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(54) **DIAGNOSTIC BIOMARKER TO PREDICT WOMEN AT RISK FOR PRETERM DELIVERY**  
DIAGNOSTISCHER BIOMARKER ZUR PROGNOSE DES RISIKOS VON FRÜHGEBURTEN  
BIOMARQUEUR DE DIAGNOSTIC POUR PRÉDIRE UN RISQUE D'ACCOUCHEMENT PRÉMATURÉ

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**Description**

**FIELD OF INVENTION**

5 [0001] This invention relates to preterm delivery. More specifically, the invention provides biomarkers and methods of using biomarkers for determining preterm delivery risk.

**BACKGROUND**

10 [0002] The following description includes information that may be useful in understanding the present invention. It is not an admission that any of the information provided herein is prior art or relevant to the presently claimed invention, or that any publication specifically or implicitly referenced is prior art.

15 [0003] Preterm delivery is one of the most important fetal health problems in the United States today. Approximately one in eight newborns is delivered preterm and the incidence of prematurity has not decreased in the last 20 years. Most preterm babies, if they survive, often have cardiac, neurologic, ophthalmic, and gastrointestinal problems that can extend even beyond childhood, and perhaps lead to adult diseases such as atherosclerosis. Currently, there are few, if any diagnostic biomarkers available that effectively identify women who are going to deliver preterm. Biomarkers that are able to identify these women at risk would be useful in the deployment of prevention/treatment strategies and to assess the effectiveness of these treatments. Thus, there is a need to develop novel diagnostics that may identify women who will deliver preterm. Especially, diagnostic biomarkers that may be detected in non-pregnant women or in women during the first trimester. The biomarkers may also be used later in the pregnancy to assess the effectiveness of treatment and/or prevention strategies.

**SUMMARY OF THE INVENTION**

25 [0004] The invention provides an *ex vivo* method of identifying a woman at risk or diagnosing susceptibility of a woman for preterm delivery, comprising:

- 30 (a) stimulating peripheral blood mononuclear cells ("PBMCs") from a woman using an antigen and/or stimulant;
- (b) determining the secretion level of a combination of cytokines from the stimulated PBMCs, wherein at least two cytokines in the combination are selected from IL-10, IL-13, and IL-1RA;
- 35 (c) comparing the secretion level of the combination measured in (b) with the secretion level of the same combination of cytokines from control PBMCs isolated from women who delivered at full term and stimulated using the same antigen and/or stimulant;
- (d) determining whether the secretion level measured in (b) is higher or lower than the secretion level from the control PBMCs;

40 thereby identifying whether the woman is at risk for preterm delivery.

[0005] The following embodiments and aspects thereof are described and illustrated in conjunction with compositions and methods which are meant to be exemplary and illustrative, not limiting in scope.

45 [0006] A method is disclosed, comprising: obtaining a biological sample from a woman who desires an assessment of her risk for preterm delivery; exposing the biological sample *ex vivo* to an antigen and/or a stimulant; and assessing an immune response to predict whether the woman is at risk for preterm delivery. In various embodiments, the biological sample can be whole blood.

[0007] In various embodiments, the method can further comprise separating blood cells from the sample and exposing the separated blood cells to the antigen.

50 [0008] In various embodiments, exposing the biological sample *ex vivo* to the antigen or the stimulant can be by collecting the biological sample into a container coated with the antigen and/or stimulant.

[0009] In various embodiments, the method can further comprise removing plasma from the biological sample and assaying the plasma to assess the immune response. In various embodiments, removing plasma from the biological sample can comprise centrifuging the tube comprising the biological sample and harvesting the plasma.

55 [0010] In various embodiments, the antigen and/or stimulant can be cortisol, LPS, phytohemagglutinin, a maternal histocompatibility antigen, a paternal histocompatibility antigen, a fetal histocompatibility antigen, a microbial antigen, a cytokine, a phorbol ester, an agent capable of inducing Th1 or Th2 type differentiation of lymphocytes or combination thereof.

[0011] In various embodiments; assessing the immune response can comprise assaying an expression level of a cytokine, cytokine receptor, cytokine receptor antagonist, chemokine, chemokine receptor, chemokine receptor antagonist, or combinations thereof. In various embodiments, the cytokine, cytokine receptor, cytokine receptor antagonist, chemokine, chemokine receptor, chemokine receptor antagonist, or combinations thereof can be selected from the group consisting of IL-10, IL-13, 1L-1RA and combinations thereof.

[0012] In various embodiments, a higher or lower expression level of the cytokine, cytokine receptor, cytokine receptor antagonist, chemokine, chemokine receptor, and/or chemokine receptor antagonist compared to a control can indicate that the woman is at an increased risk of preterm delivery. In various embodiments, the higher or lower expression level of the cytokine, cytokine receptor, cytokine receptor antagonist, chemokine, chemokine receptor, and/or chemokine receptor antagonist can be at least one to three times higher or lower compared to the levels of the control. In various embodiments, the level of the higher or lower expression of the cytokine, cytokine receptor, cytokine receptor antagonist, chemokine, chemokine receptor, and/or chemokine receptor antagonist can be indicative of a low, medium, or high risk of preterm delivery. In various embodiments, a statistically significantly higher or lower expression of the cytokine, cytokine receptor, cytokine receptor antagonist, chemokine, chemokine receptor, and/or chemokine receptor antagonist compared to a control can indicate that the woman is at an increased risk of preterm delivery.

[0013] In various embodiments, assessing the immune response can comprise using a technique selected from the group consisting of enzyme-linked immunosorbent spot (ELISpot), enzyme-linked immunosorbent assay (ELISA), microarray assay, quantitative BCP, Northern blot assay, Southern blot assay, Western blot assay, immunohistochemical assay, binding assay and combinations thereof.

[0014] A kit is disclosed, comprising: a container comprising an antigen and/or a stimulant; instructions to use the container to collect a biological sample from a woman to detect an immune response; and instructions to determine the risk of preterm delivery of the woman. The kit can further comprise a reagent to detect the immune response. The kit can further comprise a positive control and/or a negative control.

[0015] A method is disclosed, comprising: obtaining a sample of whole blood from a woman who desires an assessment of her risk for preterm birth; exposing the sample ex vivo to an antigen and/or a stimulant by collecting the sample into a container coated with the antigen and/or stimulant; and assessing an immune response to predict whether the woman is at risk for preterm birth. In various embodiments, the antigen and/or stimulant can be Cortisol, LPS, phytohemagglutinin, or combinations thereof. In various embodiments, assessing the immune response can comprise assaying an expression level of a cytokine, cytokine receptor, cytokine receptor antagonist, chemokine, chemokine receptor, chemokine receptor antagonist, or combinations thereof, wherein a statistically significantly higher or lower expression level of the cytokine, cytokine receptor, cytokine receptor antagonist, chemokine, chemokine receptor, and/or chemokine receptor antagonist compared to a control indicates that the woman is at an increased risk of preterm delivery. In various embodiments, the cytokine, cytokine receptor, cytokine receptor antagonist, chemokine, chemokine receptor, chemokine receptor antagonist, or combinations thereof can be selected from the group consisting of IL-10, IL-13, IL-1RA and combinations thereof.

[0016] Other features and advantages of the invention will become apparent from the following detailed description, taken in conjunction with the accompanying drawings, which illustrate, by way of example, various features of embodiments of the invention.

## BRIEF DESCRIPTION OF THE FIGURES

[0017] Exemplary embodiments are illustrated in referenced figures. It is intended that the embodiments and figures disclosed herein are to be considered illustrative rather than restrictive.

[0018] **Figures 1A and 1B** depict graphical representation of expression levels of various biomarkers of preterm individuals as compared to full term individuals in accordance with various embodiments of the present invention. Empirical means and standard errors for each cytokine and treatment group are presented.

## DESCRIPTION OF THE INVENTION

[0019] Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

[0020] Multiple factors lead to preterm delivery; however, immune activation is thought be the final effector pathway that leads to preterm rupture of membranes and contractions. Family and genetic studies suggest that prematurity runs in families and history of prior preterm delivery increases the risk of future preterm deliveries. The inventors believed that these observations can be explained by the inherent differences in the immune responses of women who deliver preterm compared with those who deliver full term, and that these differences can be detected even in the non-pregnant state. The inventors believed that by detecting these differences one may be able to identify those women who are at risk to deliver preterm even before they become pregnant. The inventors believed that in order to see the differences, the immune cells of the patient had to be stressed.

**[0021]** The inventors are the first to have discovered a biomarker assay that provides physicians with a tool to identify women who are at risk for preterm delivery, even before they become pregnant or while pregnant, using a nonspecific immunologic test. Identification of women at risk allows the physician to better focus on preventative strategies in these women and improve pregnancy outcome. In one embodiment, the test may be done on small amounts of blood sample obtained from the subject, thus being minimally invasive to the fetus. The blood may be drawn directly into a test tube coated with a stimulant; alternatively the blood may be sent to a laboratory to be processed and stimulated in the laboratory. Furthermore, the test may be repeated multiple times during the course of pregnancy. Thus, it provides dynamic assessment of the preterm delivery risk under the influence of changing environmental/physiologic factors, as well as requires minimal skills to draw blood as opposed to obtaining amniotic fluid.

**[0022]** For purposes of the present invention, the following terms are defined below.

**[0023]** As used herein, the term "preterm delivery" refers to a premature birth or conditions associated with a premature birth, including for example, a child delivered before 34 weeks of gestation.

**[0024]** "Biomarker," "diagnostic biomarker," or "preterm delivery biomarker" refers to a molecular indicator that is associated with a particular pathological or physiological state. The "biomarker" as used herein is an indicator for risk of preterm delivery. The indicator can be a cytokine, cytokine receptor, cytokine receptor antagonist, chemokine, chemokine receptor, chemokine receptor antagonist or an immunomodulating agent, including interleukins and interferons. Examples of "biomarkers" include but are not limited to IL-2, IL-4, IL-5, IL-8, IL-10, IL-12, IL-13, GM-CSF, IFN-g, and TNF-a. Biomarkers of the present invention include IL-10, IL-13 and/or IL-1RA. A "biomarker" of the present invention may be detected in a sample.

**[0025]** "Sample" or "a biological sample" refers to cells or component parts, or a fraction or portion thereof of body fluids, including but not limited to blood (e.g., whole blood), amniotic fluid, or cord blood. A "sample" or "biological sample" further refers to plasma, serum, and/or peripheral blood mononuclear cells (PMBC).

**[0026]** A method of diagnosing susceptibility for preterm delivery in non-pregnant women or pregnant women is disclosed, comprising obtaining a sample from the woman and assaying the sample for the presence or absence of one or more diagnostic biomarkers, where the presence or absence of one or more diagnostic biomarkers is indicative of susceptibility for preterm delivery in the woman.

**[0027]** In another embodiment, the sample comprises PMBC supernatant. In another embodiment, the sample comprises whole blood. In another embodiment, the sample comprises serum. In another embodiment, the one or more diagnostic biomarkers comprise a microbial component lipopolysaccharide (LPS) or another immune stimulant induced cytokine, cytokine receptor, cytokine receptor antagonist, chemokine, chemokine receptor, and/or chemokine receptor antagonist expression profile. In another embodiment, the one or more diagnostic biomarkers comprise inflammatory and/or anti-inflammatory cytokine. In other embodiments, the one or more diagnostic biomarkers comprise a cytokine receptor, cytokine receptor antagonist, chemokine, chemokine receptor, and/or chemokine receptor antagonist. In another embodiment, the diagnostic biomarkers are analyzed in the presence of cortisol. In another embodiment, the cortisol concentration is in the range of 1 ug/ml to 500 ug/ml, with a preferred range of 1 ug/ml to 150 ug/ml. In another embodiment, the one or more diagnostic biomarkers comprise a low expression of IL-10, IL-13 and/or IL-1RA. In another embodiment, the low expression of IL-10, IL-13 and/or IL-1RA comprises a 1 to 3 fold decrease in expression compared to levels ordinarily found in a healthy individual.

**[0028]** There are many techniques readily available in the field for detecting the presence or absence of a cytokine, cytokine receptor, cytokine receptor antagonist, chemokine, chemokine receptor, and/or chemokine receptor antagonist or other biomarkers, including protein microarrays. For example, some of the detection paradigms that can be employed to this end include optical methods, electrochemical methods (voltometry and amperometry techniques), atomic force microscopy, and radio frequency methods, e.g., magnetic resonance spectroscopy. Illustrative of optical methods, in addition to microscopy, both confocal and non-confocal, are detection of fluorescence, luminescence, chemiluminescence, absorbance, reflectance, transmittance, and birefringence or refractive index (e.g., surface plasmon resonance, ellipsometry, a resonant mirror method, a grating coupler waveguide method or interferometry) such as ELISPOT.

**[0029]** Similarly, there are any numbers of techniques that may be employed to isolate and/or fractionate biomarkers. For example, a cytokine, cytokine receptor, cytokine receptor antagonist, chemokine, chemokine receptor, and/or chemokine receptor antagonist may be captured using biospecific capture reagents, such as antibodies, aptamers or antibodies that recognize the biomarker and modified forms of it. This method could also result in the capture of protein interactors that are bound to the proteins or that are otherwise recognized by antibodies and that, themselves, can be biomarkers. The biospecific capture reagents may also be bound to a solid phase. Then, the captured proteins can be detected by SELDI mass spectrometry or by eluting the proteins from the capture reagent and detecting the eluted proteins by traditional MALDI or by SELDI. One example of SELDI is called "affinity capture mass spectrometry," or "Surface-Enhanced Affinity Capture" or "SEAC," which involves the use of probes that have a material on the probe surface that captures analytes through a non-covalent affinity interaction (adsorption) between the material and the analyte. Some examples of mass spectrometers are time-of-flight, magnetic sector, quadrupole filter, ion trap, ion cyclotron resonance, electrostatic sector analyzer and hybrids of these.

**[0030]** Alternatively, for example, the presence of biomarkers such as a cytokine, cytokine receptor, cytokine receptor antagonist, chemokine, chemokine receptor, and/or chemokine receptor antagonist may be detected using traditional immunoassay techniques. Immunoassay requires biospecific capture reagents, such as antibodies, to capture the analytes. The assay may also be designed to specifically distinguish protein and modified forms of protein, which can be done by employing a sandwich assay in which one antibody captures more than one form and second, distinctly labeled antibodies, specifically bind, and provide distinct detection of, the various forms. Antibodies can be produced by immunizing animals with the biomolecules. Traditional immunoassays may also include sandwich immunoassays including ELISA or fluorescence-based immunoassays, as well as other enzyme immunoassays.

**[0031]** Prior to detection, a cytokine, cytokine receptor, cytokine receptor antagonist, chemokine, chemokine receptor, and/or chemokine receptor antagonist may also be fractionated to isolate them from other components in a solution or of blood that may interfere with detection. Fractionation may include white blood cell isolation from other blood components, sub-cellular fractionation of white blood cell components and/or fractionation of the desired cytokine, cytokine receptor, cytokine receptor antagonist, chemokine, chemokine receptor, and/or chemokine receptor antagonist from other biomolecules found in white blood cells using techniques such as chromatography, affinity purification, 1D and 2D mapping, and other methodologies for purification known to those of skill in the art. In one embodiment, a sample is analyzed by means of a biochip. Biochips generally comprise solid substrates and have a generally planar surface, to which a capture reagent (also called an adsorbent or affinity reagent) is attached. Frequently, the surface of a biochip comprises a plurality of addressable locations, each of which has the capture reagent bound there.

**[0032]** Alternatively, for example, the presence of biomarkers such as a cytokine, cytokine receptor, cytokine receptor antagonist, chemokine, chemokine receptor, and/or chemokine receptor antagonist may be detected using PCR techniques or flow cytometry. All of the available techniques for cytokine, cytokine receptor, cytokine receptor antagonist, chemokine, chemokine receptor, and/or chemokine receptor antagonist detection may be used.

#### Diagnostic Assays of the Invention

**[0033]** The invention provides various diagnostic assays/tests to predict whether women are at risk of preterm birth.

**[0034]** As described above, in one instance of the disclosure, the diagnostic test comprises drawing blood from a patient, separating the blood cells (for example, separating the peripheral blood mononuclear cells (PBMC)), exposing the separated cells to an antigen (for example, LPS) and assessing the immune response.

**[0035]** In another embodiment, whole blood may be stimulated *ex vivo* without separating the blood cells. For example, whole blood stimulation may be carried out by drawing blood from the subject into collection tubes coated with an antigen and/or stimulant and allowing it to incubate at 37 degrees Celsius, for a desired amount of time (for example, for 16-24 hours). The immune response may be subsequently assessed by using various techniques known in the art. In one embodiment, after collecting blood from the subject and incubating with it with the antigen and/or stimulant, the collection tubes are centrifuged, the plasma is harvested and assayed for an immune response.

**[0036]** In one instance, blood from the subject is drawn into collection tubes coated with antigens as well as into positive and negative control collection tubes. The antigens and/or stimulants induce an immune response, for example by inducing expression of a cytokine, cytokine receptor, cytokine receptor antagonist, chemokine, chemokine receptor, and/or chemokine receptor antagonist. The antigens or stimulants which induce the expression of a cytokine, cytokine receptor, cytokine receptor antagonist, chemokine, chemokine receptor, and/or chemokine receptor antagonist include but are not limited to LPS, cortisol, phytohemagglutinin, maternal histocompatibility antigens, paternal histocompatibility antigens, fetal histocompatibility antigens, other microbial antigens, cytokines, phorbol esters (e.g., phorbol myristate acetate (PMA)), agents that induce Th1 or Th2 type differentiation of lymphocytes singly or combination thereof.

**[0037]** In a further embodiment, the immune response induced by the antigens and/or stimulants may be assessed by assaying the expression of a cytokine, cytokine receptor, cytokine receptor antagonist, chemokine, chemokine receptor, and/or chemokine receptor antagonist. Various techniques may be used to assess the immune response including but not limited to enzyme-linked immunosorbent spot (ELISpot), enzyme-linked immunosorbent assay (ELISA), microarray assay, quantitative PCR, Northern blot assay, Southern blot assay, Western blot assay immunohistochemical assay or binding assay. In a preferred embodiment, ELISpot is used to assess the cytokine expression and/or chemokine expression. These assays may be performed at bedside/doctors' offices, in a laboratory or during transportation to the laboratory. Antibodies against various cytokine, cytokine receptor, cytokine receptor antagonist, chemokine, chemokine receptor, and/or chemokine receptor antagonist may be used to assess an immune response. These antibodies may be any one or more of a monoclonal antibody or fragment thereof, a polyclonal antibody or a fragment thereof, chimeric antibodies, humanized antibodies, human antibodies and a single chain antibody. In one embodiment, the antibodies may be conjugated with a radiolabel, an enzyme, a chromophore or a fluorophore for immune response detection.

**[0038]** To ascertain whether a woman is at an increased risk of preterm delivery, the aforementioned assays are carried out using blood samples from test subjects who are nonpregnant women and/or pregnant women. The results obtained (for example, the immune response induced which is measured as a function of the expression of a cytokine,

cytokine receptor, cytokine receptor antagonist, chemokine, chemokine receptor, and/or chemokine receptor antagonist) from the test subjects are compared to the results obtained from control subjects. In one embodiment, the control subjects are women who delivered at full term. In another embodiment, if the expression level of a cytokine, cytokine receptor, cytokine receptor antagonist, chemokine, chemokine receptor, and/or chemokine receptor antagonist in test subjects is higher or lower than the range of the expression level of the cytokine, cytokine receptor, cytokine receptor antagonist, chemokine, chemokine receptor, and/or chemokine receptor antagonist in the control subjects, the test subject is at an increased risk of preterm delivery.

**[0039]** The disclosure further provides a diagnostic kit comprising a collection tube coated with an antigen and/or stimulant, a positive control collection tube and/or a negative control collection tube. The kit further comprises reagents to detect an immune response (for example antibodies that bind to a cytokine, cytokine receptor, cytokine receptor antagonist, chemokine, chemokine receptor, and/or chemokine receptor antagonist expressed), for example, antibodies to detect the cytokine, cytokine receptor, cytokine receptor antagonist, chemokine, chemokine receptor, and/or chemokine receptor antagonist, buffers and instructions.

**[0040]** Methods of identifying a nonpregnant woman at risk for preterm delivery are disclosed, comprising: obtaining a sample from the nonpregnant woman, determining the expression level of one or more biomarkers in the sample, and comparing the expression level of the one or more biomarkers with the expression level of biomarkers from those observed in women who delivered fullterm (controls). Since there is individual variation, the control values will constitute a range. If the level of expression of the one or more biomarkers in the sample is higher or lower than the level of expression of the same biomarkers in women who delivered fullterm (outside the range), then it is indicative that the non-pregnant woman is at risk for preterm delivery. Depending on how much the patient biomarker values are outside the range of the standard controls, the risk of preterm delivery may be determined as "low, medium or high." The biomarkers include but are not limited to IL-10, IL-13 and/or IL-1RA. The sample can comprise stimulated PBMC/whole blood supernatant (in the presence or absence of cortisol). Alternatively, the sample can comprise serum.

**[0041]** Methods of diagnosing susceptibility of preterm delivery in a woman are disclosed, comprising: obtaining a sample from the woman, determining the expression level of one or more diagnostic biomarkers in the sample, and comparing the expression level of the one or more biomarkers with the expression level of biomarkers from women who delivered fullterm (normal range). If the level of expression of the one or more diagnostic biomarkers in the sample is higher or lower than the level of expression of the same diagnostic biomarkers in the women who delivered fullterm (normal range), then it is indicative that the woman is at risk for preterm delivery. The diagnostic biomarkers can be inflammatory and/or anti-inflammatory cytokines. In other embodiments, the diagnostic biomarkers can be a cytokine receptor, cytokine receptor antagonist, chemokine receptor, and/or chemokine receptor antagonist. The diagnostic biomarkers selected include but not limited to IL-10, IL-13 and/or IL-1RA. Biomarker expression levels that are at least one to three times less than (or greater than, for other biomarkers) the biomarker expression levels of a control are indicative of preterm delivery. The sample can comprise stimulated PBMC/whole blood supernatant (in the presence or absence of cortisol). Alternatively, the sample can comprise serum. The women may be non-pregnant or pregnant.

**[0042]** The present disclosure is also directed to a kit for use to determine the risks of preterm delivery. The kit is useful for practicing the inventive method of determining the risk of preterm delivery of non-pregnant or pregnant women. The kit is an assemblage of materials or components, including a diagnostic bioassay of the present disclosure.

**[0043]** The exact nature of the components configured in the disclosed kit depends on its intended purpose. For example, some kits are configured for the purpose of diagnosing susceptibility of women for preterm delivery. In one instance, the kit is configured particularly for the purpose of determining the risk of preterm delivery of non-pregnant women. In another instance, the kit is configured particularly for the purpose of diagnosing susceptibility of women for preterm delivery of pregnant or non-pregnant women.

**[0044]** Instructions for use may be included in the kit. "Instructions for use" typically include a tangible expression describing the technique to be employed in using the components of the kit to effect a desired outcome, such as determine the risks of preterm delivery. Optionally, the kit also contains other useful components, such as, specially prepared test tubes, diluents, buffers, pharmaceutically acceptable carriers, syringes, catheters, applicators, pipetting or measuring tools, bandaging materials or other useful paraphernalia as will be readily recognized by those of skill in the art.

**[0045]** The materials or components assembled in the kit can be provided to the practitioner stored in any convenient and suitable ways that preserve their operability and utility. For example the components can be in dissolved, dehydrated, or lyophilized form; they can be provided at room, refrigerated or frozen temperatures. The components are typically contained in suitable packaging material(s). As employed herein, the phrase "packaging material" refers to one or more physical structures used to house the contents of the kit, such as inventive compositions and the like. The packaging material is constructed by well-known methods, preferably to provide a sterile, contaminant-free environment. As used herein, the term "package" refers to a suitable solid matrix or material such as glass, plastic, paper, foil, and the like, capable of holding the individual kit components. Thus, for example, a package can be a collection tube coated with an antigen and/or a stimulant for use to collect and stimulate a biological sample from a woman. The packaging material generally has an external label which indicates the contents and/or purpose of the kit and/or its components.

## EXAMPLES

[0046] The following examples are provided to better illustrate the claimed invention and are not to be interpreted as limiting the scope of the invention. To the extent that specific materials are mentioned, it is merely for purposes of illustration and is not intended to limit the invention.

### Example 1

#### Biomarker Assay

[0047] Peripheral blood mononuclear cells (PBMC) from non-pregnant (at least 5-6 years post-partum) women with history of preterm or full term delivery, in addition to the microbial component lipopolysaccharide (LPS)-induced cytokine expression profile were examined. PBMC were separated from whole blood using Ficoll gradient. The cells were counted and equal numbers of cells were plated in 24 well plates. PBMC were treated with cortisol (50 or 300 ug/ml) or media for 1 hour prior to stimulation with LPS (0, 1, or 100 ug/ml) for 24 hours. The PBMC were lysed and the supernatant was examined for inflammatory and anti-inflammatory cytokine expression by using Bioplex technology (Bio-rad). The inventors found that during the non-pregnant state IL10, IL13 and IL1Ra expression was lower in the PBMC obtained from women whom had previous preterm delivery, and that those biomarkers may be measured to identify women who were at risk for preterm delivery in the future.

[0048] In this study two patients were preterm and four were full term. There were 9 samples per patient (3x3 design) and all samples from each patient were analyzed twice for cytokine concentrations with the exception of one full term subject who only had one analysis of each sample. As a result there are a total of 99 observations for each cytokine measured. The assay simultaneously measured concentrations of 11 inflammatory markers: IL-1ra (IL1-receptor antagonist), IL-2, IL-4, IL-5, IL-8, IL-10, IL-12, IL-13, GM-CSF, IFN-g, and TNF-a.

### Example 2

#### Establishment of cut off points

[0049] Many samples were above or below the detection for some of the endpoints. Cut off points were established for each cytokine measured (**Table 1**). If a sample was out of the range of detection, a default value was assigned to the sample as indicated below.

**Table 1. The establishment of cut off points for out of range values.**

Cytokine	Limit of Detection	Out of Range Set Value	Fraction of Samples out of Detection Range
IL-1ra	<i>all samples within levels of detection</i>	<i>n/a</i>	0/99
IL-2	LL = 0.50	0.25	10/99
IL-4	LL = 0.14	0.08	6/99
IL-5	LL = 0.90	0.5	82/99
IL-8	UL = 95,000	100,000	79/99
IL-10	<i>all samples within levels of detection</i>	<i>n/a</i>	0/99
IL-12	LL = 0.33	0.15	19/99
IL-13	LL = 0.17	0.08	8/99
GM-CSF	LL = 1.18	0.60	11/99
IFN-g	LL = 1.21	0.60	5/99
TNF-a	<i>all samples within levels of detection</i>	<i>n/a</i>	0/99
LL = Lower Limit; UL = Upper Limit			

[0050] All samples that were below the range of detection for IL-2, IL-4, IL-13, and IFN-g were from the two subjects with a history of preterm delivery. Of the 11 samples assayed that were below detection limits for GM-CSF, only 1 was from a subject with a full-term delivery while the rest were from subjects with histories of preterm deliveries.

**Example 3**

**Statistical analysis**

5 [0051] Empirical means and standard errors for each cytokine and treatment group are presented herein. Here, the averages of replicate measures were calculated first for each subject. The mean and SEM were then taken across each treatment group.

[0052] All raw data was tested via the Kolmogorov-Smirnov test to determine if the data followed a normal distribution. Log-transformations were performed for all data found to have a non-normal distribution prior to further statistical analysis. 10 For each cytokine, a mixed effects model was used to examine all data for significant effects with the outcome variable as the cytokine concentration; the fixed predictor variables as LPS concentration, cortisol concentration, and delivery status (pre- or full-term); and the random effect due to the replicate data points. To test the interaction of the three fixed predictor variables on cytokine concentration, both first and second level interactions were added to each cytokine model. Post-hoc testing was performed using a Student's t-test.

**Example 4**

**Cortisol and LPS concentrations**

20 [0053] All 99 observations were initially used in the analysis to first determine which concentrations of LPS and cortisol induced the most robust effect. For all cytokines measured there was no significant difference between the effects of 1 ug/ml and 100 ug/ml LPS. For several cytokines (IL-1ra, IL-10, and IL-13), 300 ug/ml cortisol had a more significant effect to suppress cytokine levels than 50 ug/ml.

[0054] As a result of the findings, the inventors focused on the data with 0 or 300 ug/ml of cortisol pretreatment crossed with 0 or 1 ug/ml LPS. This results in 4 samples per subject, each analyzed twice (with exception of the samples from the full term subject who only had one analysis of each sample). With 8 measurements per subject (with the exception of the full term subject who only has 4 measurements) this results in a data set with 44 measurements for each of the cytokines.

**Example 5**

**Analysis of testing for cytokine differences between groups**

35 [0055] Using the same mixed model regression, estimated group means and standard errors were calculated. Differences between preterm and full-term pregnancies were considered significant where  $p < 0.10$ .

[0056] Based on the results of the statistical modeling, cortisol did suppress secretion of IL-13 and IL-1ra; LPS increased secretion of IL-4, IL-10, IL-13, TNF-a, and IL-1ra; and women who had preterm deliveries had overall lower secretion levels of IL-10, IL-13, and IL-1ra (Table 2).

40 **Table 2: Global effects of cortisol, LPS, and premature delivery on cytokine secretion levels in PBMC based on results of mixed modeling.**

Cytokine	Cortisol Effect	LPS Effect	Pre-term Effect
IL-2	n/c, p = .80	n/c, p = .50	n/c, p = .17
IL-4	n/c, p = .26	increase, p = .07	n/c, p = .59
IL-10	n/c, p = .12	increase, p = .01	decrease, p = .01
IL-13	decrease, p = .01	increase, p = .04	decrease, p = .02
IFN-g	n/c, p = .10	n/c, p = .17	n/c, p = .22
TNF-a	n/c, p = .41	increase, p = .04	n/c, p = .75
GM-CSF	n/c, p = .51	n/c, p = .19	n/c, p = .17
IL-1ra	decrease, p = .03	increase, p = .09	decrease, p = .01
n/c = No Change			

55 [0057] Women who had preterm deliveries had lower baseline IL-10, 1L13 and IL-1Ra expression compared to those who delivered full term. There were no differences in cytokine production between LPS-stimulated PBMC from preterm delivering women when compared with that from women with full-term deliveries. In other words, adjusting for unstimulated

secretion levels, the concentration of cytokines released from LPS stimulation was no different between the two groups of women. In the presence of cortisol PBMC from women who had preterm deliveries produced lower IL-13 and IFN- $\gamma$  expression when compared with that from women with full-term deliveries.

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**[0059]** Unless specifically noted, it is the intention of the inventors that the words and phrases in the specification and claims be given the ordinary and accustomed meanings to those of ordinary skill in the applicable art(s).

**[0060]** It will be understood by those within the art that, in general, terms used herein are generally intended as "open" terms (e.g., the term "including" should be interpreted as "including but not limited to," the term "having" should be interpreted as "having at least," the term "includes" should be interpreted as "includes but is not limited to," etc.).

## Claims

1. An ex vivo method of identifying a woman at risk or diagnosing susceptibility of a woman for preterm delivery, comprising:
  - (a) stimulating peripheral blood mononuclear cells ("PBMCs") from a woman using an antigen and/or stimulant;
  - (b) determining the secretion level of a combination of cytokines from the stimulated PBMCs, wherein at least two cytokines in the combination are selected from IL-10, IL-13, and IL-1RA;
  - (c) comparing the secretion level of the combination measured in (b) with the secretion level of the same combination of cytokines from control PBMCs isolated from women who delivered at full term and stimulated using the same antigen and/or stimulant;
  - (d) determining whether the secretion level measured in (b) is higher or lower than the secretion level from the control PBMCs;
 thereby identifying whether the woman is at risk for preterm delivery.
2. The method according to claim 1, wherein the combination comprises IL-1RA.
3. The method according to claim 1, wherein the combination comprises two of IL-10, IL-13, and IL-1RA.
4. The method according to claim 1, wherein the combination comprises all three of IL-10, IL-13, and IL-1RA.
5. The method according to any one of claims 1 to 4, wherein the antigen and/or stimulant is phytohemagglutinin.
6. The method according to claim 5, wherein the PBMCs are further stimulated with an antigen and/or stimulant other than phytohemagglutinin.
7. The method according to any one of claims 1 to 6, wherein the woman is not pregnant.
8. The method according to any one of claims 1 to 6, wherein the woman is pregnant.
9. The method according to claim 8, further comprising repeating steps (a) through (d) multiple times during the course

of the woman's pregnancy.

10. The method according to any one of claims 1 to 9, wherein the secretion level is determined using an immunoassay.

5 11. The method according to any one of claims 1 to 10, wherein the PBMCs are isolated from a sample of whole blood from the woman prior to stimulation.

10 12. The method according to any one of claims 1 to 10, wherein the PBMCs are stimulated in a sample of whole blood from the woman.

### Patentansprüche

15 1. Ex vivo Verfahren zum Identifizieren einer Frau mit Risiko oder Diagnostizieren einer Prädisposition einer Frau für Frühgeburt, umfassend:

(a) Stimulieren mononukleärer Zellen des peripheren Bluts ("PBMCs") von einer Frau unter Verwendung eines Antigen und/oder Stimulans;

20 (b) Bestimmen des Sekretionsniveaus einer Kombination von Cytokinen aus den stimulierten PBMCs, wobei mindestens zwei Cytokine in der Kombination ausgewählt sind von IL-10, IL-13 und IL-1RA;

(c) Vergleichen des in (b) gemessenen Sekretionsniveaus mit dem Sekretionsniveau der gleichen Cytokin-Kombination von Kontroll-PBMCs, die von Frauen isoliert wurden, die bis zum Ende ausgetragen haben, und unter Verwendung desselben Antigen und/oder Stimulans stimuliert wurden;

25 (d) Bestimmen, ob das in (b) gemessene Sekretionsniveau höher oder niedriger als das Sekretionsniveau von den Kontroll-PBMCs ist;

wodurch identifiziert wird, ob bei der Frau ein Frühgeburt-Risiko besteht.

30 2. Verfahren nach Anspruch 1, wobei die Kombination IL-1RA umfasst.

3. Verfahren nach Anspruch 1, wobei die Kombination zwei von IL-10, IL-13 und IL-1RA umfasst.

4. Verfahren nach Anspruch 1, wobei die Kombination alle drei von IL-10, IL-13 und IL-1RA umfasst.

35 5. Verfahren nach einem der Ansprüche 1 bis 4, wobei das Antigen und/oder Stimulans Phytohämagglutinin ist.

6. Verfahren nach Anspruch 5, wobei die PBMCs ferner mit einem anderen Antigen und/oder Stimulans als Phytohämagglutinin stimuliert werden.

40 7. Verfahren nach einem der Ansprüche 1 bis 6, wobei die Frau nicht schwanger ist.

8. Verfahren nach einem der Ansprüche 1 bis 6, wobei die Frau schwanger ist.

45 9. Verfahren nach Anspruch 8, ferner umfassend ein mehrfaches Wiederholen der Schritte (a) bis (d) im Verlauf der Schwangerschaft der Frau.

10. Verfahren nach einem der Ansprüche 1 bis 9, wobei das Sekretionsniveau unter Verwendung eines Immuntests bestimmt wird.

50 11. Verfahren nach einem der Ansprüche 1 bis 10, wobei die PBMCs von einer Vollblutprobe der Frau vor der Stimulation isoliert werden.

12. Verfahren nach einem der Ansprüche 1 bis 10, wobei die PBMCs in einer Vollblutprobe der Frau stimuliert werden.

55

### Revendications

1. Procédé ex vivo d'identification d'une femme à risque ou de diagnostic de la prédisposition d'une femme pour

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l'accouchement prématuré comprenant :

(a) la stimulation des cellules mononucléées du sang périphérique (« CMSP ») d'une femme en utilisant un antigène et/ou un stimulant ;

(b) la détermination du niveau de sécrétion d'une combinaison de cytokines des CMSP stimulées, au moins deux cytokines dans la combinaison étant choisie parmi IL-10, IL-13 et IL-1RA ;

(c) la comparaison du niveau de sécrétion de la combinaison mesuré en (b) avec le niveau de sécrétion de la même combinaison de cytokines de CMSP témoins isolées de femmes ayant accouché à terme et stimulées en utilisant le même antigène et/ou stimulant ;

(d) la détermination que le niveau de sécrétion mesuré en (b) est supérieur ou inférieur au niveau de sécrétion des CMSP témoins ;

déterminant ainsi si la femme est à risque d'accouchement prématuré.

2. Procédé selon la revendication 1, dans lequel la combinaison comprend IL-1RA.

3. Procédé selon la revendication 1, dans lequel la combinaison comprend deux parmi IL-10, IL-13 et IL-1RA.

4. Procédé selon la revendication 1, dans lequel la combinaison comprend les trois IL-10, IL-13 et IL-1RA.

5. Procédé selon l'une quelconque des revendications 1 à 4, dans lequel l'antigène et/ou le stimulant est la phytohémagglutinine.

6. Procédé selon la revendication 5, dans lequel les CMSP sont davantage stimulées avec un antigène et/ou un stimulant autre que la phytohémagglutinine.

7. Procédé selon l'une quelconque des revendications 1 à 6, la femme n'étant pas enceinte.

8. Procédé selon l'une quelconque des revendications 1 à 6, la femme étant enceinte.

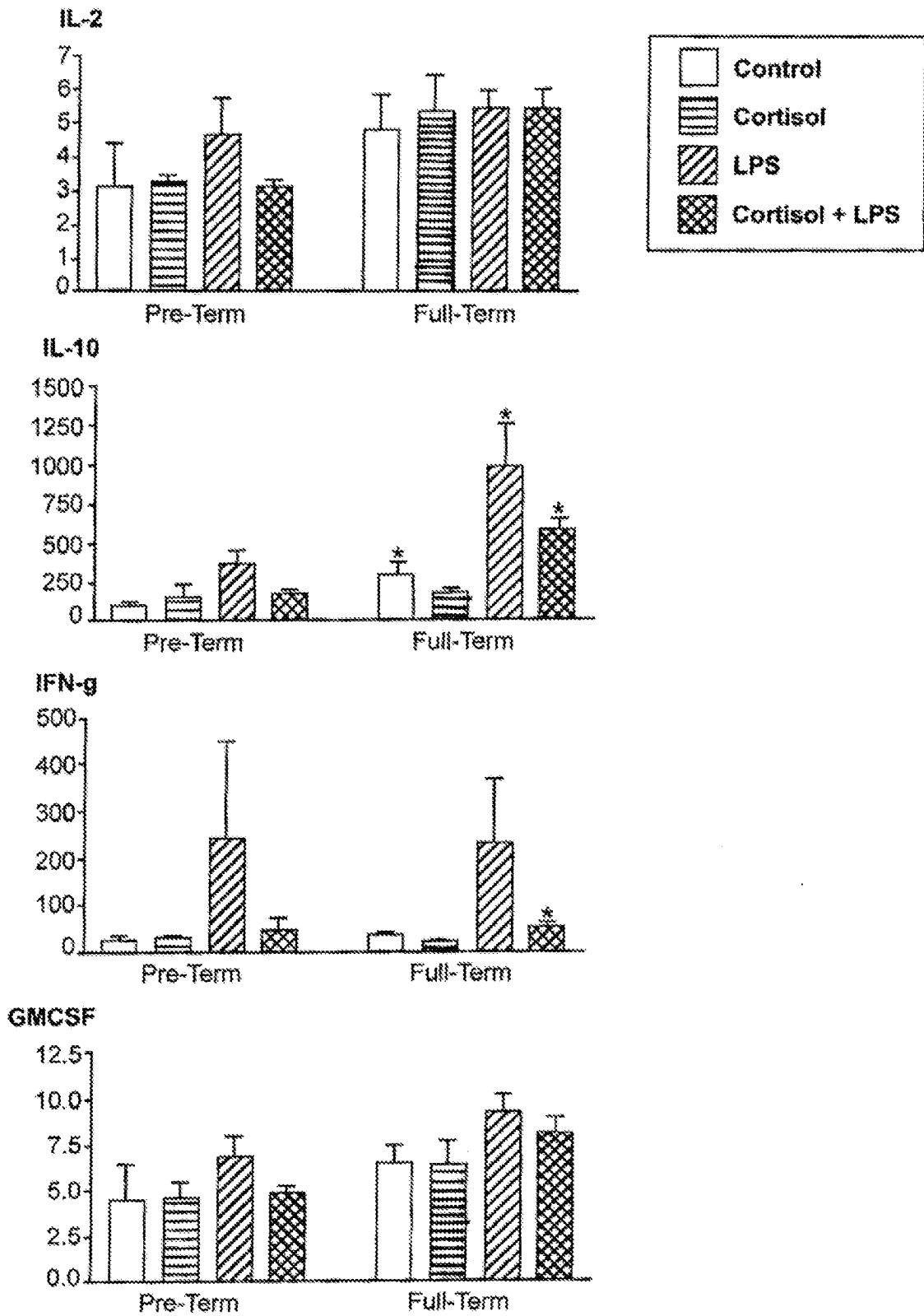
9. Procédé selon la revendication 8, comprenant en outre la répétition des étapes (a) à (d) à de multiples reprises pendant la durée de la grossesse de la femme.

10. Procédé selon l'une quelconque des revendications 1 à 9, dans lequel le niveau de sécrétion est déterminé en utilisant un immunoessai.

11. Procédé selon l'une quelconque des revendications 1 à 10, dans lequel les CMSP sont isolées à partir d'un échantillon de sang total de la femme avant la stimulation.

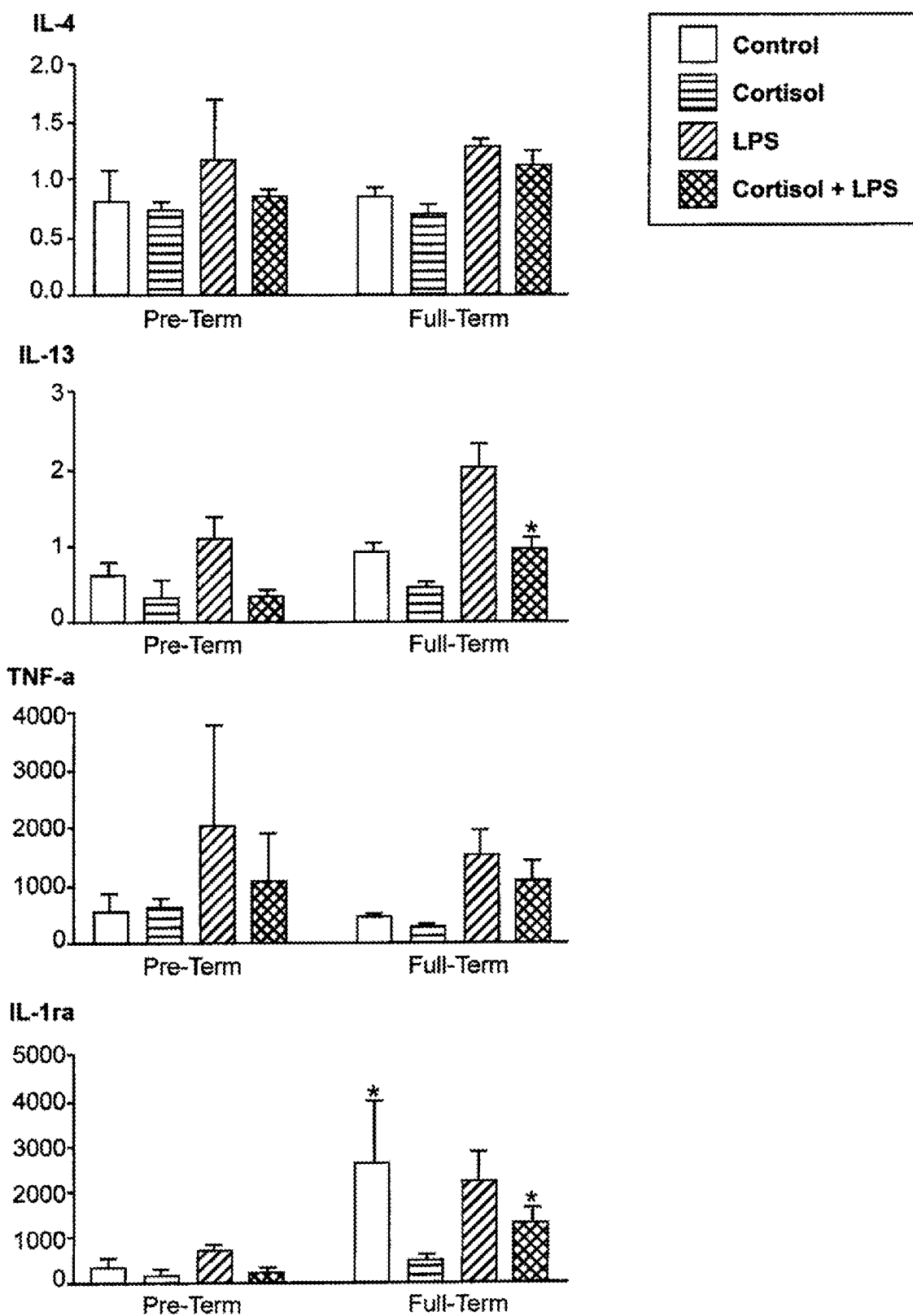
12. Procédé selon l'une quelconque des revendications 1 à 10, dans lequel les CMSP sont stimulées dans un échantillon de sang total de la femme.

FIG. 1A



\*Significant difference between similarly treated preterm group ( $p < 0.10$ )

FIG. 1B



\*Significant difference between similarly treated preterm group ( $p < 0.10$ )

## REFERENCES CITED IN THE DESCRIPTION

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专利名称(译)	诊断生物标志物预测有早产风险的妇女		
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申请号	EP2012757314	申请日	2012-03-13
[标]申请(专利权)人(译)	雪松-西奈医学中心		
申请(专利权)人(译)	雪松 - 西奈医疗中心		
当前申请(专利权)人(译)	雪松 - 西奈医疗中心		
[标]发明人	EQUILS OZLEM O SIMMONS JR CHARLES F		
发明人	EQUILS, OZLEM O. SIMMONS, JR., CHARLES F.		
IPC分类号	G01N33/53 G01N33/00 G01N33/68		
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#### 摘要(译)

本发明涉及与早产相关的生物标志物。更具体地，本发明提供了测量存在于早产风险中的女性中的生物标志物的方法，所述生物标志物包括但不限于细胞因子，细胞因子受体，细胞因子受体拮抗剂，趋化因子，趋化因子受体和/或趋化因子受体拮抗剂。诊断方法可以对全血进行。