METHODS OF PREDICTING AND DECREASING THE RISK OF PREGNANCY LOSS

Abstract: Described are methods for diagnosing and predicting the risk of pregnancy loss in a subject based on the presence of an aberrant humoral response to three proteins, Apolipoprotein B-100, alpha2macroglobulin (alpha2M), and fibronectin. The presence or a detectable level of maternal IgG antibodies to trophoblast-derived fibronectin and/or ApoB-100, and/or the absence or a non-detectable level of antibodies specifically binding to alpha2M is associated with a history of RPL and an increased risk of pregnancy loss. Also described are methods for identifying subjects at risk of pregnancy loss, selecting subjects for participation in a clinical study, and methods of decreasing the risk of pregnancy loss in a subject which include detecting the presence or absence of antibodies to one or more of trophoblast-derived ApoB-100, alpha2M, and fibronectin. Also provided are kits that contain ApoB-100, alpha2M, and fibronectin.
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METHODS OF PREDICTING AND DECREASING
THE RISK OF PREGNANCY LOSS

TECHNICAL FIELD

This invention relates to biomarkers of recurrent pregnancy loss, and methods of use thereof.

BACKGROUND

Miscarriage occurs in an estimated 10% to 15% of all pregnancies of less than 20 weeks gestation (Stirrat, Lancet 336:673-675, 1990). Recurrent miscarriage is classically defined as the occurrence of three or more consecutive losses of clinically-recognized pregnancies prior to the 20th week of gestation, exclusive of molar and ectopic pregnancies. Prospective studies have assessed the risks of subsequent miscarriage after one miscarriage to be 15%, rising to 17% to 31% after two miscarriages, and 25% to 46% after three or more miscarriages. Although the loss of one pregnancy (or sometimes even two pregnancies) is considered by many clinicians to be within the range of normal (and likely due to gamete failure), loss of three or more pregnancies is generally considered to be associated with a pathological condition. Most providers will initiate an evaluation for recurrent pregnancy loss (RPL) after two or more consecutive miscarriages.

SUMMARY

The present invention is based, at least in part, on the discovery and characterization of differences in the humoral immune responses from women with a history of recurrent pregnancy loss (RPL) compared to multiparous women with an uncomplicated obstetrical history in terms of IgG subclasses and trophoblast cell antigens recognized. Thus, the present invention includes methods for diagnosing and predicting the risk of pregnancy loss based on the presence of an aberrant humoral response, specifically to three proteins, Apolipoprotein B-100 (ApoB-100), alpha2macrogloblin (a2M), and fibronectin. The presence, a detectable level, or an increase of maternal IgG antibodies to trophoblast-derived fibronectin and/or Apolipoprotein B-100, and/or the
absence, a non-detectable level, or a decrease of antibody recognition to a2M is
associated with a history of RPL and an increased risk of future pregnancy loss.

Provided are methods of predicting the risk of pregnancy loss in a subject (i.e., a
female subject) including providing a sample containing serum from the subject; and
detecting the presence, absence, or levels of antibodies to one or more (e.g., one, two, or
three) of fibronectin (protein or nucleic acid), a2M (protein or nucleic acid), and ApoB-
100 (protein or nucleic acid) in the sample, wherein the presence or a detectable level of
antibodies to fibronectin (protein or nucleic acid) and/or ApoB-100 (protein or nucleic
acid), and/or the absence or a non-detectable level of antibodies to a2M (protein or
nucleic acid) in the sample indicates that the subject has an increased risk of pregnancy
loss. Some embodiments of these methods include providing a sample containing serum
from the subject, detecting the presence or absence of antibodies to fibronectin in the
sample, wherein the presence of antibodies to fibronectin in the sample indicates that the
subject has an increased risk of pregnancy loss. Some embodiments of these methods
further include detecting the presence or absence of antibodies to ApoB-100 in the
sample, wherein the presence of antibodies to fibronectin to ApoB-100 indicates that the
subject has an increased risk of pregnancy loss. Some embodiments of these methods
further include detecting the absence or presence of antibodies to a2M in the sample,
wherein the presence of antibodies to fibronectin or ApoB-100, or the absence of
antibodies to a2M indicates that the subject has an increased risk of pregnancy loss.

Also provided are methods of identifying a subject at risk of pregnancy loss
including providing a sample containing serum from the subject, and detecting the
presence, absence, or level of antibodies to one or more (e.g., one, two, or three) of
fibronectin (protein or nucleic acid), a2M (protein or nucleic acid), and ApoB-100
(protein or nucleic acid) in the sample, wherein a subject having antibodies to fibronectin
(protein or nucleic acid) and/or ApoB-100 (protein or nucleic acid), and/or not having or
having a non-detectable level of antibodies to a2M (protein or nucleic acid) in the sample
is identified as being at risk of pregnancy loss. Some embodiments of these methods
include providing a sample containing serum from the subject, and detecting the presence
or absence of antibodies to fibronectin in the sample, wherein a subject having antibodies
to fibronectin present in the sample is identified as being at risk of pregnancy loss. Some
embodiments of these methods further include detecting the presence or absence of antibodies to ApoB-100 in the sample, wherein a subject having antibodies to fibronectin or ApoB-100 present in the sample is identified as being at risk of pregnancy loss. Some embodiments of these methods further include detecting the presence or absence of antibodies to a2M in the sample, wherein a subject having antibodies to fibronectin or ApoB-100, or not having antibodies to a2M present in the sample is identified as being at risk of pregnancy loss.

Also provided are methods of selecting a subject for participation in a clinical study including providing a sample containing serum from the sample, and detecting the presence or absence of antibodies to one or more (e.g., one, two, or three) of fibronectin (protein or nucleic acid), a2M (protein or nucleic acid), and apolipoprotein B (protein or nucleic acid) in the sample, wherein a subject having antibodies to fibronectin (protein or nucleic acid) and/or ApoB-100 (protein or nucleic acid), and/or not having or having a non-detectable level of antibodies to a2M (protein or nucleic acid) in the sample is selected for participation in a clinical study. Some embodiments of these methods include providing a sample containing serum from the subject and detecting the presence or absence of antibodies to fibronectin in the sample, wherein a subject having antibodies to fibronectin present in the sample is selected for participation in a clinical study. Some embodiments of these methods further include detecting the presence or absence of antibodies to ApoB-100 in the sample, wherein a subject having antibodies to fibronectin or ApoB-100 present in the sample is selected for participation in a clinical study. Some embodiments of these methods further include detecting the presence of absence of antibodies to a2M in the sample, wherein a subject having antibodies to fibronectin or ApoB-100, or not having antibodies to a2M present in the sample is selected for participation in a clinical study.

Also provided are methods of decreasing the risk of pregnancy loss in a subject including providing a sample containing serum from the subject, detecting the presence or absence of antibodies to one or more (e.g., one, two, or three) of fibronectin (protein or nucleic acid), a2M (protein or nucleic acid), and ApoB-100 (protein or nucleic acid) in the sample, and administering a therapeutic treatment to a subject having antibodies to fibronectin (protein or nucleic acid) and/or ApoB-100 (protein or nucleic acid), and/or
not having or having a non-detectable level of antibodies to a2M (protein or mRNA) in the sample. Some embodiments of these methods include providing a sample comprising serum from the subject, detecting the presence or absence of antibodies to fibronectin in the sample, and administering a therapeutic treatment to a subject having antibodies to fibronectin present in the sample. Some embodiments of these methods further include detecting the presence or absence of antibodies to ApoB-100 in the sample, and administering a therapeutic treatment to a subject having antibodies to fibronectin or ApoB-100 present in the sample. Some embodiments of these methods further include detecting the presence or absence of antibodies to a2M in the sample, and administering a therapeutic treatment to a subject having antibodies to fibronectin or ApoB-100, or not having antibodies to a2M present in the sample. In some embodiments of these methods, the therapeutic treatment is selected from complement inhibitors, hormone treatment, steroid treatment, passive immunotherapy with intravenous immunoglobulins, aspirin, and tumor necrosis factor-a (TNF-a) antagonists.

In any of the methods described herein, the subject is pregnant. In any of the embodiments of all the methods described herein, the sample is obtained from the pregnant subject within the first 20 weeks (e.g., within the first 19 weeks, 18 weeks, 17 weeks, 16 weeks, 15 weeks, 14 weeks, 13 weeks, 12 weeks, 11 weeks, 10 weeks, 9 weeks, 8 weeks, 7 weeks, 6 weeks, 5 weeks, 4 weeks, 3 weeks, 2 weeks, or 1 week), within the first 13 weeks, or within the first 12 weeks of pregnancy.

In some embodiments of all of the methods described herein, the subject has had at least one (e.g., two, three, four, or five) previous pregnancy loss or is suspected of having had at least one (e.g., two, three, four, or five) previous pregnancy loss. In some embodiments of all of the methods described herein, the subject is not pregnant, but is planning or considering a future pregnancy.

In some embodiments of all of the methods described herein, the subject having had at least one previous pregnancy loss or suspected of having had at least one previous pregnancy loss may be pregnant or may not be pregnant. In some embodiments of all of the methods described herein, the sample is obtained within the first 20 weeks (e.g., within the first 19 weeks, 18 weeks, 17 weeks, 16 weeks, 15 weeks, 14 weeks, 13 weeks, 12 weeks, 11 weeks, 10 weeks, 9 weeks, 8 weeks, 7 weeks, 6 weeks, 5 weeks, 4 weeks, 3
weeks, 2 weeks, or 1 week), the first 13 weeks, or within the first 12 weeks of pregnancy from the pregnant subject that has had at least one previous pregnancy loss or is suspected of having had at least one previous pregnancy loss.

In some embodiments of all of the methods described herein, the detecting of the presence, absence, or levels of antibodies includes contacting the sample with one or more (e.g., one, two, and three) antigens selected from the group consisting of ApoB-100 (protein or nucleic acid), fibronectin (protein or nucleic acid), and a2M (protein or nucleic acid), or antigenic fragments thereof, and detecting the binding of antibodies in the sample to the antigens. In some embodiments, the antigens are immobilized on a surface, e.g., in an array or on beads. In some embodiments of all of the methods described herein, the ApoB-100 (protein or nucleic acid), fibronectin (protein or nucleic acid), and/or a2M (protein or nucleic acid) are trophoblast-derived. In some embodiments of all of the methods described herein, the subject is human.

Also provided are kits, containing essentially, one or more (e.g., one, two, or three) ApoB-100 (protein or nucleic acid), fibronectin (protein or nucleic acid), and a2M (protein or nucleic acid), or antigenic fragments thereof.

As used herein, a "subject" is a vertebrate, including any member of the class mammalia, including humans, domestic and farm animals, and zoo, sports or pet animals, such as mouse, rabbit, pig, sheep, goat, cattle, and higher primates. In preferred embodiments, the subject is a human.

By the phrase "suspected of having had a previous pregnancy loss" is meant a subject who previously experienced one or more (e.g., one, two, three, or four) symptoms of a miscarriage (e.g., vaginal bleeding, pelvic cramps, abdominal pain, persistent lower back ache, and blood clots or grayish tissue passing from the vagina), but was not diagnosed as being pregnant (e.g., not diagnosed by a health care professional or through the use of a home diagnostic kit) at the time these symptoms occurred.

By the phrase "a subject having had a previous pregnancy loss" is meant a subject that has previously had at least one (e.g., two, three, four, or five) miscarriage. For example, a subject may have been diagnosed as being pregnant by a health care professional (e.g., a physician, nurse, physician's assistant, or a laboratory technician) or through the use of a home diagnostic kit, and thereafter experienced one or more (e.g.,
two, three, four, or five) symptoms of a miscarriage (e.g., vaginal bleeding, pelvic
cramps, abdominal pain, persistent lower back ache, and blood clots or grayish tissue
passing from the vagina) or failed to carry the fetus to term. The one or more previous
miscarriages may also be confirmed by a health care professional (e.g., a physician, a
nurse, a physician's assistant, or a laboratory technician).

By the term "antigen" or "antigenic fragment" is meant any portion of a molecule
(e.g., peptide, nucleic acid (e.g., mRNA), carbohydrate, or lipid, or any combination
thereof) that is specifically recognized by an antibody. For example, an antigen or
antigenic fragment may be a peptide containing at least 5 (e.g., at least 6, 7, 8, 9, 10, 11,
12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acids) contiguous amino acids. Exemplary
peptide antigens or antigenic fragments contain at least 5 (e.g., at least 6, 7, 8, 9, 10, 11,
12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acids) contiguous amino acids of the sequence
within any one of SEQ ID NOS: 2, 4, 6, 8, 10, 12, 14, 16, and 18. The contiguous amino
acid sequence may be present within any portion of the sequence of SEQ ID NOS: 2, 4,
6, 8, 10, 12, 14, 16, or 18, for example, a sequence starting at the N-terminus, a sequence
ending at the C-terminus, or a sequence starting at any single amino acid within the
sequence (with the exception of the last four amino acids at the C-terminus of the
protein). Additional exemplary peptide antigens contain the sequence of SEQ ID NO: 2,
4, 6, 8, 10, 12, 14, 16, or 18.

Exemplary antigens or antigenic fragments that are nucleic acids contain at least 5
(e.g., at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20) contiguous
nucleotides of the sequence within any one of SEQ ID NOS: 1, 3, 5, 7, 9, 11, 13, 15, and
17. The contiguous nucleotide sequence may be present within any portion of the
sequence of SEQ ID NOS: 1, 3, 5, 7, 9, 11, 13, 15, or 17, for example, a sequence starting
at the 5'-terminus, a sequence ending at the 3'-terminus, or a sequence starting at any
single nucleotide within the sequence (with the exception of the last four nucleotides at
the 3'-terminus of the nucleic acid). Additional exemplary nucleic acid antigens contain
the sequence of SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, or 17.

By the term "at risk of pregnancy loss" is meant a subject that has an increased
risk of having a miscarriage during pregnancy as compared to a control population (e.g.,
a group of subjects of the same age, a group of subjects not diagnosed as having recurrent
pregnancy loss, a group of subjects that have never have had a miscarriage, or a group of subjects that have never experienced, at a single time, a combination of three or more symptoms of a miscarriage).

By the phrase "a subject planning or considering future pregnancy" is meant a subject who is not pregnant, but is planning a future pregnancy or considering becoming pregnant in the future.

By the phrase "therapeutic treatment" is meant a treatment that may decrease (e.g., a significant decrease (as used herein, the term "decrease" is meant a statistically significant decrease), such as by at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, or 50%) the risk of having a miscarriage in a subject. Non-limiting examples of therapeutic treatment are known in the art and include, without limitation, complement inhibitors, hormone treatment, steroid treatment, passive immunotherapy with intravenous immunoglobulins, aspirin, and TNF-α antagonists. Examples of therapeutic treatments are described herein and additional examples of therapeutic treatments are known in the art.

Unless otherwise defined, all 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. Methods and materials are described herein for use in the present invention; other, suitable methods and materials known in the art can also be used. The materials, methods, and examples are illustrative only and not intended to be limiting. All publications, patent applications, patents, sequences, database entries, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control.

Other features and advantages of the invention will be apparent from the following detailed description and figures, and from the claims.

DESCRIPTION OF DRAWINGS

Figure 1 is a schematic illustration of exemplary methods for obtaining trophoblast cellular proteins and performing Western blot analysis.

Figure 2 is a representative Western immunoblot demonstrating the reactivity profile of total circulating antibody derived from control term-derived patients compared
Western blots, sera from women with a history of RPL exhibited greater immunoreactivities compared to controls, with a total antibody reactivity 3.6-fold greater with nuclear antigens ($p=0.0044$), a 4.1-fold greater reactivity with membrane-derived antigens ($p=0.0001$), and a 1.8-fold greater recognition of cytosolic antigens ($p=0.0113$).

Figures 3A-F are a set of six Western blots showing the results of experiments performed as diagramed in Figure 1 in Term samples (3A-3C) and RPL samples (3D-3F), showing levels of IgGAM (3A and 3D), IgG2 (3B and 3E); and IgG3 (3C and 3F). MW, molecular weight; M, membrane protein fractions; N, nuclear protein fractions; and C, Cytosolic protein fractions.

Figure 4 is a bar graph showing the reactivity of antibodies from control (Term) and RPL subjects to antigens derived from the membrane or nucleus of SW-71 cells. In these experiments, the Western blot x-ray films with antibody-antigen complexes were scanned, digitized, and then converted to pixel density. Immunoreactivities for antigens from nuclear, membrane, or cytosolic compartments were standardized and the mean values and standard deviations were calculated.

Figure 5 is two Western blots and a set of six pixel density graphs correlated for each lane of Western blot antibody reactivity to trophoblast antigens: nuclear, membrane, and cytosolic. A representative Term Western blot and three correlated graphs for Term subjects are shown (top half) and a representative RPL Western blot and three correlated graphs for RPL subjects are depicted (bottom half). The RPL Western blot has 1.80-fold increased reactivity relative to the representative Term Western blot and its pixel density graphs.

Figure 6 is a schematic illustration of exemplary methods for protein expression profiling with immunoprecipitation.

Figure 7 depicts the incongruent antigen antibody complexes between the control (Term) and RPL subjects. The arrows indicate a2-macroglobulin, fibronectin, and Apolipoprotein B-100.
DETAILED DESCRIPTION

While survival of the fetal allograft in the maternal allo-reactive environment remains unexplained, suppression of cellular immunity appears to be one manifestation of pregnancy that may be a critical factor in its success. The pathophysiology of recurrent pregnancy loss (RPL) is complex with many unknown contributing factors and mechanisms. Suggested causes currently applicable to clinical evaluation include anatomical uterine or pelvic defects, genetic, or molecular abnormalities, endocrine disorders, thrombophilias and anti-phospholipid antibody syndrome. However, in up to 50% of cases, no etiology can be identified (Szekeres-Bartho et al, *Hum. Reprod. Update* 14:27-35, 2008). Increasing evidence supports the involvement of various aberrant maternal-fetal immunoregulatory mechanisms and, while survival of the fetal allograft in the maternal allo-reactive environment remains unexplained, suppression of cellular immunity appears to be one manifestation of pregnancy that may be a critical factor in its success. The etiology of pregnancy loss varies and is often controversial, with multiple factors potentially involved, including genetic, anatomic, infectious, environmental, immunologic, endocrine, and hematologic causes.

placentation may play a critical role in the pathogenesis of partial or total rejection of the fetal allograft, leading to complications, such as spontaneous miscarriage.

Successful pregnancy is linked with a shift to a Th2 immune response (e.g., an elevated Th2/Th1 immune response ratio), characterized by an increased rate of antibody production (e.g., the production of fetal reactive IgG antibodies) and decreased cell-mediated responses. The theory of immunodystrophy has been proposed to account for the dichotomous Th1- and Th2-cytokine profile associated with human pregnancy loss and success, respectively. Endometrial lymphocytes of recurrent spontaneous aborters express distinct immune-phenotypic profiles that antedate implantation and suggest that endometrial immunologic conditions are intrinsically altered in recurrent aborters.

Activation of T-lymphocytes during pregnancy can result in one of two different cytokine profiles: Th2-secreted cytokines (e.g., IL-4, IL-5, and IL-10) that suppress cellular immunity and Th1 -secreted cytokines (e.g., IFN-γ, IL-2, and TNF-a) that induce cellular immunity (e.g., T-cell activation). Failure to suppress T-cell activation may allow the generation of cellular fetal-reactive immune responses, a potential key causative factor in infertility and adverse pregnancy outcomes. An increase in the ratio of Th2 cytokines to Th1 cytokines is associated with successful pregnancy and a decrease in this ratio is associated with recurrent pregnancy loss (Jenkins et al, *Fertil. Steril.* 73:1206-1208, 2000; Hill et al, *JAMA* 273:1933-1936, 1995). Clinical studies have demonstrated the predominance of Th1-type cytokine production in patients with pregnancy complications, such as pre-eclampsia (Hill et al, *JAMA* 273:1933-1936, 1995). There is no conclusive evidence as to whether some or all of these mechanisms are functional; however, it appears that mechanisms crucial for immunosuppression would be pivotal in early pregnancy.

A failure to suppress T-cell activation may allow the generation of cellular fetal-reactive immune responses, which may represent a key causative factor in infertility and adverse pregnancy outcomes. The data also indicate that the induction of IgG in normal pregnant patients is linked with a shift to a predominant IgG2 subclass, which does not appear to occur in women with recurrent pregnancy loss. One hypothesis is that, in women who suffer from recurrent pregnancy loss, the shift to anti-fetal immune responses lacking or exhibiting weak effector function fails to occur.
As demonstrated herein, women with a history of recurrent pregnancy loss demonstrate aberrant presence or absence of antibodies to three proteins: Apolipoprotein B-100, alpha2macrogloblin, and fibronectin. Thus, the presence, a detectable level, or an increase of maternal IgG antibodies to trophoblast-derived fibronectin (protein or nucleic acid) and/or ApoB-100 (protein or nucleic acid), and/or the absence, a non-detectable level, or a decrease of antibodies that specifically bind to a2M (protein or nucleic acid) is associated with a history of RPL and in increased risk of future pregnancy loss.

**Apolipoprotein B-100**


Lipoprotein oxidation has been proposed as a key player in the pathogenesis of pregnancy complications, such as pre-eclampsia and IUGR (Sarandol et al., *Arch. Gynecol. Obstet.* 270:157-160, 2004). In normal pregnancies, physiologic hyperlipidemia is believed to be controlled by anti-oxidative defense mechanisms, hormonal, or other biochemical influences (Cekmen et al., *Clin. Biochem.* 36:575-578, 2003; Sarandol et al, *Arch. Gynecol. Obstet.* 270:157-160, 2004). Aberrances in these control mechanisms may lead to lipid peroxidation products that mediate oxidative damage and result in disseminated endothelial dysfunction (Sarandol et al., *Clin. Biochem.* 37:990-996, 2004). Perhaps, in normal pregnancy, an enzyme or other substrate/protein/molecule stabilizes and/or utilizes lipoproteins, inhibiting the common pathway of oxidation.
Some researchers have proposed a role for antioxidants such as vitamin E and/or estrogen to inhibit oxidation of lipoproteins (Sarandol et al., Arch. Gynecol. Obstet. 270:157-160, 2004). Conversely, the absence of an endogenous protection mechanism may also lead to aberrant lipoprotein oxidative damage at the uteroplacental interface.

ApoB activity has been detected in the maternal corpus luteum during early pregnancy (Yamada et al, Human Reprod. 13:944-952, 1998). Corpus luteal cells produce and secrete abundant progesterone, synthesized from serum-derived cholesterol compounds. Studies show that ApoB represents uptake of LDL in to the luteal steroid producing cells. Human chorionic gonadotropin (HCG) administration enhanced levels of mPvRNA for the LDL receptor in luteal cells (Yamada et al., Human Reprod. 13:944-952, 1998; Benyo et al, Endocrinology 133:699-704, 1993). Endogenous or exogenous HCG may play a role in preserving and/or augmenting the presence of LDL-receptors, thereby maintaining the uptake of cholesterol compounds required for substantial progesterone production. Perhaps antibody recognition of ApoB in normal pregnant patients permits or supports its utilization in the luteal production and secretion of progesterone required in early pregnancy support and development. Conversely, perhaps patients who do not display this IgG recognition are subject to dysfunctional corpus luteum and subsequent recurrent pregnancy loss.


The sequence of human Apolipoprotein B 100 can be found at NM_000384.2 (nucleic acid; SEQ ID NO: 1) and NP_000375.2 (protein; SEQ ID NO: 2).

Some embodiments of all of the methods described herein include the detection or determination of the presence, a detectable level, or an increase in the level of antibodies that specifically bind to apolipoprotein B-100 or an antigenic fragment thereof. The
detected antibodies may be antibodies that specifically bind to an apolipoprotein B-100 protein, or an antigenic fragment thereof, or an apolipoprotein B-100 nucleic acid (e.g., mRNA), or an antigenic fragment thereof. For example, an antibody may specifically bind to at least 5 (e.g., at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20) contiguous amino acids in the sequence of SEQ ID NO: 2. The at least 5 (e.g., at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20) contiguous amino acids within the sequence of SEQ ID NO: 2 may be located anywhere within the sequence, for example, the contiguous amino acid sequence may begin at the N-terminus, may end at the C-terminus, or may begin at any amino acid within the sequence of SEQ ID NO: 2 (except for the last four C-terminal amino acids). In some embodiments, the detected antibody may specifically bind to polypeptide containing the sequence of SEQ ID NO: 2.

The detected antibodies may be antibodies that specifically bind to an apolipoprotein nucleic acid (e.g., mRNA) or an antigenic fragment thereof. For example, the detected antibody may specifically bind to at least 5 (e.g., at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20) contiguous nucleotides present within the sequence of SEQ ID NO: 1. The at least 5 (e.g., at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20) contiguous nucleotides within the sequence of SEQ ID NO: 1 may be located anywhere within the sequence, for example, the contiguous nucleotide sequence may begin at the 5'-terminus, may end at the 3'-terminus, or may begin at any nucleotide within the sequence of SEQ ID NO: 1 (except for the last four 3'-terminal nucleotides). In some embodiments, the detected antibody may specifically bind to a nucleic acid containing the sequence of SEQ ID NO: 1.

Additional embodiments of all of the methods described herein (e.g., methods for determining the risk of pregnancy loss in a subject, for identifying a subject at risk of pregnancy loss, for selecting a subject for participation in a clinical study, and for decreasing the risk of pregnancy loss in a subject) involve the detection or determination of the presence, a detectable level, or an increased level of Apolipoprotein B-100 protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof, in a sample from the subject (e.g., in the serum of the subject). In these methods, the Apolipoprotein B-100 protein that is detected may be, for example, a protein containing the sequence of SEQ ID NO: 2, or any antigenic fragment thereof. For example, an antigenic fragment of
Apolipoprotein B-100 protein that may be detected can contain at least 5 (e.g., at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20) contiguous amino acids within the sequence of SEQ ID NO: 2. The at least 5 (e.g., at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20) contiguous amino acids within the sequence of SEQ ID NO: 2 may be located anywhere within the sequence, for example, the contiguous amino acid sequence may begin at the N-terminus, may end at the C-terminus, or may begin at any amino acid within the sequence of SEQ ID NO: 2 (except for the last four C-terminal amino acids).

In additional examples of these methods, the Apolipoprotein nucleic acid (e.g., mRNA) that is detected may be, for example, a nucleic acid containing the sequence of SEQ ID NO: 1, or any antigenic fragment thereof. For example, an antigenic fragment of Apolipoprotein B-100 nucleic acid that may be detected can contain at least 5 (e.g., at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20) contiguous nucleotides within the sequence of SEQ ID NO: 1. The at least 5 (e.g., at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20) contiguous nucleotides within the sequence of SEQ ID NO: 1 may be located anywhere within the sequence, for example, the contiguous nucleotide sequence may begin at the 5'-terminus, may end at the 3'-terminus, or may begin at any nucleotide within the sequence of SEQ ID NO: 1 (except for the last four 3'-terminal nucleotides).

**Fibronectin**

The maternal extracellular matrix and maternal-fetal interface have been suggested to play a pivotal role in conditions of early recurrent abortions, intrauterine growth restriction, and pre-eclampsia. Fetal fibronectin is one extracellular matrix protein that may act as "trophoblast glue," with increased concentrations at the chorionic-decidual margin and surrounding the extravillous trophoblasts (Mercorio et al., *Eur. J. Gynecol. Reprod. Biol.* 126:165-169, 2006; Guller et al, *Up-To-Date*, version 17.3, 2009). Integrin receptors for fibronectin with strong binding activity have been observed on the surface of blastocysts (Mercorio et al, *Eur. J. Gynecol. Reprod. Biol.* 126:165-169, 2006). Derangement in the signals and receptivity between cellular matrix proteins, e.g., fibronectin, and cell adhesion molecules may be responsible for pregnancy failure.
The fibronectin gene has three regions subject to alternative splicing, with the potential to produce 20 different transcript variants. The human reference sequences are as follows: NM_002026.2 (nucleic acid; SEQ ID NO: 3) and NP_002017.1 (protein; SEQ ID NO: 4) for fibronectin 1 isoform 3 preproprotein; NM_054034.2 (nucleic acid; SEQ ID NO: 5) and NP_473375.2 (protein; SEQ ID NO: 6) for fibronectin 1 isoform 7 preproprotein; NM_212474.1 (nucleic acid; SEQ ID NO: 7) and NP_997639.1 (protein; SEQ ID NO: 8) for fibronectin 1 isoform 6 preproprotein; NM_212475.1 (nucleic acid; SEQ ID NO: 9) and NP_997640.1 (protein; SEQ ID NO: 10) for fibronectin 1 isoform 2 preproprotein; NM_212476.1 (nucleic acid; SEQ ID NO: 11) and NP_997641.1 (protein; SEQ ID NO: 12) for fibronectin 1 isoform 5 preproprotein; NM_212478.1 (nucleic acid; SEQ ID NO: 13) and NP_997643.1 (protein; SEQ ID NO: 14) for fibronectin 1 isoform 4 preproprotein; and NM_212482.1 (nucleic acid; SEQ ID NO: 15) and NP_997647.1 (protein; SEQ ID NO: 16) for fibronectin 1 isoform 1 preproprotein (the longest transcript that encodes the longest isoform).

Some embodiments of all of the methods described herein include the determination of the presence, a detectable level, or an increase in the level of antibodies that specifically bind to fibronectin or an antigenic fragment thereof. The detected antibodies may be antibodies that specifically bind to a fibronectin protein or an antigenic fragment thereof, or a fibronectin nucleic acid (e.g., mRNA), or an antigenic fragment thereof. For example, an antibody may specifically bind to at least 5 (e.g., at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20) contiguous amino acids in the sequence of SEQ ID NO: 4, 6, 8, 10, 12, 14, or 16. The at least 5 (e.g., at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20) contiguous amino acids within the sequence of SEQ ID NOS: 4, 6, 8, 10, 12, 14, or 16 may be located anywhere within the sequence, for example, the contiguous amino acid sequence may begin at the N-terminus, may end at the C-terminus, or may begin at any amino acid within the sequence of SEQ ID NO: 4, 6, 8, 10, 12, 14, or 16 (except for the last four C-terminal amino acids in any one of these sequences). In some embodiments, the detected antibody may specifically bind to polypeptide containing the sequence of SEQ ID NO: 4, 6, 8, 10, 12, 14, or 16.

The detected antibodies may be antibodies that specifically bind to a fibronectin nucleic acid (e.g., mRNA). For example, the detected antibody may specifically bind to
at least 5 (e.g., at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20) contiguous nucleotides present within the sequence of SEQ ID NO: 3, 5, 7, 9, 11, 13, or 15. The at least 5 (e.g., at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20) contiguous nucleotides within the sequence of SEQ ID NO: 3, 5, 7, 9, 11, 13, or 15 may be located anywhere within the sequence, for example, the contiguous nucleotide sequence may begin at the 5'-terminus, may end at the 3'-terminus, or may begin at any nucleotide within the sequence of SEQ ID NO: 3, 5, 7, 9, 11, 13, or 15 (except for the last four 3'-terminal nucleotides of any one of these sequences). In some embodiments, the detected antibody may specifically bind to a nucleic acid containing the sequence of SEQ ID NO: 3, 5, 7, 9, 11, 13, or 15.

In additional embodiments of the methods described herein (e.g., methods for determining the risk of pregnancy loss in a subject, for identifying a subject at risk of pregnancy loss, for selecting a subject for participation in a clinical study, and for decreasing the risk of pregnancy loss in a subject) involve the detection of the presence, a detectable level, or an increased level of fibronectin protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof, in a sample from the subject (e.g., in the serum of the subject). In these methods, the fibronectin protein that is detected may be, for example, a protein containing the sequence of SEQ ID NO: 4, 6, 8, 10, 12, 14, or 16, or any antigenic fragment thereof. For example, an antigenic fragment of a fibronectin protein that may be detected can contain at least 5 (e.g., at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20) contiguous amino acids within the sequence of SEQ ID NO: 4, 6, 8, 10, 12, 14, or 16. The at least 5 (e.g., at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20) contiguous amino acids within the sequence of SEQ ID NO: 4, 6, 8, 10, 12, 14, or 16 may be located anywhere within the sequence, for example, the contiguous amino acid sequence may begin at the N-terminus, may end at the C-terminus, or may begin at any amino acid within the sequence of SEQ ID NO: 4, 6, 8, 10, 12, 14, or 16 (except for the last four C-terminal amino acids of any one of the sequences).

In additional examples of these methods, the fibronectin nucleic acid (e.g., mRNA) that is detected may be, for example, a nucleic acid containing the sequence of SEQ ID NO: 3, 5, 7, 9, 11, 13, or 15, or any antigenic fragment thereof. For example, an antigenic fragment of a fibronectin nucleic acid that may be detected can contain at least
5 (e.g., at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20) contiguous nucleotides within the sequence of SEQ ID NO: 3, 5, 7, 9, 11, 13, or 15. The at least 5 (e.g., at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20) contiguous nucleotides within the sequence of SEQ ID NO: 3, 5, 7, 9, 11, 13, or 15 may be located anywhere within the sequence, for example, the contiguous nucleotide sequence may begin at the 5'-terminus, may end at the 3'-terminus, or may begin at any nucleotide within the sequence of SEQ ID NO: 3, 5, 7, 9, 11, 13, or 15 (except for the last four 3'-terminal nucleotides).

**Alpha2-macroglobulin**

Alpha2-macroglobulin (a2M) is a major inhibitor of endoproteinases and carries a regulatory role in the protection, transport, and clearance of cytokines and growth factors (Esadeg et al., *Placenta* 24:912-921, 2003). a2M has a potential means of immunosuppression in the human uteroplacental interface and may be subject to transplacental transport to the neonate (Benyo et al., *Endocrinology* 133:699-704, 1993). a2M targets cytokines to cells expressing the a2M-receptor or lipoprotein-receptor related protein (Esadeg et al, *Placenta* 24:912-921, 2003; Shimizu et al, *Exp. Anim.* 51:361-365, 2002). Uterine a2M is thought to originate from endothelial cells lining the endometrial vessels. Small serum concentrations of a2M are found in normal healthy adults, and its concentration has been reported to double or triple during the secretory phase of the menstrual cycle suggesting a role as a decidualization protein (Esadeg et al, *Placenta* 24:912-921, 2003). During pregnancy, a receptor for the a2M-proteinase complex has been demonstrated on the human placental syncytiotrophoblasts (Thomas et al, *Placenta* 11:413-430, 1990; Jensen et al, *Placenta* 9:463-471, 1988). In addition, synthesis and secretion of a2M has also been detected in the visceral yolk sac of fetal rats. The sequence of human a2M can be found at NM_000014.4 (nucleic acid; SEQ ID NO: 17) and NP_000005.2 (amino acid; SEQ ID NO: 18).

Some embodiments of the methods described herein include the determination or detection of the absence, a non-detectable level, or a decreased level of antibodies that specifically bind to a2M or an antigenic fragment thereof. The detected antibodies may be antibodies that specifically bind to an a2M protein, or an antigenic fragment thereof,
or an a2M nucleic acid (e.g., mRNA), or an antigenic fragment thereof. For example, an antibody may specifically bind to at least 5 (e.g., at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20) contiguous amino acids in the sequence of SEQ ID NO: 18. The at least 5 (e.g., at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20) contiguous amino acids within the sequence of SEQ ID NO: 18 may be located anywhere within the sequence, for example, the contiguous amino acid sequence may begin at the N-terminus, may end at the C-terminus, or may begin at any amino acid within the sequence of SEQ ID NO: 18 (except for the last four C-terminal amino acids). In some embodiments, the detected antibody may specifically bind to polypeptide containing the sequence of SEQ ID NO: 18.

The detected antibodies may be antibodies that specifically bind to an a2M nucleic acid (e.g., mRNA). For example, the detected antibody may specifically bind to at least 5 (e.g., at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20) contiguous nucleotides present within the sequence of SEQ ID NO: 17. The at least 5 (e.g., at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20) contiguous nucleotides within the sequence of SEQ ID NO: 17 may be located anywhere within the sequence, for example, the contiguous nucleotide sequence may begin at the 5'-terminus, may end at the 3'-terminus, or may begin at any nucleotide within the sequence of SEQ ID NO: 17 (except for the last four 3'-terminal nucleotides). In some embodiments, the detected antibody may specifically bind to a nucleic acid containing the sequence of SEQ ID NO: 17.

In additional embodiments of all of the methods described herein (e.g., methods for determining the risk of pregnancy loss in a subject, for identifying a subject at risk of pregnancy loss, for selecting a subject for participation in a clinical study, and for decreasing the risk of pregnancy loss in a subject) involve the detection of the absence, a non-detectable level, or a decreased level of a2M protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof, in a sample from the subject (e.g., in the serum of the subject). In these methods, the a2M protein that is detected may be, for example, a protein containing the sequence of SEQ ID NO: 18, or any antigenic fragment thereof. For example, an antigenic fragment of a2M protein that may be detected can contain at least 5 (e.g., at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20) contiguous amino acids within the sequence of SEQ ID NO: 18. The at least 5 (e.g., at least 6, 7, 8,
9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20) contiguous amino acids within the sequence of SEQ ID NO: 18 may be located anywhere within the sequence, for example, the contiguous amino acid sequence may begin at the N-terminus, may end at the C-terminus, or may begin at any amino acid within the sequence of SEQ ID NO: 18 (except for the last four C-terminal amino acids).

In additional examples of these methods, the a2M nucleic acid (e.g., mRNA) that is detected may be, for example, a nucleic acid containing the sequence of SEQ ID NO: 17, or any antigenic fragment thereof. For example, an antigenic fragment of an a2M nucleic acid that may be detected can contain at least 5 (e.g., at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20) contiguous nucleotides within the sequence of SEQ ID NO: 17. The at least 5 (e.g., at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20) contiguous nucleotides within the sequence of SEQ ID NO: 17 may be located anywhere within the sequence, for example, the contiguous nucleotide sequence may begin at the 5'-terminus, may end at the 3'-terminus, or may begin at any nucleotide within the sequence of SEQ ID NO: 17 (except for the last four 3'-terminal nucleotides).

Methods of Predicting Pregnancy Loss

Provided herein are methods of predicting the risk of pregnancy loss in a subject that include providing a sample containing serum from the subject and detecting the presence, absence, or level of antibodies that specifically bind to one or more (e.g., one, two, or three) of a fibronectin (protein or nucleic acid), an a2M (protein or nucleic acid), and an Apolipoprotein B-100 (protein or nucleic acid), or an antigenic fragment thereof, in the sample, wherein the presence, a detectable level, or an increased level of antibodies to a fibronectin (protein or nucleic acid) and/or ApoB-100 (protein or nucleic acid), or antigenic fragment thereof, and/or the absence, a non-detectable level, or a decreased level of antibodies to an a2M (protein or nucleic acid), or an antigenic fragment thereof, in the sample, indicate that the subject has an increased (e.g., a statistically significant increase, such as an increase of at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100%) risk of pregnancy loss. Additional methods for predicting the risk of pregnancy loss in a subject may include providing a sample (e.g., a sample containing serum) from the subject and
detecting the presence, absence, or level of one or more (e.g., one, two, or three) of a fibronectin (protein or nucleic acid), an a2M (protein or nucleic acid), and an Apolipoprotein B-100 (protein or nucleic acid), or an antigenic fragment thereof, in the sample, wherein the presence, a detectable level, or an increased level of a fibronectin (protein or nucleic acid) and/or an ApoB-100 (protein or nucleic acid), or antigenic fragment thereof, and/or the absence, a non-detectable level, or a decreased level of an a2M (protein or nucleic acid), or antigenic fragment thereof, in the sample, indicate that the subject has an increased (e.g., a statistically significant increase, such as an increase of at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100%) risk of pregnancy loss.

In some embodiments of all of the methods described herein, the subject may be a pregnant woman in the first (weeks 0-12) or second (weeks 13-27) trimester of pregnancy (e.g., any time between 0 to 20 weeks, 6 to 20 weeks, 6 to 12 weeks, or 24 weeks after conception). In some embodiments of all of the methods described herein, the subject may be a pregnant subject within the first 20 weeks of pregnancy (e.g., within 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, 10 weeks, 11 weeks, 12 weeks, 13 weeks, 14 weeks, 15 weeks, 16 weeks, 17 weeks, 18 weeks, or 19 weeks of pregnancy). Early pregnancy loss is defined as the termination of pregnancy before 20 weeks gestation or with a fetal weight of <500 g.

The subject (e.g., a pregnant subject or a non-pregnant subject) may also have had at least one (e.g., two, three, four, five, or six) pregnancy loss or may be suspected of having had at least one (e.g., two, three, four, five, or six) previous pregnancy loss. In some embodiments, the subject is within the first 20 weeks of pregnancy (e.g., within 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, 10 weeks, 11 weeks, 12 weeks, 13 weeks, 14 weeks, 15 weeks, 16 weeks, 17 weeks, 18 weeks, or 19 weeks of pregnancy) and has had at least one (e.g., two, three, four, five, or six) pregnancy loss or is suspected of having had at least one (e.g., two, three, four, five, or six) pregnancy loss.

A sample (e.g., serum) from the subject may be collected from the subject prior to pregnancy, following a miscarriage or a suspected miscarriage, or at any time during pregnancy (e.g., within 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8
weeks, 9 weeks, 10 weeks, 11 weeks, 12 weeks, 13 weeks, 14 weeks, 15 weeks, 16 weeks, 17 weeks, 18 weeks, 19 weeks, or 20 weeks). Samples may be frozen or stored for a period of time (e.g., at least one day, two days, three days, four days, five days, six days, or 1 week) prior to detecting/determining the presence, absence, or level of antibodies to one or more (e.g., one, two, or three) of a fibronectin (protein or nucleic acid), an Apolipoprotein B-100 (protein or nucleic acid), and an a2M (protein or nucleic acid), and/or the presence, absence, or level of one or more (e.g., one, two, or three) of a fibronectin (protein or nucleic acid), an Apolipoprotein B-100 (protein or nucleic acid), and an a2M (protein or nucleic acid), or an antigenic portion thereof.

Any method known in the art can be used for detecting the presence of antibodies in a sample (e.g., antibodies that specifically bind to fibronectin (protein or mRNA), Apolipoprotein B-100 (protein or mRNA), or a2M (protein or mRNA), or an antigenic portion thereof). For example, a sample from a subject (e.g., a sample containing serum, such as, serum, plasma, or blood), from a subject (e.g., any of the subjects described herein, such as a pregnant subject) can be contacted with all or an antigenic fragment of a protein or nucleic acid described herein (e.g., a fibronectin protein or nucleic acid, an a2M protein or nucleic acid, and/or an ApoB-100 protein or nucleic acid, or an antigenic fragment thereof), and binding of any antibodies in the sample to these antigen(s) can be detected using methods known in the art.

For example, an array (e.g., any array, microarray, biochip, or point-of-care test as is known in the art) can be provided that comprises one or more of the proteins, nucleic acids, or antigenic fragments thereof, and the array can be contacted with the sample containing serum from the subject, and the binding of any antibodies present in the sample can be detected.

Methods for detecting binding of the antibodies are known in the art, and can include the use of secondary antibodies; alternatively, any other antibody-specific ligand can be used. The secondary antibodies are generally modified to be detectable, e.g., labeled. The term "labeled" is intended to encompass direct labeling by coupling (i.e., physically linking) a detectable substance to the secondary antibody, as well as indirect labeling of the multimeric antigen by reactivity with a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials,
luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase (HRP), alkaline phosphatase, β-galactosidase, and acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, and quantum dots, dichlorotriazinylamine fluorescein, dansyl chloride, and phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include green fluorescent protein and variants thereof, luciferase, luciferin, and aequorin; and examples of suitable radioactive material include 125I, 131I, 35S, or 3H.

Methods for producing such labeled antibodies are known in the art, and many are commercially available.

In some embodiments, the methods further include determining the subtype of the antibodies that bind to the antigens, e.g., detecting the presence of IgG3 antibodies, which as described herein are associated with an increased humoral response and increased risk of pregnancy loss. Antibodies that bind to the Fc region of IgG3 are commercially available and may be used to determine the presence, level, or absence of IgG3 antibodies in the sample.

Any method of detecting the antibodies can be used, including but not limited to radioimmunoassays (RIA), enzyme-linked immunosorbent assays (ELISA), Western blotting, surface plasmon resonance, microfluidic devices, protein array, mass spectrometry, or other assays as known in the art. In some embodiments, the antigens can be produced in tetrameric form as described in US-2009-005425-A1.

As described herein, the invention provides methods for predicting pregnancy loss by detecting the presence of aberrant humoral response; as noted above, these methods can include the use of an array. The invention provides an array (i.e., "biochip" or "microarray") that includes immobilized antigens that facilitate the detection of a particular antibody or antibodies in a biological sample. Antigens that identify the antibodies as described herein can be included in a custom array for detecting subjects predisposed to pregnancy loss, e.g., RPL. For example, a custom array can include antigens that specifically bind antibodies to one or more (e.g., one, two, or three) of a fibronectin, an a2M, and an ApoB-100. The antigens can be a full-length protein, a full-
length nucleic acid (e.g., an mRNA), or a fragment thereof (as described herein). The array can also include biomolecules that identify additional antibodies. The arrays can be used to develop a database of information using data obtained using the methods described herein.

The term "array," as used herein, generally refers to a predetermined spatial arrangement of binding ligands, antigens, or spatial arrangements of binding ligands or antigens. Arrays according to the present invention that include antigens immobilized on a surface may also be referred to as "antigen arrays." Arrays according to the present invention that comprise surfaces activated, adapted, prepared, or modified to facilitate the binding of antigens to the surface may also be referred to as "binding arrays." Further, the term "array" may be used herein to refer to multiple arrays arranged on a surface, such as would be the case where a surface bore multiple copies of an array. Such surfaces bearing multiple arrays may also be referred to as "multiple arrays" or "repeating arrays." The use of the term "array" herein may encompass antigen arrays, binding arrays, multiple arrays, and any combination thereof; the appropriate meaning will be apparent from context. An array can include antigens that detect antibodies and other proteins altered in a subject who is likely to experience pregnancy loss. The array can be contacted with one or more biological samples from a subject; the samples can include fluid or solid samples from any tissue of the body including excretory fluids such as urine. Non-urine samples include, but are not limited to serum, plasma, amniotic fluid, and placental tissue.

An array of the invention comprises a substrate. By "substrate" or "solid support" or other grammatical equivalents, herein is meant any material appropriate for the attachment of antigens and is amenable to at least one detection method. As will be appreciated by those in the art, the number of possible substrates is very large. Possible substrates include, but are not limited to, glass and modified or functionalized glass, plastics (including acrylics, polystyrene, and copolymers of styrene and other materials, polypropylene, polyethylene, polybutylene, polyurethanes, TEFLOW-, etc.), polysaccharides, nylon or nitrocellulose, resins, silica or silica-based materials including silicon and modified silicon, carbon, metals, inorganic glasses, plastics, ceramics, and a variety of other polymers. In addition, as is known the art, the substrate may be coated
with any number of materials, including polymers, such as dextrans, acrylamides, gelatins, or agarose. Such coatings can facilitate the use of the array with a biological sample derived from urine or serum.

A planar array of the invention will generally contain addressable locations (e.g., "pads," "addresses," or "micro-locations") of antigens in an array format. The size of the array will depend on the composition and end use of the array. The arrays can contain 1, 2, or more different antigens; in some embodiments, different portions of the same protein are also included, to detect antibodies that bind to different epitopes on the protein. Generally, the array will comprise from two to as many as 100,000 or more antigens, depending on the end use of the array. A microarray of the invention will generally comprise at least one antigen that identifies or "captures" an antibody present in a biological sample. In some embodiments, the compositions of the invention may not be in an array format; that is, for some embodiments, compositions comprising a single antigen may be made as well. In addition, in some arrays, multiple substrates may be used, either of different or identical compositions. Thus, for example, large planar arrays may comprise a plurality of smaller substrates.

As an alternative to planar arrays, bead-based assays in combination with flow cytometry have been developed to perform multiparametric immunoassays. In bead-based assay systems the antigens can be immobilized on addressable microspheres. Each antigen for each individual immunoassay is coupled to a distinct type of microsphere (i.e., "microbead") and the immunoassay reaction takes place on the surface of the microspheres. Dyed microspheres with discrete fluorescence intensities are loaded separately with their appropriate biomolecules. The different bead sets carrying different capture probes can be pooled as necessary to generate custom bead arrays. Bead arrays are then incubated with the sample in a single reaction vessel to perform the immunoassay.

In some embodiments, product formation of the antibody with their immobilized antigens can be detected with a fluorescence-based reporter system. The antibodies can be labeled directly by a fluorogen or detected by a second fluorescently-labeled capture biomolecule. The signal intensities derived from captured antibodies are measured in a flow cytometer. The flow cytometer first identifies each microsphere by its individual
color code. Second the amount of captured antibody on each individual bead is measured by the second color fluorescence specific for the bound target. This allows multiplexed quantitation of multiple targets from a single sample within the same experiment. Sensitivity, reliability, and accuracy are comparable to standard microtiter ELISA procedures. With bead-based immunoassay systems antibodies can be simultaneously quantified from biological samples. An advantage of bead-based systems is the individual coupling of the antibody to distinct microspheres.

Thus, microbead array technology can be used to sort antibodies bound to specific antigens using a plurality of microbeads, each of which can carry about 100,000 identical molecules of a specific antigen on its surface. Once captured, the antibody can be handled as fluid, referred to herein as a "fluid microarray."

An array can encompass any means for detecting an antibody. For example, microarrays can be biochips that provide high-density immobilized arrays of antigens, where antibody binding is monitored indirectly (e.g., via fluorescence). In addition, an array can be of a format that involves the capture of antibodies by biochemical or intermolecular interaction, coupled with direct detection by mass spectrometry (MS).

Arrays and microarrays that can be used with the methods described herein can be made according to the methods described in U.S. Patent Nos. 6,329,209; 6,365,418; 6,406,921; 6,475,808; and 6,475,809, which are incorporated herein in their entirety.

New arrays, to detect specific selections or sets of biomarkers described herein can also be made using the methods described in these patents.

The antigens can be immobilized on the surface using methods and materials that minimize the denaturing of the antigens, that minimize alterations in the structure of the antigens, or that minimize interactions between the antigens and the surface on which they are immobilized.

Surfaces usef ul in the arrays may be of any desired shape (form) and size. Non-limiting examples of surfaces include chips, continuous surfaces, curved surfaces, flexible surfaces, films, plates, sheets, tubes, and the like. Surfaces preferably have areas ranging from approximately a square micron to approximately 500 cm². The area, length, and width of surfaces according to the present invention may be varied according to the requirements of the assay to be performed. Considerations may include, for example,
ease of handling, limitations of the material(s) of which the surface is formed, requirements of detection systems, requirements of deposition systems (e.g., arrayers), and the like.

In certain embodiments, it is desirable to employ a physical means for separating groups or arrays of binding islands or immobilized antigens: such physical separation facilitates exposure of different groups or arrays to different solutions of interest. Therefore, in certain embodiments, arrays are situated within wells of 96, 384, 1536, or 3456 microwell plates. In such embodiments, the bottoms of the wells may serve as surfaces for the formation of arrays, or arrays may be formed on other surfaces and then placed into wells. In certain embodiments, such as where a surface without wells is used, binding islands may be formed or antigens may be immobilized on a surface and a gasket having holes spatially arranged so that they correspond to the islands or antigens may be placed on the surface. Such a gasket is preferably liquid-tight. A gasket may be placed on a surface at any time during the process of making the array and may be removed if separation of groups or arrays is no longer necessary.

The immobilized antigens can bind to antibodies present in a biological sample overlying the immobilized antigens. For example, an antibody present in a biological sample can contact an immobilized antigen and bind to it, thereby facilitating detection of the antibody.

Modifications or binding of antibodies to antigens in solution or immobilized on an array may be detected using detection techniques known in the art. Examples of such techniques include immunological techniques such as competitive binding assays and sandwich assays; fluorescence detection using instruments such as confocal scanners, confocal microscopes, or CCD-based systems, and techniques such as fluorescence, fluorescence polarization (FP), fluorescence resonant energy transfer (FRET), total internal reflection fluorescence (TIRF), fluorescence correlation spectroscopy (FCS); colorimetric/spectrometric techniques; surface plasmon resonance, by which changes in mass of materials adsorbed at surfaces may be measured; techniques using radioisotopes, including conventional radioisotope binding and scintillation proximity assays so (SPA); mass spectroscopy, such as matrix-assisted laser desorption/ionization mass spectroscopy (MALDI) and MALDI-time of flight (TOF) mass spectroscopy; ellipsometry, which is an

Arrays as described herein can be included in kits. Such kits may also include, as non-limiting examples, one or more of reagents useful for preparing antigens for immobilization onto binding islands or areas of an array, reagents useful in preparing a sample, or reagents useful for detecting binding of antibodies in a sample to immobilized antigens, control samples that include known antibodies and instructions for use.

For example, kits provided by the invention may essentially include one or more (e.g., one, two, three, four, five, or six) of a fibronectin (protein and/or nucleic acid), an a2M (protein and/or nucleic acid), and an Apolipoprotein B-100 (protein and/or nucleic acid), or antigenic fragments thereof. Kits may also contain one or more (e.g., one, two, three, four, five, or six) antibodies that specifically bind to a fibronectin (protein or nucleic acid), an a2M (protein or nucleic acid), and an Apolipoprotein B-100 (protein or nucleic acid), or an antigenic fragment thereof. For example, the one or more antigens or the one or more antibodies provided in the kits may be immobilized on a surface (e.g., in the form of a ELISA assay).

In some embodiments of all the methods described herein, the presence, absence, or levels of one or more (e.g., one, two, or three) of fibronectin protein or mRNA, Apolipoprotein B-100 protein or mRNA, and a2M protein or mRNA, or an antigenic fragment thereof, present in a sample (e.g., a sample containing serum) from the subject is determined. A variety of examples of fibronectin protein and nucleic acid (e.g., mRNA), Apolipoprotein B-100 protein and nucleic acid (e.g., mRNA), and a2M protein and nucleic acid (e.g., mRNA), and antigenic fragments thereof are described herein. Methods for measuring the presence, absence, or levels of an antigenic protein or peptide in a biological sample using antibodies are known in the art, including, for example,
radioimmunoassays (RIA), enzyme-linked immunosorbent assays (ELISA), Western blotting, surface plasmon resonance, microfluidic devices, protein array, and mass spectrometry. Methods for measuring the presence, absence, or levels of a nucleic acid in a biological sample are known in the art, for example, polymerase chain reaction (PCR)-based techniques (e.g., real-time quantitative PCR and gene array). Primers for use in the methods of measuring the presence, absence, or levels of a nucleic acid may be designed based on the sequence of SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, or 17 using methods known in the art.

In any of the methods described herein, one or more (e.g., one, two, three, four, five, six, seven, or eight) of any combination of the following, in a sample from the subject, indicate that the subject has an increased (e.g., a statistically significant increase, such at an increase of 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100%) risk of pregnancy loss: the absence or a non-detectable level of antibodies that specifically bind to an a2M protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof (as described herein); a decrease in the level of antibodies that specifically bind to an a2M protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof (as described herein) (e.g., as compared to a control subject of the same age or a control subject that has had one or more successful pregnancies, or a subject that has not had a miscarriage or is not suspected of having had a miscarriage); the absence or a non-detectable level of a2M protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof (as described herein); a decreased level of a2M protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof (as described herein) (e.g., as compared to a control subject of the same age, a control subject that has had one or more successful pregnancies, and/or a control subject that has not had a miscarriage or is not suspected of having had a miscarriage); the presence or a detectable level of antibodies that specifically bind to a fibronectin protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof (as described herein); an increase in the level of antibodies that specifically bind to a fibronectin protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof (as described herein) (e.g., as compared to a control subject of the same age, a control subject that has had one or more successful pregnancies, and/or a control subject that has not had a miscarriage or is not
suspected of having had a miscarriage); the presence or detectable level of a fibronectin protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof (as described herein); an increased level of a fibronectin protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof (as described herein) (e.g., as compared to a control subject of the same age, a control subject that has had one or more successful pregnancies, and/or a control subject that has not had a miscarriage or is not suspected of having had a miscarriage); the presence or a detectable level of antibodies that specifically bind to an Apolipoprotein B-100 protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof (as described herein); and an increase in the levels of antibodies that specifically bind to an Apolipoprotein B-100 protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof (as described herein) (e.g., as compared to a control subject of the same age, a control subject that has had one or more successful pregnancies, and/or a control subject that has not had a miscarriage or is not suspected of having had a miscarriage); the presence or a detectable level of an Apolipoprotein B-100 protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof (as described herein); and an increased level of an Apolipoprotein B-100 protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof (as described herein) (e.g., as compared to a control subject of the same age, a control subject that has had one or more successful pregnancies, and/or a control subject that has not had a miscarriage or is not suspected of having had a miscarriage). In any of the methods described herein, the term "decrease" is meant a statistically significant decrease (e.g., by at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95%). In any of the methods described herein, the term "increase" is meant a statistically significant increase (e.g., by at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95%). By the term "non-detectable level" is meant a level of a protein, nucleic acid, or antibody that cannot be detected by the method used to perform the measurement in a given experiment. The non-detectable level of a protein, nucleic acid, or antibody will vary depending on the particular assay used to perform the measurement. By the term "detectable level" is meant a level of a protein, nucleic, or antibody that may be detected by the method used to perform the measurement in a given experiment.
Methods of Identifying a Subject at Risk of Pregnancy Loss

Also provided are methods of identifying a subject at risk (e.g., having an increased risk or pregnancy loss relative to a control population) of pregnancy loss that include providing a sample (e.g., a sample containing serum) from the subject and detecting the presence, absence, or level of antibodies that specifically bind to one or more (e.g., one, two, or three) of a fibronectin (protein or nucleic acid), an a2M (protein or nucleic acid), and an Apolipoprotein B-100, or an antigenic fragment thereof, in the sample, wherein the presence, a detectable level, or an increased level of antibodies to a fibronectin (protein or nucleic acid) and/or an ApoB-100 (protein or nucleic acid), or an antigenic fragment thereof, and/or the absence, a non-detectable level, or a decreased level of antibodies to an a2M (protein or nucleic acid), or antigenic fragment thereof, in the sample, identifies the subject as having an increased (e.g., a statistically significant increase, such as an increase of at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100%) risk of pregnancy loss. Additional methods for identifying a subject at risk of pregnancy loss may include providing a sample (e.g., a sample containing serum) from the subject and detecting the presence, absence, or level of one or more (e.g., one, two, or three) of a fibronectin (protein or nucleic acid), an a2M (protein or nucleic acid), and an Apolipoprotein B-100 (protein or nucleic acid), or an antigenic fragment thereof, in the sample, wherein the presence, a detectable level, or an increased level of a fibronectin (protein or nucleic acid) and/or an ApoB-100 (protein or nucleic acid), or antigenic fragment thereof, and/or the absence, a non-detectable level, or a decreased level of an a2M (protein or nucleic acid), or antigenic fragment thereof, in the sample, identifies the subject as having an increased risk of pregnancy loss.

These methods may be performed on any of the subjects described herein. The method may also be performed at any of the time points described herein.

The presence, absence, or levels of antibodies that specifically bind to a fibronectin (protein or nucleic acid), an a2M (protein or nucleic acid), or an Apolipoprotein B-100 (protein or nucleic acid), or an antigenic fragment thereof, may be determined using any of the methods described herein or those known in the art. The presence, absence, or levels of a fibronectin (protein or nucleic acid), an a2M (protein or
nucleic acid), or a Apolipoprotein B-100 (protein or nucleic acid), or an antigenic
fragment thereof, may be determined using any of the methods described herein or those
known in the art.

In any of the methods described herein, one or more (e.g., one, two, three, four,
five, six, seven, or eight) of any combination of the following, in a sample from the
subject, identify the subject as being at risk (e.g., having an increased risk) of pregnancy
loss: the presence or a detectable level of antibodies that specifically bind to an ApoB-
100 protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof (as described
therein); an increase in the level of antibodies that specifically bind to an ApoB-100
protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof (as described
herein) (e.g., as compared to a control subject of the same age, a control subject that has
had one or more successful pregnancies, and/or a control subject that has not had a
miscarriage or is not suspected of having had a miscarriage); the presence or a detectable
level of ApoB-100 protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof
(as described herein); an increased level of ApoB-100 protein or nucleic acid (e.g.,
mRNA), or an antigenic fragment thereof (as described herein) (e.g., as compared to a
control subject of the same age, a control subject that has had one or more successful
pregnancies, and/or a control subject that has not had a miscarriage or is not suspected of
having had a miscarriage); the presence or a detectable level of antibodies that
specifically bind to a fibronectin protein or nucleic acid (e.g., mRNA), or an antigenic
fragment thereof (as described herein); an increase in the level of antibodies that
specifically bind to a fibronectin protein or nucleic acid (e.g., mRNA), or an antigenic
fragment thereof (as described herein) (e.g., as compared to a control subject of the same
age, a control subject that has had one or more successful pregnancies, and/or a control
subject that has not had a miscarriage or is not suspected of having had a miscarriage);
the presence or a detectable level of a fibronectin protein or nucleic acid (e.g., mRNA), or
an antigenic fragment thereof (as described herein); an increased level of a fibronectin
protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof (as described
herein) (e.g., as compared to a control subject of the same age, a control subject that has
had one or more successful pregnancies, and/or control subject that has not had a
miscarriage or is not suspected of having had a miscarriage); the absence or a non-
detectable level of antibodies that specifically bind to an a2M protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof (as described herein); and a decrease in the levels of antibodies that specifically bind to an a2M protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof (as described herein) (e.g., as compared to a control subject of the same age, a control subject that has had one or more successful pregnancies, and/or a control subject that has not had a miscarriage or is not suspected of having had a miscarriage); the absence or a non-detectable level of an a2M protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof (as described herein); and a decreased level of an a2M protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof (as described herein) (e.g., as compared to a control subject of the same age, a control subject that has had one or more successful pregnancies, and/or a subject that has not had a miscarriage or is not suspected of having had a miscarriage).

Methods of Selecting a Subject for Participation in a Clinical Study

Also provided are methods of selecting a subject for participation in a clinical study that include providing a sample (e.g., a sample containing serum) from the subject and detecting the presence, absence, or level of antibodies that specifically bind to one or more (e.g., one, two, or three) of a fibronectin (protein or nucleic acid), an a2M (protein or nucleic acid), and an Apolipoprotein B-100 (protein or nucleic acid), or an antigenic fragment thereof, in the sample, wherein the presence, a detectable level, or an increased level of one or more antibodies that specifically bind to a fibronectin (protein or nucleic acid) and/or an ApoB-100 (protein or nucleic acid), or an antigenic fragment thereof, and/or the absence, a non-detectable level, or a decreased level of antibodies that specifically bind to an a2M (protein or nucleic acid), or antigenic fragment thereof, in the sample, indicates that the subject should be selected for participation in a clinical study. Additional methods for selecting a subject for participation in a clinical study may include providing a sample (e.g., a sample containing serum) from the subject and detecting the presence, absence, or level of one or more (e.g., one, two, or three) of a fibronectin (protein or nucleic acid), an a2M (protein or nucleic acid), and an Apolipoprotein B-100 (protein or nucleic acid), or an antigenic fragment thereof, in the sample, wherein the presence, a detectable level, or an increased level of a fibronectin
(protein or nucleic acid) and/or an ApoB-100 (protein or nucleic acid), or antigenic fragment thereof, and/or the absence, a non-detectable level, or a decreased level of an a2M (protein or nucleic acid), or antigenic fragment thereof, in the sample indicates that the subject should be selected for participation in a clinical study.

These methods may be performed on any of the subjects described herein. The method may be also be performed at any of the time points described herein.

The presence, absence, or levels of antibodies that specifically bind to a fibronectin (protein or nucleic acid), an a2M (protein or nucleic acid), or an antigenic fragment thereof, may be determined using any of the methods described herein or those known in the art. The presence, absence, or levels of a fibronectin (protein or nucleic acid), an a2M (protein or nucleic acid), or a Apolipoprotein B-100 (protein or nucleic acid), or an antigenic fragment thereof, may be determined using any of the methods described herein or those known in the art.

In any of the methods described herein, one or more (e.g., one, two, three, four, five, six, seven, or eight) of any combination of the following, in a sample from the subject, indicate that the subject should be selected for participation in a clinical study: the presence or a detectable level of antibodies that specifically bind to an ApoB-100 protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof (as described herein); an increase in the level of antibodies that specifically bind to an ApoB-100 protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof (as described herein) (e.g., as compared to a control subject of the same age, a control subject that has had one or more successful pregnancies, and/or a control subject that has not had a miscarriage or is not suspected of having had a miscarriage); the presence or a detectable level of ApoB-100 protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof (as described herein); an increased level of ApoB-100 protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof (as described herein) (e.g., as compared to a control subject of the same age, a control subject that has had one or more successful pregnancies, and/or a control subject that has not had a miscarriage or is not suspected of having had a miscarriage); the presence or a detectable level of antibodies that specifically bind to a fibronectin protein or nucleic acid (e.g., mRNA), or an antigenic
fragment thereof (as described herein); an increase in the level of antibodies that specifically bind to a fibronectin protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof (as described herein) (e.g., as compared to a control subject of the same age, a control subject that has had one or more successful pregnancies, and/or a control subject that has not had a miscarriage or is not suspected of having had a miscarriage); the presence or a detectable level of a fibronectin protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof (as described herein); an increased level of a fibronectin protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof (as described herein) (e.g., as compared to a control subject of the same age, a control subject that has had one or more successful pregnancies, and/or a control subject that has not had a miscarriage or is not suspected of having had a miscarriage); the absence or a non-detectable level of antibodies that specifically bind to an a2M protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof (as described herein); and a decrease in the levels of antibodies that specifically bind to an a2M protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof (as described herein) (e.g., as compared to a control subject of the same age, a control subject that has had one or more successful pregnancies, and/or a control subject that has not had a miscarriage or is not suspected of having had a miscarriage); the absence or a non-detectable level of an a2M protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof (as described herein); and a decreased level of an a2M protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof (as described herein) (e.g., as compared to a control subject of the same age, a control subject that has had one or more successful pregnancies, and/or a control subject that has not had a miscarriage or is not suspected of having had a miscarriage).

Methods for Decreasing the Risk of Pregnancy Loss

Also provided are methods of decreasing the risk of pregnancy loss in a subject that include providing a sample (e.g., a sample containing serum) from the subject; determining the presence, absence, or level of antibodies that specifically bind to one or more (e.g., one, two, or three) of a fibronectin (protein or nucleic acid), an a2M (protein or nucleic acid), and an Apolipoprotein B-100 (protein or nucleic acid), or an antigenic fragment thereof, in the sample; and administering to the subject a therapeutic treatment
if subject has, has a detectable level, or has an increased level of antibodies that specifically bind to a fibronectin (protein or nucleic acid) and/or an ApoB-100 (protein or nucleic acid), or an antigenic fragment thereof, and/or does not have, has a non-detectable level, or a decreased level of antibodies that specifically bind to an a2M(protein or nucleic acid), or antigenic fragment thereof in the sample. Additional methods of decreasing the risk of pregnancy loss in a subject include providing a sample (e.g., a sample containing serum) from the subject; determining the presence, absence, or level of one or more (e.g., one, two, or three) of a fibronectin (protein or nucleic acid), an a2M (protein or nucleic acid), and an Apolipoprotein B-100 (protein or nucleic acid), or an antigenic fragment thereof, in the sample; and administering to the subject a therapeutic treatment if subject has, has a detectable level, or has an increased level of a fibronectin (protein or nucleic acid) and/or an ApoB-100 (protein or nucleic acid), or antigenic fragment thereof, and/or does not have, has a non-detectable level, or a decreased level of an a2M (protein or nucleic acid), or antigenic fragment thereof in the sample.

These methods may be performed on any of the subjects described herein. The method may be also be performed at any of the time points described herein. The methods may be used to select a subject for administration of a treatment to reduce the risk of a pregnancy loss.

The presence, absence, or levels of antibodies that specifically bind to a fibronectin (protein or nucleic acid), an a2M (protein or nucleic acid), or a Apolipoprotein B-100 (protein or nucleic acid), or an antigenic fragment thereof, may be determined using any of the methods described herein or those known in the art. The presence, absence, or levels of a fibronectin (protein or nucleic acid), an a2M (protein or nucleic acid), or an Apolipoprotein B-100 (protein or nucleic acid), or an antigenic fragment thereof, may be determined using any of the methods described herein or those known in the art.

In any of the methods described herein, at least one therapeutic treatment should be administered to a subject having one or more (e.g., one, two, three, four, five, six, seven, or eight) of any combination of the following features: the presence or a detectable level of antibodies that specifically bind to an ApoB-100 protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof (as described herein), in the sample; an
increase in the level of antibodies that specifically bind to an ApoB-100 protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof (as described herein), in the sample (e.g., as compared to a control subject of the same age, a control subject that has had one or more successful pregnancies, and/or a control subject that has not had a miscarriage or is not suspected of having had a miscarriage); the presence or a detectable level of ApoB-100 protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof (as described herein), in the sample; an increased level of ApoB-100 protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof (as described herein), in the sample (e.g., as compared to a control subject of the same age, a control subject that has had one or more successful pregnancies, and/or a control subject that has not had a miscarriage or is not suspected of having had a miscarriage); the presence or a detectable level of antibodies that specifically bind to a fibronectin protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof (as described herein), in the sample; an increase in the level of antibodies that specifically bind to a fibronectin protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof (as described herein), in the sample (e.g., as compared to a control subject of the same age, a control subject that has had one or more successful pregnancies, and/or a control subject that has not had a miscarriage or is not suspected of having had a miscarriage); the absence or a non-detectable level of antibodies that specifically bind to an a2M protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof (as described herein), in the sample; and a decrease in the levels of antibodies that specifically bind to an a2M protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof (as described herein), in the sample (e.g., as compared to a control subject of the same age, a control subject that has had one or more successful pregnancies, and/or a subject that has not had a miscarriage or is not suspected of having had a miscarriage); the absence or a non-detectable level of an a2M protein or nucleic
acid (e.g., mRNA), or an antigenic fragment thereof (as described herein), in the sample; and a decreased level of an a2M protein or nucleic acid (e.g., mRNA), or an antigenic fragment thereof (as described herein), in the sample (e.g., as compared to a control subject of the same age, a control subject that has had one or more successful pregnancies, and/or a control subject that has not had a miscarriage or is not suspected of having had a miscarriage).

The therapeutic treatment may be administered by a health care professional (e.g., a physician, a nurse, or a physician's assistant). The treatment may be administered in a patient's home or in a health care facility (e.g., a hospital or a clinic). In some embodiments, the therapeutic treatment is a treatment that decreases or suppresses an immune response, e.g., that decreases inflammation, or decreases a Th1-type immune response, and/or enhances a Th2-type immune response.

Non-limiting examples of therapeutic treatment include complement inhibitors (e.g., antibodies that bind to complement components, such as Cl, C3, and C5 (e.g., 5G1.1SC and 5G1.1 (Alexion), eculizumab, and pex-elifuzumab); soluble complement receptor 1, Cl-inhibitor (Cl-Inh), Cl esterase inhibitor, C3 inhibitor (POT-4), C5 complement inhibitor (Alexion), compstatin, heparin, and the complement inhibitors described in U.S. Patent Nos. 4,146,640; 4,007,270; 4,241,301; and 5,847,082; and U.S. Patent Application Publications Nos. 2007/0141573; 2009/01 7098; and 2009/0214538), hormones (e.g., progesterone), steroids (e.g., prednisone), passive immunotherapy with intravenous immunoglobulin, aspirin (e.g., low-dose aspirin), and TNF antagonists (e.g., soluble fragments of TNF-a receptors (e.g., etanercept) and antibodies that specifically bind to TNF-a (e.g., adalimumab and infliximab), and small molecule inhibitors of TNF-\(\alpha\) (e.g., pentoxyfyllene)). One or more (e.g., two, three, four, or five) therapeutic treatments may be administered to the subject. In some methods, the subject may be pregnant (e.g., within the first 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, 10 weeks, 11 weeks, 12 weeks, 13 weeks, 14 weeks, 15 weeks, 16 weeks, 17 weeks, 18 weeks, 19 weeks, or 20 weeks of pregnancy) or may be planning on becoming pregnant in the future (e.g., the therapeutic treatment is administered at least one month, at least 3 weeks, at least 2 weeks, at least 1 week prior to conception).
The dosage (e.g., 0.1 to 100 mg, 0.1 mg to 80 mg, 0.1 mg to 70 mg, 0.1 to 60 mg, 0.1 mg to 50 mg, 1 mg to 40 mg, 1 mg to 30 mg, 1 mg to 20 mg, and 1 mg to 10 mg) and administration regime (e.g., once a day, twice a day, three times a day, four times a day, once a week, twice a week, three times a week, four times a week, once every two weeks, once a month, twice a month, three times a month, or four times a month) of the therapeutic treatment may be determined by a health care professional based on the physical condition of the subject (e.g., age, health, pregnant or non-pregnant, and other health conditions) and based on the dosing and administration schedules known in the art (for a general review of exemplary treatments, see, Tincani et al., *Clinic Rev. Allerg. Immunol.* 39:153-159, 2010; Stephenson et al, *Human Reproduction* 25:2203-2209, 2010; and Dukhovny et al, *Curr. Opin. Endocrinol. Diabetes Obes.* 16:451-458, 2009). For example, a subject identified for the administration of a therapeutic treatment using the provided methods, may be intravenously administered passive immunoglobulin one or more times (e.g., two, three, four, or five times) during and/or prior to pregnancy (as described herein). A physician may monitor the subject (e.g., using the methods to determine the risk of pregnancy loss described herein) to determine whether the dosage or the frequency of therapeutic treatment should be altered (e.g., increase in the dosage and/or frequency of administration of a therapeutic treatment for those subjects indicated as having an increased risk of pregnancy loss) during a given time frame (e.g., during the term of the pregnancy (e.g., anywhere from between conception to 9 months of pregnancy, between conception and up to 8 months of pregnancy, between conception and up to 7 months of pregnancy, between conception up to 6 months of pregnancy, between conception up to 5 months of pregnancy, between conception up to 4 months of pregnancy, between conception up to 3 months of pregnancy, between conception and up to 2 months of pregnancy, between 3 and 20 weeks of pregnancy, between 5 and 20 weeks or pregnancy, or between 10 and 20 weeks of pregnancy), a period of time prior to conception (e.g., within 6 months of conception, within 5 months of conception, within 4 months of conception, within 3 months of conception, within 2 months of conception, within 1 month of conception, within 3 weeks of conception, within 2 weeks of conception, within 1 week of conception, or within 3 days of conception), or a period of time beginning prior to conception (e.g., within 6 months of conception, within 5 months
of conception, within 4 months of conception, within 3 months of conception, within 2
months of conception, within 1 month of conception, within 3 weeks of conception,
within 2 weeks of conception, within 1 week of conception, or within 3 days of
conception) to the end of the term or a time point during the term of the pregnancy (e.g.,
anywhere from between conception to 9 months of pregnancy, between conception and
up to 8 months of pregnancy, between conception up to 7 months of pregnancy, between
conception up to 6 months of pregnancy, between conception up to 5 months of
pregnancy, between conception up to 4 months of pregnancy, between conception up to 3
months or pregnancy, between conception and up to 2 months of pregnancy, between 3
and 20 weeks of pregnancy, between 5 and 20 weeks or pregnancy, or between 10 and 20
weeks of pregnancy).

**EXAMPLES**

The invention is further described in the following examples, which do not limit
the scope of the invention described in the claims.

**Example 1. Alterations in Immune Responses in Women with Recurrent Pregnancy Loss.**

Current literature supports the concept that failure to suppress maternal lymphoid
activation pathways and aberrant auto-antibody production is associated with pregnancy
complications, from infertility to spontaneous recurrent pregnancy loss (RPL). These
experiments were designed to enhance understanding of the human immunologic
responses and antigen recognition patterns that develop during the first trimester in
women with a history of RPL compared to first-trimester, multi-parous women with an
uncomplicated obstetrical history.

Western immunoblotting using human serum-derived antibodies from RPL and
healthy subjects and trophoblast-derived antigens was used to characterize a distinct
difference in the total IgG recognition profiles among healthy pregnant controls and RPL
patients (see, schematic diagram of the experimental method in Figure 1). These antigens
were obtained from the first trimester trophoblast-derived cell line, SW.71 (Yale
University, New Haven, CT, USA), which was maintained in DMEM/F12 (Gibco
Invitrogen) media supplemented with 2mL L-glutamine, 10% fetal bovine serum, ImM sodium pyruvate, O.lmM non-essential amino acids, 100 units/mL penicillin-streptomycin at 37 °C and 5% CO₂ in 75-cm² tissue culture flasks. This cell line was isolated from a seven-week placenta immortalized by ectopic expression of the catalytic subunit of human telomerase.

Nuclear, cellular, and cytoplasmic proteins were extracted from the Sw.71 cell-line derived using a cell fraction kit (BioVision, Mountain View, California, USA) using the manufacturer's instructions. The protein concentrations of each fraction were determined using Bio-Rad DC protein quantification assay (Bio-Rad Laboratories, Hercules, California, USA).

To visualize subject autoantibody reactivity patterns, these extracted, solubilized nuclear, cytosolic, and cellular membrane proteins (40 µg/lane) were applied to 10% SDS-PAGE gels and electrophorectically separated by the method of Laemili (Nature 227:680-685, 1970). The reactive proteins were analyzed by Western immunoblotting (Brown et al, Int. J. Cancer 55:678-684, 1993). Nitrocellulose membranes were probed overnight at 4 °C with patient serum (diluted 1:100) and then washed three times in Tris-buffered saline (TBS). Western blotting was completed using peroxidase-conjugated anti-human IgG2, IgG3, and whole IgG (AbD Sertotec, Raleigh, NC). Bound antibody-antigen complexes were visualized using enhanced chemiluminescence (Immun-Star, Bio-Rad, Hercules, California). The resulting x-ray film was scanned as a 16-bit grayscale JPEG image. This grayscale image was digitized and converted into pixel density using Un-scan-it software (Silk Scientific Corp., Orem, UT). On each gel image, the number of pixels for all visualized bands was quantitated using Un-Scan-It and the total number of pixels for all bands within each lane was calculated. This total number of pixels for all bands in specific lanes was determined and the mean (average) total pixels for the specific lane for patients within each population (controls versus RPL) were calculated for antigens derived from the nuclear and membrane fractions.

The serum samples used in the experiments described herein were obtained from first trimester pregnant women who either had had ≥ 2 recurrent spontaneous abortions without a successful pregnancy (n = 8) or had had two or more term, uncomplicated deliveries (n = 2). Patients with histories of RPL were in their first trimester of
pregnancy with a history of two or more recurrent consecutive miscarriages of unknown etiology (i.e., with documented normal maternal and paternal karyotypes, normal uterine cavity imaging and/or assessment, and normal thrombophilia profile). Venous blood samples were obtained, allowed to clot, and sera isolated. These samples were obtained from volunteers in the private gynecology offices and clinics of the Department of Obstetrics, Gynecology and Women's Health at the University of Louisville School of Medicine, under an informed consent protocol approved by the Institutional Review Board at the University. For this proteomics study, eight patients with a history of recurrent spontaneous abortions were enrolled in the study (Table 1). All patients were in good general health and were not taking any medications, except for one patient (subject #8), who was euthyroid while receiving replacement medication. All were Caucasian except for one (subject #6), who was Chinese. None patients with a history of RPL had anticardiolipin antibodies or lupus anticoagulant. Seven women had a normal uterine contour by evaluation with either hysterosalpingography or saline infu-
sonohysterography. Seven women had either a normal serum progesterone level (10 ng/mL) or an in-phase luteal biopsy result.

The data from these experiments demonstrate a distinct difference in the total IgG recognition profiles among healthy pregnant controls and RPL patients (Figures 2 and 3A-F). The data in Figures 2 and 3A-3F, indicate that sera from women with a history of RPL exhibited greater immunoreactivities compared to controls, with a total antibody reactivity 3.6-fold greater with nuclear antigens (p=0.0044), a 4.1-fold greater reactivity with membrane-derived antigens (p=0.0001), and a 1.8-fold greater recognition of cytosolic antigens (p=0.01 13). Among IgG subclasses, a notably enhanced recognition pattern was observed in IgG3, which revealed an increase of 1.8-fold greater immunoreactivity than controls. This increase was consistently noted across all three antigen sources (nuclear, membrane, and cytosolic antigens), with antigens ranging from 15 to 250 kDa.

Western blots of antibody-antigen complexes, resulting from the use of patient serum as the source of primary antibodies, were scanned, digitized, and converted to pixel density. The pixel densities for these two groups of patients were compared for total IgG reactivity for antigens derived from the membrane, nuclear, and cytoplasmic
fractions (Figure 4). Immunoreactivities for antigens from each cellular compartment were standardized using the pixel values of control standard (HRP-anti-mouse IgG) included in each gel. Duplicate gels were run for each subject and the resulting ratios from the gels were averaged. The mean values and standard deviations were calculated using InStat Graph Pad. Sera from women with a history of RPL exhibited greater immunoreactivities compared to controls, with a total antibody reactivity 1.48-fold greater with nuclear antigens (p=0.0190), a 1.57-fold greater reactivity with membrane-derived antigens (p=0.0056), and a 1.90-fold greater recognition of cytosolic antigens (p=0.0162).

Among IgG subclasses, a notable enhanced recognition pattern was observed in IgG3 with an increase of 1.8-fold greater immunoreactivity compared to controls (Figure 5). Digitization of the reactive bands demonstrated that this increase was consistently noted across all three antigen sources (nuclear, membrane, and cytosolic antigens), with antigens ranging from 15 to 250 kDa. The enhanced reactivity was linked with the recognition of additional antigenic proteins and not simply greater reactivity with the same components.
Antigen recognition was also determined by immunoprecipitation and protein separation by gel electrophoresis, followed by mass spectrometry, substantially as shown in Figure 6. In these experiments, Sw.71 cell-line derived nuclear and cellular solubilized proteins (5μg) were individually sonicated in RIPA buffer (260μL, containing protease and phosphatase inhibitor cocktails, Sigma Chemical) and incubated with serum-derived immunoglobulins (100μL) from control (n = 2) and test subjects (n = 5). The individual samples were then incubated in agarose-bound anti-human IgG (40μL), centrifuged, and washed to obtain a pellet of immunoaffinity-isolated cellular and nuclear proteins. This was done for each control and test subject. The antigen-antibody complexes were reduced and solubilized using 2x Laemili buffer. Samples were then applied to an 18-well 4-15% Tris-HCL, 1.0 mm, CriterionTM Precast Gel (Bio-Rad, Hercules, California), and separated by electrophoresis. Each gel was then stained using ImperialTM Protein Stain and scanned using PharosFX™ Molecular Imager System (Bio-Rad, Hercules, California).

Specific trophoblast cellular antigens recognized in antibody-antigen binding complexes were defined by mass spectrometry sequencing. The incongruent control and test immunoprecipitation gel spots were removed, washed to remove staining of dye and inhibitory chemicals, and dried to absorb maximum volume of digestion buffer. The dried gel spots were rehydrated in digestion buffer containing sequencing grade modified trypsin (1:30 by mass) and proteins were digested in-gel at 37 °C. Digested peptides were extracted from the gel with trifluoroacetic acid extraction buffer and digested tryptic peptides were desalted using C-18 Zip-tips (Millipore). The desalted peptides were mixed with a-cyano-4-hydroxycinnamic acid matrix (CHCA) and spotted into wells of a MALDI plate. Mass spectra (MS) of the peptides in each sample were obtained using Applied Biosystems 4700 Proteomics Analyzer. A minimum of 10 of the most abundant peptides for each sample were further subjected to fragmentation and tandem mass spectrometry (MS/MS) analysis. Protein identification were based on peptide fingerprint mass mapping and peptide fragmentation mapping (using MS/MS spectra). Combined MS and MS/MS spectra were submitted for database search using GPS Explorer software equipped with MASCOT search engine to identify proteins from primary sequence databases.
Specific trophoblast cellular antigens recognized in antibody-antigen binding complexes were defined by immunoprecipitation (Figure 7) and subsequent mass spectrometry sequencing (Table 2). SDS-PAGE of the immunoprecipitated proteins derived from membrane and nuclear fractions derived from Sw.71 trophoblast cells revealed numerous qualitative and quantitative differences, as defined by the presence of specific bands (Figure 7). Subsequent analyses focused on three major bands exhibiting unique association with RPL. Mass spectra (MS) of the peptides in each sample were obtained using Applied Biosystems 4700 Proteomics Analyzer. Protein identification was based on peptide fingerprint mass mapping and peptide fragmentation mapping (using MS/MS spectra). Combined MS + MS/MS analysis was performed using Mascot v 2.1.04 from Matrix Science Ltd and proteins were identified using SwissProt database. Each matched peptide was characterized by an ion score; a high confidence in peptide to protein match was reached when two or more ion scores indicated identity (Table 2). The results include three differently recognized trophoblast antigens: Apolipoprotein B-100 (ApoB-100), fibronectin, and a2-macroglobulin (a2-M). Specifically, recognition of maternal IgG antibodies to trophoblast-derived fibronectin and ApoB-100 were noted when serum was obtained from women who suffer RPL. This antibody recognition was absent when serum was obtained from pregnant, multiparous women with an uncomplicated obstetrical history. Notably, serum from these same control, multiparous subjects revealed antibody recognition to a2M, a pattern that was contrarily absent in serum from RPL subjects. These findings suggest that perhaps an aberrant maternal antibody recognition of fibronectin and a2M leads to dysfunctional development of the maternal-fetal interface with possible subsequent pregnancy loss or other advanced-gestation obstetrical complications. Concurrently, a combination of the three previously-described functions and mechanisms of action of ApoB may play a vital role in the sustainability of early pregnancy. Perhaps this lack of antibody-ApoB binding, as demonstrated from the serum of healthy controls, alters the intended function of ApoB at the level of the uterine endothelium, the steroid-producing corpus luteal cells, and/or the nutrient-rich embryo yolk sac.

Since paternal genetic material determines at least half the antigenic array of the fetus, expression of these components are capable of eliciting an immune response that
can result in the spontaneous loss (abortion) of the fetus. Antibodies that recognize the fetus have been demonstrated in the maternal circulation, and IgG that is reactive with paternal antigens can be eluted from the placenta (Creus et al., Humn. Reprod. 13:39-43, 1998; Wilson et al, Fertil. Steril. 76:915-917, 2001). In this study, we investigated the antigenic recognition patterns of circulating IgG obtained from women with RPL.

Table 2. Mass Spectrometry (MS) Protein Identification.

<table>
<thead>
<tr>
<th>Serum Source</th>
<th>Protein Identification</th>
<th>Molecular Weight (Da)</th>
<th>Protein Score</th>
<th>Confidence Interval (100%)</th>
<th>Ion Score Notes</th>
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</thead>
<tbody>
<tr>
<td>Term</td>
<td>α2-Macroglobulin</td>
<td>164614</td>
<td>514</td>
<td>100</td>
<td>7 ion scores indicated identity</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>353</td>
<td>100</td>
<td>5 ion scores indicated identity</td>
</tr>
<tr>
<td>RPL</td>
<td>Apolipoprotein B 100</td>
<td>516666</td>
<td>239</td>
<td>100</td>
<td>3 ion scores indicated identity</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>409</td>
<td>100</td>
<td>5 ion scores indicated identity</td>
</tr>
<tr>
<td>RPL</td>
<td>Fibronectin</td>
<td>266034</td>
<td>593</td>
<td>100</td>
<td>7 ion scores indicated identity</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>647</td>
<td>100</td>
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</tbody>
</table>

Combined MS + MS/MS analysis performed using Mascot v 2.1.04 from Matrix Science Ltd. Proteins were identified using SwissProt database. Protein significance level was 56 by Mascot (p<0.05). The Ion Score Notes refers to matched peptides using Mascot. High confidence in peptide to protein match when two or more ion scores indicate identity. * = MS also detected serum albumin by 2 ion scores; + = MS also detected Ig gamma-2 chain C region & Ig gamma 3 chain C region by 1 ion score.
compared with those of pregnant women in the first trimester of uncomplicated pregnancies.

Pregnancy has been shown to produce significant changes within the immune system, generally noted as a shift to a Th2-biased (humoral) immune response. Many of these alterations are not observed in women experiencing RPL. Pregnancy has been associated with the production of Th2 type cytokines (such as IL-10 and IL-4), while RPL has been linked with the production of Th1 type cytokines (such as IFN-γ, IL-12). Previous studies have shown that normal uncomplicated pregnancy is associated with significant changes in IgG subclasses (Wilson et al, *Fertil. Steril.* 76:915-917, 2001).

Normal pregnancy-associated IL-4 production can induce peripheral blood mononuclear cells to become activated and increase total IgG production, as well as enhanced IgG4. In contrast, RPL-associated IFN-γ production can inhibit these events. RPL is generally associated with reduced levels of IL-10 and these patients exhibit diminished levels of total IgG (Eblen et al, *Fertil. Steril.* 73:305-313, 2000; Wilson et al, *Fertil. Steril.* 76:915-917, 2001).

The present data show that uncomplicated pregnancy is linked with changes in the production of IgG reactive with trophoblast-derived antigens. Pregnant women who subsequently abort exhibited a different IgG subset patterns compared with healthy pregnant women, e.g., increased levels of IgG3. The IgG class of antibody predominates in the blood and interstitial fluids and is the most multi-functional of the all antibody classes. The IgG molecule consists of two antigen binding regions (Fab) and one ligand binding region (Fc) through which various effector activities are initiated (e.g., activation of the classical complement pathway, phagocytosis, and antibody-dependent cellular cytotoxicity) (Jefferis et al, *Ann. Biol. Clin.* 52:57-65, 1994). While generally representing only 7% of total circulating IgG, the IgG3 subclass exhibits the highest complement activation and high affinity for Fc receptors on immune effector cells. Results from this study demonstrate an overall increase in antibody recognition of trophoblastic antigens, as well as distinct antigen-antibody binding patterns (Figures 2, 3A-3F, and 4), particularly for IgG3 subclasses (Figure 5), in women experiencing RPL compared to controls. This increase in IgG3 immunoreactivity, recognized in sera obtained from RPL subjects compared to controls, suggests a higher degree of Th2
immune cell activation and subsequent fetal allograft rejection. Perhaps an atypical ratio of the IgG subclasses in RPL patients, favoring the more immunoreactive IgG3, is a potential link to the mechanism and etiology of recurrent aborters.

In addition to the enhanced recognition of trophoblast-derived antigens by IgG3, patients experiencing RPL, exhibited the recognition of distinct antigenic proteins. Of the trophoblast-derived proteins, we isolated and defined two proteins exhibiting unique antibody recognition in RPL patients: fibronectin and Apolipoprotein B-100, while RPL patients did not have antibodies that recognize alpha2-macroglobulin. Additional trophoblast-derived antigens recognized in patients experiencing RPL are listed in Table 2.

Alpha2-macroglobulin (a2M) is homo-tetramer of 180 kDa subunits. It is a major inhibitor of endoproteinases and plays a regulatory role in the transport and clearance of cytokines and growth factors. It also may protect against the cytotoxic effects of various cytokines while inhibiting the degradation of other cytokines (Esadeg et al., Placenta 24:912-921, 2003). It exists in low serum concentrations in normal healthy adults and, in mammalian blood, it targets cytokines to cells expressing the a2M-receptor or lipoprotein-receptor related protein (Esadeg et al, Placenta 24:912-921, 2003; Shimizu et al., Exp. Anim. 51:361-365, 2002). In humans, uterine a2M is thought to originate from endothelial cells lining the endometrial vessels. Its concentration has been reported to double or triple during the secretory phase of the menstrual cycle suggesting a role as a decidualization protein (Esadeg et al, Placenta 24:912-921, 2003). During pregnancy, a receptor for the a2M-proteinase complex has been demonstrated on the human placental syncytiotrophoblasts (Jensen et al, Placenta 9:463-477, 1988; Thomas et al, Placenta 11:413-430, 1990). Exhibiting immuno-suppressive activity, a2M is believed to be a potential means of immunosuppression in the human uteroplacental interface and may be subject to transplacental transport to the neonate (Benyo et al., Endocrinology 133:699-704, 1993). In this study, serum obtained from healthy control subjects revealed antibody recognition to the a2M tetramer; whereas, serum obtained from pregnant women afflicted with RPL did not (Figure 7, Table 2). With its regulatory role in the activities of leukocytic and non-leukocytic derived cytokines, a2M may be a key component in the anomalous processes resulting in RPL. The antibody recognition and binding to this
protein, as demonstrated from the serum of healthy subjects, may influence a2-M activities from various involved sites, including uterine decidualization, endothelial structure, trophoblast invasion and growth, and transplacental transport.

Apolipoprotein B is a core protein of LDL, which mediates the interaction between low density lipoproteins (LDL) and its receptor (Yamada et al., *Hum. Reprod.* 13:944-952, 1998). The principal function of Apolipoprotein B (ApoB-100 and ApoB-48) is to provide a structural framework for packaging neutral lipids, such as triglycerides and cholesterol esters, into lipoproteins for their transportation in an aqueous circulation (Farese et al, *J. Lipid Res.* 37:347-360, 1996). It, furthermore, contains ligands for the receptor-mediated endocytosis of various lipoproteins. Mutations in the LDL-receptor and related proteins have been shown to result in aberrant uptake of ApoB and other lipoproteins into cells. A lack of appropriate lipoprotein control mechanisms ultimately leads to lipoprotein oxidation products that mediate oxidative damage and result in endothelial dysfunction and premature atherosclerosis (Cekmen et al., *Clin. Biochem.* 36:575-578, 2003; Sarandol et al, *Clin. Biochem.* 37:990-996, 2004; Sarandol et al, *Arch. Gynecol. Obstet.* 270:157-160, 2004). In normal pregnancies, there appears to be factors that promote ApoB utilization via receptor mediated endocytosis while protecting it from oxidation and subsequent destructive effects. Trophoblast cells, in particular, express high levels of LDL-receptor and related proteins giving rise to the idea that growth restriction or other vascular obstetrical complications may be associated with a chronic pattern of atherogenic or aberrant lipoprotein metabolism. Perhaps, in normal pregnancy, a specific enzyme or other substrate, protein, or molecule plays a role in stabilizing lipoproteins, inhibiting the common pathway of oxidation. Some researchers have proposed a role for antioxidants such as vitamin E and/or estrogen to inhibit oxidation of lipoproteins (Sarandol et al, *Arch. Gynecol. Obstet.* 270:157-160, 2004). Conversely, the absence of an endogenous protection mechanism may also lead to aberrant lipoprotein oxidative damage at the uteroplacental interface. Such a process may be involved in the circumstances of complicated pregnancies (i.e., RPL, pre-eclampsia, IUGR, etc.).

The expression of ApoB mRNA has been localized in the human embryo yolk endodermal cells by in situ hybridization (Cekmen et al, *Clin. Biochem.* 36:575-578,
While its physiologic purpose in the human yolk sac remains unclear, detection of ApoB in the yolk sac of mice and rats has led to a probable model for transport and packaging of maternally-derived, nutrient rich ApoB-containing lipoproteins into the yolk sac of developing embryo (Cekmen et al, Clin. Biochem. 36:575-578, 2003). The humoral recognition of ApoB in affected RPL subjects may play a hostile role in the nutrition of the maturing embryo, hindering normal embryo development.

This study observed maternal IgG antibody recognition of trophoblast-derived ApoB-100 when serum was obtained from pregnant women with history of RPL. This same antibody recognition was not observed when serum was obtained from healthy pregnant controls (Figure 7). These data suggests that the recognition pattern from test subjects, and lack of recognition by control subjects, may play an aberrant role in lipoprotein metabolism, oxidative destruction, and impairment of endovascular function at the uteroplacental interface. The data show serum antibody recognition of trophoblast-derived ApoB-100 from early pregnancy subjects experiencing RPL. A lack of this recognition was noted when serum was obtained from subjects with a normal obstetrical history. The antibody-ApoB recognition may alter the intended function of ApoB whether at the level of the uterine endothelium, the steroid-producing corpus luteal cells, or the nutrient-rich embryo yolk sac.

Fetal fibronectin is an extracellular matrix protein that is thought of as "trophoblast glue" and is found in increased concentrations at the chorionic-decidual margin and surrounding the extravillous trophoblasts (Guller et al, Up-to-Date 17.3, 2009; Mercorio et al, Eur. J. Obstet. Gynecol. Reprod. Biol. 126:165-169, 2006). A tightly-regulated balance exists between the activity of the receptive maternal decidua, the invading trophoblast, and developing chorion. Indeed, the maternal extracellular matrix and maternal-fetal interface are thought to play a pivotal role in conditions of early recurrent abortions, intrauterine growth restriction, and pre-eclampsia. Furthermore, derangement in the autocrine and paracrine signals and receptivity between cellular matrix proteins, such as fibronectin, and cell adhesion molecules may be responsible for pregnancy failure. Acquisition of adhesion-competent invading trophoblast cells is characterized by apical accumulation of integrin receptors for fibronectin and strong fibronectin binding activity on the surface of blastocysts (Mercorio et al., Eur. J. Obstet.
Reprod. Biol. 126:165-169, 2006). The data herein show the recognition of maternal IgG antibodies to trophoblast-derived fibronectin when serum was obtained from women who suffer a history of RPL (Figure 7, Table 1). This recognition was absent in healthy, multiparous control subjects. These findings suggest that perhaps aberrant maternal antibody recognition of fibronectin leads to dysfunctional development of the maternal-fetal interface with possible subsequent pregnancy loss or other advanced-gestation obstetrical complications. A growing bulk of evidence suggests an active role of fetal fibronectin in implantation. The autocrine/paracrine control mechanism operating within the decidua has been implicated in the regulation of trophoblast invasion, possibly via modulations of extracellular matrix proteins as fibronectin and its specific integrin trophoblast receptor.

Of particular immunologic importance, fibronectin can regulate production and release of IL-1β. Due to the profound effects of IL-1β on immune cell function during inflammation, investigations have focused on the factors that regulate IL-1β expression. Extracellular matrix components (ECM) can induce the expression of IL-1β (Roman et al., Cytokine 12:1581-1596, 2000). One component well-studied is fibronectin (FN) and this high molecular weight adhesive molecule is expressed by tissue macrophages and fibroblasts. Thus, FN appears to be well positioned to affect the expression of IL-1β. In vitro studies have demonstrated that FN can stimulate the expression of IL-1β mRNA, and its translation into the 31 kDa intracellular precursor protein, as well as the secretion of the 17 kDa active form in human mononuclear cells (Roman et al., Cytokine 12:1581-1596, 2000). Thus, the production of effector IgG3 reactive with fibronectin may block the FN-induced IL-1β production. Since IL-1β serves as a "master" pro-inflammatory regulator associated with early pregnancy, its blockage may prevent the induction of pro-inflammatory environment.

It is likely that these specific trophoblast cellular responses activate various pro-inflammatory or other immunoregulatory activities that inhibit proper implantation and ultimately inhibit growth and survival of the invading trophoblast and developing embryonic cells. This data is clinically useful for screening for women afflicted with RPL and, more importantly, for developing treatment strategies during pre-conceptual and prenatal care.
OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.
WHAT IS CLAIMED IS:

1. A method for predicting risk of pregnancy loss in a subject, the method comprising:
   (a) providing a sample comprising serum from the subject; and
   (b) detecting the presence or absence of antibodies to fibronectin in the sample;
   wherein the presence of antibodies to fibronectin in the sample indicates that the subject has an increased risk of pregnancy loss.

2. The method of claim 1, further comprising in (b) detecting the presence or absence of antibodies to Apolipoprotein B-100 (ApoB-100) in the sample, wherein the presence of antibodies to fibronectin or ApoB-100 indicates that the subject has an increased risk of pregnancy loss.

3. The method of claim 1 or 2, further comprising in (b) detecting the absence or absence of antibodies to alpha2-macroglobulin (a2M) in the sample, wherein the presence of antibodies to fibronectin or ApoB-100, or the absence of antibodies to a2M indicates that the subject has an increased risk of pregnancy loss.

4. The method of any one of claims 1-3, wherein detecting the presence or absence of antibodies comprises contacting the sample with one or more antigens selected from the group consisting of ApoB-100, fibronectin, and a2M, or antigenic fragments thereof, and detecting binding of antibodies in the sample to the antigens.

5. A method of identifying a subject at risk of pregnancy loss comprising:
   (a) providing a sample comprising serum from the subject; and
   (b) detecting the presence or absence of antibodies to fibronectin in the sample;
   wherein a subject having antibodies to fibronectin present in the sample of (a) is identified as being at risk of pregnancy loss.
6. The method of claim 5, further comprising in (b) detecting the presence or absence of antibodies to Apolipoprotein (ApoB-100) in the sample, wherein a subject having antibodies to fibronectin or ApoB-100 present in the sample of (a) is identified as being at risk of pregnancy loss.

7. The method of claims 5 or 6, further comprising in (b) detecting the presence or absence of antibodies to alpha2-macroglobulin (a2M) in the sample, wherein a subject having antibodies to fibronectin or ApoB-100, or not having antibodies to a2M present in the sample of (a) is identified as being at risk of pregnancy.

8. A method of selecting a subject for participation in a clinical study comprising: (a) providing a sample comprising serum from the subject; and (b) detecting the presence or absence of antibodies to fibronectin in the sample, wherein a subject having antibodies to fibronectin present in the sample of (a) is selected for participation in a clinical study.

9. The method of claim 8, further comprising in (b) detecting the presence or absence of antibodies to Apolipoprotein B-100 (ApoB-100) in the sample, wherein a subject having antibodies to fibronectin or ApoB-100 present in the sample of (a) is selected for participation in a clinical study.

10. The method of claim 8 or 9, further comprising in (b) detecting the presence or absence of antibodies to alpha2-macroglobulin (a2M) in the sample, wherein a subject having antibodies to fibronectin or ApoB-100, or not having antibodies to a2M present in the sample of (a) is selected for participation in a clinical study.

11. The method of any one of claim 8-10, wherein detecting the presence or absence of antibodies comprises contacting the sample with one or more antigens selected from the group consisting of ApoB-100, fibronectin, and a2M, or antigenic fragments thereof, and detecting binding of antibodies in the sample to the antigens.
12. A method of decreasing the risk of pregnancy loss in a subject comprising:
(a) providing a sample comprising serum from the subject;
(b) detecting the presence or absence of antibodies to fibronectin in the sample; and
(c) administering a therapeutic treatment to a subject having antibodies to fibronectin present in the sample of (a).

13. The method of claim 12, further comprising in (b) determining the presence or absence of antibodies to Apolipoprotein B-100 (ApoB-100) in the sample, and administering a therapeutic treatment to a subject having antibodies to fibronectin or ApoB-100 present in the sample of (a).

14. The method of claim 12 or 13, further comprising in (b) determining the presence of absence of antibodies to alpha2-macroglobulin (a2M) in the sample, and administering a therapeutic treatment to a subject having antibodies to fibronectin or ApoB-100, or not having antibodies to a2M present in the sample of (a).

15. The method of any of the preceding claims, wherein the subject has had at least one previous pregnancy loss or is suspected of having had at least one previous pregnancy loss.

16. The method of any of the preceding claims, wherein the subject is not pregnant, but is planning or considering a future pregnancy.

17. The method of any of the preceding claims, wherein the subject is pregnant.

18. The method of any of the preceding claims, wherein the sample in (a) is obtained from the subject within the first 20 weeks, within the first 13 weeks, or within the first 12 weeks of pregnancy.
19. The method of any one of claims 12-14, wherein detecting the presence or absence of antibodies comprises contacting the sample with one or more antigens selected from the group consisting of ApoB-100, fibronectin, and a2M, or antigenic fragments thereof, and detecting binding of antibodies in the sample to the antigens.

20. The method of any of the proceeding claims, wherein the subject is human.

21. The method of any one of claims 12-14, wherein said therapeutic treatment is selected from the group consisting of: complement inhibitors, hormone treatment, steroid treatment, passive immunotherapy with intravenous immunoglobulins, aspirin, and tumor necrosis factor (TNF)-a antagonists.

22. A kit consisting essentially of ApoB-100, fibronectin, and a2M, or antigenic fragments thereof.
FIG. 1

1. Resolve protein samples on SDS-PAGE

2. Electrophoretically transfer fractionated proteins from gel onto PVDF membrane

3. Block the membrane with neutral protein (BSA or milk casein)

4. Incubate the membrane with primary antibodies obtained from different Patient serum (Control and RPL)

5. Incubate the membrane labeled secondary with HRP-antibody specific to primary antibody

6. Incubate the blot with chemiluminescent HRP substrate and expose to film

Western Blot

X-Ray Films Converted to Pixel Density for Quantification and Analysis
Term

RPL

<table>
<thead>
<tr>
<th></th>
<th>RPL Immunoreactivity</th>
<th>P value</th>
</tr>
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<tbody>
<tr>
<td>Nuclear</td>
<td>3.6 fold greater</td>
<td>p = 0.0044</td>
</tr>
<tr>
<td>Membrane</td>
<td>4.1 fold greater</td>
<td>P = 0.0001</td>
</tr>
<tr>
<td>Cytosol</td>
<td>1.8 fold greater</td>
<td>P = 0.0113</td>
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FIG. 2
Antigen Source

FIG. 4
RECURRENT PREGNANCY LOSS

N Nuclear Pixel Density
M Membrane Pixel Density
C Cytosol Pixel Density

FIG. 5
1. SW 71 Cell Line Protein Fractionation and Quantification

2. Incubate with patient sera for antibody targeting of fractionated cellular proteins

3. Incubate with Agarose Anti-human IgG for precipitation of Ag-Ab complexes

4. Centrifuge and wash the precipitated pellet (x3)

5. Add 2X reducing buffer and boil to solubilize Ag-Ab complexes

6. Electrophoretically separate samples in 4-15% 18-well SDS-Gel

7. Remove gel from the apparatus and stain for proteins

Antigen Recognition by Mass Spectrometry

FIG. 6