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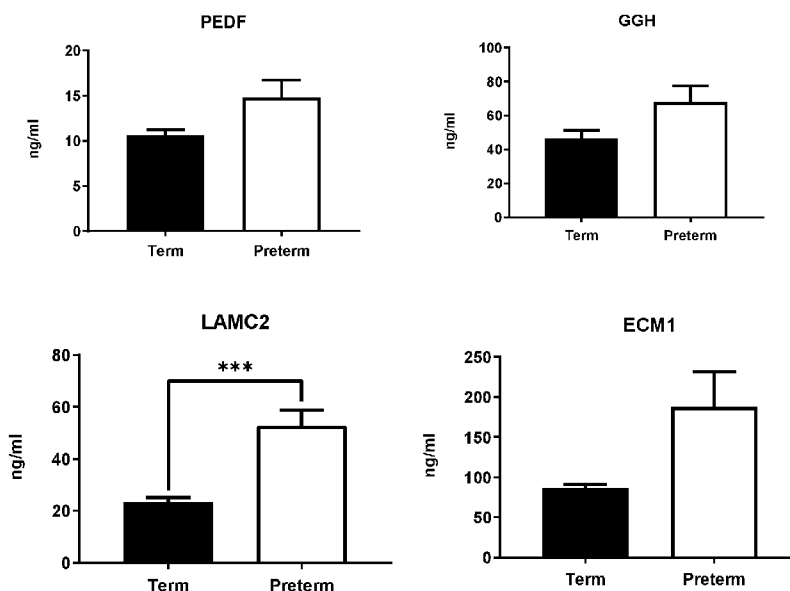


Figure 1

(57) Abstract: The present invention relates to biomarker pairs, and particularly, although not exclusively, to pairs of biomarkers that are markers of preterm birth. The biomarker pairs are useful, several weeks or months prior to birth, for distinguishing individuals at risk of experiencing preterm birth.

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BIOMARKER PAIRS OF PRETERM BIRTH

Field of the Invention

The present invention relates to biomarkers, and particularly, although not exclusively, to biomarkers of preterm birth. The biomarkers are useful, several weeks or months prior to birth, for distinguishing individuals at risk of experiencing preterm birth.

Background

Preterm birth is defined by birth that takes place before the completion of 37 weeks of gestation. It is estimated that over 15 million babies are born preterm annually. Globally, preterm birth is one of the leading causes of death for children under the age of five with an estimated one million preterm birth- related mortalities. Many of the survivors face a lifetime of challenging disabilities which include learning disabilities and visual and hearing problems. Although neonatology advances in the past decades has increased survival rates for preterm birth, above 20% of preterm neonates will suffer at least one major disability including chronic lung disease, impaired mental development, cerebral palsy, deafness, or blindness.

There is a significant need to identify pregnant women who are at risk of preterm birth. In most cases, classification of pregnancies as high-risk is attributed to prior medical history or clinical examinations, identifying only a small subsection of the true high-risk pregnancies prone to preterm birth. In the current paradigms, treatment for high-risk pregnancies involves prophylactic treatment or enhanced surveillance or close monitoring of the pregnancy, which reduces preterm birth rates. Still, the majority of pregnancies, including first-time pregnancies, that are prone to spontaneous preterm birth are not identified at early stages and hence early medical intervention for such cases is not possible.

There are several tests in the market for risk assessment of preterm birth for women presenting risk symptoms. One such example is the Fetal Fibronectin (fFN) test which provides a risk assessment for symptomatic women. Fetal fibronectin is present in the vagina if a preterm delivery is likely to occur; hence the fFN test is commonly used in pregnant women with symptoms indicating a possibility for preterm birth, such as contractions, vaginal bleeding, fluid leaking from the vagina, increased vaginal discharge, backache and cramp in lower abdomen. The strength of the fFN test lies in its high negative predictive value for up to 10 days following the test (i.e. a negative result means that there is a low possibility of preterm labour within the next 7 to 10 days following the test). However, when the fFN test is positive, the results are less conclusive.

Patient management varies based on risk factors. Prophylactic treatment such as progesterone has been shown to reduce preterm birth rates in numerous clinical studies profiling women with short cervical length or prior history of preterm birth as a high-risk population. Symptomatic women may receive treatments such as tocolytics or steroids based on the risk factors. The limitation of current clinical practice is that the correlation of treatment and outcomes is very low.

Thus, there is an urgent need for effective identification of pregnancies at high-risk of preterm birth, so that appropriate treatment can be administered promptly to reduce preterm birth rates. The present invention provides biomarkers and methods for predicting risk of preterm birth to overcome at least in part some of the disadvantages. In particular, the present invention seeks to provide a risk assessment for classification of women with high-risk for preterm birth several weeks or even months before symptoms of preterm birth appear.

The present invention has been devised in light of the above considerations.

Summary of the Invention

The present invention provides novel combinations of biomarker, in particular pairs of biomarkers formed from any pair combination of PEDF, GGH, LAMC2 and ECM1, and methods for predicting risk or likelihood (particularly increased risk or likelihood) of preterm birth in a subject based on determining the levels of one, each or both biomarkers of a biomarker pair. Methods disclosed herein involve the determination of the presence or absence of, or quantification of the level of, any two biomarkers selected from the group consisting of PEDF, GGH, LAMC2 and ECM1.

The biomarker combinations disclosed herein were determined from patient-derived samples. Biomarker combinations disclosed herein differentiate samples from individuals who experience term and preterm birth, weeks or months before the individual is symptomatic. Such biomarker combinations may be useful for identifying an individual at risk of preterm birth, and thus may be useful for guiding clinical decisions such as the initiation of treatment to prolong gestation and/or prevent or reduce the risk of preterm birth.

Methods disclosed herein can be used to determine the risk or likelihood of preterm birth in asymptomatic or symptomatic individuals. In particular embodiments the individual is asymptomatic.

As disclosed herein, the following pairs of biomarkers are markers of preterm birth: PEDF and GGH; PEDF and LAMC2; PEDF and ECM1; GGH and LAMC2; GGH and ECM1; LAMC2 and ECM1 (each pair being referred to as a "biomarker pair" herein). Methods described herein may involve measuring or determining the level or amount of

one, each or both biomarkers in a biomarker pair in a sample. The biomarker pairs may be used in methods for identifying individuals at risk of preterm birth, and methods for determining whether an individual is at risk of preterm birth, or for predicting whether an individual is at risk of preterm birth.

- 5 Variation of the level of one or each biomarker in a biomarker pair, as compared to a control or reference level for one or each biomarker in a biomarker pair, may indicate that the individual is at increased risk of preterm birth. Such methods involve determining the level of each biomarker in a biomarker pair in a sample obtained from the individual being tested. In some aspects, the methods involve determining the level
10 of more than one biomarker pair, e.g. 2, 3, 4, 5 or 6 biomarker pairs, and predicting the risk of preterm birth.

Either under-expression or over-expression of a biomarker or biomarker pair may indicate that the individual is at risk of preterm birth.

- 15 In some cases, the biomarker or biomarker pair may indicate that the individual is at risk of preterm birth if it is over-expressed at a certain point of gestation, or under-expressed at a different point of gestation.

- Provided herein is a method for predicting whether an individual is at risk of preterm birth, the method comprising determining in a sample obtained from the individual the level of any two biomarkers selected from the group consisting of PEDF, GGH, LAMC2
20 and ECM1.

- Also provided herein is a method for predicting whether an individual is at risk of preterm birth, the method comprising determining the level of one, each or both biomarkers of a biomarker pair in a sample obtained from the individual, and predicting whether the individual is at risk of preterm birth based on the level of the biomarker(s),
25 wherein the biomarker pair is selected from: PEDF and GGH; PEDF and LAMC2; PEDF and ECM1; GGH and LAMC2; GGH and ECM1; and/or LAMC2 and ECM1.

- Also provided herein is a method for predicting whether an individual is at risk of preterm birth, the method comprising determining the level of one, each or both biomarkers of a biomarker pair in a sample obtained from the individual, and classifying
30 the individual as at risk of preterm birth or not at risk of preterm birth, based on the biomarker pair values, wherein the biomarker pair is selected from PEDF and GGH; PEDF and LAMC2; PEDF and ECM1; GGH and LAMC2; GGH and ECM1; LAMC2 and ECM1.

- 35 In some embodiments the level of one, each or both biomarkers is compared to a reference level, wherein the reference level is derived from the level of one, each or both biomarkers in a sample obtained from an individual known to have experienced preterm or term birth.

In some embodiments the method further comprises predicting the risk of preterm birth with one or more other indicators of preterm birth, selected from the Fetal Fibronectin (fFN) test, a short cervical length, contractions, vaginal bleeding, fluid leaking from the vagina, increased vaginal discharge, backache and cramping in lower abdomen.

- 5 Also provided herein is progesterone for use in the treatment of an individual predicted to be at risk of preterm birth, wherein the individual has been predicted to be at risk of preterm birth by a method described herein.

Also provided herein is a method for selecting an individual for treatment to reduce the risk of preterm birth, the method comprising predicting the risk of preterm birth in the
10 individual using a method as described herein and, if the individual is determined to be at risk of preterm birth, administering a treatment to reduce the risk of preterm birth, wherein the treatment to reduce the risk of preterm birth comprises progesterone and/or cervical cerclage and/or vaginal pessary.

Also provided herein is a method for predicting whether an individual is at risk of
15 preterm birth, the method comprising determining in a sample obtained from the individual the level of any two biomarkers selected from the group consisting of PEDF, GGH, LAMC2 and ECM1 and transmitting the determined levels to a physician involved in the treatment of the individual, wherein the risk of preterm birth is predicted based on the level of the biomarkers in the sample.

20 Also provided herein is a method for predicting whether an individual is at risk of preterm birth, the method comprising determining the level of one, each or both biomarkers of a biomarker pair in a sample obtained from the individual, and transmitting the determined levels to a physician involved in the treatment of the individual, wherein the risk of preterm birth is predicted based on the level of the
25 biomarkers in a biomarker pair in the sample, and wherein the biomarker pair is selected from: PEDF and GGH; PEDF and LAMC2; PEDF and ECM1; GGH and LAMC2; GGH and ECM1; and/ or LAMC2 and ECM1.

Also provided herein is a method for detecting a biomarker pair selected from PEDF and GGH; PEDF and LAMC2; PEDF and ECM1; GGH and LAMC2; GGH and ECM1; or
30 LAMC2 and ECM1, the method comprising:

- a. obtaining a sample from an individual;
- b. detecting whether one, each or both biomarkers in a said biomarker pair is present in the sample by contacting the sample with one or more antibodies selected from an anti-PEDF antibody, an anti-GGH antibody, an anti-LAMC2
35 antibody, and/or an anti-ECM1 antibody, and detecting binding between at least one of PEDF, GGH, LAMC2 or ECM1 and a respective said antibody.

Also provided herein is a method for determining that an individual is at risk of preterm birth, said method comprising:

- a. obtaining a sample from an individual;
- b. detecting whether one, each or both biomarkers in a biomarker pair is present in the sample, by contacting the sample with one or more antibodies selected from an anti-PEDF antibody, an anti-GGH antibody, an anti-LAMC2 antibody, and/or an anti-ECM1 antibody, and detecting binding between at least one of PEDF, GGH, LAMC2 or ECM1 and a respective said antibody, wherein said biomarker pair is selected from PEDF and GGH; PEDF and LAMC2; PEDF and ECM1; GGH and LAMC2; GGH and ECM1; or LAMC2 and ECM1; and
- c. determining that the individual is at risk of preterm birth when the presence of a said biomarker pair in the sample is detected.

Also provided herein is a method of determining that an individual is at risk of preterm birth and prolonging gestation in that individual, the method comprising:

- a. obtaining a sample from an individual;
- b. detecting whether one, each or both biomarkers in a biomarker pair is present in the sample, wherein said biomarker pair is selected from PEDF and GGH; PEDF and LAMC2; PEDF and ECM1; GGH and LAMC2; GGH and ECM1; or LAMC2 and ECM1;
- c. determining that the individual is at risk of preterm birth when the presence of said biomarker pair in the sample is detected; and
- d. administering an effective amount of progesterone to the individual determined to be at risk of preterm birth or selecting the individual for treatment with an effective amount of one or more agents selected from a progesterone or an analogue thereof, a tocolytic, a corticosteroid, an antibiotic, a non-steroidal anti-inflammatory drug (NSAID) or an Omega 3 fatty acid or derivative thereof if the individual is determined to be at risk of preterm birth; and/or
- e. performing cervical cerclage on the individual determined to be at risk of preterm birth or selecting the individual for cervical cerclage, if the individual is determined to be at risk of preterm birth.

Also disclosed herein is a method of determining the likelihood of an individual experiencing a preterm birth, the method comprising detecting, in a sample from the individual, biomarker values for one, each or both biomarkers in a biomarker pair selected from PEDF and GGH; PEDF and LAMC2; PEDF and ECM1; GGH and LAMC2; GGH and ECM1; LAMC2 and ECM1, and determining the percentage

likelihood that an individual will experience a preterm birth, based on the biomarker values.

Also disclosed herein is a computer implemented method for predicting whether an individual is at risk of preterm birth, the method comprising retrieving on a computer
5 biomarker information for an individual, wherein the biomarker information comprises biomarker values corresponding to one, each or both biomarkers in a biomarker pair selected from PEDF and GGH; PEDF and LAMC2; PEDF and ECM1; GGH and LAMC2; GGH and ECM1; LAMC2 and ECM1, and with the computer generating a classification of the one, each or both biomarker values; and indicating a likelihood that
10 the individual is at risk of preterm birth, based on the classification.

Certain aspects disclosed herein describe methods, optionally computer-implemented methods, for determining the risk of preterm birth in an individual. The methods may involve providing data corresponding to the level of at least two of PEDF, GGH, LAMC2, ECM1, in a sample obtained from the individual; performing, with the computer, a
15 classification of the biomarker value; and determining the risk of preterm birth in the individual, based on the classification.

In the methods described herein, the sample is preferably a vaginal fluid sample. The vaginal fluid sample may be a cervicovaginal fluid sample. Alternatively, the sample is an amniotic fluid sample.

20 In methods described herein each biomarker determined may preferably be a protein or a polypeptide.

In methods described herein the level of one, each or both biomarkers in a biomarker pair may be determined by enzyme-linked immunosorbent assay (ELISA), optionally using one or a pair of antibodies each respectively binding to one of the members of a
25 biomarker pair.

In some embodiments both biomarkers in a said biomarker pair are detected by contacting the sample with two different antibodies, wherein one antibody binds a first member of the biomarker pair and another antibody binds a second member of the biomarker pair. In some other embodiments, one member of a biomarker pair is
30 detected by contacting the sample with an antibody that binds said member and the other member of the biomarker pair is detected by contacting the sample with a non-antibody binding entity, e.g. an aptamer, that binds said other member. Each member of the biomarker pair may be detected simultaneously, e.g. as part of the same assay, or separately, e.g. as part of separate assays.

35 In some cases, the antibody is derived from, mouse, rabbit or goat, preferably, mouse or rabbit. The antibody may be human, humanised or chimeric.

Any method for predicting or determining whether an individual is at risk of preterm birth described herein may be a computer implemented method.

Also provided herein is a kit, optionally for use in predicting the risk or likelihood of preterm birth in a subject, wherein the kit comprises two different antibodies each
5 selected from the group consisting of: an anti-PEDF antibody, an anti-GGH antibody, an anti-LAMC2 antibody, an anti-ECM1 antibody.

In some embodiments the kit comprises, or consists of, one or more pairs of antibodies. For example, in some embodiments the kit comprises an anti-PEDF antibody and an anti-GGH antibody; in some embodiments the kit comprises an anti-PEDF antibody and
10 an anti-LAMC2 antibody; in some embodiments the kit comprises an anti-PEDF antibody and an anti-ECM1 antibody; in some embodiments the kit comprises an anti-GGH antibody and an anti-LAMC2 antibody; in some embodiments the kit comprises an anti-GGH antibody and an anti-ECM1 antibody; and/or in some embodiments the kit comprises an anti-LAMC2 antibody and an anti-ECM1 antibody. The kit may comprise
15 more than one of said pairs of antibodies. Alternatively, the kit may comprise only one antibody pair, and may therefore contain no more than two antibodies.

Methods described herein may each further comprise administering a treatment to an individual determined or predicted to be at risk. The treatment may comprise cervical cerclage or administration of one or more agents selected from a progesterone or an
20 analogue thereof, a tocolytic, a corticosteroid, an antibiotic, an NSAID or an Omega 3 fatty acid or derivative thereof. In any embodiment, the progesterone may be a synthetic progesterone, such as 17- α -hydroxyprogesterone caproate, the tocolytic may be magnesium sulfate, indomethacin or Nifedipine, the antibiotic may be erythromycin or penicillin, the NSAID may be indomethacin and the Omega 3 fatty acid derivative
25 may be docosahexaenoic acid (DHA).

Also disclosed herein is one or more agents selected from a progesterone or an analogue thereof, a tocolytic, a corticosteroid, an antibiotic, an NSAID or an Omega 3 fatty acid or derivative thereof for use in a method of treating an individual determined or
30 predicted to be at risk of preterm birth (optionally wherein the individual has been determined to be at risk of preterm birth) based on one, each or both biomarker values for a biomarker pair selected from PEDF and GGH; PEDF and LAMC2; PEDF and ECM1; GGH and LAMC2; GGH and ECM1; LAMC2 and ECM1.

Also disclosed herein is a method of treatment comprising administration of one or more agents selected from a progesterone or an analogue thereof, a tocolytic, a
35 corticosteroid, an antibiotic, an NSAID or an Omega 3 fatty acid or derivative thereof to an individual determined or predicted to be at risk of preterm birth (optionally wherein the individual has been determined to be at risk of preterm birth) based on one, each or both biomarker values in a biomarker pair selected from PEDF and GGH; PEDF and

LAMC2; PEDF and ECM1; GGH and LAMC2; GGH and ECM1; LAMC2 and ECM1. Progesterone for use in such a method is also disclosed, along with the use of progesterone in the manufacture of a medicament for use in such a method.

There is also provided the use of one or more agents selected from a progesterone or an analogue thereof, a tocolytic, a corticosteroid, an antibiotic, an NSAID or an Omega 3 fatty acid or derivative thereof in the manufacture of a medicament for the treatment of an individual determined or predicted to be at risk of preterm birth (optionally wherein the individual has been determined to be at risk of preterm birth) based on one, each or both biomarker values for a biomarker pair selected from PEDF and GGH; PEDF and LAMC2; PEDF and ECM1; GGH and LAMC2; GGH and ECM1; LAMC2 and ECM1.

Also disclosed herein is a method of treatment comprising cervical cerclage to an individual determined or predicted to be at risk of preterm birth (optionally wherein the individual has been determined to be at risk of preterm birth) based on one, each or both biomarker values in a biomarker pair selected from PEDF and GGH; PEDF and LAMC2; PEDF and ECM1; GGH and LAMC2; GGH and ECM1; LAMC2 and ECM1. Methods for selecting an individual for treatment with cervical cerclage are also disclosed.

The invention includes the combination of the aspects and preferred features described except where such a combination is clearly impermissible or expressly avoided.

Summary of the Figures

Embodiments and experiments illustrating the principles of the invention will now be discussed with reference to the accompanying figures in which:

Figure 1. Charts showing the difference in levels of individual biomarkers PEDF, GGH, LAMC2 or ECM1 between samples derived from term (≥ 37 weeks) and preterm (< 35 weeks) deliveries. The mean biomarker protein concentration was calculated based on the outcomes of an ELISA immunoassay. Concentration of PEDF, GGH, LAMC2 and ECM1 were quantified by ELISA on a cohort of term samples ($n=185$) and preterm samples ($n=7$). Data were analysed using Student's t-test. $P = p$ -value. *** indicates p -value ≤ 0.001

Figure 2A to C. Charts showing ability of biomarker pair values to predict preterm birth (< 35 weeks), for PEDF and GGH. A combinatorial algorithm consisting of PEDF and GGH was performed (Panel 1 PEDF*GGH) (A,B). The algorithm resulted in a significant stratification of term and preterm samples with a P -value of 0.0177. (C) Receiver Operating Characteristics (ROC) curve indicated an area under the curve of 0.7931.

Figure 3A to C. Charts showing ability of biomarker pair values to predict preterm birth (<35 weeks), for PEDF and ECM1. A combinatorial algorithm consisting of PEDF and ECM1 was performed (Panel 1 PEDF*ECM1) (A,B). The algorithm resulted in a significant stratification of term and preterm samples with a *P*-value of 0.0011. (C) Receiver Operating Characteristics (ROC) curve indicated an area under the curve of 0.8069.

Figure 4A to C. Charts showing ability of biomarker pair values to predict preterm birth (<35 weeks), for LAMC2 and ECM1. A combinatorial algorithm consisting of LAMC2 and ECM1 was performed (Panel 1 LAMC2*ECM1) (A,B). The algorithm resulted in a significant stratification of term and preterm samples with a *P*-value of ≤ 0.0001 . (C) Receiver Operating Characteristics (ROC) curve indicated an area under the curve of 0.9035.

Figure 5A to C. Charts showing ability of biomarker pair values to predict preterm birth (<35 weeks), for GGH and ECM1. A combinatorial algorithm consisting of GGH and ECM1 was performed (Panel 1 GGH*ECM1) (A,B). The algorithm resulted in a significant stratification of term and preterm samples with a *P*-value of 0.0090. (C) Receiver Operating Characteristics (ROC) curve indicated an area under the curve of 0.8517.

Figure 6A to C. Charts showing ability of biomarker pair values to predict preterm birth (<35 weeks), for PEDF and LAMC2. A combinatorial algorithm consisting of PEDF and LAMC2 was performed (Panel 1 PEDF*LAMC2) (A,B). The algorithm resulted in a significant stratification of term and preterm samples with a *P*-value of ≤ 0.0001 . (C) Receiver Operating Characteristics (ROC) curve indicated an area under the curve of 0.7722.

Figure 7A to C. Charts showing ability of biomarker pair values to predict preterm birth (<35 weeks), for GGH and LAMC2. A combinatorial algorithm consisting of GGH and LAMC2 was performed (Panel 1 GGH*LAMC2) (A,B). The algorithm resulted in a significant stratification of term and preterm samples with a *P*-value of ≤ 0.0001 . (C) Receiver Operating Characteristics (ROC) curve indicated an area under the curve of 0.7614.

Figure 8. Charts showing the difference in levels of individual biomarkers PEDF, GGH, LAMC2 or ECM1 between samples derived from term (≥ 37 weeks) and preterm (<37 weeks) deliveries. The mean biomarker protein concentration was calculated based on the outcomes of an ELISA immunoassay. Concentration of PEDF, GGH, LAMC2 and ECM1 were quantified by ELISA on a cohort of term samples ($n=180$) and

preterm samples (n=12). Data were analysed using Student's t-test. $P = p$ -value. * indicates p -value ≤ 0.05 . **** indicates p -value ≤ 0.0001

Figure 9A to C. Charts showing ability of biomarker pair values to predict preterm birth (<37 weeks), for PEDF and GGH. A combinatorial algorithm consisting of PEDF and GGH was performed (Panel 1 PEDF*GGH) (A,B). The algorithm resulted in a significant stratification of term and preterm samples with a P -value of 0.0067. (C) Receiver Operating Characteristics (ROC) curve indicated an area under the curve of 0.7542.

Figure 10A to C. Charts showing ability of biomarker pair values to predict preterm birth (<37 weeks), for PEDF and ECM1. A combinatorial algorithm consisting of PEDF and ECM1 was performed (Panel 1 PEDF*ECM1) (A,B). The algorithm resulted in a significant stratification of term and preterm samples with a P -value of 0.0025. (C) Receiver Operating Characteristics (ROC) curve indicated an area under the curve of 0.7319.

Figure 11A to C. Charts showing ability of biomarker pair values to predict preterm birth (<37 weeks), for GGH and ECM1. A combinatorial algorithm consisting of GGH and ECM1 was performed (Panel 1 GGH*ECM1) (A,B). The algorithm resulted in a significant stratification of term and preterm samples with a P -value of 0.0115. (C) Receiver Operating Characteristics (ROC) curve indicated an area under the curve of 0.7931.

Figure 12A to C. Charts showing ability of biomarker pair values to predict preterm birth (<37 weeks), for LAMC2 and ECM1. A combinatorial algorithm consisting of LAMC2 and ECM1 was performed (Panel 1 LAMC2*ECM1) (A,B). The algorithm resulted in a significant stratification of term and preterm samples with a P -value of ≤ 0.0001 . (C) Receiver Operating Characteristics (ROC) curve indicated an area under the curve of 0.8389.

Figure 13A to C. Charts showing ability of biomarker pair values to predict preterm birth (<37 weeks), for PEDF and LAMC2. A combinatorial algorithm consisting of PEDF and LAMC2 was performed (Panel 1 PEDF*LAMC2) (A,B). The algorithm resulted in a significant stratification of term and preterm samples with a P -value of ≤ 0.0001 . (C) Receiver Operating Characteristics (ROC) curve indicated an area under the curve of 0.7794.

Figure 14A to C. Charts showing ability of biomarker pair values to predict preterm birth (<37 weeks), for GGH and LAMC2. A combinatorial algorithm consisting of GGH and LAMC2 was performed (Panel 1 GGH*LAMC2) (A,B). The algorithm resulted in a significant stratification of term and preterm samples with a P -value of ≤ 0.0001 . (C)

Receiver Operating Characteristics (ROC) curve indicated an area under the curve of 0.7454.

Detailed Description of the Invention

5 Aspects and embodiments of the present invention will now be discussed with reference to the accompanying figures. Further aspects and embodiments will be apparent to those skilled in the art. All documents mentioned in this text are incorporated herein by reference.

10 Methods and biomarkers described herein may be useful for determining whether an individual is at risk of preterm birth, identifying an individual at risk of preterm birth, or predicting the risk of preterm birth in an individual.

The term “predicting” is used interchangeably with “determining” herein, and is used to say or estimate that preterm birth will happen in an individual. Methods disclosed herein may be used to determine or predict the likelihood (e.g. risk or a risk-score) that
15 an individual will experience preterm birth.

Preterm birth is birth that occurs before the mother has reached 37 weeks of gestation. Preterm birth is subdivided late preterm birth 35 weeks +0 days to 36 weeks +6 days of gestation, moderate preterm birth 32 weeks +0 days to 34 weeks +6 days of gestation and early preterm is prior to 32 weeks of gestation.

20 The cause of preterm birth is often not known. Risk factors include diabetes, high blood pressure, being pregnant with more than one baby, IVF pregnancy, being either obese or underweight, a number of vaginal infections, tobacco smoking, illicit drug use, extremes of maternal age and psychological stress, among others.

Preeclampsia is clinically indicated as hypertension and proteinuria manifesting
25 between 20 weeks of gestation and up to 6 weeks post-partum. Whilst preeclampsia can lead to preterm birth, in many cases it does not. Preeclampsia is just one factor that may contribute to an increased risk of preterm birth, and thus factors that are known to cause or be associated with preeclampsia are not necessarily causative or associated with preterm birth.

30

Biomarkers

The methods disclosed herein involve the determination of the presence or absence of, or quantification of the level of, one or more biomarkers selected from the group consisting of PEDF, GGH, LAMC2 and ECM1. In particular, the methods disclosed
35 herein involve the determination of the presence or absence of, or quantification of the

level of, any two biomarkers selected from the group consisting of PEDF, GGH, LAMC2 and ECM1.

As such, the methods disclosed herein involve the determination of the presence or absence of, or quantification of the level of, each biomarker in a biomarker pair selected from the group consisting of PEDF and GGH; PEDF and LAMC2; PEDF and ECM1; GGH and LAMC2; GGH and ECM1; and LAMC2 and ECM1 (the order in which biomarkers are presented in these pairs is not intended to be of significance to the invention herein disclosed).

A biomarker pair refers to two biomarkers, determination of the level of each biomarker in the pair being useful in methods according to the present invention. Reference to determining the level of a biomarker pair refers to determining the level of each individual biomarker in the pair. Detection/determination of each individual biomarker in a biomarker pair may be performed separately, e.g. in separate assays, or may be performed simultaneously, e.g. in a single multianalyte assay.

Each of the biomarker pairs used herein may be used alone or in combination with other biomarkers of preterm birth. Moreover, the biomarker pairs may be used together with one or more other indicators of preterm birth, including the Fetal Fibronectin (fFN) test, short cervical length, contractions, vaginal bleeding, fluid leaking from the vagina, increased vaginal discharge, backache and cramping in lower abdomen.

Pigment Epithelium-Derived Factor (PEDF); UniProtKB – P36955 [Aliases: SERPINF1, Cell proliferation-inducing gene 35 protein, EPC-1]

PEDF is a neurotrophic protein, which induces extensive neuronal differentiation in retinoblastoma cells, as well as a potent inhibitor of angiogenesis. As it does not undergo the S (stressed) to R (relaxed) conformational transition characteristic of active serpins, it exhibits no serine protease inhibitory activity.

As described PCT/EP2018/079639, which is hereby incorporated by reference in its entirety, PEDF is increased in samples obtained from individuals that experienced preterm birth than in individuals that experienced term birth. This indicates that over-expression of PEDF in a sample indicates that the individual is at increased risk of preterm birth. Over-expression of PEDF in a sample may indicate that the individual is likely to experience preterm birth, irrespective of the sampling time. Over-expression of PEDF may indicate that the individual is likely to experience preterm birth within the next 1-4 weeks.

Gamma-glutamyl hydrolase (GGH); UniProtKB – Q92820 [Aliases: Conjugase, GH, Gamma-Glu-X carboxypeptidase]

GGH hydrolyzes the polyglutamate side chains of pteroylpolyglutamates, which progressively removes gamma-glutamyl residues from pteroylpoly-gamma-glutamate to

yield pteroyl-alpha-glutamate (folic acid) and free glutamate. It may play an important role in the bioavailability of dietary pteroylpolyglutamates and in the metabolism of pteroylpolyglutamates and antifolates.

As described in PCT/EP2018/079639, the overall mean concentration of GGH from all samples (collected between 19 and 37 weeks gestation) was increased in samples obtained from individuals that experienced preterm birth than in individuals that experienced term birth. This indicates that over-expression of GGH in a sample indicates that the individual is at increased risk of preterm birth. GGH is particularly over-expressed in samples obtained 1-4 weeks prior to preterm birth, or at 32-37 weeks gestation. This indicates that over-expression of GGH in a sample obtained at 32-37 weeks gestation indicates that the individual is at risk of preterm birth. Conversely, under-expression of GGH in a sample obtained prior to 32 weeks, or at around 26 weeks or less gestation may indicate that the individual is at risk of preterm birth. In an individual suspected of being at risk of preterm birth, an increased level of GGH may indicate that birth will occur in 4 weeks or less.

Laminin subunit gamma-2 (LAMC2); UniProtKB – Q13753 [Aliases: Cell-scattering factor 140 kDa subunit, Kalinin subunit gamma, Ladsin 140 kDa subunit, Laminin B2t chain, Laminin-5 subunit gamma, Large adhesive scatter factor 140 kDa subunit, Nicein subunit gamma, LAMB2T, LAMNB2, Epiligrin subunit gamma, Kalinin/nicein/epiligrin 100 kDa subunit]

LAMC2 is a heparin binding protein that binds to the cells via a high affinity receptor. Long and short isoforms are produced by alternative splicing. Laminin is thought to mediate the attachment, migration, and organization of cells into tissues during embryonic development by interacting with other extracellular matrix components.

Ladsin, a laminin variant containing the laminin gamma-2 chain exerts cell-scattering activity toward a wide variety of cells, including epithelial, endothelial, and fibroblastic cells.

As described in PCT/EP2018/079639, LAMC2 was increased in samples obtained from individuals that experienced preterm birth than in individuals that experienced term birth. This indicates that over-expression of LAMC2 in a sample indicates that the individual is at increased risk of preterm birth. LAMC2 was over-expressed in preterm birth samples obtained prior to 32 weeks of gestation or in samples obtained less than 8 weeks before birth in preterm birth samples.

Extracellular matrix protein 1 (ECM1); UniProtKB – Q16610 [Aliases: Secretory component p85]

The ECM1 gene encodes an ~85 KDa soluble protein that is involved in endochondral bone formation, proliferation of endothelial cells, angiogenesis, and tumour biology. It also interacts with a variety of extracellular and structural proteins, contributing to the

maintenance of skin integrity and homeostasis. ECM1 acts as a “biological glue” in a variety of tissues contributing to the organization and scaffolding of collagen.

Four alternatively spliced transcript variants encoding distinct isoforms have been described for this gene. Any of these may be used as a biomarker according to the present disclosure. In some cases, variant 1 is detected. As described in PCT/EP2018/079639, the overall mean concentration of ECM1 from all samples (collected between 19 and 37 weeks gestation) was lower in samples obtained from individuals that experienced preterm birth than in individuals that experienced term birth ($p = 0.025$). This indicates that under-expression of ECM1 in a sample indicates that the individual is at increased risk of preterm birth. ECM1 was under-expressed in samples from individuals that experienced preterm birth, at all time points sampled. In an individual suspected at being at risk of preterm birth, a lower level of ECM1 may indicate birth will occur in less than 12 weeks, less than 9 weeks, or less than 4 weeks.

Certain methods disclosed herein involve detecting the presence or absence or a biomarker, a biomarker value or biomarker level for each of a biomarker pair. These terms refer to a measurement that is made using any appropriate analytical method for detecting the biomarker in a biological sample and that indicates the presence, absence, absolute amount or concentration, relative amount or concentration, titer, level, expression level, ratio or other measurement corresponding to the biomarker in the sample. The exact nature of the value or level depends on the specific design and components of the particular analytical method employed to detect the biomarker.

Biomarkers that indicate that an individual is at risk of preterm birth may be over-expressed or under-expressed, as compared to a reference value or level or the biomarker that indicates or is a sign of term birth. “up-regulation”, “over-expression”, increased and related terms are used to refer to a value or level in a sample that is greater than a value or level (or range of values or levels) of the biomarker that is typically detected in similar samples from individuals that are known to have experienced term birth. “down-regulation”, “under-expression”, “reduced” and related terms are used to refer to a value or level in a sample that is less than a value or level (or range of values or levels) of the biomarker that is typically detected in similar samples from individuals that are known to have experienced term birth.

A biomarker that is over-expressed or under-expressed may also be referred to as being “differentially expressed” or as having a “differential” level or value as compared to the expression level or value observed in individuals known to have experienced term birth. Differential expression can also be referred to as a variation from a “normal” expression level of the biomarker.

The term “differential gene expression” and “differential expression” are used interchangeably to refer to a gene (or its corresponding protein expression product)

whose expression is activated to a higher or lower level in a subject at risk of preterm birth, relative to its expression in an individual known to have experienced term birth. The terms also include genes (or the corresponding protein expression products) whose expression is activated to a higher or lower level at different stages of the same

5 disease. It is also understood that a differentially expressed gene may be either activated or inhibited at the nucleic acid level or protein level, or may be subject to alternative splicing to result in a different polypeptide product. Such differences may be evidenced by a variety of changes including mRNA levels, surface expression, secretion or other partitioning of a polypeptide. Differential gene expression may include a

10 comparison of expression between two or more genes or their gene products; or a comparison of the ratios of the expression between two or more genes or their gene products; or even a comparison of two differently processed products of the same gene, which differ between individuals at risk of preterm birth or individuals that experience term birth. Differential expression includes both quantitative, as well as qualitative,

15 differences in the temporal or cellular expression pattern in a gene or its expression products in individuals who experience preterm and term birth.

Reference herein to an individual biomarker includes genetic variants, isoforms, mutants, homologs, or orthologs thereof.

20 **Method outcomes**

Methods disclosed herein are useful for identifying individuals at risk of preterm birth, or for determining whether an individual is, or is not, at risk of preterm birth. Methods may also be used to predict the risk of preterm birth in an individual.

In some cases, the method may involve a step of recording the level of the biomarkers.

25 In some cases, the methods may involve a step of transmitting the level of the biomarkers disclosed herein to a physician involved in the care of the individual being tested. In some cases, the method may also involve transmitting a reference level of the biomarker, for comparison with the level of biomarker in the individual. In some cases, the level of risk of preterm birth determined in the individual is transmitted to the

30 physician. For example, a level of risk may be allocated a percentage (where 100% indicates that the individual will certainly experience preterm birth, and 0% indicates that the individual will certainly experience term birth). Thus, some methods disclosed herein involve allocating a percentage value to the level of risk that the individual is determined to have. The method may involve the step of transmitting that percentage

35 to a physician involved in the care of that individual.

The methods disclosed herein may be used to select an individual for treatment or other management. Certain methods disclosed herein involve the administration of a treatment to an individual identified as at risk of preterm birth.

Treatments useful in the methods disclosed herein include the administration of a progesterone, synthetic progesterone or progesterone analogue, one or more agents selected from a progesterone or an analogue thereof, a tocolytic, a corticosteroid, an antibiotic, an NSAID or an Omega 3 fatty acid or derivative thereof. The progesterone may be a synthetic progesterone, such as 17- α -hydroxyprogesterone caproate. The tocolytic may be magnesium sulfate, indomethacin or Nifedipine. The antibiotic may be erythromycin or penicillin. The NSAID may be indomethacin. The Omega 3 fatty acid derivative may be docosahexaenoic acid (DHA).

The individual may be selected for treatment with progesterone. Progesterone has been shown to reduce preterm birth rates in numerous clinical studies profiling women with short cervical length or prior history of preterm birth as a high-risk population.

Treatment with progesterone may comprise the administration of natural progesterone, or synthetic progestin such as 17- α -hydroxyprogesterone caproate. The progesterone may be P4 micronized (natural) progesterone. 17- α -hydroxyprogesterone caproate is also known by the brand names DelalutinTM, ProlutonTM, Proluton DepotTM and MakenaTM. Natural micronized progesterone, a natural progesterone, is similar to that produced in the corpus luteum and placenta. Micronized progesterone can be utilised as oral capsule, vaginal gel or vaginal suppository. Synthetic Progestins include medroxyprogesterone acetate (MPA, also known as depot medroxyprogesterone acetate (DMPA)) and norethindrone acetate (NETA). They are typically given by injection. Synthetic progestins are also known by the brand names (MPA) ProveraTM, Depo-ProveraTM, Depo-SubQ Provera 104TM, CurretabTM, CycrinTM, FarlutalTM, GestapuranTM, PerlutexTM, VeramixTM and (NETA) Primolut-NorTM, AygestinTM, GestakadinTM, MilligynonTM, MonogestTM, NorlutateTM, Primolut NTM, SH-420TM, SovelTM, StyptinTM. The micronized progesterone may be self-administered by the patient. Natural micronized progesterone is also known by the brand names PrometriumTM, UtrogestanTM, EndometrinTM and CrinoneTM. Administration may be orally, vaginally, or intramuscularly. Progesterone, progestin or 17- α -hydroxyprogesterone caproate for use in such methods, or the use of progesterone, progestin or 17- α -hydroxyprogesterone caproate for use in the manufacture of a medicament for use in such a method are also disclosed.

Individuals identified at risk of preterm birth may be treated with cervical cerclage. Cervical cerclage may also be referred to as a cervical stitch. Cervical cerclage is used to treat cervical incompetence or insufficiency, where the cervix starts to shorten and open too early during a pregnancy. Cervical cerclage may involve the insertion of a strong suture into and around the cervix.

Any known form of cervical cerclage may be used. For example, the cervical cerclage may be a McDonald cerclage, a Shirodkar cerclage or an abdominal cerclage. Cervical cerclage may be particularly useful where the individual is determined to have cervical incompetence. Cervical incompetence may be determined by transvaginal ultrasound scan.

Alternatively, the treatment may comprise a cervical pessary. In some cases, the treatment may be an Arabin Pessary.

In some cases, the individual may be selected to receive tocolytics or steroids, such as corticosteroids. Tocolytics may be used to arrest uterine contraction during preterm labor. Steroids may aid in fetal lung development. The method may involve a step of administering the tocolytic and/or steroid to the individual. Tocolytics and steroids have been used for women presenting with contractions. Examples of tocolytic agents suitable in the invention are magnesium sulfate, indomethacin and nifedipine.

In some cases, the methods are used to select an individual for further, regular or intensive monitoring. For example, the methods may be used to determine that a further sample should be obtained from that individual, and the biomarker presence or absence and/or level should be determined in the future. The further sample may be obtained 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35 or 36 weeks after the first sample. The further sample may be obtained at gestational week 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36 or 37. The method may involve further sampling every 1, 2, 3, 4, or 5 weeks during the pregnancy.

In some cases, the individual may be treated with antibiotics. Antibiotics may be particularly used in individuals with preterm premature rupture of the membranes (PPROM). Suitable antibiotics include erythromycin and penicillin.

In some cases, the treatment may be administration of an NSAID. NSAIDs may inhibit prostaglandin to reduce uterine contractions. The NSAID may be indomethacin. In some cases, the treatment may be Omega 3 Fatty Acid or a derivative of Omega 3 Fatty acid. For example, the treatment may be DHA (docosahexaenoic acid).

Monitoring may comprise monitoring for fetal distress, such as monitoring fetal heartbeat, monitoring fetal movement or meconium monitoring.

Measurement of Biomarkers

Biomarkers disclosed herein are preferably protein biomarkers. Any method of detecting and/or quantifying a protein known in the art may be used.

Methods according to the present invention may be performed, or products may be present, in vitro, ex vivo, or in vivo. The term "in vitro" is intended to encompass experiments with materials, biological substances, cells and/or tissues in laboratory conditions or in culture whereas the term "in vivo" is intended to encompass

5 experiments and procedures with intact multi-cellular organisms. "Ex vivo" refers to something present or taking place outside an organism, e.g. outside the human or animal body, which may be on tissue (e.g. whole organs) or cells taken from the organism.

The methods disclosed herein preferably relate to the determination of protein
10 expression. Protein expression can be measured by quantifying the amount of protein in a cell, tissue or sample, or by observing the localisation of the protein within cells and tissues.

In some cases, immunoassays are used to detect the biomarker target in a sample from the subject. Immunoassays use antibodies or other entities with specific affinity for the
15 target molecule in conjunction with a detectable molecule. In some cases, the antibody is conjugated to the detectable molecule. The detectable molecule may be referred to as a label. The detectable molecule produces a detectable signal when the antibody is bound to the target molecule. The detectable signal may be a quantifiable signal. In some cases, an aptamer is used instead of, or together with, the antibody.

20 Immunoassays include enzyme-linked immunosorbent assays (ELISA), immunoblotting, flow cytometry and immunohistochemistry. In certain aspects described herein, the assay is an immunohistochemistry assay. Such assays commonly use antibodies, although other target specific molecules such as aptamers or other ligands may be used. Antibody arrays or protein chips may also be used.

25 The method may be approved for use by a regulatory agency. The method may be an FDA approved method.

Antibodies

Antibodies which will bind to the biomarkers of the invention are already known. In view
30 of today's techniques in relation to monoclonal antibody technology, antibodies can be prepared to most antigens.

The antigen-binding portion may be a part of an antibody (for example a Fab fragment) or a synthetic antibody fragment (for example a single chain Fv fragment [ScFv]).

Suitable monoclonal antibodies to selected antigens may be prepared by known
35 techniques, for example those disclosed in "Monoclonal Antibodies: A manual of techniques ", H Zola (CRC Press, 1988) and in "Monoclonal Hybridoma Antibodies: Techniques and Applications ", J G R Hurrell (CRC Press, 1982). Chimeric antibodies

are discussed by Neuberger et al (1988, 8th International Biotechnology Symposium Part 2, 792-799).

Monoclonal antibodies (mAbs) are useful in the methods of the invention and are a homogenous population of antibodies specifically targeting a single epitope on an antigen. Suitable monoclonal antibodies can be prepared using methods well known in the art (e.g. see Köhler, G.; Milstein, C. (1975). "Continuous cultures of fused cells secreting antibody of predefined specificity". *Nature* 256 (5517): 495; Siegel DL (2002). "Recombinant monoclonal antibody technology". Schmitz U, Versmold A, Kaufmann P, Frank HG (2000); "Phage display: a molecular tool for the generation of antibodies--a review". *Placenta* 21 Suppl A: S106–12. Helen E. Chadd and Steven M. Chamow; "Therapeutic antibody expression technology," *Current Opinion in Biotechnology* 12, no. 2 (April 1, 2001): 188-194; McCafferty, J.; Griffiths, A.; Winter, G.; Chiswell, D. (1990). "Phage antibodies: filamentous phage displaying antibody variable domains". *Nature* 348 (6301): 552–554; "Monoclonal Antibodies: A manual of techniques ", H Zola (CRC Press, 1988) and in "Monoclonal Hybridoma Antibodies: Techniques and Applications ", J G R Hurrell (CRC Press, 1982). Chimaeric antibodies are discussed by Neuberger et al (1988, 8th International Biotechnology Symposium Part 2, 792-799)).

Polyclonal antibodies are useful in the methods of the invention. Monospecific polyclonal antibodies are preferred. Suitable polyclonal antibodies can be prepared using methods well known in the art.

Fragments of antibodies, such as Fab and Fab2 fragments may also be used as may genetically engineered antibodies and antibody fragments. The variable heavy (VH) and variable light (VL) domains of the antibody are involved in antigen recognition, a fact first recognised by early protease digestion experiments. Further confirmation was found by "humanisation" of rodent antibodies. Variable domains of rodent origin may be fused to constant domains of human origin such that the resultant antibody retains the antigenic specificity of the rodent parented antibody (Morrison et al (1984) *Proc. Natl. Acad. Sci. USA* 81, 6851-6855).

That antigenic specificity is conferred by variable domains and is independent of the constant domains known from experiments involving the bacterial expression of antibody fragments, all containing one or more variable domains. These molecules include Fab-like molecules (Better et al (1988) *Science* 240, 1041); Fv molecules (Skerra et al (1988) *Science* 240, 1038); single-chain Fv (ScFv) molecules where the VH and VL partner domains are linked via a flexible oligopeptide (Bird et al (1988) *Science* 242, 423; Huston et al (1988) *Proc. Natl. Acad. Sci. USA* 85, 5879) and single domain antibodies (dAbs) comprising isolated V domains (Ward et al (1989) *Nature* 341, 544). A general review of the techniques involved in the synthesis of antibody

fragments which retain their specific binding sites is to be found in Winter & Milstein (1991) Nature 349, 293- 299.

By "ScFv molecules" we mean molecules wherein the VH and VL partner domains are covalently linked, e.g. directly, by a peptide or by a flexible oligopeptide.

- 5 Fab, Fv, ScFv and dAb antibody fragments can all be expressed in and secreted from E. coli, thus allowing the facile production of large amounts of the said fragments.

Whole antibodies, and F(ab')₂ fragments are "bivalent". By "bivalent" we mean that the said antibodies and F(ab')₂ fragments have two antigen combining sites. In contrast, Fab, Fv, ScFv and dAb fragments are monovalent, having only one antigen combining
10 site. Synthetic antibodies which bind to the biomarker may also be made using phage display technology as is well known in the art (e.g. see "Phage display: a molecular tool for the generation of antibodies--a review". Placenta. 21 Suppl A: S106–12. Helen E. Chadd and Steven M. Chamow; "Phage antibodies: filamentous phage displaying antibody variable domains". Nature 348 (6301): 552–554).

- 15 In some preferred embodiments the antibody is detectably labelled or, at least, capable of detection. For example, the antibody may be labelled with a radioactive atom or a coloured molecule (chromophore) or a fluorescent molecule or a molecule which can be readily detected in any other way. Suitable detectable molecules include fluorescent proteins, luciferase, enzyme substrates, and radiolabels. The antibody may be directly
20 labelled with a detectable label or it may be indirectly labelled. For example, the antibody may be unlabelled and can be detected by another antibody which is itself labelled. Alternatively, the second antibody may have bound to it biotin and binding of labelled streptavidin to the biotin is used to indirectly label the first antibody.

An aspect disclosed herein is two complexes, each complex being of an antibody and a
25 (different) biomarker selected from the group consisting of PEDF, GGH, LAMC2 and ECM1. The complex may further comprise a second, different antibody. The complex may further comprise a detectable moiety. The complex may be present in a sample of cervicovaginal fluid. The complex may be isolated.

30 **Aptamers**

As an alternative to the use of antibodies to detect biomarkers other target-specific binding agents may optionally be used, one example being the class of molecule known as aptamers.

- 35 Aptamers, also called nucleic acid ligands, are nucleic acid molecules characterised by the ability to bind to a target molecule with high specificity and high affinity. Almost every aptamer identified to date is a non-naturally occurring molecule.

Aptamers to a given target (e.g. one of PEDF, GGH, LAMC2 or ECM1) may be identified and/or produced by the method of Systematic Evolution of Ligands by EXponential enrichment (SELEXTM). Aptamers and SELEX are described in Tuerk and Gold (Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. Science. 1990 Aug 3;249(4968):505-10) and in WO91/19813.

Aptamers may be DNA or RNA molecules and may be single stranded or double stranded. The aptamer may comprise chemically modified nucleic acids, for example in which the sugar and/or phosphate and/or base is chemically modified. Such modifications may improve the stability of the aptamer or make the aptamer more resistant to degradation and may include modification at the 2' position of ribose.

Aptamers may be synthesised by methods which are well known to the skilled person. For example, aptamers may be chemically synthesised, e.g. on a solid support.

Solid phase synthesis may use phosphoramidite chemistry. Briefly, a solid supported nucleotide is detritylated, then coupled with a suitably activated nucleoside phosphoramidite to form a phosphite triester linkage. Capping may then occur, followed by oxidation of the phosphite triester with an oxidant, typically iodine. The cycle may then be repeated to assemble the aptamer.

Aptamers can be thought of as the nucleic acid equivalent of monoclonal antibodies and often have K_d's in the nM or pM range, e.g. less than one of 500nM, 100nM, 50nM, 10nM, 1nM, 500pM, 100pM. As with monoclonal antibodies, they may be useful in virtually any situation in which target binding is required, including use in therapeutic and diagnostic applications, in vitro or in vivo. In vitro diagnostic applications may include use in detecting the presence or absence of a target molecule.

Aptamers according to the present invention may be provided in purified or isolated form. Aptamers according to the present invention may be formulated as a pharmaceutical composition or medicament.

Suitable aptamers may optionally have a minimum length of one of 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 nucleotides

Suitable aptamers may optionally have a maximum length of one of 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, or 80 nucleotides

Suitable aptamers may optionally have a length of one of 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40,

41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, or 80 nucleotides.

Detection and Labelling

5 Methods disclosed herein involve the detection and/or quantification of biomarkers.

In the methods disclosed herein, each biomarker (the “target”) may be directly detected. The target may be detected by an anti-target antibody.

Alternatively, detection of the target may be indirect. That is to say that the target may be detected by the anti-target antibody, and the anti-target antibody is subsequently
10 detected by a secondary detectable antibody. The secondary antibody is preferably labelled. Suitable secondary antibodies may be raised against the antibody isotype of the animal species in which the primary antibody has been raised. For example, the secondary antibody may be an anti-mouse antibody, capable of binding to mouse antibodies. Methods using a secondary antibody may be more sensitive than direct
15 detection methods, due to signal amplification from multiple secondary antibodies binding to each primary antibody.

Suitable labels include enzymes such as horseradish peroxidase, alkaline phosphatase, glucose oxidase and luciferase, and colorimetric agents, including quantum dots, fluorophores and chromophores. Suitable fluorophores include FITC, TRITC, Cy5,
20 Texas Red, Alexa Fluor and others. The label may be a radiolabel.

A variety of detectable enzymatic substrates are available for use with enzymatically labelled antibodies. These include chromogenic detection systems, such as Horseradish peroxidase(HRP), pNPP, BCIP/NBT (5-bromo-4-chloro-3'-indolylphosphate/nitro-blue tetrazolium), TMB (tetramethybenzidine), DAB (3,3'-
25 diaminobenzidine), OPD (ortho-phenylenediamine dihydrochloride) and ABTS (2,2'-azinobis[-ethylbenzothiazoline-6-sulfonic acid]), and chemiluminescent substrates such as an ECL (enhanced chemiluminescent) label or Acridinium ester (AE).

Methods may involve the use of an antibody or antibody- derived binding agent, such as a scFv or Fab fragment. Alternatively, or in combination with the antibody, the method
30 may involve the use of an aptamer.

ELISA

In some cases, the target may be detected by ELISA (enzyme-linked immunosorbent assay). Target molecules (such as the biomarker proteins disclosed herein) from a
35 sample are attached to a surface and detected using a specific antibody. The target may be attached to the surface non-specifically (via adsorption to the surface) or

specifically (using a specific capture agent such as an antibody). ELISA may be used to quantify target in a sample. The surface may be a solid support, such as a multiwell plate, microbead, or dipstick.

5 Commercially available ELISA assays may be used. The ELISA may be an indirect ELISA, Sandwich ELISA or competitive ELISA.

ELISA may involve the use of first, capture, antibody to bind the target molecule. A second, detection, antibody to the target molecule is then added. Binding of the second antibody indicates the presence and/or level of the target.

10 The first antibody may be bound to a solid support. The first and second antibodies are not identical. Usually, the first and second antibodies bind to different epitopes on the target molecule. In some cases, the second antibody binds to a complex of the first antibody and the target, but not to either the first antibody or the target when not in complex. The second antibody may be labelled.

15 As such, in some embodiments a biomarker may be detected by contacting the sample with two different antibodies that each bind to the biomarker. One antibody may bind to a first epitope on the biomarker and another antibody may bind to a second, different, epitope on the biomarker.

20 In some embodiments a biomarker may be detected by contacting the sample with an antibody that binds to the biomarker, e.g. to a first epitope, and with a non-antibody binding entity, e.g. an aptamer, that also binds the biomarker, e.g. at a second, different, epitope on the biomarker.

25 In some embodiments a biomarker may be detected by contacting the sample with two different non-antibody binding entities that each bind to the biomarker. One said entity may bind to a first epitope on the biomarker and another said entity may bind to a second, different, epitope on the biomarker.

Kits and methods may therefore provide pairs of binding entities, e.g. antibodies, aptamers or a combination, that bind to the same biomarker, preferably at different sites or epitopes on the biomarker. Each pair provides the basis of a sandwich assay format.

30 Further information on ELISA may be found in *Enzyme-linked Immunosorbent Assay (ELISA) From A to Z* Hosseini et al published by Springer Singapore ISBN: 978-981-10-6765-5.

Immunoblotting

35 In some aspects, the target is detected by immunoblotting, or western blotting. In such methods, proteins in a sample are separated based on their electric charge or size. They may be separated by an electrophoresis- based method. The separated proteins

are transferred to a membrane, where they are stained with an antibody that is specific to the target. The antibody is then detected, either directly by virtue of the antibody being conjugated to a detectable label, or indirectly, by adding a labelled secondary antibody.

5

Mass Spectrometry

In some aspects, the methods disclosed herein involve the detection and/or quantification of protein using mass spectrometry. Mass spectrometry may use peptides with sequences unique to the target protein as surrogates for the target.

10 Measurements are made with respect to the mass and intensity of the peak due to the protein, protein fragment or partial peptide of interest. Prior to the measurements a fixed amount of substance serving as the internal standard is added to the original biological material and the intensity of its peak is also measured. The concentration of the target in the original biological material can be calculated from the ratio of peak intensity of the
15 target to the peak intensity of the internal standard. Various mass-spectrometry methods are known and may be used for detecting and/or quantifying biomarkers as disclosed herein, including MALDI-TOF (time of flight), SELDI/TOF, liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS), high performance liquid chromatography- mass spectrometry (HPLC-MS),
20 capillary electrophoresis-mass spectrometry, nuclear magnetic resonance spectrometry, or tandem mass spectrometry.

In vitro Diagnostics and Kits

An aspect of the present disclosure includes in vitro diagnostic methods, and in vitro
25 diagnostic kits for performing such methods. A kit as described herein may include one or more antibodies (or other target binding entity/entities) such as an anti-biomarker antibody or fragment thereof. The kit may be suitable for selecting a subject at risk of preterm birth.

The kit may be suitable for a point-of-care in vitro diagnostic test. It may be a kit for
30 laboratory-based testing. The kit may include instructions for use, such as an instruction booklet or leaflet. The instructions may include a protocol for performing any one or more of the methods described herein. The instructions may include a protocol for performing an immunoassay or immunochromatographic assay. They may describe methods and suggestions for adapting the test for different types of sample. They may
35 provide methods and suggestions for optimising the results obtained from the test, such as minimising the signal to noise ratio.

The kit may be suitable for performing an immunoassay or immunochromatographic assay. In some cases, the in vitro diagnostic test involves a lateral flow device, or “dipstick” test. In some cases, the kit includes a multiwell plate or other solid support that is pre-coated with a capture agent, such as an anti-biomarker antibody.

- 5 The kit may comprise one or more suitable receptacles, preferably in a microtitre format, such as one or more microtitre plates, disks, platforms or cartridges.

The kit may further comprise immunoassay components, whereby the immunoassay components are pre-stored in said receptacles, disks, platforms and/or cartridges, which are preferably covered with a sealing film and/or foil suitable for sterile packaging.

- 10 In some embodiments a suitable kit may comprise a plate, e.g. multi-well plate or cartridge suitable for performing an immunoassay such as an enzyme-linked immunosorbent assay (ELISA) when used together with a suitable analyser device or system. As such, disclosed herein is a plate or cartridge having disposed thereon and/or adhered thereto at least one pair of antibodies, e.g. any pair described herein,
15 configured to permit binding to the respective antigen/analyte biomarker as part of an immuno-assay, such as an ELISA format assay. The antibodies may be attached to the plate or cartridge at defined locations, e.g. defined wells.

Suitable plates may be multi-well plates, e.g. having 4, 8, 12, 16, 32, 48, 64, 80 or 96 wells. Plates or cartridges may incorporate microfluidic channels.

- 20 The kit may be provided for use as part of a system, the system comprising a said receptacle and an analyser configured to analyse the output signal(s) from an immunoassay performed using the receptacle. The plate, cartridge and/or analyser may be configured to perform an ELISA assay, which may be a single assay or multiple assays. The analyser may comprise a detection unit which may be configured to detect
25 output signal(s) from the assay, e.g. defined wavelengths of visible or non-visible light.

The analyser may be loaded with software, e.g. for signal or image acquisition, and may comprise or be compatible for use with, a computing device configured for processing an acquired signal or image.

- 30 A kit may further comprise wash buffers and/or reagents useful in an immunoassay method that involves detection of the analyte-antibody interaction.

The kit may additionally include standards or controls. The kit may additionally include buffers, diluents or other reagents, such as stop buffer, sample preparation buffer, colour development reagents, streptavidin conjugates, substrates or wash buffer.

- 35 The kit may be adapted for use with dry samples, wet samples, frozen samples, fixed samples, urine samples, saliva samples, tissue samples, blood samples, or any other type of sample, including any of the sample types disclosed herein.

Immunoassay technology, such as ELISA, provides a basis for the development of *in vitro* diagnostic (IVD) kits for healthcare, industrial, food safety, environmental monitoring and many other bioanalytical applications. This assay technology and format enables the person of ordinary skill in the art to perform sandwich assays, e.g. ELISA, chemiluminescent immunoassay, fluorescent immunoassay and nanoparticle/beads-based immunoassay. Such technology can be provided at the point-of-care, point-of-need, bioanalytical, home or remote settings, useful in the fields of both medicine and diagnosis.

Examples of commercially available immunoassay systems include the Quanterix Simoa®, Meso Scale Discovery (MSD) MSD® QuickPlex SQ 120® SECTOR S 600®, Luminex® Luminex® 100/200™ System Dynex® Multiplier® SmartPLEX® technology and the ProteinSimple® Ella® multiplex assay system.

The kit may comprise a device for obtaining or processing a vaginal fluid sample. The kit may comprise vaginal fluid extraction buffer, for example a buffer containing approximately 50mM HEPES, 150mM NaCl, 0.1% SDS, 1mM EDTA, 1mM Pefabloc SC 4-(2-aminoethyl)_benzene sulfonyl fluoride (AEBSF). The kit may comprise a sample collection device, such as a swab, cervicovaginal wick, diaphragm-like device, cervical aspirator, or cytobrush. The kit may comprise a container suitable for storing a vaginal fluid sample.

Swabs suitable for use in the kits include foam swabs, Dacron swabs, rayon swabs, flocked swabs and cotton swabs. Suitable foam swabs include MW942 (Sigma-Swab Duo), Polyurethane foam swab (Catch-All; Epicenter) and CultureSwab EZ polyurethane foam swabs (BD). Suitable Dacron swabs include Deltalab Eurotubo 300263 (Fisher Scientific, UK), Sterile G-in, Dacron-tipped plastic applicators (Solon, Skowhegan, ME), Dacron swab (Cardinal Health, McGraw Park, IL) and Dacron swabs (Puritan Medical, Guilford, ME, USA). Suitable rayon swabs include BBL CultureSwab (Becton Dickinson, Oxford, UK) and MW167 (Duo-Transtube®). Suitable flocked swabs (nylon) include Seacliff Packaging, BD, COPAN. Suitable cotton swabs include Sterile dry swabs (Eurotubo, Rubi, Spain), Cotton-tipped swabs (Falcon™ Screw Cap Single SWUBE™ applicator, Becton Dickinson and Co., Sparks, MD), Falcon™ Screw Cap Single SWUBE™ applicator (BD).

Wicks suitable for use in the kit include tampons, strips or sponges, including ophthalmic PVA sponge (Eyetec™, Network Medical Ltd.), Tear-Flo™ Strips (Wilson Ophthalmic), Weck-Cel® sponges (Xomed Surgical Products, Jacksonville, FL), Sno-strips (Akorn Inc., Abita Springs LA) and Polywicks (Polyfiltronics, Rockland, MA, USA).

Diaphragm like devices suitable for use in the kit include Instead SoftCup (Ultrafem), Sterile gauze, or a Menstrual cup (SoftCup, EuroFemPro, Netherlands, or the SoftCup, Instead Inc., San Diego, CA).

Suitable cervical aspirators include Vaginal Specimen Aspirators (CarTika), or long tuberculin syringes.

Certain kits disclosed herein comprise antibodies that each bind to a biomarker of preterm birth and a device or buffer for obtaining or processing a vaginal fluid sample.

- 5 Antibodies that bind to a biomarker of preterm birth may be an anti-PEDF, anti-GGH, anti-LAMC2, or anti-ECM1 antibody.

Also disclosed herein is a composition comprising vaginal fluid and two antibodies selected from the group consisting of: an anti-PEDF, anti-GGH, anti-LAMC2, or anti-ECM1 antibody.

10

Sampling methods

Methods and agents described herein involve the analysis of certain biomarkers in cervicovaginal fluid. Several methods of sampling cervicovaginal fluid are known, and may be used in the methods.

- 15 The methods may involve sampling by cervicovaginal lavage. This involves obtaining cervicovaginal washings by rinsing the cervicovagina with washing buffer and collecting the fluid after the rinsing.

In some methods, cervicovaginal swabs are taken. Suitable swabs are known in the art. Preferred swabs for use in the methods and kits disclosed herein include foam
20 swabs, Dacron swabs, rayon swabs, flocked swabs and cotton swabs. Suitable foam swabs include MW942 (Sigma-Swab Duo), Polyurethane foam swab (Catch-All; Epicenter) and CultureSwab EZ polyurethane foam swabs (BD). Suitable Dacron swabs include Deltalab Eurotubo 300263 (Fisher Scientific, UK), Sterile G-in, Dacron-tipped plastic applicators (Solon, Skowhegan, ME), Dacron swab (Cardinal Health,
25 McGraw Park, IL) and Dacron swabs (Puritan Medical, Guilford, ME, USA). Suitable rayon swabs include BBL CultureSwab (Becton Dickinson, Oxford, UK) and MW167 (Duo-Transtube®). Suitable flocked swabs (nylon) include Seaclyff Packaging, BD, COPAN. Suitable cotton swabs include Sterile dry swabs (Eurotubo, Rubi, Spain), Cotton-tipped swabs (Falcon™ Screw Cap Single SWUBE™ applicator, Becton
30 Dickinson and Co., Sparks, MD), Falcon™ Screw Cap Single SWUBE™ applicator (BD).

In other methods, cervicovaginal fluid is sampled with a wick. Wicks suitable for use in the methods disclosed herein include tampons, strips or sponges, including opthalmic PVA sponge (EyeteC™, Network Medical Ltd.), Tear-Flo™ Strips (Wilson Ophthalmic),
35 Weck-Cel® sponges (Xomed Surgical Products, Jacksonville, FL), Sno-strips (Akorn Inc., Abita Springs LA) and Polywicks (Polyfiltronics, Rockland, MA, USA).

In other methods, diaphragm like devices are used to sample cervicovaginal fluid. Suitable diaphragm like devices are placed over the cervix to collect the cervicovaginal fluid and include Instead SoftCup (UltraFem), Sterile gauze, or a Menstrual cup (SoftCup, EuroFemPro, Netherlands, or the SoftCup, Instead Inc., San Diego, CA).

- 5 The method may involve the use of a cervical aspirators such as a Vaginal Specimen Aspirators (CarTika), or long tuberculin syringe.

In some methods, cervicovaginal fluid is sampled with a cytobrush.

Certain kits disclosed herein comprise an antibody that binds to a biomarker of preterm birth and a device or buffer for obtaining or processing a vaginal fluid sample.

10

Controls

In some methods disclosed herein the level of the biomarker is compared to the level of a control or a reference value or level.

- 15 In some cases, the control may be a reference sample or reference dataset. The reference may be derived from one or more samples that have been previously obtained from a subject known to have undergone preterm birth. Alternatively, the reference may be derived from one or more samples that have been previously obtained from a subject known to have undergone term birth. The reference may be a dataset obtained from analyzing a reference sample.

- 20 Controls may be positive controls in which the target molecule is known to be present, or expressed at high level, or negative controls in which the target molecule is known to be absent or expressed at low level.

- The control may be a sample or level from a patient known to have experienced a preterm or term birth. The control value may be obtained by performing analysis of the biomarker in parallel with a sample from the individual to be tested. Alternatively, the control value may be obtained from a database or other previously obtained value.
- 25

Samples

- Methods disclosed herein relate to the detection of biomarkers in a sample obtained from an individual or patient. The method may be performed in vitro. Preferably, the method involves a sample that has been obtained from an individual. Thus, the method may, but preferably does not, involve a step of obtaining a sample from an individual.
- 30

- Preferably, the sample is a sample of vaginal fluid, such as cervicovaginal (cervico-vaginal; cervical-vaginal) fluid (CVF) or cervical fluid. Alternatively, the sample may be a blood sample, such as whole blood, plasma or serum sample, a lymph sample, a
- 35

urine sample or an amniotic fluid sample, a saliva sample or a sample of any body fluid. The sample may be a protein sample derived from a vaginal fluid or cervicovaginal fluid sample, or a protein sample derived from a blood sample, such as whole blood, plasma or serum sample, a lymph sample, a urine sample, an amniotic fluid sample, a saliva sample or a sample of any body fluid.

The sample may have been pre-treated. For example, the sample may have been contacted with one or more preservative agents or buffers. The sample may have been frozen, lyophilized, or dried.

Although the individual or patient may be mammalian, such as a cat, dog, horse, or ape, the individual is preferably a human. The terms "patient", "individual" and "subject" are used interchangeably herein.

The individual may be a female individual. The individual may be pregnant. The individual may be symptomatic or asymptomatic of labor. Preferably, the individual is asymptomatic.

Symptomatic individuals are individuals who present with one or more symptoms of preterm birth, such as contractions, particularly regular contractions, back ache, including back ache in the lower back, cramping in the lower abdomen or menstrual-like cramps, fluid leaking from the vagina, flu-like symptoms, nausea, vomiting, increased pressure in the pelvis or vagina, increased vaginal discharge and/or vaginal bleeding.

Asymptomatic individuals may not present with any symptoms of preterm birth, or with symptoms that may or may not be indicative of preterm birth, such as backache, including backache in the lower back, cramping in the lower abdomen or menstrual-like cramps, fluid leaking from the vagina, flu-like symptoms, nausea, vomiting, increased pressure in the pelvis or vagina, increased vaginal discharge and/or vaginal bleeding.

Commonly, asymptomatic individuals do not present with any symptom of preterm birth.

In some cases, the individual may be suspected of being at high risk of preterm birth prior to obtaining the sample. For example, the sample may be obtained and/or the presence or level of the biomarker may be determined because the individual is suspected to be of high risk of preterm birth. An individual may be suspected to have a high risk of preterm birth based on their prior medical history of premature births or miscarriages. Alternatively, or additionally, the individual may be suspected to have high risk of preterm birth based on the results of a Fetal Fibronectin (fFN) test, a short cervical length or based on symptoms such as contractions, vaginal bleeding, fluid leaking from the vagina, increased vaginal discharge, backache or cramping in lower abdomen. Alternatively, or additionally, the individual may be considered to be of high risk of preterm birth due to the presence of one or more risk factors such as diabetes, high blood pressure, being pregnant with more than one baby, IVF pregnancy, body-mass index [BMI] (too high or too low), a number of vaginal infections, tobacco smoking,

drug use, extremes of maternal age, psychological stress, ethnic background, and socio-economic status or income.

Samples may be obtained from an individual weeks or months prior to birth, or prior to the expected date of term birth. For example, samples may be obtained 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35 or 36 weeks prior to birth. In some cases, samples are taken 1-4, 5-8, 9-12 or more than 12 weeks prior to the expected normal birth date.

Samples may be obtained at a time point which, based on a 37 -week expected term, is predicted to be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35 or 36 weeks prior to normal birth. In some cases, samples are taken 1-4, 5-8, 9-12 or more than 12 weeks prior to the expected normal birth date.

Alternatively, samples may be taken at around 1 month, around 2 months, around 3 months, around 4 months, around 5 months, around 6 months, around 7 months, around 8 months, or around 9 months prior to the expected normal birth date.

Looked at another way, samples may be taken at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, or 37 weeks of gestation.

Samples may be taken 10 weeks to 13 weeks plus 6 days, 14 weeks to 21 weeks plus 6 days, 22 weeks to 25 weeks plus 6 days, 26 weeks to 29 weeks plus 6 days, 30 weeks to 33 weeks plus 6 days, or more than 34 weeks gestational age. The first sample may be taken around 12-14 weeks. The second sample may be taken between 16-24 weeks.

Samples may be taken in the first, second or third trimester. The first trimester lasts from zero to 13 weeks plus 6 days. The second trimester lasts from 14 weeks to 27 weeks plus 6 days. The third trimester lasts from 28 weeks until birth.

The skilled person will appreciate that it may be difficult to precisely determine the number of weeks of gestation. Methods for estimating the number of weeks of gestation are known in the art, and any of these may be used in the methods disclosed herein. For example, weeks of gestation are commonly estimated based on the date of the last menstrual period (LMP). The weeks of gestation may be determined based on the date on which the last menstrual period began. Alternatively, weeks of gestation may be based on the date of ovulation, if known. Commonly, the date of ovulation is two weeks after the date on which the last menstrual period began. Length of gestation may be determined based on a dating scan. A dating scan is commonly performed between 10 and 13 weeks plus 6 days, based on the date of the first day of the last menstrual period.

Different biomarkers may be more appropriate at different sample times. For example, a biomarker may be useful for determining whether an individual is at risk of preterm birth in a sample obtained from that individual at an early stage, whereas a different biomarker may be useful for determining that an individual is at risk in a sample
5 obtained from that individual at a later stage.

In some cases, samples may be obtained from an individual at multiple time points. For example, a first sample may be obtained in the first trimester, and a second sample may be obtained in the second trimester. Multiple samples may be obtained in order to identify trends or changes in biomarker expression. In some cases, a sample may be
10 obtained early in the pregnancy, such as in the first trimester, so as to establish a control or baseline level of biomarker for that individual.

Proteins and Polypeptides

Whilst the methods of the present invention may involve the detection of full-length
15 protein sequences, this is not always necessary. As an alternative, homologues, mutants, derivatives, isoforms, splice-variants or fragments of the full-length polypeptide may be detected.

Derivatives include variants of a given full-length protein sequence and include naturally occurring allelic variants and synthetic variants which have substantial amino
20 acid sequence identity to the full-length protein.

Protein fragments may be up to 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 or 150 amino acid residues long. Minimum fragment length may be 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or 30 amino acids or a number of amino acids between 3 and 30.

25 Mutants may comprise at least one modification (e.g. addition, substitution, inversion and/or deletion) compared to the corresponding wild-type polypeptide. The mutant may display an altered activity or property, e.g. binding.

Mutations may occur in any of the biomarker proteins and components containing such fragments may serve the purpose of modulating the activity of the mutant to restore,
30 completely or partially the activity of the wild-type polypeptide.

Derivatives may also comprise natural variations or polymorphisms which may exist between individuals or between members of a family. All such derivatives are included within the scope of the invention. Purely as examples, conservative replacements which may be found in such polymorphisms may be between amino acids within the
35 following groups: alanine, serine, threonine; glutamic acid and aspartic acid; arginine

and leucine; asparagine and glutamine; isoleucine, leucine and valine; phenylalanine, tyrosine and tryptophan.

In this specification, a biomarker may be any peptide, polypeptide or protein having an amino acid sequence having a specified degree of sequence identity to one of the biomarker sequences, or to a fragment of one of these sequences. The specified degree of sequence identity may be from at least 60% to 100% sequence identity. More preferably, the specified degree of sequence identity may be one of at least 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% 99% or 100% identity.

The features disclosed in the foregoing description, or in the following claims, or in the accompanying drawings, expressed in their specific forms or in terms of a means for performing the disclosed function, or a method or process for obtaining the disclosed results, as appropriate, may, separately, or in any combination of such features, be utilised for realising the invention in diverse forms thereof.

While the invention has been described in conjunction with the exemplary embodiments described above, many equivalent modifications and variations will be apparent to those skilled in the art when given this disclosure. Accordingly, the exemplary embodiments of the invention set forth above are considered to be illustrative and not limiting. Various changes to the described embodiments may be made without departing from the spirit and scope of the invention.

For the avoidance of any doubt, any theoretical explanations provided herein are provided for the purposes of improving the understanding of a reader. The inventors do not wish to be bound by any of these theoretical explanations.

Any section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described.

Throughout this specification, including the claims which follow, unless the context requires otherwise, the word "comprise" and "include", and variations such as "comprises", "comprising", and "including" will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

It must be noted that, as used in the specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Ranges may be expressed herein as from "about" one particular value, and/or to "about" another particular value. When such a range is expressed, another embodiment includes from the one particular value and/or to the other particular value. Similarly, when values are expressed as approximations, by the use of the antecedent

“about,” it will be understood that the particular value forms another embodiment. The term “about” in relation to a numerical value is optional and means for example +/- 10%.

Examples

5 EXAMPLE 1

CVF (cervicovaginal fluid) samples were collected from pregnant women at 19-37 weeks of gestational age. A sterile bivalve speculum was inserted into patients' vagina. A dual-tipped swab was placed in the posterior fornix of the vagina for 30 seconds and then placed into 1 mL of chilled CVF extraction buffer (50mM HEPES, 150mM NaCl,
10 0.1% SDS, 1mM EDTA, 1mM Pefabloc SC 4-(2-aminoethyl)benzene sulfonyl fluoride (AEBSF)). Samples were vortexed for 10s, after which the swab was inverted and was centrifuged for 5min at 1000xg. The swab was discarded, and the sample tube was vortexed for 10s before centrifugation at 1000xg for 5min. The extracted CVF (supernatant) was aliquoted into tubes and stored at -80°C until required.

15 Seven protein biomarkers were tested in the 200 CVF samples which were obtained from 86 patients who eventually had term and preterm deliveries (ECM1, GGH, LAMC2, EFEMP1, PTN, FGA and PEDF). The samples were collected longitudinally from 19-38 weeks of gestation. The biomarker expression level in the CVF samples were measured using commercial ELISA kits, namely, PEDF (DuoSet, #DY1177-05, R&D
20 Systems, Minneapolis, MN), ECM1 (#ELH-ECM1-1, Raybiotech), GGH (#EH4206, Wuhan Fine Biotech), LAMC2 (#SEC083Hu, Cloud-clone), PTN (#23437, LSBIO), FGA (#11466, LSBIO), EFEMP1 (#MBS178533, MyBioSource). Samples were run as duplicates in a standard 96- well plate alongside a reference control and a standard protein at known concentration.

25 ELISA protocols were assayed based on the manufacturers' manual. Below described the general protocol:

Coat 96 wells with 100 µl Capture Antibody, at a concentration of between 0.8-10µg/ml in coating buffer. Cover the plate and incubate overnight at 4°C.

30 Add 300 µl of blocking solution to each well. Incubate for 60 minutes. Wash plate three times with wash buffer and dry by tapping inverted plate on dry paper.

Add 100 µl of Standard protein in serial dilutions and properly diluted samples. Samples or standards are run in duplicates and incubated for 90 min at 37°C. Wash plate three times with wash buffer and dry by tapping inverted plate on dry paper.

35 Add 100 µl of biotin-conjugated detection antibody, diluted in reagent diluent or appropriate buffer and incubate for 1 hour at 37°C. Wash plate three times with wash buffer.

Add 100 µl of enzyme-conjugated streptavidin, diluted in reagent diluent or appropriate buffer and incubate for 60 minutes at 37°C. Wash plate three times with wash buffer and dry by tapping inverted plate on dry paper.

5 Add 100 µl of the appropriate substrate solution to each well. Incubate at 37°C for up to 20 minutes, or until desired colour change is attained.

Read absorbance values immediately at the appropriate wavelength or add 50 µl of "stop solution". Gently tap plate to ensure thorough mixing. Measure absorbance at 450nm and referenced at 540nm.

10 The biomarker concentration was determined based on the standard curve run on every plate as either a linear or 4 Parameter Logistic (4PL) standard curve. The final concentration was normalized based on the total protein concentration determined by bicinchoninic acid assay (BCA assay).

The values and data of samples were then assembled to compare the Term and Preterm results stratifying based on 3 principal methods:

- 15
- Entire group in which the entire cohort (n=200) of Term (n=136) and Preterm (n=64) were assessed for difference in mean and demonstrating p-values obtained from Student's t-test analysis.
 - Gestational week- samples grouped based on the gestational week at sampling
 - 20 • Time from delivery- Samples were grouped based on the number of days between the sample and delivery.

Statistical analysis.

25 For statistical analysis, two-tailed unpaired Student's t-test was performed at confidence interval (CI)= 0.95 using the Microsoft Excel software, with p-value (P) of less than 0.05 considered significant. All numerical data including error bars represent the mean +/- Standard error of mean (SEM).

Results and discussion.

30 The biomarker quantification on the 200 clinically- derived samples demonstrated a difference between term and preterm samples. Further stratification of the samples enabled the emphasis for potential time points in gestation that will enable a better understanding of preterm birth risk base.

35 ECM1 was differentially expressed between all 200 term and preterm samples collected with a p-value of $P=0.0025$. Upon stratification of the samples into different gestational

ages and different time from sampling to delivery, the differential expression remained in the same direction (i.e. ECM1 was expressed less on average in all preterm samples regardless of gestational age and time to delivery). As ECM1 is a known marker in several skin-related disorder and angiogenesis, it is therefore somewhat surprising for its correlation with preterm birth.

GGH was differentially expressed between term and preterm samples. The expression of GGH was on average elevated in samples from preterm women vs samples from term women. Interestingly, in both term and preterm cases, there was an incremental increase in expression levels as gestational age progressed and time to delivery declined. GGH is not a widely known biomarker, it is involved in immune pathways and extracellular matrix regulation.

LAMC2 was differentially expressed between all 200 term and preterm samples. In contrast to the other markers, the difference was more pronounced towards the last days before delivery. LAMC2 is involved in epithelial transition pathways and is known for its involvement in several skin disease indications. Intriguingly, it has never been associated with changes in cervical vaginal space.

EFEMP1 was also differentially expressed between all 200 term and preterm samples. However, more interestingly there was a transition between elevated expression and reduced expression of EFEMP1 throughout gestation between term and preterm samples. In the early timepoints of both gestational age and time to delivery, EFEMP1 term samples were elevated in comparison to the preterm samples. However, this trend was reversed in later timepoints of gestational ages and time to delivery.

PTN was differentially expressed between all 200 term and preterm samples. The most pronounced difference in the expression profile of PTN between term and preterm samples was observed in the earliest gestational ages, and in the earliest as well as the latest timepoints in time to delivery.

FGA was differentially expressed between all 200 term and preterm samples. Differences in FGA expression was more pronounced in late stages of gestation and towards the last days before delivery. FGA as a protein is involved in inflammation and immune response pathways and thus might relate to preterm birth cases initiated by such pathways.

PEDF was differentially expressed between all 200 term and preterm samples. PEDF was consistently elevated in the preterm samples in all stratifications, indicating that this would be a robust biomarker at any time point. PEDF is a protein tightly related to angiogenesis and thus remodelling of tissue. We hypothesize that its involvement in preterm birth is related to cervical remodelling.

The current state of predicting women at risk for preterm birth is quite limited. The two most common ways to achieve a risk profile are based on prior history and cervical length measurements. These methods fail to correctly assess the risk of preterm birth in the majority of women even when used in combination. Thus, a tool that would accurately predict women at risk of preterm birth would be a great asset to the clinical community in managing pregnancies, and would further allow the reduction of preterm birth cases and saving on the significant health care costs. Here, we demonstrate the corner stones for such a tool via individual biomarkers. As we see it, these biomarkers, ECM1, FGA, EFEMP1, GGH, PEDF, PTN and LAMC2 lay the foundation for a kit that would combine the predictive values of the individual biomarkers, generating a highly accurate tool.

EXAMPLE 2:

CVF (cervicovaginal fluid) samples were collected from pregnant women at 16-24 weeks of gestational age.

CVF samples are collected by research midwives from consenting pregnant women prior to any cervical examination or procedure. The cervix is visualized using a sterile speculum and a sterile double-tipped swab is inserted into the posterior vaginal fornix for 30s. To extract the proteins from the CVF, both tips of the swab are placed into a 5mL tube containing 1mL of CVF extraction buffer (100mM Tris, 150mM NaCl, 1mM EGTA, 1mM EDTA, 0.1% Triton X-100, 1mM Pefabloc SC 4-(2-aminoethyl)benzene sulfonyl fluoride (AEBSF)) followed by a brief vortex. The double-tipped end is inverted with the tube using sterile forceps and centrifuged at 1000xg for 5mins at 4°C. The swab is removed and the sample briefly vortexed and centrifuged at 1000xg for 5mins at 4°C, followed by aliquoting into 8 PCR tubes for storage at -80°C.

Individual biomarker levels were determined by ELISA and results stratified by patient delivery date of less than 35 weeks or less than 37 weeks, both being preterm.

To determine effectiveness of biomarker pairs vs. individual biomarkers a combinatorial algorithm was performed (level of biomarker 1 * level of biomarker 2), and *p*-value determined using Student's *t*-test. Receiver Operating Characteristics (ROC) curve and area under the curve were also determined.

Results for subjects who delivered at less than 35 weeks are shown in Figures 1 to 7, and are summarised in Table 1. Results for subjects who delivered at less than 37

weeks are shown in Figures 8 to 14, and are summarised in Table 2. All pair combinations demonstrated an improvement in *P*-value that is more than additive compared to the respective individual biomarkers.

- 5 **Table 1:** *P*-values of individual biomarkers and pair combinations, gestational age at delivery < 35 weeks.

Biomarker	<i>P</i> -value
PEDF	0.187
GGH	0.0747
LAMC2	0.000892
ECM1	0.0641
PEDF*GGH	0.0177
PEDF*LAMC2	0.000000343
PEDF*ECM1	0.0011
GGH*LAMC2	0.000000757
GGH*ECM1	0.0090
LAMC2*ECM1	0.0000000254

- 10 **Table 2:** *P*-values of individual biomarkers and pair combinations, gestational age at delivery < 37 weeks.

Biomarker	<i>P</i> -value
PEDF	0.150
GGH	0.0134
LAMC2	0.0000489
ECM1	0.047783
PEDF*GGH	0.0067
PEDF*LAMC2	0.000000329
PEDF*ECM1	0.0025
GGH*LAMC2	0.000000772
GGH*ECM1	0.0115
LAMC2*ECM1	0.00000192

References

A number of publications are cited above in order to fully describe and disclose the invention and the state of the art to which the invention pertains. Full citations for these references are provided below. The entirety of each of these references is incorporated herein.

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- 40

For standard molecular biology techniques, see Sambrook, J., Russel, D.W. *Molecular Cloning, A Laboratory Manual*. 3 ed. 2001, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press

Claims:

1. A method for predicting whether an individual is at risk of preterm birth, the method comprising determining in a sample obtained from the individual the level of any two
5 biomarkers selected from the group consisting of PEDF, GGH, LAMC2 and ECM1.
2. A method for predicting whether an individual is at risk of preterm birth, the method comprising determining the level of each biomarker of a biomarker pair in a sample obtained from the individual, and predicting whether the individual is at risk of
10 preterm birth based on the level of the biomarkers, wherein the biomarker pair is selected from:
 PEDF and GGH;
 PEDF and LAMC2;
 PEDF and ECM1;
15 GGH and LAMC2;
 GGH and ECM1; or
 LAMC2 and ECM1.
3. The method of claim 1 or 2, wherein the sample is a sample of vaginal fluid.
20
4. The method of any one of claims 1 to 3, wherein each biomarker determined is a protein or polypeptide.
5. The method of any one of claims 1 to 4, wherein the level of one, each or both
25 biomarkers is compared to a reference level, wherein the reference level is derived from the level of one, each or both biomarkers in a sample obtained from an individual known to have experienced preterm or term birth.
6. The method according to any one of claims 1 to 5, wherein the method further
30 comprises predicting the risk of preterm birth with one or more other indicators of preterm birth, selected from the Fetal Fibronectin (fFN) test, a short cervical length, contractions, vaginal bleeding, fluid leaking from the vagina, increased vaginal discharge, backache and cramping in lower abdomen.

7. Progesterone for use in the treatment of an individual predicted to be at risk of preterm birth, wherein the individual has been predicted to be at risk of preterm birth by a method according to any one of claims 1 to 5.

8. A method for selecting an individual for treatment to reduce the risk of preterm birth, the method comprising predicting the risk of preterm birth in the individual using a method according to any one of claims 1 to 5 and, if the individual is determined to be at risk of preterm birth, administering a treatment to reduce the risk of preterm birth, wherein the treatment to reduce the risk of preterm birth comprises progesterone and/or cervical cerclage and/or vaginal pessary.

9. A method for predicting whether an individual is at risk of preterm birth, the method comprising determining in a sample obtained from the individual the level of any two biomarkers selected from the group consisting of PEDF, GGH, LAMC2 and ECM1 and transmitting the determined levels to a physician involved in the treatment of the individual, wherein the risk of preterm birth is predicted based on the level of the biomarkers in the sample.

10. A method for predicting whether an individual is at risk of preterm birth, the method comprising determining the level of each biomarker of a biomarker pair in a sample obtained from the individual, and transmitting the determined levels to a physician involved in the treatment of the individual, wherein the risk of preterm birth is predicted based on the level of the biomarkers in a biomarker pair in the sample, and wherein the biomarker pair is selected from:

PEDF and GGH;
PEDF and LAMC2;
PEDF and ECM1;
GGH and LAMC2;
GGH and ECM1; or
LAMC2 and ECM1.

11. The method according to any one of claims 1 to 6, 9 or 10, wherein the method for predicting whether the individual is at risk of preterm birth is a computer implemented method.

12. A method for detecting a biomarker pair selected from PEDF and GGH; PEDF and LAMC2; PEDF and ECM1; GGH and LAMC2; GGH and ECM1; or LAMC2 and ECM1, the method comprising:

a. obtaining a sample of vaginal fluid from an individual;

b. detecting whether each biomarker in a said biomarker pair is present in the vaginal fluid sample by contacting the vaginal fluid sample with one or more antibodies selected from an anti-PEDF antibody, an anti-GGH antibody, an anti-LAMC2 antibody, and/or an anti-ECM1 antibody, and detecting binding between at least one of PEDF, GGH, LAMC2 or ECM1 and a respective said antibody.

13. A method for determining that an individual is at risk of preterm birth, said method comprising:

a. obtaining a sample from an individual;

b. detecting whether each biomarker in a biomarker pair is present in the sample, by contacting the sample with one or more antibodies selected from an anti-PEDF antibody, an anti-GGH antibody, an anti-LAMC2 antibody, and/or an anti-ECM1 antibody, and detecting binding between at least one of PEDF, GGH, LAMC2 or ECM1 and a respective said antibody, wherein said biomarker pair is selected from PEDF and GGH; PEDF and LAMC2; PEDF and ECM1; GGH and LAMC2; GGH and ECM1; or LAMC2 and ECM1; and

c. determining that the individual is at risk of preterm birth when the presence of a said biomarker pair in the sample is detected.

14. The method of claim 13 wherein the sample is a vaginal fluid sample.

15. A method of determining that an individual is at risk of preterm birth and prolonging gestation in that individual, the method comprising:

a. obtaining a vaginal fluid sample from an individual;

b. detecting whether each biomarker in a biomarker pair is present in the vaginal fluid sample, wherein said biomarker pair is selected from PEDF and GGH; PEDF and LAMC2; PEDF and ECM1; GGH and LAMC2; GGH and ECM1; or LAMC2 and ECM1;

c. determining that the individual is at risk of preterm birth when the presence of said biomarker pair in the vaginal fluid sample is detected; and

d. administering an effective amount of progesterone to the individual determined to be at risk of preterm birth or selecting the individual for treatment with an effective amount of one or more agents selected from a progesterone or an analogue thereof, a tocolytic, a corticosteroid, an

antibiotic, an NSAID or an Omega 3 fatty acid or derivative thereof if the individual is determined to be at risk of preterm birth; and/or

- e. performing cervical cerclage on the individual determined to be at risk of preterm birth or selecting the individual for cervical cerclage, if the individual is determined to be at risk of preterm birth.

16. The method according to any one of claims 12 to 15, wherein the level of one or both biomarkers in a biomarker pair is determined by enzyme-linked immunosorbent assay (ELISA).

17. A kit, optionally for use in predicting the risk or likelihood of preterm birth in a subject, wherein the kit comprises two different antibodies each selected from the group consisting of: an anti-PEDF antibody, an anti-GGH antibody, an anti-LAMC2 antibody, an anti-ECM1 antibody.

18. A kit according to claim 17, wherein the kit comprises:

an anti-PEDF antibody and an anti-GGH antibody;

an anti-PEDF antibody and an anti-LAMC2 antibody;

an anti-PEDF antibody and an anti-ECM1 antibody;

an anti-GGH antibody and an anti-LAMC2 antibody;

an anti-GGH antibody and an anti-ECM1 antibody; or

an anti-LAMC2 antibody and an anti-ECM1 antibody.

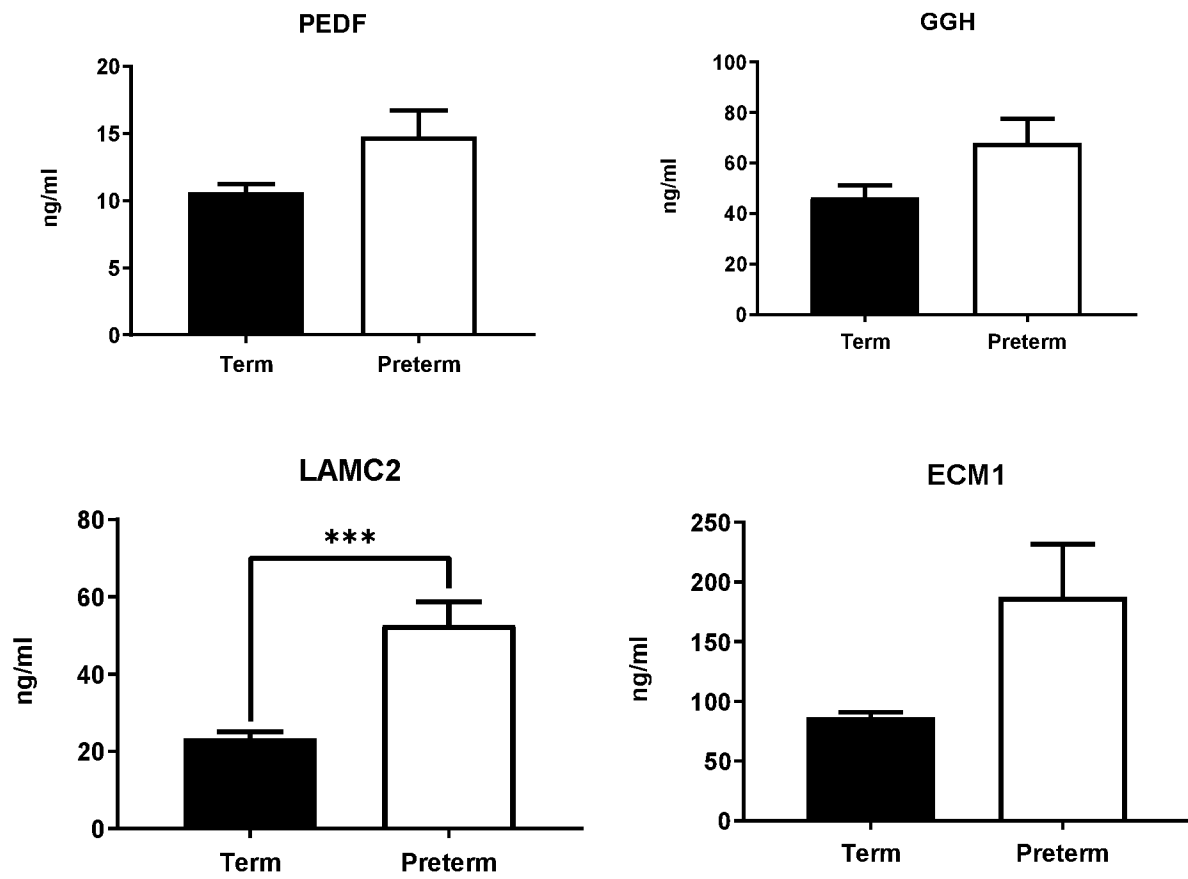
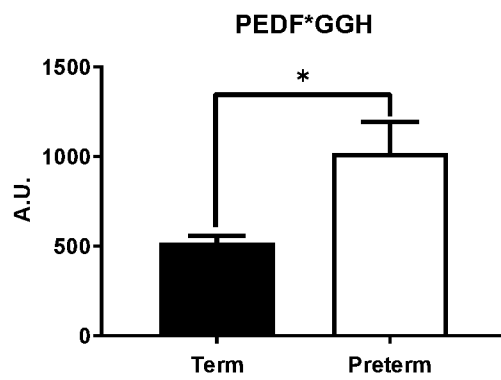
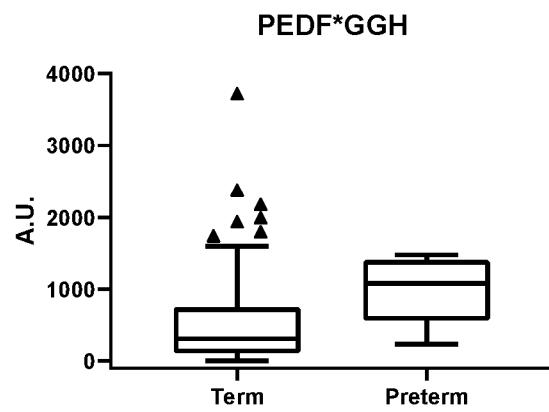


Figure 1

(A)



(B)



(C)

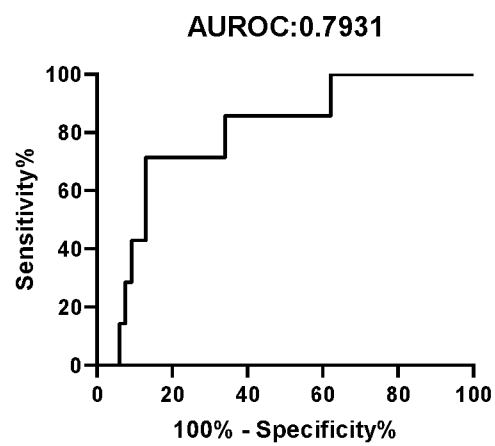
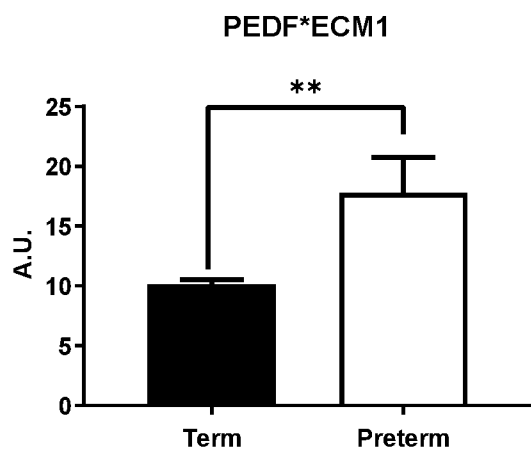
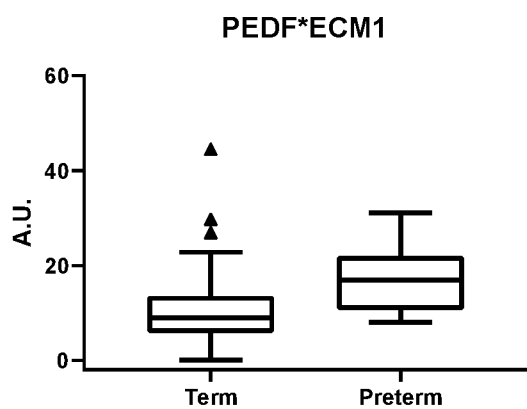


Figure 2

(A)



(B)



(C)

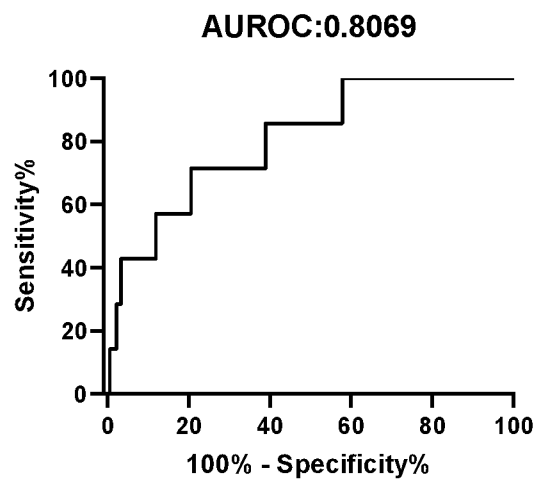
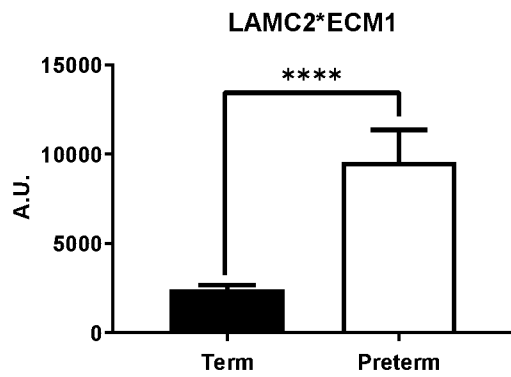
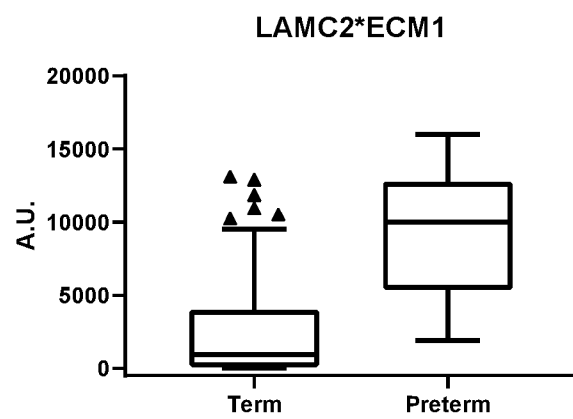


Figure 3

(A)



(B)



(C)

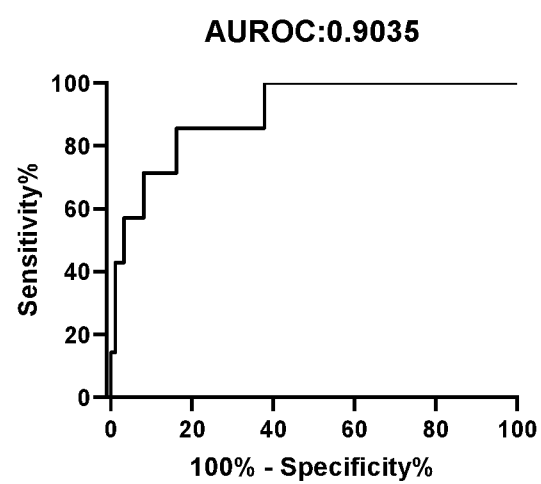
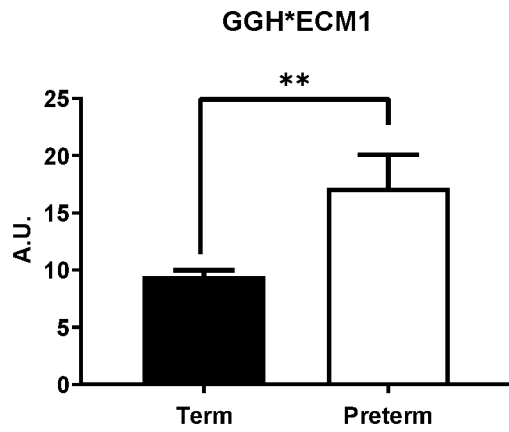
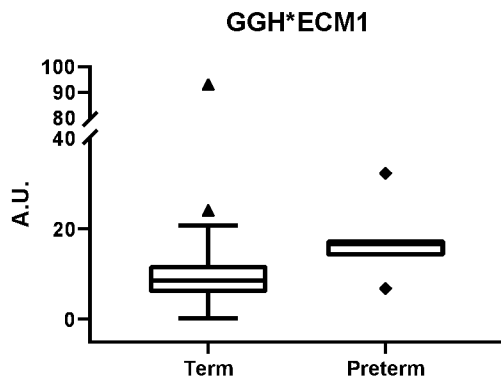


Figure 4

(A)



(B)



(C)

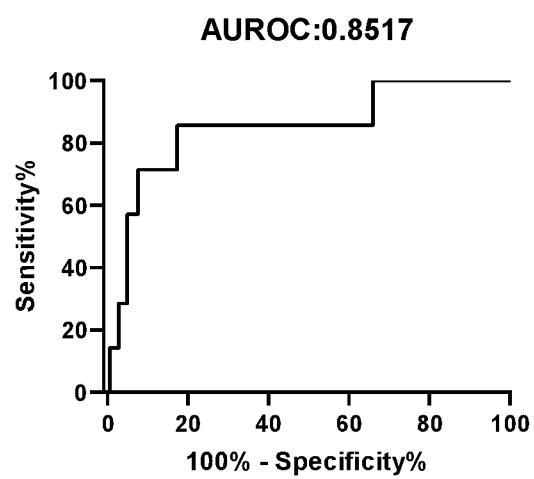
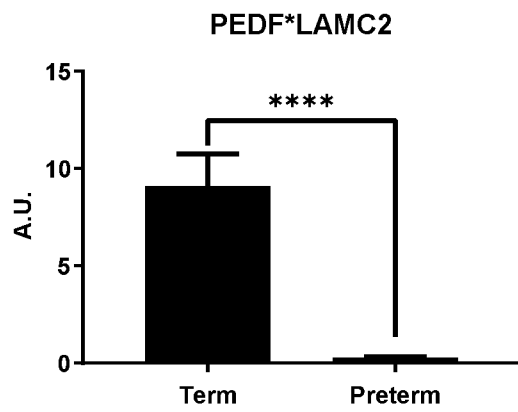


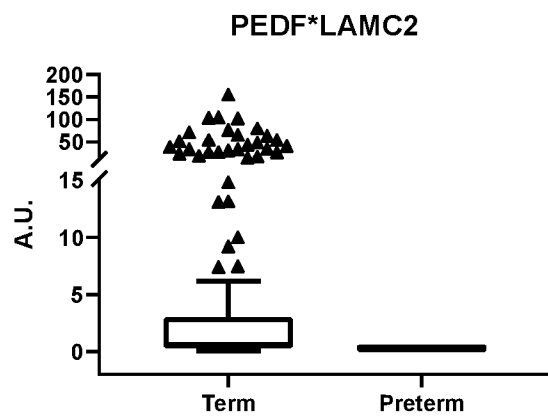
Figure 5

6/14

(A)



(B)



(C)

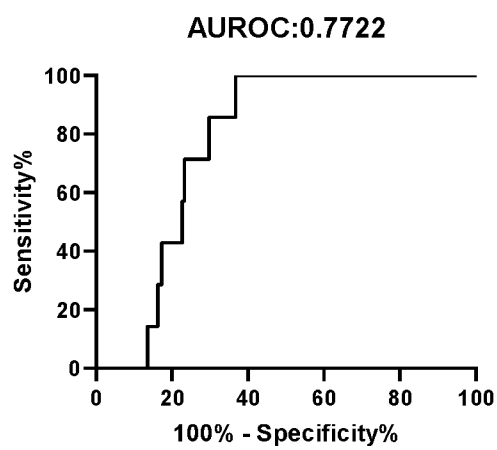
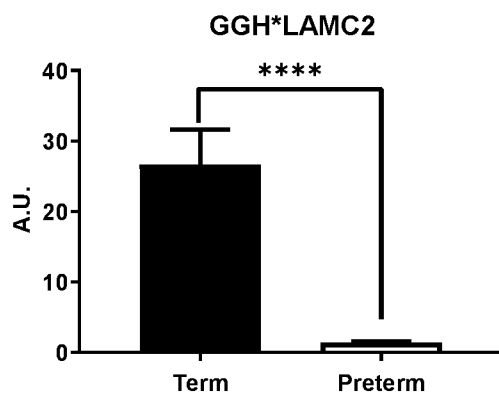


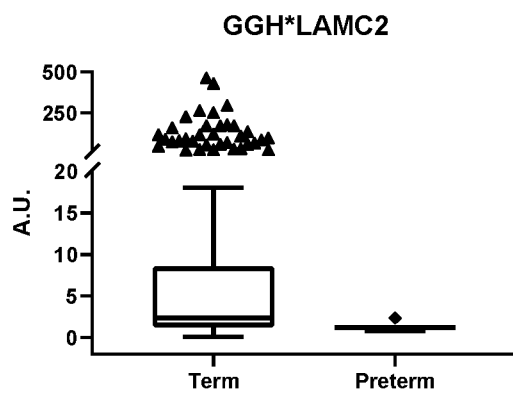
Figure 6

7/14

(A)



(B)



(C)

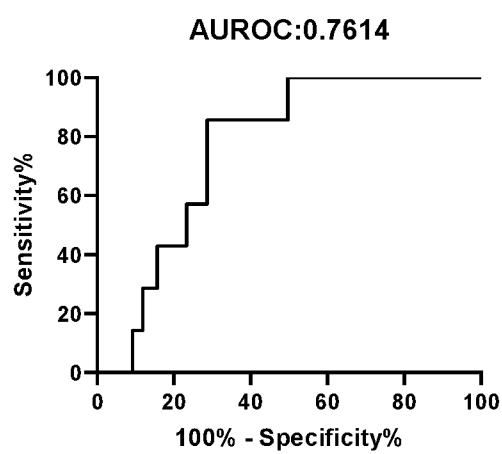


Figure 7

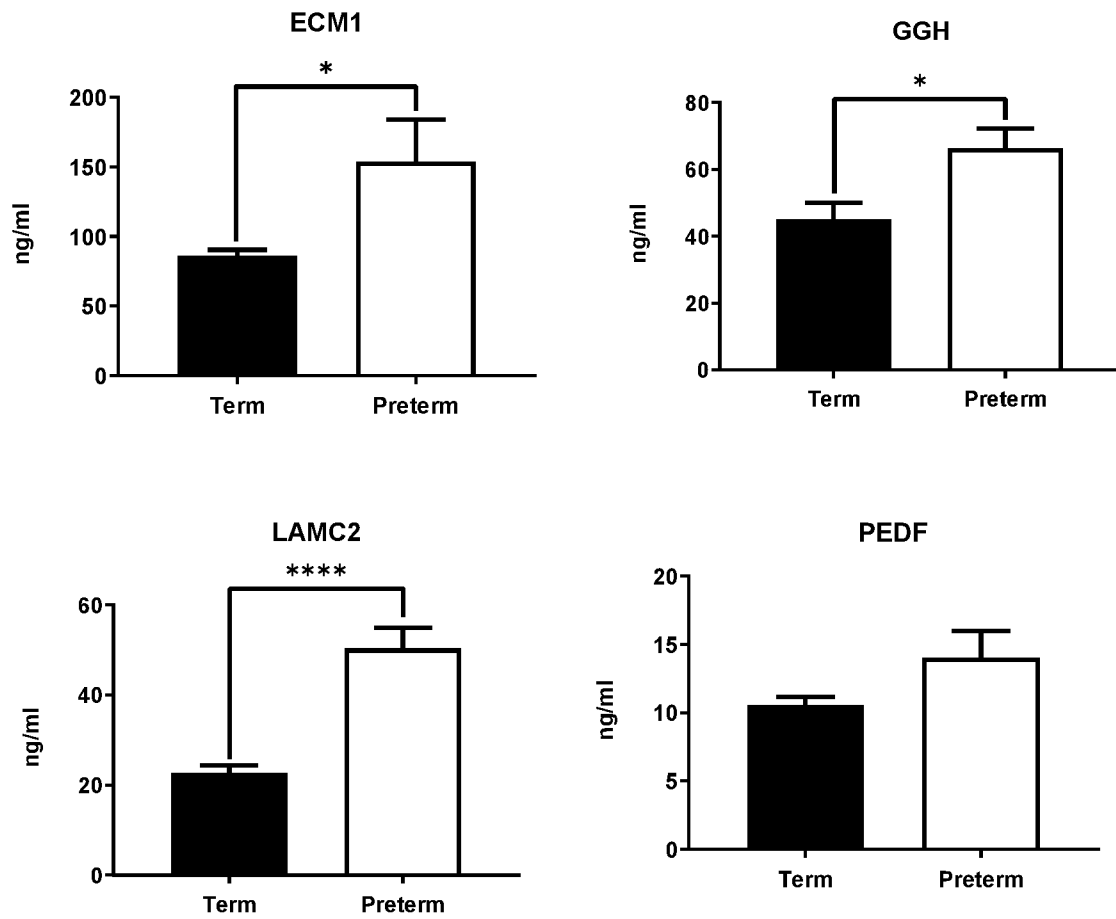
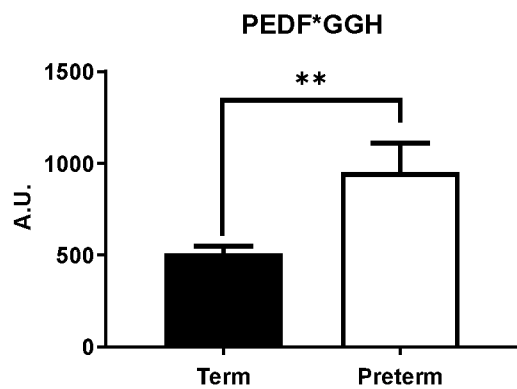


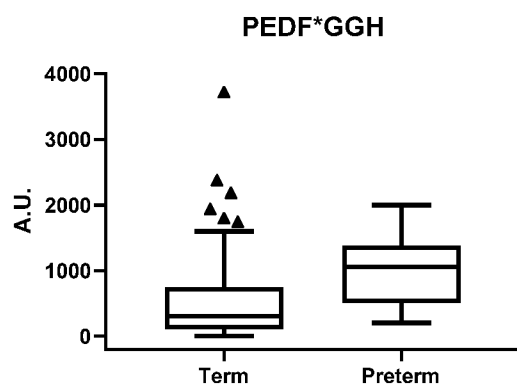
Figure 8

9/14

(A)



(B)



(C)

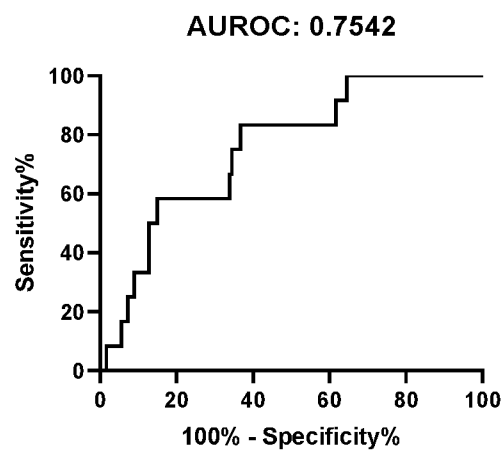
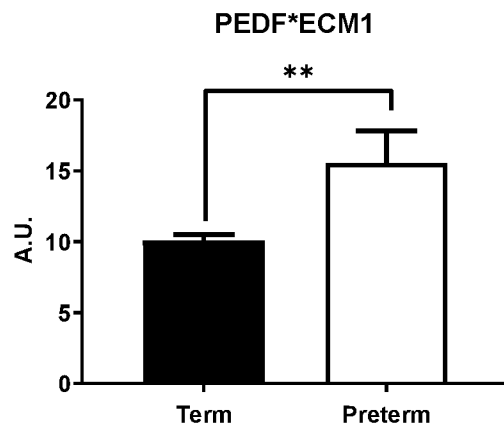


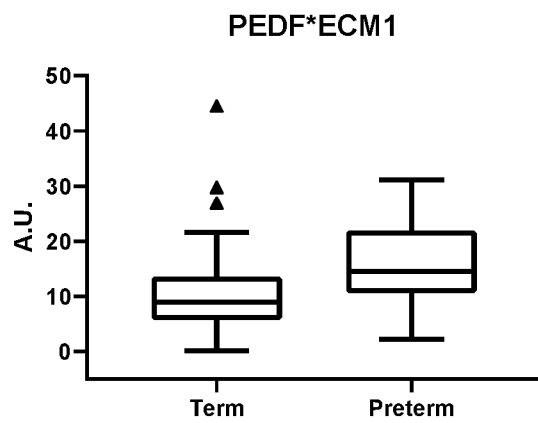
Figure 9

10/14

(A)



(B)



(C)

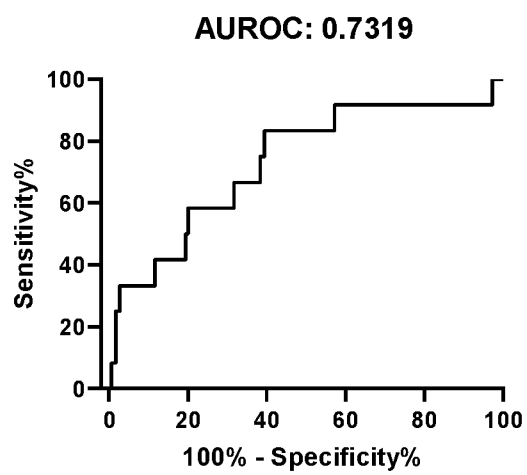
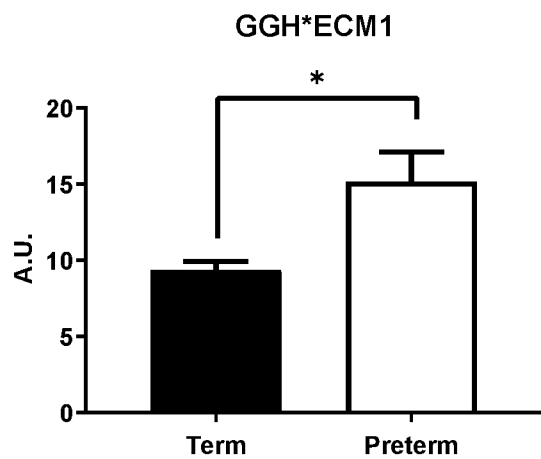


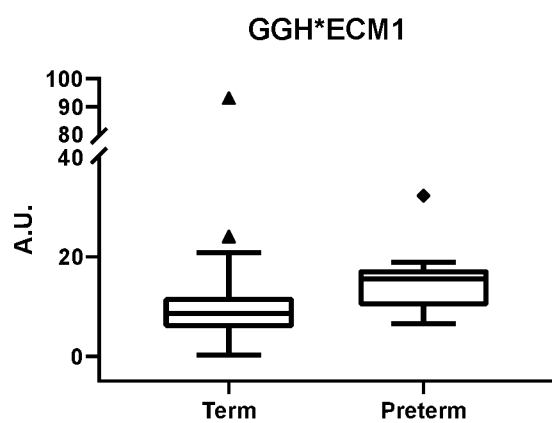
Figure 10

11/14

(A)



(B)



(C)

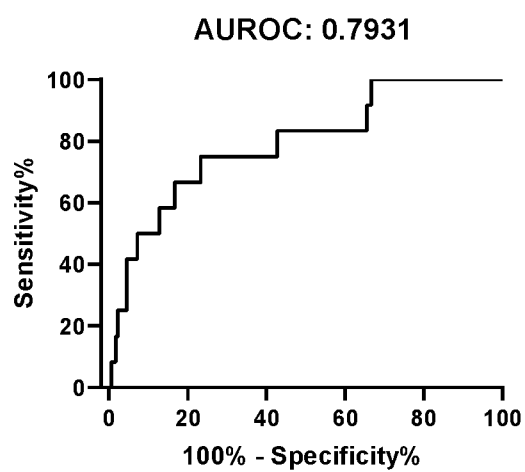
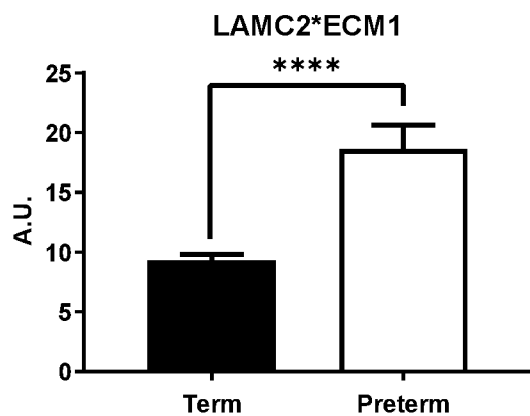


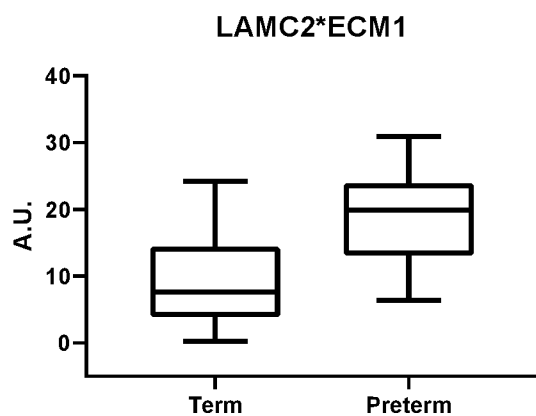
Figure 11

12/14

(A)



(B)



(C)

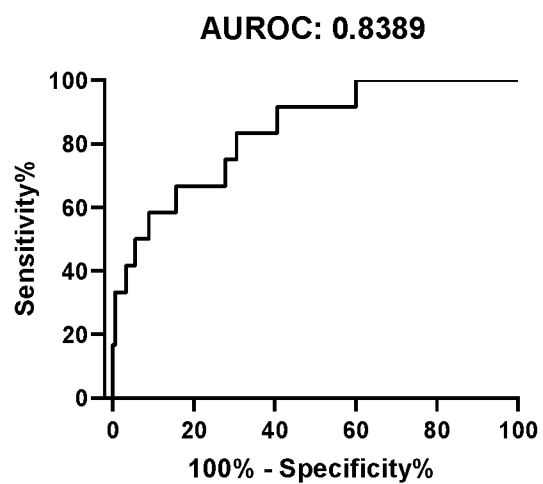
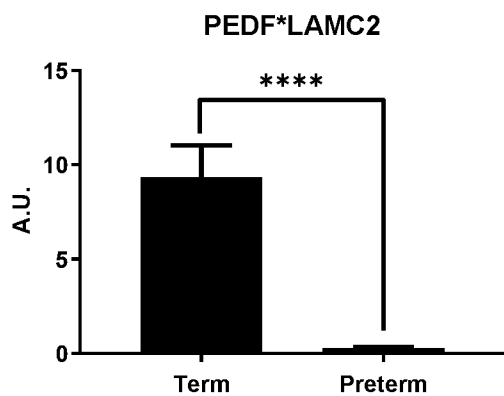
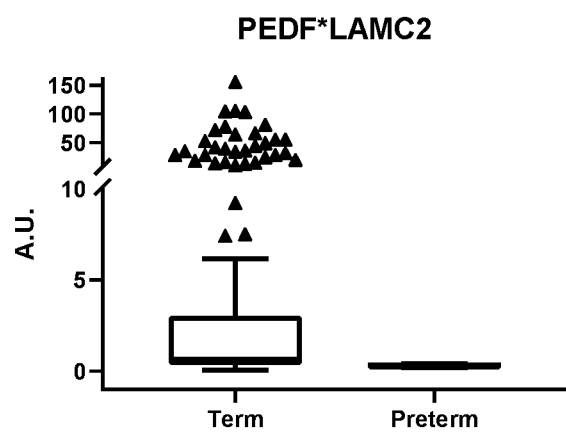


Figure 12

(A)



(B)



(C)

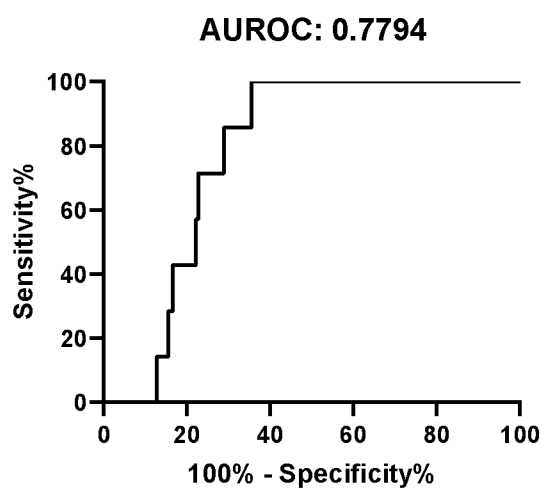
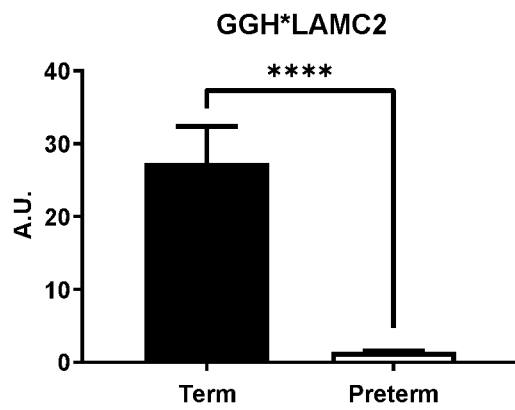
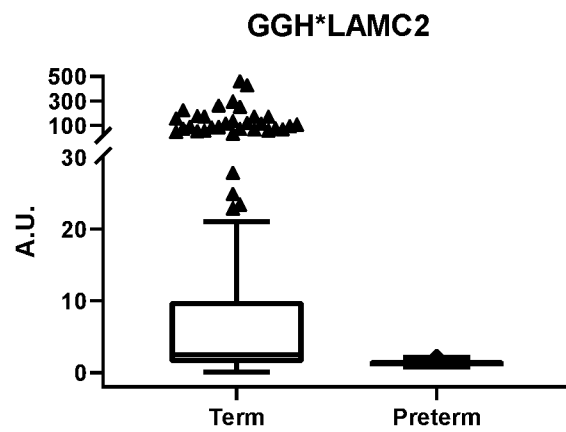


Figure 13

(A)



(B)



(C)

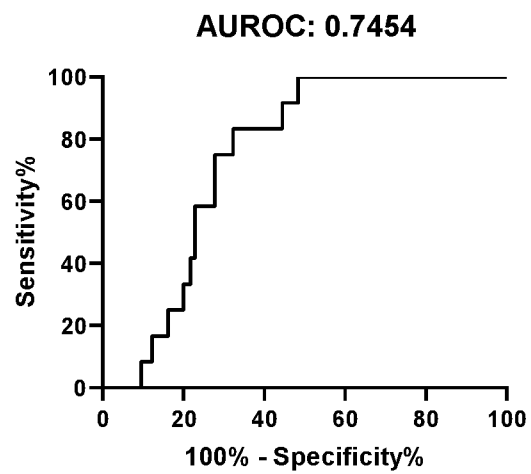


Figure 14

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2020/059597

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	S LIONG ET AL: "New biomarkers for the prediction of spontaneous preterm labour in symptomatic pregnant women: a comparison with fetal fibronectin", BJOG: AN INTERNATIONAL JOURNAL OF OBSTETRICS AND GYNAECOLOGY, vol. 122, no. 3, 24 July 2014 (2014-07-24), pages 370-379, XP055706983, GB ISSN: 1470-0328, DOI: 10.1111/1471-0528.12993 -----	1-16
X	US 2017/022565 A1 (BONIFACE JOHN JAY [US] ET AL) 26 January 2017 (2017-01-26) par.158, 159, 165, examples 5 and 11, figures, claims) ----- -/-	1-16



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

3 July 2020

Date of mailing of the international search report

08/09/2020

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Behrens, Ralf

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2020/059597

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SULLIVAN S APEREIRA L MDUFFORD M T ET AL: "Second trimester prediction of SPTB phenotypes: PPR0M vs. PTL", REPRODUCTIVE SCIENCES, SAGE PUBLICATIONS, INC, US, vol. 25, no. 1, 1 March 2018 (2018-03-01), page 184A, XP009511922, ISSN: 1933-7205 abstract -----	1-16

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2020/059597

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-16(partially)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-16(partially)

Method for predicting whether an individual is at risk of PTB based on the detection the pair of biomarkers of PEDF and GGH.

2. claims: 1-16(partially)

Method for predicting whether an individual is at risk of PTB based on the detection the pair of biomarkers of PEDF and LAMC2.

3. claims: 1-16(partially)

Method for predicting whether an individual is at risk of PTB based on the detection the pair of biomarkers of PEDF and ECM1.

4. claims: 1-16(partially)

Method for predicting whether an individual is at risk of PTB based on the detection the pair of biomarkers of GGH and another protein selected from the list of claim 1.

5. claims: 1-16(partially)

Method for predicting whether an individual is at risk of PTB based on the detection the pair of biomarkers of LAMC2 and another protein selected from the list of claim 1

6. claims: 1-16(partially)

Method for predicting whether an individual is at risk of PTB based on the detection the pair of biomarkers of ECM1 and another protein selected from the list of claim 1.

7-9. claims: 17, 18(partially)

Kits for the detection of PEDF and one other biomarker selected from the list of claim 17.

10. claims: 17, 18(partially)

Kits for the detection of GGH and one other biomarker selected from the list of claim 17.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

11. claims: 17, 18(partially)

Kits for the detection of LAMC2 and one other biomarker
selected from the list of claim 17.

12. claims: 17, 18(partially)

Kits for the detection of ECM1 and one other biomarker
selected from the list of claim 17.

INTERNATIONAL SEARCH REPORT

Information on patent family members

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