



Combination of vaginal pH with vaginal sialidase and prolidase activities for prediction of low birth weight and preterm birth

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KEY WORDS

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Objective: The purpose of this study was to assess if easy to measure vaginal fluid biomarkers are predictive for low birth weight (LBW, <2500 g), very LBW (VLBW, <1500 g), spontaneous preterm at <37 weeks' gestation, and total preterm deliveries (at <37, <35, <32 weeks' gestation).

Study design: Low and high cutoffs for vaginal fluid pH, sialidase, and prolidase activities were examined in a nested case-control study of 579 Danish women (from a study population of 2846 women) with samples collected at mean 17 weeks' gestation. One hundred sixteen LBW (17 VLBW), 117 preterm deliveries (85 spontaneous), and 418 normal term deliveries were analyzed.

Results: Vaginal pH ≥ 4.7 or pH ≥ 5 by itself was not associated with LBW or prematurity. Conversely, combination of pH ≥ 5 and high sialidase activity demonstrated OR 17 (CI 1.8-150) for LBW; OR 31 (CI 1.8-516) for VLBW; along with OR 18 (CI 1.6-204) for preterm at <35 weeks'; and OR 31 (CI 1.9-542) for preterm at <32 weeks' gestation. The combination of pH ≥ 5 and high prolidase activity demonstrated OR 13 (CI 1.3-122) for LBW; OR 33 (CI 2.0-553) for VLBW, as well as OR 9.2 (CI 0.6-150) for preterm at <35 weeks'; and OR 35 (CI 2.0-586) for preterm at <32 weeks' gestation. In this population, no woman having high sialidase and high prolidase activity had a term birth, or a baby weighting ≥ 2500 g at birth.

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Conclusion: In this Danish population, mid-gestation findings of vaginal fluid elevated pH with sialidase and/or prolidase were associated with LBW, VLBW, and early preterm at <35 or <32 weeks' gestation.

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Low birth weight (LBW, <2500 g), including very LBW (VLBW, <1500 g), resulting primarily from short gestation and preterm delivery remains the most common determinant for subsequent neonatal mortality, morbidity, and long-term neurodevelopmental disorders, including cerebral palsy, in industrialized countries.¹⁻⁴ Many cases of LBW have no readily recognizable etiologically linked risk factors identifiable in asymptomatic mothers before mid gestation.^{2,3} Among the multiple pathophysiologic pathways to LBW and prematurity, several investigations have shown that abnormal vaginal flora, including bacterial vaginosis (BV), is associated with preterm delivery (PTD), LBW, early and late miscarriage, and maternal complications.¹⁻⁵ However, only limited proportions of women with BV actually have adverse pregnancy outcomes. In one study, only 6.3% had a LBW preterm infant, and 3.4% had a LBW full-term infant.⁴ Thus, more specific predictive markers for LBW and preterm delivery are needed among women with BV and other cases of LBW/PTD-associated altered vaginal flora.¹⁻⁴

BV is clinically characterized by a vaginal pH ≥ 4.5 , amine odor, adherent white discharge, clue cells (Amsel's criteria), and decrease in lactobacilli.⁶ Thorsen et al⁵ described a microbial pathologic "core" for BV, resulting from synergistic relations between *Gardnerella vaginalis* and anaerobic bacteria, and this observation has been supported by further studies.⁷⁻⁸ BV-associated anaerobic microorganisms produce hydrolytic enzyme activities detectable in vaginal fluid, including sialidase^{9,10} and prolidase.¹¹ Sialidase (neuraminidase) and prolidase (proline aminopeptidase) activities in pregnant women with BV have been previously correlated with increased risks of adverse pregnancy outcomes.^{10,12,13}

Some, but not all, US studies demonstrated that an elevated vaginal pH (≥ 4.5 or ≥ 5.0) by itself,^{14,15} or combined with an elevated Nugent Gram stain score,¹⁴ or with elevated neutrophils¹⁵ was associated with preterm delivery and LBW. In a previous nested case-control study performed in the same European population, we found that sialidase or prolidase activity as single vaginal markers were associated with subsequent LBW, but not with spontaneous preterm delivery at <37 weeks' gestation.¹³

The intent of the present analysis was to determine whether combinations of readily measured objective parameters of vaginal fluid, ie, pH, sialidase, and prolidase activities, can detect and quantify increased risks for LBW or VLBW, spontaneous preterm at <37 weeks, and total preterm deliveries at <37 or <35 or

<32 weeks' gestation among European women evaluated in the second trimester of gestation.

Material and methods

Study population

We selected 579 women from a cohort totaling 2846 singleton pregnant women at their first prenatal visit (before the 24th week of gestation) enrolled prospectively from November 1992 to February 1994 at Odense Hospital (Denmark). The purpose of the original study was to evaluate associations between maternal infections and adverse pregnancy outcomes. Inclusion and exclusion criteria were previously described (appropriate informed consent was obtained, and clinical research was conducted in accordance with the guidelines for human experimentation of the authors' institutions).^{5,13}

LBW and VLBW were defined as birth weight <2500 g and <1500 g, respectively. PTD was defined as spontaneous delivery (non-medically indicated), including spontaneous rupture of membranes or labor before 37 weeks' gestation. In addition, total deliveries (spontaneous and induced) that occurred at <37 weeks' or <35 weeks', or <32 weeks' gestation were examined.¹⁴

Nested case-control study sample

A total of 117 prematurity (85 were spontaneous, ie, PTD) cases and 116 LBW cases were obtained. Among LBW cases, 72 were from preterm deliveries at <37 weeks' gestation, and 44 were from term deliveries. A randomly selected group of 418 women delivering normal birth weight babies at term (NTD) was used as control group. The rationale for a number of 418 controls was to get at least 3 controls per case in any circumstance and included was also an oversampling of 20%. None of the deliveries among NTD were medically induced. Among participants in the nested case-control study, mean age at predicted date of delivery was 29 years, (18-42 years); mean gestational age at enrollment was 16w + 5d (16 full weeks' gestation plus 5 days) (range, 7w + 4d to 24w + 0d); mean gestational age of 161 cases and 418 controls were 17w + 2d and 16w + 1d, respectively. Among the 579 women enrolled, 43 had preeclampsia, 11 had pregnancy-induced hypertension, and 5 had essential hypertension.

Table I Characteristics of women included in the nested-case control study

Characteristic	NBW	LBW	VLBW	NTD	PTD	<37 wk	<35 wk	<32 wk
Number of women	463	116	17	418	85	117	49	15
Age (y)	28.8 (18.5-41.9)*	28.1 (20.9-40.9)*	29.9 (23.8-38.4)*	28.9 (18.5-40.1)*	27.4 (18.7-41.9)*	27.8 (18.7-41.9)*	27.5 (21.7-38.4)*	29.9 (21.7-38.4)*
Gestational weeks at enrollment	16.3 (7.6-23.4)*	18.0 (9.6-24.3)*	18.6 (9.6-20.1)*	16.1 (7.6-23.4)*	17.9 (9.3-21.9)*	18.3 (9.3-22.3)*	17.6 (9.6-21.6)*	16.6 (9.6-19.0)*
Gestational weeks at delivery	40.1 (32-43.3)*	35.8 (26.3-42.3)*	30.1 (26.3-35.3)*	40.3 (37.0-43.3)*	35.6 (26.3-36.9)*	35.4 (26.3-36.9)*	33.3 (26.3-34.9)*	29.9 (26.3-31.6)*
Infant weight at birth (g)	3490 (516) [†]	2011 (449) [†]	1136 (252) [†]	3554 (494) [†]	2439 (621) [†]	2257 (657) [†]	1760 (574) [†]	1192 (330) [†]
BV positive	14.5 [‡]	20.7 [‡]	17.6 [‡]	14.6 [‡]	12.9 [‡]	13.7 [‡]	16.3 [‡]	13.3 [‡]
Sialidase > +1 positive	14.9 [‡]	28.7 [‡]	25.0 [‡]	15.3 [‡]	20.2 [‡]	19.0 [‡]	27.1 [‡]	21.4 [‡]
Sialidase > +2 positive	0.86 [‡]	3.48 [‡]	6.3 [‡]	0.96 [‡]	2.38 [‡]	1.72 [‡]	4.2 [‡]	7.1 [‡]
Prolidase > +1 positive	33.2 [‡]	42.5 [‡]	33.3 [‡]	33.9 [‡]	35.4 [‡]	33.3 [‡]	45.7 [‡]	38.5 [‡]
Prolidase > +2 positive	1.09 [‡]	4.43 [‡]	6.7 [‡]	0.96 [‡]	3.66 [‡]	3.51 [‡]	4.3 [‡]	7.7 [‡]
Vaginal pH ≥5	12.3 [‡]	19.0 [‡]	11.8 [‡]	12.0 [‡]	14.1 [‡]	15.4 [‡]	18.4 [‡]	13.3 [‡]
Previous PTD	4.97 [‡]	15.5 [‡]	11.8 [‡]	3.35 [‡]	15.3 [‡]	17.1 [‡]	10.2 [‡]	6.7 [‡]
Previous spontaneous abortion	19.7 [‡]	22.4 [‡]	5.9 [‡]	19.6 [‡]	23.5 [‡]	23.1 [‡]	22.4 [‡]	13.3 [‡]
Previous induced abortion	18.8 [‡]	15.5 [‡]	17.6 [‡]	18.7 [‡]	22.4 [‡]	20.5 [‡]	24.5 [‡]	13.3 [‡]
LBW in last pregnancy	3.02 [‡]	13.8 [‡]	11.8 [‡]	2.63 [‡]	7.06 [‡]	9.40 [‡]	6.1 [‡]	6.7 [‡]
Serious bleeding in pregnancy	1.51 [‡]	2.59 [‡]	0.0 [‡]	1.44 [‡]	2.35 [‡]	2.56 [‡]	2.0 [‡]	0.0 [‡]
≥ 6 h work walking daily	42.8 [‡]	50.0 [‡]	47.1 [‡]	42.1 [‡]	55.3 [‡]	52.1 [‡]	55.1 [‡]	46.7 [‡]
≥ 6 h work standing daily	46.9 [‡]	46.6 [‡]	41.2 [‡]	45.7 [‡]	56.5 [‡]	52.1 [‡]	49.0 [‡]	40.0 [‡]
≥ 4 alcoholic drinks weekly	1.95 [‡]	4.35 [‡]	5.9 [‡]	1.92 [‡]	4.71 [‡]	4.27 [‡]	4.1 [‡]	0.0 [‡]
≥ 10 cigarettes daily at enrollment	6.97 [‡]	13.0 [‡]	0.0 [‡]	7.23 [‡]	7.14 [‡]	9.57 [‡]	8.3 [‡]	0.0 [‡]
Body mass index	21.8 (15.8-40.9)*	21.3 (17.2-39.7)*	22.6 (17.8-28.1)*	21.8 (15.8-40.9)*	21.9 (17.6-36.4)*	22.3 (17.6-39.7)*	22.8 (17.8-39.7)*	21.3 (17.8-28.1)*

The following subsets of pregnant women were examined: NBW (≥2500 g at birth); LBW (<2500 g at birth); VLBW (<1500 g at birth); NTD (control women who delivered an infant weighting ≥2500 g at birth at ≥37 weeks' gestation); PTD (spontaneous delivery at <37 weeks' gestation); <37 weeks (total deliveries at <37 weeks' gestation); <35 weeks (total deliveries at <35 weeks' gestation); <32 weeks (total deliveries at <32 weeks' gestation).

* Median (range).

[†] Mean (SD).

[‡] Percent of women within outcome category (NBW, LBW, etc).

Vaginal sample collection

Samples were collected by a physician at enrollment visit.¹³ BV was clinically diagnosed by Amsel's criteria.^{5,6,13} Samples of vaginal fluid were collected as previously published.¹³

Sialidase activity

Sialidase activity was determined by incubation of 50 μL of the vaginal sample with 50 μL of the substrate at pH 5.0.⁷ Specific activity was expressed as nanomoles of methoxyphenol produced by comparison with

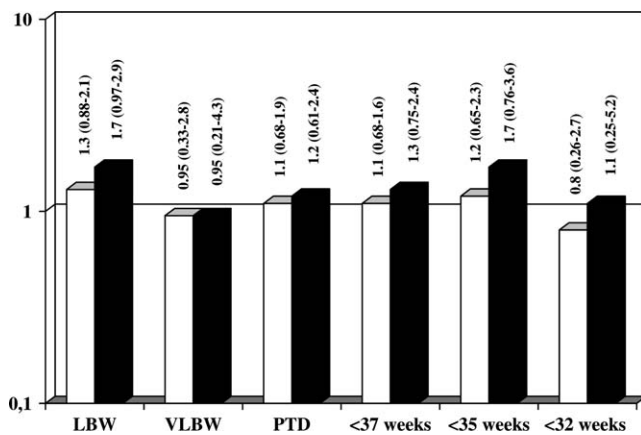


Figure 1 ORs (95% CI) of elevated vaginal pH ≥ 4.7 or ≥ 5.0 for prediction of LBW (<2500 g), and VLBW (<1500 g) vs newborn infant weighting ≥ 2500 g at birth (NBW). Vaginal pH was measured on a total of 579 women: 463 women who had NBW, 116 LBW, 17 VLBW, 418 NTD, 85 PTD, 117 women who had a delivery at <37 weeks', 49 women who had a delivery at <35 weeks', and 15 women who had a delivery at <32 weeks' gestation. ORs for spontaneous delivery at <37 weeks' gestation (PTD), and total preterm deliveries at <37, <35, and <32 weeks' gestation were evaluated vs NTD (delivery at ≥ 37 weeks of gestation of an infant with ≥ 2500 g weight at birth).

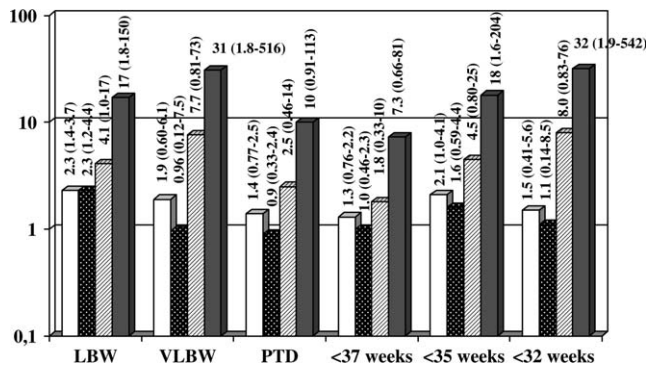


Figure 2 ORs (95% CI) of progressive sialidase activity levels +1 and +2 combined with vaginal pH ≥ 5.0 for prediction of LBW (<2500 g), and VLBW (<1500 g) vs newborn infant weighting ≥ 2500 g at birth (NBW). ORs for spontaneous delivery at <37 weeks' gestation (PTD), and total preterm deliveries at <37, <35, and <32 weeks' gestation were evaluated vs NTD (delivery at ≥ 37 weeks of gestation of an infant with ≥ 2500 g weight at birth). Sialidase activity and vaginal pH were measured on a total of 578 women: 463 women who had NBW, 115 LBW, 16 VLBW, 418 NTD, 84 PTD, 116 women who had a delivery at <37 weeks', 48 women who had a delivery at <35 weeks', and 14 women who had a delivery at <32 weeks' gestation.

a standard curve of pure methoxyphenol. Sialidase levels were defined as: no activity, <0.19 nmol; +1 cutoff, ≥ 0.19 nmol; +2 cutoff, ≥ 5.00 nmol of methoxyphenol.^{13,16}

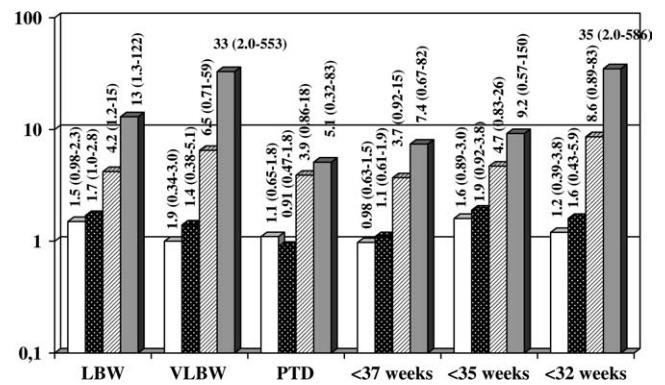


Figure 3 ORs (95% CI) of progressive prolidase activity levels +1 and +2 combined with vaginal pH ≥ 5.0 for prediction of LBW (<2500 g), and VLBW (<1500 g) vs newborn infant weighting ≥ 2500 g at birth (NBW). ORs for spontaneous delivery at <37 weeks' gestation (PTD), and all preterm deliveries at <37, <35, and <32 weeks' gestation were evaluated vs NTD (delivery at ≥ 37 weeks of gestation of an infant with ≥ 2500 g weight at birth). Prolidase activity and vaginal pH were measured on a total of 574 women: 461 women who had NBW, 113 LBW, 15 VLBW, 416 NTD, 82 PTD, 114 women who had a delivery at <37 weeks', 46 women who had a delivery at <35 weeks', and 13 women who had a delivery at <32 weeks' gestation.

Prolidase activity

Prolidase activity was determined as described.⁷ Absorbance (mOD) was read at 405 nm. Prolidase levels were defined as: no activity, <22 mOD; +1 cutoff, ≥ 22 mOD; +2 cutoff, ≥ 2000 mOD.^{13,16}

Statistical analysis

Odds ratios (ORs) and 95% CIs were calculated using logistic regression to estimate the relative risk for each adverse pregnancy outcome. Various factors were examined as potential confounders, and those that qualified were included in the model to adjust the ORs. Stata (StataCorp, College Station, Tex) and SPSS (Statistical Package for Social Sciences; Chicago, Ill) were used for data analyses.

Results

Of the 2846 eligible women, 579 were examined in this nested case-control study of LBW, VLBW, and prematurity. Characteristics of study subsets of women are described in Table I.

Figure 1 illustrates findings associated with vaginal pH. Study women at approximately 17 weeks' gestation with vaginal pH ≥ 4.7 or ≥ 5.0 did not demonstrate a significantly increased incidence of subsequent LBW, VLBW, spontaneous, or total preterm deliveries.

Figure 2 illustrates findings associated with progressive levels of sialidase activity alone or in combination

Table II Combinations of progressive sialidase levels with progressive prolidase and pH levels for prediction of LBW (<2500 g at birth), and VLBW (<1500 g at birth) vs newborn infant weighting ≥ 2500 g at birth (no LBW)

Combination of 2 enzyme markers and pH	> 2500 g n	> 2500 g %	LBW n	LBW %	Odds ratio	95% CI	VLBW n	VLBW %	Odds ratio	95% CI
Sialidase > +1 and prolidase > +1	52	11	22	19	1.8 [‡]	1.0-3.2	3	20	2.0	0.5-7.2
Sialidase > +1 and prolidase > +2	1	0.2	3	2.7	10 [‡]	1.0-100	1	6.7	33	2.0-553
Sialidase > +2 and prolidase > +1	4	0.9	4	3.5	3.5 [‡]	0.8-15	1	6.7	8.2	0.9-78
Sialidase > +2 and prolidase > +2	0		2	1.8	*		1	6.7	*	
pH ≥ 5.0 , sialidase > +1 and prolidase > +1	30	6.5	15	13	2.0 [‡]	1.0-4.0	1	6.7	1.0	0.1-8.1
pH ≥ 5.0 , sialidase > +1 and prolidase > +2	1	0.2	3	2.7	10 [‡]	1.0-100	1	6.7	33	2.0-553
pH ≥ 5.0 , sialidase > +2 and prolidase > +1	1	0.2	4	3.5	13 [‡]	1.4-121	1	6.7	33	2.0-553
pH ≥ 5.0 , sialidase > +2 and prolidase > +2	0		2	1.8	*		1	6.7	*	

Sialidase and prolidase activity was measured in a total of 461 women who had no LBW, 113 women who had LBW, and 15 women who had VLBW.

* No woman with sialidase > +2 and prolidase > +2 had an infant having ≥ 2500 g weight at birth; thus, ORs could not be calculated.

[‡] In these cases, there was confounding by smoking for which the ORs was consequently adjusted.

Table III Combinations of progressive sialidase levels with progressive prolidase levels for prediction of PTD, and all preterm deliveries at <37 weeks' gestation

Combination of 2 enzyme markers and pH	NTD n	NTD %	PTD n	PTD %	Odds ratio	95% CI	<37 wk (n)	<37 wk (%)	Odds ratio	95% CI
Sialidase > +1 and prolidase > +1	48	12	11	13	1.2	0.6-2.4	14	12	1.1	0.6-2.0
Sialidase > +1 and prolidase > +2	1	0.2	1	1.2	5.1	0.3-83	2	1.8	7.4	0.7-82
Sialidase > +2 and prolidase > +1	4	1.0	2	2.4	2.6	0.5-14	2	1.8	1.8	0.3-10
Sialidase > +2 and prolidase > +2	0		1	1.2	*		1	0.9	*	
PH ≥ 5.0 , sialidase > +1 and prolidase > +1	28	6.7	5	6.1	0.9	0.3-2.4	8	7.0	1.0	0.5-2.4
PH ≥ 5.0 , sialidase > +1 and prolidase > +2	1	0.2	1	1.2	5.1	0.3-83	2	1.8	7.4	0.7-82
PH ≥ 5.0 , sialidase > +2 and prolidase > +1	1	0.2	2	2.4	10	0.9-116	2	1.8	7.4	0.7-82
PH ≥ 5.0 , sialidase > +2 and prolidase > +2	0		1	1.2	*		1	0.9	*	

Sialidase and prolidase activity was measured on a total of 416 women who had NTD (delivery of an infant with ≥ 2500 g weight at birth at >37 weeks' gestation), 82 women who had a PTD, and 114 women who had a delivery at <37 weeks' gestation.

* No woman with sialidase > +2 and prolidase > +2 had a NTD.

with pH ≥ 5.0 . A positive +1 sialidase activity was associated with significantly increased (OR 2.3) risk of LBW, irrespective of pH. Sialidase values of +2 demonstrated crude OR 4.1 (CI 1.0-17) for LBW. The crude OR for LBW increased 4-fold with a concomitant vaginal pH ≥ 5 , crude OR 17 (CI 1.8-150). An even larger crude OR 31 (CI 1.8-516) was observed for VLBW.

Results for prematurity differed somewhat from LBW; Figure 2 shows that an elevated sialidase and vaginal pH ≥ 5.0 was not significantly associated with spontaneous PTD, as well as total preterm deliveries at <37 weeks' gestation (vs NTD). However, a positive +1 sialidase activity demonstrated OR 2.1 (CI 1.0-4.1) for preterm at <35 weeks' gestation. Dramatically, the combination of pH ≥ 5 with high sialidase (+2) had large statistically significant ORs for early preterm delivery: OR 18 (CI 1.6-204) for preterm delivery at

<35 weeks', and OR 32 (CI 1.9-542) for preterm delivery at <32 weeks' gestation.

Figure 3 illustrates results for prolidase activities. A positive prolidase activity (+1) did not demonstrate a statistically significant OR for LBW, whether or not combined with pH ≥ 5 . However, high levels of prolidase activity (+2) demonstrated statistically significant associations (OR 4.2, CI 1.2-15) for LBW. The OR was dramatically increased 3-fold by combination with pH ≥ 5 , OR 13 (CI 1.3-122). Consistently large ORs for VLBW were observed in women with high prolidase (+2) and pH ≥ 5 (OR 33, CI 2.0-553, Figure 3). High prolidase (+2) in combination with pH ≥ 5 demonstrated OR 35 (CI 2.0-586, Figure 3) for prematurity at <32 weeks' gestation.

A number of potential confounders were examined. Those included previous PTD, previous spontaneous abortion, previous induced abortion, serious bleeding in

Table IV Combinations of progressive sialidase levels with progressive prolidase and pH levels for prediction of preterm delivery.

Combination of 2 vaginal markers and pH	<35 wks n	<35 wks %	Odds ratio	95% CI	<32 wks n	<32 wks %	Odds ratio	95% CI
Sialidase > +1 and prolidase > +1	8	17	1.6	0.7-3.7	2	15	1.4	0.3-6.5
Sialidase > +1 and prolidase > +2	1	2.2	9.2	0.6-150	1	7.7	35	2.0-586
Sialidase > +2 and prolidase > +1	2	4.3	4.7	0.8-26	1	7.7	8.6	0.9-83
Sialidase > +2 and prolidase > +2	1	2.2	*		1	7.7	*	
pH \geq 5.0, sialidase > +1 and prolidase > +1	5	11	1.7	0.6-4.6	1	7.7	1.2	0.2-9.2
pH \geq 5.0, sialidase > +1 and prolidase > +2	1	2.2	9.2	0.6-150	1	7.7	35	2.0-586
pH \geq 5.0, sialidase > +2 and prolidase > +1	2	4.3	19	1.7-212	1	7.7	35	2.0-586
pH \geq 5.0, sialidase > +2 and prolidase > +2	1	2.2	*		1	7.7	*	

Sialidase and prolidase activities were measured on a total of 416 women who had NTD, 46 women who had a delivery at <35 weeks', and 13 women who had a delivery at <32 weeks' gestation.

* No woman with sialidase > +2 and prolidase > +2 had a NTD.

pregnancy, 6 or more work hours walking daily, 6 or more work hours standing daily, more than 4 alcoholic drinks per week, smoking more than 10 cigarettes a day at enrollment, LBW in the last delivery, BMI, and age. In most cases, no association with both outcome and marker variables were found. Only for LBW as outcome was there confounding by smoking. In those cases, the estimates were controlled for that.

Table II details combinations of positive sialidase and prolidase activities that demonstrated statistically significant associations with LBW. In particular, the combination of high prolidase (+2) with a positive sialidase (+1) had crude OR 13 (CI 1.3-122) for LBW (adjusted for smoking the OR was 10 [CI 1.0-100]). This combination demonstrated an OR 33 (CI 2.0-553) for VLBW. No woman having the combination of high prolidase (+2) with high sialidase (+2) had an infant weighting \geq 2500 g at birth (thus, ORs for this profile could not be calculated).

Table III shows that all combinations of sialidase and prolidase activities were not statistically significant for PTD or total preterm deliveries at <37 weeks' gestation (versus NTD). No woman having the combination of the highest values of both sialidase (+2) and prolidase (+2) activity had a NTD.

Table IV shows that the combination of high prolidase (+2) with a positive sialidase (+1) demonstrated an OR 35 (CI 2.0-586) for prematurity at <32 weeks' gestation. The determination of a pH \geq 4.7 in addition to all combinations of positive and/or elevated sialidase and prolidase activities as reported in Tables II, III, and IV did not substantially increase the ORs for study outcomes (data not shown). Determination of a vaginal pH \geq 5 in women with sialidase +2 and prolidase +1 increased 4-fold the risk for LBW (OR 17, CI 1.9-153, Table II); VLBW (OR 33, CI 2.0-553, Table II); delivery at <35 weeks' (OR 19, CI 1.7-212, Table IV), and at <32 weeks' gestation (OR 35, CI 2.0-586, Table IV).

In this study, 91 women were BV positive. No statistically significant findings were obtained for BV association with LBW (OR 1.5, CI 0.9-2.6), nor with any other study adverse pregnancy outcomes.

Comment

Much attention has recently focused on use of vaginal pH and presence of BV as a tool to identify women at risk for LBW/PTD caused by reproductive tract altered microflora.^{2,4,14,15} Abnormal vaginal flora disorders appear heterogeneous with respect to clinical presentation and response to therapy. It is increasingly recognized that many instances of altered vaginal microflora do not fulfill criteria for inclusion in the BV group. On the other hand, BV-positive women appear to be highly heterogeneous when evaluated by presence of vaginal biomarkers. Specific BV subgroups have been recently described based on host immune responses and levels of microbial enzymes.^{12,13,16} Thus, it appears that in some BV-positive women the alteration of vaginal ecology is a harmless condition.

Vaginal biomarkers have been suggested to play an important role in risk stratification in women with abnormal microflora. We applied a multimarker strategy that incorporates pH and microbial enzyme activities in a nested case-control study in a low-risk population of Danish pregnant women. We observed that evaluation of vaginal pH in combination with other readily measurable markers in vaginal fluid (sialidase and prolidase activities) is associated with increased risks of LBW, VLBW, and total early prematurity at <35 or <32 weeks' gestation.

Previous studies on this cohort demonstrated that the clinical diagnosis of BV was not significantly associated with increased risk for LBW nor PTD.^{5,13} These previous studies focus attention on how to best identify women at risk of subsequent adverse outcome by

evaluation of microbial flora and/or a single biochemical parameter. Our present results highlight that use of combined objective and easy to use quantifiable biochemical markers in the vaginal fluid may identify women who are at significant risk of LBW, VLBW, and prematurity.

A recent study performed on US women reported an increased incidence of preterm birth, LBW, and VLBW in women with vaginal pH ≥ 4.5 and Nugent Gram score of 9 to 10 early in pregnancy.¹⁴ Another study¹⁵ showed that among US pregnant women with vaginal pH ≥ 5 had OR 1.3 and OR 2.0 for preterm at <37 weeks' and <32 weeks' gestation, respectively. We were unable to confirm these findings regarding vaginal pH ≥ 5 , although we found similar trends especially for LBW (OR 1.7, not statistically significant), and total deliveries at <35 weeks' gestation (OR 1.7, not statistically significant). These differing results may derive in part from the size and study methods of our and the US trial. Discrepancies could possibly be due to population determined differences in vaginal pH values among European vs US pregnant women.^{17,18}

Sialidases are enzymes involved in the pathogenesis of several diseases. Sialidases cleave sialic acid off from various glycoproteins such as IgA,⁷ innate immune factors,¹⁶ mucins and cellular receptors, thus altering both innate and acquired local immunity mechanisms. Some bacteria typically present in the BV milieu, particularly *Bacteroides* and *Prevotella* produce sialidases.⁹ It is of note that sialidase in vaginal fluid has a maximal hydrolytic activity at pH 5,⁸ suggesting possible synergism between sialidase and elevated pH.

Previous studies of vaginal sialidase activity on US pregnant women have not yielded consistent results.^{10,19} In a previous study performed on the same Danish population, sialidase activity, per se, was a better predictive marker than BV and was associated with LBW.¹³

In the present study, we found that sialidase (+1, +2) activity itself may be a better marker for LBW than an elevated vaginal pH ≥ 4.7 or ≥ 5 . However, by combining biochemical parameters we found that the determination of a vaginal pH ≥ 5 increases 4-fold the risk for LBW of women at the highest level of sialidase activity (from OR 4.1 to 17). Confirmingly, the combination of high sialidase and pH ≥ 5 demonstrated a statistically significant OR 31 for VLBW. We observed that high sialidase and pH ≥ 5 had significant consistently elevated ORs for total early preterm deliveries: OR 18 for delivery at <35 weeks', and OR 32 for delivery at <32 weeks' gestation, and was associated with a trend for elevated risk for spontaneous PTD (OR 10).

Prolidases are proteolytic enzymes that facilitate matrix remodeling and cellular infiltration, and can modulate cytokines and other immune mediators. Increased prolidase hydrolysis in the vagina and cervix

could result in the breakdown of the protective mucosal barriers.^{10,11}

G. vaginalis is a strong in vitro producer of prolidase activity. Multiple other BV-associated bacteria including: *Mobiluncus* spp., *Peptostreptococcus* spp., *Streptococcus intermedius*, *Bifidobacterium* spp. can produce prolidases.¹¹

In a previous study, presence of prolidase activity constituted a single marker for LBW, and high prolidase values were more predictive for LBW than BV.¹³ In the present study we demonstrate that high prolidase activity is a marker for both LBW and prematurity when combined with an elevated vaginal pH. The concomitant finding of vaginal ≥ 5 and high prolidase activity was associated with elevated risk for LBW (OR 13), for VLBW (OR 33), and total deliveries at <32 weeks' gestation (OR 35).

Our present findings support the notion of synergistic inter-relationships among virulence factors produced by bacteria present in altered vaginal microflora. Combinations of microbial enzyme activities were associated with higher risk of adverse outcome than a single biochemical marker positivity. Importantly, among the few subjects with the highest levels of both microbial enzyme activities, each (2/2) of the infants were born preterm and weighting less than 2500 g.

Potential strengths of the present study include: (1) study population is homogenous with universal access to health care, and relatively stable; (2) the biomarkers are easily and objectively measurable on both fresh and frozen samples; and (3) neither clinical evaluation of Amsel's criteria nor interpretation of Gram slides is necessary. It is of note that diagnosis of BV by Amsel's criteria in this population was not statistically associated with any of the study adverse outcomes. Differentiated misclassification among outcome variables was minimal, as information on outcomes was collected after exposure variables, and laboratory analyses were blinded to all other study variables.

Our findings are consistent with the hypothesis that only a subgroup of women with abnormal vaginal flora are really at risk of adverse pregnancy outcome, and identification of women colonized by bacteria producing sialidases and/or prolidases is a more selective tool than BV.

Weakness of our study include the limited number of pregnancy adverse outcomes obtained from the original cohort of 3596 women,⁵ which yielded wide ranges in the observed CI, and prevented further stratification of subsets of women. Other weaknesses include the homogeneity of the Danish study population.

We speculate that sialidase and prolidase microbial activities, perhaps increased by elevated pH, in vaginal fluid may synergistically contribute to lower and impair defenses and cause tissue damage.^{2,10-13} Studies by us and others demonstrate that high enzymatic activity

levels in vaginal fluid suppress factors of the adaptive and innate vaginal immune response.^{7,16} Enzymatic perturbations of lower genital tract defenses may correlate with capability of microbes and/or virulence factors to penetrate into the upper genital tract, causing intrauterine infection and inflammation that can impair fetal growth as well as cause prematurity.^{1-5,15}

In the present study we found a stronger association of combinations of biomarkers with total early preterm deliveries (at 35 or 32 weeks' gestation) than with spontaneous or total preterm delivery at <37 weeks' gestation. These findings support observations of other authors that the relationship of lower tract infection markers to preterm birth is strongest at the earliest gestational ages, and that infection is estimated to be more likely the pathogenesis of early preterm births (around 60%) than that preterm at <37 weeks' gestation (around 20%).^{2,15}

Our results suggest that combinations of easily measured vaginal biomarkers (pH, sialidase, and proli-dase activities) are associated with clinically important risks of LBW and prematurity when measured in the second trimester.

Future controlled trials are needed to replicate our findings, and to evaluate strategies of early pregnancy screening and selection of patients to possibly abrogate hydrolytic enzyme activities and restore "healthy" vaginal flora. If effective, this strategy of early identification and treatment (antimicrobial, and/or probiotic, and/or antiproteolytic) could reduce burdens of disease associated with prematurity and LBW caused by abnormal reproductive tract microflora.

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