

# Prediction of Spontaneous Preterm Birth in At-risk Women Using Thrombospondin 1 from Cervicovaginal Fluid: A Prospective Observational Study

## Thrombospondin 1 im Zervikovaginalsekret als Prädiktor einer spontanen Frühgeburt bei Frauen mit erhöhtem Risiko: eine prospektive Observationsstudie



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### Key words

biomarker, cervical length, desmoplakin, preterm birth, preterm labor, stratifin, test characteristics, thrombospondin 1

### Schlüsselwörter

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### ABSTRACT

**Introduction** Thrombospondin 1, desmoplakin and stratifin are putative biomarkers for the prediction of preterm birth. This study aimed to validate the predictive capability of these biomarkers in patients at risk of preterm birth.

**Materials and Methods** We included 109 women with symptoms of threatened spontaneous preterm birth between weeks 20 0/7 and 31 6/7 of gestation. Inclusion criteria were uterine contractions, cervical length of less than 25 mm, or a personal history of spontaneous preterm birth. Multiple gestations were also included. Samples of cervicovaginal fluid were taken before performing a digital examination and transvaginal ultrasound. Levels of cervicovaginal thrombospondin 1, desmoplakin and stratifin were quantified by enzyme-linked immunosorbent assays. The primary endpoint was spontaneous preterm birth before 34 + 0 weeks of gestation.

**Results** Sixteen women (14.7%) delivered before 34 + 0 weeks. Median levels of thrombospondin 1 were higher in samples where birth occurred before 34 weeks vs.  $\geq 34$  weeks of gestation (4904 vs. 469 pg/mL,  $p < 0.001$ ). Receiver operator characteristics analysis resulted in an area under the curve of 0.86 ( $p < 0.0001$ ). At an optimal cut-off value of 2163 pg/mL, sensitivity, specificity, positive predictive value and negative predictive value were 0.94, 0.77, 0.42 and 0.99, respectively, with an adjusted odds ratio of 32.9 (95% CI: 3.1–345,  $p = 0.004$ ). Multiple gestation, cervical length, and preterm labor had no impact on the results. Survival analysis revealed a predictive period of more than eight weeks. Levels of desmoplakin and stratifin did not differ between groups.

**Conclusion** Thrombospondin 1 allowed long-term risk estimation of spontaneous preterm birth.

## ZUSAMMENFASSUNG

**Einleitung** Thrombospondin 1, Desmoplakin und Stratifin sind putative Biomarker für die Vorhersage einer Frühgeburt. Ziel dieser Studie war es, das prädiktive Potenzial dieser Biomarker in Patientinnen mit erhöhtem Frühgeburtenrisiko zu überprüfen.

**Material und Methoden** Insgesamt wurden 109 Frauen mit Symptomen einer drohenden spontanen Frühgeburt im Zeitraum zwischen 20 0/7 und 31 6/7 SSW in die Studie aufgenommen. Einschlusskriterien waren uterine Kontraktionen, eine Zervixlänge von weniger als 25 mm oder spontane Frühgeburt in der Anamnese. Mehrlingsschwangerschaften wurden auch eingeschlossen. Es wurde ein zervikovaginaler Abstrich entnommen, gefolgt von einer manuellen und einer Ultraschalluntersuchung. Die zervikovaginalen Konzentrationen von Thrombospondin 1, Desmoplakin und Stratifin wurden mithilfe von ELISA quantifiziert. Der primäre Endpunkt war eine spontane Frühgeburt vor 34 + 0 SSW.

**Ergebnisse** Bei 16 Frauen (14,7%) kam es vor 34 + 0 SSW zur Entbindung. Die mittleren Thrombospondin-1-Konzentrationen waren höher in den Proben, bei denen die Geburt unter 34 SSW stattfand, verglichen mit einer Geburt  $\geq 34$  SSW (4904 vs. 469 pg/ml,  $p < 0,001$ ). Die ROC-Analyse ergab eine AUC von 0,86 ( $p < 0,0001$ ). Für den optimalen Cut-off von 2163 pg/ml betrug die Sensitivität, Spezifität sowie der positive und negative prädiktive Wert jeweils 0,94, 0,77, 0,42 und 0,99 bei einer adjustierten Odds Ratio von 32,9 (95%-KI 3,1–345,  $p = 0,004$ ). Mehrlingsschwangerschaft, Zervixlänge und vorzeitige Wehen hatten keinen Einfluss auf die Ergebnisse. Bei der Überlebensanalyse zeigte sich ein prädiktiver Zeitraum von mehr als 8 Wochen. Es gab keine Unterschiede zwischen den Gruppen hinsichtlich der Desmoplakin- und Stratifin-Konzentrationen.

**Schlussfolgerung** Thrombospondin 1 erlaubt die langfristige Risikoabschätzung für eine spontane Frühgeburt.

## Abbreviations

ART	assisted reproductive technique
AUC	area under the curve
CI	confidence interval
CVF	cervicovaginal fluid
DSP	desmoplakin
fFN	fetal fibronectin
IQR	interquartile range
NPV	negative predictive value
OR	odds ratio
PAMG-1	placental alpha microglobulin-1
piGFBP-1	phosphorylated insulin-like growth factor binding protein-1
POCT	point-of-care test
PPV	positive predictive value
PTB	preterm birth
ROC	receiver operating characteristics
SFN	stratifin
sPTB	spontaneous preterm birth
THBS-1	thrombospondin 1

## Key Message

In this study, significantly higher thrombospondin 1 levels were observed in cervicovaginal fluid up to eight weeks before preterm delivery. ROC analysis revealed an AUC of 0.86 ( $p < 0.0001$ ) for the prediction of preterm birth before 34 weeks of gestation in at-risk women.

## Introduction

The worldwide burden of preterm birth (PTB) has been estimated to be 14.8 million live-born infants per year, which corresponds to 10.6% of all births [1]. Fifteen percent of these children are born before week 32 of gestation. In Europe, 57.3% of neonatal deaths

occurred when newborns were delivered before 32 weeks of gestation [2]. Approximately 70% of PTB occur spontaneously (sPTB) [3]. However, the prediction of sPTB based on clinical symptoms such as uterine contractions or measurement of cervical ripening using digital examination is imprecise and accuracy is poor [4, 5]. The positive predictive value (PPV) of regular painful uterine contractions for sPTB before 35 weeks of gestation varies between 15 and 25% [4, 6]. The risk of delivery is about 18-fold higher compared to the population-based prevalence of PTB of 2.7% [7]. However, the majority of symptomatic women will not have a PTB. High false-positive rates and low PPV for the prediction of sPTB result in unnecessary treatment which contributes to the low success rates of efforts to reduce the prevalence of sPTB [6, 8]. For example, more than half of the women included in a study on the use of nifedipine for maintenance tocolysis, delivered after 34 weeks of gestation, even if they only received placebo [9].

Cervical length measurement by transvaginal ultrasound has improved the prediction of sPTB, but the overall accuracy of test characteristics remains unsatisfactory [8, 10–12]. In a meta-analysis, the predictive performance of cervical length measurement in symptomatic women with threatened preterm labor was computed for various cut-off values [13]. A cervical length  $< 25$  mm increased the probability of sPTB before 34 weeks of gestation from 11.4% (pre-test probability) to 20.7%, with a sensitivity of 64.3% [13]. Reduction of the cut-off to  $< 15$  mm increased the PPV to 62%, but sensitivity declined to 46.2%. Predictive performance is further limited by differences in agreement about the reliability of transvaginal cervical length measurement, with a positive inter-observer agreement on a cervical length of  $\leq 25$  mm of just 59% [14].

Risk estimation using biomarkers obtained from cervicovaginal fluid (CVF) has the potential to resolve this dilemma. CVF contains a myriad of proteins, and during pregnancy the biochemical composition CVF is affected by the physiological and pathophysiological changes in the vagina, endocervix, endometrial decidua, adjacent fetal membrane and placenta [15]. The processes involved in

human labor such as cervical remodeling, myometrial activation or rupture of fetal membranes influence the composition of the CVF [15]. Commercially available point-of-care tests (POCT) of the biomarkers fetal fibronectin (fFN), phosphorylated insulin-like growth factor binding protein-1 (pIGFBP-1) and placental alpha microglobulin-1 (PAMG-1) allow them to be quantified in CVF to estimate the risk of subsequent sPTB. All three proteins originate from the decidua, or rather the chorioamniotic membranes, and are released in great quantities during disintegration of the fetal membranes [16–21]. As this is an event which occurs during the common pathway of parturition [22], these biomarkers have been shown to offer the best results for the short-term prediction of sPTB within 7–10 days, opening a window of opportunity for the administration of antenatal corticosteroid prophylaxis and in-utero transfer of the fetus to a tertiary care center [8, 23, 24]. However, the predictive performance was disappointing, as a direct comparison of cervical length (cut-off: 15 mm) with fFN did not result in a better prediction of sPTB within seven days in symptomatic women [23]. Moreover, the use of fFN did not result in a longer duration of pregnancy or a better neonatal outcome [25].

Thus, there is an ongoing need to identify biomarkers which could improve the prediction of sPTB in symptomatic women. A proteome study which analyzed the secretome of human columnar epithelial endocervical cells (End1) and human vaginal cells (Vk2) revealed a panel of 15 candidate biomarkers for PTB [26]. The proteins were tested on previously self-collected CVF specimens from five women with subsequent sPTB between week 28 and week 32 of gestation and five controls who delivered at term. Three proteins were more highly expressed in women with PTB: desmoplakin (DSP) isoform 1 peptide (70.7-fold), stratifin (SFN) peptide (42.4-fold) and the thrombospondin-1 (THBS1) precursor peptide (5.1-fold). The aim of our study was to validate the predictive capability of these candidate biomarkers in the CVF of women at increased risk of sPTB.

## Material and Methods

### Study design and participants

We included women who contacted our tertiary care center (Rostock, Germany) between January 2017 and April 2019 with symptoms of threatened preterm birth. Eligibility criteria were the existence of at least one of the following risk factors for preterm birth between week 20 0/7 and week 31 6/7 of gestation: uterine contractions (> 3/30 min or painful labor), cervical length < 25 mm measured by transvaginal ultrasound, or a personal history of preterm birth or abortion after 16 weeks of gestation. Multiple gestations were allowed. Exclusion criteria were cervical dilatation > 3 cm, previous insertion of an Arabin pessary or surgical cerclage, preterm rupture of membranes, vaginal bleeding, maternal temperature of more than 37.5 °C, vaginal pH higher than 5, sexual intercourse during the last 24 h or vaginal examination in the last 6 h, tocolysis in the previous 7 days, hypertensive disorders, and iatrogenic indication for termination of pregnancy within 7 days after study inclusion. The CVF sample was collected during speculum examination and prior to digital palpation and transvaginal ultrasound of the cervix. Measurement of the cervical

length on admission was part of the standard operating procedure of the department.

The condition of the cervix was evaluated by digital palpation and classified using the gestational age-dependent Westin score [27]. A ratio of palpated score/maximum physiological score > 1 represented preterm maturation of the cervix.

The clinician on duty was responsible for clinical examinations and further treatment decisions, including the indication for tocolysis, corticosteroid prophylaxis, progesterone, cerclage or pessary depending on the clinical situation and subject to national guideline recommendations. PAMG-1, fFN or pIGFBP-1 in the CVF were not analyzed.

The primary outcome was sPTB < 34 0/7 weeks, including patients with subsequent preterm rupture of membranes. Secondary outcome parameters were sPTB < 37 0/7 weeks and delivery within seven days after study enrollment. Gestational age was calculated from the first day of the last menstrual cycle and was corrected based on ultrasound findings if measurements of the crown-rump length in the first trimester differed by more than seven days.

### Preparation and processing of CVF samples

CVF samples were collected using a sterile nylon<sup>®</sup> flocked swab (ESwap 480C, Copan, Brescia, Italy) that was rotated in the posterior fornix of the vagina. After that, the swab was transferred to a tube filled with 1000 µl RIPA buffer (Merck, Darmstadt, Germany) containing 10 µg aprotinin (Roth, Karlsruhe, Germany). After dilution, the swab was removed and tubes with 250 µl aliquots were stored at – 80 °C until further processing. To quantify the investigated proteins, the ELISA assays listed in Supporting Information Table S1 were used according to manufacturer's instructions (all from Cloud-Clone Corp., Wuhan, China). Measurements were done in triplicate.

### Statistical analysis

All data were stored and analyzed using the IBM SPSS statistical package 25 (SPSS Inc. Chicago, IL, USA), Excel 2013 (Microsoft Corporation, Redmond, WA, USA) and the R 4.0.0 RStudio statistical software and ggplot2 package. Testing for differences in continuous variables between groups was done using Student's t-test, Mann-Whitney U-test or Kruskal-Wallis test as appropriate; comparisons of categorical variables between groups was done with Fisher's exact test. Survival analysis (time to delivery) was carried out with the Kaplan–Meier method, with statistical comparison of groups done using log-rank test. The impact of multiple gestations, preterm labor, and cervical length < 25 mm on THBS-1 levels depending on gestational age at delivery (both as fixed factors) was computed with two-factorial ANOVA. All p-values were obtained using two-sided statistical tests, and values < 0.05 were considered statistically significant.

Receiver operating characteristics (ROC) curves and the area under the curve (AUC) were computed, and the optimal cut-off value (minimal distance to sensitivity and specificity of 1) was calculated using the following equation:  $(1 - \text{sensitivity})^2 + (1 - \text{specificity})^2$ . To calculate the diagnostic criteria, a second diagnostic cut-off with a specificity of just under 0.9 was selected. A logistic regression model was used to assess the independence of specific

► **Table 1** Patient characteristics.

Characteristic	All patients n = 109	Delivery < 34 weeks n = 16	Delivery ≥ 34 weeks n = 93	p-value
Maternal age, years (mean ± SD)	30.4 ± 4.7	31.0 ± 4.4	30.3 ± 4.8	0.597*
Maternal weight before pregnancy, kg (mean ± SD)	70.5 ± 17.4	82.1 ± 25.7	68.8 ± 14.3	0.062*
Body mass index before pregnancy (BMI), kg/m <sup>2</sup> (mean ± SD)	26.5 ± 16.1	29.4 ± 9.9	24.4 ± 4.9	0.063*
Obesity (BMI ≥ 30 kg/m <sup>2</sup> )	21 (19.6%)	7 (43.8%)	14 (15.4%)	<b>0.015‡</b>
Gravidity, n (median, min – max)	2 (1–7)	2 (1–7)	2 (1–6)	0.230†
Parity, n (median, min – max)	0 (0–5)	0 (0–5)	0 (0–3)	0.470†
History of preterm birth, n (%)	21 (19.3%)	5 (31.3%)	15 (16.1%)	0.171‡
Multiple gestation, n (%)	14 (12.8%)	8 (50.0%)	6 (6.5%)	<b>&lt; 0.001‡</b>
Assisted reproductive technique, n (%)	11 (10.1%)	7 (43.8%)	4 (4.3%)	<b>&lt; 0.001‡</b>
Gestational diabetes	16 (14.7%)	6 (37.5%)	10 (10.9%)	<b>0.014‡</b>
Cervical length, mm (median, IQR)	20.0 (14.5–32.5)	14.5 (6.0–21.25)	20.0 (15.0–34.0)	<b>0.021*</b>
Cervical length ≤ 25 mm, n (%)	70 (64.2%)	14 (87.5%)	56 (60.2)	<b>0.047‡</b>
Cervical length ≤ 20 mm, n (%)	61 (56.0%)	12 (75.0%)	49 (52.7)	0.111‡
Preterm uterine contractions, n (%)	78 (71.6%)	11 (68.8%)	67 (72.0%)	0.771‡
Palpatory premature cervix, n (%)	53 (52.5%)	14 (87.5%)	39 (45.9%)	<b>0.002‡</b>
Gestational age at study inclusion, weeks (median, IQR)	26 (24–29)	26 (23.25–28.75)	26 (24–29)	0.796†
Gestational age at delivery, weeks (median, IQR)	38 (35–39)	30.5 (28–31)	39 (37–39)	<b>&lt; 0.001†</b>
Interval to delivery, days (median, IQR)	76.0 (56.5–98.5)	20.5 (5.25–48.0)	81.0 (63.0–102.5)	<b>&lt; 0.001†</b>
Preterm rupture of membranes (after study inclusion), n (%)	20 (18.3%)	5 (31.3%)	15 (16.7%)	0.178‡
Arabin pessary, n (%)	12 (11.0%)	2 (12.5%)	10 (10.8%)	1.000‡
Surgical cerclage, n (%)	2 (1.8%)	1 (6.3%)	1 (1.1%)	0.278‡
Micronized vaginal progesterone, n (%)	77 (70.6%)	9 (56.3%)	68 (73.1%)	0.142‡
Tocolysis, n (%)	55 (50.1%)	14 (87.5%)	41 (45.1%)	<b>0.002‡</b>
Antenatal steroid prophylaxis, n (%)	60 (55.0%)	14 (87.5%)	46 (50.5%)	<b>0.006‡</b>

\* Student's t-test for independent samples, † ranked Mann-Whitney U-test, ‡ Fisher's exact test, IQR = interquartile range, SD = standard deviation, bold = significant differences

outcome parameters. We used a sequential method with the addition of variables in order of magnitude of the crude odds ratios (ORs) and starting with the largest estimate. In the first adjusted model, the covariates “multiple gestation”, “premature cervical ripening”, “obesity” and a “history of preterm birth” were included; the second model included the interventional variables “progesterone treatment”, “use of an Arabin pessary”, “tocolysis” and “antenatal corticosteroid prophylaxis”.

We performed a pre-study sample size calculation with a regression model using the statistical G\*Power 3.1.9.2 software. The estimated a priori probability for delivery < 34 weeks was set to 15%, which resulted in a calculated sample size of 139 patients for an assumed post-test probability of 30%, an  $\alpha$ -error of 0.05, and a power of 90%. Study recruitment was limited a priori to a period of two years. At this time point, the sample size was 109 patients, which corresponds to a statistical power of > 99% in a two-tailed post-hoc power analysis.

### Ethical approval

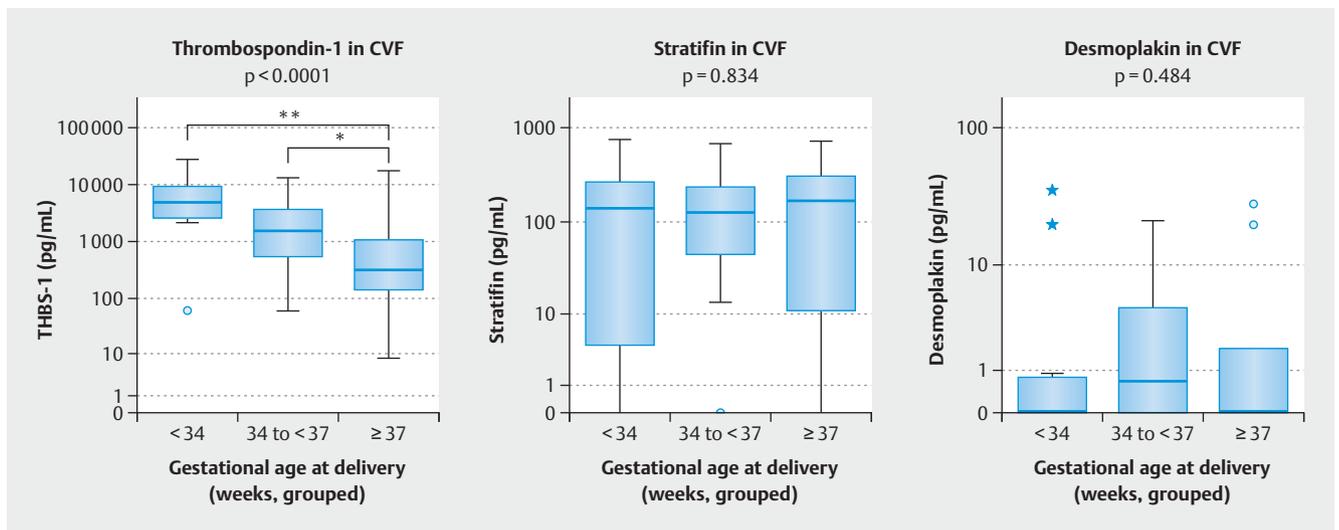
This study was conducted as part of the “Analysis of diagnostic accuracy of predictive biomarkers in risk assessment of threatening

preterm birth” (ADAPROB) study. The protocol was approved by the local ethics committee of the University of Rostock (IRB-No. A2016-0162), and the study was registered with the German Clinical Trials Register (DRKS-ID: DRKS00010763). Written informed consent was obtained from all participants.

## Results

### Characterization of study population

One hundred and nine women were included in the study. All participants were either symptomatic for preterm birth or had a shortened cervix. None of the women was included based only on a history of preterm birth or abortion. An episode of preterm uterine contractions was the most common reason for consulting a physician (n = 78, 71.6%) and for study inclusion (► **Table 1**). In women without labor, a cervix < 25 mm (n = 26/31, 83.9%) was the leading criterion for inclusion. The median gestational age at study entry was 26 weeks (interquartile range [IQR] 24–29) and the median gestation age at delivery was 38 weeks (IQR 35–39). Sixteen women (14.7%) delivered before 34 weeks and 36 women



**Fig. 1** Thrombospondin 1, stratifin and desmoplakin protein concentrations in cervicovaginal fluid (CVF) measured by ELISA. Boxplots are grouped by gestational age at delivery. Thrombospondin 1 and stratifin were measured in the total study cohort and subsequently grouped as: < 34 weeks (n = 16), 34 to < 37 weeks (n = 20) and ≥ 37 weeks (n = 73); measurement of desmoplakin was restricted to 40 women: < 34 weeks (n = 12), 34 to < 37 weeks (n = 15) and ≥ 37 weeks (n = 13). P-values were computed by Kruskal-Wallis test. Post-hoc analysis (Dunn-Bonferroni) for thrombospondin 1 revealed significant intergroup differences between < 34 weeks and ≥ 37 weeks (adjusted  $p < 0.0001 = **$ ) and between 34 to < 37 weeks and ≥ 37 weeks (adjusted  $p = 0.013 = *$ ).

(33.0%) before 37 weeks. Six patients (5.5%) gave birth within seven days, with a median interval between study inclusion and delivery of 2.5 days (IQR 0–6.25). The majority of patients who gave birth before 34 weeks of gestation (62.5%) delivered after seven days.

### Levels of THBS-1, SFN and DSP in CVF

Median concentrations of THBS-1 in CVF were higher in women with sPTB (► Fig. 1). The median concentration was 4904 pg/mL (IQR 2617–9236) for delivery < 34 weeks and 469 pg/mL (IQR 152–1770) for delivery ≥ 34 weeks ( $p < 0.001$ ). The median level for PTB < 37 weeks was 2633 pg/mL (IQR 925–7058) vs. 314 pg/mL (IQR 141–1077) for delivery ≥ 37 weeks ( $p < 0.001$ ). There were no correlations between THBS-1 levels and gestational age at CVF sampling, maternal age, maternal weight or body mass index when only women who delivered at term (n = 73) were evaluated. Multiple gestation, preterm labor, and cervical length < 25 mm had no influence on THBS-1 levels in the two-factorial ANOVA model, neither as a main effect nor in interaction with the time of delivery (Supporting Information, Figs. S1–S3).

The median level of SFN in CVF was 156 pg/mL (IQR 18–306) and there were no differences between groups with regard to gestational age at delivery (< 34 weeks: 141 pg/mL [IQR 6–318] vs. ≥ 34 weeks: 167 pg/mL [IQR 18–306],  $p = 0.731$ ). DSP was not or only minimally detectable in CVF (median concentration: 0.2 ng/mL [IQR 0.0–2.8]) irrespective of gestational age at delivery (< 34 weeks: 0.0 ng/mL [IQR 0.0–0.9] vs. ≥ 34 weeks: 0.4 ng/mL [IQR 0.0–5.5],  $p = 0.320$ ) (► Fig. 1).

### ROC analysis and definition of cut-off values

ROC and AUCs of THBS-1 for the prediction of PTB < 34 weeks, < 37 weeks and within seven days were 0.86 (95% CI: 0.75–0.96,

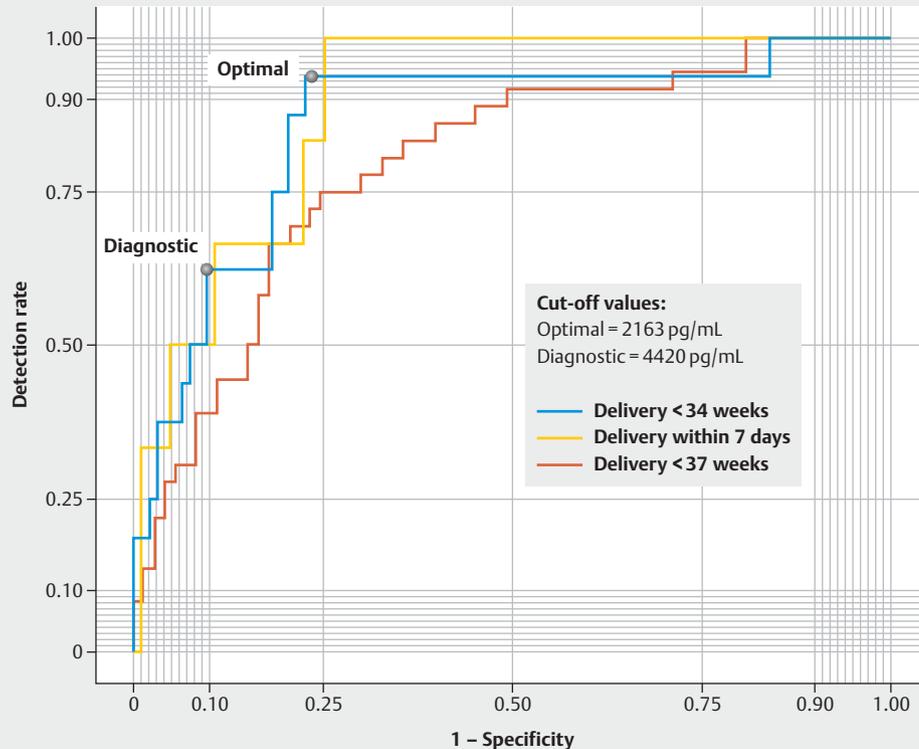
$p < 0.0001$ ), 0.80 (95% CI: 0.71–0.88,  $p < 0.0001$ ) and 0.89 (95% CI: 0.80–0.98,  $p = 0.001$ ), respectively (► Fig. 2). Calculations of test characteristics were based on the optimal (2163 pg/mL) and a diagnostic (4420 pg/mL) cut-off value for the prediction of birth < 34 weeks. Test characteristics for secondary endpoints were computed using the same cut-offs (► Table 2). The optimal cut-off was positive in 33.0% of all women (n = 36/109), of whom 41.7% (95% CI: 26–58%) delivered < 34 weeks. The diagnostic cut-off was positive in 17.4% (n = 19/109), of whom 52.6% (95% CI: 30–75%) delivered < 34 weeks. This corresponds to a positive likelihood ratio of 6.5 (95% CI: 3.1–13.4) and a negative likelihood ratio of 0.4 (95% CI: 0.2–0.8). A negative test result decreased the probability from 14.7% to 7% for the diagnostic cut-off value and 1% for the optimal cut-off value. The corresponding unadjusted ORs were 51.4 (95% CI: 6.4–412,  $p < 0.001$ ) for the optimal and 15.6 (95% CI: 4.6–52.9,  $p < 0.001$ ) for the diagnostic cut-off value.

Test characteristics of the endpoint-specific cut-off levels are presented in Supporting Information, Table S2.

### Risk factors for PTB and adjusted ORs

Diagnostic ORs for preterm birth < 34 weeks were calculated for maternal age > 34 years, obesity (body mass index ≥ 30 kg/m<sup>2</sup>), history of PTB, use of assisted reproductive technology (ART), multiple gestation, premature cervical ripening assessed by digital palpation, cervical length ≤ 20 mm, painful uterine contractions (> 3/30 min), positive cardiotocography, genital infection with *Ureaplasma spp.*, vaginal dysbiosis, and nulliparity. Of these parameters, ART, multiple gestation, premature cervical ripening and obesity were significantly associated with PTB (► Fig. 3, Supporting Information Table S3).

Multiple gestation, premature cervical ripening, obesity, and a history of preterm birth were explanatory variables in multiple lo-



► **Fig. 2** Receiver operating characteristics (ROC) curves for the prediction of spontaneous preterm birth in symptomatic women before 34 weeks of gestation (blue line), within 7 days (yellow line) and before 37 weeks of gestation (red line), based on thrombospondin-1 levels. The corresponding areas under the curves are 0.859, 0.892 and 0.795. The optimal cut-off value for delivery before 34 weeks of gestation corresponds to the point with the minimal distance to a sensitivity and specificity of 1. The diagnostic cut-off value corresponds to a specificity of 0.9.

► **Table 2** Test characteristics of THBS-1 for the prediction of preterm birth using two different cut-off values. The addition of cervical length to THBS-1 levels did not improve predictive capability.

Endpoint	Cut-off value (pg/mL)	Sensitivity	Specificity	PPV	NPV	LR+	LR-	Odds ratio (95% CI)
Delivery < 34 weeks	2163	0.94	0.77	0.42	0.99	4.2	0.1	51.4 (6.4–412.4)
Delivery < 37 weeks	2163	0.64	0.82	0.64	0.82	3.6	0.4	8.2 (3.3–20.2)
Delivery within 7 days	2163	1.00	0.71	0.17	1.00	3.4	0.0	n. a.
Delivery < 34 weeks	4420	0.63	0.90	0.53	0.93	6.5	0.4	15.6 (4.6–52.9)
Delivery < 37 weeks	4420	0.36	0.92	0.68	0.74	4.4	0.7	6.3 (2.2–18.5)
Delivery within 7 days	4420	0.67	0.85	0.21	0.98	4.6	0.4	11.7 (2.0–69.8)
Delivery < 34 weeks	4420 or CL < 14 mm	0.81	0.74	0.35	0.96	3.2	0.3	12.5 (3.3–47.5)
Delivery < 34 weeks	2163 and CL < 25 mm	0.81	0.83	0.45	0.96	4.7	0.2	20.9 (5.3–81.7)
Delivery < 34 weeks	2163 and CL < 20 mm	0.69	0.84	0.42	0.94	4.3	0.4	11.4 (3.5–37.7)
Delivery < 34 weeks	2163 and CL < 14 mm	0.50	0.91	0.50	0.91	5.8	0.5	10.6 (3.1–36.0)

n. a. = not available, CI = confidence interval, CL = cervical length, LR+ = positive likelihood ratio, LR- = negative likelihood ratio, PPV = positive predictive value, NPV = negative predictive value

gistic regression analysis and improved the predictive model (► Fig. 4). The remaining parameters did not fit the model and were excluded from analysis. ART showed multicollinearity with multiple gestation and was also removed. After adjustment, THBS-1 remained a significant independent predictor of PTB with an adjusted OR of 32.9 (95% CI: 3.1–345,  $p = 0.004$ ) for the optimal and 12.3 (95% CI: 3.1–71.6,  $p = 0.005$ ) for the diagnostic cut-off value.

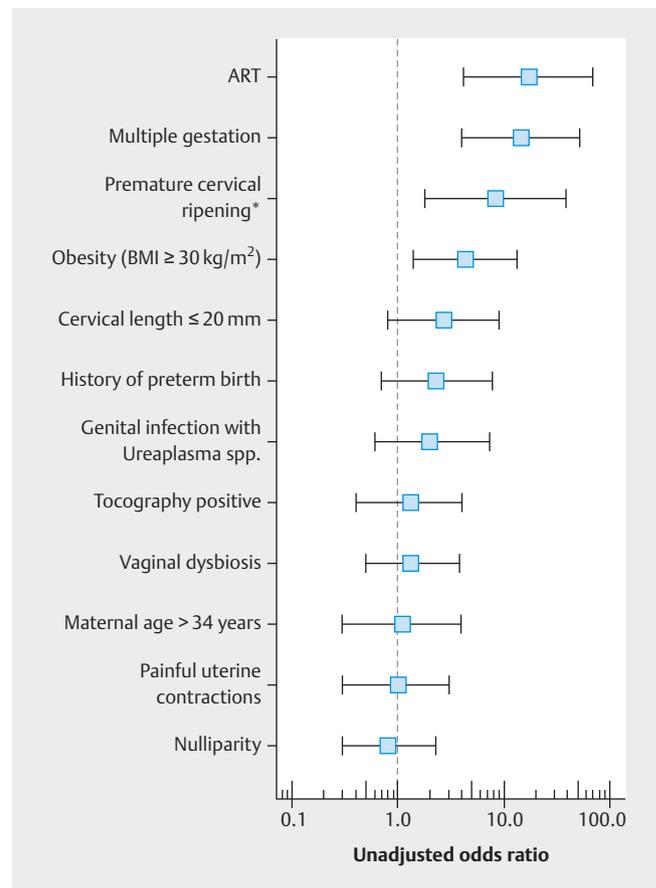
In the second adjustment model, the following variables of treatment were added: application of vaginal progesterone, tocolysis, use of an Arabin pessary, and corticosteroid prophylaxis. In this model, only tocolysis remained a significant explanatory variable, resulting in an adjusted OR of 43.8 (95% CI: 5.3–361,  $p < 0.001$ ) for the optimal and 12.7 (95% CI: 3.5–46,  $p < 0.001$ ) for the diagnostic cut-off value.

### Interval between inclusion in the study and delivery

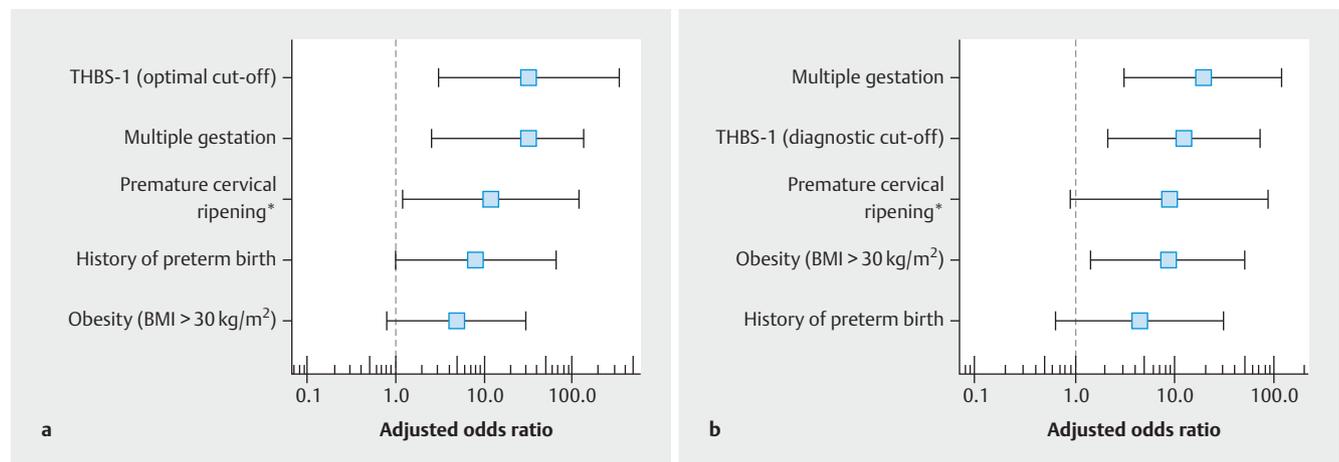
Median time-to-delivery interval was 21 days (IQR 6–47) for delivery < 34 weeks, 51 days (IQR 21–71) for delivery < 37 weeks, and 90 days (IQR 67–108) for delivery  $\geq 37$  weeks. Kaplan-Meier plots revealed a nearly linear increase in the risk of delivery < 34 weeks in THBS-1 test-positive women within an interval of more than eight weeks (► Fig. 5). The interval at which 50% of test-positive women (diagnostic cut-off) delivered < 34 weeks was 58 days (8.3 weeks).

## Discussion

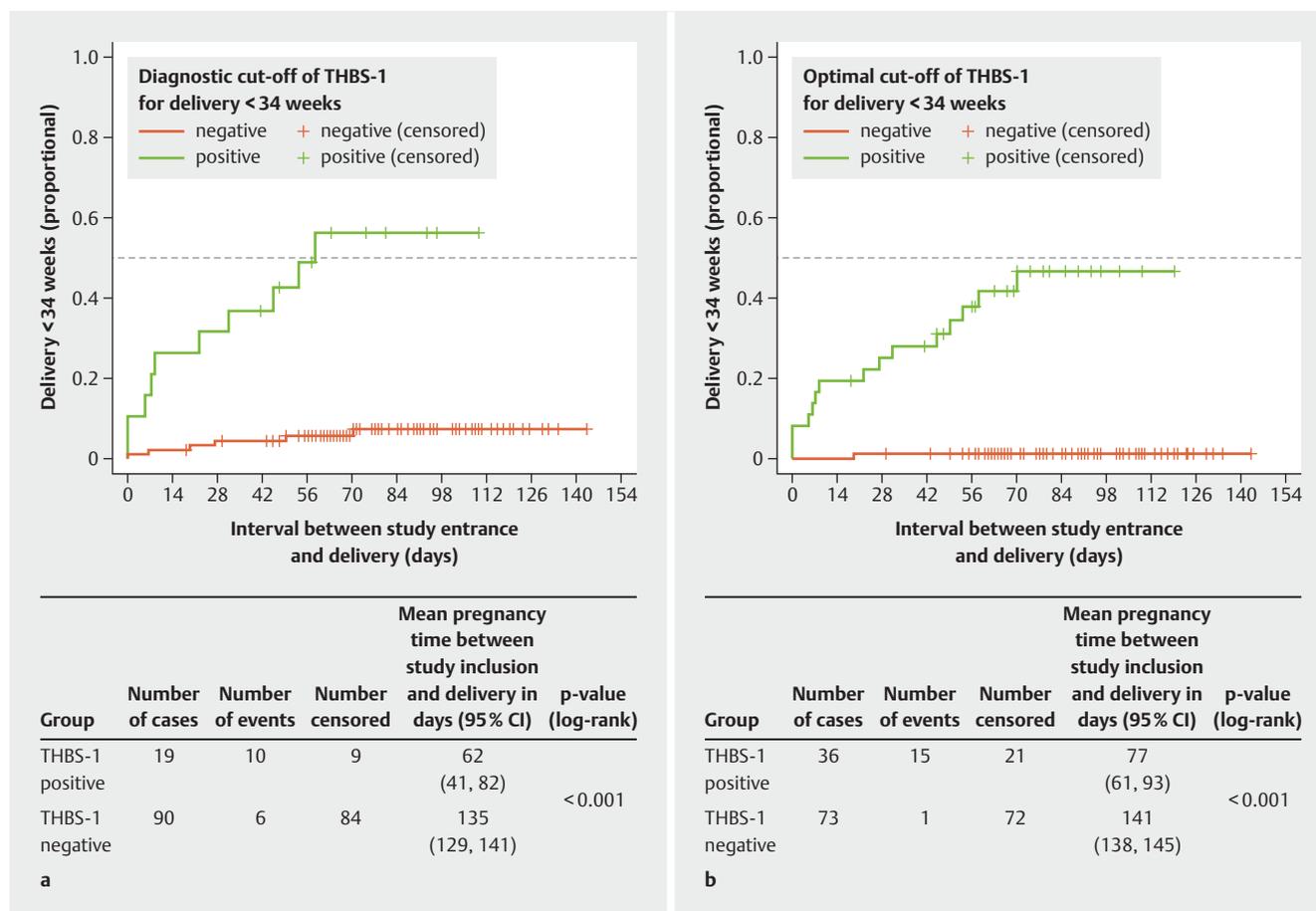
In contrast to DSP and STN, the amount of THBS-1 in CVF was found to be higher in women with subsequent PTB. The quantification was based on an immunodetection assay, which showed a 15-fold increase in sPTB < 34 weeks and an 8-fold increase < 37 weeks compared to delivery at term. Most of the test-positive women had a PTB even if they did not give birth within the first seven days. As THBS-1 allowed subsequent PTB in at-risk women



► Fig. 3 Analysis of risk factors for preterm birth below 34 weeks of gestation. Shown here are the unadjusted odds ratios (box) with 95% confidence intervals (whiskers). ART = assisted reproductive technology. \* assessed by digital examination and using a gestational age-dependent cervical score.



► Fig. 4 Results of the multiple logistic regression analysis. Factors that did not fit the model were excluded. **a** Results of the model using the optimal THBS-1 cut-off of 2163 pg/mL, and **b** results of the model using the diagnostic THBS-1 cut-off of 4420 pg/mL. The adjusted odds ratios (box) with 95% confidence intervals (whiskers) are depicted. THBS-1 = thrombospondin 1, \* assessed by digital examination and using a gestational age-dependent cervical score.



► **Fig. 5** Kaplan-Meier curves showing the risk of preterm birth < 34 weeks of gestation **a** using the optimal THBS-1 cut-off of 2163 pg/mL, and **b** the diagnostic THBS-1 cut-off of 4420 pg/mL. Patients who delivered  $\geq 34$  weeks were censored (indicated by bars). Upper curves correspond to test-positive women. The risk of delivery showed an almost linear increase for the period of more than 8 weeks.

to be identified over the course of more than eight weeks, it seems to be an “intermediate-term” predictor.

The matricellular glycoprotein THBS-1 is an extracellularly secreted homotrimeric protein which mediates signal transduction between the extracellular matrix and cell surface receptors (e.g., CD47, CD36 and integrins) by binding a variety of ligands [28]. The mature THBS-1 homomer results from a precursor peptide after removal of the N-terminal signal sequence [29]. THBS-1 is involved in several functional pathways such as anti-angiogenesis, tissue remodeling, inflammation and myometrial contractions [28]. Upregulation of THBS-1 was observed in term labor placenta [30, 31] with higher levels of expression in sPTB compared to term birth [32], myometrium in labor [33, 34] and cervical ripening with and without labor [35, 36]. It is detected in CVF and amniotic fluid [26, 37]. The expression of THBS-1 in the human myometrium is strongly upregulated at the onset of labor at term [34]. Upregulation occurs in both spontaneous and oxytocin-induced labor and is accompanied by deposition of THBS-1 in the extracellular matrix [34]. In animal models, an upregulation of uterine THBS-1 expression has also been observed in preterm labor [38, 39]. This increase was independent of the etiology of preterm labor and induced by both suspension of progesterone and infection-associated inflammation [39]. Treatment with nimesulide, a

selective cox-2-inhibitor, reversed the contraction-dependent effect [38].

A transcriptome analysis of human cervical tissue revealed a 1.6-fold increase of THBS-1 expression following cervical ripening before the onset of labor compared to the unripe cervix at term [36], and vaginal parturition was associated with a 10-fold increase [35]. Sha et al. selected their candidate biomarkers from the standard secreted proteins of a human endocervical (End1) and a human vaginal (Vk2) cell line [26]. In this proteome study, the findings were proven by liquid chromatography and tandem mass spectrometry of five pooled CVF samples collected from asymptomatic women who delivered preterm compared to five term controls [26]. However, a subsequent proteome study from the same laboratory using the same protein candidates was not able to confirm the predictive capacity of THBS-1 [40]. In the nested case-control study, the CVF samples of 33 women with subsequent sPTB < 34 weeks were compared to 32 term deliveries. CVF samples were previously collected from asymptomatic women with a history of preterm birth between 19 and 23 weeks of gestation. Unfortunately, the authors failed to discuss the putative reasons for the differences in results in more detail. As we only included symptomatic women at risk for PTB, we hypothesize

that the onset of preterm labor or ripening of the cervix is the critical trigger for the increase of THBS-1 levels.

PAMG-1, fFN and pIGFBP-1 are widely proven biomarkers for the prediction of PTB, and POCT assays for the semi-quantitative detection of these biomarkers in CVF are available [24,41,42]. In a recent meta-analysis comparing test characteristics, PAMG-1 was superior to fFN and pIGFBP-1 with corresponding summary ROC and AUCs of 0.96, 0.87 and 0.80 for the endpoint sPTB within 7 days in symptomatic women [24]. In the meta-analysis, PAMG-1 had an overall PPV of 0.76 (95% CI: 0.69–0.84) and a sensitivity of 0.76 (95% CI: 0.57–0.89), even if the two largest studies of symptomatic women only showed PPVs between 0.23 and 0.35 and a corresponding sensitivity of 0.50 [43,44]. However, the prevalence of sPTB within seven days was low in both studies (1.3 and 3.2%), which may explain the poorer performance. In a recent prospective observational study, the predictive capability of qualitative fFN, quantitative fFN and qualitative PAMG-1 was compared in 128 women with symptoms of preterm labor between 24 and 34 weeks of gestation [45]. The prevalence of sPTB < 34 weeks was 5.5% and was therefore lower than in our study (14.7%). Unexpectedly, PAMG-1 showed a lower predictive accuracy than fFN for the prediction of sPTB < 34 weeks. PPVs were 17.9% for qualitative fFN, 20.0% for quantitative fFN, and only 12.5% for PAMG-1. The corresponding sensitivities were 71.4%, 71.4% and 28.6%. The results for qualitative fFN and PAMG-1 were concordant in 74%. Independently of the different test performances, all results suggest that the predictive capability of PAMG-1, fFN and pIGFBP is optimal within seven days of testing [24]. The increase of these biomarkers seems to be a “late” event during the common pathway of sPTB, limiting the available options to intervene and delay or avoid preterm birth [8]. In contrast, an earlier identification, possibly by THBS-1, might help to initiate interventions (e.g., vaginal progesterone, cerclage or pessary) which could subsequently improve neonatal outcomes by the meaningful prolongation of pregnancy.

One of the strengths of our study is its prospective design and clearly defined inclusion criteria. Inclusion of multiple pregnancies was allowed and, as expected, a significant proportion of preterm births consisted of multiple pregnancies (50% of all deliveries < 34 weeks). This may be a limitation, but we were not able to identify differences in THBS-1 levels in CVF for multiple gestations and believe that the collective evaluation was feasible. Previous studies used complex methods for biomarker detection that are not very practicable for routine diagnosis [26,35,40]. The use of an immunodetection-based assay in our study suggests that it could have wider clinical applications. Even if the intended sample size of 139 women was missed after two years of inclusion, the definitive results have sufficient statistical power, as was shown in the post-hoc analysis. However, the results of our exploratory study did not allow statements regarding test accuracy to be made.

## Conclusion

In the present study, we were able to validate the predictive capability of THBS-1 levels in CVF to estimate the risk of PTB. The results were encouraging, and confirmation in a large multicenter study will be useful.

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## Conflict of Interest

The authors declare that they have no conflict of interest.

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