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(54) Title: PRENATAL URINARY SCREENING FOR DOWN SYNDROME AND OTHER ANEUPLOIDIES

(57) Abstract

Herein disclosed are methods for prenatally assessing the risk of a pregnancy being affected by Down syndrome or other fetal aneuploidies by testing first trimester maternal urine samples for levels of urinary gonadotropin peptide (UGP) elevated above normal. Risk assessments can be made based upon UGP levels alone or in conjunction with levels of other urinary and/or serum markers, ultrasound parameters and other factors, such as, maternal age. Among other benefits, first trimester prenatal screening provides the opportunity to terminate a pregnancy at an early gestational age, in the case of an unfavorable outcome.

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PRENATAL URINARY SCREENING FOR DOWN SYNDROME AND OTHER ANEUPLOIDIES

FIELD OF THE INVENTION

The present invention is in the field of prenatal diagnosis. It concerns non-invasive methods to screen prenatally for fetal Down syndrome and other aneuploidies by determining the levels of urinary gonadotropin peptide (UGP) [also known as β-core, β-core fragment, β-core-hCG, or urinary gonadotropin fragment (UGF)] in maternal urine samples, alone or in conjunction with other markers.

BACKGROUND OF THE INVENTION

Trisomy 21, commonly known as Down syndrome, is characterized by an extra copy of chromosome 21 and is one of the most common serious congenital abnormalities. People afflicted with Down syndrome have severe mental retardation, reduced life expectancies, and abnormal immune responses that predispose them to serious infections as well as thyroid autoimmunity. Further, 40% of Down syndrome patients have congenital heart disease and a 10 to 20-fold increased risk of developing leukemia over the general population. All Down syndrome patients older than 40 develop neuropathological changes characteristic of Alzheimer's disease.

Prenatal tests to detect fetal aneuploidies, such as Down syndrome (trisomy 21), Turner syndrome (monosomy X), Klinefelter syndrome (47, XXY), triple-X (47, XXX) among other aneuploidies, by amniocentesis or chorionic villus sampling (CVS) have been available since the late 1960s. Amniocentesis is the most common invasive prenatal diagnostic procedure. In amniocentesis, amniotic fluid is sampled by inserting a hollow needle through the mother's anterior abdominal and uterine walls into the amniotic cavity by piercing the chorion and amnion. It is usually performed in the second trimester of pregnancy. CVS is performed primarily during the first trimester, and involves collecting cells from the chorion which develops into the placenta.
Another invasive prenatal diagnostic technique is cordocentesis or percutaneous umbilical cord blood sampling, commonly known as fetal blood sampling. Fetal blood sampling involves obtaining fetal blood cells from vessels of the umbilical cord, and is often performed about the 20th gestational week.

Amniocentesis is used selectively because it presents a risk of about 1% of inducing spontaneous abortion. CVS and fetal blood sampling carry a similar or higher risk of inducing abortion, and there is also concern that those procedures may lead to fetal limb malformations in some cases. Thus, amniocentesis, CVS and fetal blood sampling are procedures that are only employed if a pregnancy is considered at high risk for a serious congenital anomaly. Thus, some means is required to select those pregnancies that are at a significant risk of Down syndrome or other fetal aneuploidies to justify the risks of such invasive prenatal diagnostic procedures, as amniocentesis, CVS and fetal blood sampling.

Prior to 1983, the principal method for selecting pregnancies that had an increased risk for genetic defects, such as Down syndrome, was based on maternal age, that is, the older the age of the mother, the higher the risk that the pregnancy would be affected by aneuploidy. In 1974, biochemical screening for neural tube defects by measuring alpha-fetoprotein (AFP) began. In 1984, the use of the AFP screen was additionally adopted for the detection of Down syndrome. Since the early 1990s, a multiple marker blood test has been used to screen for this disorder. A common version of this test is the three marker triple test. The triple screen measures AFP, human chorionic gonadotropin (hHCG) and unconjugated estriol (uE3) in the serum of pregnant women.

The triple screen provides a means to screen the population of pregnant women to determine which pregnancies are at risk for Down syndrome and other serious genetic defects. The risk is calculated based on the results of the screen, along with other cofactors, such as, maternal age, to determine if the risk is high enough to warrant an invasive diagnostic procedure, such as, amniocentesis, CVS or fetal
blood sampling. Such prenatal screens, as the triple screen, can be used either to reduce the need for amniocentesis or to increase Down syndrome detection for the same amount of amniocentesis. "The efficiency of the Triple test is projected to be one case of fetal Down syndrome detected for every 50 amniocenteses performed." [Canick and Knight, "Multiple-marker Screening for Fetal Down Syndrome," Contemporary OB/GYN, pp. 3-12 (April 1992).]

Although pregnant women who are 35 years or older are the standard high risk group for fetal Down syndrome, screening also needs to be applied to young women because although they are at lower risk, most affected pregnancies are in young women. Approximately 80% of babies born with Down syndrome are born to mothers under 35. ["Down Syndrome Screening Suggested for Pregnant Women under 35," ACOG Newsletter, 38(8): 141 (Aug. 1994).]

The triple screen combines the analysis of three markers from serum to reduce false positive results (which result in the performance of unnecessary invasive procedures) and false negatives (in which serious genetic defects, such as, trisomy 21, go undetected). In women under 35, the double screen (AFP and hCG) can pick up about half of Down syndrome cases and a large proportion of other chromosome defects during the second trimester. The triple screen (AFP, hCG and \( uE_3 \)) increases the detection rate by 5-10% of Down syndrome and a further increase in the detection of all other serious chromosome defects, thus decreasing the number of false-positives. Such rates mean that the double and triple screens still fail to detect a significant number of Down syndrome affected pregnancies.

Although the triple screen has a suggested screening period of 15 to 20 weeks gestation, such screening has been recommended between weeks 16-18 to maximize the window for spinal bifida detection. [Canick and Knight, supra (April 1992).] A 1992 survey of prenatal maternal serum screening for AFP alone or for multiple analyses reported that very few such screenings occurred in the thirteenth or earlier week of gestation. [Palomaki et al., "Maternal Serum Screening for
Fetal Down Syndrome in the United States: A 1992 Survey," *Am. J. Obstet. Gynecol.*, 169(6): 1558-1562 (1992).] The triple screen thus suffers from the additional problem that once a risk of Down syndrome is predicted, and amniocentesis or another invasive prenatal definitive diagnostic procedure is performed to diagnose Down syndrome, it is at an advanced date of gestation, when termination of a pregnancy can be more physically and emotionally trying for the mother, and when certain less traumatic abortion procedures, such as, vacuum curettage, may not be available.

The limitations of the triple screen and the adverse consequences of unnecessary, potentially harmful and expensive invasive prenatal diagnostic procedures, such as, amniocentesis, have led to a search for more discriminatory markers for prenatal Down syndrome screening. Of the maternal serum markers in routine use, human chorionic gonadotropin (hCG) is the most discriminatory. [Cuckle et al., *Prenatal Diagnosis*, 14: 953-958 (1994).]

Human chorionic gonadotropin (hCG) is a glycopeptide hormone produced by the syncytiotrophoblasts of the fetal placenta, and has a molecular weight of about 38 kilodaltons (kd). It can be detected by immunoassay in the maternal urine within days after fertilization and thus provides the basis of the most commonly used pregnancy tests. The intact hCG molecule is a dimer comprising a specific β subunit non-covalently bound to an α subunit, which is common to other glycoproteins.


The terminal degradation product of the β-subunit of hCG is called urinary gonadotropin peptide (UGP), or

Urinary gonadotropin peptide (UGP) has an amino acid sequence related to the β-subunit of hCG. UGP is comprised of β-subunit residues 6 through 40 attached by disulfide linkages to residues 55 through 92. UGP is glycosylated but lacks the sialic acid and O-linked carbohydrate residues present on hCG β subunit.


Cuckle et al., Prenatal Diagnosis, 14: 953-958 (1994) showed that UGP levels are elevated on average in the second trimester of singleton pregnancies affected by fetal Down syndrome and reduced in the first trimester of singleton pregnancies affected with other serious, but less common aneuploidies such as, Edwards syndrome and triploidy, and of a twin pregnancy discordant for Down syndrome. The observed median level in Down syndrome (6.11 MOM: 95% confidence
interval 3.7 to 10.0) was greater than the corresponding median level for intact hCG in maternal serum (2.0 MOM; 1.9-2.1) and free β-hCG (2.3 MOM; 2.1-2.5).

There are important advantages to using urinalysis for prenatal screening for Down syndrome. Urine tests are less expensive than serum testing, avoid the safety issues and handling risks associated with the collection and storage of blood samples, as well as the invasiveness and discomfort of phlebotomy. Urine samples can be easily collected and shipped, if necessary, where women have limited access to medical testing facilities because of geography or socioeconomic status. UGP is stable to changes in temperature, pH, and storage time at -20 and 40°C.

However, the UGP assay results described in Cuckle et al., supra showed elevated UGP levels in maternal urine samples taken between the 19th week and the 22nd week plus 4 days of gestation from Down syndrome affected pregnancies. As indicated above, there are disadvantages to second trimester testing, in that delays in confirming a fetal Down syndrome diagnosis result in more traumatic abortion procedures being necessitated. Also, the emotional attachment and expectations of the pregnant woman and her family for a healthy baby, grow during the pregnancy, making the abortion decision more difficult later in the gestational term.

The instant invention provides the benefits of urinalysis and avoids the problems of second trimester prenatal screening by providing methods to screen first trimester urine samples for fetal Down syndrome and other aneuploidies. According to the methods of this invention, a UGP level in a first trimester maternal urine sample when elevated above normal levels indicates a significant risk of the pregnancy under analysis being affected by Down syndrome or another fetal aneuploidy, such as, Turner syndrome, Klinefelter syndrome or triple-X. Preferred prenatal screening methods of the instant invention are highly specific for UGP and minimally cross-reactive with intact hCG, with β-subunit hCG and with α-subunit hCG.
SUMMARY OF THE INVENTION

The instant invention provides methods for prenatally determining whether there is a significant risk of a pregnancy being affected by Down syndrome or other fetal aneuploidies by testing first trimester maternal urine samples for elevations of urinary gonadotropin peptide (UGP) levels above normal. Antibody and non-antibody methods are disclosed to detect and quantitate UGP levels in maternal urine samples. One representative embodiment of the invention employs immunoassays that are specific for UGP and have molar cross-reactivities of less than about 10% with intact hCG, with β-subunit hCG, and with α-subunit hCG. More preferably such immunoassay methods of this invention have a molar cross-reactivity of less than about 5%, more preferably less than about 3%, and still more preferably less than about 1%, with intact hCG, with β-subunit hCG, and with α-subunit hCG.

The UGP level in a first trimester maternal urine sample is related to the median for unaffected pregnancies at approximately the same gestational age, wherein a UGP level above that median indicates a risk of Down syndrome or another fetal aneuploidy such as, Turner syndrome, Klinefelter syndrome or triple-X, affecting the pregnancy under analysis, the degree of elevation above that median being considered. Results from the prenatal screening methods of this invention are generally expressed as multiples of the gestation-specific median value (MOM) for unaffected pregnancies of the same gestation. Exemplary positive results from the screening methods according to this invention are those wherein the UGP level is from about 1.1 MOM to higher multiples, from about 1.5 MOM to higher multiples, from about 2 MOM and higher multiples.

The UGP screening result from the methods of this invention is used to assess the fetal Down syndrome or other fetal aneuploidies either alone or in conjunction with results from other screening tests with other serum and/or urinary markers, and/or other factors, such as, maternal age, maternal health, maternal weight and ultrasound parameters among other factors. For example, maternal age and UGP levels are
independent predictors of Down syndrome risk, as is true for each of the commonly used serum markers. Therefore, after performing the prenatal screening methods of this invention, the risk of a Down syndrome affected pregnancy can be calculated by multiplying the age-related risk by a likelihood ratio derived from the UGP level found in the maternal urine sample in relation to control samples.

Other urinary markers which could be preferred for assessing the risk of a Down syndrome affected pregnancy in conjunction with UGP levels, include pregnancy-associated plasma protein A (PAPP-A), dimeric inhibin, total estrogen (tE), unconjugated estriol (uE3), total estriol (tE3), AFP and proform of eosinophilic major basic protein (proMBP), among other urinary marker possibilities.

In general, a positive result from the screening methods of this invention is an indicator that a more invasive prenatal diagnostic procedure, such as, amniocentesis, CVS or fetal blood sampling, should be performed to determine definitively whether the pregnancy is affected with Down syndrome or another fetal aneuploidy.

Gestation-specific medians for UGP can be calculated by weighted non-linear regression from the values for control urine samples. To account for variations in the concentrations of urine samples, UGP levels can be expressed in terms of creatinine. Gestational ages of cases and controls can be determined by ultrasound parameters and by last menstrual period dating.

The control samples are preferably taken from a population of pregnant women that are matched as well as practicable to the population from which the pregnant woman who provided the test sample comes. For example, population parameters could include race, ethnicity, and geographical location, among other parameters.

The immunoassay methods of this invention employ UGP standards. Blithe et al., U.S. Patent No. 5,445,968 (issued August 29, 1995) discloses methods of purifying UGP.

The prenatal screening methods of this invention using immunoassays can be in any standard immunoassay format,
for example, a competitive radioimmunoassay, a sandwich EIA or sandwich radioimmunoassay (RIA), among other known formats. A sandwich assay is a preferred format of this invention, and a sandwich EIA is a further preferred embodiment.

The prenatal screening methods of this invention can be automated. A preferred automated immunoassay system is Ciba Corning Diagnostic Corp.'s (CCD's) ACS-180™ Automated Chemiluminescence System [CCD; Medfield, MA (USA)].

The prenatal screening methods of this invention employ antibodies, that are defined herein to include whole antibodies or biologically active fragments of antibodies. The antibodies used in the immunoassay methods can be monoclonal and/or polyclonal, preferably monoclonal and/or affinity-purified polyclonal antibodies. The specificity of the immunoassay methods is provided by antibodies which specifically bind to UGP; generally such antibodies are monoclonal antibodies.

Tracer antibodies that can be used in the immunoassay methods of this invention can be directly or indirectly linked to a detectable marker. The signal from said marker can indicate the level of UGP in the sample tested. The signal's intensity may be directly proportional to the level of UGP in the sample.

Exemplary detectable markers can be selected from the group consisting of radionuclides, fluorescers, bioluminescers, chemiluminescers, dyes, enzymes, coenzymes, enzyme substrates, enzyme cofactors, enzyme inhibitors, enzyme subunits, metal ions, and free radicals.

Antibodies used in the immunoassay methods may be linked to a solid phase, for example, the wall of a container or the surface of magnetic or paramagnetic particles, among other solid phases.

DETAILED DESCRIPTION

The following abbreviations are used herein:

Abbreviations
AFP = alpha-fetoprotein
Definitions

Alternative terms used in the art for urinary gonadotropin peptide (UGP) are β-core fragment, β-core-hCG, and urinary gonadotropin fragment (UGF).
The first trimester is herein defined as 14 completed weeks (14 weeks, 6 days) from the onset of a pregnant woman's last normal menstrual period (LNMP). Intact hCG is a term that defines hCG in its dimeric form when its α and β subunits are non-covalently bound together.

Total hCG is a term that includes intact hCG and either its free α subunit or its free β subunit. "Aneuploidy" is defined herein to refer to any deviation from the human diploid number of 46 chromosomes. The term "antibodies" is defined herein to include not only whole antibodies but also biologically active fragments of antibodies, preferably fragments containing the antigen binding regions.

**Representative Embodiments**

Herein are disclosed methods for prenatally assessing risks of a pregnancy being affected by Down syndrome or other fetal aneuploidies by testing maternal urine samples for elevations above normal of urinary gonadotropin peptide (UGP). The methods employ antibody and non-antibody procedures for detecting and quantitating UGP in such samples. Elevated UGP levels above normal in a maternal urine sample indicate a significant risk of Down syndrome or other aneuploidies, such as, Turner syndrome, triple-X or Klinefelter syndrome among other aneuploidies.

The incidence of Turner syndrome (monosomy X) in new-born females is about one in 5,000 live female births [Thompson et al., Thompson and Thompson Genetics in Medicine, (5th ed.; WB Saunders; Philadelphia, PA, USA; 1991)]. The incidence of triple-X (47, XXX) is one in 960 live births, and the incidence of Klinefelter syndrome (47, XXY) is one in 1080 live births [Hook and Hamerton, "The frequency of chromosome abnormalities detected in consecutive newborn studies ..." in: Population Cytogenetics: Studies in Humans [Hook and Porter (eds.); Academic Press; NY, USA (1977)].

One preferred embodiment of the prenatal screening methods of this invention is expressed as follows.
A method for prenatally determining whether there is a significant risk of a pregnancy being affected by fetal aneuploidy during the pregnancy's first trimester comprising:
(a) taking a maternal urine sample during the first trimester of said pregnancy;
(b) testing said maternal urine sample to determine the level of UGP in said sample;
(c) determining whether the level of UGP in said sample is elevated above a level of UGP that is normal in urine samples from women whose pregnancies are unaffected by aneuploidy, and whose pregnancies are at about the same gestational age as the pregnancy under analysis; and
(d) determining whether there is a significant risk of fetal aneuploidy based upon the level of UGP in said sample, wherein a level elevated above said normal level indicates that there is a significant risk of the pregnancy being affected by fetal aneuploidy.

Many methods can be used to detect and quantitate UGP in maternal urine samples. Antibody and non-antibody methods can be used. A variety of immunoassay formats can be used as set forth below. Non-antibody methods include, for example, chromatographic procedures that separate UGP from other components of urine, such as, high pressure liquid chromatography (HPLC) and mass spectrometry, alone or in combination; fluorometric detection means; nuclear magnetic resonance (NMR); and the use of non-antibody receptors and other binding proteins that may exist celluilarly or in serum.

Preferably an immunoassay is used to detect and quantitate UGP in maternal urine samples in the methods of this invention. Such immunoassays can be in any standard immunoassay format, such as, sandwich assays, competition assays, bridge immunoassays, among other formats well known to those of skill in the art. [See, for example, U.S. Patent Nos. 5,296,347; 4,233,402; 4,034,074; and 4,098,876.] For example, a competitive radioimmunoassay (RIA), a sandwich EIA or sandwich RIA may be preferred immunoassay formats. A sandwich assay is a preferred format of this invention, and a sandwich RIA or EIA is a further preferred format.
A particularly preferred immunoassay to detect and quantitate UGP is the radioimmunoassay described herein in Example 4 [a modification of the method described in Lee et al., J. Endocrinol., 130: 481-489 (1991)]. A further particularly preferred immunoassay for use in the methods of this invention is the Triton® UGP EIA Kit from Ciba Corning Diagnostics Corp. (Alameda, CA; USA), which was used in Examples 1-3, infra.

In one representative embodiment of the instant invention, an immunoassay that is highly specific for UGP is used, which has a molar cross-reactivity of less than about 10% with each of the following: intact hCG, β-subunit hCG, and with α-subunit hCG. Preferably, such an immunoassay specific for UGP preferably has molar cross-reactivities of less than 5% with intact hCG, with β-subunit hCG, and with α-subunit hCG; more preferably those cross-reactivities are less than 3%; and still more preferably those cross-reactivities are less than 1%.

The Triton® UGP EIA Kit from Ciba Corning Diagnostics Corp. (CCD; Alameda, CA; USA) is such a preferred immunoassay that is highly specific for UGP. That commercially available immunoassay exhibits the following molar cross-reactivities: hCG, 0.11 per cent; free beta subunit of hCG, 0.043 per cent, and free alpha subunit of hCG, 0.009 per cent. Other representative immunoassays highly specific for UGP can comprise, for example, antibodies described in Blithe et al., U.S. Patent No. 5,445,868 (issued August 29, 1995).

In a preferred embodiment of the invention, monoclonal antibodies specific to UGP are coupled to paramagnetic particles (PMP) and incubated with the urine sample to be tested. The immobilized complex is washed to remove unbound materials. Anti-UGP antibodies conjugated with a chemiluminscent tag, preferably acridinium ester (AE), are then incubated with the immunocomplex immobilized on the PMP. A step to separate bound tracer from the unbound tracer is then performed. The bound fraction is then incubated with the light reagent and the amount of light photons generated is determined in a luminometer, such as, for example, the Ciba
Corning ACS-180™ Automated Chemiluminescent System [Ciba
Corning Diagnostics Corp. (CCD) E. Walpole, MA (USA)], which
is further described infra. The values obtained are directly
proportional to the concentration of UGP in the urine sample.

The embodiments outlined above are representative of
a wide number of assay methods that can be used in accordance
with this invention. There are many variations and
modifications of such outlined embodiments within the skill of
one in the art. Variations of the representative embodiments
of the methods of this invention within conventional knowledge
of those of skill in the art are considered to be within the
scope of the instant invention. Preferred variations and more
detailed embodiments are identified in the following sections.

Reference is made hereby to standard textbooks of
immunology that contain methods for carrying out various
immunoassay formats that can be adapted from those
specifically represented herein. See, for example, Molecular
Biology and Biotechnology: A Comprehensive Desk Reference
(Ed. R. A. Meyers) [VCH Publishers, Inc., New York, N.Y.
(1995)]; Moore and Persaud, The Developing Human: Clinically
Oriented Embryology, 5th Edition [W. B. Saunders Company;
Philadelphia/London/Toronto/Montreal/Sydney/Tokyo (1993)];
Darnell et al., Molecular Cell Biology, W. H. Freeman and
Company (N.Y. 1990); Colowick et al., Methods in Enzymology,
Volume 152 [Academic Press, Inc. (London) Ltd. (1987)]; and
Goding J. W., Monoclonal Antibodies: Principles and Practice:
Production and Application of Monoclonal Antibodies in Cell
Biology, Biochemistry, and Immunology [Academic Press Inc.
(London) Ltd.; 1983].

Timing of Sample Taking

Generally, maternal urine samples can be taken for
testing, according to this invention, during the fifth week to
the 14th completed week, that is, 14 weeks plus six days, of
gestation. Preferably, maternal urine samples can be taken
for testing during the sixth week to the 14th completed week
of gestation; more preferably during the seventh week to the
14th completed week of gestation; still more preferably during
the eighth week to the 14th completed week of gestation; further preferably, during the ninth week to the 14th completed week of gestation; alternatively, during the 10th week to the 14th completed week of gestation; further alternatively during the ninth week to the 13th week of gestation; and still further also preferably from the ninth week to the twelfth completed week of gestation.

Other preferred gestational periods from the first trimester, during which maternal urine samples can be taken for testing according to this invention include the following: the gestational periods from the beginning of the seventh week to the end of the 14th week; from the beginning of the eighth week to the end of the 14th week; from the beginning of the ninth week to the end of the 14th week; from the beginning of the tenth week to the end of the 14th week; from the beginning of the fifth week to the end of the 13th week; from the beginning of the fifth week to the end of the 12th week; from the beginning of the sixth week to the end of the 13th week; from the beginning of the sixth week to the end of the 12th week; from the beginning of the seventh week to the end of the 13th week; from the beginning of the seventh week to the end of the 12th week; from the beginning of the eighth week to the end of the 13th week; from the beginning of the eighth week to the end of the 12th week; from the beginning of the eighth week to the end of the 11th week; from the beginning of the eighth week to the end of the 10th week; from the beginning of the ninth week to the end of the 11th week; from the beginning of the ninth week to the end of the 10th week; from the beginning of the tenth week to the end of the 13th week; and from the beginning of the tenth week to the end of the twelfth week.

Urine Concentration

The prenatal screening methods of this invention do not require uniformity in the volume of urine obtained from each pregnant woman of the control or case groups or the time of voiding. The UGP concentration can be corrected for
creatinine, for example, expressed in mmol/mmol or nmol/mmol creatinine.

The CCD UGP kit expresses UGP levels as pmol per mg urinary creatinine. UGP concentrations may be reported in International Units as mmol UGP/L and expressed as nmol/mmol creatinine as exemplified in Example 2, infra.

Creatinine content in urine can be measured by the Jaffe method using a Monarch 200 centrifugal analyser as described in Cuckle et al., *Prenatal Diagnosis*, 14: 953-958 (1994). Creatinine levels can also be measured on a Synchron CX-5 chemistry analyser (Beckman Instruments; Brea, CA, USA).

**Other Markers**

"Population screening is the identification, among apparently healthy individuals, of those who are sufficiently at risk of a specific disorder to justify a subsequent diagnostic test or procedure. This implies the testing of all pregnancies in order to identify those few at a great enough risk to warrant an invasive diagnostic procedure, such as amniocentesis." [Canick et al., "Maternal Serum Screening for Aneuploidy and Open Fetal Defects," *Prenatal Diagnosis*, 20(3): 443 (Sept. 1993).] A positive result from a screen indicates a higher risk of fetal Down syndrome, and a negative result does not necessarily mean that a fetus is free of Down syndrome but that the fetus is at a lower risk of the syndrome.

Screening detection rates can be increased and false positives can be decreased by combining the results of a prenatal screening method with results of screening with other markers and assessing the results in conjunction with other factors. When considering the cost of a second or third marker, it has to be weighed against the extra costs incurred without their use in additional amniocentesis tests for the affected pregnancies. [Cuckle, H. S., *Clin. Chem.*, 38(9): 1687-1689 (1992).]

As indicated above, the results from the prenatal screening methods of this invention can be used alone or in conjunction with results from other screening tests with other
serum and/or urinary markers, and/or other factors, such as, ultrasound parameters, maternal age, maternal health, maternal weight, among other factors to assess the risks of a pregnancy being affected by Down syndrome or other fetal aneuploidies. For example, maternal age and UGP level are independent predictors of Down syndrome risk, as is true for each of the commonly used serum markers.

Other markers which could be preferred urinary markers for assessing the risks of a Down syndrome affected pregnancy include PAPP-A, dimeric inhibin, tE, tE₃, uE₃, AFP, 16α-OH-DHAS and proMBP, among other urinary marker possibilities. Particularly preferred potential urinary markers to be used in conjunction with UGP to assess fetal Down syndrome risks are PAPP-A, dimeric inhibin and tE.

Example 4 illustrates testing urine samples for UGP, tE and α-hCG. As illustrated in Example 4, the levels of tE and α-hCG in maternal urine samples when reduced below respective normal levels for those markers indicate a significant risk of fetal aneuploidy, such as, Down syndrome or Turner syndrome.

PAPP-A may be found in first trimester urine samples, preferably taken during the ninth to the 13th or 14th completed week of gestation, at levels significantly reduced below normal in Down syndrome affected pregnancies.


Total estrogen can be measured by a continuous flow system based on the Kober reaction [Lever et al., "Improved estriol determination in a continuous flow system," Biochem. Med., 8: 188-198 (1973)]. Total estrogen could be measured as μmol/mmol creatinine.

By the second trimester, maternal estrogen is largely derived from fetal hepatic and placental metabolism of precursors secreted by the fetal adrenal glands [Oakey, R.E., Vitamins and Hormones, 28: 1 (1970)]. Thus, measurement of
estrogen production reflects fetal and placental function. The unconjugated estrogens formed by the placenta are rapidly converted to conjugated forms by addition of glucuronic and sulphuric acid residues, before excretion. The inventors measured those urinary conjugated estrogens, mostly estriol conjugates. By contrast, in maternal serum screening for Down syndrome, unconjugated estriol is measured rather than the more abundant conjugated forms.

The method used in Example 4 herein assesses excretion of a combination of 16α-hydroxyestrogens (e.g. estriol) and deoxysterogens (e.g. estrone, estradiol). A different method would be to target one particular estrogen (e.g., estriol-16α-glucosiduronate).

Improved discrimination may be achieved by measuring precursors directly in the urine. A preferred precursor is 16α-hydroxydehydroepiandrosterone sulphate (16α-OH-DHAS). Through a placental process known as aromatization, this compound is the precursor of more than 85% of estrogens in late pregnancy urine. 16α-OH-DHAS has a relatively low affinity for the series of placental enzymes which catalyze its conversion to estriol [Purohit and Oakey, J. Ster. Biochem., 33: 439-448 (1989)]. Any decline in placental function in Down syndrome might well exaggerate the quantity which escapes aromatization. In the extreme condition of steroid sulphatase deficiency, for example, sufficient 16α-OH-DHAS avoids aromatization and is excreted in maternal urine to provide a secure diagnostic test for the condition [Wilmot et al., Ann. Clin. Biochem., 25: 155-161 (1988)].

Also, physical measurements, such as ultrasound markers, can be used in combination with UGP levels to assess prenatally the risk of fetal aneuploidy. Exemplary ultrasound markers include nuchal translucency [Nicolaides et al., Br. Med. J., 304: 867 (1992)], femur length, humerus, cephalic index, ventricular size, and ultrasound detected defects in the heart, gut, and organs, among other physical markers.
Antibodies

As indicated above, the term "antibodies" is defined to include not only whole antibodies but also biologically active fragments of antibodies, preferably fragments containing the antigen binding regions. Such antibodies may be prepared by conventional methodology and/or by genetic engineering.

Antibody fragments may be genetically engineered, preferably from the variable regions of the light and/or heavy chains (VH and VL), including the hypervariable regions, and still more preferably from both the VH and VL regions. For example, the term "antibodies" as used herein comprehends polyclonal and monoclonal antibodies and biologically active fragments thereof including among other possibilities "univalent" antibodies [Glennie et al., Nature, 295: 712 (1982)]; Fab proteins including Fab' and F(ab')2 fragments whether covalently or non-covalently aggregated; light or heavy chains alone, preferably variable heavy and light chain regions (VH and VL regions), and more preferably including the hypervariable regions [otherwise known as the complementarity determining regions (CDRs) of said VH and VL regions]; Fc proteins; "hybrid" antibodies capable of binding more than one antigen; constant-variable region chimeras; "composite" immunoglobulins with heavy and light chains of different origins; "altered" antibodies with improved specificity and other characteristics as prepared by standard recombinant techniques and also by oligonucleotide-directed mutagenesis techniques [Dalbadie- McFarland et al., PNAS (USA), 79: 6409 (1982)].

It may be preferred for many immunoassays of this invention that biologically active fragments rather than whole antibodies be used. Fab fragments are particularly preferred fragments in accordance with this invention to avoid non-specific binding.

Antibodies for use in the instant invention can be genetically engineered. [See, for example, Morrison et al., Clin. Chem., 34: 1668 (1988); Morrison and Oi, Adv. Immunol., 44: 65 (1989); Rodwell, Nature, 342: 99 (1989); Pluckthun,

Standards

Purified reference preparations can be obtained by established procedures. [See, for example, Blithe et al., "Purification of β-core fragment from pregnancy urine and demonstration that its carbohydrate moieties differ from those of native human chorionic gonadotropin-β," Endocrinol., 122: 173-180 (1988).]

Automated Immunoassay System

The methods of this invention can be readily adapted to automated immunochemistry analyzers. To facilitate automation of the methods of this invention and to reduce the turnaround time, anti-UGP antibodies may be coupled to magnetizable particles.

A preferred automated/immunoassay system is the Ciba Corning ACS:180™ Automated Chemiluminescence System [CCD; Medfield, MA (USA)]. The Ciba Corning ACS:180™ Automated Immunoassay System is described in Dudley, B.S., J. Clin. Immunoassay, 14(2): 77 (Summer 1991). The system uses chemiluminescent labels as tracers and paramagnetic particles as solid-phase reagents. The ACS:180 system accommodates both competitive binding and sandwich-type assays, wherein each of the steps are automated. The ACS:180 uses micron-sized paramagnetic particles that maximize the available surface
area, and provide a means of rapid magnetic separation of bound tracer from unbound tracer without centrifugation. Reagents can be added simultaneously or sequentially. Other tags, such as an enzymatic tag, can be used in place of a chemiluminescent label, such as, acridinium ester.

Solid Phase

The solid phase used in the assays of this invention may be any surface commonly used in immunoassays. For example, the solid phase may be particulate; it may be the surface of beads, for example, glass or polystyrene beads; or it may be the solid wall surface of any of a variety of containers, for example, centrifuge tubes, columns, microtiter plate wells, filters, membranes and tubing, among other containers.

When particles are used as the solid phase, they will preferably be of a size in the range of from about 0.4 to 200 microns, more usually from about 0.8 to 4.0 microns. Magnetic or magnetizable particles such as, paramagnetic particles (PMP), are a preferred particulate solid phase, and microtiter plate wells are a preferred solid wall surface. Magnetic or magnetizable particles may be particularly preferred when the steps of the methods of this invention are performed in an automated immunoassay system.

Preferred detection/quantitation systems of this invention may be luminescent, and a luminescent detection/quantitation system in conjunction with a signal amplification system could be used, if necessary. Exemplary luminescent labels, preferably chemiluminescent labels, are detailed below, as are signal amplification systems.

Signal Detection/Quantitation Systems

The complexes formed by the assays of this invention can be detected, or detected and quantitated by any known detection/quantitation systems used in immunoassays. As appropriate, the antibodies of this invention used as tracers may be labeled in any manner directly or indirectly, that
results in a signal that is visible or can be rendered visible.

Detectable marker substances include radionuclides, such as $^3$H, $^{125}$I, and $^{131}$I; fluorescers, such as, fluorescein isothiocyanate and other fluorochromes, phycoobiliproteins, phycoerythin, rare earth chelates, Texas red, dansyl and rhodamine; colorimetric reagents (chromogens); electron-opaque materials, such as colloidal gold; bioluminescers; chemiluminescers; dyes; enzymes, such as, horseradish peroxidase, alkaline phosphatase, glucose oxidase, glucose-6-phosphate dehydrogenase, acetylcholinesterase, α-, β-galactosidase, among others; coenzymes; enzyme substrates; enzyme cofactors; enzyme inhibitors; enzyme subunits; metal ions; free radicals; or any other immunologically active or inert substance which provides a means of detecting or measuring the presence or amount of immunocomplex formed. Exemplary of enzyme substrate combinations are horseradish peroxidase and tetramethyl benzidine (TMB), and alkaline phosphatase and paranitrophenyl phosphate (pNPP).

Preferred detection, or detection and quantitation systems according to this invention produce luminescent signals, bioluminescent (BL) or chemiluminescent (CL). In chemiluminescent (CL) or bioluminescent (BL) assays, the intensity or the total light emission is measured and related to the concentration of the analyte. Light can be measured quantitatively using a luminometer (photomultiplier tube as the detector) or charge-coupled device, or qualitatively by means of photographic or X-ray film. The main advantages of using such assays is their simplicity and analytical sensitivity, enabling the detection and/or quantitation of very small amounts of analyte.

Exemplary luminescent labels are acridinium esters, acridinium sulfonyl carboxamides, luminol, umbelliferone, isoluminol derivatives, photoproteins, such as aequorin, and luciferases from fireflies, marine bacteria, Vargulla and Renilla. Luminol can be used optionally with an enhancer molecule, preferably selected from the group consisting of 4-iodophenol or 4-hydroxy- cinnamic acid. Acridinium esters
are one of the preferred types of CL labels according to this invention. A signal is generated by treatment with an oxidant under basic conditions.

Also preferred luminescent detection systems are those wherein the signal is produced by an enzymatic reaction upon a substrate. CL and BL detection schemes have been developed for assaying alkaline phosphatase (AP), glucose oxidase, glucose 6-phosphate dehydrogenase, horseradish peroxidase (HRP), and xanthine-oxidase labels, among others. AP and HRP are two preferred enzyme labels which can be quantitated by a range of CL and BL reactions. For example, AP can be used with a substrate, such as an adamantyl 1,2-dioxetane aryl phosphate substrate (e.g. AMPPD or CSPD; [Kricka, L.J., "Chemiluminescence and Bioluminescence, Analysis by," at p. 167, Molecular Biology and Biotechnology: A Comprehensive Desk Reference (ed. R.A. Meyers) (VCH Publishers; N.Y., N.Y.; 1995)]; preferably a disodium salt of 4-methoxy-4-(3-phosphatephenyl)spiro[1,2-dioxetane-3,2'-adamantane], with or without an enhancer molecule, preferably, 1-(triocytlyphosphonium methyl)-4-(tributylphosphonium methyl) benzene dichloride. HRP is preferably used with substrates, such as, 2',3',6'-trifluorophenyl 3-methoxy-10-methylacridan-9-carboxylate.

CL and BL reactions can also be adapted for analysis of not only enzymes, but other substrates, cofactors, inhibitors, metal ions and the like. For example, luminol, firefly luciferase, and marine bacterial luciferase reactions are indicator reactions for the production or consumption of peroxide, ATP, and NADPH, respectively. They can be coupled to other reactions involving oxidases, kinases, and dehydrogenases, and can be used to measure any component of the coupled reaction (enzyme, substrate, cofactor).

The detectable marker may be directly or indirectly linked to an antibody used in an assay of this invention.

Exemplary of an indirect linkage of the detectable label is the use of a binding pair between the antibody and the marker, or the use of well known signal amplification signals, such
as, using a biotinylated antibody complexed to UGP and then adding strepavidin conjugated to HRP and then TMB.

Exemplary of binding pairs that can be used to link antibodies of assays of this invention to detectable markers are biotin/avidin, streptavidin, or anti-biotin; avidin/anti-avidin; thyroxine/thyroxine-binding globulin; antigen/antibody; antibody/anti-antibody; carbohydrate/lectins; hapten/anti-hapten antibody; dyes and hydrophobic molecules/hydrophobic protein binding sites; enzyme inhibitor, coenzyme or cofactor/enzyme; polynucleic acid/homologous polynucleic acid sequence; fluorescein/anti-fluorescein; dinitrophenol/anti-dinitrophenol; vitamin B12/intrinsic factor; cortisone, cortisol/cortisol binding protein; and ligands for specific receptor protein/membrane associated specific receptor proteins. Preferred binding pairs according to this invention are biotin/avidin or streptavidin, more preferably biotin/streptavidin.


Depending upon the nature of the label, various techniques can be employed for detecting, or detecting and quantitating the label. For fluorescers, a large number of fluorometers are available. For chemiluminescers, luminometers or films are available. With enzymes, a fluorescent, chemiluminescent, or colored product can be determined or measured fluorometrically, luminometrically, spectrophotometrically or visually.
The following examples are presented to help in the better understanding of the subject invention and are for purposes of illustration only. The examples are not to be construed as limiting the invention in any manner.

Example 1

In this example, one maternal urine sample from a Down syndrome affected pregnancy at the gestational age of 12 weeks, 0 days, as determined by ultrasound, was assayed for UGP according to the methods of this invention. [The case sample (karyotype: 47, XX, +21) was kindly provided by Nancy C. Rose, M.D. of the Department of Obstetrics and Gynecology at the Hospital of the University of Pennsylvania (PA, USA).] The reason for the prenatal diagnosis has advanced maternal age.

Six controls from gestational ages plus or minus one week from that of the Down syndrome case sample were tested by the same method to establish the MOM. [Five of the control samples and one other sample were kindly provided respectively by Britta Stirnai, M.D. (Germany), and Leonard H. Kellner, M.S. (Montefiore Medical Center, Albert Einstein College of Medicine; New York, N.Y. USA). The tests and analyses were performed by or under the direction of Leonard H. Kellner, M.S. (Montefiore Medical Center) and Jacob A. Canick, Ph.D. (Women and Infants Hospital, Brown University School of Medicine; Providence, Rhode Island, USA).]

Case and control samples were stored at -20°C for from 1 week to 8 months prior to assay. While the fetal karyotype of the control samples was not always known, it was assumed that none of the controls was from an aneuploid pregnancy. Gestational age of the control samples were determined by either ultrasound parameters or by last menstrual period dating.

The Triton® UGP EIA Kit from Ciba Corning Diagnostics (Alameda, CA; U.S.A.) was used to determine the UGP levels in the case and control samples. The protocol used for the assay was essentially as provided with the kit but appropriate dilutions for the first trimester samples were made. The
assay range for UGP was 0.4-16.0 fmol/ml. Because of the high levels of UGP in the maternal urine samples, the samples prior to assay were diluted 50,000 to 100,000 (50k to 100k) with diluent provided with the kit as indicated in the results shown in Table 1.

UGP levels were ultimately expressed as pmol per mg urinary creatinine to account for variations in the concentration of the urine samples. There had been no attempt to control the time of day that the urine samples were taken. Creatinine levels were measured on a Synchrom CX-5 chemistry analyser (Beckman Instruments; Brea, CA; USA).

<table>
<thead>
<tr>
<th>Samples</th>
<th>gest. age</th>
<th>creatinine (mg/ml)</th>
<th>UGP raw (fmol/ml)</th>
<th>dilution factor</th>
<th>UGP (pmol/mg creat.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 1</td>
<td>13.2</td>
<td>1.01</td>
<td>2.34</td>
<td>100K</td>
<td>234.0</td>
</tr>
<tr>
<td>Control 2</td>
<td>11.6</td>
<td>0.63</td>
<td>1.48</td>
<td>50K</td>
<td>74.0</td>
</tr>
<tr>
<td>Control 3</td>
<td>12.1</td>
<td>0.52</td>
<td>2.54</td>
<td>50K</td>
<td>127.0</td>
</tr>
<tr>
<td>Control 4</td>
<td>11.4</td>
<td>1.10</td>
<td>4.05</td>
<td>50K</td>
<td>202.5</td>
</tr>
<tr>
<td>Control 5</td>
<td>11.0</td>
<td>0.90</td>
<td>3.06</td>
<td>50K</td>
<td>153.0</td>
</tr>
<tr>
<td>Control 6</td>
<td>12.0</td>
<td>1.04</td>
<td>0.83</td>
<td>50K</td>
<td>41.5</td>
</tr>
</tbody>
</table>

Case:

<table>
<thead>
<tr>
<th>Samples</th>
<th>gest. age</th>
<th>creatinine (mg/ml)</th>
<th>UGP raw (fmol/ml)</th>
<th>dilution factor</th>
<th>UGP (pmol/mg creat.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Down Syndrome Case</td>
<td>12.0</td>
<td>1.60</td>
<td>6.24</td>
<td>100K</td>
<td>624.0</td>
</tr>
</tbody>
</table>

The control values ranged from 39.9 to 244.2 pmol UGP per mg creatinine. The median value for the six controls was 177 pmol per mg creatinine. The level of UGP in the Down syndrome case was 390 pmol UGP per mg creatinine. Thus, the result for this case expressed as multiples of the control median (MOM) was 390/177 = 2.20 MOM, i.e., slightly more than twice the control median, a positive result.
Example 2

In this example, seven first trimester maternal urine samples from Down syndrome affected pregnancies, that is, seven case samples, and 214 first trimester control urine samples were assayed for UGP. The assay protocol with the Triton UGP EIA Kit (CCD; Alameda, CA; USA) used was essentially as described above in Example 1. However, the samples were diluted 10,000-fold with the kit diluent, and if the UGP levels were still too high for the kit's assay range, a repeat assay was done at a higher dilution.

Dilutions were performed with a Hamilton Microlab 100 semi-automatic diluter. The concentrations reported in this example are in mmol UGP/L and expressed as nmol/mmol creatinine.

Controls

A regression curve from the UGP/creatinine values for the 214 control samples from pregnancies of 9 to 13 weeks gestation was prepared. The following quadratic equation basically describes the data:

\[ a + bx + cx^2 + dx^3 \]

wherein \( x \) represents the gestational age in days; and wherein the coefficients are as follows:

\[ a = -527.7; \]
\[ b = 19.13; \]
\[ c = -0.2230; \text{ and} \]
\[ d = 0.0008558. \]

The observed versus the fitted values were as follows:

<table>
<thead>
<tr>
<th>Gestational Week</th>
<th>No. of Samples</th>
<th>Observed</th>
<th>Fitted</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>9</td>
<td>12</td>
<td>9.9758</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>18</td>
<td>12.6759</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>77</td>
<td>14.5134</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>29</td>
<td>11.4615</td>
</tr>
<tr>
<td>35</td>
<td>13</td>
<td>78</td>
<td>10.9880</td>
</tr>
</tbody>
</table>
There appeared to be a peak urinary UGP level at about 11 weeks and then a decline in the levels thereafter.

**MOMs in unaffected pregnancies.** The spread of the MOM values for unaffected pregnancies are exemplified as follows.

<table>
<thead>
<tr>
<th>Controls</th>
<th>UGP MOM</th>
</tr>
</thead>
<tbody>
<tr>
<td>10th centile</td>
<td>0.41</td>
</tr>
<tr>
<td>Median</td>
<td>0.99</td>
</tr>
<tr>
<td>90th centile</td>
<td>1.95</td>
</tr>
</tbody>
</table>

Assuming a Gaussian distribution, the SD of log UGP based on the 10th-90th range would be 0.26, which is similar to that of free beta hCG in serum.

**Down Syndrome Affected Pregnancies**

The results of testing the seven urine samples from the Down syndrome affected pregnancies are expressed below as multiples of the gestation-specific median (MOM) and shown below in Table 2. Case No. 3 is from a twin pregnancy discordant for Down syndrome.

**TABLE 2**

<table>
<thead>
<tr>
<th>Down Syndrome</th>
<th>Gestational</th>
<th>MOM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case No.</td>
<td>Age in Weeks</td>
<td></td>
</tr>
<tr>
<td>(1)</td>
<td>10</td>
<td>1.90215</td>
</tr>
<tr>
<td>(2)</td>
<td>11</td>
<td>0.34949</td>
</tr>
<tr>
<td>(3) (twin)</td>
<td>12</td>
<td>2.65054</td>
</tr>
<tr>
<td>(4)</td>
<td>12</td>
<td>0.92861</td>
</tr>
<tr>
<td>(5)</td>
<td>12</td>
<td>2.79836</td>
</tr>
<tr>
<td>(6)</td>
<td>12</td>
<td>0.49450</td>
</tr>
<tr>
<td>(7)</td>
<td>13</td>
<td>1.71604</td>
</tr>
</tbody>
</table>

Four out of the seven case samples extremely elevated, namely Case Nos. 1, 3, 5 and 7. The distribution in Down syndrome cases may be bimodal with the low values being
from non-viable pregnancies. About one-half of first trimester Down syndrome affected fetuses are non-viable.

Two of the urine samples -- Case Nos. 3 and 7 -- were taken after CVS. The remainder were prospective samples taken before CVS or any other invasive prenatal diagnostic procedure was performed.

The mean approximated the observed log median of the seven cases, the log of 1.72 MOM or 0.24. The observed SD of log UGP is 0.36.

**Covariates**

There appears to be no correlation between the UGP concentrations in the first trimester urine samples with the following covariates: maternal age ($r = 0.04615$ based on 72 samples); maternal weight ($r = -0.21217$ based on 65 samples); serum AFP ($r = 0.02184$ based on 67 samples); and serum uE3 ($r = 0.00305$ based on 67 samples). However, there appears to be a statistically significant correlation between the urinary UGP levels and serum free beta hCG levels, although the correlation coefficient is very low ($r = 0.42888$ based on 67 samples; $p = 0.0003$).

**Example 3**

Four hundred and sixty-three samples were obtained from apparently normal pregnancies at 9-13 weeks gestation. Five of those pregnancies were subsequently found to be affected with aneuploidy. An additional 7 samples were obtained after chorionic villus sampling (CVS) had confirmed aneuploidy. Of the 12 cases, 10 were affected by Down syndrome (trisomy 21) and 2 had Edwards syndrome (trisomy 18). There were three sources of samples from apparently normal pregnancies: 165 were obtained prior to CVS because of increased risk of aneuploidy, 44 at the time of the first antenatal hospital visit and 249 concurrently with maternal serum screening which in Leeds [Centre for Reproduction, Growth & Development; Research School of Medicine; University of Leeds (Leeds, U.K.)] is done at 13-18 weeks gestation although some women provide earlier samples in error.
Urine samples from cases and controls were stored at -20°C or lower until retrieved for assay. Each sample was assayed without knowledge of whether it was from a case or a control. UGP was measured using the enzyme immunoassay method [Triton® UGP EIA Ciba Corning Diagnostics Corp., Alameda, CA (USA)] essentially as described above in Example 2. To allow for variable urine concentrations, UGP levels were expressed as nmol per mmol creatinine and to account for gestational differences, levels were also expressed as multiples of the normal median (MOM) level for the appropriate gestation. The normal gestation-specific median levels were derived by regression of the observed weekly medians against gestation among the controls after weighting for the number at each week. Gestational age was based on ultrasound biometry in each case and in 87% of controls.

The median UGP level increased to a peak at 10-11 weeks and fell thereafter. A log quadratic regression fitted this reasonably well: the observed and regressed values were 10.9 and 12.2 nmol/mmol at 9 weeks (15 samples); 13.3 and 13.2 at 10 weeks (58 samples); 14.6 and 13.5 at 11 weeks (85 samples); 10.5 and 13.0 at 12 weeks (38 samples); and 11.4 and 11.3 at 13 weeks (262 samples). The regression equation was log₁₀ UGP = -1.079 + 0.05592*ga - 0.0003537*ga² where ga is the gestation in days. Using this equation to calculate MOMs gave a normal median of 1.00, a 10-90th centile range of 0.40-2.08 and a 25-75th centile range of 0.61-1.51.

Table 3 shows the individual MOM values for each of the 12 affected pregnancies together with clinical details. The UGP levels for both cases of Edwards’ syndrome were below the normal 25th centile, a statistically significant reduction (p<0.05, Wilcoxon Rank Sum Test). In four of the 10 cases of Down syndrome the level was raised above the normal 90th centile, and in two it was reduced (one below the 10th centile and one below the 25th). Overall the distribution of values in Down syndrome did not quite differ significantly from controls (P = 0.09, Wilcoxon Rank Sum Test).
These results indicate that first trimester maternal urine UGP levels are raised in a proportion of pregnancies affected with Down syndrome and reduced in Edward syndrome.

Table 3

<table>
<thead>
<tr>
<th>Aneuploidy</th>
<th>MOM</th>
<th>Gestation (wks)</th>
<th>Maternal Age at Term (yrs)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Down</td>
<td>3.57</td>
<td>13</td>
<td>28</td>
<td>Post CVS</td>
</tr>
<tr>
<td>Down</td>
<td>3.27</td>
<td>11</td>
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<td>26</td>
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<td>1.37</td>
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<td>32</td>
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<tr>
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<td>1.06</td>
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<td>10</td>
<td>36</td>
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<td>0.89</td>
<td>12</td>
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<td>Pre CVS</td>
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<tr>
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<td>Down</td>
<td>0.48</td>
<td>12</td>
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<td>Pre CVS</td>
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<tr>
<td>Down</td>
<td>0.36*</td>
<td>11</td>
<td>37</td>
<td>Pre CVS</td>
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<tr>
<td>Edwards</td>
<td>0.10*</td>
<td>11</td>
<td>40</td>
<td>Post CVS</td>
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* The samples marked with an asterisk were also included in experiments using a different assay for UGP and yielded levels of 0.48, 0.48, 1.01 and 0.06 MOM respectively. The assay used was a modification of a radioimmunoassay method described in Lee et al., J. Endocrinol., 13: 481-489 (1991) as described in Cuckle et al., Prenatal Diagnosis, 14: 953-958 (1994).
Example 4

Multiple Marker Urinary Screening -- One First Trimester Down Syndrome Case and One First Trimester Turner Syndrome Case, Tested for UGP and Free α-hCG by RIA, and tE by Kober Reaction

In this example, UGP levels were detected and quantitated in urine along with two other markers -- tE and free α-subunit of hCG (α-hCG). First trimester maternal urine samples were tested from two pregnancies affected by aneuploidy -- one with Down syndrome (trisomy 21) and one with Turner syndrome (45,X/46,X+ring -- a mosaic). Maternal urine samples were also taken from 291 controls, of which 49 were at a gestational age of <13 weeks, and 77 were at a gestational age of 13-14 weeks.

Methods

In the case of the Down syndrome affected pregnancy, the urine sample was obtained at 11 weeks gestational age before prenatal diagnosis by chorionic villus sampling (CVS). CVS was performed because of family history or chromosomal abnormality. In the case of the Turner syndrome affected pregnancy, the urine sample was taken at 13 weeks gestational age after CVS that was performed because of advanced maternal age.

Patients were recruited in the area served by the Yorkshire Regional Cytogenetics Laboratory [Yorkshire, UK] and from those attending selected antenatal clinics at Queen Charlotte’s Maternity Hospital [London, UK]. A control series was obtained from the urine samples which are routinely taken for sugar, protein and bacterial analysis from women attending antenatal clinics in the Leeds General Infirmary [Leeds, UK] and at Queen Charlotte’s Hospital.

Urine samples from cases and controls were stored at -40°C until retrieved for assay. Each sample was assayed without knowledge of whether it was from a case or a control. UGP was measured using a modification of a previously published radioimmunoassay method [Lee et al., J. Endocrinol..]
130: 481-489 (1991)]. The UGP specific antisera (S504-
Immunogen International, Llandyssul, Dyfed SA44 5JT, UK) was
retitrated against UGP tracer in order to give a desensitized
assay range of 6-600 pmol/L. This assay has a partial mole
per mole cross-reaction with intact hCG (6.9%) and free β-hCG
(18%) but negligible cross-reactivity with free α-subunit hCG,
luteinizing hormone (LH), thyroid stimulating hormone (TSH)
and follicle stimulating hormone (FSH) (<0.7%). All samples
were diluted 1/100 and 1/1000 prior to assay.

te was measured by a continuous flow system based on
the Kober reaction [Lever et al., Biochem. Med., 8: 188-198
(1973)], sensitized by doubling the sample volume and having
the concentration of the calibrators previously used for late
pregnancy samples.

Free α-hCG was measured using an in-house
desensitized radioimmunoassay with a standard range of 25-500
µg/L, thus requiring samples to be diluted 1/5 or 1/10. The
assay uses a polyclonal sheep antibody raised against free α-
hCG isolated from a crude commercial preparation of pregnancy
urine hCG by gel exclusion and hydrophobic interaction
chromatography. An immunoglobulin preparation from the
resultant antisera (S781; Polyclonal Antibodies Ltd,
Llandyssul, Dyffed, UK) was extensively absorbed against a
solid phase of intact hCG conjugated to activated sepharose.

This removed antibodies to epitopes present on free α-hCG
exposed when it combines with the β-subunit. The NIH
reference preparation CR123 was used to prepare assay
standards and to form the tracer, by iodination using the
chloramine T method. Cross-reactivity with intact hCG, free
β-hCG, UGP, luteinizing hormone (LH), follicle stimulating
hormone (FSH) and thyrotropin (TSH) was assessed at 50% of
B/Bo. Purified reference preparations were obtained from the
National Institutes of Health (Bethesda, Maryland, USA) for
intact hCG (CR123), free β-hCG (CR123) and UGP [donated by
Drs. R. Wehmann and D. Blithe; Blithe et al., Endocrinol.,
122: 173-180 (1988)] and WHO International Reference
Preparations from the National Institute for Biological
Standards and Control (Potters Bar, Hertfordshire, UK) for LH
(68/40), FSH (83/575) and TSH (80/558). Molecular weights were calculated from the established primary structures: intact hCG 38 kd; free β-hCG 23 kd; free α-hCG 15 kd [Bellisario et al., J. Biol. Chem., 248: 6796 (1973)]; UGP 10 kd [Birken et al., Endocrinol., 123: 572 (1988)]; LH 29 kd; FSH 33 kd and TSH 30 kd (Professor S. Jeffcoate, personal communication). On a molar basis the cross-reactivity was <0.01% with free β-hCG and UGP but higher with intact hCG (10%), LH (8%), FSH (8%) and TSH (6%), possibly caused by dissociation of the purified preparations which would free the α-subunit common to all the glycoprotein hormones.

Since random urines were obtained and there was no uniformity in the time of voiding, the creatinine concentration was measured (Jaffe method using a Monach 200 centrifugal analyser), and the concentrations of the markers were expressed per mmol creatinine. To allow for gestational differences, levels were also expressed as multiples of the normal median (MOM) level for the appropriate gestation derived from the 291 singleton controls. Because of the small number of controls at any week of gestation, and so that MOMs could be calculated to the exact day of gestation, the observed medians in different gestational bands were regressed after weighting for the number in each band. Gestational age was based on ultrasound biparietal diameter or crown-rump length measurement for all cases and controls.

Standard statistical modeling techniques were used to examine the screening potential of each marker and of different multi-marker combinations [Royston and Thompson, Stats. Med., 11: 257 (1992)]. A multivariate log Gaussian frequency distribution was assumed, provided no significant deviation from fit was found in the Shapiro-Wilks Test. If the fit was poor in the extreme tails of the distribution, truncation limits were used. The marker means were estimated by the observed medians, the standard deviations by the 10–90th centile differences divided by 2.563 and the correlation coefficients by the observed values after excluding outliers exceeding 3 standard deviations from the mean. The expected Down syndrome detection rate was calculated for a given false-
positive rate from the log Gaussian model, assuming that the maternal age distribution is that of England and Wales in 1989–92 (Office of Population Censuses & Surveys (1991–1994)).

Results

The observed median level of each marker in unaffected singleton pregnancies according to gestational age are shown in Table 4. The regressed medians used to calculate MOMs were $10^{0.871-0.01505\times GA}$ for UGP, $10^{-1.271+0.01341\times GA}$ for tE and $10^{-0.170+0.00184\times GA}$ for $\alpha$-hCG, where GA is the gestation in days.

Table 5 shows the individual MOM values for each of the two aneuploidy affected pregnancies together with the clinical details. For comparison, Table 6 shows selected centiles in the 291 singleton controls. There was a wide spread of UGP values in the singleton controls exemplified by the 3-fold range between the 25th and 75th centiles or by the 7-fold 10th to 90th centile range.
<table>
<thead>
<tr>
<th>Gestation (wks)</th>
<th>Preganacies</th>
<th>UGP (nmol/1)</th>
<th>UGP (nmol/mmol creatinine)</th>
<th>tE (μmol/1)</th>
<th>tE (μmol/mmol creatinine)</th>
<th>α-hCG (nmol/1)</th>
<th>α-hCG (nmol/mmol creatinine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 13</td>
<td>49</td>
<td>273</td>
<td>33.7</td>
<td>7.0</td>
<td>0.71</td>
<td>9.7</td>
<td>1.06</td>
</tr>
<tr>
<td>13-14</td>
<td>77</td>
<td>235</td>
<td>23.6</td>
<td>11.0</td>
<td>1.12</td>
<td>12.4</td>
<td>1.06</td>
</tr>
</tbody>
</table>

**TABLE 5**

Individual Marker Level (MOM) in 1 Singleton Down Syndrome Pregnancy and 1 Turner Syndrome Pregnancy

<table>
<thead>
<tr>
<th>Aneuploidy</th>
<th>Gestation (wks)</th>
<th>Maternal age (yrs)</th>
<th>Diagnosis*</th>
<th>UGP</th>
<th>tE</th>
<th>α-hCG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Down syndrome</td>
<td>11</td>
<td>37</td>
<td>FH → CVS</td>
<td>1.01</td>
<td>0.54</td>
<td>1.09</td>
</tr>
<tr>
<td>Turner syndrome</td>
<td>13</td>
<td>42</td>
<td>MA → CVS</td>
<td>3.35</td>
<td>0.37</td>
<td>0.60</td>
</tr>
</tbody>
</table>

* Reasons for carrying out prenatal diagnosis → method used: FH = family history or chromosomal abnormality; MA = advanced maternal age; CVS = chorionic villus sampling.

+ Urine sample obtained before the prenatal diagnosis.
TABLE 6
Selected Centiles of Marker Level (MOM)
in 291 Singleton Unaffected Pregnancies

<table>
<thead>
<tr>
<th>Centile</th>
<th>UGP</th>
<th>tE</th>
<th>α-hCG</th>
</tr>
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<tbody>
<tr>
<td>5</td>
<td>0.38</td>
<td>0.60</td>
<td>0.54</td>
</tr>
<tr>
<td>25th</td>
<td>0.66</td>
<td>0.80</td>
<td>0.68</td>
</tr>
<tr>
<td>50th</td>
<td>1.04</td>
<td>0.99</td>
<td>1.00</td>
</tr>
<tr>
<td>75th</td>
<td>1.66</td>
<td>1.35</td>
<td>1.61</td>
</tr>
<tr>
<td>90th</td>
<td>2.50</td>
<td>1.77</td>
<td>3.22</td>
</tr>
</tbody>
</table>

The results shown in Table 5 indicate a false-negative result for UGP level in the Down syndrome case, but the level of UGP for the Turner syndrome case, more than three times above normal, indicates a positive result. The tE level in the Down syndrome case is a positive result being about one-half of the normal level. The tE level in the Turner syndrome case being at 0.37 MOM is also indicative of a positive result. The α-hCG level for the Down syndrome case is a false-negative result at 1.09 MOM, whereas the α-hCG result for the Turner syndrome case is a positive result as 0.60 MOM is below normal levels.

The descriptions of the foregoing embodiments of the invention have been presented for purposes of illustration and description. They are not intended to be exhaustive or to limit the invention to the precise form disclosed, and obviously many modifications and variations are possible in light of the above teachings. The embodiments were chosen and described in order to best explain the principles of the invention and its practical application to enable thereby others skilled in the art to best utilize the invention in various embodiments and with various modifications as are suited to the particular use contemplated. It is intended that the scope of the invention be defined by the claims appended hereto.

All references cited herein are hereby incorporated by reference.
What we claim is:

1. A method for prenatally determining whether there is a significant risk of a pregnancy being affected by fetal aneuploidy during the pregnancy's first trimester comprising:
   (a) taking a maternal urine sample during the first trimester of said pregnancy;
   (b) testing said maternal urine sample to determine the level of UGP in said sample;
   (c) determining whether the level of UGP in said sample is elevated above a level of UGP that is normal in urine samples from women whose pregnancies are unaffected by aneuploidy, and whose pregnancies are at about the same gestational age as the pregnancy under analysis; and
   (d) determining whether there is a significant risk of fetal aneuploidy based upon the level of UGP in said sample, wherein a level elevated above said normal level indicates that there is a significant risk of the pregnancy being affected by fetal aneuploidy.

2. The method according to Claim 1 wherein an immunoassay is used to determine the level of UGP in said sample.

3. The method according to Claim 1 wherein said fetal aneuploidy is selected from the group consisting of Down syndrome, Klinefelter syndrome, Turner syndrome and triple-X.

4. The method according to Claim 2 wherein said fetal aneuploidy is selected from the group consisting of Down syndrome and Turner syndrome.

5. The method according to Claim 2 wherein said immunoassay is specific for UGP and has a molar cross-reactivity of less than about 10% with each of the following: intact hCG, β-subunit hCG, and α-subunit hCG.
6. The method according to Claim 5 wherein said immunoassay has a molar cross-reactivity of less than about 5% with each of the following: intact hCG, β-subunit of hCG, and α-subunit hCG.

7. The method according to Claim 5 wherein said immunoassay has a molar cross-reactivity of less than about 3% with each of the following: intact hCG, β-subunit hCG, and α-subunit hCG.

8. The method according to Claim 5 wherein said immunoassay has a molar cross-reactivity of less than about 1% with each of the following: intact hCG, β-subunit hCG, and α-subunit hCG.

9. The method according to Claim 8 wherein said fetal aneuploidy is selected from the group consisting of Down syndrome, Klinefelter syndrome, Turner syndrome and triple-X.

10. The method according to Claim 1 wherein said maternal urine sample was taken during the eighth week to the thirteenth completed week of said pregnancy.

11. The method according to Claim 2 wherein said immunoassay is automated.

12. The method according to Claim 1 wherein the UGP levels are corrected for variability in urine concentrations by being expressed relative to creatinine content in said urine samples.

13. The method according to Claim 2 wherein said immunoassay is a sandwich enzyme immunoassay or a sandwich radioimmunoassay.

14. The method according to Claim 1 wherein said maternal urine sample is also tested to determine another marker’s level;
wherein the level of said other urinary marker in said sample is compared to a level of said other urinary marker that is normal in urine samples from women whose pregnancies are unaffected by aneuploidy, and whose pregnancies are at about the same gestational age as the pregnancy under analysis; and

wherein in step (d) the risk of fetal aneuploidy is based upon the level of UGP in said sample in conjunction with the level of said other urinary marker, wherein a level of said other urinary marker above or below the normal level for said marker indicates an increased risk of fetal aneuploidy.

15. The method according to Claim 14 wherein said other urinary marker is selected from the group consisting of tE, 16α-OH-DHAS, PAPP-A, α-hCG, dimeric inhibin, uE₂, tE₃, AFP and proMBP.

16. The method according to Claim 1 wherein step (d) further comprises determining maternal age and factoring the maternal age into determining whether there is a significant risk of fetal aneuploidy, wherein an older maternal age indicates an increased risk of fetal aneuploidy.

17. The method according to Claim 1 further comprising taking a maternal serum sample during the pregnancy’s first trimester from the same pregnant woman from whom the urine sample is taken;

testing said maternal serum sample to determine the level of a serum marker in said serum sample;

determining whether the level of said serum marker is above or below a level of said serum marker that is normal in serum samples from women whose pregnancies are unaffected by aneuploidy, and whose pregnancies are at about the same gestational age as the pregnancy under analysis; and

wherein in step (d) the risk of fetal aneuploidy is based upon the level of UGP in said sample in conjunction with the level of said serum marker, wherein a level of said serum
marker above or below the normal level for said serum marker indicates an increased risk of fetal aneuploidy.

18. A method according to Claim 17 wherein said other serum marker is selected from the group consisting of: PAPP-A, major basic protein, proMBP, intact hCG, free α-hCG, free β-hCG, AFP, uE₃, 16α-OH-DHAS, dimeric inhibin and nondimeric inhibin.

19. The method according to Claim 1 wherein step (d) further comprises considering one or more ultrasound markers for said pregnancy, wherein an abnormal ultrasound marker indicates an increased risk of fetal aneuploidy.

20. The method according to Claim 1 wherein said UGP level is determined by methods selected from the group consisting of: chromatography, NMR, fluorometric detection, and with non-antibody receptors and/or with binding proteins.
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/76 G01N33/543

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

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>PRENATAL DIAGNOSIS. vol. 14, no. 10, October 1994, GB, pages 953-958, XP000609894</td>
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<td>H. S. CUCKLE ET AL.: &quot;Urinary beta-core human chorionic gonadotrophin: A new approach to Down's syndrome screening.&quot; cited in the application see the whole document</td>
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<td>PRENATAL DIAGNOSIS. vol. 15, no. 1, January 1995, GB., pages 11-16, XP000609863</td>
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<td>M. HAYASHI ET AL.: &quot;Maternal urinary beta-core fragment of hCG/creatinine ratios and fetal chromosomal abnormalities in the second trimester of pregnancy.&quot; see the whole document</td>
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* D document referring to an oral disclosure, use, exhibition or other means
* P document published prior to the international filing date but later than the priority date claimed

Date of the actual completion of the international search 18 November 1996

Date of mailing of the international search report 26-11-1996

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Authorized officer
Griffith, G.
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