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The effect of blood staining on cervicovaginal quantitative fetal fibronectin concentration and prediction of spontaneous preterm birth☆

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ABSTRACT

Objective: Spontaneous preterm birth is the leading cause of neonatal morbidity and mortality. Cervicovaginal fetal fibronectin (fFN) has enhanced prediction of preterm birth and, more recently, quantified results have become available so that management can planned more effectively and targeted to individual women. Manufacture guidelines stipulate that fetal fibronectin (fFN) samples should be discarded in the presence of moderate to heavy vaginal bleeding but there hasn't yet been any formal investigation into the effect of blood staining on fetal fibronectin concentration and subsequent preterm birth prediction. The objective for this study was to determine the impact of blood stained swabs on quantitative fetal fibronectin (qfFN) concentration and prediction of spontaneous preterm birth (sPTB) in asymptomatic high-risk women.

Study design: Predefined blinded sub-analysis of a larger prospective study of qfFN in asymptomatic women at high-risk of preterm labour. Women with and without blood stained swabs were matched for gestational age at testing and delivery, risk factors and cervical length measurement.

Results: Median fFN concentration in blood stained swabs (n = 58) was 66 ng/ml vs. 7.5 ng/ml in the controls (n = 58) ($p < 0.0001$). At ≥50 ng/ml threshold the false positive ratio (FPR) in blood stained was 25/33 (75.8%) vs. 8/15 (53%) in controls, (risk difference 22.4; −6.8 to 51.6, p = 0.18). At ≥50 ng/ml threshold the false-negative ratio (FNR) in blood stained was 2/25 (8.0%) vs. 1/43 (2.3%) in controls (risk difference −5.7; −17.2 to 5.9, p = 0.55).

At each threshold 10, 50 and 200 ng/ml blood stained swabs had higher sensitivity but lower specificity for predicting preterm birth. Receiver Operating Characteristic (ROC) curve, the strongest global measure of test performance, for prediction of delivery at <34 weeks gestation was similar in blood stained vs. control groups. (0.78 vs. 0.84) in blood stained vs. control groups respectively.

Conclusion: Blood stained swabs have elevated qfFN concentrations but may still have predictive value, and clinical utility. Very low fFN values (<10 ng/ml) are especially reassuring and indicate lower risk of delivery than non-blood stained swabs. The higher false positive rate must be noted and explained to the patient.

Introduction

Spontaneous preterm birth (sPTB), birth before 37 completed weeks' of gestation), is the leading cause of neonatal morbidity and mortality [1]. Prediction of sPTB in symptomatic and asymptomatic high-risk women has been enhanced in recent years by the use of cervicovaginal fluid (CVF) fetal fibronectin (fFN) testing, now widely used in clinical practice. fFN is a glycoprotein found at the interface between chorion and decidua [2] which is usually present in low levels in CVF from 18 weeks of gestation; high levels after

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this time may indicate choriodecidual disruption preceding preterm labour.

fFN has repeatedly been shown to have a high negative predictive value; an excellent 'rule out' test for spontaneous delivery between 23 and 34 weeks gestation. In contrast, the positive predictive value is sub-optimal (<20%) [3]. Traditionally a qualitative test (positive/negative at a threshold concentration of 50 ng/ml), we have now demonstrated improved accuracy in symptomatic [4] and asymptomatic [5] women using a novel bedside analyser (Hologic, Marlborough MA, USA) allowing rapid quantification of fFN concentration; quantitative fetal fibronectin (qfFN), with alternative concentration thresholds of 10 ng/ml and 200 ng/ml more accurately defining those at low and high risk respectively. This has enabled more accurate risk prediction amongst women who would have all traditionally been classified as 'positive', enhancing the positive predictive value of the test (up to 50%), whilst maintaining strong negative prediction.

Manufacture guidelines for both qualitative and quantitative tests stipulate that they should not be used with 'moderate or heavy vaginal bleeding' as plasma fFN can interfere with the CVF fibronectin assay giving potential false positive tests [6]. This is undesirable for any diagnostic test, especially one with modest positive prediction. Similarly, it could be hypothesised that blood-staining of the swab, which independently of fFN can indicate preterm birth risk, could give rise inappropriately to a false negative fFN test. However, incidental macroscopic blood-staining on a cervicovaginal swab is not uncommon, often attributed to the disruption of friable cervical tissue or due to a cervical ectropion. Yet we have not been able to locate any published studies describing the effect of blood-staining on fetal fibronectin results; it is not known whether blood increases false positive rates randomly due to assay cross-over, or whether a test taken from those who had visible blood-staining may have value, but at a different threshold than those currently used. The introduction of the quantified test may allow this to have clinical utility in practice.

The aim of this study was to compare qfFN concentration in a group of asymptomatic high-risk women, with visibly blood-stained swabs, taken between 18^{+0} and 27^{+6} weeks of gestation ('cases'), to a matched group of high-risk asymptomatic women with normal swabs ('controls'). Predictive statistics for sPTB <34 weeks of gestation were calculated and compared.

Materials and methods

A sub-analysis of a larger prospective blinded observational study (**E**valuation of **Q**uantitative Fetal Fibronectin **i**n **P**rediction of **P**reterm Birth, EQUIPP) evaluating the prediction of sPTB using qfFN in high-risk asymptomatic women [5]. The study took place between October 2012–September 2013 at five teaching hospitals in the United Kingdom and was approved by the South East London Research Ethics Committee (REC no: 10/H0806/68 London, UK). Written informed consent was obtained from all participants. Gestational age (GA) was confirmed by early obstetric ultrasound (11–14 weeks' gestation). Participant baseline demographics, obstetric history and risk factors were entered onto an online secure study specific database (www.medscinet.net/ptbstudies). Women were considered high risk if they were 18 + 0–27 + 6 weeks' gestation (the clinically recognized gestational window for fFN testing) [5,7] with one or more of: previous sPTB, previous premature preterm rupture of membranes (PPROM), previous late miscarriage ($16–23^{+6}$), previous cervical surgery (LLETZ, cone biopsy), uterine abnormality or a cervical length <25 mm in this pregnancy. Women presenting with moderate or heavy vaginal bleeding were not included.

Participants with 'macroscopically blood stained' qfFN swabs were matched (1:1) with women from the same database with normal swabs, according to gestational age at testing and delivery (±7 days) and risk factors for PTB (previous sPTB, previous late miscarriage, previous cervical surgery, uterine abnormality or cervical length <25 mm in the current pregnancy). Women with no suitable matched control were excluded.

The qfFN samples were collected as per manufacturer's instructions (Hologic). At speculum examination, Dacron swabs were rotated in the posterior fornix of the vagina for approximately 10 s. Swabs were placed in a test buffer (200 μl aliquots) which were then analysed simultaneously by the qualitative Rapid fFN TLI_{IQ} analyser (Hologic) and quantitative Rapid fFN 10Q analyser (Hologic). Clinicians were blinded to the quantitative result (a result code was generated by the analyzer) but the qualitative result was made available. The 10Q analyser has a range between 0 and 500 ng/ml (upper limit). The reliability of the Rapid 10Q analyser has previously been published [8]. Test thresholds (cut offs) of 10, 50 and 200 ng/ml were pre-defined prior to study data analysis based on the literature [9]. Pregnancy outcome details were obtained from handheld note review by trained research midwives and data entered onto the study database. Data entry was checked for inaccuracies contemporaneously by senior research midwives. Women were considered to have the outcome of interest (sPTB) if they had spontaneous onset of labour, or experienced PPROM, with subsequent premature delivery. Women with iatrogenic delivery <34 weeks' were excluded. Samples from women reporting prior sexual intercourse (within 48 h) were excluded from analysis due to known interference with the assay [10], as were results from women with PPROM, multiple pregnancy or cervical dilation ≥3 cm. A 'true positive' result was defined as spontaneous onset of labour (or PPROM) <34 weeks' with qfFN >50 ng/ml. A 'false positive' result was CVF qfFN ≥50 ng/ml at testing, and delivery $>33^{+6}$ weeks' gestation. Predictive statistics using alternative thresholds (10 ng/ml and 200 ng/ml) were also explored.

Statistical analysis was conducted using the Stata software (version 11.2; StatCorp LP, College Station, TX). Standard distributional checks were carried out, asymmetric qfFN values logged and checks repeated. Geometric means were generated after transformation of log-normal distributions. Quantitative fFN values were compared between groups using Student's *t*-tests on log transformed values and (nonparametric) area under the Receiver Operating Characteristic (ROC) curves. Medians were compared using the Wilcoxon rank sum test. Results are reported as ratios of geometric means. To check for a difference in performance between the blood stained and normal swabs, interaction between swab status and test result was compared using logistic regression with a correction to the standard errors for matching [10].

Results

A total of 63 asymptomatic high-risk participants with singleton pregnancies and blood stained swabs between 18^{+0} and 27^{+6} weeks' were identified. Of these, 2 participants who underwent iatrogenic deliveries (both pre-labour induction for pre-eclampsia) were excluded, and 1 was excluded due to an 'invalid' qfFN result (the bedside analyser was unable to provide a result). Two more were excluded due to lack of appropriate matched control, leaving 58 participants fulfilling criteria for analysis. These were matched with 58 controls according to gestational age at testing, gestational age at delivery, and risk factors for premature birth. Demographic, background, and obstetric characteristics for study participants are described in Table 1 and were comparable for cases and controls. Mean gestational age at testing for both groups was 23^{+1} weeks, and mean gestational age at delivery was 37^{+1} weeks. sPTB rate <34 weeks and <37 weeks gestation in the blood stained cases was 10/

Table 1
Demographic and Obstetric characteristics of study population. Results are mean (SD) or n (%), as indicated.

Characteristic	Blood stained swabs	Controls
Age	32 ± 4	33 ± 5
Body mass index, kg/m^2	25 ± 6	25 ± 5
Ethnicity, n (%)		
White	29 (50)	27 (47)
Black	16 (28)	22 (38)
Other	13 (22)	9 (15)
Preterm birth, n (%)	20 (35)	19 (33)
Previous Preterm rupture of membranes, n (%)	9 (16)	4 (7)
Previous second trimester miscarriage, n (%)	19 (33)	18 (31)
Previous cervical surgery, n (%)	26 (44)	26 (44)
Smoking history%		
Current	2 (4)	3 (5)
Ex-smoker	14 (24)	10 (17)
Never	42 (72)	45 (78)
History of domestic violence, n (%)	0 (0)	3 (6)
Gestational age at testing	$23^{+1} \pm 3^{+2}$	$23^{+1} \pm 3^{+1}$
Gestational age at delivery	$37^{+1} \pm 4^{+3}$	$37^{+1} \pm 4^{+4}$

58 (17%) and 13/58 (22%) respectively; in the control group this was 8/58 (13.8%, p = 0.61) and 11/58 (19.0%, p = 0.65).

The median concentration of qfFN in the blood stained cases was 66 ng/ml (quartiles 13, 270), vs. 7.5 ng/ml (3, 52) in the controls (p < 0.0001). Average qfFN concentration was 3.7 (95% confidence Interval (CI) 2.1-6.7) times higher in the blood stained swab group vs. controls (ratio of the geometric means 53.9 and 14.4 ng/ml, p < 0.0001). Only 11/58 (19.0%) blood stained swabs fell into the lowest qfFN category <10 ng/ml, vs. 32/58 (55%) for controls. In comparison 15/58 (26%) blood stained swabs measured CVF concentrations ≥200 ng/ml, vs 5/58 controls (9%) (Table 2, Fig. 1).

Table 3 illustrates the predictive statistics for prediction of sPTB <34 week of gestation, at pre-defined qfFN thresholds. For prediction of sPTB <34 weeks of gestation, the specificity of the test was reduced in the blood stained swab group compared with the control group when using the traditional 'positive' threshold of ≥50 ng/ml (difference −36.1%; −53.5 to −18.7, p < 0.01), as well as when using a threshold of ≥200 ng/ml (difference −12.7; −24.5 to −8.1, p < 0.05), and the lower threshold of ≥10 ng/ml (difference −39.1; −57.4 to −21.1, p < 0.01). The overall false positive rate (FPR) in the blood stained swabs groups were consistently higher than the control groups, although these differences did not meet statistical significance. For the threshold of ≥50 ng/ml FPRs were 25/33 (75.8%) in the blood stained swabs vs. 8/15 (53%) in the control swabs, (risk difference 22.4; −6.8 to 51.6, p = 0.18). For a threshold of ≥200 ng/ml, FPR was 8/15 (53.3%) vs. 2/5 (40%), (risk difference 13.3; −36.5 to 63.1, p = 1.00); and for a threshold of ≥10 ng/ml FPR was 37/47 (78.7%) vs. 19/26 (73.1%) (risk difference 5.6%, CI −15.0 to 26.3, p = 0.58).

Table 2
Spontaneous preterm birth in asymptomatic high-risk women according to quantitative fetal fibronectin (qfFN) categories, for the 58 blood stained swabs (non-shaded), and 58 controls (shaded).

qfFN category (ng/ml)	Test	N (%)	Spontaneous Preterm Birth < 34 weeks of gestation N (%)
Less than 10	Blood stained	11 (19)	0 (0.0)
	Control	32 (55)	1 (3)
10-49	Blood stained	14 (24)	2 (14)
	Control	11(19)	0 (0)
50-199	Blood Stained	18 (31)	1 (6)
	Control	10 (17)	4 (40)
≥200	Blood stained	15 (26)	7 (47)
	Control	5 (9)	3 (60)
All	Blood stained	58 (100)	10 (17)
	Control	58 (100)	8 (14)

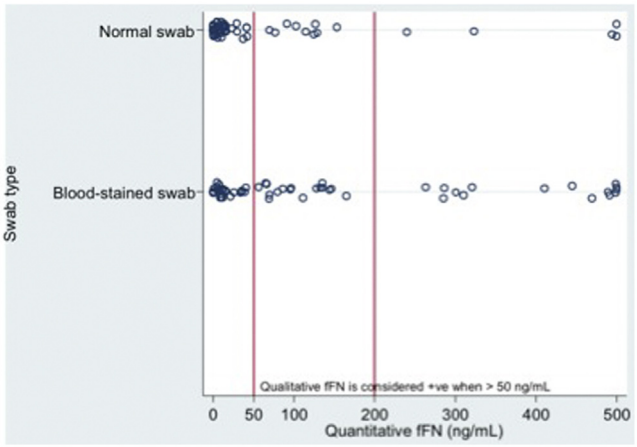

Fig. 1. Strip plot of quantitative fetal fibronectin concentration results in the blood stained swabs and control groups.

Overall sensitivities for prediction of sPTB <34 weeks were generally high in both groups (Table 3); apparent numerical differences did not reach statistical significance but, of note, overall sensitivity for delivery <34 weeks was higher (70% [34.8 to 93.3, 7 of 10])in the blood stained swab group when using a qfFN threshold of ≥200 ng/ml compared with 37.5% (8.5 to 75.5, 3 of 8) in the control group, (risk difference 32.5%; −11.5 to 76.5, p = 0.34). The false negative rate was slightly higher in the blood stained swabs than control swabs at the traditional threshold of 50 ng/ml; 2/25 (8.0%) vs 1/43 (2.3%) for the blood stained and control swabs respectively, though this difference was not statistically significant (risk difference −5.7; −17.2 to 5.9, p = 0.55). This was similar at a threshold of ≥200 ng/ml; 3/43 (7.0) vs 5/53 (9.4%) in the cases and controls (risk difference 2.5; −8.5 to 13.4, p = 0.73). At a threshold of ≥10 ng/ml the FNR was unsurprisingly low in both groups blood vs. control; 0/11 (0.0%) in the blood stained group vs. 1/32 (3.1%) in the control group (risk difference 3.1, −2.9 to 9.2, p = 1.0).

ROC curves for the performance of qfFN for sPTB prediction prior to 34 and 37 weeks of gestation in the blood stained and control groups are shown in Fig. 2A and B. For the prediction of sPTB <34 weeks of gestation, area under the curve (AUC) was 0.78 (0.62–0.95) in the blood stained swab group, compared with 0.84 (0.61–1.0) in the control group (p = 0.70). For prediction of sPTB >37 weeks of gestation, the AUC was 0.71 (0.53–0.88) vs. 0.80 (0.62–0.98) (p = 0.46) in the blood stained vs. the control groups respectively.

Table 3
Prediction of spontaneous preterm birth at <34 weeks' gestation according to cervicovaginal fluid fetal fibronectin concentration for both the blood stained swabs (non-shaded) and control swabs (18–27^{+6} weeks) (shaded).

		Fetal fibronectin threshold (ng/ml)		
Predictive variable	**Test**	**≥10**	**≥50**	**≥200**
Sensitivity	Blood Stained	100.0 (69.2 to 100.0)	80.0 (44.4 to 97.5)	70.0 (34.8 to 93.3)
	Control	87.5 (47.3 to 99.7)	87.5 (47.3 to 99.7)	37.5 (8.5 to 75.5)
Specificity	Blood Stained	22.9 (12.0 to 37.3)	47.9 (33.3 to 62.8)	83.3 (69.8 to 92.5)
	Control	62.0 (47.2 to 75.3)	84.0 (70.9 to 92.8)	96.0 (86.3 to 99.5)
PPV	Blood Stained	21.3(10.7 to 35.7)	24.2 (11.1 to 42.3)	46.7 (21.3 to 73.4)
	Control	26.9(11.6 to 47.8)	46.7 (21.3 to 73.4)	60.0 (14.7 to 94.7)
NPV	Blood Stained	100.0 (71.5 to 100.0)	92.0 (74.0 to 99.0)	93.0 (80.9 to 98.5)
	Control	96.9 (83.8 to 99.9)	97.7 (87.7 to 99.9)	90.6 (79.3 to 96.9)
Positive LR	Blood Stained	1.3 (1.11 to 1.51)	1.54 (1.0 to 2.3)	4.2 (2.0 to 8.9)
	Control	2.3 (1.48 to 3.58)	5.5 (2.8 to 10.9)	9.4 (1.8 to 46.7)
Negative LR	Blood Stained	0.0	0.4 (0.1 to 1.5)	0.4 (0.1 to 0.9)
	Control	0.2 (0.03 to 1.3)	0.15 (0.02 to 0.9)	0.7 (0.4 to 1.1)

PPV, positive predictive value; NPV, negative predictive value; ROC, receiver operating curve; LR, likelihood ratio.Data are% (95% confidence interval) unless otherwise specified.
*Specificity comparisons (risk difference) for the blood stained vs. the control test at each fibronectin threshold were significantly different at each threshold (P < 0.05). Sensitivity comparisons did not reach statistical significance at each threshold (P > 0.05).

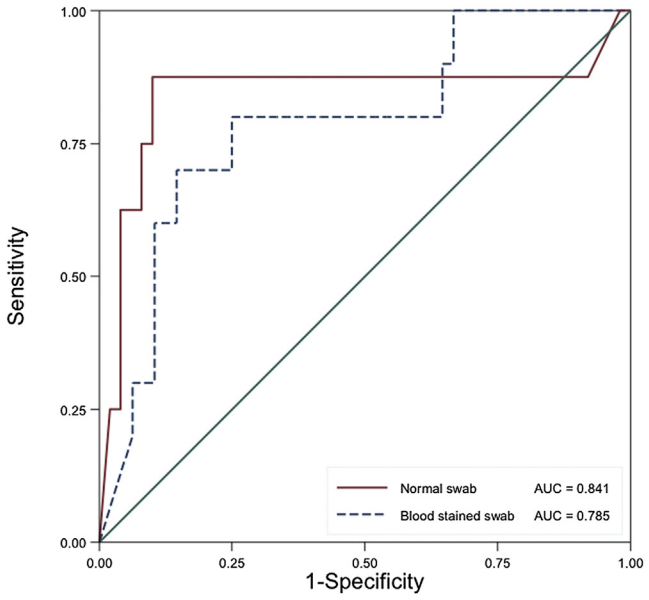

Fig. 2. ROC curve comparison of performance of fetal fibronectin concentration for prediction of delivery <34 weeks' gestation in blood stained swabs and control group.

Comment

Matsuura and co-workers [11,12] originally described the IgG monoclonal antibody FDC-6 in fibronectins from fetal tissues, which specifically recognise III-CS, the region defining the fetal isoform of fibronectin. The plasma form of fibronectin, synthesised by hepatocytes, circulates in the blood of pregnant women, men and non-pregnant women. Approximately 1–4% of circulating plasma fibronectin [6] contains the reactive III-CS region which has the capacity to bind to the FDC-6 antibody and may therefore be detected by conventional CVF fFN assays. In the seminal study of fFN and prediction of sPTB, Lockwood et al. [2] not only revealed that the presence of fFN >50 ng/ml in CVF secretions identified a group of women with symptoms of sPTB at high risk of subsequent preterm delivery, but found that mean (±SD) concentrations of fFN in maternal plasma was 2000 ±2300 ng/ml in the second trimester. For this reason, they excluded women with visible blood staining on their swab from their primary analysis. Thus, manufacturers of CVF fFN bedside tests recommend that visibly blood stained swabs are discarded, due to risk of a false positive result. This is the first study to prospectively quantify the difference in qfFN concentration *in vivo* between blood stained swabs and matched controls, and evaluate how this affects prediction of preterm birth in asymptomatic women.

Women with blood stained swabs had nearly four times higher mean fFN concentrations when measured using the same antibody as the ELISA in the rapid bedside qfFN analyser (Hologic). Blood-staining reduced the ability of the test to correctly identify women who did not delivery prematurely; both the traditional 'positive' test threshold of 50 ng/ml and lower threshold of 10 ng/ml had significantly reduced specificity to predict sPTB <34 weeks' in the presence of blood staining, and correspondingly higher false positive rates when compared to matched controls.

However, rather than reinforcing the manufacturers recommendation to avoid fFN testing in women with macroscopic blood stained swabs, we have demonstrated potential value for the use of qfFN testing to predict sPTB as the ROC area under the curve (AUC) of 0.78 (0.62–0.95) for prediction of spontaneous delivery >34 weeks' was only slightly (and not statistically) lower than that of the matched controls (AUC 0.84, 0.61 to 1.0). A qfFN concentration of >200 ng/ml had good sensitivity (70%) and specificity (83%) for prediction of sPTB <34 weeks of gestation with blood stained samples, and the FPR was only slightly higher than in the control group (53% vs. 40%, p = 1.0). Even when blood stained samples are obtained, asymptomatic high-risk women should be warned that their sPTB risk is higher than expected, and continued monitoring and surveillance (e.g. longitudinal cervical length screening or other gestation appropriate interventions such as admission to hospital or administration of corticosteroids). The mechanism of risk could be different, i.e; may indicate placental pathology. However, as sensitivity remains high, in the presence of visual blood, the fact remains that fFN is an important predictor.

Conversely, we have shown that high risk women with very low concentrations of qfFN when measured from a blood stained swab, can be reassured of a very low risk of sPTB <34 weeks; none of the 11 women in the blood stained cohort with fFN <10 ng/ml delivered <34 weeks' and the FNR was not shown to be significantly higher at the ≥10, ≥50 or ≥200 ng/ml 'positive' qfFN thresholds, when compared with the matched control group.

In a similar study of the traditional qualitative fFN (50 ng/ml threshold) in women symptomatic of preterm labour, Bruijn et al. [13] found that blood staining on the swab increased the proportion of positive results, but that conversely, blood-staining was significantly associated with a lower chance of a false positive result, when compared to non blood stained swabs. This is likely because for women in preterm labour, vaginal bleeding is independently associated with risk of preterm delivery. In contrast, this study aimed to evaluate whether there is value in testing for CVF fFN when a blood stained swab was obtained, usually a result of disruption of friable cervical tissue or an ectropion, rather than in the presence of moderate of heavy frank vaginal bleeding (these women were considered symptomatic and seen in the acute department, rather than in the out-patient prematurity clinic). The relatively small sample size limited the predicted power to detect statistically significant differences between prediction of sPTB in the blood-stained and control population but more than 3000 qfFN swabs from over 1000 women at high risk of preterm birth were required to obtain this number of macroscopically blood stained samples.

In conclusion, if a qfFN swab is blood stained, then the sample may still have predictive value for the clinician and patient; very low levels of qfFN (<10 ng/ml) are still reassuring of a low risk of delivery, and high concentrations (>200 ng/ml) indicate higher risk of delivery <34 weeks of gestation, though the increased risk of false positive results must be recognized and explained fully to the patient, and repeated testing may be of value.

Conflict of interest

Drs. Hezelgrave and Shennan received financial assistance to provide educational talks on preterm birth from Hologic, USA. Drs. Hezelgrave, Tribe and Shennan received funding for research paid to their institution.

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