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ORIGINAL RESEARCH ARTICLE

Prenatal screening for fetal aneuploidies during the first and second trimester of pregnancy

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Abstract

Prenatal screening for chromosomal defects during the first and second trimesters of pregnancy has become an established part of obstetric practice in many countries. The goal of current maternal serum screening programmes is to identify women who are at an increased risk of having a baby affected with Down syndrome (trisomy 21), Edwards syndrome (trisomy 18) or neural tube defects and who will benefit from such diagnostic tests. The most commonly used test for genetic diagnosis is amniocentesis; however, the rate of spontaneous fetal loss caused by this test averages at about 1 in every 200 procedures. Because of this risk, serum analyte testing has become an important and non-invasive first step in detecting patients at risk of carrying a child with congenital abnormalities. The non-invasive screening options which are currently available to patients include combining maternal age with one of the following: first-trimester serum screening [nuchal translucency (NT) and maternal serum biochemistry markers]; second-trimester serum screening (maternal serum biochemistry markers such as the triple test and the quadruple test); or the two-step integrated screening, which comprises first- and second-trimester serum screening with or without NT.

Introduction

Pregnancy screening for fetal aneuploidy began during the 1960s,[1] using maternal age as the screening test. With the development of new serum biochemistry markers in the 1980s,[2] the triple screening test (conducted during the second trimester of pregnancy) became the most prominent screening tool and demonstrated a substantial improvement in detection rates of Down syndrome compared with the previous method of screening using maternal age alone. The detection rate improved from 30% by screening using maternal age alone to 65% by combining maternal age and the triple test, with a false-positive rate (FPR) of 5%. In the 1990s, the emphasis shifted from second-trimester to first-trimester screening when it was realized that the majority of fetuses with aneuploidies could be identified by screening based on a combination of maternal age, fetal nuchal translucency (NT) and maternal serum biochemistry [i.e. levels of free beta-human chorionic gonadotropin (free β-hCG) and pregnancy-associated plasma protein A (PAPP-A)].

The risk of developing some of the fetal chromosomal abnormalities increases with maternal age (Table 1), and pregnant women who will be older than 35 years at time of parturition are routinely offered invasive prenatal diagnostic testing. The most commonly used tests for genetic diagnosis are chorionic villous sampling (CVS) in the first trimester and amniocentesis in the second trimester; however, the rate of spontaneous fetal loss related to amniocentesis or CVS averages at about 1 in every 200 procedures.[3] Because of this risk, serum analyte testing has become an important and non-invasive first step in detecting women at risk of having a baby with congenital abnormalities. The goal of prenatal screening is to identify women who are at an increased risk of having a baby affected with aneuploidy and who will benefit from diagnostic testing. Maternal screening has the added benefit of reducing the number of normal pregnancies lost because of the complication of an invasive procedure.

Currently available non-invasive screening options include maternal age combined with one of

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TABLE 1 Estimated risk of a fetus developing Down syndrome, Edwards syndrome or Patau syndrome in relation to maternal age and number of weeks' gestation (adapted from references 4–7)

	Maternal age (years)														
Syndrome	**20**	**25**	**30**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**	**39**	**40**	**41**	**42**
Down syndrome															
Number of weeks' gestation															
12[a]	1068	946	626	543	461	383	312	249	196	152	117	89	68	51	38
16[a]	1200	1062	703	610	518	430	350	280	220	171	131	100	76	57	43
20[a]	1295	1147	759	658	559	464	378	302	238	185	142	108	82	62	46
40[a]	1527	1352	895	776	659	547	446	356	280	218	167	128	97	73	55
Edwards syndrome															
Number of weeks' gestation															
12[a]	2484	2200	1456	1263	1072	891	725	580	456	354	272	208	157	118	89
16[a]	3590	3179	2103	1825	1549	1287	1047	837	659	512	393	300	227	171	128
20[a]	4897	4336	2869	2490	2114	1755	1429	1142	899	698	537	409	310	233	175
40[a]	18 013	15 951	10 554	9160	7775	6458	5256	4202	3307	2569	1974	1505	1139	858	644
Patau syndrome															
Number of weeks' gestation															
12[a]	7826	6930	4585	3980	3378	2806	2284	1826	1437	1116	858	654	495	373	280
16[a]	11 042	9778	6470	5615	4766	3959	3222	2576	2027	1575	1210	922	698	526	395
20[a]	14 656	12 978	8587	7453	6326	5254	4277	3419	2691	2090	1606	1224	927	698	524
40[a]	42 423	37 567	24 856	21 573	18 311	15 209	12 380	9876	7788	6050	4650	3544	2683	2020	1516

[a]Table numbers refer to 1 in every number presented e.g. at maternal age 20 and 12 weeks' gestation, the risk of having a baby with Down syndrome is 1 in every 1068 pregnancies.

the following: first-trimester serum screening (NT and maternal serum biochemistry markers); second-trimester serum screening (maternal serum biochemistry markers – triple test or the quadruple test); or two-step integrated screening, which comprises first- and second-trimester serum screening with or without NT (Figure 1).

First-trimester screening test

First-trimester screening is performed at a gestation of between 10 weeks and 13 weeks 6 days and was first suggested by Brizot *et al.*[8] in 1994. The markers used to calculate the risk of the fetus developing abnormalities are two serum markers: PAPP-A and free β-hCG. A third marker is fetal NT (a fluid-containing area at the back of the fetal neck), which is identified by ultrasonography. NT measurement needs to be carried out by experienced sonographers at a gestation of between 10 weeks and 13 weeks 6 days.

First-trimester screening allows early diagnosis of chromosomal abnormalities such as Down syndrome (trisomy 21), Edwards syndrome (trisomy 18) and Patau syndrome (trisomy 13). The detection rate for Down syndrome is 91% with an associated FPR of 5%. For an equivalent FPR, the detection rate of screening using maternal age alone is 30% and screening using maternal age plus the serum biochemistry test gives a detection rate of 65% for Down syndrome (Table 2).[9,10] This method also identifies 94% of all major chromosomal defects, such as Edwards syndrome, Patau syndrome, triploidy and Turner syndrome, and 60% of other chromosomal defects such as deletions, partial trisomies, unbalanced translocations and sex chromosome aneuploidies other than Turner syndrome.[11] To assess the patient-specific risks, the a priori maternal age-related risk is multiplied by a likelihood ratio, which is determined from the deviation of the measured NT, free β-hCG and PAPP-A from their expected medians.

Biochemical markers during the first trimester of pregnancy

A number of changes have been observed in the level of hormones in the body during a normal human pregnancy, from very early gestation to parturition, and even beyond. Many hormonal proteins are

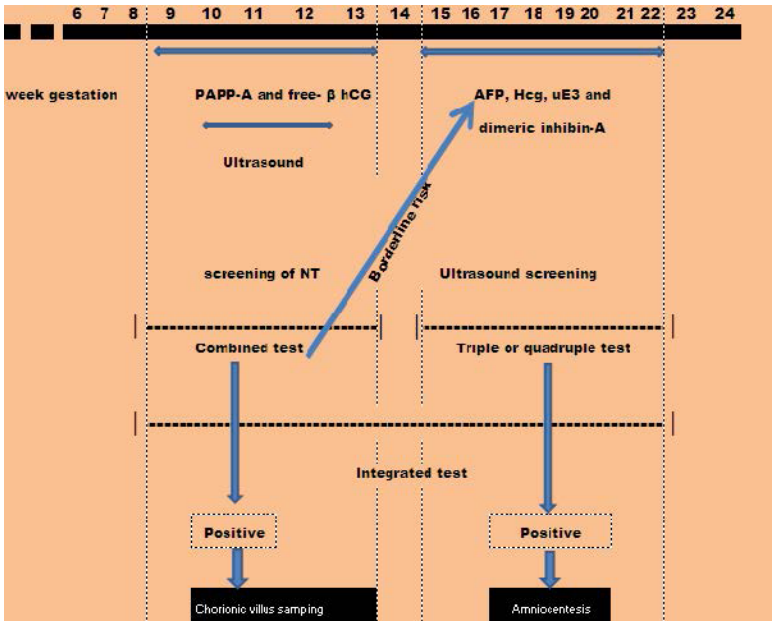

FIGURE 1 Maternal serum screening during first and second trimester of pregnancy.

TABLE 2 Detection rate of Down syndrome, Edwards syndrome and Patau syndrome during the first trimester of pregnancy (adapted from reference 9)

Screening policy	Down syndrome (FPR 5%)	Edwards syndrome (FPR 0.5%)	Patau syndrome (FPR 0.5%)
Maternal age and fetal NT	80	68	61
Maternal age and serum free β-hCG and PAPP-A	65	80	59
Maternal age, NT and serum free β-hCG and PAPP-A	91	97	84

created, or modified, by the placenta; however, the level of these hormonal proteins differs in cases of pathological pregnancies and may be monitored to provide a diagnosis or a risk prediction for developing gestational diseases, taking into account hormone levels as well as any pre-existing maternal risk factors. The excessive release of some placental hormones in association with gestational diseases may be part of an adaptive response from the placenta and fetal membranes to adverse environmental conditions, such as hypertension, hypoxia and infection, or to malformations of the fetus and placenta. A high concentration of these hormones in the maternal peripheral blood, in the fetal (cord) blood and in the amniotic fluid is a clinical sign of increased placental hormone synthesis. The discovery of these slight differences in protein levels in pathological and non-pathological pregnancies forms the basis for using these proteins as biochemical markers in screening protocols. During biochemical testing, each element being measured is first converted in to a multiple of the expected normal median (MoM), which is specific to fetal gestational age, maternal weight, ethnicity, smoking status, method of conception and parity as well as the machine and reagents used for the assay. In euploid pregnancies, the average adjusted value for both free β-hCG and PAPP-A is 1.0 MoM at all gestational ages.[12]

Pregnancy-associated plasma protein A

Plasma-associated protein A is a large zinc-containing glycoprotein that is produced by both the placental syncytiotrophoblast[13] and the decidua and is located on human chromosome 9q33.1.[14] It belongs to the alpha-macroglobulin plasma protein group, which was first identified in 1974 in the plasma of a pregnant woman. The protein is secreted as a disulphide-bound homodimer with a molecular weight of 400 kDa. In the plasma, PAPP-A circulates either in the free form or as a heterotetrameric complex of the proform of eosinophil major basic protein (proMBP)

forming PAPP-A/proMBP with a molecular weight of approximately 500 kDa.[14,15] During pregnancy, both PAPP-A and proMBP are expressed in abundance in the placenta but they are each expressed in different cell types. Most of the PAPP-A is synthesized in the placental syncytiotrophoblast whereas all of the proMBP is synthesized in the extravillous cytotrophoblasts, from where it is secreted without propeptide cleavage. The processes of the PAPP–A/proMBP complex occur in the extracellular environment and, to the best of our knowledge, at the surface of syncytiotrophoblast. During a normal pregnancy, the concentration of PAPP-A in the maternal circulation increases with increasing fetal gestational age. PAPP-A becomes detectable soon after zygote implantation and increases throughout the pregnancy, doubling every 3–4 days during the first trimester. As a result of the rapid increase in PAPP-A during the first trimester, the level of this protein is closely related to gestational age and it is common practice to express PAPP-A concentration in terms of MoM. In addition to gestational age, a number of maternal and pregnancy-associated characteristics such as smoking, multiple parity, diabetes mellitus and maternal weight also affect the maternal serum concentration of PAPP-A.

The most important clinical use of PAPP-A is the first-trimester screening for chromosomal abnormalities that are characterized by a low concentration of PAPP-A. Decreased levels of PAPP-A are found in association with abnormal placental function, which has formed the basis for the first-trimester screening for fetal Down syndrome. The purpose of these programmes is to identify pregnant woman who should be offered diagnostic CVS or amniocentesis. PAPP-A concentrations are 100-fold and 1000-fold lower in fetal blood and amniotic fluid, respectively, than in maternal blood and the amniotic fluid of a non-Down syndrome fetus. Low PAPP-A values during the first trimester have been observed and are typically 0.15 MoM for Down syndrome, 0.18 MoM for Edwards syndrome, 0.25 MoM for Patau syndrome and 0.49 for Turner syndrome.[16] Low levels of PAPP-A have also been associated with preterm delivery, intrauterine growth retardation, miscarriage, stillbirth, small for gestational age infants and ectopic gravidity.[17,18]

Free beta-human chorionic gonadotropin

In 1990, Macri *et al*. reported that maternal serum β-hCG is elevated in Down syndrome pregnancies.[19] In 1995, Eldar-Geva *et al*. reported that, although the production of each subunit's hCG messenger RNA (mRNA) is increased in Down syndrome pregnancies, β-subunit production is more markedly increased.[20] These findings suggests that the free β-hCG subunit might be superior to hCG for detection of Down syndrome. Various authors have reported that screening for elevated levels of free β-hCG is more effective than screening for increased hCG in the maternal serum;[21,22] therefore, β-hCG has been introduced in first-trimester screening.

Human chorionic gonadotropin hormone is composed of two non-covalently linked subunits, alpha (α) and β, and is produced by the syncytiotrophoblast cells of the placenta. hCG has a single β-subunit that contains 145 amino acids linked by six disulphide bridges and an α-subunit that contains 92 amino acids linked by five disulphide bridges. Five hCG-related molecules are present in the maternal serum: non-nicked hCG (which represents the active form), nicked hCG, a free α-subunit, a free β-subunit and the nicked free β-subunit.[23] The free β-subunit can be derived from three sources: direct trophoblast cell production, dissociation of hCG into a free α-subunit and a free β-subunit or by macrophage or neutrophil enzymes nicking the hCG molecule.[12] The free β-hCG circulating in the maternal serum accounts for only about 0.3–4% of the total hCG.[12]

Maternal serum hCG peaks at 8–10 weeks' gestation and then declines to reach a plateau at 18–20 weeks' gestation and remains relatively constant until term. Molecular biology studies[12,20] have demonstrated that Down syndrome trophoblasts show a marked increase in the production of β-hCG mRNA and a smaller increase in α-hCG mRNA, suggesting that one of the causes of high hCG levels in the maternal serum is the increased hCG production and secretion by the placenta. hCG is also used to predict complications, especially in early pregnancy, such as miscarriage and ectopic pregnancy. A failing pregnancy is usually associated with a slower than normal increase in the maternal serum hCG, which gradually turns into a decrease. In a successful pregnancy, the increase in the maternal serum hCG level is exponential, i.e. the concentration doubles in approximately 1.5–2 days. In ectopic pregnancies resulting from *in vitro*

fertilization, the increase in maternal serum hCG is delayed by an average of 1.5 days, but the rate of increase usually normalizes during the first 4 weeks after embryo transfer.

Nuchal translucency

In 1992, Schulte-Valentin and Schindler[24] first reported on non-echogenic nuchal oedema, which is currently known as NT (Figure 2). It is the most important marker on an ultrasound scan during first-trimester screening for chromosomal abnormalities and is measured between 10 and 14 weeks' gestation. NT measures the subcutaneous fluid-filled space between the back of the spine and the skin on the back of the neck of the fetus. Increased NT is associated with Down syndrome, Turner syndrome and other chromosomal abnormalities as well as many fetal malformations and genetic syndromes. The prevalence of these abnormalities is related to the thickness, rather than the appearance, of NT. Fetal NT increases with crown–rump length (CRL) and increases at about 17% a week; hence, it is important to take gestational age into account when analysing NT. The translucent area disappears after 14 weeks' gestation, when the subcutaneous tissue becomes more echogenic, and NT is therefore a transient phenomenon.[25] The NT measurement is converted into MoM for the relevant gestational age, based on CRL, for risk calculation. Using the NT measurement plus maternal age, 73% of affected pregnancies can be identified with a 5% FPR.[26] The NT measurement needs to be performed by experienced sonographers and should be obtained at a gestation of between 10 weeks and 13 weeks 6 days, which is equivalent to a CRL of between 38 and 84 mm. NT can be detected in 99% of fetuses at the end of the first trimester. The 50th percentile NT increases from 1.2 mm in week 11 (CRL of 45 mm) to 1.5 mm in week 13, day 6 (CRL of 82 mm). The 95th percentile ranges from 2 mm in week 11 to 2.6 mm in week 13, day 6.

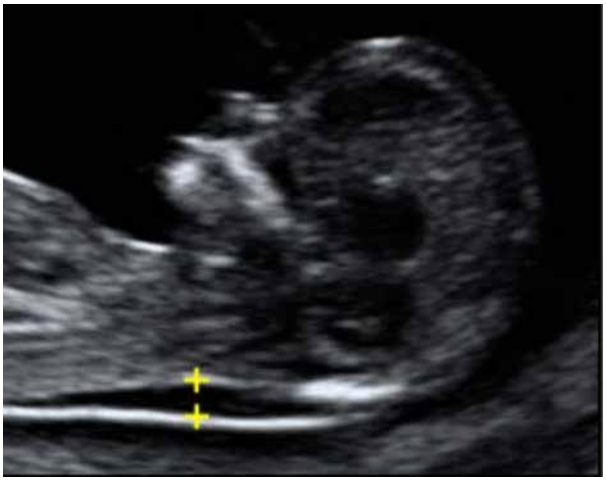

FIGURE 2 An ultrasound scan at 11–14 weeks' gestation for measurement of fetal NT thickness.

In the majority of fetuses with Down syndrome, NT is increased compared with normal fetuses of the same gestational age. Increased NT measurement may also be associated with miscarriage and an abnormal karyotype. In addition to Down syndrome, the NT measurement can also indicate the risk of triploidies, Edwards syndrome, Patau syndrome and monosomy X. Special attention should be given to those cases in which the NT is increased and the karyotype is normal. When NT levels increase above the 95th percentile, the chance that a healthy baby will be born decreases; however, when the NT level is above the 95th percentile and the karyotype is normal, this may be indicative of different malformations and genetic syndromes, the most frequent of which are defects of the heart and great arteries and a wide range of structural defects and genetic conditions. Increased NT has also been associated with other anomalies, including diaphragmatic hernia, omphalocele, neural tube defects, body stalk anomalies, a number of rare genetic syndromes and skeletal dysplasias. These include rare genetic syndromes such as Smith–Lemli–Opitz syndrome, Noonan syndrome, arthrogryposis, Pena–Shokeir syndrome, multiple pterygium syndrome, spinal muscular atrophy, Jarcho–Levin syndrome, thanatophoric dysplasia and thalassaemia.

Patient-specific risk for chromosomal abnormalities

All pregnancies are associated with some risk that the fetus has a chromosomal defect. To calculate the individual risk, it is necessary to take into account the a priori risk, which depends on maternal age and gestational age, and to multiply this by a likelihood ratio, which depends on the results of the ultrasonography and/or maternal serum biochemical tests that may have been performed during the course of the pregnancy to determine the patient-specific risk. Every time a test is carried out, the a priori risk is multiplied by the likelihood ratio derived

from that test to calculate a new risk, which then becomes the a priori risk for the next test.

Fetal nuchal translucency and maternal serum testing in the first trimester

In Down syndrome pregnancies, the maternal serum concentration of free β-hCG is about twice as high as in euploid pregnancies whereas PAPP-A is reduced to half compared with euploid pregnancies (approximately 1 MoM and 0.5 MoM, respectively)[10] (Table 3). There is no significant association between fetal NT and maternal serum free β-hCG or PAPP-A in either Down syndrome or chromosomally normal pregnancies; therefore, the ultrasonography results and the biochemical markers can be combined to provide more effective screening than either method individually. One option for first-trimester combined screening for Down syndrome is to perform biochemical and ultrasonography testing at 12 weeks' gestation. The detection rate of Down syndrome at 12 weeks' gestation is about 90% with an FPR of 5%.[10] An alternative strategy for first-trimester combined screening is for biochemical testing and ultrasonography scanning to be carried out in two separate patient visits, with the biochemical testing conducted at 9 to 10 weeks' gestation and the ultrasonography scanning at 12 weeks' gestation. It has been estimated that this approach would improve the detection rate from 90% to 93–94%.[10] The tests are better at 9–10 weeks' gestation rather than at 13 weeks' gestation because the difference in PAPP-A between trisomic and euploid pregnancies is greater during the earlier gestational period. Although the difference in free β-hCG between trisomic and euploid pregnancies increases with gestation length, the magnitude of the difference is smaller than that of the difference in PAPP-A levels between trisomic and euploid pregnancies. A third option would be to perform the scan at 12 weeks' gestation and optimize the performance of biochemical testing by measuring PAPP-A at 9 weeks' gestation and free β-hCG at the time of the first ultrasonography scanning session at 12 weeks' gestation, or even later, which gives an estimated detection rate of 95% with an FPR of 5%.[12]

All three syndromes are associated with increased maternal age, increased fetal NT and decreased maternal serum PAPP-A, but whereas maternal serum free β-hCG is increased in a Down syndrome pregnancy it is decreased in in pregnancies affected by Edwards and Patau syndromes. In cases of sex chromosomal anomalies, the level of maternal serum free β-hCG is normal whereas the level of PAPP-A is low. In diandric triploidy, the level of maternal serum free β-hCG is greatly increased, whereas the level of PAPP-A is mildly decreased. Digynic triploidy is associated with markedly decreased levels of maternal serum free β-hCG and PAPP-A. Screening for chromosomal defects in the first, rather than the second, trimester provides earlier reassurance for those with normal results and a less traumatic termination for those choosing this option. A potential disadvantage of early detection of chromosomal anomalies is that an earlier assessment of risk and prenatal diagnosis identifies the pregnancies that are more likely to miscarry. Approximately 30% of Down syndrome fetuses miscarry between 12 weeks' gestation and full term. This issue of intrauterine lethality for chromosomally defected fetuses is a potential criticism of all methods of antenatal screening, including second-trimester maternal serum biochemistry.

TABLE 3 Ultrasonography and biochemical characteristics of fetuses with normal chromosomes and of those with Down syndrome, Edwards syndrome and Patau syndrome. Reproduced from Kagan KO, Wright D, Valencia C, Maiz N, Nicolaides KH. Screening for trisomy 21, 18 and 13 by maternal age, fetal nuchal translucency, fetal heart rate, free beta hCG and pregnancy-associated plasma protein-A. *Hum Reprod* 2008; 23:1968–75[9] by permission of Oxford University Press

	Fetal NT (mm)	PAPP-A MoM	Free β-hCG MoM
Normal karyotype	2.0	1.0	1.0
Down syndrome	3.4	0.5	2.0
Edwards syndrome	5.5	0.2	0.2
Patau syndrome	4.0	0.3	0.5

Second-trimester screening

Two screening tests are performed during the second trimester: the triple and the quadruple screening test. The triple screening test has been available since the late 1980s as a cost-effective serum pregnancy screening test for Down syndrome, neural tube defects and Edwards syndrome.[27] A combination of maternal age with serum α-fetoprotein (AFP), total hCG and unconjugated estriol (uE3) can identify approximately 60–65% of affected pregnancies with a 5% FPR[28] (Table 4). Although the triple screening test can be performed between

TABLE 4 Screening strategies for Down syndrome

Method of screening	Detection rate (%)
Maternal age	30
First-trimester screening (10–14 weeks)	
Maternal age plus NT measurement (by ultrasound)	75–80
Maternal age plus first-trimester double test (PAPP-A, β-hCG)	60–70
Maternal age plus first-trimester combined test (NT, PAPP-A, β-hCG)	85–96
Second-trimester screening (14–22 weeks)	
Maternal age plus second-trimester double test (AFP, hCG)	55–60
Maternal age plus triple test (AFP, hCG, uE3)	60–65
Maternal age plus quadruple test (AFP, hCG, uE3, inhibin A)	65–80
Integrated test (first trimester: NT, PAPP-A and β-hCG; second trimester: quadruple test)	90–95
Prenatal diagnosis	
Amniocentesis (14–16 weeks' gestation)	100
CVS (10–12 weeks' gestation)	100
Fluorescence *in situ* hybridization	100

15 and 22 weeks' gestation, it is most effective when conducted at 15–16 weeks' gestation as this allows simultaneous assessment of AFP as an additional screen for neural tube defects. This method of risk calculation was devised by Wald *et al*.[28] and involves multiplying the age-specific risk, as an odds ratio, by the likelihood ratio which was derived from the approximate trivariate Gaussian frequency distribution of the serum markers. Maternal weight, twin pregnancy and the presence of insulin-dependent diabetes mellitus also affect the serum markers for the triple test. Increasing maternal weight is associated with a lowering of maternal serum AFP (MSAFP), uE3 and hCG levels. Wald *et al.*[28] have reported that routine maternal weight adjustments for the serum marker levels can increase the detection rate by approximately 0.5% for a given FPR, or can reduce the FPR by 0.1% for a given detection rate. In comparison with singleton pregnancies, in twin pregnancies a median MSAFP is 2.1 times higher, median uE3 level is 1.7 times higher and median hCG level is 1.8 times higher. In women with insulin-dependent diabetes mellitus, the MSAFP, uE3 and hCG levels are 0.7, 0.92 and 0.95 times higher, respectively, than in women who do not suffer from insulin-dependent diabetes mellitus. Several studies[27,28] have shown a significant correlation between the results of the triple screening test showing abnormal markers and preterm labour, pre-eclampsia, intrauterine growth restriction and premature rupture of the membranes. Recently, the maternal serum quadruple screen has been replacing the triple screen as it provides greater sensitivity (80% detection rate with an FPR of 5% for Down syndrome).[29] The quadruple test can be performed on antenatal patients between 15 and 22 weeks' gestation and includes the triple-screen serum markers with an additional marker, inhibin A, which is initially produced by the corpus luteum and later by the placenta.

Alpha-fetoprotein

Alpha-fetoprotein is a glycoprotein with a molecular weight of 68 kDa produced by the fetal yolk sac, liver and gastrointestinal tract. This protein was first discovered by Bergstrand and Czar in 1956[30] and subsequently named by Gitlan[31] as α-fetoprotein. AFP has approximately 4% carbohydrate moiety represented by one oligosaccharide residue.[32] The protein moiety has been completely determined and consists of one polypeptide chain of 590 amino acids arranged in three well-defined domains.[33]

Alpha-fetoprotein is produced by the fetal yolk sac in small quantities and by the fetal liver in large quantities as the yolk sac degenerates. The fetal liver produces AFP until 30 weeks' gestation and then stops abruptly. Fetal serum levels peak at about 9 weeks' gestation (at approximately 3 million µg/l) and decline progressively until term, when the final level of fetal serum is approximately 20 000 µg/l. AFP is initially detectable at a level of approximately 5 µg/l in the maternal serum at about 10 weeks' gestation.[34] The concentration increases at an approximate rate of 15% per week to peak at approximately 180 µg/l at about 25 weeks' gestation.[34] The concentration of AFP in the maternal serum subsequently declines slowly until term. After birth, the MSAFP rapidly decreases to < 2 µg/l and the levels of serum AFP in the baby decline exponentially to reach adult concentration by the 10th month of life.[34]

Laboratory measurements of AFP levels are reported as MoM. Maternal AFP is said to be elevated when the value is ≥ 2.5 MoM for a single fetus and ≥ 4.5MoM for a pregnancy with two or more fetuses. The MSAFP is elevated in 85–95%[35] of cases in which the fetus has open neural tube defects whereas the MSAFP

is lowered in approximately 30%[36,37] of cases where the fetus has Down syndrome. In addition to neural tube defects, such as spina bifida and anencephaly (failure of brain and skull development), the causes of elevated MSAFP to a level of > 2.5 MoM include fetal death, misdated pregnancies, other anatomical abnormalities (such as abdominal wall defects), placental abnormalities (such as chorioangiomas, placental lakes and placental oedemas), fetal growth restriction, pre-eclampsia, preterm delivery, stillbirth, infection and hypoxia.[38,39] In fetuses with open neural tube defects (such as spina bifida) the normal integumentary covering at the site of the lesion is absent, which allows abnormally large quantities of AFP to leak into the amniotic fluid and subsequently into the maternal serum. As this phenomenon also occurs in other non-neural tube lesions, other morphological abnormalities, such as abdominal wall defects and cystic hygromas, also demonstrate an elevated level of AFP in the amniotic fluid and the maternal serum that can be detected during screening.

In 1984, Merkatz *et al.* reported that the MSAFP in the second trimester of pregnancies affected by fetal Down syndrome was lower (< 0.7 MoM) than that of normal pregnancies.[37] Lower levels of AFP (< 0.25 MoM) may be seen not only in patients who are not pregnant, but also in those affected by fetal death, spontaneous abortion, preterm birth, stillbirth or macrosomia or have a misdated pregnancy, a hydatidiform mole or trisomic fetus or who are going through a normal pregnancy.

Human chorionic gonadotropin

Bogart *et al.*[40] reported an elevation of maternal serum hCG levels in Down syndrome pregnancies, and since then the levels of hCG have been measured in most screening programmes. hCG is a complex glycoprotein produced exclusively by the syncytiotrophoblast shortly after zygote implantation into the uterine wall. The level of hCG increases rapidly in the first 8 weeks of gestation and then decreases steadily until 20 weeks' gestation, when it plateaus. Maternal weight and parity have an effect on hCG levels and high maternal serum levels of hCG accompanied by low levels of MSAFP have been associated with an increased risk of carrying a fetus with Down syndrome. The geometric mean MoM for Down syndrome pregnancies determined from the results of 18 studies, comprising a total of 559 Down syndrome cases, was 2.03 MoM.[41] Although the precise explanation for this finding is unknown, it may be due to fetuses with Down syndrome showing delayed development. Since maternal serum levels of hCG decline between 12 and 20 weeks' gestation, an immature fetus with Down syndrome will be associated with a higher concentration of hCG than an unaffected pregnancy. A low hCG level is associated with Edwards syndrome and normal hCG levels are associated with neural tube defects. Increased hCG levels are found in placental anomalies (such as molar pregnancies), multiple pregnancy or fetal demise. Unexplained elevation of second-trimester hCG has been seen in hypoxic cytotrophoblasts; however, the exact cause is not known.[21]

Unconjugated estriol

Estriol is produced in very large quantities during the final trimester of pregnancy. The biosynthesis pathway requires three organs to be fully functioning: the fetal adrenal glands, the fetal liver and the placenta. uE3 is produced by the placenta from precursors that are created in the fetal adrenal glands and the fetal liver. Estriol diffuses from the placenta into the maternal blood, where it can be measured as uE3. The levels of maternal serum uE3 rise above non-pregnancy levels by 7–9 weeks' gestation and continue to increase throughout pregnancy. The level of maternal serum uE3 is approximately 4 nmol/l at 15 weeks' gestation and increases to approximately 40 nmol/l by parturition.[38,42] Any disruption in the biosynthesis pathway will lead to very low level of maternal serum uE3. Conditions that cause such disruption include fetal anencephaly, molar pregnancy, placental steroid sulphatase deficiency, fetal death, and chromosomal or congenital anomalies such as Down syndrome, Smith–Lemli–Opitz syndrome and Edwards syndrome.[43,44]

Low levels of maternal uE3 in the third trimester have also been reported in newborns with low birthweight and have been found to indicate fetal distress. Decreased uE3 levels (< 0.5 MoM) have been found to be significantly associated with pregnancy-induced hypertension, miscarriage, intrauterine growth restriction and intrauterine fetal death.[45] Maternal serum uE3 levels are significantly lower in Down syndrome pregnancies than non-Down syndrome pregnancies, ranging from 0.65[46] to 0.79 MoM.[47,48] Amniotic fluid and placental tissue uE3 levels are

also significantly lower in a Down syndrome-related pregnancy. uE3 is a variable that is independent of maternal age and therefore can be used alone or in combination with maternal age for the determination of the relative risk of Down syndrome.

Dimeric inhibin A

Inhibins are circulating dimeric glycoprotein hormones synthesized by the gonads and the placenta. These glycoproteins were first isolated from the ovarian follicular fluid and named after their ability to inhibit the pituitary secretion of follicle-stimulating hormone. The α-subunit can combine with one of the two β-subunits (βA or βB) to form inhibin A or inhibin B. Dimeric inhibin A (DIA) concentrations exhibit a complex pattern during the course of pregnancy, rising to a peak at 8–10 weeks' gestation and then declining to a minimum at 17 weeks' gestation, before beginning to slowly increase towards term. The average inhibin levels do not change greatly from 15 to 20 weeks' gestation and a typical value of DIA at 17 weeks' gestation is 175 ng/l.[34]

The maternal serum levels of inhibin A in the second trimester of pregnancy are twice as high in pregnancies affected by Down syndrome as in those that are not affected. Studies[49,50] have reported DIA values ranging from 1.53 to 2.60 MoM in Down syndrome pregnancies. Inhibin A is as effective a marker for Down syndrome as hCG, and yet it also provides information that hCG and the other markers do not.[51–54] The level of inhibin is significantly decreased in the presence of primary antiphospholipid antibodies syndrome (median MoM 0.6) and it has also been described as extremely elevated in pregnancies complicated by triploidy, HELLP syndrome (where the patient suffers from haemolysis, elevated liver enzymes and a low platelet count) and following the loss of one twin in the first trimester.[17]

Clinical application of the triple and quadruple analyte-screening test

Many biochemical markers have been studied during the second trimester of pregnancy, but the triple test, which analyses serum AFP, hCG, uE3 and maternal age, is the most popular combination. The triple test has a sensitivity of approximately 65% for Down syndrome and 70% for Edwards syndrome.[28] In Down syndrome pregnancies, the levels of hCG increase whereas levels of AFP and uE3 decrease (Table 5). In the case of Edwards syndrome, the levels of all three analytes are low. In twin pregnancies, second-trimester serum screening detects approximately 50% of affected fetuses and can be difficult to interpret because a normal twin may mask the results of an affected twin.[55] Recently, a quadruple-screening test has been developed, so named because it uses four biochemical markers (AFP, hCG, uE3 and DIA).[56] The combination of maternal age and the quadruple screening test detects approximately 75% of Down syndrome fetuses in women who are younger than 35 years with an FPR of 5%, and it detects >80% of the Down syndrome fetuses in women who are over the age of 35 years with an FPR of 5%.[29] In most cases of Down syndrome, the AFP and uE3 levels are low, whereas hCG and DIA levels are high compared with a non-Down syndrome pregnancy.

TABLE 5 The second-trimester biochemical markers

	AFP	uE3	Total hCG	Inhibin A
Neural tube defects	↑	NA	NA	NA
Down syndrome	↓	↓	↑	↑
Edwards syndrome	↓	↓	↓	NA

NA, not applicable.

Screening in the second trimester using multiple markers does not reliably detect the other forms of aneuploidy that can occur as result of increasing maternal age, such as Patau syndrome (1 in 20 000 live births) and Klinefelter syndrome (47,XXY; 1 in 1000 live births). These forms of aneuploidy would be detected by amniocentesis and CVS.

The laboratory conducting the tests must be informed of the gestational age of the fetus at the time the sample was taken to ensure an accurate interpretation. Gestational age is usually calculated from the first day of the last menstrual period; however, ultrasonographic measurement of the CRL in the first trimester, or measuring the biparietal diameter of the fetus in the second trimester, provides a more reliable estimate of gestational age, accurate to within 7 and 10 days, respectively. If ultrasonography results in a change in gestational age of more than10 days, then the test results must be reinterpreted. If the sample was taken at

< 15 weeks' gestation then a new sample should be obtained and analysed in accordance with the correct gestational age.

First- and second-trimester screening for aneuploidy

There have been several different approaches proposed to improve screening detection rates, many of which take advantage of both first- and second-trimester screening tests: first, the integrated screening test, which combines maternal age with NT measurement, PAPP-A and free β-hCG in the first trimester; and, secondly, triple and quadruple screening in the second trimester. These tests are reported to be more effective and accurate than all other tests, yielding very good results, with detection rates of 94% for the first-trimester tests and 85% for the second-trimester tests, with an FPR of 5% and 1%, respectively.[57] The disadvantage of these screening methods is that a portion of woman may fail to attend the second-trimester test. Additionally, the results of these tests are not obtained until after 16 weeks' gestation and a termination of the pregnancy at this stage can be traumatic since it usually requires a medical abortion rather than surgical and the mother may have already felt the fetal movements. Another screening method is stepwise sequential screening, in which all patients have a first-trimester NT test, serum PAPP-A and free β-hCG tests and the resulting high-risk patients are offered CVS whereas low- or intermediate-risk patients have a second-trimester AFP test, a uE3 test, free β-hCG and inhibin tests. If the combined risk from first- and second-trimester testing is high, patients are offered second-trimester amniocentesis. An alternative option is the contingent screening method, which is similar to stepwise sequential screening except that second-trimester biochemical testing is performed only in women in whom first-trimester screening suggests an intermediate risk of fetal anomalies.[10] The estimated performance of the three approaches is similar, with an overall detection rate of 90–94% and an FPR of 5%. The advantages of the contingent screening method are twofold: first, second-trimester testing can be avoided in 75–80% of patients; and, secondly, approximately 60% of fetuses with aneuploidies can be identified during the first trimester.[10]

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