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R. Ecochard

Hospices Civils de Lyon, Service de Biostatistique

R. Leiva

Bruyère Research Institute

Thomas Bouchard

University of Calgary

H. Boehringer

Diagnostic Consulting Network

A. Direito

Hospices Civils de Lyon, Service de Biostatistique

See next page for additional authors

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Authors

R. Ecochard, R. Leiva, Thomas Bouchard, H. Boehringer, A. Direito, A. Mariani, and Richard Fehring

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Use of Urinary Pregnanediol 3-glucuronide to Confirm Ovulation

[R. Ecochard](#)

Hospices Civils de Lyon, Service de Biostatistique, Lyon, France

Université de Lyon, Lyon, France

Université Lyon 1, Villeurbanne, France

CNRS, UMR5558, Laboratoire de Biométrie et Biologie Evolutive, Equipe Biostatistique-Santé, Villeurbanne, France

[R. Leiva](#)

Bruyère Research Institute, CT Lamont Primary Health Care Research Centre, Ottawa, Canada

University of Ottawa, Department of Family Medicine, Ottawa, Canada

[T. Bouchard](#)

Department of Family Medicine, University of Calgary, Calgary, Alberta, Canada

[H. Boehringer](#)

Diagnostic Consulting Network, Carlsbad, CA

[A. Direito](#)

Hospices Civils de Lyon, Service de Biostatistique, Lyon, France

Université de Lyon, Lyon, France

Université Lyon, Villeurbanne, France

Laboratoire de Biométrie et Biologie Evolutive, Equipe Biostatistique-Santé, France

[A. Mariani](#)

Service de Pédiatrie, Centre hospitalier Bassin de Thau, France

[R. Fehring](#)

College of Nursing, Marquette University, Milwaukee, WI

Abstract

Objective

Urinary hormonal markers may assist in increasing the efficacy of Fertility Awareness Based Methods (FABM). This study uses urinary pregnanediol-3 α -glucuronide (PDG) testing to more accurately identify the infertile phase of the menstrual cycle in the setting of FABM.

Methods

Secondary analysis of an observational and simulation study, multicentre, European study. The study includes 107 women and tracks daily first morning urine (FMU), observed the changes in cervical mucus discharge, and [ultrasonography](#) to identify the day of ovulation over 326 menstrual cycles. The following three scenarios were tested: (A) use of the daily pregnandi-3 α -glucuronide (PDG) test alone; (B) use of the PDG test after the first positive urine [luteinizing hormone](#) (LH) kit result; (C) use of the PDG test after the disappearance of fertile type mucus. Two models were used: (1) one day of PDG positivity; or (2) waiting for three days of PDG positivity before declaring infertility.

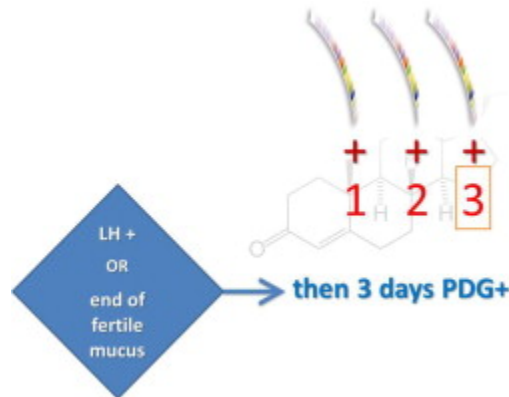
Results

After the first positivity of a LH test or the end of fertile mucus, three consecutive days of PDG testing over a threshold of 5 μ g/mL resulted in a 100% specificity for ovulation confirmation. They were respectively associated an identification of an average of 6.1 and 7.6 recognized infertile days.

Conclusions

The results demonstrate a clinical scenario with 100% specificity for ovulation confirmation and provide the theoretical background for a future development of a competitive lateral flow assay for the detection of PDG in the urine.

Graphical abstract



Keywords

Menstrual cycle, Pregnanediol-3-glucuronide, Luteinizing hormone, Natural family planning, Ovulation, Fertility awareness methods

1. Introduction

Since the mid-20th century, urinary hormone assays have been proposed to help identify the fertile phase of the menstrual cycle [\[1\]](#), [\[2\]](#). These assays can be used by women wishing to postpone pregnancy by using Fertility Awareness Based Methods (FABM). Three urinary hormonal testing methods have long been proposed in scientific literature to help identify the ovulatory period: oestrone-3-glucuronide (E1G), pregnanediol-3 α -glucuronide (PDG), and [luteinizing hormone](#) (LH) [\[3\]](#), [\[4\]](#). In addition to urinary markers, cervical mucus is one of the most widely used biological markers for self-estimating the beginning and end of the fertile phase in a menstrual cycle [\[5\]](#), [\[6\]](#), [\[7\]](#). Furthermore, two clinical indicators of ovulation are broadly known, the mucus peak symptom [\[6\]](#), [\[8\]](#), [\[9\]](#), [\[10\]](#), [\[11\]](#) and the [basal body](#) temperature (BBT) rise. Instead of mucus or BBT as indicators, a hormonal marker of ovulation would be useful. Some home-based ovulation predictor kits based on LH identification in the urine have been marketed for this purpose [\[12\]](#), [\[13\]](#). However, in a previous study, it was discovered that ovulation may sometimes be missed with LH kits if their threshold are above 20 mIU/mL [\[11\]](#). Furthermore, there are many different amplitudes, configurations, and durations of the [LH surge](#) that might erroneously predict ovulation [\[14\]](#), [\[15\]](#).

A more direct and objective measure to confirm ovulation is the urinary measure of the metabolite of post ovulatory [progesterone](#). Several authors have suggested that the use of single morning urinary samples of PDG above a threshold would be a better indicator of ovulation [\[16\]](#), [\[17\]](#), [\[18\]](#). Even more, devices using this concept were at one time considered for marketing [\[19\]](#). However, this approach was vulnerable to error due to the nature of the assays of urinary PDG and the variability in PDG concentration throughout the menstrual cycle [\[20\]](#). Traditionally, PDG concentrations have been corrected for [creatinine](#) to avoid these problems; however, this correction adds a technical difficulty to

develop simple-to-use, home-based-point of care devices. As a result, other methods combining electronic urinary monitors are being studied to address this problem [\[21\]](#), although they are likely to be cost prohibitive for many women. Despite the latter looking very promising, it is clear that other more affordable, easy to use, and versatile methods would be welcomed by FABM users.

The combination of robust markers of ovulation, namely urinary hormones and cervical mucus, could synergistically improve the identification of the fertile and infertile phases. In the mid-nineteen nineties, researchers collected information from normally ovulating women regarding daily urinary hormone measures, recordings of basal body temperature, cervical mucus observations, and serial ovarian ultrasound in order to study the possibility for a PDG urine hormonal assay [\[11\]](#). A database of information was created but due to legal-commercial disclosure agreements, the results regarding the role of PDG in confirming post-ovulatory infertility were not published until now; this paper will present these results. In this study we assessed the potential diagnostic qualities of these markers, focusing on a given urinary PDG concentration threshold to identify the post-ovulatory infertile phase of the cycle.

2. Experimental

2.1. Subjects

This European prospective study was conducted between 1996 and 1997 in eight fertility centers: Aix-en-Provence, Dijon, and Lyon (France), Milano and Verona (Italy), Düsseldorf (Germany), Liège (Belgium), and Madrid (Spain). It included healthy menstruating women aged 18–45 years with previous menstrual cycles of 24–34 days who had experience recording [basal body](#) temperature and monitoring cervical mucus. However, for the purpose of the current analysis, no women were excluded based on the duration of the cycle.

Women with a history of infertility, pelvic inflammatory disease, cycle disturbances, disturbed follicular development, or current hormone therapy were excluded from the study. We also excluded women who had had gynaecological surgery, a delivery within the last three months, women who were breastfeeding, and competitive athletes.

The study included 107 women and analyzed an average of three cycles per woman for a total of 326 cycles. The original study that collected the data [\[11\]](#) was approved by the local ethics committee (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale de Lyon). All participants gave their written informed consent. The study procedures were carried out in accordance with the Ethical Standards for Human Experimentation established by the Declaration of Helsinki.

2.2. Methods

2.2.1. Mucus symptoms

Study participants checked for changes in cervical mucus two or three times daily, recording the sensation (dry, moist, wet, or slippery) and the consistency (tacky, creamy, or stretchy) of the mucus. This information allowed for the ability to distinguish between (i) days with no mucus felt or seen; (ii) days with mucus felt or seen but not having the characteristics of high fertility; and (iii) days with mucus

that felt wet or slippery or that resembled an egg-white and had a stretchy appearance. The last day of clear, stretchy and/or lubricative mucus discharge was called the peak symptom [\[9\]](#), [\[22\]](#)

2.2.2. Hormone assessments

Assays were carried out on the first morning urine (FMU) with two 10–12 mL aliquots frozen on the day of collection at $-20\text{ }^{\circ}\text{C}$ in tubes containing [gentamicin sulphate](#). On the day of analysis, the aliquots were thawed in a single laboratory and tested in duplicates for quantitative detection of oestrone-3-glucuronide (E1G-ng/mL), pregnanediol-3 α -glucuronide (PDG- $\mu\text{g/mL}$), [follicle stimulating hormone](#) (FSH-mIU/mL), and [luteinizing hormone](#) (LH-mIU/mL) using time-resolved [fluorometric](#) immunosorbent assays (Delfia). Each hormonal sample was repeated twice: the relative difference (i.e. CV) was respectively 5.96%, 10.79%, 8.66% and 7.17% for PDG, FSH, LH and E1G. We cannot provide detailed information on assay performance except the intra-assay CV's. This data remains within the property of the funding company.

2.2.3. Ultrasound scans

The serial transvaginal ovarian ultrasounds (with follicle measurements) started either at the onset of the fertile cervical mucus or at the detection of the [LH surge](#) by the home test. These scans were carried out every other day until the largest follicle reached 16 mm, then every day until evidence of ovulation. To note that while there is increasing evidence to indicate that multiple ovarian follicular waves develop during the human menstrual cycle [\[22\]](#), the evidence always point towards the last wave being the ovulatory single event of a given cycle [\[23\]](#). The same physician at each centre performed the scans. The ultrasound-determined day of ovulation (US-DO) was the 24-h period that separated the sight of a mature follicle on one scan and either of the following on the second scan: (i) a change in the follicle size, shape, or sonographic density; (ii) follicle rupture; (iii) the presence of an early corpus luteum; (iv) the presence of free fluid in the cul-de-sac. If a woman missed an ultrasound examination, the US-DO was the first day after the last pre-ovulatory ultrasound with a follicle ≥ 18 mm or the second day with a follicle < 18 mm.

2.3. Measured outcomes

2.3.1. Fertility definitions

The *fertile phase* was estimated during the pre-ovulatory phase as the period stretching from the first day of menses to the end of the US-DO. The *infertile phase* was defined as the day after ovulation day, up to the following menses.

Positive PDG test was defined as a test result above a defined concentration threshold. A *negative PDG test* was defined as a test result below that threshold. Different PDG concentration thresholds in FMU samples were analyzed for specificity, sensitivity, true negative and true positive cycles. A cycle with at least one day with a positive PDG test in the fertile phase was classified as a *false positive*: i.e. PDG concentration was high despite being during the potentially fertile phase. A cycle with all days with negative PDG tests in the fertile phase was classified as a *true negative*: i.e. PDG was appropriately low during the potentially fertile phase. A cycle with days in the infertile phase with positive PDG tests

was classified as a *true positive*: i.e. PDG was appropriately high during the infertile phase. A cycle without at least one day in the infertile phase with a positive PDG test was classified as a *false negative*: i.e. PDG was always low despite being in the infertile phase.

The *sensitivity* was estimated as the proportion of true positives, that is, cycles with appropriate recognition of the post-ovulatory infertile phase. The *specificity* was estimated as the proportion of true negatives, that is, cycles with appropriate recognition of the pre-ovulatory fertile phase. Lack of specificity creates the risk of unplanned pregnancy, and therefore, a high specificity for ovulation confirmation was the main aim of the study. Achieving a specificity of 100% would mean that there is no positive test in the absence of ultrasound-confirmed ovulation, in other words, we would not want a woman to think she was infertile if she has not yet ovulated.

2.3.2. Tested scenarios for different PDG thresholds

The following scenarios were tested: (A) Use of the daily PDG test alone starting the first day of the cycle; (B) Use of the daily PDG test only after a first positive urine LH kit result (LH threshold of 20 mIU/mL); and (C) Use of the PDG test only after the disappearance of highly fertile type mucus at the vulva, i.e. return to absence of mucus or mucus without the characteristics of high fertility. In the event of a second wave of highly fertile type mucus during the testing, the test was re-started following the disappearance of the second wave of highly fertile type mucus. The chosen LH threshold of 20 mIU/mL was based on data from previous published analysis [\[15\]](#). The two most common used thresholds of commercially available urinary LH kits are 20 and 25. The respective specificity and sensitivity were found to be 0.95 and 0.43 for a threshold of 20; and, 0.97 and 0.34 for 25. Given on these results, we chose 20 to be the best as it had the higher sensitivity.

2.3.3. PDG test models and interpretation of the results

We postulated two models of using the PDG test based on current FABM practices: (1) One day of positivity above a certain threshold is considered to be sufficient to declare infertility; or (2) Three consecutive days of positivity are observed before infertility is declared.

2.4. Statistical analysis

The best threshold was obtained through secondary analysis by performing a statistical analysis in a range from 0.5 to 15 µg/mL and determining the threshold based on specificity and sensitivity. Sensitivity and specificity were estimated with their 95% confidence intervals. Then a ROC (the Receiver Operating Characteristic) curve was used to describe the evolution of sensitivity and specificity according to the given threshold. A descriptive analysis of the cycle characteristics was performed using geometric mean, standard deviation, minimum, median, and maximum for quantitative data. Frequency was used for categorical variables. All statistical analyses were performed using the R software version 2.13.0 (The R Foundation for Statistical Computing). A p -value <0.05 was considered for statistical significance.

3. Results

[Table 1](#) depicts the participants' characteristics including hormonal profiling. The 107 subjects studied were 19–44 years old. Sixty-nine of them (64%) had at least one child before the study. The BMIs ranged between 17.1 and 28.3. Eleven women reported current smoking. The mean cycle length was 28.1 days (range 22–44 days). The mean time to ovulation was 14.8 days (range 9–33 days) and the mean post-ovulatory phase length was 13.3 days (range 7–17 days). In 28 cycles out of the 326 monitored, the first ultrasound was performed after ovulation and, in 15 others, it was not possible to determine exactly the day of ovulation by ultrasound. This left 283 ovulatory cycles for analysis. In a sub-analysis, out of the 206 available cycles with complete records on mucus coding, eight cycles (4%) showed two waves of fertile-type cervical mucus separated by a vaginal dry phase. Ovulation followed the last wave. It is well recognized that this is a product of variations in hormonal patterns within the menstrual cycle [\[24\]](#). In addition, as previously published [\[32\]](#), no differences were observed between different BMI groups and PDG mean levels: 12.41(0.56), 13.09 (0.53), 11.70 (0.80) µg/mL for BMI ranges of <19.2, 19.2–23.4, and >23.4 respectively.

Table 1. Women and cycles characteristics.

Characteristics	Mean (\pm sem)	Minimum	Maximum
<i>Women</i>			
Age (years)	32.42 (0.35)	19	44
Age at Menarche (years)	13.17 (0.1)	9	17
Body mass index (kg/m ²)	21.27 (0.15)	17.12	28.34
Sport activity (h/week)	1.16 (0.13)	0	9
Regular smokers (%)	11		
Vegans (%)	4		
<i>Cycles</i>			
Cycle length (days)	28.07 (0.17)	22	44
Follicular phase (days)	14.76 (0.17)	9	33
Luteal phase (days)	13.35 (0.1)	7	17
<i>FSH (mIU/mL)</i>			
Early follicular phase	3.61 (0.19)	0.06	26.26
Periovulatory phase	5.24 (0.26)	0.08	21.42
Luteal phase	1.73 (0.1)	0.06	10.75
<i>LH (mIU/mL)</i>			
Early follicular phase	3.56 (0.13)	0.08	11.77
Periovulatory phase	15.5 (0.66)	0.54	51.82
Luteal phase	6.19 (0.37)	0.08	52.99
<i>E1–3-G (ng/mL)</i>			
Early follicular phase	10.61 (0.34)	1.17	45.44
Periovulatory phase	50.76 (1.87)	4.44	281.18

Characteristics	Mean (\pm sem)	Minimum	Maximum
Luteal phase	29.72 (1.21)	2.39	213.52
<i>Pd-3α-G</i> (μ g/mL)			
Early follicular phase	2.34 (0.09)	0.18	19.62
Periovulatory phase	3.02 (0.1)	0.31	14.68
Luteal phase	12.66 (0.37)	1.53	64.84

Average hormonal concentrations were estimated at the three phases of the cycle: at days 2, 3, and 4 of the cycle for the early follicular phase, at US-DO \pm 1 for the periovulatory phase, and at US-DO +5,+7, and +9 for the luteal phase.

The specificity and sensitivity of the PDG test in a range from 0.5 to 15 μ g/mL with all various proposed scenarios is depicted in the Receiver Operating Characteristic curves in [Fig. 1](#). The main goal of this study was to achieve a low false positive rate, that is, highest specificity, to ensure the lowest simulated pregnancy rate and to confirm ovulation. The ideal concentration threshold in a FMU sample for PDG positivity was found to be at 5 μ g/mL.

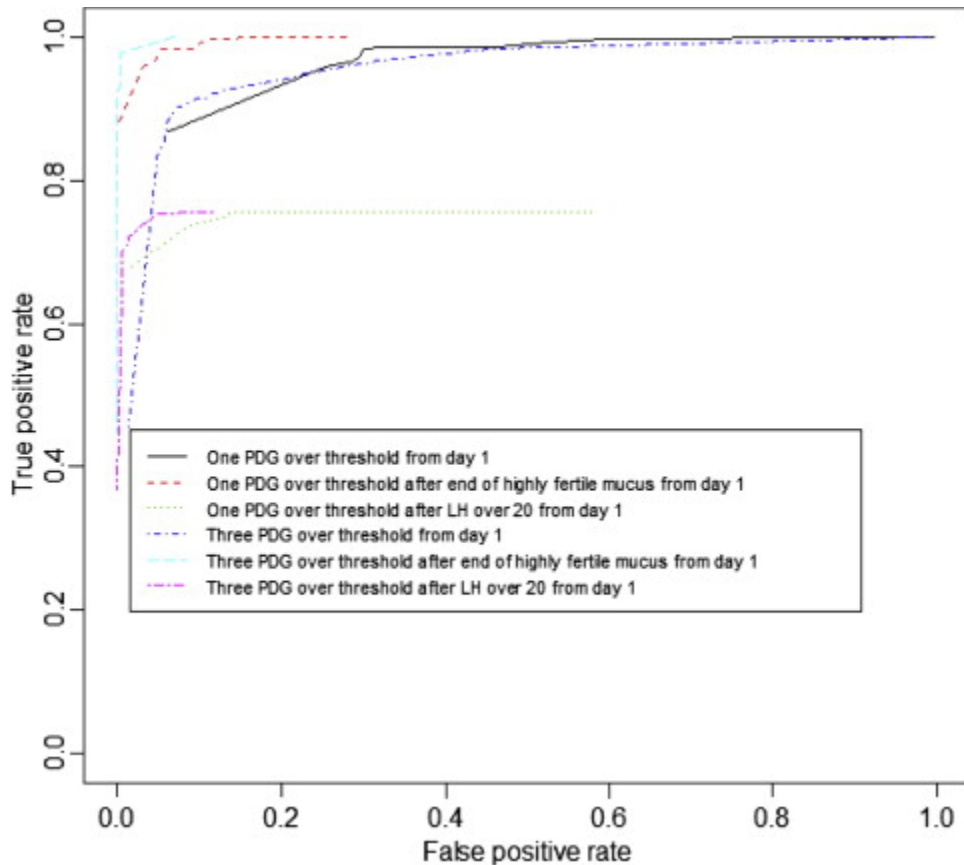


Fig. 1. ROC curves presenting the evolution of true and false positive rates according to the chosen threshold of PDG tests; one ROC curve per scenario.

The two scenarios with the highest specificity are Scenario B; using the PDG test for three consecutive days after a first positive urine LH kit result and Scenario C which is the most specific; using the PDG test for three consecutive days after high fertility cervical mucus is detected.

[Table 2](#), [Table 3](#) show the number of daily PDG tests used for each of the three scenarios, the number of recognized true infertile days by the testing, the test's specificity and sensitivity.

Table 2. Estimated measures of the performance of PDG positivity in the one-day model (threshold: 5 µg/mL).

Testing scenarios	Number of daily testing One positive PDG result			
	Number of days tested	Number of recognized true infertile days	Sensitivity %	Specificity %
(A) Daily PDG testing alone from day 1	12.1	5.5	0.986	0.534
(B) PDG daily testing only after a positive LH test	2.3	6.5	0.756	0.776
(C) PDG testing following peak fertility (i.e. presence of high fertile type mucus at the vulva followed by a change to non-fertile type)	2.8	9.5	0.984	0.907

Table 3. Estimated measures of the performance of PDG positivity in the 3-day model (threshold: 5 µg/mL).

Testing scenarios	Number of daily testing Three daily consecutive positive PDG results			
	Number of days tested	Number of recognized true infertile days	Sensitivity %	Specificity %
(A) Daily PDG testing alone from day 1	18.6	6.6	0.929	0.862
(B) PDG daily testing only after a positive LH test	5.2	6.1	0.721	0.986
(C) PDG testing following peak fertility (i.e. presence of high fertile type mucus at the vulva followed by a change to non-fertile type)	5.9	7.6	0.922	1

[Table 2](#) shows the results associated with scenarios using the one-day model of positive PDG testing above a specific threshold. [Table 3](#) refers to the three-day model (three days of consecutive positive tests). The three scenarios were applied to both models.

The most specific scenario, which combined the use of three positive PDG tests after the identification of high fertility mucus (Scenario C), resulted in 92% of sensitivity for ovulation. In this scenario, 6 days were tested per cycle and 7.6 days are recognized as infertile during the post-ovulatory phase. If we ignore the 8% of cycles with lack of sensitivity, 8.1 days are recognized as infertile during the post-ovulatory phase.

The other scenarios were all limited in some way. First, when using LH positivity as the starting point for the PDG test (Scenario B), there was limited sensitivity with only 76% of the cycles achieving a positive LH test and thus precluding the use of the PDG test. Second, when using the PDG test alone (Scenario A), there was limited specificity and the PDG test occasionally read positive before ovulation had taken place.

In a sub-analysis to take into account sport activity, data was available for 76 women. We did it to assess whether sport activity may be associated with a reduced luteal PDG. First, we divided participants in three groups: no sport-activity, intensive sport-activity for less than 3 h per week and for more than 3 h per week. The sport activity was assessed only once per woman and we analyzed the data accordingly. Within each group, PDG values from three days of each cycle were averaged to obtain one value per cycle (i.e. the mean PDG level of days US-DO +5, +7, and +9). We then averaged all these mean PDG levels for all the cycles within a given group. In this analysis, the results obtained were 11.23, 12.01 and 11.14 for the three groups respectively ($p = 0.77$, i.e. the difference was not statistically significant).

4. Discussion

The present study predicts that the use of three consecutive days of PDG testing over a threshold of 5 $\mu\text{g/mL}$ in a FMU sample will result in a perfect specificity for ovulation confirmation following fertility-type mucus identification. The 100% confirmation would allow women to identify the absolute infertile period after ovulation for the sake of avoiding pregnancy. On the other hand, the rationale behind the fact that the combination of LH/PDG did not give a perfect specificity can be found on recent LH research [15]. It is now known that LH levels present some variability in amplitude, duration and configuration with ovulation sometimes occurring later than one day after the [LH surge](#). It is then possible that our proposed PDG test protocol may give a false negative since it may test too early to show a PDG surge. However, we believe the high specificity obtained is still within the clinically relevant range ($Sp = 0.986$).

The beginning of the fertile phase still requires the use of a first indicator; either, a count of days from the onset of the cycle, or the presence of mucus, felt or seen, at the vulva [6] or the detection of a significant rise in urinary E1G, either alone or in various combinations [25], [26], [27]. To identify the end of the fertile phase, our results confirm the interest of using either a LH test first or to rely on days following the presence of cervical mucus for FABM users, and then confirm the infertility using three consecutive days of PDG testing over a threshold of 5 $\mu\text{g/mL}$ in a FMU sample. A noon sample of urinary LH above a set threshold has been considered sufficient to identify the imminence of ovulation [28]. Alternatively, instead of relying on the same threshold for every woman, electronic devices have been developed to identify a significant increase over a given woman's baseline hormonal levels during the previous days [29]. Our results show that three consecutive days of PDG positivity above a specific threshold following LH positivity or the end of fertile-type cervical mucus will confirm infertility with a high predictive specificity.

Clinical an-ovulatory scenarios such as pre-menopause and polycystic ovarian disease (PCOD), has been mentioned as challenges for FABM use. Firstly, the effect of these scenarios should be negligible

in the interpretation of the PDG method; since, it is only with those ovulatory cycles that the rise of urinary PDG occurs. However, it is been long reported that there are clinical scenarios when luteal [progesterone](#) levels, and consequently PDG levels as well, may be found at lower level than normal: in women with unexplained infertility, after ovulation induction with clomiphene [citrate](#) or [gonadotropins](#), in women with hyperprolactinemia, recurrent miscarriages, [luteinizing](#) unruptured follicles, in oligomenorrhic obese women with or without hirsutism such as PCOD, and in perimenopausal women [\[30\]](#), [\[31\]](#), [\[32\]](#). Our current proposed protocol lacks the power to discriminate for all these conditions because it is based on one single threshold, yet, it could be the source of future research on the relationship between cervical mucus, LH and PDG surges. Secondly and even more importantly, it is the combination of two markers (cervical mucus or LH) plus PDG that may provide the safety net needed in these conditions with reduced PDG level. For instance, it is recognized that use of cervical mucus monitoring in FABM can help to identify approaching ovulation even in pre-menopause [\[24\]](#). Thirdly, in clinical situations such as vaginal infections when the cervical mucus essentially becomes non-interpretable, the use of urinary LH testing could be used as substitute as mentioned in our protocols. Finally, despite the fact that the clinical question of accurately identifying the infertile phase of the menstrual cycle is an old one (1, 2, and 20), the proposed model is new. No quantitative assessment of the use of PDG as adjunct to the concurrent use of FABM has ever been published, nor a study has used such a large dataset.

With these results, the next major challenge will be the development of a simple competitive lateral flow assay for the detection of PDG in the urine. The ability to develop such assays has been available for several years and the manufacturing processes of these tests are well known. Similar urinary tests such as those used to identify commonly abused substances could be adapted for this purpose given that PDG and these substances have comparable molecular weights [\[33\]](#).

One further theoretical limitation of our study is that the algorithms used were based on multiple cycles per participants, leading to potentially to overestimates of specificity and sensitivity. To quantify for this effect, a mixed regression model for repeated measurement was used to describe the PDG level during the luteal phase (at US-DO +5, +7, and +9). The inter-women and intra-women coefficients of variation were respectively 30% and 11%. However, we did not use a validation dataset to confirm the sensitivity and specificity: our estimates of performance can be overly optimistic. It would be wise to confirm these results using other datasets in future research. This fact might indeed contribute to an overestimate of specificity, but not necessarily invalidate the results in a clinical setting. Likewise, the fact that our PDG assays were tested only among European women, it may potentially limits the study's results yet not necessarily invalidating our findings [\[34\]](#). A clinical study based on the proposed PDG devices would again need to address concerns such a different population and racial differences.

Given that this test has a dual purpose (to confirm the end of the fertile period for women wishing to get pregnant and identifying the infertile phase for those wishing to avoid pregnancy) it is very versatile. The 100% specificity for ovulation in these scenarios would be helpful to identify those women with adequate ovulation for the purpose of an infertility work-up, providing a home-based alternative for serum progesterone testing. For those wishing to postpone pregnancy, the high specificity for ovulation demonstrated here provides a simple and very reliable identification for the post-ovulatory infertile phase. In order to make this approach practical, the cost of the simple PDG test ought to remain low.

Future studies could assess the two best scenarios (B and C) for women who are seeking to avoid and achieve pregnancy.

On a side note, it was noted that in 2% of the cycles (6/283), menses occurred beyond the generally accepted 16 days post-ovulation, in these few cycles, we question whether the ultrasound determination of the day of ovulation was off by one day or two or an early pregnancy loss occurred. Our assessment of these rare events led us to conclude that the potential bias would not be significant.

In order to provide women with a simple, home-based test to identify the absolute infertile period after ovulation, we have demonstrated a new objective measure that is 99–100% specific for the ovulation event as confirmed by ultrasound. In order to avoid the previous challenge of the individual woman's menstrual cyclic variations in urinary concentrations of PDG, we have proposed a novel model that employs three consecutive days of PDG tests above a threshold of 5 µg/mL after either LH positivity or highly fertile mucus. This model is 99–100% specific for ovulation depending of the scenario used and is thus a very promising tool for women wanting a conservative and reliable measure to complement their Fertility Awareness Based Method.

Potential conflict of interest

H.B. is employed for a company that specializes on the development of competitive lateral flow assays.

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