A rapid amniotic fluid Interleukin-6 assessment for the identification of intra-amniotic inflammation in women with preterm labor and intact membranes

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Running title: Rapid diagnosis of intra-amniotic inflammation

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Abstract

Objective: A multivariable predictive model has recently been developed with good accuracy to predict spontaneous preterm delivery within 7 days in women with preterm labor and intact membranes (PTL). However, this model measures amniotic fluid interleukin (IL)-6 concentrations using an ELISA method, thereby limiting clinical implementation.

The main objective of this study was to validate the automated immunoassay as a quantitative method to measure amniotic fluid IL-6 in women with PTL and to evaluate the diagnostic performance of amniotic fluid IL-6 alone and as part of a multivariable predictive model to predict spontaneous delivery in 7 days with this automated method.

Study design: Retrospective observational study in women with PTL below 34 weeks that underwent amniocentesis to rule out microbial invasion of the amniotic cavity. Women with clinical signs of chorioamnionitis, cervical length measurement at admission >5th centile, maternal age < 18 years and no consent to perform amniocentesis for this indication were excluded. The local Institutional Review Boards approved the study (HCB/2019/0940).

Analysis of amniotic fluid IL-6 concentrations: Amniotic fluid IL-6 concentrations were measured using an automated Cobas e602 electrochemiluminescence immunoanalyzer and Human IL-6 Quantikine ELISA.

Results: Of the entire study group (n=100), 38 women spontaneously delivered within 7 days after admission. Both laboratory methods showed good agreement (intraclass correlation coefficient: 0.937 (95% Confidence Interval (CI) 0.908 – 0.957); p<0.001).

Diagnostic performance of amniotic fluid IL-6 to predict spontaneous delivery within 7 days when it was included in the multivariable predictive model showed an area under curve of 0.894 (95% CI 0.799 – 0.955), sensitivity of 97%, specificity of 74%, positive predictive value of 73%, negative predictive value of 97%, positive likelihood ratio (LR) of 3.7 and negative LR of 0.045.

Conclusion: While both analytical methods were comparable for measuring amniotic fluid IL-6 concentrations in women with PTL, the Cobas immunoanalyzer provided rapid diagnosis of IAI within minutes. The predictive model showed a good diagnostic performance to target women at high-risk of spontaneous delivery within 7 days.

Main text

Microbial invasion of the amniotic cavity (MIAC) and intra-amniotic inflammation (IAI) are involved in the etiology of early spontaneous preterm delivery(1, 2). Thus, earlier onset of symptoms, earlier gestational age at delivery and a shorter latency to delivery have been widely reported in women with MIAC and/or IAI admitted with symptoms of preterm labor and intact membranes (PTL)(1, 3, 4).

Our group recently developed and validated a multivariable predictive model of spontaneous preterm delivery within 7 days in women with PTL that included clinical variables such as gestational age, ultrasound cervical length measurement and amniotic fluid glucose and interleukin (IL)-6 concentrations (2). However, IL-6 was measured using an ELISA method, thereby limiting clinical translation. Although some authors have previously proposed rapid bedside tests to measure IL-6 (5-7), there have been no clinical efforts to implement these tests in the clinical setting.

Automated electrochemiluminescence immunoassays are available in most hospitals and can measure IL-6 within only a few minutes and have demonstrated utility for optimizing the clinical management of women with preterm prelabor rupture of membranes (8). However, these immunoassays have not been validated as a method to measure amniotic fluid IL-6 in women with PTL.

Since instrument validation is important in laboratory work before clinical implementation of a new method, the objectives of this study were to validate an automated immunoassay as a quantitative method to measure amniotic fluid IL-6 in women with PTL and evaluate the diagnostic performance of amniotic fluid IL-6 alone

and as part of a predictive model of spontaneous preterm delivery within 7 days (2) with this automated method.

Material and methods

Patient selection

This retrospective observational study included women admitted with PTL below 34 weeks from 2015 to 2017 at BCNatal (Hospital Clinic and Hospital Sant Joan de Déu, Barcelona) that underwent amniocentesis to rule out MIAC. Gestational age was established according to crown-rump length in the first-trimester ultrasound (US) scan (9).

Women with the following conditions were excluded: clinical signs of chorioamnionitis (10) at admission, cervical length measurement at admission >5th centile (11), maternal age < 18 years and no consent to perform amniocentesis for this indication. The maternal characteristics of the women who declined amniocentesis were similar to those of our study population.

Patient selection and sampling procedures were performed in accordance with the Declaration of Helsinki and applicable local regulatory requirements after approval from the Institutional Review Boards (HCB/2019/0940).

Clinical management

Briefly as reported previously (2), standard management of women diagnosed with PTL included US transvaginal cervical length measurement and maternal blood analysis for evaluation of maternal C-reactive protein and white blood cell count at admission. As part of the institutional clinical protocols, women with singleton pregnancies

admitted with a diagnosis of PTL below 34 weeks were offered amniocentesis to rule out MIAC.

A complete course of antenatal steroids was administered until 34.6 weeks for fetal maturation. If there was no clinical contraindication, a course of 48h of tocolysis was administered to prolong pregnancy during steroid administration. Broad-spectrum antibiotics were administered in women with amniotic fluid glucose concentrations < 5 mg/dL and/or with microorganisms identified by amniotic fluid Gram staining and/or positive amniotic fluid cultures. In women with advanced cervical dilatation (Bishop > 6) we also started prophylactic broad-spectrum antibiotics that were discontinued if amniotic fluid cultures were negative. In the case of the onset of uterine contractions after 48 h of steroid administration, tocolysis was re-introduced only if MIAC or clinical chorioamnionitis were excluded.

MIAC was defined based on amniotic fluid culture results for genital mycoplasma (Mycoplasma IST 2, bioMérieux for *Ureaplasma* spp. or *Mycoplasma hominis)* or aerobic (Chocolate agar) and anaerobic bacteria (Schaedler agar for anaerobes and thioglycollate broth). Amniotic fluid samples were also analyzed in the Department of Microbiology by specific polymerase chain reaction (PCR) amplification of the *16S ribosomal RNA* gene using the primers: 5´- AGA GTT TGA TCC TGG CTC AG - 3´and 5´- GGA CTA CCA GGG TAT CTA AT - 3´ followed by Sanger sequencing. Sequences were identified using the Blast algorithm in the NCBI database, with minimum 98% sequence identity.

Amniotic fluid IL-6 analysis

Amniotic fluid IL-6 concentrations were measured using an automated Cobas e602 electrochemiluminescence immunoanalyzer (Cobas 8000 platform, Roche Diagnostics, Basel, Switzerland)(12) and Human IL-6 Quantikine ELISA (R&D Systems Inc., Minneapolis, MN, USA).

IL-6 concentrations ranged from 1.5-5000 pg/mL and were extended to 50,000 pg/mL with a 10-fold dilution of the sample with Cobase602. Coefficients of variation for inter-assay and intra-assay precision were 1.1-4.2 % and 0.9-3.4 %, respectively.

The sensitivity of the human IL-6 Quantikine ELISA method was < 0.70 pg/mL, with coefficients of variation for inter-assay and intra-assay precision < 10%.

Classification of outcomes

The outcome was spontaneous delivery within 7 days after admission. Women who delivered because of maternal or fetal indications were censored.

Sample size calculation

We selected a total sample size of 100 amniotic fluid aliquots in our research biobank from women recruited in an observational study within a common research line for the prediction of adverse outcomes in PTL at BCNatal (Hospital Clinic and Hospital Sant Joan de Déu, Barcelona). These 100 amniotic fluid aliquots were distributed into groups of different ranges of amniotic fluid IL-6 concentration including 10-15 aliquots in each group.

Statistical analysis

Statistical analysis was performed using SPSS 22.0 for MAC OS (IBM Corporation, USA). The interclass correlation coefficient (ICC) and Passing-Bablok regression (13) analyses

were calculated to measure agreement between the two methods. The diagnostic performance of amniotic fluid IL-6 alone and included in a multivariable predictive model (including gestational age at admission, cervical length measurement, amniotic fluid glucose and IL-6in a log scale)(2) was calculated (area under the receiver operating characteristic (AUC) curve, sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (LR+), and negative likelihood ratio (LR-)) for spontaneous preterm delivery outcome within 7 days. Multivariable analysis was performed by backward stepwise logistic regression using LR method. The regression formula for spontaneous delivery within 7 days was -7.588+0.132*gestational age at admission -0.051*US cervical length – 0.055* amniotic fluid glucose + 1.432* amniotic fluid log (IL-6) (2).

Results

Of the entire study group (n=100), 38 women spontaneously delivered within 7 days after admission. Table 1 shows the maternal characteristics and perinatal outcomes between women who spontaneously delivered within 7 days from admission and those who did not.

Higher maternal CRP and WBC concentrations at admission, shorter cervical length measurement, lower amniotic fluid glucose concentrations and higher amniotic fluid IL-6 were observed in women who spontaneously delivered within 7 days. The occurrence of MIAC was also higher in this group (47% vs. 5%; p<0.001). A polimicrobial infection was detected in 3 cases. The most common microorganism isolated was *Ureaplasma* spp. (n 9).

Table 2 shows the centiles of amniotic fluid IL-6 measured by ELISA and the automated

Cobas e602 electrochemiluminescence immunoanalyzer method.

When we compared both laboratory methods they showed good agreement (ICC:

0.937 (95% Confidence Interval (CI) 0.908 – 0.957); p<0.001). The results of the

passing-Bablok regression analysis are presented in Figure 1 and also show good

agreement between the two analytical methods.

The diagnostic performance of IL-6 measured with the Cobas method alone or

included in the multivariable predictive model for predicting spontaneous delivery

within 7 days was assessed using receiver operating characteristic (ROC) curves. The

AUC of amniotic fluid IL-6 as a stand-alone predictive parameter to predict

spontaneous delivery within 7 days was 0.83 (95% CI 0.75-0.92), with 2385 pg/mL

being the best cut-off with a sensitivity of 86.8%, false-positive rate of 35.5%, PPV of

60%, NPV of 88.9%, LR+ of 2.45 and LR- of 0.2039.

The AUC of the multivariable model for predicting spontaneous delivery within 7 days

was 0.894 (95% CI 0.799 – 0.955) with a sensitivity of 97%, false-positive rate of 26%,

PPV of 73%, NPV of 97%, LR+ of 3.7 and LR- of 0.045.

Diagnostic performance of amniotic fluid glucose (cut-off 16 mg/dL), IL-6 as stand-

alone (cut-off 2385 pg/mL) and the multivariable predictor model to predict

spontaneous delivery within 7 days was presented in Table 3.

Discussion

The main findings of this study showed that Cobas and ELISA methods were

comparable for measuring amniotic fluid IL-6 concentrations in women with PTL. In

addition, we ratified the good diagnostic performance observed in our multivariable

predictive model of spontaneous preterm delivery within 7 days by measuring IL-6

concentrations with this automated method.

Both quantitative methods showed good agreement for assessing amniotic fluid IL-6 in women with PTL. However, from a clinical perspective, Cobas has more advantages.

ELISA method requires a manual and labor-intensive handling process. Control reagents and aliquots needs to be manually processed. In addition, ELISA method requires a manual calibration each time, and this limits time results (around 46 hours). On contrary, Cobas is a modular fully automated analyzer; the Cobas IL-6 reagent is ready for use; calibration is not needed each time because it is stable for 28 days; and more interestingly, the results are obtained in 18 minutes.

Although both techniques require a diluting process when IL-6 concentrations are very high, this process is faster and less time-consuming with Cobas (minutes vs. days, respectively).

Finally, the degree of variation (CV) with Cobas is lower than with ELISA, making more precise the estimation.

Although other IL-6 rapid tests (5, 6) have shown a good correlation with the ELISA

method, the Cobas immunoanalyzer is able to simultaneously run different and

heterogeneous immunoassays, including 80 different assays for hormones, bone,

cardiac and tumor markers, anemia and infectious diseases. This property efficiently

increases the testing capacity of this automated method in the clinical setting.

We observed that the diagnostic performance of amniotic fluid IL-6 to predict

spontaneous delivery within 7 days was good when it was included in a multivariable

predictive model. Indeed, we ratified the good diagnostic performance observed in the

predictive model of spontaneous preterm delivery (2) by measuring IL-6

concentrations with the Cobas method. Our findings suggest that, in those centers that integrate the amniocentesis as part of clinical management of women admitted with early symptoms of PTL, the predictive model might help to target women at high-risk of spontaneous preterm delivery within 7 days who might benefit from antenatal strategies that have shown to improve perinatal outcomes (14, 15).

Similarly, the model might allow the low-risk group to deliver within 7 days to be rapidly ruled out. In this regard, only 30% of women with symptoms of preterm labor deliver within 7 days (16). Cardiovascular and metabolic abnormalities have been observed in large animal models and cohorts of children exposed to antenatal corticosteroids that are consistent with fetal programming for adult diseases (17). Moreover, an association among term infants exposed to antenatal corticosteroids during pregnancy and neurocognitive disorders has also been reported (18). A future randomized control trial to evaluate whether the implementation of this predictive model based on amniotic fluid information optimizes antenatal management in women with early symptoms of PTL and is a cost-effective strategy is warranted.

Conclusion

In conclusion, the Cobas immunoanalyzer is a reliable method for analyzing amniotic fluid IL-6 concentrations in women with PTL. The good diagnostic performance observed in the predictive model might help, in those centers that integrate the amniocentesis as part of the clinical management of women admitted with early symptoms of PTL, to target women at high-risk of spontaneous delivery within 7 days.

Statement of Ethics

Patient selection and sampling procedures were performed in accordance with the Declaration of Helsinki and applicable local regulatory requirements after approval from the Institutional Review Boards (HCB/2019/0940). Written informed consent was obtained for sample collection from all subjects.

Conflict of interest statement

The authors have no conflicts of interest to declare.

Author Contributions

TC., MK conceived the project. TC., MK., VA designed the experiment. TC collected the samples and coded data. CA, MH., processed and analyzed samples. TC., VA analyzed and interpreted data. TC., MK., BJ., XF., VA co-wrote and revised the manuscript. MK., BJ supervised the project.

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Table 1 Maternal and perinatal characteristics.

	sPTD 7 days (n 38)	No sPTD 7days (n 62)	*p*
Maternal age (years)	33.1 (26.3; 36.5)	33.7 (29.4; 37.5)	0.312
Caucasian ethnicity	27 (71)	14 (23)	0.238
Prior preterm birth	3 (8)	1 (2)	0.642
GA at admission (weeks)	25.7 (23.8; 29.9)	27.1 (24.6; 29.4)	0.500
Cervical length (mm)	2 (0; 15)	11 (5; 23)	0.013
CRP (mg/L)	2.1 (0.8; 5.0)	0.7 (0.3; 1.6)	0.002
WBC (x10^9/L)	14420 (11760 16770)	11785 (9947.5; 13515)	0.002
GA at amniocentesis (weeks)	25.7 (23.8; 30.03)	27.1 (24.8; 29.5)	0.472
AF glucose (mg/dL)	7 (0.8; 23)	30 (22.5; 41)	<0.001
AF IL-6 (ng/dL)	40.4 (7.1; 50.0)	1.3 (0.5; 5.5)	<0.001
MIAC	18 (47)	3 (5)	<0.001
GA at delivery (weeks)	25.9 (24.1; 30.6)	35.6 (30.7; 38.5)	<0.001
Latency to delivery (days)	1 (0; 2)	52 (15; 74)	<0.001

sPTD: Spontaneous preterm delivery; GA: Gestational age; CRP: C-reactive protein; WBC: White blood cells; AF: Amniotic fluid; MIAC: Microbial invasion amniotic cavity.Continuous variables were compared using a nonparametric Mann Whitney U test presented as medians (25th; 75th interquartile percentile). Categorical variables were compared using Chi-square or Fisher exact tests and presented as number (%).

Table 2 Centiles of amniotic fluid IL-6 using the Cobas e602 immunoanalyzer and ELISA method.

| AF IL-6	5 th percentile	10th percentile	25th percentile	50th percentile	75th percentile	90th percentile	95th percentile
Cobas e602 (pg/mL)	236.09	358.03	624.60	3255.50	23244.75	50000.00	50000.00
ELISA (pg/mL)	263.60	407.0	954.25	2769.50	14607.50	48258.50	51475.0

AF: amniotic fluid

Table 3 diagnostic performances of amniotic fluid glucose (cut-off 16 mg/dL), IL-6 as stand-alone (cut-off 2385 pg/mL) and the multivariable predictor model to predict spontaneous delivery within 7 days

	AF glucose (cut-off 16 mg/dL)	**IL-6 as stand-alone (cut-off 2385 pg/mL)**	**Multivariable predictor model**
Sensitivity (%)	60	86.8	97
FP rate	4.5	35.5	26
PPV (%)	90	60	73
NPV (%)	77.8	88.9	97
LR +	26.4	2.45	3.7
LR -	0.4093	0.2039	0.045

AF: Amniotic fluid; AUC: Area under curve, CI: Confidence interval; FP: False positive; PPV: Positive predictive value; NPV: Negative predictive value; LR: Likelihood ratio.

Figure 1. Comparison of Cobas e602 immunoanalyzer and ELISA methods using

Passing-Bablok regression analysis
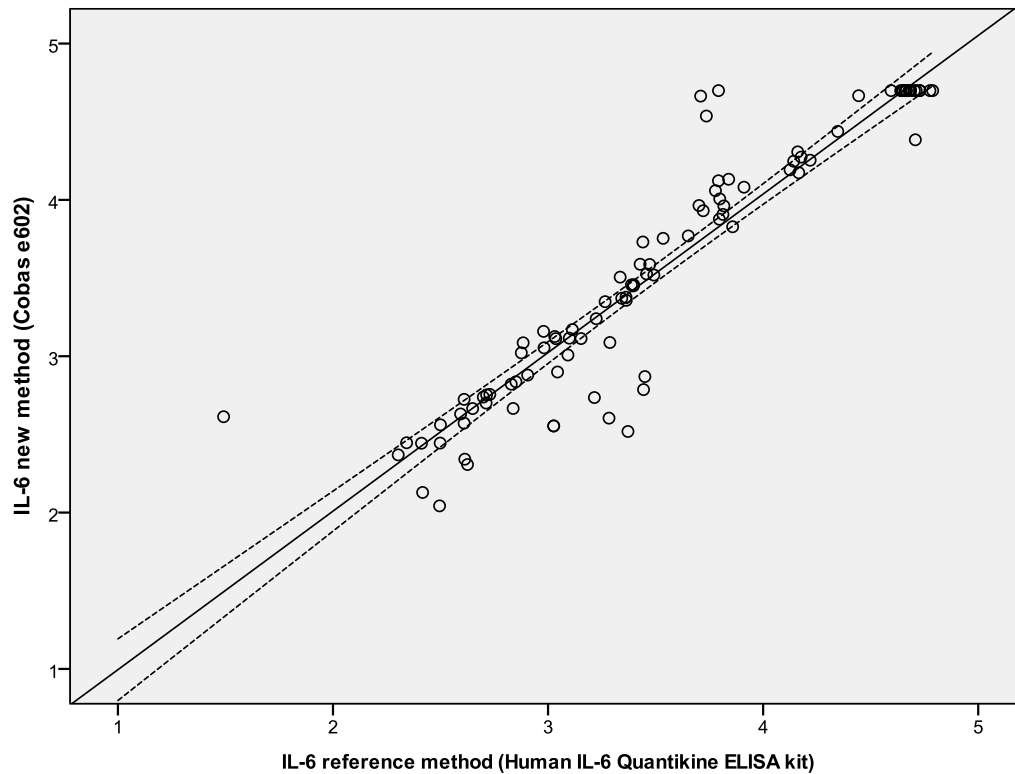
Passing-Bablok regression analysis shows good agreement between the two analytical methods assessing IL-6 levels in amniotic fluid (logarithmic scale). Linear regression is represented as a solid black line; the dashed line represents the 95% Confidence Interval. The regression equation is: IL-6 Cobas e602 = -0.109+1.041*Human IL-6 Quantikine ELISA kit; slope=-0.109 (95% CI -0.306-0.021); intercept=1.041 (95% CI 1.004 -1.101).