate (1:10,000; ##Promega##). Detection of Endo180 was performed using BCIP/NBT Universal Substrate Working Solution (Promega) as described (Sturge et al., 2003, 2006, 2007; Huijbers et al., 2010).

**Figure 1.** Details of patients included in the study. Number of patients recruited and excluded is shown together with those for whom index and reference standard tests were performed. Numbers of patients with recurrent/locoregional disease, visceral metastasis, bone metastasis alone or bone metastasis with visceral metastasis in lung, liver or more than one visceral site are indicated. Numbers of patients who were bisphosphonate naive, had previously received, or were currently receiving, bisphosphonates are shown.

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**Patients.** Ethical approval for the study was obtained from Imperial College Healthcare NHS Trust Tissue Bank Committee (REC reference 07/MRE09/54). The study population was restricted to a consecutive series of 92 patients with histologically confirmed BCa who attended breast oncology clinics at Imperial College Healthcare NHS Trust between 4 March 2011 and 29 August 2011 and was designed following the guidelines of Standards for Reporting of Diagnostic accuracy studies (STARD) (Bossuyt et al., 2003). Three patients were excluded from the study due to incorrect completion of consent forms and one patient who originally agreed to enter the study withdrew consent (Figure 1). Treatment followed the standard of care that patients normally receive at Imperial College Healthcare NHS Trust. Early BCa was defined as a localized tumour previously treated with definitive surgery, systemic therapy and adjuvant radiotherapy (as clinically indicated) or receiving on-going (neo)-adjuvant systemic therapy. All early BCa patients had a staging investigation that was negative with no evidence of recurrent disease at outpatient review when blood samples were collected. Metastatic disease sites were determined from the most recent radiological evaluation. The definition of visceral metastasis was radiological evidence of recurrent disease involving the pleura, peritoneum or lymph nodes (paratracheal, axillary, mediastinal, supraclavicular, infraclavicular) or lesions in visceral organs (liver, lung, spleen). The definition of bone metastasis was the detection of osseous lesions by bone scintigraphy, computed tomography and/or magnetic resonance imaging.
imaging. Exclusion criteria were inadequate haematological and/or biochemical parameters.

**Plasma preparation and analysis.** Plasma samples were collected and Endo180 and CA 15-3 antigen levels were analysed as described in Supplementary Materials and methods.

**Statistical analysis.** The Statistical Advisory Service (Imperial College London) used SPSS software to predict that 17 patients would be necessary for each patient group to detect low-to-moderate correlation between variables (α = 5% probability, type I error significance level; 95% power; 50% confidence interval (CI); 40% standard deviation). Pearson’s correlation coefficient (r) was calculated to determine assumed linearity. Kolmogorov–Smirnov goodness of fit was used to test likelihood of a normal distribution at 95% confidence. John Tukey criteria defined outliers as values that were 1.5 times above the third quartile (Q3) or below the first quartile (Q1). Non-parametric tests were used to calculate the probability (P) of a significant difference (95% CI). Mann–Whitney U-test was used for comparison between two groups. Kruskal–Wallis one-way analysis of variance was used for comparison between more than two groups. Where statistical differences were found, Bonferronni multiple comparisons adjustment was applied to confirm pairwise differences. For Bonferroni correction, the overall type I error constraint was set to 0.05 and the level of statistical significance was set to 0.05/n, where n is the number of tests. P-values < 0.05/n were considered statistically significant. All tests were two-sided. The test used for each analysis is cited in the text and figure legends.

**Figure 2.** Endo180 is cell membrane-associated or released in soluble form in monolayers of human BCa cell lines cultured under serum-free conditions. (A) The domain structure of cell membrane-associated Endo180. The cysteine-rich domain (CRD), fibronectin type II domain (FNII) and C-type lectin-like domains (CTLDs), of which there are eight (1–8); and binding sites of the known ligands for FNII (native collagen) and CTLD-2 (glycosylated collagen and sugar moieties); are shown. Three anti-Endo180 antibodies and their respective epitopes are depicted: mouse anti-human Endo180 monoclonal antibody 39.10 is mapped to bind CTLD-4 (unpublished); mouse anti-human Endo180 monoclonal antibody A5/158 is mapped to bind CTLD-2 (Sturge et al, 2003); and rabbit anti-mouse CAT-2 monoclonal antibody was raised against the conserved 42 amino-acid intracellular domain of mouse Endo180 and has equal affinity for the detection of the receptor in human cell lysates (Sturge et al, 2007; Caley et al, 2011). The domain structure of soluble Endo180 ectodomain following its juxtamembrane cleavage and predicted detection by 39.10 and A5/158 antibodies, but not CAT-2 antibody, is also shown. (B) MDA-MB-231 cells and (C) MCF-7 cells that overexpress Endo180 (MCF-7-Endo180) were cultured in DMEM without fetal bovine serum for 0 (time zero), 6, 12, 24 and 48 h at which time whole-cell lysates and corresponding conditioned medium were collected. Samples were resolved on 8% w/v SDS polyacrylamide gels, transferred to polyvinylidene fluoride membranes and immunoblot analysis was performed using the three anti-Endo180 monoclonal antibodies: 39.10 (1 mg ml⁻¹), A5/158 (1 mg ml⁻¹) and CAT-2 (1.5 mg ml⁻¹); and α-tubulin monoclonal antibody (1/10000 dilution) and horse radish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (1/4000 dilution). Representative immunoblots from 10 experimental repeats are presented with corresponding graphs showing relative Endo180 levels calculated by the densitometric analysis of 39.10, A5/158 and CAT-2 immunoreactive bands. Relative Endo180 levels in conditioned media (black bars) were calculated by normalisation against relative Endo180 levels at time zero (relative Endo180 level = 1.0) for all subsequent time points (6, 12, 18 and 24 h). Relative Endo180 levels in cell lysates (white bars) were adjusted against the level of α-tubulin and normalised against the relative Endo180 levels at time zero (relative Endo180 level = 1.0) for all subsequent time points (6, 12, 18 and 24 h). The markers of molecular weight in kilodaltons (kDa) are indicated using black arrowheads for each immunoblot.
RESULTS

Release of soluble Endo180. Viable disease markers are released into the circulation in appreciable and easily detectable amounts (Diamandis, 2010). Antibodies that bind epitopes in the ectodomain (A5/158 and 39.10) (Sturge et al, 2003) or intracellular domain (CAT-2) (Sturge et al, 2007) of Endo180 (Figure 2A) were used to immunoblot whole-cell lysates and corresponding conditioned media from MDA-MB-231 and MCF-7-Endo180 cells, in which the receptor enhances their pro-tumorigenic and metastatic behaviour (Sturge et al, 2003, 2006; Wienke et al, 2003, 2007). Endo180 detection by all three antibodies in MDA-MB-231 (Figure 2B) and MCF-7-Endo180 (Figure 2C) whole-cell lysates; and by A5/158 and 39.10, but not CAT-2, in corresponding conditioned media confirmed that Endo180 ectodomain is released in a soluble form from BCa cells. Soluble Endo180 levels were increased in MDA-MB-231 and MCF-7-Endo180 cell conditioned media by 9.5-fold (after 12 h) and 42-fold (after 24 and 48 h) culture in serum-free medium (Figure 2B and C).

Detection of soluble Endo180 in plasma. Immunodetection of Endo180 by 39.10 antibody in human tissue and cell lysates has been well characterised (Wienke et al, 2007; Kogianii et al, 2009; Huijbers et al, 2010; Caley et al, 2011) and was adapted here as a research assay in accordance with recommendations for initial biomarker discovery where no commercial test exists (Pepe et al, 2008). A single and easily quantifiable 180 kDa immunoreactive band could be detected by 39.10 antibody in plasma resolved using SDS–PAGE (Supplementary Figure 1).

Elevated Endo180 in metastatic BCa. Endo180 levels in plasma collected from 88 BCa patients (Figure 1) did not correlate with their clinicopathological characteristics or current treatment (Supplementary Data; Supplementary Tables 1–3). The median time from diagnosis of patients with early BCa (n = 29) was 5 months (range 0–180 months; Q1: 3 months; Q3: 25 months) and with metastatic BCa (n = 59) was 60 months (range 0–300 months; Q1: 17 months; Q3: 156 months). The range of relative Endo180 plasma levels in early BCa cases (n = 29) was consistent with a normal distribution (P = 0.69; mean ± s.d. = 0.92 ± 0.37; range 0.32–1.78; Q1: 0.63; Q3: 1.13; 95% CI: 0.78–1.06, Kolmogorov–Smirnov goodness of fit), indicating that Endo180 release into plasma during early BCa is a tightly regulated event. Relative Endo180 levels in recurrent/metastatic BCa (n = 59) were significantly elevated compared with early BCa (P < 0.0001, Mann–Whitney U-test) and inconsistent with a normal distribution (P = 0.01; mean ± s.d. = 1.71 ± 0.87; range 0.32–4.37; Q1: 1.13; Q3: 2.30; 95% CI: 1.49–1.94, Kolmogorov–Smirnov goodness of fit) (Figure 3A), indicating that dysregulated Endo180 release occurs at this advanced disease stage. As expected, CA 15-3 antigen levels were significantly higher (P < 0.0001, Mann–Whitney U-test) in recurrent/metastatic (n = 59) compared with early (n = 27) BCa (Figure 3B).

Plasma Endo180 levels are increased in locoregional disease, visceral metastasis and bone metastasis. To investigate whether Endo180 was predictive of recurrent and/or metastatic disease in visceral and/or osseous tissue, the 59 recurrent/metastatic patients were stratified according to tissue site(s) affected by secondary lesions. Kruskal–Wallis one-way analysis of variance with Bonferroni correction (P = 0.05/n, n = 7) revealed that relative Endo180 levels were significantly different (P = 0.0005) in early BCa (mean ± s.d.: 0.92 ± 0.37, n = 29), recurrent locoregional disease (mean ± s.d.: 1.53 ± 0.46, n = 5), metastases in one or more visceral tissues (mean ± s.d.: 1.78 ± 1.20, n = 12), bone metastasis alone (mean ± s.d.: 1.48 ± 0.64, n = 14) and bone metastasis with additional dissemination in lung(s) (mean ± s.d.: 1.72 ± 0.61, n = 34). A single and easily quantifiable 180 kDa immunoreactive band could be detected by 39.10 antibody in plasma resolved using SDS–PAGE (Supplementary Figure 1).

Figure 3. Endo180 levels are elevated in the plasma of patients with metastatic compared with early BCa. Plasma samples from BCa patients (n = 88) were resolved on 8% w/v SDS polyacrylamide gels, transferred to polyvinylidene fluoride membranes and immunoblot analysis was performed using 1 mg ml–1 mouse anti-human Endo180 monoclonal 39.10 antibody and 1/4000 dilution of horse radish peroxidase-conjugated goat anti-mouse IgG (a representative blot is presented in Supplementary Figure 1). Relative plasma Endo180 levels were calculated from the mean measurement obtained from three analytical repeats. Plasma from the same early BCa patient was used as a reference control (relative Endo180 level = 1.0) and the normalisation of densitometric measurements on all analytical gels. CA 15-3 antigen concentrations (U ml–1) were measured using a non-competitive two-step immunoassay in plasma samples (n = 86) collected in parallel. The box and whiskers plots shown depict the total range, interquartile distribution, outliers (open or closed circles) and median value (horizontal line) of relative plasma Endo180 levels (A, C) and plasma concentrations of CA 15-3 antigen (Log10 [U ml–1] B, D). (A) The graph compares relative plasma Endo180 levels in cases of early BCa (n = 29) and metastatic BCa (n = 59). (B) The graph compares plasma concentrations of CA 15-3 antigen (Log10 [U ml–1]) in cases of early BCa (n = 27*) and metastatic BCa (n = 59). (C) The graph compares relative plasma Endo180 levels in cases of early BCa (n = 29) and recurrent locoregional disease (n = 5) or metastasis in visceral tissue alone (n = 12), bone alone (n = 14) or bone with dissemination in lung (n = 5), liver (n = 8) or two or more visceral organs (n = 15). (D) The graph compares plasma concentrations of CA 15-3 antigen (Log10 [U ml–1]) in cases of early BCa (n = 27*) and metastatic sites as described above (C). The P-values shown were determined using Mann–Whitney U-test for comparison between cases of early BCa and metastatic BCa (A, B). or Kruskal–Wallis one-way analysis of variance for comparison between early BCa and recurrent/metastatic BCa stratified according to affected tissue sites (C, D), and confirmed by Bonferroni adjustment (P = 0.05/n, n = 7). *Data for CA 15-3 antigen were missing for 2 out of the 29 early BCa patients included in the study.
Diagnostic accuracy of Endo180. The performance of Endo180 and CA 15-3 antigen, alone and in combination, for the classification of recurrent/metastatic vs early/localised BCa was assessed. The respective sensitivity (% true-positive rate) and specificity (% false-positive rate) for the classification of metastasis by CA 15-3 antigen alone (fixed cutoff: 28 U ml\(^{-1}\)) (as used in the Department of Medical Oncology at Imperial College NHS Trust) was 85% and 50%. For a consecutive range of values for Endo180 alone (variable cutoff: 0.95–1.65 relative plasma levels), the respective range of true-positive and false-positive rates for the classification of metastasis was 82–97% and 36–54%. For CA 153 antigen (fixed cutoff: 28 U ml\(^{-1}\)) combined with Endo180 (variable cutoff: 0.95–1.65 relative levels), the respective true-positive and false-positive rates for classification of metastasis was 94–97% and 32–48% (Supplementary Figure 2).

Modulation of Endo180 by bisphosphonates. Stratification of the 42 patients with bone metastasis (with or without additional visceral metastasis) according to bisphosphate treatment status (Figure 4A) and Kruskal–Wallis one-way analysis of variance with Bonferroni correction (P = 0.05/n, n = 3) revealed that Endo180 levels were significantly higher in bisphosphonate-naive cases (mean ± s.d. = 2.17 ± 0.79, n = 13) compared with those having previous (mean ± s.d.: 1.83 ± 0.33, n = 5) or current (mean ± s.d.: 1.37 ± 0.74, n = 24) bisphosphate treatment.

Figure 4. Endo180 improves the prediction of metastatic BCa in the bisphosphonate-naive setting. (A) Stratification of all patients for which the index test and reference standard test were performed (n = 86) according to low or high CA 15-3 antigen and Endo180 status with respective cutoffs of 28 U ml\(^{-1}\) and relative plasma level of 1.15 indicated with grey arrowheads. Disease status for each patient is indicated using the following colour code: early BCa (pink), locoregional disease (yellow), visceral metastasis alone (purple), bone metastasis alone (green) and bone plus visceral metastasis (orange). The sensitivity (% true positives) and specificity (% false negatives) of Endo180 and CA 15-3 antigen for classification of metastasis are indicated as fractional numbers of patients and corresponding percentages. Patients currently (+) or previously (-) receiving bisphosphonate treatment are indicated. Box and whiskers plots depicting the total range, interquartile distribution, outliers (open and closed circles) and median value (horizontal line) of: (B) Endo180 and CA 15-3 antigen; in the 42 patients with bone metastasis with or without visceral tissue metastasis classified as bisphosphate naive (n = 13), having previously received bisphosphonates (n = 5) or currently being treated with bisphosphonates (n = 24). The P-values shown were determined using Kruskal–Wallis one-way ANOVA, and confirmed by Bonferroni adjustment (P = 0.05/n, n = 3). (C) Evaluation of the sensitivity (% true positives) and specificity (% false negatives) of Endo180 and CA 15-3 antigen in all 57 bisphosphonate-naive patients was conducted using the same criteria as used in (A). The Endo180 cutoff (1.15) chosen for the analyses presented in (A, C) was close to the Q1 value for recurrent/metastatic BCa (1.13) and the Q3 value for early BCa (1.13) and was higher than the upper 95% CI for early BCa (1.06). *Data for CA 15-3 antigen were missing for 2 out of the 29 early BCa patients included in the study.
(P = 0.011) (Figure 4B). In contrast, CA 15-3 antigen levels were unaltered by bisphosphonates (P = 0.71) (Figure 4B).

The true-positive and false-positive rates of 68% and 45% for CA 15-3 antigen (fixed cutoff: 28 U ml$^{-1}$); 70–95% and 20–31% for Endo180 alone (variable cutoff: 0.95–1.65 relative levels); and 87–92% and 21–28% for Endo180 in combination with CA 15-3 antigen in bisphosphonate-naïve cases (n = 57) indicates that Endo180 improves the specificity for classifying metastasis where there is no current or prior exposure to these agents (Figure 4C).

**DISCUSSION**

This study provides evidence that Endo180 has the potential to fulfil the official National Institutes of Health definition of a biomarker as a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes or pharmacologic responses to a therapeutic intervention (Diamandis, 2010). The accuracy of Endo180 for the classification determined in this study is aligned with biomarker discoveries made by homing-in on aberrant collagen turnover in metastatic breast disease (Lipton et al, 2011) where Endo180 is proposed to play a fundamental role (Caley et al, 2011; Sturge et al, 2011). In addition, Endo180 also has a direct molecular (Behrendt et al, 2000) and functional (Sturge et al, 2003; Messaritou et al, 2009) relationship with urokinase-type plasminogen activator, which is currently recommended as a prognostic marker for recurrent/metastatic BCa (Harris et al, 2007; Cardoso et al, 2011).

CA 15-3 antigen is normally present on luminal epithelial cells lining breast ducts and its ectodomain is proteolytically cleaved by sheddases and membrane-type-1 matrix metalloproteinase (MT1-MMP) during metastasis (Thathiah et al, 2003; Thathiah and Carson, 2004). Interestingly a co- functional relationship between Endo180 and MT1-MMP has been reported in vivo (Wagenaar-Miller et al, 2007) and in vitro (Messaritou et al, 2009). Together with the observation that Endo180 and MT1-MMP are co-expressed on epithelial and stromal cells in primary tumours (Kogianni et al, 2009), this suggests that MT1-MMP could facilitate Endo180 release and that the cellular source of Endo180 is tumour and/or stromal in origin. These independent cellular mechanism(s) and/or sources may explain the weak linear correlation between Endo180 and CA 15-3 antigen (Supplementary Figure 3).

The elevated levels of Endo180 observed during locoregional recurrence, visceral metastasis and bone metastasis suggests that increased ectodomain shedding occurs when the disease becomes active at a range of tissue sites. However, for the diagnosis of early invasive cancer, the release of Endo180 from small asymptomatic tumours – either from the tumour cells themselves and/or their stromal microenvironment – will need to be detectable above background levels in the normal population. This could be feasible given that Endo180 participates in tumour cell and stromal cell collagen turnover in primary tumours and osteolytic metastases (Curino et al, 2005; Wienke et al, 2007; Caley et al, 2011), indicating that its release could be linked to bidirectional and heterotypic interactions between tumour and stromal cells. Since 25, out of the 29, BCa patients currently or previously receiving bisphosphonates had metastatic lesions in their visceral tissue, as well as bone, it is also feasible that bisphosphonates modulate Endo180 activity at non-bone sites.

The role of bisphosphonates as an adjuvant therapy has recently been explored in the ABCSG-12 (Austrian Breast and Colorectal Cancer Study Group 12) and AZURE (Adjuvant Treatment with Zoledronic Acid in Stage II/III Breast Cancer) trials. ABCSG-12 reported that addition of zoledronic to endocrine therapy reduced disease-free survival but not overall survival in premenopausal women with endocrine responsive BCa (Gnant et al, 2009, 2011).

In the AZURE trial, zoledronic acid did not improve disease-free or overall survival but significantly reduced visceral metastases and locoregional recurrence in a low oestrogen environment (Coleman et al, 2011). Given the important role of Endo180 in collagen remodelling – and the demonstration here that Endo180 is suppressed by bisphosphonates – it is possible that the elevated activity of Endo180 at locoregional and visceral sites helps drive the bisphosphonate-sensitive mechanism identified in disseminated tumour cells (Solomayer et al, 2012); and/or adjacent stromal cells; in the collagen-rich microenvironments of primary and secondary tumours.

Prospective-sample collection and retrospective-blinded-evaluation will be necessary to ascertain how Endo180 levels change as metastatic disease develops and progresses; and to validate whether Endo180 is an accurate diagnostic, screening or prognostic marker in metastatic BCa. The observation that Endo180 is suppressed by bisphosphonates suggests that it could be an indicator of response to bisphosphonates; and should be tested in the setting of metastatic bone disease for its prognostic accuracy in predicting skeletal-related events in correlation with bone-targeted treatment. In a broader context, Endo180 could also be considered as a generalised marker for metastatic disease in other solid tumours.

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**AUTHOR CONTRIBUTIONS**

JS conceived the study. CP recruited patients for inclusion in the study. JS, CP, MCP, A-VF and GK designed the experiments. A-VF and GK conducted cell line experiments. KP, LW, JO and KE collected and processed the samples. KP and MCP conducted the index test. MR-T performed epitope mapping. CP retrieved clinicopathological and treatment data. CP and JS cross-referenced data and performed the data analysis. JS, CP and JW discussed and interpreted the data. JS and CP wrote the manuscript. All authors reviewed the final version of the manuscript.

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