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(54) Title: COMBINED METHODS FOR THE DETECTION OF CHROMOSOMAL ANEUPLOIDY

(57) Abstract: The invention relates to methods for the detection and/or diagnosis of fetal chromosomal abnormalities. In particular, the invention concerns the diagnosis of fetal chromosomal abnormalities by combining free, fetal nucleic acid-based tests with other one or more non-free, fetal nucleic acid-based chromosomal abnormality tests.

COMBINED METHODS FOR THE DETECTION OF CHROMOSOMAL ANEUPLOIDY

RELATED PATENT APPLICATION

This patent application claims the benefit of U.S. Provisional Patent Application No. 60/944,331 filed June 15, 2007, entitled COMBINED METHODS FOR THE DETECTION OF CHROMOSOMAL ANEUPLOIDY, naming Chari Georgiou Stylli as an inventor, and designated by Attorney Docket No. SEQ-6011-PV. The content of this patent application is incorporated herein by reference in its entirety.

BACKGROUND

Prenatal diagnosis has been routinely conducted using cells isolated from the fetus through procedures such as chorionic villus sampling (CVS) or amniocentesis. These conventional methods are, however, invasive and present an appreciable risk to both the mother and the fetus.

Alternatives to these invasive approaches have been developed for prenatal screening following the discoveries that several types of fetal cells can be found in maternal circulation (Johansen et al., Prenat. Diagn. 15:921-931, 1995). Circulating cell-free fetal DNA can be detected in maternal plasma and serum (Lo et al., Lancet 350:485-487, 1997). The amount of fetal DNA in maternal blood has been shown to be sufficient for genetic analysis without complex treatment of the plasma or serum and without isolating and enriching fetal cells.

SUMMARY

The present invention relates to methods for early, reliable and non-invasive testing of pregnancy-associated disorders, such as Down's syndrome and other chromosomal abnormalities (e.g., aneuploidies), based on a combination of (i) free, fetal nucleic acid-based tests and (ii) more conventional non-fetal nucleic acid-based tests and/or non-free, fetal nucleic acid-based tests. Such methods may offer improved accuracy and/or precision and/or reliability in non-invasive testing of pregnancy-associated disorders.

The present invention provides methods for combining different techniques for diagnosing, monitoring, or predicting fetal chromosomal abnormalities (e.g., aneuploidy), which may be more accurate and/or reliable and may be performed early during the fetal gestation period (e.g., in the first trimester) at minimal risk to the fetus and mother. In one aspect, the present invention combines free, fetal nucleic acid-based tests with other non-nucleic acid based tests.

Free, fetal nucleic acid-based tests include non-invasive tests for the analysis of fetal nucleic acid present in a maternal sample. Examples of free, fetal nucleic acid-based tests include, but are not limited to, RNA-based tests, allele-based tests, methylation-based tests and paralog-based

tests. The invention utilizes in part screening techniques described, for example, in the following U.S. Patents and Applications: U.S. Patent Application No. 09/380,696, which issued July 10, 2001 as U.S. Patent No. 6,258,540; U.S. Patent Application No. 10/759,783, which published October 14, 2004 as Application Publication No. 20040203037; U.S. Patent Application No. 11/378,598, which published November 9, 2006 as Application Publication No. 20060252068; U.S. Patent Application No. 11/384,128, which published November 9, 2006 as Application Publication No. 20060252071; U.S. Patent Application No. 10/661,165, which published July 15, 2004 as Application Publication No. 20040137470; U.S. Patent No. 6,927,028, which issued August 9, 2005; U.S. Patent Application No. 10/346,514, which published November 13, 2003 as Application Publication No. 20030211522; U.S. Patent Application No. 09/944,951, which issued August 9, 2005 as U.S. Patent No. 6,927,028; U.S. Patent Application No. 11/144,951, which published January 26, 2006 as Application Publication No. 20060019278; U.S. Patent Application No. 10/575,119, which published March 15, 2007 as Application Publication No. 20070059707; U.S. Patent Application No. 10/852,943, which published February 17, 2005 as Application Publication No. 20050037388; U.S. Patent Application No. 11/232,335, which published May 4, 2006 as Application Publication No. 20060094039, all of which are hereby incorporated in their entirety by reference.

Non-nucleic acid-based tests include, but are not limited to, tests based on visual cues provided by ultrasonography, maternal age-based tests, amniocentesis or chorionic villus sampling-based tests, and detection of pregnancy-associated proteins and hormones from maternal serum or plasma or whole blood. In one embodiment, non-nucleic acid-based tests may include the detection pregnancy-associated RNA transcript molecules (e.g., biomarkers) from proteins or hormones present in maternal serum or plasma or whole blood.

Mathematical models have suggested that a combined first-trimester screening program utilizing maternal age (MA), nuchal translucency (NT) thickness, serum-free beta-hCG, and serum PAPP-A will detect more than 80% of fetuses with Down's syndrome for a 5% invasive testing rate (Wald and Hackshaw, Prenat Diagn 17(9):921-9 (1997)). However, the combination of commonly used aneuploidy detection methods when combined with newer, non-invasive free fetal nucleic acid-based methods may offer improved accuracy with a lower false positive rate.

A combination of different screening methods, as provided herein, might significantly improve diagnostic accuracy. For example, individual biomarkers can typically detect a fetal aneuploidy, such as Down's syndrome, in about 30% to 80% of occurrences. A combination of diagnostic or screening techniques comprising biomarkers, ultrasonography-based tests, maternal age, and free, fetal nucleic acid-based tests may increase diagnostic accuracy to at least about 80%, more preferably at least about 85%, even more preferably at least about 90%, even more preferably at least about 95%, most preferably at least about 98%. The combination of different

screening methods which act independently, through distinct biological pathways may be particularly advantageous, since such combinations are expected to significantly increase diagnostic sensitivity.

The present invention in part relates to analyzing nucleic acids such as DNA, RNA, mRNA, oligonucleosomal, mitochondrial, epigenetically modified, single-stranded, double-stranded, circular, plasmid, cosmid, yeast artificial chromosomes, artificial or man-made DNA, including unique DNA sequences, and DNA that has been reverse transcribed from an RNA sample, such as cDNA, and combinations thereof. In a preferred embodiment, the nucleic acid is cell-free nucleic acid. In another embodiment, the nucleic acid is derived from apoptotic cells.

The present invention in part relates to analyzing nucleic acid from a sample such as whole blood, serum, plasma, umbilical cord blood, chorionic villi, amniotic fluid, cerebrospinal fluid, spinal fluid, lavage fluid (*e.g.*, bronchoalveolar, gastric, peritoneal, ductal, ear, arthroscopic), biopsy material (e.g., from a preimplantation embryo), fetal nucleated cells or fetal cellular remnants isolated from maternal blood, washings of the female reproductive tract and a sample obtained by celocentesis, urine, feces, sputum, saliva, nasal mucous, lymphatic fluid, bile, tears, sweat, breast milk, breast fluid, embryonic cells and fetal cells. In a preferred embodiment, the biological sample is plasma. In another preferred embodiment, the biological sample is cell-free or substantially cell-free. In a related embodiment, the biological sample is a sample of previously extracted nucleic acids. In another embodiment, the sample is procured by non-invasive means (e.g., maternal blood draw).

In another preferred embodiment, the sample is from a pregnant human. In a related embodiment, the biological sample is collected from a pregnant human at 1-4, 4-8, 8-12, 12-16, 16-20, 20-24, 24-28, 28-32, 32-36, 36-40, or 40-44 weeks of fetal gestation, and preferably between 5-28 weeks of fetal gestation. In another embodiment, the preferred gestational age for testing may depend on the particular test being offered (e.g., the RNA marker used in testing). In another embodiment, the pregnant human has a relatively elevated concentration of free fetal nucleic acid in her blood, plasma or amniotic fluid. In another embodiment, the pregnant human has a relatively decreased concentration of maternal apoptotic nucleic acid in her blood, plasma or amniotic fluid. The methods of the present invention may be performed in conjunction with any known method to elevate fetal nucleic acid in maternal blood, plasma or amniotic fluid. Likewise, the methods of the present invention may be performed in conjunction with any known method to decrease maternal apoptotic nucleic acid in maternal blood, plasma or amniotic fluid.

In another embodiment, the chromosomal abnormality being detected, diagnosed or monitored is a fetal aneuploidy. In a related embodiment, the fetal aneuploidy is Down's syndrome, trisomy 13, trisomy 18, X chromosome trisomy, X chromosome monosomy, Kleinfelter's syndrome

(XXY genotype), or XYY syndrome (XYY genotype). In a particular embodiment, the invention involves the detection of a fetal aneuploidy that is an autosomal aneuploidy.

The invention in part relates to methods for detecting, diagnosing or monitoring chromosomal abnormalities in the fetus of a pregnant woman using the ratio of alleles of RNA transcript molecules from the pregnant woman's fetus in comparison to other fetal non-aneuploidy chromosomes or in pregnant women with a chromosomally normal fetus, wherein this method is combined with free, fetal nucleic acid-based tests. The use of the ratio in this manner provides superior sensitivity in detecting fetal chromosomal disorders, especially when compared to merely quantifying the amount of a particular allele or the total concentration of a particular RNA transcript present.

The first step of the method involves determining the ratio of alleles of the RNA transcripts in the fetus of a pregnant woman. This is accomplished by obtaining an RNA-containing biological sample from the pregnant woman, wherein the RNA-containing biological sample contains fetal RNA. The alleles are then discriminated from RNA transcribed from at least one genetic locus from at least one chromosome of concern, followed by determination of the ratio of the alleles of the RNA transcripts. The second step involves comparing the ratio from the pregnant woman to a standard control representing an average ratio of alleles from comparable biological samples obtained from pregnant women each carrying a chromosomally normal fetus, wherein an increase or decrease in the ratio from the standard control indicates an increased risk of having a fetus with a chromosomal disorder.

In some embodiments, the present invention provides a method in which the alleles of the steps involving discriminating the alleles of RNA, determining the ratio of the different alleles, and comparing the ratio from the previous step to a standard control are differentiated by sequence variation. In another embodiment, the sequence variation is a single nucleotide polymorphism (SNP). In a further embodiment, the sequence variation is an insertion/deletion polymorphism. In still another embodiment, the sequence variation is a simple tandem repeat polymorphism.

In another embodiment, the present invention provides a method in which the RNA is expressed in the placenta at a level which is two-fold or more than that of maternal blood. In some embodiments, the RNA is expressed in the placenta at a level which is five-fold or more than that of maternal blood. In other embodiments, the RNA is expressed in the placenta at a level which is ten-fold or more than that of maternal blood.

In a further embodiment, the present invention provides a method in which the RNA is mRNA. In another embodiment, the RNA is transcribed from at least one genetic locus selected from the group consisting of collagen VI alpha 1 (COL6A1), superoxide dismutase 1 (SOD1), collagen VI alpha 2 (COL6A2), mitochondrial ATP synthase O subunit (ATP5O), BTG family,

member 3 (BTG 3), a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 1 (ADAMTS1), beta-site APP-cleaving enzyme 2 (BACE2), intersectin 1 (ITSN1), amyloid beta (A4) precursor protein (APP), ATP synthase, H+ transporting, mitochondrial F0 complex, subunit F6 (ATP5J), Down syndrome critical region gene 5 (DSCR5), placenta-specific 4 (PLAC4), hypotheticalprotein BC005107 (LOC90625), ribosomalprotein L17 (RPL17), serpin peptidase inhibitor lade B (ovalbumin) member 2 (SERPINB2) and collagen type IV alpha 2 (COL4A2). In yet another embodiment, the RNA is transcribed from a genetic locus which contains a single nucleotide polymorphism (SNP). In other embodiments, the RNA is transcribed from at least one genetic locus selected from the group consisting of collagen VI alpha 1 (COL6A1) and collagen VI alpha 2 (COL6A2). In still other embodiments, the SNP in the RNA transcribed from the genetic locus of the COL6A1 is .sup.Arg850.sub.His or .sup.Ser932.sub.Ser. In yet another embodiment, the SNP in the RNA transcribed from the genetic locus of the COL6A2 is .sup.Val728.sub.Val.

In a preferred embodiment, the present invention provides a method in which the RNA is transcribed from the genetic locus for placenta-specific 4 (PLAC4). In yet another embodiment, the RNA is any variant transcribed from the PLAC4 gene, such as AF269287, AK027868, AK092431, BC093685, BC101615, BC101617, L13197, NM.sub.--182832 and LOC191585. In still yet another embodiment, the RNA transcribed from the genetic locus of the PLAC4 gene contains a single nucleotide polymorphism, or an insertion-deletion polymorphism selected from the group consisting of rs3804026, rs4818219, rs9977003, rs7844, rs9015, rs13643, rs9305729, rs9305730, rs5019195, rs5019194, rs5844069, rs1049904, rs16998089, rs12482116, rs1909439, rs7278659, rs12106409, rs12106395, rs12106401, rs12106434, rs2183584, rs3949725, rs8130833, rs10222145 and rs9981478, or other polymorphisms located within the PLAC4 gene locus such as PLAC4-41471145 and PLAC4-41476236.

In another embodiment, the invention in part provides a methylation-based test, wherein the following steps are performed: (a) obtaining a blood sample from the woman; (b) determining the methylation status of at least a portion of a differentially methylated gene in the blood sample, wherein the portion of the differentially methylated gene from the fetus and the portion from the woman are differentially methylated, thereby distinguishing the gene from the woman and the gene from the fetus in the blood sample; (c) determining the level of the fetal gene; and (d) comparing the level of the fetal gene with a standard control. In some cases, an increase from the standard control indicates the presence or progression of a pregnancy-associated disorder. In other cases, a decrease from the standard control indicates the presence or progression of a pregnancy-associated disorder.

In another embodiment, the methylation-related method comprises the following steps: (a) obtaining DNA from a blood sample from the woman; (b) treating the DNA from step (a) with a reagent that differentially modifies methylated and non-methylated DNA; and (c) determining the levels of different alleles of a differentially methylated gene from the fetal DNA, thereby determining the ratio of the alleles, wherein the different alleles have different methylation profile in at least portion of the differentially methylated gene, and wherein an increase or a decrease in the ratio from a standard control indicates the presence of a chromosomal abnormality in the fetus.

In another embodiment, the methods of the present invention are combined with a paralog-based test. Paralog-based tests provide a universal method to detect the presence of chromosomal abnormalities by using paralogous genes as internal controls in an amplification reaction. The method comprises providing a pair of primers which can specifically hybridize to each of a set of paralogous genes under conditions used in amplification reactions, such as PCR. Paralogous genes are preferably on different chromosomes but may also be on the same chromosome (e.g., to detect loss or gain of different chromosome arms). By comparing the amount of amplified products generated, the relative dose of each gene can be determined and correlated with the relative dose of each chromosomal region and/or each chromosome, on which the gene is located.

In another embodiment, the methods of the present invention are combined with detection of maternal biomarkers for the testing of fetal Down's Syndrome and other chromosomal aneuploidies, based upon the proteomic profile of a maternal biological fluid. In an embodiment, the biomarker is detectable in maternal serum. In another embodiment, one or more maternal biomarkers may be represented as a proteomic profile. As used herein, proteomic profile refers to a representation of the expression pattern of a plurality of biomarkers (e.g., proteins) in a biological sample, e.g. a biological fluid at a given time. The proteomic profile can, for example, be represented as a mass spectrum, but other representations based on any physicochemical or biochemical properties of the proteins, or fragments thereof, are also included.

In another embodiment, the biomarker whose level of transcribed mRNA or level of translated protein is being detected is selected from the group consisting of PAPP-A, alpha-fetoprotein (AFP), human chorionic gonadotropin (bhCG), unconjugated estriol (uE3), and inhibin A. In another embodiment, the biomarkers are selected from any one of the markers disclosed in US Patent Application No. 20060094039, filed September 20, 2005, which is hereby incorporated by reference.

In another embodiment, the methods of the present invention are combined with ultrasonography-based tests to detect a chromosomal abnormality. In a preferred embodiment, the ultrasonography-based test is a nuchal translucency (NT) measurement.

DETAILED DESCRIPTION

Fetal aneuploidies are aberrations in chromosome number and commonly arise as a result of a meiotic nondisjunction during oogenesis or spermatogenesis. In other instances, such as trisomy 8, the aneuploidy results from postzygotic mitotic disjunction (Nicolaidis & Petersen, Human Reproduction, 13(2):313-319, (1998)). Such aberrations include both reductions and increases in the normal chromosome number and can involve autosomes as well as the sex chromosomes. An example of a reduction aneuploidy is Turner's syndrome, which is typified by the presence of a single X sex chromosome. Examples of increases in chromosome number include Down's syndrome (trisomy of chromosome 21), Patau syndrome (trisomy of chromosome 13), Edwards syndrome (trisomy of chromosome 18), and Kleinfelter's syndrome (an XXY trisomy of the sex chromosomes). Aneuploidies commonly lead to significant physical and neurological impairments which result in a large percentage of affected individuals failing to reach adulthood. In fact, fetuses having an autosomal aneuploidy involving a chromosome other than 13, 18, or 21 generally die in utero. However, certain aneuploidies, such as Kleinfelter's syndrome, present far less pronounced phenotypes and those affected with other trisomies, such as XXY & XXX, often will mature to be fertile adults.

Down's syndrome is the most common single pattern of malformation in man, and is one of the most common serious congenital abnormalities found at birth, with a prevalence of one in 660 live births (Jones, K., Down's Syndrome in Smith's recognizable patterns of human malformation, Jones, K., Editor, 1997, Philadelphia, Pa., pp. 8-13). Approximately a third of all fetuses with Down's syndrome who are alive in the second trimester will not survive to term; thus, the true prevalence of Down's syndrome in the second trimester is closer to 1 in 500 pregnancies (Cuckle, H., Epidemiology of Down Syndrome, in Screening for Down Syndrome in the First Trimester, J. Grudzinkas and R. Ward, Editors, 1997, RCOG Press, London, UK, pp. 3-13.). A majority of infants with Down's syndrome have serious cardiac, gastrointestinal, or other abnormalities that lead to significant morbidity and mortality. In addition, most have an IQ of less than 50, making this syndrome one of the leading causes of mental deficiency in the United States. Approximately 2.5 million pregnant women undergo serum biomarker screening for Down's syndrome each year in the United States, and, in the absence of screening, approximately 4,000 of these pregnancies may result in birth of a baby with Down's syndrome (Palomaki, G. E., et al. Am. J. Obstet. Gynecol. 176(5):1046-1051 (1997)).

While Down's syndrome is the most prevalent aneuploidy in live births, aneuploidies of chromosomes 13, 18, and the sex chromosomes affect a significant number of individuals. Trisomy 18, for example, has a prevalence of approximately 1 in 7000 births and Trisomy 13 has a

prevalence of approximately 1 in 29,000 births (Nicolaidis & Petersen, Human Reproduction, 13(2):313-319, (1998)). Other aneuploidies occur at significant rates during pregnancy, but result in spontaneous abortion before the fetus reaches term, usually within the first 15 weeks of pregnancy (Nicolaidies & Petersen, supra). For example, Trisomy 16 is the single most prevalent human trisomy and is thought to affect 1.5% of all recognized pregnancies; however, it is a lethal chromosomal aberration. Trisomies 15 and 8 occur at much lower rates (approximately 1.4% and 0.7% of all spontaneous abortions, respectively) but are also lethal aberrations (Nicoladies & Petersen, Human Reproduction, 13(2):313-319, (1998)). The present invention provides combined methods for more accurately diagnosing, monitoring, or predicting fetal aneuploidies.

Definitions

The terms “nucleic acid” and “nucleic acid molecule” may be used interchangeably throughout the disclosure. The terms refer to oligonucleotides, oligos, polynucleotides, deoxyribonucleotide (DNA), genomic DNA, mitochondrial DNA (mtDNA), complementary DNA (cDNA), bacterial DNA, viral DNA, viral RNA, RNA, message RNA (mRNA), transfer RNA (tRNA), ribosomal RNA (rRNA), siRNA, catalytic RNA, clones, plasmids, M13, P1, cosmid, bacteria artificial chromosome (BAC), yeast artificial chromosome (YAC), amplified nucleic acid, amplicon, PCR product and other types of amplified nucleic acid, RNA/DNA hybrids and polyamide nucleic acids (PNAs), all of which can be in either single- or double-stranded form, and unless otherwise limited, would encompass known analogs of natural nucleotides that can function in a similar manner as naturally occurring nucleotides and combinations and/or mixtures thereof. Thus, the term “nucleotides” refers to both naturally-occurring and modified/non-naturally-occurring nucleotides, including nucleoside tri, di, and monophosphates as well as monophosphate monomers present within polynucleic acid or oligonucleotide. A nucleotide may also be a ribo; 2'-deoxy; 2', 3'-deoxy as well as a vast array of other nucleotide mimics that are well-known in the art. Mimics include chain-terminating nucleotides, such as 3'-O-methyl, halogenated base or sugar substitutions; alternative sugar structures including nonsugar, alkyl ring structures; alternative bases including inosine; deaza-modified; chi, and psi, linker-modified; mass label-modified; phosphodiester modifications or replacements including phosphorothioate, methylphosphonate, boranophosphate, amide, ester, ether; and a basic or complete internucleotide replacements, including cleavage linkages such a photocleavable nitrophenyl moieties.

As used herein, the term "RNA-containing biological sample" refers to a sample that contains ribonucleic acid (RNA). RNA refers to a polymer of ribonucleotides that has a sequence corresponding to at least a portion of a pre-selected location in the human genome. RNA as used herein includes, but is not limited to, mRNA, ribosomal RNA and micro RNA. RNA can be protein

encoding sequences such as mRNA, or non-coding sequences such as ribosomal RNA, microRNA or other transcribed sequences without well-defined functions. Messenger RNA (mRNA) is an RNA molecule transcribed from the DNA of a gene, and from which a protein is translated by the action of ribosomes. Ribosomal RNA (rRNA) is a non-coding RNA that is not translated into a protein. One of skill in the art will appreciate that other types of RNA are useful in the present invention.

As used herein, the terms "fetal", "placental derived" and "placental expressed" refer to the origin of certain nucleic acid species that are detectable in a biological sample from a pregnant woman, e.g., blood. For example, a fetal RNA species is one that has been transcribed from a fetal DNA sequence. Placental-derived or placental expressed RNA is one type of fetal RNA. One of skill in the art will appreciate that other fetal RNA are useful in the present invention. A placental derived or placental expressed RNA species is one that is transcribed in the placenta.

As used herein, the term "discriminating alleles from RNA transcribed from at least one genetic locus from at least one chromosome of concern" refers to the detection and quantification of particular RNA alleles transcribed from a particular genetic locus on a chromosome. The detection and quantification of alleles can be carried out by a variety of methods, including the use of hybridization probes and quantitative real time polymerase chain reaction (QRT-PCR). Other methods include the use of mass spectrometry (MS), electrophoresis, pyrosequencing, primer extension microarrays, chips and sequencing.

As used herein, the term "standard control" refers to a sample suitable for the use of a method of the present invention, in order for determining the ratio of the RNA-SNP alleles transcribed from a particular genetic locus, e.g., COL6A1, SOD1, COL6A2, ATP5O, BTG3, ADAMTS1, BACE2, ITSN1, APP, ATP5J, DSCR5, PLAC4, LOC90625, RPL17, SERPINB2 or COL4A2. Such sample contains a known ratio of the RNA-SNP alleles transcribed from a particular genetic locus that closely reflects the average ratio of such RNA-SNP alleles in pregnant women who each carries a chromosomally normal fetus. The standard control can also represent the mean ratio, the median ratio, or another useful ratio known to one of skill in the art.

The term “sample” as used herein includes a specimen or culture (e.g., microbiological cultures) that comprises biological material for the detection of a chromosomal abnormality. Samples include whole blood, serum, plasma, umbilical cord blood, chorionic villi, amniotic fluid, cerebrospinal fluid, spinal fluid, lavage fluid (e.g., bronchoalveolar, gastric, peritoneal, ductal, ear, arthroscopic), biopsy material (e.g., from a preimplantation embryo), fetal nucleated cells or fetal cellular remnants isolated from maternal blood, urine, feces, sputum, saliva, nasal mucous, lymphatic fluid, bile, tears, sweat, breast milk, breast fluid, washings of the female reproductive tract and a sample obtained by celocentesis, embryonic cells and fetal cells. In an embodiment of the invention, the sample comprises a mixture of nucleic acids. For example, the mixture may

comprise nucleic acid from different species or from different individuals. In a further embodiment, the biological sample contains cellular elements or cellular remnants in maternal blood. In another embodiment, a sample may include a specimen of synthetic origin.

The term “non-invasive” as used herein refers a method for collecting a sample that poses minimal risk to an individual (e.g., the mother and/or fetus). An example of a non-invasive method is a blood draw; whereas examples of invasive methods include amniocentesis and chorionic villus sampling, both of which constitute a finite risk to the fetus.

The term "pregnancy-associated disorder," as used herein, refers to any condition or disease that may affect a pregnant woman, the fetus the woman is carrying, or both the woman and the fetus. Such a condition or disease may manifest its symptoms during a limited time period, e.g., during pregnancy or delivery, or may last the entire life span of the fetus following its birth. Some examples of a pregnancy-associated disorder include ectopic pregnancy, preeclampsia, preterm labor, and fetal chromosomal abnormalities such as trisomy 13, 18, or 21.

As used herein, the term "chromosomal abnormality" refers to a state where the number of chromosomes is not an exact multiple of the usual haploid number. Most often, there is either an additional chromosome or one missing. A common chromosomal disorder is aneuploidy. A common form of chromosomal aneuploidy is a trisomy, where a single additional chromosome is present. For example, trisomy 18 is a chromosomal abnormality where a third chromosome 18 is found in a cell, whereas a third chromosome 21 is present in the cells of a patient suffering from trisomy 21. “Chromosomal abnormality” may also refer to a state where a proportion of one or more chromosomes is not an exact multiple of the usual haploid number, due to, for example, chromosome translocation. Chromosomal translocation (*e.g.* translocation between chromosome 21 and 14 where some of the 14th chromosome is replaced by extra 21st chromosome) may cause partial trisomy 21. In one embodiment, a chromosomal abnormality is numerical or structural, and includes but is not limited to aneuploidy, polyploidy, inversion, a trisomy, a monosomy, duplication, deletion, deletion of a part of a chromosome, addition, addition of a part of chromosome, insertion, a fragment of a chromosome, a region of a chromosome, chromosomal rearrangement, and translocation. A chromosomal abnormality can be correlated with presence of a pathological condition or with a predisposition to develop a pathological condition.

As used herein, the term “chromosomal abnormality test” means a test which may directly detect a chromosomal abnormality at the nucleic acid level, for example, a FISH-based test or other nucleic acid-level based test or may mean a test which detects the phenotypic result of a chromosomal abnormality (at any level, from transcription and translation effects through effects on a cell, an organ or structure, a multi-organ system or structure).

As used herein, “free, fetal nucleic acid-based test” refers to a method for detecting, diagnosing or monitoring a fetal disease, fetal condition or fetal chromosomal abnormality by analyzing a nucleic acid of fetal origin which is present in a maternal biological sample. Preferably the biological sample is maternal serum or plasma or whole blood. The test may rely on quantifying the relative amount of fetal nucleic acid suspected of being abnormal (*i.e.*, part of an aneuploidy) relative to a standard control (*e.g.*, the control may be maternal nucleic acid or normal fetal nucleic acid from a different chromosome). In contrast, fetal nucleic acid may be obtained from fetal cells which are circulating in the maternal plasma, serum, whole blood or other biological fluids. Fetal nucleic acid from these sources would not be considered free, fetal nucleic acid. Fetal nucleic acid from these cell-based sources may be combined with any of the other methods or sample sources set forth herein.

As used herein, an “allele-based test” refers to a method for detecting, diagnosing or monitoring a chromosomal abnormality that compares the ratio between alleles of a polymorphic site from fetal nucleic acid and from maternal nucleic acid.

As used herein, an “RNA-based test” refers to a method for detecting, diagnosing or monitoring a chromosomal abnormality that compares the ratio between alleles of a polymorphic site on locus- and tissue-specific RNA transcripts from an aneuploid fetus to a euploid fetus.

As used herein, a “methylation-based test” refers to a method for detecting, diagnosing or monitoring a chromosomal abnormality based on differences in the epigenetic status between fetal nucleic acid and maternal nucleic acid, wherein the difference may be used to detect a chromosomal abnormality.

The term "epigenetic state" or "epigenetic status" as used herein refers to any structural feature at the molecular level of a nucleic acid (e.g., DNA or RNA) other than the primary nucleotide sequence. For instance, the epigenetic state of a genomic DNA may include its secondary or tertiary structure determined or influenced by, e.g., its methylation pattern or its association with cellular proteins.

As used herein, a “parolog-based test” refers to a method for detecting, diagnosing or monitoring a chromosomal abnormality by using paralogous genes as internal controls in an amplification reaction. By comparing the amount of amplified products generated, the relative dose of each gene can be determined and correlated with the relative dose of each chromosomal region and/or each chromosome, on which the gene is located.

Sources of Nucleic Acids

Samples comprising nucleic acid are obtained by procedures such as amniocentesis (e.g., Barter, Am. J Obstet. Gynecol. 99: 795-805; U.S. Pat. No. 5,048,530), chorionic villus sampling

(e.g., Imamura et al., 1996, Prenat. Diagn. 16(3): 259-61), or by maternal peripheral blood sampling (e.g., Iverson et al., 1981, Prenat. Diagn. 9: 31-48; U.S. Pat. No. 6,210,574). Fetal cells also can be obtained by cordocentesis or percutaneous umbilical blood sampling, although this technique is technically difficult and not widely available (see Erbe, 1994, Scientific American Medicine 2, section 9, chapter IV, Scientific American Press, New York, pp 41-42).

Nucleic acid isolation from blood, plasma, or serum of the pregnant mother can be performed using any method known to one skilled in the art. Any standard nucleic acid isolation technique can be used to isolate the fetal nucleic acid and the maternal nucleic acid including, but not limited to, QIAamp DNA Blood Midi Kit supplied by QIAGEN. Other standard methods of nucleic acid isolation are described, for example, in (Sambrook et al., Molecular Biology: A laboratory Approach, Cold Spring Harbor, N. Y. 1989; Ausubel, et al., Current protocols in Molecular Biology, Greene Publishing, Y, 1995). A preferred method for isolation of plasma DNA is described in Chiu et al., 2001, Clin. Chem. 47: 1607-1613, which is herein incorporated by reference in its entirety. Other suitable methods are provided in Example 2 of PCT International Application Publication Number 2007/028155, filed on September 1, 2006; PCT International Application Number PCT/US07/69991, filed May 31, 2007; US Provisional Application No. 60/805,073, filed June 16, 2006; and US Provisional Application No. 60/908,167, filed March 26, 2007, all of which are hereby incorporated in their entirety by reference.

Some of the methods of the present invention may be practiced using free, fetal nucleic acid from a maternal sample, while other methods may require nucleic acid procured through invasive means (e.g., CVS, amniocentesis). (See International Patent Application Publication No. WO2004/076653, which is hereby incorporated by reference). Still other methods do not require nucleic acid (e.g., ultrasound-based tests).

Chromosomal Abnormality Detection Methods

Recently, much interest has been focused on the biology and diagnostic applications of nucleic acids that are present at very low concentrations in humans. In particular, fetal DNA has been found to exist in maternal plasma (Lo et al. *Lancet*. 1997 Aug 16; 350 (9076):485-7). This discovery has facilitated the development of non-invasive prenatal diagnostic approaches based simply on the analysis of a maternal blood sample (Lo et al. *Am J Hum Genet*. 1998 Apr; 62(4):768-75). The non-invasive nature of maternal plasma-based approaches represents a major advantage over conventional methods of prenatal diagnosis, such as amniocentesis and chorionic villus sampling, which are associated with a small but finite risk of fetal loss. Tests that utilize free, fetal nucleic acid rely on quantifying the relative amount of fetal nucleic acid compared to a standard

control. This is often achieved by differentiating fetal nucleic acid from control nucleic acid based on sequence differences (e.g., allelic differences, epigenetic differences, paralogous genes).

Allele-based methods for diagnosing, monitoring, or predicting chromosomal abnormalities rely on determining the ratio of the alleles found in maternal sample comprising free, fetal nucleic acid. The ratio of alleles refers to the ratio of the population of one allele and the population of the other allele in a biological sample. In some cases, it is possible that in trisomies a fetus may be tri-allelic for a particular locus, and these tri-allelic events may be detected to diagnose aneuploidy. For an allele at a polymorphic site to be informative, the nucleic acid must comprise a paternal allele. Further, the mother must be homozygous at the polymorphic site and the fetus is heterozygous at the polymorphic site. In an embodiment, the maternal genotype is determined in conjunction with the methods provided herein. In a related embodiment, the mother is first genotyped (for example, using peripheral blood mononuclear cells (PBMC) from a maternal whole blood sample) to determine the non-target allele that will be targeted by the cleavage agent.

RNA-based methods for diagnosing, monitoring, or predicting chromosomal abnormalities rely on the use of pregnancy-specificity of fetal-expressed transcripts to develop a method which allows the genetic determination of fetal chromosomal aneuploidy and thus the establishment of its diagnosis non-invasively. In one embodiment, the fetal-expressed transcripts are those expressed in the placenta. Specifically, the present invention detects single nucleotide polymorphisms (SNPs) from RNA transcripts with tissue-specific expression patterns that are encoded by genes on the aneuploid chromosome. Other polymorphisms are also detectable by the methods of the present invention, such as an insertion/deletion polymorphism and a simple tandem repeat polymorphism. The status of the locus is determined through the assessment of the ratio between informative SNPs on the RNA transcribed from the genetic loci of interest. Genetic loci of interest may include, but are not limited to, COL6A1, SOD1, COL6A2, ATP5O, BTG3, ADAMTS1, BACE2, ITSN1, APP, ATP5J, DSCR5, PLAC4, LOC90625, RPL17, SERPINB2 or COL4A2.

The present invention, therefore, can be applied to the prenatal diagnosis of trisomy 21 which involves the analysis of informative SNPs on RNA transcripts with placental tissue expression that are derived from loci on chromosome 21. Fetal trisomy 21 is then determined by comparing the ratios between the informative SNPs through the detection of the placenta-expressed RNA transcripts in maternal blood. The fetal-specificity of the markers in maternal blood is conferred by their placental tissue expression, while the aneuploid status is determined by the abnormal ratios between the informative SNPs on the RNA transcripts.

Methylation-based tests offer another strategy for the development of a generic fetal-specific DNA marker for detection in maternal plasma. It has been demonstrated that fetal and maternal DNA can be distinguished by their differences in methylation status (see U.S. Patent No. 6,927,028,

which issued August 9, 2005). Methylation is an epigenetic phenomenon, which refers to processes that alter a phenotype without involving changes in the DNA sequence. Poon et al. further showed that epigenetic markers can be used to detect fetal-derived maternally-inherited DNA sequence from maternal plasma (*Clin. Chem*. 48:35-41, 2002). Epigenetic markers may be used for non-invasive prenatal diagnosis by determining the methylation status of at least a portion of a differentially methylated gene in the blood sample, wherein the portion of the differentially methylated gene from the fetus and the portion from the woman are differentially methylated, thereby distinguishing the gene from the woman and the gene from the fetus in the blood sample; determining the level of the fetal gene; and comparing the level of the fetal gene with a standard control. In some cases, an increase from the standard control indicates the presence or progression of a pregnancy-associated disorder. In other cases, a decrease from the standard control indicates the presence or progression of a pregnancy-associated disorder.

Parolog-based tests provide a universal method to detect the presence of chromosomal abnormalities by using paralogous genes as internal controls in an amplification reaction. The method comprises providing one or more pair of primers which can specifically hybridize to each of a set of paralogous genes under conditions used in amplification reactions, such as PCR. Paralogous genes are preferably on different chromosomes but may also be on the same chromosome (e.g., to detect loss or gain of different chromosome arms). By comparing the amount of amplified products generated, the relative dose of each gene can be determined and correlated with the relative dose of each chromosomal region and/or each chromosome, on which the gene is located.

Other molecular methods for the diagnosis of aneuploidies are also known (Hulten et al., 2003, Reproduction, 126(3):279-97; Armour et al., 2002, Human Mutation 20(5):325-37; Eiben and Glaubitz, J Histochem Cytochem. 2005 Mar; 53(3):281-3); and Nicolaides et al., J Matern Fetal Neonatal Med. 2002 Jul; 12(1):9-18)). Alternative molecular methods include PCR based methods such as QF-PCR (Verma et al., 1998, Lancet 352(9121):9-12; Pertl et al., 1994, Lancet 343(8907):1197-8; Mann et al., 2001, Lancet 358(9287):1057-61; Adinolfi et al., 1997, Prenatal Diagnosis 17(13):1299-311), multiple amplifiable probe hybridization (MAPH) (Armour et al., 2000, Nucleic Acids Res 28(2):605-9), multiplex probe ligation assay (MPLA) (Slater et al., 2003, J Med Genet 40(12)907-12; Schouten et al., 2002 30(12:e57), all of which are hereby incorporated by reference.

Non PCR-based technologies such as comparative genome hybridization (CGH) offer another approach to aneuploidy detection (Veltman et al., 2002, Am J Hum Genet 70(5):1269-76; Snijders et al., 2001 Nat Genet 29(3):263-4).

In an embodiment, free fetal nucleic acid-based tests include the alternative molecular methods and non-PCR-based methods described above.

The present invention in part comprises combining free, fetal nucleic acid-based tests with non-fetal nucleic acid-based chromosomal tests. Non-fetal nucleic acid-based-tests may include, but are not limited to, invasive amniocentesis or chorionic villus sampling-based test, a maternal age-based test, a biomarker screening test, and an ultrasonography-based test. It should be noted that biomarker screening tests may be performed wherein nucleic acid (either fetal or maternal) is detected. However, for the purposes of this disclosure “biomarker tests” are still considered a non-fetal nucleic acid based test.

Amniocentesis and chorionic villus sampling (CVS)-based tests offer definitive prenatal diagnosis of fetal aneuploidies, but requires invasive sampling by amniocentesis or Chorionic Villus Sampling (CVS). These sampling methods are associated with a 0.5% to 1% procedure-related risk of pregnancy loss (D'Alton, M. E., Semin Perinatol 18(3):140-62 (1994)).

While different approaches have been employed in connection with specific aneuploidies, in the case of Down's syndrome, screening was initially based entirely on maternal age, with an arbitrary cut-off of 35 years used to define a population of women at sufficiently high risk to warrant offering invasive fetal testing.

Maternal biomarkers offer another strategy for the testing of fetal Down's syndrome and other chromosomal aneuploidies, based upon the proteomic profile of a maternal biological fluid. “Maternal biomarkers” as used herein refer to biomarkers present in a pregnant female whose level of transcribed mRNA or level of translated protein is being detected and can be correlated with the presence or absence of a chromosomal abnormality.

Second-trimester serum screening techniques were introduced in order to improve detection rate and to reduce the invasive testing rate. Current standard-of-care for screening for Down's syndrome requires offering all patients a triple-marker serum test between 15 and 18 weeks gestation, which, together with maternal age (MA), is used for risk calculation. This test assays (alpha-fetoprotein (AFP), human chorionic gonadotropin (beta-hCG), and unconjugated estriol (uE3). The current standard-of-care serum "triple screen" for Down's syndrome is now evolving into a "quad test", in which the serum marker inhibin-A is added to the other three analytes.

There are also data suggesting that first-trimester concentrations of a variety of pregnancy-associated proteins and hormones differ in chromosomally normal and abnormal pregnancies. The two most promising first-trimester serum markers with regards to Down's syndrome and Edwards syndrome appear to be PAPP-A and free .beta.hCG (Wapner, R., et al., N Engl J Med 349(15):1405-1413 (2003)). It has been reported that first-trimester serum levels of PAPP-A are significantly lower in Down's syndrome, and this decrease is independent of nuchal translucency

(NT) thickness (Brizot, M. L., et al., Obstet Gynecol 84(6):918-22 (1994)). In addition, it has been shown that first-trimester serum levels of both total and free .beta.-hCG are higher in fetal Down's syndrome, and this increase is also independent of NT thickness (Brizot, M. L., Br J Obstet Gynaecol 102(2):127-32 (1995)).

Ultrasonography-based tests provide a non-molecular-based approach for diagnosing chromosomal abnormalities. The identification of certain major fetal structural abnormalities significantly increases the risk of Down's syndrome and other aneuploidies, and is then considered an indication for invasive fetal testing. Further work has been performed evaluating the role of sonographic markers of aneuploidy, which are not structural abnormalities per se, and, in the presence of a normal karyotype, may not confer any risks to the fetus. Such sonographic markers employed in Down's syndrome screening include choroid plexus cysts, echogenic bowel, short femur, short humerus, minimal hydronephrosis, and thickened nuchal fold. Investigators from the Fetal Medicine Foundation in London have suggested an 80% detection rate for Down's syndrome from screening using a combination of MA and first-trimester ultrasound evaluation of the fetus (Pandya, P. P. et al., Br J Obstet Gyneacol 102(12):957-62 (1995); Snijders, R. J., et al., Lancet 352(9125):343-6 (1998)). This relies on the measurement of the translucent space between the back of the fetal neck and overlying skin, which has been reported to be increased in fetuses with Down's syndrome and other aneuploidies. This nuchal translucency (NT) measurement is reportedly easy to obtain by transabdominal or transvaginal ultrasonography between 10 and 14 weeks gestation (Snijders, R. J., et al., Ultrasound Obstet Gynecol 7(3):216-26 (1996)).

Nucleic Acid Detection

Whether detecting sequence differences, detecting amplification products or primer extension products, any detection method known in the art may be utilized. Polymorphism detection methods known in the art include, for example, primer extension or microsequencing methods, ligase sequence determination methods (*e.g.*, U.S. Pat. Nos. 5,679,524 and 5,952,174, and WO 01/27326), mismatch sequence determination methods (*e.g.*, U.S. Pat. Nos. 5,851,770; 5,958,692; 6,110,684; and 6,183,958), allele specific oligonucleotide (ASO) analysis, methylation-specific PCR (MSPCR), pyrosequencing analysis, acycloprime analysis, Reverse dot blot, Dynamic allele-specific hybridization (DASH), Peptide nucleic acid (PNA) and locked nucleic acids (LNA) probes, Molecular Beacons, Intercalating dye, FRET primers, AlphaScreen, SNPstream, genetic bit analysis (GBA), Multiplex minisequencing, SNaPshot, GOOD assay, Microarray miniseq, arrayed primer extension (APEX), Microarray primer extension, Tag arrays, Coded microspheres, Template-directed incorporation (TDI), fluorescence polarization, Colorimetric oligonucleotide ligation assay (OLA), Sequence-coded OLA, Microarray ligation, Ligase chain reaction, Padlock probes, and

Invader assay, microarray sequence determination methods, restriction fragment length polymorphism (RFLP) procedures, PCR-based assays (*e.g.*, TAQMAN® PCR System (Applied Biosystems)), nucleotide sequencing methods, hybridization methods, conventional dot blot analyses, single strand conformational polymorphism analysis (SSCP, e.g., U.S. Patent Nos. 5,891,625 and 6,013,499; Orita *et al., Proc. Natl. Acad. Sci. U.S.A 86*: 27776-2770 (1989)), denaturing gradient gel electrophoresis (DGGE), heteroduplex analysis, mismatch cleavage detection, and techniques described in Sheffield *et al., Proc. Natl. Acad. Sci. USA 49*: 699-706 (1991), White *et al., Genomics 12*: 301-306 (1992), Grompe *et al., Proc. Natl. Acad. Sci. USA 86*: 5855-5892 (1989), and Grompe, *Nature Genetics 5*: 111-117 (1993), detection by mass spectrometry, for example, primer extension method (e.g., iPLEX™, Sequenom Inc.), (*e.g.*, US 20050079521, which is hereby incorporated by reference), real time-PCR (*e.g.*, US Patent Nos. US 5,210,015, US 5,487,972, both of which are hereby incorporated by reference), or hybridization with a suitable nucleic acid primer specific for the sequence to be detected. Suitable nucleic acid primers can be provided in a format such as a gene chip, or any combination thereof.

Primer extension polymorphism detection methods, also referred to herein as "microsequencing" methods, typically are carried out by hybridizing a complementary oligonucleotide to a nucleic acid carrying the polymorphic site. In these methods, the oligonucleotide typically hybridizes adjacent to the polymorphic site. As used herein, the term "adjacent" refers to the 3' end of the extension oligonucleotide being sometimes 1 nucleotide from the 5' end of the polymorphic site, often 2 or 3, and at times 4, 5, 6, 7, 8, 9, or 10 nucleotides from the 5' end of the polymorphic site, in the nucleic acid when the extension oligonucleotide is hybridized to the nucleic acid. The extension oligonucleotide then is extended by one or more nucleotides, often 1, 2, or 3 nucleotides, and the number and/or type of nucleotides that are added to the extension oligonucleotide determine which polymorphic variant or variants are present. Oligonucleotide extension methods are disclosed, for example, in U.S. Patent Nos. 4,656,127; 4,851,331; 5,679,524; 5,834,189; 5,876,934; 5,908,755; 5,912,118; 5,976,802; 5,981,186; 6,004,744; 6,013,431; 6,017,702; 6,046,005; 6,087,095; 6,210,891; and WO 01/20039. The extension products can be detected in any manner, such as by fluorescence methods (*see, e.g.*, Chen & Kwok, *Nucleic Acids Research 25*: 347-353 (1997) and Chen *et al., Proc. Natl. Acad. Sci. USA 94/20*: 10756-10761 (1997)) and by mass spectrometric methods (*e.g.*, MALDI-TOF mass spectrometry). Oligonucleotide extension methods using mass spectrometry are described, for example, in U.S. Patent Nos. 5,547,835; 5,605,798; 5,691,141; 5,849,542; 5,869,242; 5,928,906; 6,043,031; 6,194,144; and 6,258,538.

Microsequencing detection methods often incorporate an amplification process that precedes the extension step. The amplification process typically amplifies a region from a nucleic

acid sample that comprises the polymorphic site. Amplification can be carried out by utilizing a pair of oligonucleotide primers in a polymerase chain reaction (PCR), in which one oligonucleotide primer typically is complementary to a region 3' of the polymorphism and the other typically is complementary to a region 5' of the polymorphism. A PCR primer pair may be used in methods disclosed in U.S. Patent Nos. 4,683,195; 4,683,202, 4,965,188; 5,656,493; 5,998,143; 6,140,054; WO 01/27327; and WO 01/27329 for example. PCR primer pairs may also be used in any commercially available machines that perform PCR, such as any of the GENEAMP® Systems available from Applied Biosystems.

A microarray can be utilized for determining whether a polymorphic variant is present or absent in a nucleic acid sample. A microarray may include any oligonucleotides described herein, and methods for making and using oligonucleotide microarrays suitable for prognostic use are disclosed in U.S. Pat. Nos. 5,492,806; 5,525,464; 5,589,330; 5,695,940; 5,849,483; 6,018,041; 6,045,996; 6,136,541; 6,142,681; 6,156,501; 6,197,506; 6,223,127; 6,225,625; 6,229,911; 6,239,273; WO 00/52625; WO 01/25485; and WO 01/29259. The microarray typically comprises a solid support and the oligonucleotides may be linked to this solid support by covalent bonds or by non-covalent interactions. The oligonucleotides may also be linked to the solid support directly or by a spacer molecule. A microarray may comprise one or more oligonucleotides complementary to a polymorphic site within a nucleotide sequence.

* * *

The entirety of each patent, patent application, publication and document referenced herein hereby is incorporated by reference. Citation of the above patents, patent applications, publications and documents is not an admission that any of the foregoing is pertinent prior art, nor does it constitute any admission as to the contents or date of these publications or documents.

Modifications may be made to the foregoing without departing from the basic aspects of the invention. Although the invention has been described in substantial detail with reference to one or more specific embodiments, those of ordinary skill in the art will recognize that changes may be made to the embodiments specifically disclosed in this application, yet these modifications and improvements are within the scope and spirit of the invention.

The invention illustratively described herein suitably may be practiced in the absence of any element(s) not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising," "consisting essentially of," and "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and use of such terms and expressions do not exclude any

equivalents of the features shown and described or portions thereof, and various modifications are possible within the scope of the invention claimed. The term “a” or “an” can refer to one of or a plurality of the elements it modifies (e.g., “a device” can mean one or more devices) unless it is contextually clear either one of the elements or more than one of the elements is described. The term “about” as used herein refers to a value sometimes within 10% of the underlying parameter (i.e., plus or minus 10%), a value sometimes within 5% of the underlying parameter (i.e., plus or minus 5%), a value sometimes within 2.5% of the underlying parameter (i.e., plus or minus 2.5%), or a value sometimes within 1% of the underlying parameter (i.e., plus or minus 1%), and sometimes refers to the parameter with no variation. For example, a weight of “about 100 grams” can include weights between 90 grams and 110 grams. Thus, it should be understood that although the present invention has been specifically disclosed by representative embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and such modifications and variations are considered within the scope of this invention.

Embodiments of the invention are set forth in the claim that follows.

What is claimed is:

1. A method for detecting a chromosomal abnormality, comprising the steps of:

a) performing one or more free, fetal nucleic acid-based tests to detect a chromosomal abnormality, wherein the free, fetal nucleic acid-based test is selected from the group consisting of an allele-based test, an RNA-based test, a methylation-based test and a parolog-based test; and

b) performing one or more non-free, fetal nucleic acid-based chromosomal abnormality tests to detect a chromosomal abnormality, wherein the one or more other chromosomal abnormality tests are selected from the group consisting of, a maternal age-based test, a maternal biomarker screening test, and an ultrasonography-based test.