Prediction of preterm labour from a single blood test: the role of the endocannabinoid system in predicting preterm birth in high-risk women

by

P. Bachkangi[a], A. H. Taylor [a,b], Monica Bari[c], Mauro Maccarrone[d], Justin C. Konje[a,e*]

[a] Endocannabinoid Research Group, Reproductive Sciences Section, Department of Cancer Studies and Molecular Medicine, University of Leicester, Leicester, UK; [b]Department of Molecular and Cell Biology, University of Leicester, Leicester, UK, Department of Medicine; [c]Università di Roma Tor Vergata, Italy [d]Università Campus Bio-Medico di Roma, Italy; [e]Department of Obstetrics and Gynaecology, Sidra Medicine, Doha and Wellness Women's Research Center, HMC, Doha, Qatar.

To whom correspondence should be addressed:

Professor Justin C. Konje

JCK4@le.ac.uk or jkonje@sidra.org

Tel direct: +974 4012-5810; Mobile: +974 7785-3765

Running Title: The ECS in the preterm birth prediction

Keywords: Anandamide, Endocannabinoids, *N*-acylethanolamine, Oleoylethanolamide, Palmityolethanolamide, Preterm labour, Prediction

Conflict of interest statement: None

Number of words: 3485 Number of Figures: 2 Number of Tables: 2

Abstract

Objective: To determine if plasma concentrations of the *N*-acylethanolamines (NAEs) N-arachidonoylethanolamine (AEA), *N*-oleoylethanolamide (OEA) and *N*-palmitoylethanolamide (PEA) increase in women at high risk for preterm birth (PTB) and whether these could be used to predict preterm delivery and if so, how they compare with current methods.

Design: Prospective cohort study.

Setting: A large UK teaching hospital.

Population: 217 pregnant women were recruited between 24 and 34 gestational weeks at 'high-risk' for PTB, recruited from a prematurity prevention clinic or antenatal wards.

Methods: Plasma AEA, OEA, and PEA concentrations were measured using ultra-high performance liquid chromatography-tandem mass spectrometry whilst FAAH enzyme activity was measured by fluorometric radiometric assay and CL by ultrasound scan. The clinical usefulness of these measurements were determined by ROC and multivariate analyses.

Results: AEA and PEA concentrations were significantly higher in women who delivered prematurely. An AEA concentration >1.095 nM predicted PTB, the gestational age at delivery and the recruitment to delivery interval (RTDI). A PEA concentration >17.50 nM only predicted PTB; FAAH enzyme activity was not related to these changes. Multivariate analysis (all variables) generated an equation to accurately predict the RTDI.

Conclusions: A single plasma AEA or PEA measurement can predict PTB. A single AEA measurement predicts the gestational age of delivery and the remaining period of pregnancy with reasonable

accuracy and better than existing conventional tests thus offering a better window for primary prevention of PTB.

Funding:

The ERG and University of Leicester.

Introduction

Preterm birth (PTB), defined as delivery before 37 completed weeks of gestation (1), constitutes 9.6% of all births worldwide (1), 7.3% in the UK (2), and is responsible for 75% of all perinatal mortalities and many long-term morbidities for the surviving infants (3). Its aetiology is poorly defined but threatened preterm labour (PTL), which is multifactorial (4-8) results in preterm birth in a significant number of cases. Of the many factors that have been investigated as predictors of PTB, onco-fetal fibronectin (ofFN), and insulin-like growth factor-binding protein-1 (IGFBP-1) in cervico-vaginal swabs are commonly used in clinical practice on symptomatic women, but shown only to identify those who are unlikely to go into PTL (9-15). The best predictive test for PTB in high-risk women is, however, sonographic cervical length measurement (CL) (16-22), with a long cervix (≥ 30 mm) indicative of low risk, while a cervix of ≤15 mm indicative of high risk. The actual risk of PTB, however, is dependent on when the measurement is made and the skill of the sonographer making that measurement; e.g. for a length of <15mm there is a 90% risk at ≤28 weeks of gestation and 50-60% at 28 to 32 weeks of gestation (16, 17, 21). Furthermore, CL measurement is often used in combination with a cervico-vaginal tests (either ofFN or IGFBP-1), especially when the clinical or sonographic findings are equivocal (23-25). Thus, no approved biochemical predictive test for PTB currently exists.

Previously it has been shown that plasma *N*-arachidonylethanolamine (anandamide, (AEA)) concentrations increase in the third trimester, reaching a peak in labour (15, 26, 27) leading to the hypothesis that plasma AEA concentrations too would increase in the plasma of women with 'threatened PTL'. Indeed, Nallendran *et al*. showed that plasma AEA concentrations increase in women at 'high risk' for PTB who subsequently deliver prematurely (28). Furthermore, a correlation between elevated plasma AEA levels in pregnancy and spontaneous miscarriage has also been demonstrated (29) and thought to be mediated through the reciprocal actions of the main AEA degrading enzyme, fatty acid amide hydrolase [FAAH; (30)] supporting the suggestion that increased FAAH activity might be absent during or just prior to PTL. Additionally, two less commonly studied *N*-

acylethanolamines (NAEs) *N*-oleoylethanolamine (OEA) and *N*-palmitoylethanolamine (PEA), may also be involved in the aetiology of PTL. . These NAEs increase plasma AEA concentrations by inhibiting its catabolism by FAAH and by reducing its cellular uptake (31), a phenomenon known as 'the entourage effect'. AEA in turn is an important source of prostaglandins which increase prior to and during labour. This could be the mechanism responsible for the observations of Nallendran *et al.* (28) in that *a prior* increase in FAAH activity and/or increased OEA and PEA concentrations might precede PTL and thus add to the predictive value of AEA in PTB.

Hence, our hypothesis here was that a single blood test of NAEs would predict the risk of preterm birth, the gestational age (GA) at delivery and the interval from sampling to delivery (recruitment to delivery interval: RTDI) in women at 'high-risk' for PTB. The establishment of a test where the prediction of PTB can be achieved in both symptomatic and asymptomatic women would be of value in the prevention and early management of PTB.

Materials and Methods

Subjects

Participants in this prospective cohort study were recruited either from the Prematurity Prevention Clinic (PPC) or from the Obstetric Wards at the University Hospitals of Leicester NHS Trust, at Leicester Royal Infirmary. Inclusion criteria were: women at 'high risk' of PTB seen in the PPC (i.e. previous PTB, a family history of PTB, history of 2 or more LLETZ procedures, cervical length <25mm on transvaginal ultrasound scan (national guidelines (2)), or had a cervical cerclage inserted in a previous pregnancy) or presented with symptoms of PTL but were not in active labour (i.e. had abdominal pains and cervical dilatation was less than 3 cm with intact fetal membranes). The gestational age at the time of recruitment was between 24^{+0} to 34^{+0} weeks of gestation, except for one patient who was 23^{+5} weeks pregnant on admission but whose sample was taken at 24^{+0} weeks. Women with multiple pregnancies, or those with medical conditions (such as hypertension, connective tissue or endocrine disorders, etc.), or suspected to have chorioamnionitis and/or placental abruption, were excluded. All volunteers gave signed informed consent and the study was approved by the Leicestershire, Northamptonshire and Rutland Research Ethics Committee (IRB Reference Number: 06/Q2501/48). Attendees at the PPC were asymptomatic patients who were approached on either their first or second visit to the clinic (in either the first or second trimesters). Emergency admissions were recruited either on the labour ward, in the Maternity Assessment Centre, or on the antenatal wards. Cervical length (CL) measurements were obtained by a well-trained sonographer and only from the women recruited from the PPC. All measurements and assessments were made before the patients delivered.

N-acylethanolamine purification and quantification

N-acylethanolamine measurements were initially performed by column extraction and ultra-high-pressure liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) as previously described (34), but later by using modifications described by Lam *et al.* (32). Briefly, blood collected in EDTA tubes was centrifuged at 1200 g for 30 minutes at 4°C within an hour of collection. Plasma (0.5ml) was

placed into Kimble vials in duplicate and diluted with 0.5ml of deionized (HPLC-grade) water; 20µl of internal standard was then added and vortex mixed for approximately 10 sec. Lipids were extracted using Oasis HLB cartridges (Waters UK Ltd) of 1cc capacity, attached to a Vacmaster vacuum manifold (Biotage, Uppsala, Sweden). The vacuum flow-rate was adjusted to 1ml/minute and each Oasis HLB cartridge activated with 1ml 100% methanol and cleared with 1ml of HPLC-grade water. At this point, 1ml of the premixed plasma sample was added to the column, washed with 40% methanol (1ml). Lipids were then eluted into plastic test tubes (Test Tube PP Push Cap 2.5ml, Sarstedt, Leicester, Leicestershire, UK**)** by adding acetonitrile (1ml). Samples were next dried gently at 40°C under a constant stream of nitrogen gas on a FSC400D Sample Concentrator (Techne®, Barber Insys, Sandy, Bedfordshire, UK). Acetonitrile (80µl) was then added to solubilise the lipids and the mixture transferred to a HPLC vial. Triplicate measurements of the NAEs were made using an Acquity system (Waters Ltd, Hertfordshire, UK) as detailed by Lam *et al*. (33).

Cervical length measurements and cervico-vaginal swabs

Cervical length (CL) measurements were obtained by trans-vaginal ultrasound, performed as part of the patient's normal clinical assessment and were made as described by Kegan and Sonek (34). A cut-off value of <25mm for increased risk of PTB was used. Cervico-vaginal swabs were collected after the TVS (if clinically indicated or if the volunteer consented). These swabs were collected from the cervical os and the results interpreted as per the manufacturer's instructions (35, 36). The ofFN swabs were purchased from Fullterm® (Hologic UK Ltd, Crawley, West Sussex, UK) and the IGFBP-1 Actim® Partus swabs from Medix Biochemica, UnaHealth Ltd., Stoke-on-Trent, Staffs, UK.

FAAH activity measurement

Whole blood was collected in plain tubes (Sarstedt Ltd., Leicester, UK) prefilled with acid citrate dextrose (ACD) anticoagulant, placed on ice, transferred to the laboratory and stored at −80 °C until

111 the time for their transport to a laboratory in Rome (Italy), where lymphocytic membranes were
112 prepared and FAAH enzyme activity measured using radiolablled AEA, as described (30, 37).

Statistical Analyses

113 Data that were not normally distributed were normalised by logarithmic transformation. Each
114 predictive variable was studied by univariate linear regression and then with multivariate analysis. In
115 the univariate analyses, unpaired Student's *t*-test (GraphPad Prism version 6:00 for windows,
116 GraphPad Software, La Jolla California USA, www.graphpad.com) was used to determine if plasma
117 NAE concentrations were significantly different. To determine predictor specificity, sensitivity,
118 negative predictive value (NPV) and positive predictive value (PPV), Receiver-Operator Curve (ROC)
119 analyses were performed (GraphPad Prism version 6:00 for windows). Plasma AEA data from
120 previously unpublished work on a similar patient cohort (27) were combined with the data obtained
121 when all 3 NAEs were measured simultaneously to improve confidence in the multivariate analyses,
122 where plasma AEA was an inclusive variable. These data were analysed using Stata Statistical Software
123 version 14, (StataCorp LP, Texas, USA) and statistical significance accepted when $p<0.05$.

Results

Analysis of clinical data

A total of 217 patients were recruited. Neither maternal age (p=0.33; Mann-Whitney U test) nor BMI (p=0.98; Mann-Whitney U test) were significantly different between the women who delivered prematurely and those who delivered at term (data not shown). Plasma AEA measurements were performed on all patients but those for OEA and PEA were only made on the last 51 patients recruited. This was because simultaneous measurement of the three NAEs was only possible midway through the study (33). CL assessment was performed on 64 patients, whilst IGFBP-1 and ofFN measurements were undertaken in 41 and 79 patients, respectively (Table 1), due to a change from using ofFN to IGFBP-1 being implemented in the Clinical Department of Obstetrics during the study period. FAAH enzyme activity was assessed in 43 samples recruited from the final 51 patients (Table 1).

Plasma NAE concentrations

Plasma AEA concentrations (0.61 ± 0.25nM; mean ± SD) in the women who delivered preterm (n=71) were statistically (p=0.026) higher than those (0.40 ± 0.38nM; Figure 1A) in the women who delivered at term (n=146). Similarly, plasma PEA concentrations in the women who delivered preterm (15.25 ± 5.38nM) were statistically higher (p=0.042) than in the women who delivered at term (12.01 ± 6.32nM; Figure 1B). Although plasma OEA concentrations in the women who delivered preterm (4.24 ± 2.82nM) were higher than in the women who delivered at term (3.11 ± 1.51nM; Figure 1C), this was not statistically significant (p=0.087).

Cervical length measurements and cervico-vaginal swabs

Of 64 volunteers who had ultrasonic CL measurement, 10 delivered prematurely and 54 delivered at term. A ROC curve generated from the data demonstrated a sensitivity of 20.0%, specificity of 85.4%, a PPV of 30.0% and a NPV of 77.4 % when CL length of <25mm was used as the cut-off for PTB prediction.

There was no significant difference in IGFBP-1 values between the PTB and term groups (p=0.3; 95% CI: -0.82 to 2.18). The value of ofFN for the prediction of PTB and the recruitment to delivery interval (RTDI) was examined by univariate analysis. There was a significance in predicting PTB (p=0.03, 95% CI 0.13 to 2.90), but not for RTDI (p=0.09, 95% CI: -26.74 to 1.81). Since IGFBP-1 and ofFN had similar NPVs and are supposed to be useful predictors of prematurity in this cohort, the data from both measurements were combined into a single binary variable ('swab test'). Logistic regression was performed to examine the swab test's association with prematurity and was found to be significant (p=0.02, 95% CI: 0.17 to 2.18). ROC analysis using the combined swab test as a single variable indicated a sensitivity of 33.33%, a specificity 78.79%, a NPV of 86.67% and a PPV of 22.22% for the prediction of PTB.

FAAH activity measurements

The FAAH enzyme activity in lymphocytic membranes was not statistically different (p=0.29; 95% CI: -0.01 to 0.03) between women who delivered prematurely (mean ± SEM: 110.3 ± 6.1pmol/min/mg) and those that delivered at term (96.6 ± 8.8pmol/min/mg).

Combined data analysis and prediction of PTB, RTDI and GA at delivery

For further investigation of the various predictors of prematurity, the data were subjected to univariate and multivariate analyses. In univariate analyses, there was a significant difference for AEA (p=0.001, 95% CI: 0.34 to 1.41) and PEA plasma concentrations for the prediction of preterm birth. AEA concentrations (0.405 ± 0.04nM; mean ± SEM) were higher in the preterm group than in the term group (0.608 ± 0.09nM). PEA concentrations were also significantly (p=0.04, 95% CI: -0.29 to -0.005) higher in the preterm group (15.25 ± 1.26nM) when compared to those of the term group (12.01 ± 1.1nM). Although the mean plasma OEA concentration was also higher (4.24 ± 0.66nM) in the preterm group than in the term group (3.11 ± 0.26nM), the values were not statistically different (p=0.09, 95%

CI: -0.18 to 2.28). In multivariate logistic regression analysis, no combinations of predictors were able to predict the risk of preterm birth.

In multivariate analyses, CL measurement was a lone predictor of RTDI (Table 2). Accordingly, in univariate analysis CL was also demonstrated to be a predictor of RTDI. None of the other parameters provided evidence of being a predictor of PTB, RTDI or GA at delivery using multivariate analysis. Although plasma PEA concentration [PEA] was a significant predictor of PTB alone, plasma AEA concentration [AEA] was a significant predictor of PTB, gestational age at delivery and the RTDI (Table 2).

Regression analysis of GA at delivery as a continuous outcome variable showed a statistically significant correlation only for AEA in univariate (p=0.004, 95% CI: -2.27 to -0.43) but not in multivariate analysis (p=0.19, 95% CI: -3.61 to 0.77).

Logistic regression analyses for the prediction of RTDI in days showed statistically significant prediction of this by AEA concentrations (p=0.02, 95% CI: -20.19 to -2.13) and CL (p=0.002; 95% CI: 0.50 to 2.03) in the univariate analysis. In the multivariate analysis, only the CL (p=0.04; 95% CI: 0.04 to 1.70) was predictive and while the logAEA measurment lost its ability to predict PTB (p=0.1, 95% CI -41.60 to 4.08) (Table 2).

Plasma NAE concentrations, cervical swab measurements and FAAH activity as predictors of PTB risk

For the 51 samples, where all the variables were available, ROC analysis indicated that CL at a cut-off of 25mm had 0% sensitivity, 76% specificity, a PPV of 0%, and a NPV of 89% for the prediction of PTB. ROC analysis of IGFBP-1 as a positive or negative test, had a sensitivity of 44.4%, a specificity of 61.2%, with a PPV of 52% and a NPV of 79%.

ROC analysis of plasma AEA as a predictor of PTB using a cut-off point of 0.428nM, had a sensitivity of 67%, a specificity of 67%, a PPV of 52%, and a NPV of 79%, with an odds ratio of 2.00 for PTB. Similar analyses for plasma PEA concentrations with a cut-off point of 17.50nM, had a sensitivity of 33%, a specificity of 88%, with a PPV of 60%, and a NPV of 71% (Figure 2). The odds ratio of a PTB for a plasma PEA above 17.5nM was 2.75. At the same time, ROC analysis for plasma OEA at a cut-off point of 4.31nM, revealed an assay sensitivity of 44% and specificity of 85%, with a PPV of 62%, and NPV of 74%, with an odds ratio of 2.93 (Figure 2).

Discussion

The data presented here indicate that it may be possible to predict whether women at 'high risk' for PTB are actually going to deliver prematurely from a single blood test. Univariate analysis of plasma NAE concentrations indicated that AEA is the only reliable predictor of PTB (p=0.001), the GA at delivery (p=0.004) and the RTDI in days (p=0.02). Cervical length was shown to be a predictor of RTDI: in both univariate (p=0.002) and multivariate (p=0.04) analyses, confirming it to be a predictor of PTB (16-22). It also showed that the cohort under study was suitable for these studies.

Analysis of ofFN showed it to have a significant predictive value for PTB (p=0.03) but not for RTDI (p=0.09). In clinical settings, ofFN is often exchanged for IGFBP-1 (as happened here), so it was important to determine if these can be used interchangeably or together to predict PTB. Consequently, ofFN and IGFBP-1 results were combined to produce a single new variable (swab test). By doing so, this improved their combined predictive value, from p=0.03 for ofFN and p=0.3 for IGFBP-1 to p=0.02 when combined. This observation suggests that clinicians can manage patients with a sole positive ofFN measurement but not with a sole positive IGFBP-1 measurement, and that prediction of PTB risk is improved when these test are combined.

Neither ofFN nor IGFBP-1 were useful in predicting the RTDI. This observation is not unexpected because ofFN is known to have a high specificity in predicting PTB only before 34 weeks (9). Clinically, the window of this test for predicting for PTB is between 7 and 21 days (39), implying it is not useful when the RTDI is more than 21 days, hence it is mainly used for secondary or tertiary prevention. Similarly, IGFBP-1 though with a higher predictive ability than ofFN (40, 41) (sensitivity: 74-100% and specificity: 77-98.2%), is not useful beyond 7 days from testing (42) and here again, the value of that prediction is imprecise. One feature of this study was that our participants were 'unwilling' to have a swab test performed, considering it to be invasive, but were 'happy' to have a blood sample for NAE

measurement to predict PTB. This suggests that measurement of NAEs would be acceptable for both the women at 'high risk' of PTB and any woman presenting with threatened PTL.

The reason why the high-risk women had higher plasma AEA concentrations, even when asymptomatic, remains unclear. Although the same analysis conducted for plasma OEA and PEA concentrations (the NAEs involved in the "entourage effect" and thus might promote the observed plasma AEA concentrations), showed both of these NAEs to also be non-significantly increased in the women who subsequently went on to deliver prematurely, most likely due to the limited sample size. We had hypothesised that increased FAAH activity in the lymphocytic cell membrane might be responsible for this but that clearly was not the case as there was no change in our quantified lymphocytic FAAH activity.

The calculated sensitivity, specificity, NPV and PPV for all three NAEs were much better when compared to those for CL, ofFN and IGFBP-1 suggesting that these observations offer hope for a more reliable predictive test for PTB. Taken together, we showed that plasma AEA concentration > 1.095nM has a specificity of 87.14%, a sensitivity of 25.93%, a NPV of 70.24% and a PPV of 61.2%, and thus providing a better test than the currently available predictive tests.

Predicting PTB, GA at delivery and the RTDI with a single blood test could have a profound impact in clinical practice, because the measurement of the plasma NAEs can then allow for initiation of interventions months in advance of birth, something that current tests cannot do (they are reliable for only two weeks from the time of the examination and have poor sensitivities and PPVs). Furthermore, the opportunity to make appropriate arrangements for patients who are at risk of imminent PTB, whether by the administration of steroids, inserting a cervical cerclage, or arranging an *in-utero* transfer may be determined with this new test. Both ofFN and IGFBP-1 measurements provide good NPVs, but they are poor predictors of PTB (9-15) and as a result one third of pregnant women

are admitted with 'threatened PTL' and receive unnecessary treatment, even though most of their pregnancies reach term (43-45).

The goal of the multivariate analysis undertaken here was to generate an equation that uses all the variables to predict RTDI. We omitted OfFN because it was considered to be insignificant. The following equation to determine the RTDI was derived:

RTDI (Days) = 1.20 – (18.76 x **log[AEA]**) + (13.12 x **log[OEA]**) – (0.25 x **PEA**) + (0.87 x **CL**) + (0.10 x **FAAH**) + (16.03 x **IGFBP-1**)

From the available data arising from all 217 plasma AEA samples, plasma AEA offered a novel predictor of PTB at a concentration > 1.095nM (with a specificity of 87.1%, a sensitivity of 25.9%, a NPV of 70.2% and a PPV of 61.2%). This is a much better test than any currently available predictive test. Similarly, despite the limited number (n=51) of samples, plasma PEA concentrations >17.50nM offered a predictive accuracy with a specificity of 88%, a sensitivity of 33%, an NPV of 71% and a PPV of 60% justifying the inclusion of plasma PEA in a predictive model for PTB.

Limitations

The main limitation for this study was that many patients did not wish to consent of TVS or speculum examination because they considered it to be an invasive procedure. The measurement of plasma NAEs was a more appealing prospect, however, because measurement of OEA and PEA analysis concentrations only occurred later in the study and smaller number of participants had these important measurements made. Future studies should use measurement of all 3 NAEs within all patient groups, not only those at high risk of PTB, to determine how good these lipids are at predicting PTB and the RTDI.

Conclusion

Plasma NAEs from a single blood test offer great promise as a predictors of PTB. The non-invasive nature of the test appeals to patients. While this shows great potential, there is the need for larger scale studies to confirm these exciting observations and then undertake clinical trials of this application. We believe that this test, or something similar, could transform the management of PTB, since it provides a first biochemical test for the primary prevention of the condition.

Disclosure of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Funding

This work was funded by the University Hospitals of Leicester and the MRCOG Part II Revision Course run by Professor Konje.

Acknowledgements

The authors thank Dr. Maria Viskaduraki, (biostatistician in the Bioinformatics and Biostatistics Analysis Support Hub (BBASH), University of Leicester) who performed the multivariate analyses and the midwives and clinicians at Leicester Royal Infirmary for their assistance with patient recruitment.

References

1. Beck S, Wojdyla D, Say L, Betran AP, Merialdi M, Requejo JH, et al. The worldwide incidence of preterm birth: a systematic review of maternal mortality and morbidity. Bull World Health Organ. 2010;88(1):31-8.

2. Preterm labour and birth. NICE guideline [Internet]. 2015. Available from: http://nice.org.uk/guidance/ng25.

3. Goldenberg RL, Culhane JF, Iams JD, Romero R. Epidemiology and causes of preterm birth. Lancet. 2008;371(9606):75-84.

4. Messer LC, Vinikoor LC, Laraia BA, Kaufman JS, Eyster J, Holzman C, et al. Socioeconomic domains and associations with preterm birth. Soc Sci Med. 2008;67(8):1247-57.

5. Messer LC, Kaufman JS, Mendola P, Laraia BA. Black-white preterm birth disparity: a marker of inequality. Ann Epidemiol. 2008;18(11):851-8.

6. Messer LC, Kaufman JS, Dole N, Savitz DA, Laraia BA. Neighborhood crime, deprivation, and preterm birth. Ann Epidemiol. 2006;16(6):455-62.

7. Abel EL, Kruger M, Burd L. Effects of maternal and paternal age on Caucasian and Native American preterm births and birth weights. Am J Perinatol. 2002;19(1):49-54.

8. Dolk H. Paternal age and preterm birth. Epidemiology. 2006;17(5):593; author reply -4.

9. Honest H, Bachmann LM, Gupta JK, Kleijnen J, Khan KS. Accuracy of cervicovaginal fetal fibronectin test in predicting risk of spontaneous preterm birth: systematic review. BMJ. 2002;325(7359):301.

10. Akercan F, Kazandi M, Sendag F, Cirpan T, Mgoyi L, Terek MC, et al. Value of cervical phosphorylated insulinlike growth factor binding protein-1 in the prediction of preterm labor. J Reprod Med. 2004;49(5):368-72.

11. Eroglu D, Yanik F, Oktem M, Zeyneloglu HB, Kuscu E. Prediction of preterm delivery among women with threatened preterm labor. Gynecol Obstet Invest. 2007;64(2):109-16.

12. Paternoster D, Riboni F, Vitulo A, Plebani M, Dell'Avanzo M, Battagliarin G, et al. Phosphorylated insulin-like growth factor binding protein-1 in cervical secretions and sonographic cervical length in the prediction of spontaneous preterm delivery. Ultrasound Obstet Gynecol. 2009;34(4):437-40.

13. Lembet A, Eroglu D, Ergin T, Kuscu E, Zeyneloglu H, Batioglu S, et al. New rapid bed-side test to predict preterm delivery: phosphorylated insulin-like growth factor binding protein-1 in cervical secretions. Acta Obstet Gynecol Scand. 2002;81(8):706-12.

14. Kekki M, Kurki T, Karkkainen T, Hiilesmaa V, Paavonen J, Rutanen EM. Insulin-like growth factor-binding protein-1 in cervical secretion as a predictor of preterm delivery. Acta Obstet Gynecol Scand. 2001;80(6):546-51.

15. Altinkaya O, Gungor T, Ozat M, Danisman N, Mollamahmutoglu L. Cervical phosphorylated insulin-like growth factor binding protein-1 in prediction of preterm delivery. Arch Gynecol Obstet. 2009;279(3):279-83.

16. Iams JD, Goldenberg RL, Meis PJ, Mercer BM, Moawad A, Das A, et al. The length of the cervix and the risk of spontaneous premature delivery. National Institute of Child Health and Human Development Maternal Fetal Medicine Unit Network. N Engl J Med. 1996;334(9):567-72.

17. Hassan SS, Romero R, Berry SM, Dang K, Blackwell SC, Treadwell MC, et al. Patients with an ultrasonographic cervical length < or =15 mm have nearly a 50% risk of early spontaneous preterm delivery. Am J Obstet Gynecol. 2000;182(6):1458-67.

18. Leitich H, Brunbauer M, Kaider A, Egarter C, Husslein P. Cervical length and dilatation of the internal cervical os detected by vaginal ultrasonography as markers for preterm delivery: A systematic review. Am J Obstet Gynecol. 1999;181(6):1465-72.

19. Iams JD, Paraskos J, Landon MB, Teteris JN, Johnson FF. Cervical sonography in preterm labor. Obstet Gynecol. 1994;84(1):40-6.

20. Berghella V, Ness A, Bega G, Berghella M. Cervical sonography in women with symptoms of preterm labor. Obstet Gynecol Clin North Am. 2005;32(3):383-96.

21. Heath VC, Southall TR, Souka AP, Elisseou A, Nicolaides KH. Cervical length at 23 weeks of gestation: prediction of spontaneous preterm delivery. Ultrasound Obstet Gynecol. 1998;12(5):312-7.

22. Andersen HF, Nugent CE, Wanty SD, Hayashi RH. Prediction of risk for preterm delivery by ultrasonographic measurement of cervical length. Am J Obstet Gynecol. 1990;163(3):859-67.

23. Schmitz T, Maillard F, Bessard-Bacquaert S, Kayem G, Fulla Y, Cabrol D, et al. Selective use of fetal fibronectin detection after cervical length measurement to predict spontaneous preterm delivery in women with preterm labor. Am J Obstet Gynecol. 2006;194(1):138-43.

24. Danti L, Prefumo F, Lojacono A, Corini S, Testori A, Frusca T. The combination of short cervical length and phIGFBP-1 in the prediction of preterm delivery in symptomatic women. J Matern Fetal Neonatal Med. 2011;24(10):1262-6.

25. Azlin MI, Bang HK, An LJ, Mohamad SN, Mansor NA, Yee BS, et al. Role of phIGFBP-1 and ultrasound cervical length in predicting pre-term labour. J Obstet Gynaecol. 2010;30(5):456-9.

26. Habayeb OM, Taylor AH, Evans MD, Cooke MS, Taylor DJ, Bell SC, et al. Plasma levels of the endocannabinoid anandamide in women--a potential role in pregnancy maintenance and labor? J Clin Endocrinol Metab. 2004;89(11):5482-7.

27. Nallendran V, Lam PM, Marczylo TH, Bankart MJ, Taylor AH, Taylor DJ, et al. The plasma levels of the endocannabinoid, anandamide, increase with the induction of labour. BJOG. 2010;117(7):863-9.

28. Nallendran V LP, McParland PC, Taylor AH, Konje JC Prediction of preterm labour among asymptomatic high risk patients using plasma anandamide levels. Reprod Sci 2009;16(3): (Suppl.220A):529.

29. Maccarrone M, Bari M, Battista N, Finazzi-Agro A. Estrogen stimulates arachidonoylethanolamide release from human endothelial cells and platelet activation. Blood. 2002;100(12):4040-8.

30. Maccarrone M, Valensise H, Bari M, Lazzarin N, Romanini C, Finazzi-Agro A. Relation between decreased anandamide hydrolase concentrations in human lymphocytes and miscarriage. Lancet. 2000;355(9212):1326-9.

31. Bambang Katerina, Tulay Karasu, Alpha Gebeh, Anthony H. Taylor, Timothy H. Marczylo, Patricia Lam, et al. From Fertilisation to Implantation in Mammalian Pregnancy— Modulation of Early Human Reproduction by the Endocannabinoid System. pharmaceuticals. 2010(3):2910-29.

32. Lam PM, Marczylo TH, Konje JC. Simultaneous measurement of three N-acylethanolamides in human bio-matrices using ultra performance liquid chromatography-tandem mass spectrometry. Anal Bioanal Chem. 2010;398(5):2089-97.

33. Lam PM, Marczylo TH, El-Talatini M, Finney M, Nallendran V, Taylor AH, et al. Ultra performance liquid chromatography tandem mass spectrometry method for the measurement of anandamide in human plasma. Anal Biochem. 2008;380(2):195-201.

34. Kagan KO, Sonek J. How to measure cervical length. Ultrasound Obstet Gynecol. 2015;45(3):358-62.

35. Actim Partus - The reliable way to identify and rule out the risk of preterm delivery 2017 [Available from: https://www.medixbiochemica.com/wp-content/uploads/2017/06/Actim-Partus-brochure-022017.pdf

36. QuikCheck fFN™ Test Kit 2017 [Available from: https://www.hologic.com/sites/default/files/2018-05/AW-05842-002_004_02.pdf.

37. Fezza F, Gasperi V, Mazzei C, Maccarrone M. Radiochromatographic assay of N-acyl-phosphatidylethanolamine-specific phospholipase D activity. Anal Biochem. 2005;339(1):113-20.

38. Appiah-Saky K, Konje JC. Prevention of preterm labour. Obstetrics, Gynaecology & Reproductive Medicine. 2015;25(9).

39. Leitich H, Kaider A. Fetal fibronectin--how useful is it in the prediction of preterm birth? BJOG. 2003;110 Suppl 20:66-70.

40. Audibert F, Fortin S, Delvin E, Djemli A, Brunet S, Dube J, et al. Contingent use of fetal fibronectin testing and cervical length measurement in women with preterm labour. J Obstet Gynaecol Can. 2010;32(4):307-12.

41. Ting HS, Chin PS, Yeo GS, Kwek K. Comparison of bedside test kits for prediction of preterm delivery: phosphorylated insulin-like growth factor binding protein-1 (pIGFBP-1) test and fetal fibronectin test. Ann Acad Med Singapore. 2007;36(6):399-402.

42. Kwek K, Khi C, Ting HS, Yeo GS. Evaluation of a bedside test for phosphorylated insulin-like growth factor binding protein-1 in preterm labour. Ann Acad Med Singapore. 2004;33(6):780-3.

43. Lucovnik M, Chambliss LR, Garfield RE. Costs of Unnecessary Admissions and Treatments for "Threatened Preterm Labor". Am J Obstet Gynecol. 2013.

44. Rose CH, McWeeney DT, Brost BC, Davies NP, Watson WJ. Cost-effective standardization of preterm labor evaluation. Am J Obstet Gynecol. 2010;203(3):250 e1-5.

383 45. Murphy DJ. Epidemiology and environmental factors in preterm labour. Best Pract Res Clin
384 Obstet Gynaecol. 2007;21(5):773-89.
385