SEQUENTIAL TESTING STRATEGIES IN PRENATAL SCREENING FOR DOWN'S SYNDROME

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ABSTRACT

Down's syndrome is a classic chromosomal disorder with an incidence rate of one in every 750 live births. Early detection of Down's syndrome pregnancies through screening will provide the option of early termination of pregnancy and better obstetric care to women with affected pregnancies. Some of the screening policies which have been implemented in the UK are second trimester double, triple or quadruple marker tests, first trimester combined ultrasound and biochemical (CUB) screening, and integrated screening. Screening performance can be optimized by applying appropriate correction factors for variables such as maternal smoking, ethnicity and assisted conception. Typical screening performance is around 70% detection of Down's syndrome pregnancies at a 5% false positive rate for second trimester quadruple marker screening, 90% detection at a 5% false positive rate for CUB screening and 90% detection at a 1-2% false positive rate for integrated screening. The NHS Fetal Anomaly Screening Programme Committee has set a current performance target for Down's syndrome screening of at least 75% detection at a 3% or lower false positive rate and this can be achieved by CUB or integrated testing by setting a threshold (cut-off) risk of 1 in 150 at term. However, further improvements in performance proposed by the Committee to meet a detection rate of 90% at a false positive rate of 2% or less are unlikely to be reached by single stage testing, and protocols which include some element of sequential testing are required. The Health Technology Assessment Programme is currently reviewing two new approaches to screening, namely, repeated measure and cross trimester testing to evaluate their potential to meet the more challenging standard.

In the present study, using various combinations of maternal serum marker and ultrasound measurements, several screening strategies and refinements are explored to establish their potential for improving detection rates and reducing false positive rates in Down's syndrome screening. Extensive use has been made of routinely collected screening data from the west of Scotland Regional Screening programme for retrospective analysis using standard Gaussian methods, statistical modeling and SPSS and S-PLUS statistical software. The performance of within- and across-trimester contingent screening programmes have been evaluated and the effects of ethnicity, maternal smoking habit and assisted reproductive technology (ART) on screening markers has been assessed using first and second trimester samples.

Screening within the first trimester

The standard approach to CUB screening is to carry out maternal serum marker measurements (PAPP-A and fβhCG) and ultrasound Nuchal Translucency measurements at 11-13+6 weeks of gestation. This study had also shown that in the CUB screened population in the west of Scotland, adopting a within-trimester contingent screening protocol where all women have serum marker testing but only those women with intermediate risks from the serum markers are offered NT, would have achieved a detection rate of 88.7% at a false positive rate of 5.8% with 29% of women requiring an NT measurement. Using LMP based gestational age this screening protocol would have achieved a detection rate of 83.3% at a false positive rate of 7.4% with 25.9% of women requiring an NT measurement. When analysis was performed only on pregnancies with certain LMP dates, the contingent screening protocol would have achieved a detection rate of 88.9% at a false positive rate of 7.0% with 25.3% of women requiring an NT measurement. Where ultrasound resources are scarce within-trimester contingent screening

has the potential to maintain screening performance whilst reducing the number of NT scans required.

Across –trimester screening

Evidence suggests that sequential testing strategies can improve screening performance. This has been explored in this study by statistical modelling using S-PLUS. Various combinations of markers were tested. It was estimated that optimal performance could be achieved by a cross-trimester contingent screening protocol with repeat measures of PAPP-A (NT, PAPP-A, fβhCG in the first trimester followed by AFP, hCG, InhA, uE3, PAPP-A in the second trimester in a sub-set of women with intermediate risks). This could achieve a detection rate of 92.2% at a false positive rate of 1.4% but with only 9.7% of women requiring a second trimester screening test. This meets the aspirational performance standard proposed by the UK NSC. Without NT measurements (i.e. serum only screening), the model indicates that this screening protocol would achieve a detection rate of 86.2% at a false positive rate of 3.0% with 22.3% of women requiring a second trimester screening test. Therefore, the inclusion of NT measurement at the first stage of testing is necessary to achieve the desired performance.

The Effects of Smoking and Ethnicity

Many maternal and pregnancy factors are known to affect serum marker concentrations and small but useful improvements in screening performance can be made by correcting for these. Changes however, vary between trimesters and in this study paired first and second trimester samples have been used to measure the changes in serum marker levels in smokers and between different ethnic groups at each stage of pregnancy.

In this study, the AFP level in smokers was increased in the first trimester by 16.3% when compared with the non-smokers. The hCG level in smokers was decreased by 27.6% and 30.5% in the first and second trimesters respectively. The fβhCG level was decreased in smokers in the second trimester by 17.1% when compared with non-smokers. The PAPP-A level was decreased by 14% and 22.8% in first and second trimesters respectively when compared with non-smokers. These results demonstrate that the effect of smoking is gestation dependant and without appropriate correction factors being applied, these serum marker changes would result in inappropriate risks being estimated for individual women.

The study on the effect of ethnicity on screening markers has shown that South Asian women had higher hCG levels in the first trimester compared with Caucasian women. They also had lower fβhCG and PAPP-A in the second trimester. Oriental women had higher first and second trimester hCG levels when compared with Caucasian women. They also had higher fβhCG and PAPP-A levels in the first trimester. Middle East women had lower first trimester AFP when compared with Caucasian women. Black women had higher hCG in the first trimester when compared with Caucasian women. In Black women, the PAPP-A level was also elevated in both trimesters. While this study confirms that correction for ethnicity is clearly indicated, appropriate correction factors are difficult to derive as there is likely to be some variation in the classification of ethnicity between studies.

Assisted Reproductive Technology

The growing use of ART in developed countries and the variety of different methods employed make accurate correction factors desirable but difficult to derive. In this study, women pregnant after ART had larger NT measurements compared with women who had conceived spontaneously. The PAPP-A level was lower in the IVF or ICSI with fresh eggs

group when compared with the controls. Among the ART treatment groups, the NT was higher in the IVF or ICSI with fresh eggs group when compared with the controls. The AFP level was higher in the IVF with donor's egg group when compared with the controls. The hCG level was higher in the ART group overall when compared with the controls. Women pregnant after IVF or ICSI with fresh eggs and frozen eggs had higher hCG level.

Smoking frequency, birthweight and prematurity

In addition to its effects on serum marker concentrations, smoking in pregnancy is known to be associated with low birth weight and prematurity. It is important therefore that maternal smoking is accurately recorded on screening request forms and in this study, the accuracy of self reported smoking status was assessed by analysis of cotinine in serum. Results showed that the percentage of self-reported smokers (24.1%) at booking was significantly lower than the cotinine-validated estimate of 30.1%. Also, smoking was associated with low birth weight, delivery prior to 39 weeks, increased AFP level (3.1%) and reduced hCG level (28.7%) in the second trimester. An increasing AFP level (but not hCG level) was associated with lower birth weight and delivery prior to 39 weeks in both smokers and non smokers but the effect was most marked in smokers. The difference in birth weight between the highest and the lowest AFP category for non-smokers was 448.3g and for smokers was 619.2g, suggesting that smoking exacerbates the effect of an elevated AFP on birth weight. Overall the difference in birth weight between the lowest AFP category in non smokers and the highest AFP category in smokers was 931.6g.

Summary

In summary, this study has shown that a cross-trimester contingent screening protocol with repeat measures has the potential to meet the UK NSC aspirational standard of 90% detection of Down's syndrome pregnancies with a screen positive rate of less than 2%. Around 90% of women would complete screening in the first trimester without the need for a second stage sequential test. Correcting for factors such as maternal smoking habits, ethnicity and ART would further improve screening performance. Also it has been shown that where ultrasound resources are scarce, within-trimester and across-trimester protocols can reduce the need for NT measurement in all women and still deliver excellent screening performance although this falls short of the higher performance standard. The potential of these new screening protocols now need to be tested in prospective multicentre trials to confirm their performance in prospective practice.

DECLARATION

I declare that this thesis has been composed, along with the work described herein, by myself, Thenmalar Vadiveloo.

The contents of this thesis have not been submitted for any other degree or professional qualification. All sources of information have been acknowledged.

THENMALAR VADIVELOO
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SPECIALLY DEDICATED AT THE LOTUS FEET OF BHAGAVAN SRI SATHYA SAI BABA

"Thank you swami for leading and guiding me in completing this thesis"

"The end of education is character" - Sri Sathya Sai Baba

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PUBLICATIONS

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ORAL PRESENTATIONS

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POSTER PRESENTATIONS

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LIST OF ABBREVIATIONS

ADAM12	-	A Disintegrin And Metalloprotease 12
AFP	-	Alpha-fetoprotein
ART	-	Assisted reproductive techniques
BPD	-	Bi-parietal diameter
CRL	-	Crown rump length
CUB	-	Combined ultrasound and biochemical
CV	-	Coefficient of variance
CVS	-	Chorionic villus sampling
DHEA	-	Dehydroepiandrosterone
DR	-	Detection rate
ECR	-	Early completion rate
FASTER	-	First- and Second-Trimester Evaluation of Risk
fβhCG	-	Free beta human chorionic gonadotrophin
FMF	-	Fetal Medicine Foundation
FPR	-	False positive rate
hCG	-	Human chorionic gonadotropin
HRP	-	Horseradish peroxide
ICSI	-	Intracytoplasmic sperm injection
InhA	-	Inhibin A
IVF	-	*In vitro* fertilization
kD	-	Kilodalton

LMP	-	Last menstrual period
MoM	-	Multiple of the median
mL	-	Millilitre
mU/L	-	Milliunits per litre
ng/ml	-	Nanograms per millilitre
NS	-	Non-smoker
NSC	-	National Screening Committee
NT	-	Nuchal translucency
OSCAR	-	One-stop clinic assessment of risk
PAPP-A	-	Pregnancy associated plasma protein - A
PIV	-	Pulsatility index for veins
proMBP	-	Proform of eosinophil major basic protein
QF-PCR	-	Quantitative fluorescence polymerace chain reaction
Rpm	-	Revolutions per minute
S	-	Smoker
SD	-	Standard deviation
SMR	-	Scottish Morbidity Records
SP-1	-	Pregnancy-specific-beta-1-glycoprotein
STDUR	-	Stopped smoking during pregnancy
STPR	-	Stopped smoking prior to pregnancy
SURUSS	-	Serum Urine and Ultrasound Screening Study
uE3	-	Unconjugated estriol
U/ml	-	Unit per millilitre
µL	-	Microlitre

CHAPTER 1: INTRODUCTION

1.1 BACKGROUND

Down's syndrome, a classic chromosomal disorder resulting in mental retardation and severe congenital disorders, was the first medical condition to be associated with a chromosomal abnormality. With the incidence rate of one in every 750 live births, early detection through screening is imperative to help in prenatal diagnosis of Down's syndrome. This will provide the option of early termination of pregnancy and better obstetric care to the women with Down's syndrome pregnancies (Gardner and Sutherland, 2004; Roper and Reeves, 2006).

The Down's Syndrome Screening Programme was started under the UK National Screening Committee (NSC). The UK NSC sets standards and oversees the implementation of screening programmes in England. The committee was set up in 1996. The recommended screening strategies from 2007 are the first trimester combined ultrasound and biochemical (CUB) screening, integrated testing and serum integrated testing. The Health Technology Assessment is currently reviewing two new strategies for screening, namely, repeated measure and cross trimester testing. These tests are expected to further improve the performance of Down's syndrome screening programmes in the period after 2010 (NHS Fetal Anomaly Screening Programme, 2008).

1.2 DOWN'S SYNDROME

The earliest mention of this disorder was made by John Langdon Down in 1866. Down described this disorder as 'Mongolian Idiocy' in an essay classifying mental handicaps. However, the cause of the disorder remained unknown until 1959, when a French cytogeneticist, Jerome Lejeune, discovered trisomy 21 as the cause of this genetic

abnormality. Subsequently, the condition was renamed as 'Down's Syndrome' in 1961, after John Langdon Down (Chudley and Chodirker, 2003)

1.2.1 INCIDENCE RATE OF DOWN'S SYNDROME

Down's syndrome, a classic chromosomal disorder, was the first medical condition to be associated with a chromosome abnormality in 1959 (Lejeune *et al*., 1959). In the absence of prenatal intervention, one in 750 live births in a typical population is affected by this chromosomal disorder (Gardner & Sutherland, 2004; Roper and Reeves, 2006). According to the Scottish Perinatal and Infant Mortality and Morbidity Report 2007, the rate of Down's syndrome in Scotland was 1.02 in 1000 births (1 in 980), during the period of 2002 to 2006 (Information Services Division NHS Scotland, 2008) and this lower incidence reflects the impact of screening and prenatal diagnosis. A large number of Down's syndrome pregnancies are sufficiently viable to survive to term (Cuckle, 2005). At conception, the frequency of Down's syndrome is much higher. Nearly 75% of the Down's syndrome fetuses identified during the first trimester, and about 50% of those identified during the second trimester are lost before the completion of the pregnancy term (Roper and Reeves, 2006). Advanced maternal age is the strongest risk factor linked to the cause of Down's syndrome pregnancies. The birth prevalence increases from 0.6 to 4.1 per 1,000 between the age of 15 and 45. This risk increases even more with a previous history of a Down's syndrome pregnancy (Cuckle, 2005).

1.2.2 PHENOTYPE OF DOWN'S SYNDROME

Down's syndrome is associated with variable phenotypes. However, mental retardation, neonatal hypotonia, small and hypocellular brain and minor facial dysmorphic features such as small nose, up-slanting palpebral fissures, speckling of iris (Brushfield spots), flat facial profile, low set ears, single palm crease, wide gap between the first and second toes and shortened fifth finger can be seen in almost all individuals with Down's syndrome (Korenberg *et al*., 1994).

Those with Down's syndrome also suffer from other congenital abnormalities such as heart defects and gastrointestinal abnormalities. A study conducted by Hayes *et al* (1997) in Dublin showed that heart defect is the most common abnormality among children (found in 45.8%) with Down's syndrome followed by gastrointestinal disorders. This finding was consistent with another study conducted in Strasbourg by Stoll *et al* (1998). Other abnormalities such as urinary tract malformation, limb defects and congenital cataract have also been reported along with Alzheimer disease in those surviving beyond the age of 40 (Hayes *et al*., 1997; Stoll *et al.*, 1998; Noble, 1998; Baliff and Mooney, 2003).

1.2.3 CYTOGENETICS OF DOWN SYNDROME

Over 95% of Down's syndrome cases are caused by trisomy 21, where the cells in the body have three copies of chromosome 21 instead of the normal two. Studies have shown that non-disjunction at maternal meiosis 1 is the primary cause of most trisomy 21 cases (Robinson, 1977; Sherman *et al*., 1994; Noble, 1998). Non-disjunction occurs when homologous chromosomes fail to segregate symmetrically at cell division. This causes one daughter cell to have two copies of chromosome 21 and the other have none (Gardner & Sutherland, 2004). Figure 1.1 illustrates the classic view of the mechanism of non-disjunction. The other causes of Down's syndrome are Mosaicism and Robertsonian chromosomal translocation.

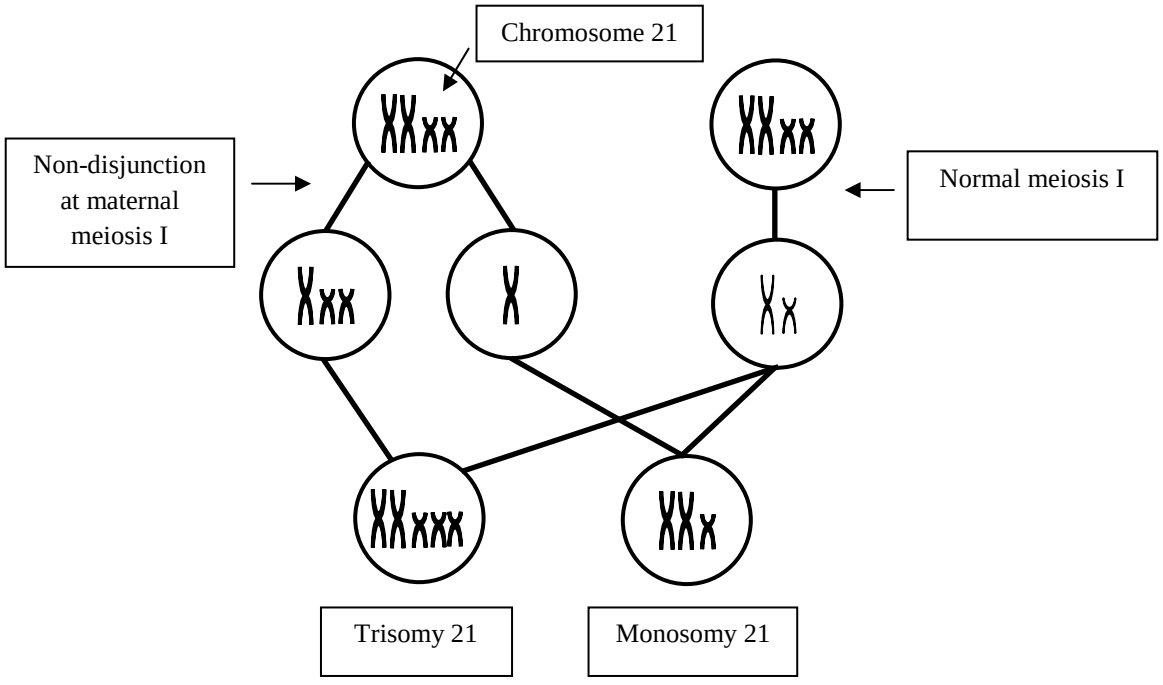


Figure 1.1: The mechanism of non-disjunction in Trisomy 21

(Reproduced from http://www.perinatal.nhs.uk/car/anomaly/chromosome/downs.htm)

1.3 PRENATAL DIAGNOSIS

Prenatal diagnosis allows the option of termination of pregnancy or better obstetric care and planned delivery for the pregnancy. Prenatal diagnosis may be offered to women who are identified as high risk through a screening test, who are in advanced childbearing age, or who have had a previous child with a chromosome abnormality.

1.3.1 AMNIOCENTESIS

Prenatal diagnosis of chromosomal disorders using amniocentesis has been well established since the early 1970s. In second trimester amniocentesis, which is performed around 16 weeks of gestation, a needle is inserted through the abdominal wall ideally under ultrasound guidance into the amniotic cavity and a sample of amniotic fluid (20mls) is collected. The fetal cells from the amniotic fluid can then be cultured and karyotyping performed. The disadvantage of this diagnostic procedure is that the results are available only after 16 weeks of gestation as cell culture and karyotyping may take 2 to 3 weeks. The long waiting period for the diagnostic results can cause anxiety among the pregnant women and termination of pregnancy is more difficult and traumatic at late stages of pregnancy (Alfirevic *et al*., 2003; Gardner & Sutherland, 2004).

Early amniocentesis, which is performed at 9 to 14 weeks of gestation, was first introduced in the late 1980s. This diagnostic procedure is the same as the second trimester amniocentesis. Ultrasound was considered essential to guide the needle into the amniotic cavity due to the small target area (Alfirevic *et al*., 2003; Gardner & Sutherland, 2004). Studies however have found that fetal loss rate in early amniocentesis (2.2%) was greater than in second trimester amniocentesis (0.6%) (Nicolaides *et al*., 1994b; Daniel *et al*., 1998; Collins *et al*., 1998) and this method has generally been abandoned. Early

amniocentesis also has an adverse effect on perinatal lung function. Yuksel *et al* (1997) reported that infants whose mothers had had early amniocentesis during pregnancies had higher thoracic gas volume (TGV) and lower functional residual capacity (FRC) than infants whose mother had undergone no invasive diagnosis procedure.

1.3.2 CHORIONIC VILLUS SAMPLING

Chorionic villus sampling (CVS) is a first trimester diagnostic procedure performed at 10 to 11 weeks of gestation. This procedure was first developed in China in the mid 1970s and then expanded to the Western countries in 1980s (Alfirevic *et al.,* 2003). In CVS, the sampling of placental tissue is done using percutaneous transabdominal or transvaginal / transcervical method with ultrasound guidance. The transabdominal technique is the most commonly used method now. The early diagnosis of chromosomal abnormalities permits pregnant women to access early pregnancy termination (Alfirevic *et al*., 2003; Gardner & Sutherland, 2004). However, this diagnostic procedure has a risk of fetal loss of 1.5-2% (Brun *et al.,* 2003).

1.3.3 RAPID DIAGNOSTIC TECHNIQUES APPLIED TO AMNIOCENTESIS & CVS

The standard karyotype analysis involves cell culture, harvesting of dividing cells, staining and the analysis of chromosome banding. In the UK, the average reporting time using this analytical method is 13 to 14 days (NEQAS, 2000). It was the need for a quick and rapid method for the detection of chromosomal abnormalities that led to the development of fluorescence in situ hybridisation (FISH) and quantitative fluorescence polymerase chain reaction (QF-PCR) techniques. FISH uses chromosome-specific probes with fluorescent labels attached for detection of fetal chromosomal abnormalities. QF-PCR is based on the

amplification of repeat sequences at the polymorphic loci. The application of FISH and QF-PCR enables diagnosis and reporting of chromosomal abnormalities within 24-48 hours of sample receipt (Pertl *et al.,* 1999; Mann *et al*., 2001; Grimshaw *et al*., 2003; Nicolini *et al*., 2004).

1.4 PRENATAL SCREENING

The development of screening for fetal abnormalities has greatly improved the prenatal care in many developed countries. According to Wald (1994), screening is defined as 'The systematic application of a test or inquiry, to identify individuals at sufficient risk of a specific disorder to benefit from further investigation or direct preventative action, among persons who have not sought medical attention on account of symptoms of the disorder'. Women who are screened positive are generally offered counselling and a diagnostic test.

In the 1970s, screening for Down's syndrome was performed based on advanced maternal age. The women, who were pregnant at the age of 35 or above, were offered diagnostic testing through amniocentesis (Benn, 2002; Powell and Grudzinskas, 1995). Due to the small but distinct risk of pregnancy loss following amniocentesis and the inability to detect Down's syndrome pregnancies in women who were aged less than 35 years, efforts were made to develop a screening test which could be offered to all women and identify those who are at high risk of fetal aneuploidy (Powell and Grudzinskas, 1995). The estimated rate of Down's syndrome rises from about 0.6 per 1000 (1 in 1667) at age 20 to about 1.1 per 1000 (1 in 909) at age 30, 3.2 per 1000 (1 in 313) at age 35, 11.1 per 1000 (1 in 90) at age 40 and 40.5 per 1000 (1 in 25) at age 45 (Hook, 1981).

All pregnant women are therefore at risk of having a pregnancy with a chromosomal abnormality. When a pregnant woman opts into a screening programme, her individual risk

is calculated based on the 'a priori risk', which depends on maternal age, gestational age, and the screening test results. The 'a priori risk' is multiplied by the likelihood ratio derived from the screening test, to determine the patient-specific risk. The 'a priori risk' generally increases with maternal age and decreases with advancing gestation. This is because fetuses with chromosome abnormalities are more likely to die in utero compared to normal fetuses (Hook, 1981; Ferguson-Smith and Yates, 1984; Snijders *et al*., 1994; Snijders *et al*., 1999; Nicolaides, 2004).

1.4.1 MATERNAL AGE RISK

With the development of prenatal screening, a need for maternal age-specific prevalence rates arose. A maternal age-specific rate schedule developed by Cuckle *et al* (1987) is widely employed for the purpose. The maternal age-specific risk schedule was developed by plotting a regression curve using the combined results of eight large, published surveys of Down's syndrome in live births. It was widely used in risk calculation and was embedded in many computer programmes used in routine screening. The widespread use of this rate schedule and the need for accurate maternal age-specific rates of Down's syndrome, led to further critical re-evaluations of this data (Hecht and Hook, 1994). Subsequently, Hecht and Hook (1996) reported that the schedule in their study predicted higher rates than those predicted by Cuckle *et al* (1987), particularly in older women and proposed an alternate rate schedule. This finding was confirmed by Bray *et al* (1998) using meta-analysis of nine data sets to estimate maternal age-specific risk. In 1998, Cuckle investigated the effect of using different maternal age-specific prevalence curves on detection rate, for three second trimester screening protocols. Cuckle (1998) concluded that the inaccuracy caused by the use of different maternal age curves is unlikely to markedly influence the Down's syndrome screening result.

Pregnancies with Down's syndrome are likely to end in spontaneous fetal loss. Therefore, the risk of having pregnancy with Down's syndrome changes with gestational age. In 1999, Morris *et al* investigated the fetal loss rates in Down's syndrome pregnancies using data from National Down's syndrome Cytogenetics Register. Based on this study together with two other previous studies (Macintosh *et al.*, 1995; Halliday *et al.*, 1995), Morris *et al* (1999) reported that nearly 43% of pregnancies ended in a miscarriage or still birth between the time of CVS and term, and about 23% of miscarriages or still births occurred between the time of amniocentesis and term and 12% of births were stillborn or resulted in a neonatal death. A later study by Savva *et al* (2006) on the relationship between maternal age and the risk of spontaneous fetal loss in Down's syndrome pregnancies confirmed that the fetal loss rate in Down's syndrome pregnancies increases with maternal age.

1.4.2 SCREENING MARKERS

Nuchal translucency (NT), alpha fetoprotein (AFP), human chorionic gonadotropin (hCG), free β human chorionic gonadotropin (fβhCG), pregnancy associated plasma protein A (PAPP-A), unconjugated estriol (uE3) and inhibin A (InhA) are commonly used markers in Down's syndrome screening. The concentrations of these biochemical markers changes with gestation. Therefore, in order to remove the fluctuation caused by gestation in the marker levels, the concentrations of the markers are normally expressed as 'multiple of the median' (MoM) where the observed concentration is expressed as a ratio of the median value observed in a normal pregnancy of the same gestation. When the MoM values are transformed to log, the distributions in both normal and Down's syndrome pregnancies are Gaussian. However, there is no complete separation between the normal and Down's syndrome pregnancies (Spencer, 2007; Aitken *et al.*, 2007). Cuckle *et al* (1987) proposed the use of Gaussian distribution to derive the likelihood that a particular marker level is

associated with Down's syndrome pregnancy. Likelihood ratio is the proportion of affected pregnancies with a given marker level divided by the proportion of unaffected pregnancies with the same marker level. Using the Gaussian distribution, the likelihood ratio can be derived from the ratio of the heights of the two log Gaussian frequency distributions at the given marker level (Cuckle *et al.*, 1987).

1.4.3 MARKER PREDICTIVE VALUE

The efficiency of a marker in screening depends on two factors;1) the shift of the mean or median level in affected cases and 2) the spread of the values (the standard deviation (SD)) in affected and unaffected cases. The marker with greater median shift in affected pregnancies and/or with smaller spread of values will have better predictive value and be more effective. Mahalanobis distance is normally used to evaluate the effectiveness of a marker in screening for Down's syndrome. Mahalanobis distance is calculated using the following equation:

$$(Mean\ [unaffected] - Mean\ [affected]) / SD\ [unaffected]^2$$

Table 1.1 shows the estimated Mahalanobis distance for Down's syndrome screening markers in first and second trimesters. Using this calculation, PAPP-A, fβhCG and NT measurement are the best markers for first trimester screening and hCG, InhA and fβhCG are the best markers for second trimester screening (Aitken *et al*., 2007).

Table 1.1: Mahalanobis Distance of Down's syndrome screening markers (Aitken *et al.*, 2007)

Markers	Mahalanobis Distance	
	First trimester	**Second trimester**
AFP	0.23	0.69
hCG	0.38	1.86
InhA	0.35	1.65
uE3	0.68	1.20
PAPP-A	2.08	-
FβhCG	1.45	2.04
NT	6.46	-

1.4.4 SECOND TRIMESTER SCREENING

1.4.4.1 ALPHA-FETOPROTEIN (AFP)

Second trimester screening is performed between 15 and 20 weeks of gestation. In 1984, alpha-fetoprotein (AFP) was discovered to be a potential biochemical marker to identify pregnancies with increased risk of Down's syndrome and other trisomies (Merkatz *et al.*, 1984). AFP is a 69kD protein that belongs to the albuminoid family. AFP is synthesized by the yolk sac and the fetal liver (Powell *et al.*, 1995, Seppala, 1975, Mizejewski, 2001). During pregnancy, fetal AFP enters the maternal circulation via two possible pathways; transplacental diffusion and transamniotic membrane diffusion (Mizejewski, 2001). AFP concentration in the maternal circulation increases progressively to peak at 32 weeks (Macintosh and Chard, 1993).

According to several studies, a reduction in the maternal serum AFP level occurs in Down's syndrome pregnancies, in the second trimester (Merkatz *et al*., 1984; Cuckle *et al*., 1984; Fuhrmann *et al*., 1984; Tabor *et al*., 1984). A study by Newby *et al* (1997) on biochemical markers and pathophysiology of Down's syndrome pregnancies indicated that the unchanged level of AFP in fetal liver homogenates and the significant elevation of AFP in placental tissue from Down's syndrome pregnancies suggest a possible transport defect specific to AFP which reduces the amount of AFP reaching the maternal circulation to about 75% of the level in unaffected pregnancies.

In 2002, Spencer *et al* studied the trend of marker median levels in Down's syndrome pregnancies between 6 and 20 weeks of gestation. Figure 1.2 illustrates the trend of multiple of the median (MoM) of AFP in Down's syndrome pregnancies between 6 and 20 weeks of gestation. The AFP measurement does not separate unaffected pregnancies from Down's syndrome pregnancies for gestational ages below 10 weeks. The optimum gestational age for AFP measurement for Down's syndrome screening is at approximately 16 weeks as there is the maximum separation at that gestational age (Spencer *et al*., 2002).

In the 1970s, screening for Down's syndrome was performed based on advanced maternal age alone. In 1987, Cuckle and co-workers estimated the risk of having a Down's syndrome pregnancy by combining maternal age and maternal serum AFP level. Cuckle *et al* (1987) reported that screening for Down's syndrome using both maternal age and maternal serum AFP level was more efficient than using maternal age alone. For an example, using maternal age and AFP level, a detection rate of 28% with a false positive rate of 2.8% would be achieved for a risk cut-off of 1:200. Using maternal age alone, the same detection rate (28%) could be achieved with a higher false positive rate (4.3%) (Cuckle *et al*., 1987).

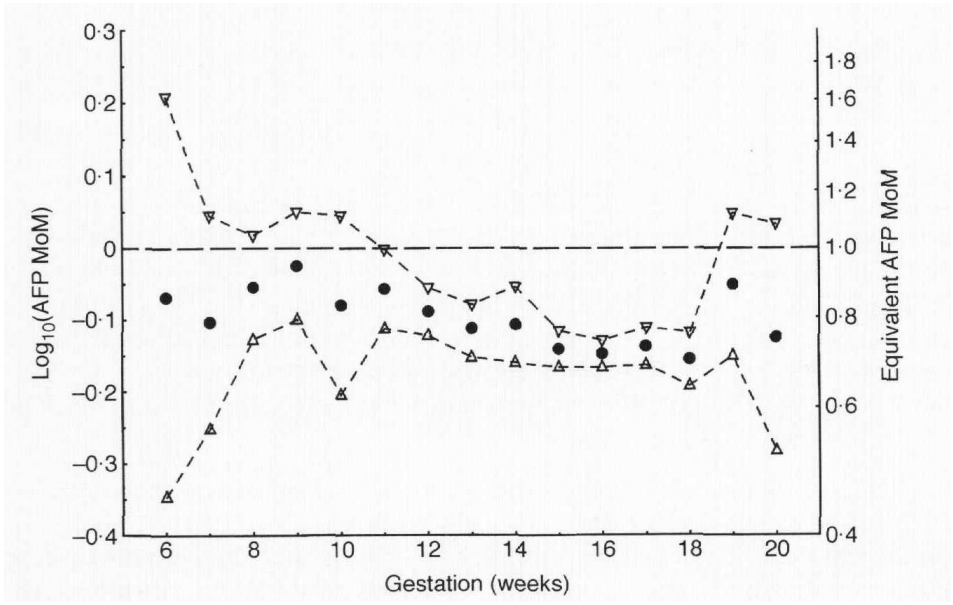


Figure 1.2: Mean $\log_{10}$ (AFPMoM) for each gestational age
(reproduced from Spencer *et al.*, 2002)

1.4.4.2 HUMAN CHORIONIC GONADOTROPIN (hCG)

In 1987, Bogart *et al* discovered an association between elevated second trimester human chorionic gonadotropin (hCG) levels and Down's syndrome pregnancies. Human chorionic gonadotropin is a glycoprotein hormone with a molecular weight of 36,000 to 46,000 daltons. Human chorionic gonadotropin is synthesized in the syncytiotrophoblast cells and composed of two subunits (alpha and beta). The alpha subunit has a structure similar to that of luteinizing hormone, follicle stimulating hormone and thyroid stimulating hormone. Whereas, the beta subunit is a unique glycoprotein specific to hCG. In the circulation, hCG is mostly in the intact form and 0.3% to 4% exists as free beta human chorionic gonadotrophin (fβhCG) (Powell and Grudzinskas, 1995; Albertini *et al*., 1982; Macintosh and Chard, 1993).

Spencer (1991) investigated the analytical and clinical performance of the measurement of second trimester fβhCG in pregnancies affected by Down's syndrome. The study demonstrated that fβhCG is elevated (0.99 MoM in unaffected, 2.06 MoM in Down's syndrome) in pregnancies affected by Down's syndrome. Studies by Newby *et al* (1997) also showed that hCG and fβhCG levels in second trimester placental tissue from Down's syndrome pregnancies were higher than those in placental tissues from unaffected pregnancies. The similar changes of these markers both in the maternal serum and the placental tissue from Down's syndrome pregnancies suggest that the transport of these markers from their site of synthesis to the maternal circulation is not affected in Down's syndrome pregnancies.

Later studies of Spencer *et al* (2002) showed that optimum efficiency of screening using hCG can be achieved at 16 weeks of gestation. hCG level was found to be similar in both affected and unaffected pregnancies between 10 to 12 weeks of gestation (Figure 1.3) (Spencer *et al*., 2002).

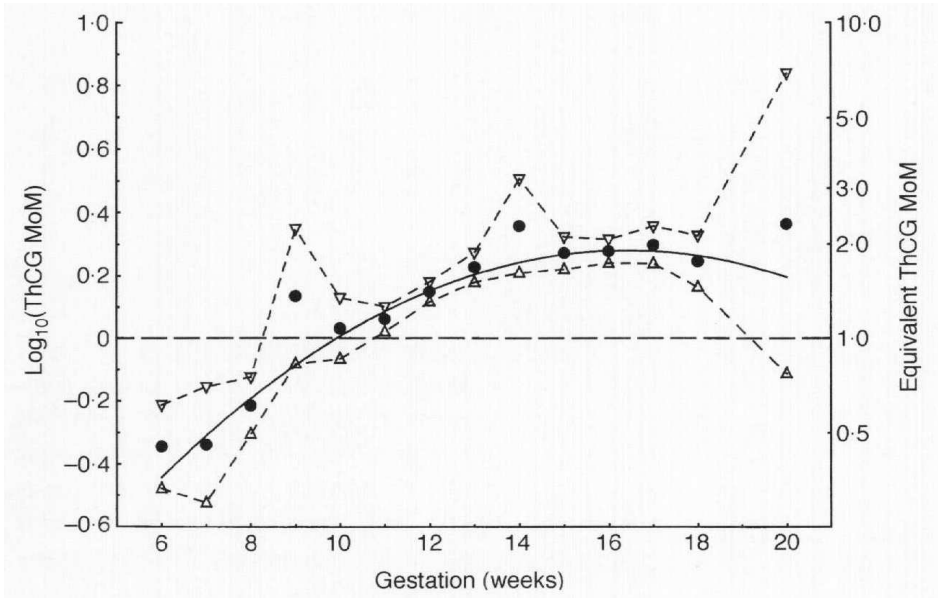


Figure 1.3: Mean $\log_{10}$ (hCGMoM) for each gestational age
(reproduced from Spencer *et al.*, 2002)

FβhCG level was found to be a viable marker between 10 to 20 weeks of gestation. However the optimum efficiency can be achieved when screening is performed at 15 weeks of gestation (Figure 1.4) (Spencer *et al*., 2002).

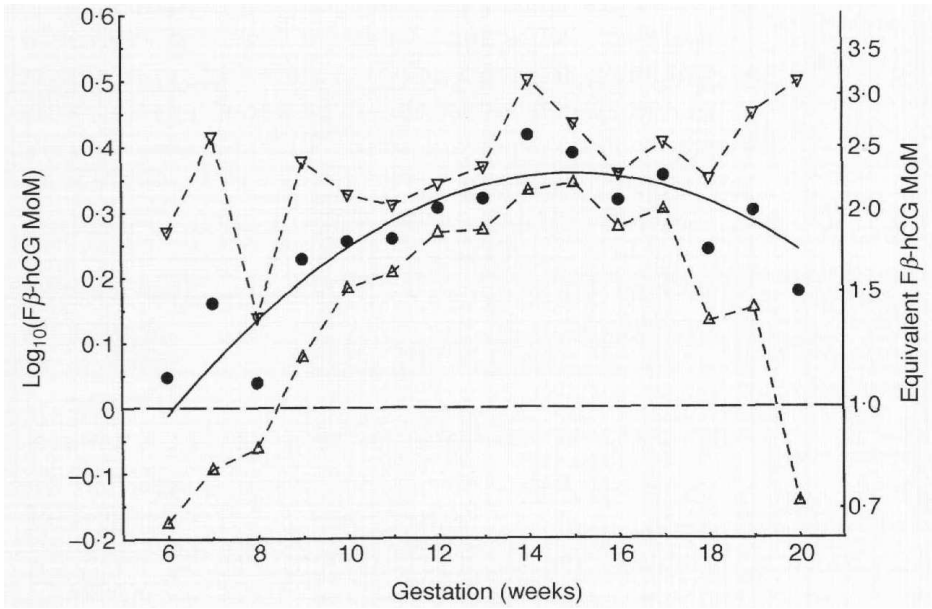


Figure 1.4: Mean $\log_{10}$ (FβhCGMoM) for each gestational age
(reproduced from Spencer *et al*., 2002)

1.4.4.3 UNCONJUGATED ESTRIOL (uE3)

The reduction in secretion of AFP by the fetal liver in Down's syndrome led Canick and co-workers (1988) to investigate other fetal liver products which might also be associated with Down's syndrome. Unconjugated estriol (uE3), a steroid product of the fetoplacental unit, requires the participation of the fetal liver for its synthesis. It is synthesized in the syncytiotrophoblast from fetal precursors. Dehydroepiandrosterone (DHEA) is produced by the fetal adrenal and is converted to 16OH-DHEA by the fetal liver. These compounds circulate in the fetus as sulphate conjungates. The newly formed 16OH-DHEA sulphate is deconjugated by the placenta and converted to estriol by an aromatase. Estriol can be measured as unconjugated steroid in maternal circulation (Wald *et al*., 1988; Macintosh and Chard, 1993).

The studies by Canick *et al* (1988) indicated that maternal serum uE3 was decreased in Down's syndrome pregnancies with a median MoM of 0.79. This finding was later confirmed by other studies on uE3 (Wald *et al*., 1988; Wald *et al*., 1991; Crossley *et al*., 1993). Although uE3 was found to be a useful marker for Down's syndrome screening, there was concern regarding the high correlation between AFP and uE3 (Crossley *et al*., 1993; Powell and Grudzinskas, 1995) and imprecision of uE3 assay (Powell and Grudzinskas, 1995).

1.4.4.4. INHIBIN A (InhA)

In 1992, Van Lith *et al* published a report showing that inhibin may be a useful marker for Down's syndrome screening. Inhibin , a heterodimeric glycoprotein with a molecular weight of 32 000D, composed of an α-subunit and one of the two β subunits (β_A or β_B). When the β subunit combined with the α subunit, it gives rise to either dimeric inhibin-A or inhibin-B.In early pregnancy, the feto-placental unit is the major source of inhibin (InhA) (Florio *et al*., 2001). InhA levels have a profile similar to hCG and are lowest in the maternal serum from unaffected pregnancies at 17 weeks of gestation (Aitken and Crossley, 2005).

Maternal serum inhibin level was reported to be elevated in Down's syndrome pregnancies in the second trimester (Van Lith *et al*., 1992; Spencer *et al*., 1993; Cuckle *et al*., 1994a). However the degree of elevation of inhibin levels in Down's syndrome pregnancies varied from study to study. Inhibin was initially studied using non-specific assays that utilizes antibodies directed towards the α subunit of inhibin. Such an assay measured total immunoreactive inhibin and failed to specifically detect intact dimeric InhA. The development of new assay enabled to detect intact dimeric InhA rather than non-specific immunoreactive inhibin.

In 1996, Aitken *et al* investigated the level of InhA in pregnancies using a new assay specific for dimeric InhA. Their studies showed that InhA levels were significantly elevated in Down's syndrome pregnancies in the second trimester and measuring the levels of InhA together with AFP and fβhCG significantly improved the detection rate. This finding was confirmed by subsequent studies on InhA (Wallace *et al*., 1996; Haddow *et al*., 1998; Renier *et al*., 1998).

However, the value of InhA as the fourth marker in the second trimester screening had remained debatable, until recently. Although there have been previous reports showing that the second trimester maternal serum InhA level is elevated in Down's syndrome pregnancies (Aitken *et al*., 1996; Renier *et al*., 1998), InhA was not widely used as part of screening programs due to issues relating to assays and standardization. The assay is now on a new platform (Access – Beckman Coulter) with reduced inter- and intra-kit lot variation.

In 2001, Spencer *et al* reported that although InhA level was increased in Down's syndrome pregnancies in the first trimester, it does not improve the detection rate of screening by a combination of pregnancy associated plasma protein A (PAPP-A), fβhCG and nuchal translucency (NT) measurement at 10 to 14 weeks of gestation. Christiansen and Norgaard-Pedersen (2005) suggested that combination of InhA in early first trimester (prior to 11 weeks) screening can be as good as integrated and second trimester screening.

1.4.4.5 ADAM12

In 2003, Laigaard *et al* reported ADAM12 as a promising marker for Down's syndrome screening. The ADAMs belongs to a family of membrane-anchored cell-surface proteins. Earlier report by Gilpin *et al* (1998) shows that human ADAM12 exist in two forms;

ADAM12-S (short) and ADAM12-L (long). The study also revealed that both forms of ADAM12 are found in abundance in the human term placenta.

In the studies by Laigaard *et al* (2003), it was found that ADAM12 level in the maternal serum was 60-fold increase from early to late pregnancy whereas it is seen to decrease significantly in Down's syndrome pregnancies, in the first trimester. Laigaard *et al* (2006a) later reported that ADAM12 was not reduced in Down's syndrome pregnancies in the late first trimester. A further large scale study conducted by Laigaard and co-workers (2006b), for assessing the performance of ADAM12 as first trimester Down's syndrome marker, confirmed the findings from the two previous studies (Laigaard *et al*., 2003; Laigaard *et al*., 2006a). ADAM12 was concluded to have the best discriminatory efficiency early in the first trimester and the discriminatory power was found to decrease from week 10-11 to week 12-13 (Laigaard *et al*., 2006b).

Recent studies have showed that ADAM12 levels are reduced in pregnancies prior to 10 weeks but not to the extent observed by Laigaard *et al* (2003) (Spencer *et al*., 2008a; Spencer *et al*., 2008b; Spencer *et al*., 2008c). These studies indicate that ADAM12 is unlikely to be of much value when screening for Down's syndrome is performed between 11 to 13 weeks of gestation (Spencer *et al*., 2008a; Spencer *et al*., 2008b). However, certain reports have been made by Christiansen *et al* (2007) that maternal serum ADAM12 level is significantly elevated in Down's syndrome pregnancies in the second trimester. Though this finding was confirmed by Donalson *et al* (2008), the magnitude of increase was smaller. More prospective studies are required to establish whether ADAM12 is in fact a useful marker for Down's syndrome screening.

The meta-analysis and distributions of maternal serum biochemical markers in Down's syndrome cases in the second trimester are shown in Table 1.2 and Figure 1.5. The largest shift in median MoM in Down's syndrome pregnancies is found for fβhCG, following intact hCG and InhA.

Table 1.2: Meta-analysis of maternal serum biochemical markers in Down's syndrome cases in the second trimester (From Aitken *et al*., 2007)

Biochemical marker	**Down's syndrome cases**	**Median MoM**
AFP	1559	0.75
fβhCG	649	2.26
hCG	1138	2.07
uE3	963	0.72
InhA	930	1.99

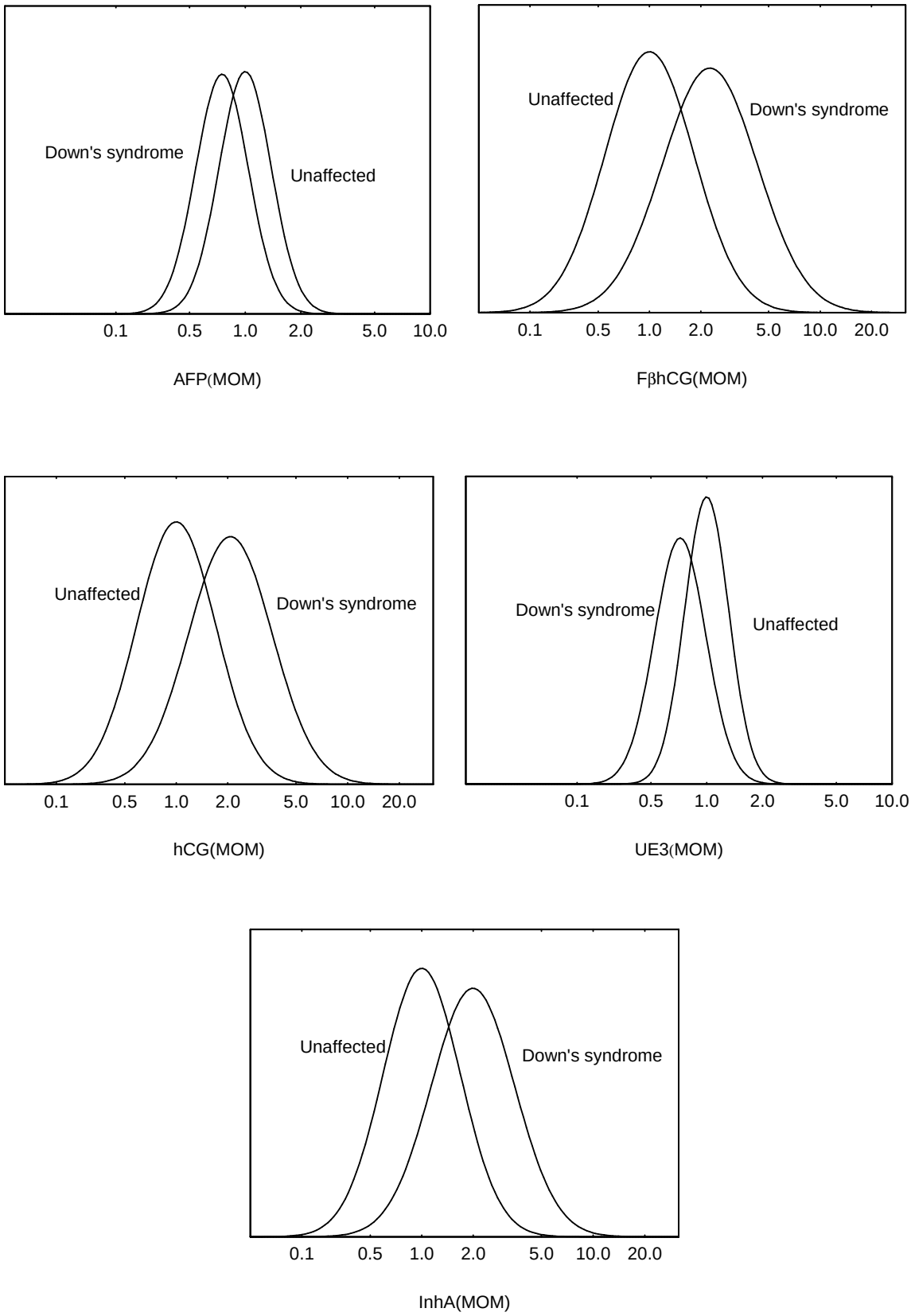


Figure 1.5: The distributions of second trimester markers in unaffected and Down's syndrome pregnancies.

The risk of a pregnancy being affected by Down's syndrome is calculated from the maternal age risk in combination with the AFP, hCG, uE3 and/or other marker levels. Table 1.3 shows the predicted screening performance using statistical modelling for various marker combinations (Cuckle, 2001).

Table 1.3: Predicted detection rate for a fixed false positive rate of 5% of various second trimester marker combinations using statistical modelling (Cuckle, 2001).

Marker combinations	**Detection rate (%)**
AFP & hCG	59.3
AFP, hCG & uE3	62.7
AFP, hCG, uE3 & InhA	69.0
AFP & fβhCG	63.2
AFP, fβhCG, uE3	66.8
AFP, fβhCG, uE3, InhA	72.1

In Scotland, maternal serum AFP was first used for Down's syndrome screening in 1987. Maternal serum hCG measurements was included in the screening protocol in 1991. A risk cut-off of 1:250 at term is currently used to identify high and low risk pregnancies. The results of those women with 'high risk' is either faxed or telephoned to the referring source as soon as it is available so that patients can be called in for a counselling session. All results, including the 'low risk' and 'high risk' ones, are sent by post to the antenatal clinic, in order to inform the patients about the results and to file in the patient record (personal communication with Dr. Jenny Crossley).

1.4.5 FIRST TRIMESTER SCREENING

Second trimester screening has the disadvantage of a relatively low detection rate with a high false positive rate and it is carried out relatively late in pregnancy. This, combined

with the fact that CVS can be carried out as a diagnostic test in the first trimester of pregnancy, led to research interest in first trimester screening. As a result of research efforts around the world, the two most effective first trimester serum markers were identified; PAPP-A and fβhCG.

1.4.5.1 PREGNANCY ASSOCIATED PLASMA PROTEIN A (PAPP-A)

PAPP-A is a pregnancy specific glycoprotein of 750 000 to 820 000 molecular weight which exists in pregnancy serum as a heterotetrameric 2:2 complex with the proform of eosinophil major basic protein (proMBP). This complex is called PAPP-A/proMBP and weights approximately 500kDa. PAPP-A is synthesized in the trophoblast and is detected in the maternal circulation about 28 days after implantation (Bischof, 1979; Fialova and Malbohan , 2002; Macintosh and Chard, 1993; Powell and Grudzinskas, 1995).

Earlier studies have shown that first trimester PAPP-A levels are significantly decreased in Down's syndrome pregnancies (Brambati *et al*., 1993; Brambati *et al.*, 1991). Later studies by Newby *et al* (1997) show that PAPP-A levels in both placental tissues and maternal circulation are not significantly altered in the second trimester. However, recent reports by Spencer *et al* (2002) indicate that optimum efficiency can be achieved when screening is performed in the earlier stages of pregnancy, at about 8 weeks (Figure 1.6).

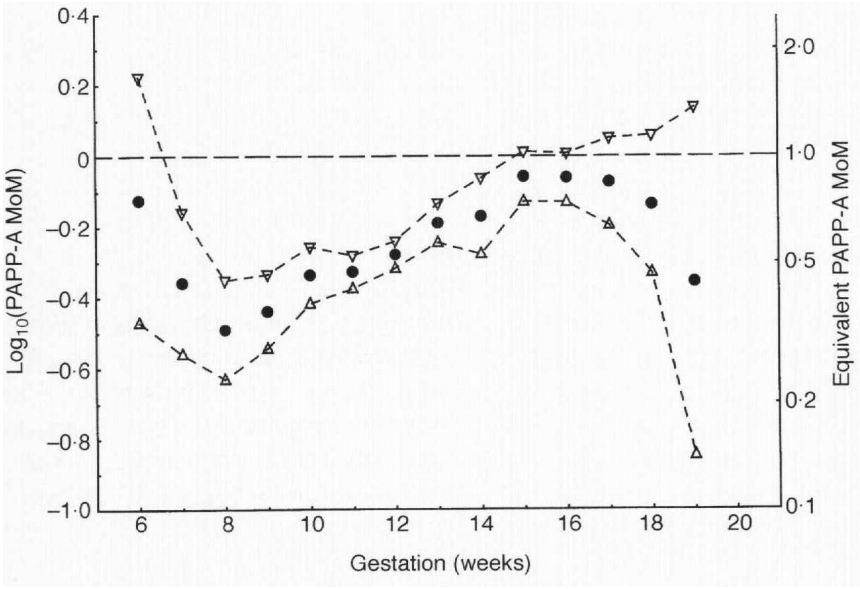


Figure 1.6: Mean $\log_{10}$ (PAPP-AMoM) for each gestational age
(reproduced from Spencer *et al*., 2002)

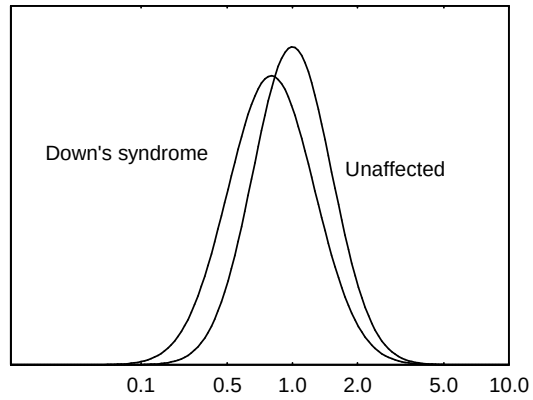
1.4.5.2 FREE β HUMAN CHORIONIC GONADOTROPIN (FβhCG)

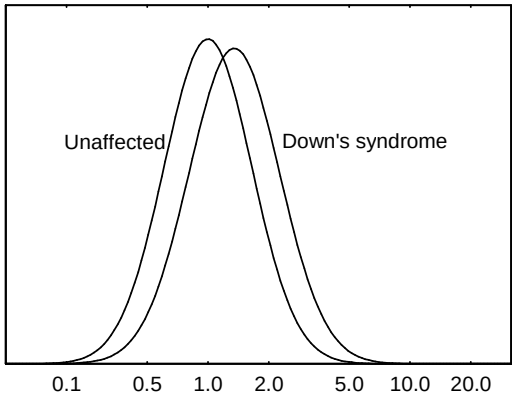
FβhCG level is a viable marker between 10 to 20 weeks of gestation and the optimum efficiency using this marker can be achieved when screening is performed at 15 weeks of gestation (Figure 1.4) (Spencer *et al*., 2002). Previous studies have reported that first trimester FβhCG levels are significantly increased in Down's syndrome pregnancies (Macri *et al.*, 1993; Spencer *et al*., 1992) and FβhCG is a better marker than intact hCG in the first trimester (Hallahan *et al.*, 2000).

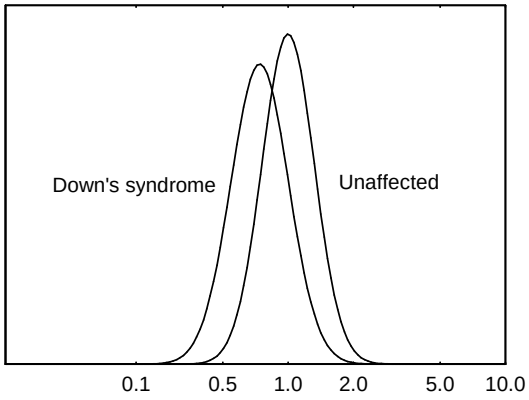
The meta-analysis and distributions of maternal serum biochemical markers in Down's syndrome cases in the first trimester are shown in Table 1.4 and Figure 1.7. In the first trimester, the largest shift in median MoM in Down's syndrome pregnancies is found for PAPP-A, following fβhCG and InhA.

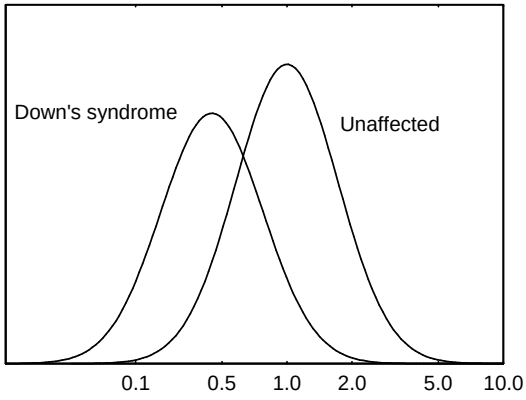
Table 1.4: Meta-analysis of maternal serum biochemical markers in Down's syndrome cases in the first trimester (From Aitken *et al*., 2007)

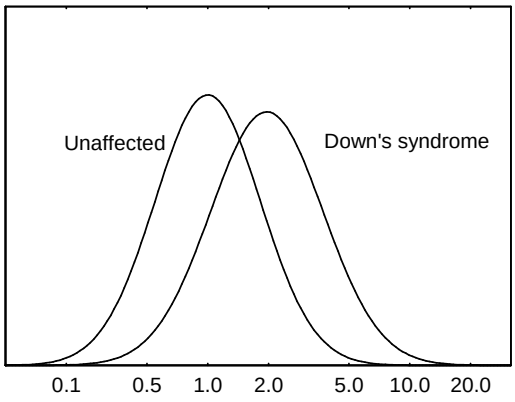
Biochemical marker	Down's syndrome cases	Median MoM
AFP	637	0.8
hCG	772	1.35
uE3	294	0.74
PAPP-A	1057	0.45
FβhCG	1190	1.96
InhA	317	1.47











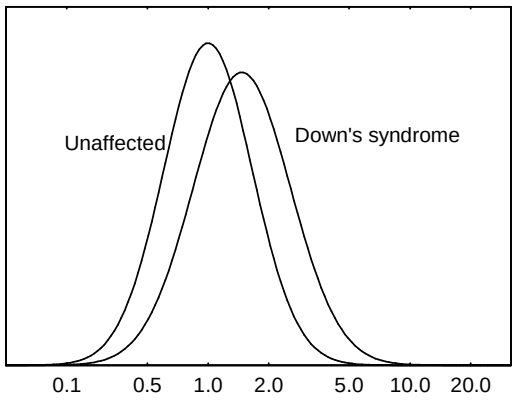


Figure 1.7: The distributions of first trimester markers in unaffected and Down's syndrome pregnancies.

1.4.5.3 NUCHAL TRANSLUCENCY (NT)

Another effective marker for Down's syndrome screening is ultrasound measurement of fetal nuchal translucency (NT) (Nicolaides *et al*., 1994a). 'Nuchal translucency' is a term used by Nicolaides *et al* (1992) to describe accumulation of fluid between the fetal skin and soft tissues overlying the cervical spine. In normal fetuses, the average maximum thickness of NT is about 1.4 to 1.5mm at 13 weeks of gestation. Figures 1.8 and 1.9 illustrate NT in normal and Down's syndrome fetuses at 12 weeks of gestation. Collection of fluid in this ultrasound-translucent area may be caused by various mechanisms including cardiac failure and venous congestion. The fetus with increased NT is at high risk of an adverse outcome like choromosomal abnormalities (Nicolaides, 2004). Previous studies have shown that increased NT (≥2.5mm) is associated with Down's syndrome pregnancy (Nicolaides *et al*., 1992; Pandya *et al*., 1995; Taipale *et al*., 1997).

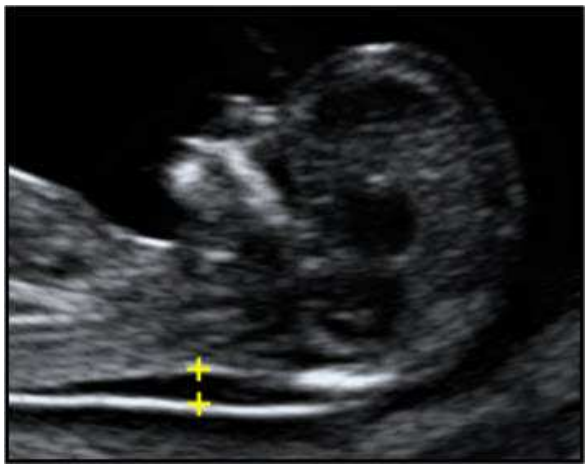


Figure 1.8: Ultrasound picture of fetus with normal NT thickness.

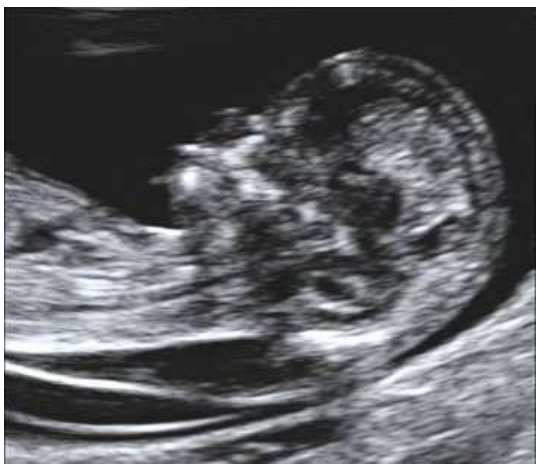


Figure 1.9: Ultrasound picture of fetus affected with Down's syndrome with increased NT thickness.

Reproduced from:
http://www.fetalmedicine.com/fmf/training-certification/certificates-of-competence/11-13-week-scan/nuchal/

The optimum gestational age for NT measurement is between 11 weeks and 13 weeks and 6 days. Nicolaides *et al* (2002) stated some of the essential criteria in order to achieve accurate and uniform NT measurement among different ultrasound operators:

1. Providing appropriate training to all sonographers and auditing of their results.
2. Good quality ultrasound equipment with video-loop function and callipers which will be able to provide measurement to one decimal point (0.1mm).
3. Transabdominal ultrasound examination can successfully measure NT in about 95% of cases and transvaginal sonography examination in other cases.
4. The fetal crown rump length (CRL) should be between 45mm and 84mm.
5. It is essential to take into account the gestational age when determining whether the NT measurement is increased because fetal NT increases with CRL.
6. A good sagittal section of the fetus is required for the measurement of the CRL. NT should be measured when the fetus is in the neutral position.
7. It is important to distinguish between fetal skin and amnion because both structures appear as thin membranes at this gestation.

Therefore, in order to achieve a reliable measurement of NT the above criteria should be adhered to. The studies by Evans *et al* (2007) show that inaccuracies in NT measurement of 25% or 0.5mm can reduce the detection rate by 18%.

Two methods are commonly used for standardizing NT measurements in the first trimester for Down's syndrome screening. The first method is the parametric method of multiples of the median (MoM). This method involves dividing the measured value by the median of the normal population. The second method is the non-parametric method of the delta-NT differential. This method involves subtracting the median from the measured value (Wald and Hackshaw, 1997; Spencer *et al*., 2003c).

In 2003, Spencer *et al* reported that the use of the NT MoM approach in the Down's syndrome risk calculation was inaccurate and inappropriate. This was because the three underlying assumptions for the Gaussian MoM approach to be valid were not valid. The three basic assumptions were:

1. Either NT MoM or some transformation of NT MoM has a Gaussian distribution;
2. The standard deviation (SD) of the MoM in the transformed domain is constant;
3. The median MoM in trisomy 21 pregnancies is a constant proportion of the median for unaffected pregnancies.

Spencer *et al* (2003c) found that the distributions of NT MoM (Figure 1.10) and $\log_{10}$(NT MoM) were not Gaussian, the SDs did not remain constant with gestation, and the median MoM in the trisomy 21 pregnancies was not a constant proportion of the median for unaffected pregnancies. Therefore, Spencer *et al* (2003c) proposed that the delta-NT approach is the best approach to calculate accurate patient-specific risks. Delta-NT takes into account the gestational variation in NT by expressing the measured fetal NT as the difference from the normal median NT at the measured CRL.

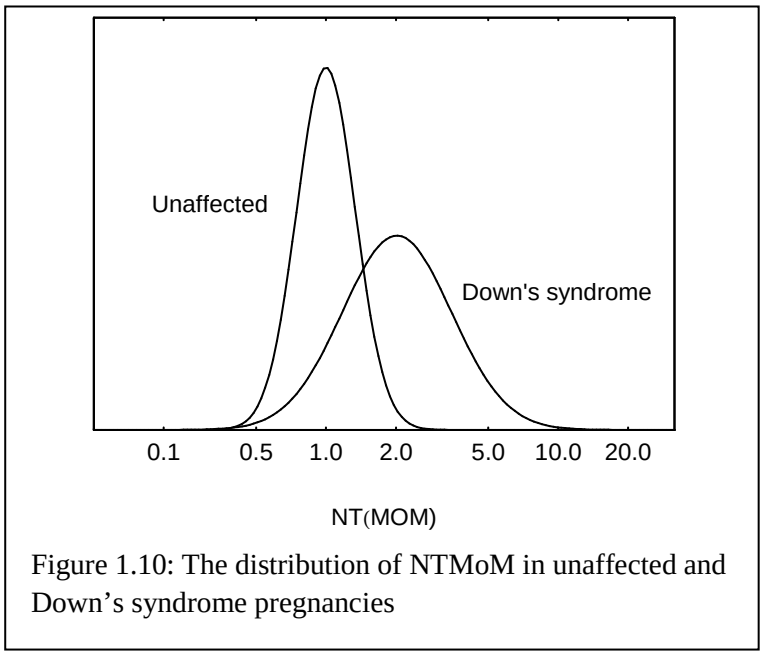


Figure 1.10: The distribution of NTMoM in unaffected and Down's syndrome pregnancies

In 1998, Nicolaides and co-workers derived parameters for NT screening based on 95,476 singleton unaffected pregnancies and 326 Down's syndrome pregnancies. The median NT in Down's syndrome pregnancies was 2.02 MoM. The $\log_{10}$ standard deviation of the distribution was 0.120 in the unaffected pregnancies and 0.235 in the Down's syndrome. This large difference in standard deviation between unaffected and Down's syndrome pregnancies creates an anomaly in the calculated risk at smaller NT measurements. The likelihood ratio decreases as NT MoM reduces to about 0.8 MoM but thereafter begin to increase again at lower NT levels. Therefore, a lower truncation limit of 0.8 MoM should be applied in the risk calculation to avoid giving incorrect risks for small NT measurements (Crossley and Aitken, 1999).

When calculating patient-specific risk for Down's syndrome, NT measurements can be incorporated into maternal age-related risk and biochemical markers. This is done by multiplying the likelihood ratios for NT and for the biochemical markers with maternal age-related risk at the time of screening.

1.4.5.4 OTHER ULTRASOUND MARKERS

Recently new ultrasound markers have been shown to improve the performance of Down's syndrome screening. Three markers; assessment of nasal bone, tricuspid regurgitation and abnormal flow velocity patterns in the ductus venosus appear to be promising (Spencer, 2007). Cicero *et al* (2006) reported that the nasal bone was absent in 62.1% of fetuses with Down's syndrome and 0.6% of normal fetus (Figures 1.11 & 1.12). Cicero *et al* (2005) reported that there is no association between an absent fetal nasal bone and PAPP-A or fβhCG. A detection rate of 90% at a false positive rate of 2.5% can be achieved by incorporating nasal bone assessment to combined ultrasound and biochemical (CUB) screening (Cicero *et al*., 2006).

Tricuspid regurgitation is another potential marker determined by pulsed wave Doppler ultrasonography. Previous studies have shown that tricuspid regurgitation is found in more than 65% of Down's syndrome fetuses and less than 8.0% of normal fetuses (Faiola *et al*., 2005; Falcon *et al*., 2006a; Falcon *et al*., 2006b). Falcon *et al* (2006b) reported that there is no association between tricuspid regurgitation and biochemical markers and incorporating tricuspid regurgitation to CUB screening would be expected to achieve a detection rate of 95% at a false positive rate of 5%.

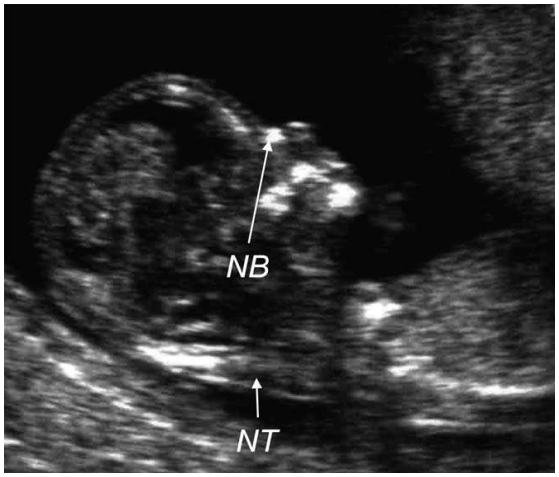


Figure 1.11: Ultrasound picture of fetus with normal NT thickness and a present nasal bone.

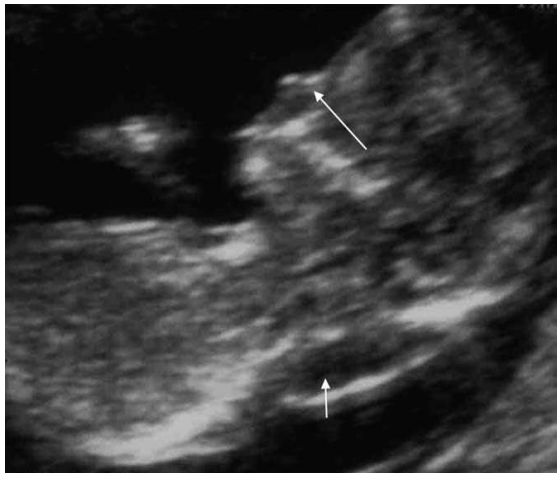


Figure 1.12: Ultrasound picture of fetus affected with Down's syndrome with increased NT thickness and an absent nasal bone.

Reproduced from Nicolaides, 2004

Many Down's syndrome fetuses have abnormal blood flow through the ductus venosus due to congenital heart diseases. Studies conducted by Borrell *et al* (2005) show that there is no correlation between the pulsatility index for veins (PIV) and serum markers. Thus, addition of PIV to NT alone would be expected to increase the detection rate from 76% to 85% and

combined with serum markers, the modelled detection rate increased to 92% at a 5% false positive rate (Borrell *et al*., 2005)

Although the new ultrasound markers improve the performance of Down's syndrome screening, the usage of these markers is time consuming and requires highly skilled operators with much experience. Therefore, it is unlikely these ultrasound markers will be incorporated in the routine first trimester screening programme (Spencer, 2007).

1.4.6 METHODS OF SCREENING

Screening identifies those women who are at high risk of carrying a Down's syndrome fetus. Each pregnant woman who is screened for Down's syndrome is given a patient-specific risk based on her age, family history and screening marker levels. A variety of methods of combining biochemical and ultrasound markers to give risks of Down's syndrome is in use or has been proposed. The performance of a screening test is normally evaluated in terms of 'detection rate', the proportion of affected pregnancies that are screened-positive using the screening test, the 'false positive rate', the proportion of unaffected pregnancies that are screened-positive using the screening test, and the 'screen positive rate', the proportion of pregnancies that are screened positive using the screening test. For the best screening test the marker combination should give the highest detection rate for the lowest false positive rate and be acceptable to women (Cuckle, 2002).

1.4.6.1 COMBINED ULTRASOUND AND BIOCHEMICAL MARKERS (CUB) SCREENING

Combined ultrasound and biochemical (CUB) screening for Down's syndrome using NT measurements, maternal serum PAPP-A and fβhCG is offered routinely in many centres. Due to the low or no correlation between the three markers in both normal pregnancies and

Down's syndrome pregnancies, CUB screening appears to be an effective screening procedure. The effectiveness of CUB screening in clinical practice is well documented with detection rates of 85-91% at a 4-5% screen positive rate being typically reported (Spencer *et al*., 2000a; Stenhouse *et al*., 2004, Perni *et al*., 2006).

In Scotland, CUB screening for Down's syndrome started in 2000. Maternal blood samples are collected from 9 weeks of gestation and NT measurement are normally obtained from 11 weeks to 13 weeks and 6 days of gestation. A fetal scan is carried out to measure CRL or bi-parietal diameter (BPD) measurement to determine the gestational age. A risk cut-off of 1 in 250 at term is used to identify high and low risk pregnancies. The combined risk will be reported to the antenatal clinic after a couple of days and women with risk $\geq$ 1 in 250 will be re-called for counselling and offered a diagnostic test (Stenhouse *et al.*, 2004).

One-stop clinic for assessment of risk (OSCAR) is one way of implementing first trimester screening for Down's syndrome. In the one-stop clinic, the ultrasound examination of the fetus and biochemical testing on maternal serum are carried out simultaneously and patients will receive their combined risk at their antenatal clinic visit (Spencer *et al*., 2000a; Bindra *et al*., 2002; Spencer *et al*., 2003a; Avgidou *et al*., 2005). The advantage of this type of approach is that the patients can be counselled regarding their combined risk and the diagnostic options available, if required at the same visit.

1.4.6.2 INTEGRATED TESTING

In the integrated testing protocol, women are offered NT measurement and maternal serum PAPP-A test in the first trimester and maternal serum AFP, hCG or fβhCG, uE3 and InhA test in the second trimester. The first trimester test results will not be interpreted or disclosed to the patients until the second trimester test is performed. A study by Wald *et al*

(1999a) has showed that integrated testing (PAPP-A and NT in the first trimester and AFP, hCG, uE3 and InhA in the second trimester) could potentially achieve a detection rate of 94% at a false positive rate of 5%. This finding was consistent with a recent study conducted in Australia where integrated screening was reported to have a detection rate of 91% at a false positive rate of 2.5% (Cocciolone *et al.*, 2008). In serum integrated testing, NT measurement is excluded from the screening protocol. The detection rate reduces from 94% to 85% at a false positive rate of 5% when NT measurement is omitted from the screening protocol (Wald *et al*., 1999a).

In 2003, Wald and co-workers reported the results of the Serum Urine and Ultrasound Screening Study (SURUSS), funded by the UK National Health Technology Assessment Program. The objective of SURUSS trial was to identify the most effective, safe and cost-effective method of antenatal screening for Down's syndrome using NT, maternal serum and urine markers in the first and second trimesters of pregnancy, and maternal age in various combinations. Twenty-five maternity units offering second trimester screening participated in this study and the results were based on 47,053 singleton pregnancies, including 101 Down's syndrome pregnancies. Wald *et al* (2003) reported that integrated testing is the most effective screening method for Down's syndrome with detection rates of 93% at a 5% false positive rate.

Although integrated testing has been reported to have a high detection rate, the non-disclosure of the first trimester screening results is a major disadvantage of this screening protocol. As the results from the first trimester test will not be interpreted or given to the patients until the second trimester test is performed, many pregnant women could be deprived of the chance of getting early diagnostic tests. Moreover, it also increases the anxiety due to the long wait for the test results till the second trimester. It is particularly

problematic when a larger NT measurement has been seen, as it can be difficult not to disclose this to the patient. A study conducted by Spencer and Aitken in 2004 on women's preferences for prenatal screening testing reported that only 24% of women preferred the integrated test compared to 75% of women favouring first trimester screening (Spencer and Aitken, 2004). Apart from these issues, integrated test has also been reported to be more expensive that other types of screening protocols (Gilbert *et al*., 2001).

1.4.6.3 CONTINGENT SCREENING

The concept of contingent screening is illustrated in figure 1.13. All pregnant women are offered the first stage of screening. A risk is calculated and women are divided into three groups; high, intermediate and low risk, depending on the level of risk. Those falling in the 'high risk' group are offered a diagnostic test while those under 'low risk' do not have to undergo any further testing. Those who fall in the 'intermediate risk' category are also advised to take a second stage of screening. A likelihood ratio is then derived from the second stage of screening. This ratio is then combined with the risk at the first stage of screening and the composite risk is assessed against a final cut-off risk. Those women with a final risk greater than the final risk cut-off are classified as 'screen positive' and added to the initial high risk group. Whereas, the women with final risks lower that the final risk cut-off are categorised as 'screen negative' and are listed among the initial low risk group.

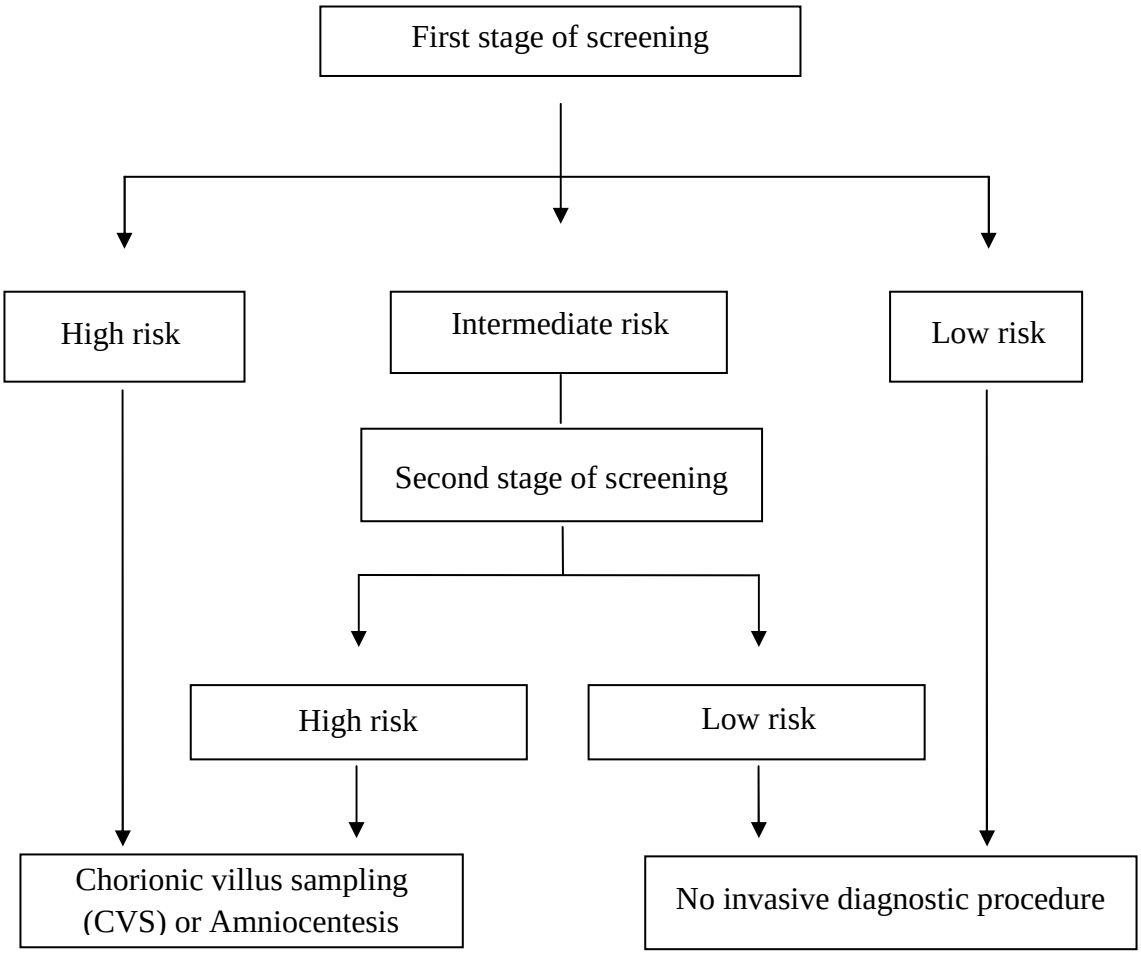


Figure 1.13: Contingent screening protocol.

Risks derived from first stage of screening are estimated for all women and used to triage the population into high, low and intermediate risk groups. Women with high risk would be offered a diagnostic test and those women with low risk would not be offered any further testing. Women with an intermediate risk would be offered second stage of screening and those with composite risk greater than the cut-off would be offered diagnostic testing.

Within First Trimester Contingent Screening

One of the critical factors in maintaining the performance of CUB screening is consistent and accurate NT measurement. This requires ultrasonographers with specific training and a system of on-going monitoring within a quality assured programme. This has hampered the adoption of CUB screening in some centers which lack the ultrasound resources to provide high quality NT measurements to the entire booking population.

A possible solution to this problem was proposed by Christiansen and Larsen (2002) who suggested a within-trimester contingent testing approach in the first trimester. In this protocol, the women initially undergo a biochemical testing (PAPP-A and fβhCG) and then go on to have NT measurement only if the risk calculated from maternal age and serum markers falls within an intermediate risk range. Women who fall within the high risk group are offered diagnostic testing, whilst those in the low risk group do not have to undergo any further tests. Based on mathematical modelling and with initial high and low cut-off risks of 1 in 65 and 1 in 1000 respectively and a final risk cut-off of 1 in 400, Christiansen and Larsen (2002) estimated that only 19.4% of women would require an NT scan to yield a detection rate of 78.9% for a 4% false positive rate. This small reduction in detection rate compared to full CUB screening in all women is offset by an increase in the cost-effectiveness of CUB screening due to a significant decrease in the number of NT measurements required.

In 2006, Laigaard *et al* conducted a study on within trimester contingent screening where women were selected for NT and fβhCG measurement at 11 to 12 weeks of gestation based on PAPP-A and ADAM 12 (A Disintegrin And Metalloprotease 12) measurements at 8 to 9 weeks of gestation. This study based on mathematical modelling has estimated that this

screening protocol can achieve a detection rate of 92% for a false positive rate of 1% with only 5.6% of women requiring NT and fβhCG measurement (Laigaard *et al*., 2006b).

Contingent Screening Across The First and Second Trimesters

In this model (Figure 1.14), women were selected for second trimester screening based on NT and PAPP-A measurement in the first trimester. Wright *et al* (2004) using data from SURUSS suggested that at the cost of a small reduction in overall performance, this screening model offers considerable psychological and clinical advantages over integrated screening with early diagnosis of a proportion of the affected cases. Wright *et al* (2004) also showed that by changing the initial and final cut-off risks, the early detection and completion rates can be varied. For example, increasing the early completion rate from 75% to 80%, with a 30% early detection rate and 85% overall detection rate means lowering second trimester cut-off from 1 in 126 to 1 in 155 for a small increase in the false positive rate by an estimated 0.1% (Wright *et al*., 2004). As reported by Maymon *et al* (2004) this model obviates the ethnical and clinical implication of non-disclosure of first trimester results and also the financial implication of unnecessary second trimester testing for the whole population.

In 2005, Benn *et al* (2005) had estimated the performance of contingent screening in the UK and USA, using statistical modelling. The contingent screening policy was based on the commonly used markers, cut-offs and gestational age at testing in both countries. For the UK, women were selected for second trimester screening based on PAPP-A and fβhCG measurements at 10 weeks of gestation and NT measurement at 11 weeks of gestation. In the second trimester screening, AFP, fβhCG, uE3 and InhA levels were measured at 14 to 20 weeks of gestation. While for the US, the first stage of screening was based on PAPP-A, hCG and NT measurements at 12 weeks of gestation and the second stage of screening

was based on AFP, hCG, uE3 and InhA measurements at 14 to 20 weeks of gestation (Benn *et al*., 2005). The studies showed that, in the UK and US, this screening protocol could achieve a detection rate of 91.4% and 89.1% at a false positive rate of 2.1% and 3.1% respectively but with only 19% of women requiring second trimester screening.

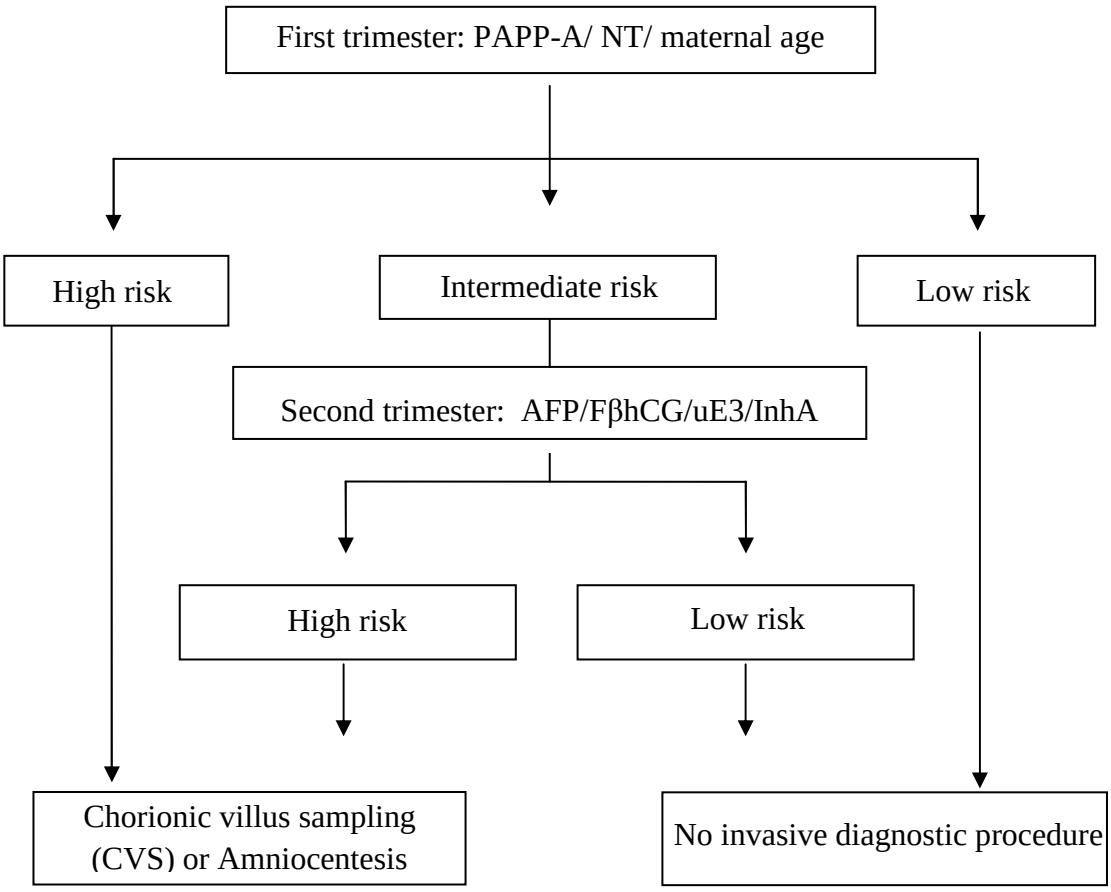


Figure 1.14: Contingent screening across trimester protocol.

Risks derived from first trimester screening are estimated for all women and used to triage the population into high, low and intermediate risk groups. Women with high risk would be offered a diagnostic test and those women with low risk would not be offered any further testing. Women with an intermediate risk would be offered second trimester screening and those with composite risk greater than the cut-off would be offered diagnostic testing.

Later in 2008, Cuckle *et al* conducted a comparison of the performance of contingent screening with integrated testing using First- and Second-Trimester Evaluation of Risk (FASTER) trial data. The conclusion was that the contingent screening detection rate for a fixed false positive rate is comparable with integrated testing, and this can be achieved with a significant reduction in second trimester screening requirement. However, Wald *et al* (2006) had a different viewpoint, reporting integrated testing as the simplest, most efficient and the safest screening policy and contingent screening as the most complex and least efficient screening policy.

Three Stage Contingent Screening

In this model (Figure 1.15), the first stage of screening is based on PAPP-A and fβhCG measurement at 10 weeks of gestational age. Those with a risk above the cut-off will proceed to the second stage to have NT measurement. And, a risk will be calculated based on maternal age, NT and first trimester biochemical markers (in the first stage). Women who fall within the high risk group are offered diagnostic testing whilst those in the low risk group will not have to undergo any further testing. Those with intermediate risk will be offered the second trimester screening. In the second trimester screening, AFP, fβhCG, uE3 and InhA levels would be measured. The combined risk will be assessed against a final risk cut-off and the pregnancies are classified as screen negative or positive (Wright *et al*., 2006).

The study by Wright *et al* (2006) based on statistical modelling showed that if 40% of women proceed to the second stage of screening and 20% of these women continue to stage three of screening, this screening policy can achieve a detection rate of 85% for a false positive rate of 0.7%. In this screening strategy, 60% of women complete screening after the first stage and 80% of women complete screening in the first trimester.

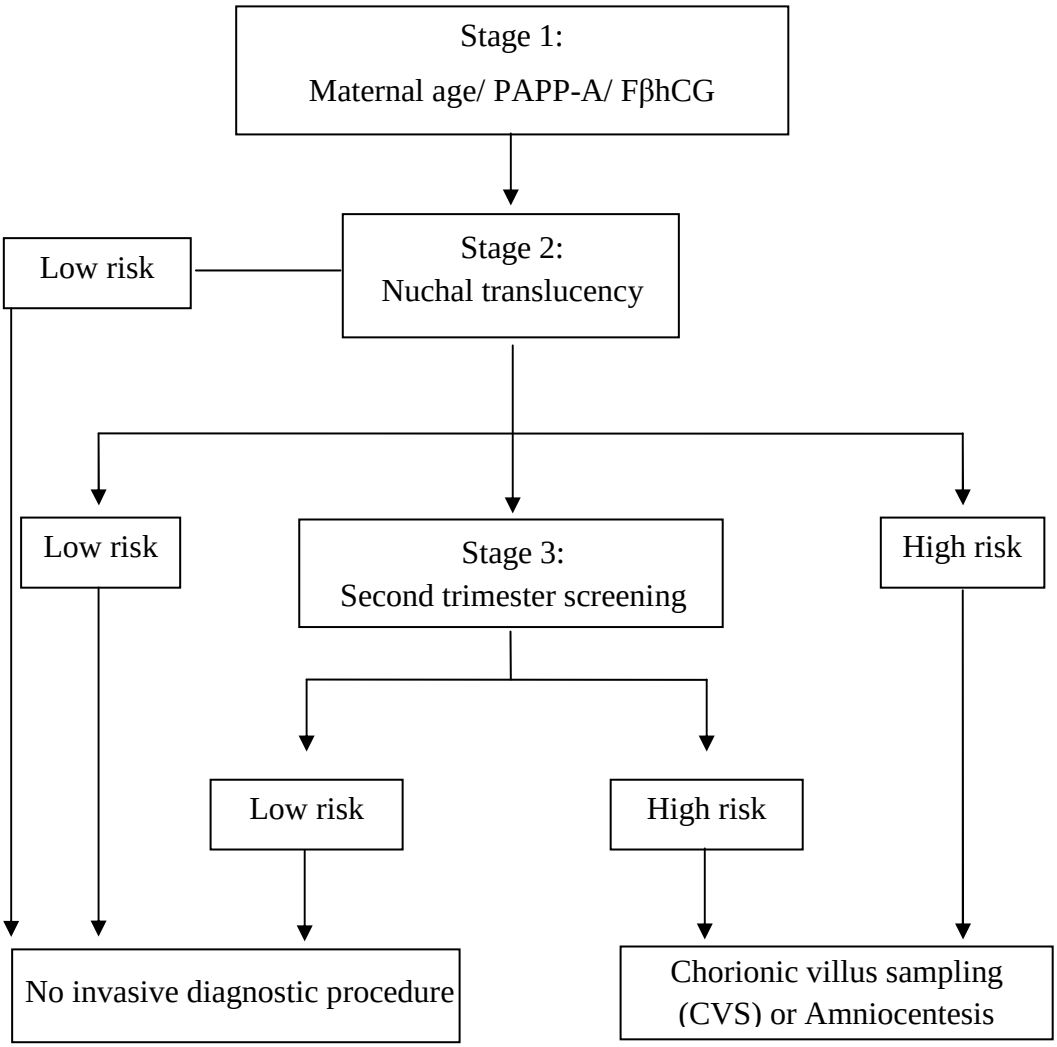


Figure 1.15: Three-stage contingent screening protocol.

Risks derived from first trimester serum screening are estimated for all women. Those women with low risk would not be offered any further testing and NT would be measured on the remainder and the risk would be reassessed. Those with very low risk would be screened negative and would not be offered any further testing. Those with very high risk would be offered early diagnostic test. Women with an intermediate risk would be offered second trimester screening and those with composite risk greater than the cut-off would be offered diagnostic testing.

(reproduced from Wright *et al*., 2006).

Contingent Screening With Advanced Ultrasound Examination In The Second Stage

In 2005 Nicolaides *et al* proposed another variant of contingent screening where complex first trimester ultrasound examination is offered at the second stage of screening. As per this screening protocol, all women were offered CUB screening (NT, PAPP-A and fβhCG) at 11+0 to 13+6 weeks' gestation. Those with intermediate risk are further assessed for risk using first-trimester ultrasound examinations for detecting the presence/absence of the nasal bone, the presence/absence of tricuspid regurgitation or normal/abnormal Doppler velocity waveform in the ductus venosus. The detection rate and false positive rate achieved varies with the method used in the second stage of screening. The detection rate using this protocol has been found to range from 92% at false positive rate of 2.1% for presence/absence of nasal bone, 94.2% at 2.7% for increased impedance in the ductus venosus and 91.7% at 2.7% for tricuspid regurgitation (Nicolaides *et al*., 2005). A similar study conducted by Gyselaers *et al* (2006) concluded that contingent screening reduces the number of pregnancies requiring ultrasound scan.

1.4.6.4 REPEAT MEASUREMENT

Wright and Bradbury (2005) demonstrated the potential value of using highly correlated repeated measures of serum markers taken in the first and second trimester of pregnancy. This contradicts the conventional thinking where the choice of markers in multimarker screening test has been influenced by the extent to which the markers provide independent information as characterized by low correlations between markers and the univariate properties of markers (Wright and Bradbury, 2005).

Using mathematical modelling and the marker parameters published by Wald *et al* (2003) (SURUSS study), they estimated the false positive rate required to give a detection rate of

85% for various combinations of repeat marker measurements. For example, measuring PAPP-A, uE3 and InhA at 10 weeks of gestation and again in a second blood sample at 15 weeks was estimated to give 85% detection at a 0.3% false positive rate. The same performance was also estimated for a PAPP-A, uE3 and NT measurement at 10 weeks followed by repeat PAPP-A and uE3 measurements at 15 weeks (Wright and Bradbury, 2005). The corresponding figures for the integrated test using the same marker parameters are 85% detection at a 1.2% false positive rate (Wald *et al*., 2003). This shows repeat measure screening using serum markers (without NT measurements) is able to achieve similar screening performance as integrated screening (with NT measurement). Wright and Bradbury (2005) has demonstrated that certain combination of highly correlated markers, some of which individually have poor discriminatory power, do have substantial benefits over the established combinations of markers used in the integrated test.

The underlying mechanism of this approach is illustrated using PAPP-A. Even though the discriminatory power of an individual PAPP-A measurement is good in the first trimester and poor in the second trimester, the joint distribution of PAPP-A measurements in the first and second trimesters effectively separates the Down syndrome and unaffected populations. This separation is maximized when the measurement of the marker in the two trimester are highly correlated.

The reports published by Palomaki *et al* in 2006, confirm that measuring PAPP-A in first and second trimester improves Down's syndrome screening. Using paired first and second trimester serum samples from 34 Down's syndrome pregnancies and 514 unaffected pregnancies, Palomaki *et al* (2006) reported that, for a fixed false positive rate of 1%, repeat measures of PAPP-A in addition to the serum integrated test had a detection rate of 86% compared with 82% using integrated testing.

1.4.7 FACTORS INFLUENCING MATERNAL SERUM MARKERS

Studies have shown that there are a number of factors affecting the level of biochemical markers used in Down's syndrome screening. Some of these factors are used to correct results or MoMs in order to derive a more precise risk estimate.

1.4.7.1 GESTATIONAL AGE

Risk estimations for Down's syndrome is critically dependent on accurate gestational age due to the variation of maternal serum concentrations with gestational age. AFP, uE3 and pregnancy-specific-beta-1-glycoprotein (SP-1) levels increase with advancing gestational age in the second trimester. Meanwhile, hCG, fβhCG and InhA levels decreases with advancing gestational age. In order to correct for this variation, the marker concentrations are converted to MoM which will be used to derive likelihood ratios (Aitken *et al*., 2007).

Gestational age can be estimated either from last menstrual period (LMP) date or ultrasound scans (BPD, CRL or head circumference). Wald *et al* (1992a) reported that the detection rate for Down's syndrome increases from 58% to 67% at a fix false positive rate of 5% when ultrasound scan was used to estimate gestational age. The use of ultrasound scan to determine gestational age reduces the variation of MoM values for AFP, hCG and uE3 in unaffected and Down's syndrome pregnancies. This decreases the extend of overlap in these distribution and improves the Down's syndrome screening performance (Wald *et al*., 1992a).

In the second trimester the performance of uE3 improved the most with ultrasound based estimation of gestational age because uE3 concentration changes the most with gestational age (Wald *et al*., 1992a). PAPP-A concentration increases exponentially in the first trimester and continues to increase throughout pregnancy right up to term (Fialova and

Malbohan, 2002). FβhCG concentration increases to a peak at approximately 64 days in the first trimester and then the marker concentration starts decreasing (Berry *et al*., 1995). Similar concentration profiles are found for intact hCG and InhA. When gestational age is overestimated, hCG, fβhCG and InhA MoM values will be higher than expected and AFP and uE3 MoM values will be lower. This will have the effect of increasing the derived risks. An underestimation of gestational age will have the reverse effect (Aitken *et al*., 2007).

1.4.7.2 MATERNAL WEIGHT

Previous studies have reported that heavier women tend to have lower serum marker concentration and lighter women tend to have higher serum marker concentration (Haddow *et al*., 1981; Wald *et al*., 1981; Bartels *et al*., 1993). This occurs because of dilution effect in heavy women who tend to have greater blood volume compared to lighter women. Correction for maternal weight is performed by dividing the MoM value by the expected MoM value calculated from the adjustment equation for her weight (Neveux *et al*., 1996).

According to Neveux *et al* (1996) the reciprocal-linear equation fits second trimester AFP and hCG data better than the classic log-linear equation, for weight correction. In contrast, the reports by Reynolds *et al* (2006) suggest that the log-linear equation gives a better fit compared to the reciprocal-linear equation. Therefore, it is important for screening centres to construct their own weight correction equation based on data from their own population and these should be reviewed to take into account the changing weight profile of the pregnant population. For the first trimester markers, both log-linear and reciprocal-linear equation fit the data well (Spencer *et al*., 2003b). Log-linear equations were found to give a marginally better fit than reciprocal-linear equation for fβhCG and reciprocal-linear equation were found to be marginally better than reciprocal-linear for PAPP-A.

1.4.7.3 MATERNAL SMOKING HABITS

A study by Thomsen *et al,* in 1983, showed that maternal serum AFP level was 20% higher in smokers compared to non-smokers and suggested that this might be due to the increase permeability of the placental barrier caused from smoking. This was followed by Bernstein *et al* (1989), who reported that maternal serum oestradiol level was 17.6% lower and hCG level was 21.5% lower in smoking women in early pregnancy. Many studies since have been conducted on the effect of maternal smoking habit on serum markers and the impact on screening for Down's syndrome.

Cuckle *et al* (1990) reported that there were significant difference in AFP and hCG levels between smokers and non-smokers, in the second trimester. This finding was confirmed by Bartels *et al* (1993) who reported a 21% decrease in hCG level and 3% decrease in uE3 level in smokers compared to non-smokers. Bartels *et al* (1993) also reported that AFP level is significantly increased in smokers compared to non-smokers and that there is a dose-response association. The studies by Ferriman *et al* (1999) also indicate that InhA level is significantly increased in smokers compared to non-smokers. Reports by Rudnicka *et al* (2002) show that smokers had 5% higher of AFP level, 4% lower of uE3 level, 20% lower of fβhCG level and 62% higher of InhA level compared to non-smokers.

The studies by Spencer (1999a) show that PAPP-A level in the first trimester is significantly reduced in smokers compared to non-smokers, and there is no significant change in fβhCG level. This finding was consistent with a study by Niemimaa *et al* (2003) who reported a 20% decrease in PAPP-A level among smokers and no significant changes in fβhCG level. Kagan *et al* (2007) found a reduction of 20% in PAPP-A level and 3% in fβhCG level among smokers. These findings are similar to other first trimester studies on smoking (Spencer *et al*., 2004; de Graaf *et al*., 2000). The report by Miron *et al* in 2008

shows that smoking has similar effects on PAPP-A and fβhCG levels in dried blood samples.

Niemimaa *et al* (2003) also reported a small increase in NT measurement in smokers, but suggested that this finding probably has no clinical relevance to the performance of NT screening due to the small difference between the groups. In contrast, the study by Spencer *et al* (2004) shows that there is no significant difference in NT measurements between smokers and non-smokers.

In 1998, Spencer reported that the second trimester Down's syndrome screening detection rate and false positive rate in smokers were 10% and 2% lower respectively than those in non-smokers. Correcting for smoking will result in overall 2% increase in detection rate for a 0.4% increase in false positive rate. Later studies by Crossley *et al* (2002b) showed that correction for smoking in the second trimester had little effect on the overall detection rate of Down's syndrome but it reduced the false positive rate by 20%. It is found that correcting for smoking gives more accurate risks for individual women. In 2004, Spencer *et al* reported a similar finding on first trimester screening where the false positive rate was reduced from 4.48% to 3.46% after correction in the smoking group.

1.4.7.4 ETHNICITY

Studies have shown that ethnic origin has an impact on the biochemical marker levels, which cannot be explained by differences in maternal weight. Previous studies on first trimester Down's syndrome marker has shown that PAPP-A and fβhCG levels were increased in Afro-Caribbean and Oriental women (Spencer *et al*., 2005b; Spencer *et al*., 2000e; Leung *et al*., 2006). The studies by Spencer *et al* in 2005 show that the PAPP-A levels are higher and the fβhCG lower in South Asian women. Similar studies by Krantz *et*

al (2005) indicate that fβhCG was 16% higher for African Americans, 6% higher for Asians and 9% lower for Hispanics as compared to Caucasians. PAPP-A was 35% higher in African-American women but no significant difference was found in other ethnic groups (Krantz *et al*., 2005). Delta NT was reported to be significantly lower in Afro-Caribbean and South Asian women (Spencer *et al*., 2005b).

The various studies by Canick *et al*., 1990; Bogart *et al*., 1991; Burton and Nieb, 1991; O'Brien *et al*., 1997; Benn *et al*., 1997 confirm that ethnic origin has an impact on second trimester Down's syndrome markers. In 1996, Watt *et al* published a report that black women had 22% higher AFP levels, 19% higher total hCG levels and 12% higher fβhCG levels compared to the Caucasian women. Higher hCG levels were also reported in black women by Kulch *et al* (1993). No significant changes were found in uE3 levels. Muller *et al*, reported in 1994 that Asian women had higher hCG levels compared to the Caucasian women. According to Hseih *et al*. (1995) and Onda *et al*. (1996), Oriental women have higher levels of AFP and hCG compared to Caucasian women.

Correcting biochemical markers for ethnicity would have a significant impact on individual patient-specific risks which could affect a patient's decision on whether or not to have a diagnostic test (Spencer *et al*., 2000e; Spencer *et al*., 2005b). However for NT, although there is significant difference among ethnic groups (Chen *et al*., 2002; Thilaganathan *et al*., 1998; Spencer *et al*., 2005b), correcting NT for ethnicity appears unnecessary (Krantz *et al*., 2005).

1.4.7.5 ASSISTED REPRODUCTIVE TECHNOLOGY

One of the factors known to affect marker levels in Down's syndrome screening is assisted reproductive techniques (ART). In 1996, Barkai *et al* reported a significant increase in

maternal serum hCG level and reduction in uE3 level among pregnancies with ovulation induction compared to pregnancies which are conceived unassisted. Pregnancies with in vitro fertilization (IVF) were found to have decreased levels of AFP, hCG and uE3 but only uE3 levels were significantly decreased. Pregnancies with egg donation were reported to have elevated AFP, hCG and uE3 levels (Barkai *et al*., 1996b).

The changes in marker levels in assisted reproductive pregnancies vary from study to study. The studies conducted by Lambert-Messerlian *et al* (2006), show that pregnancies with IVF had elevated levels of hCG and InhA and decreased levels of uE3 in the second trimester. In assisted reproductive pregnancies with egg donation, AFP and InhA levels were elevated but there were no changes in uE3 and hCG levels. IVF pregnancies with egg donation had higher levels of AFP and InhA compared to IVF pregnancies without egg donation. The studies by Maymon and Shulman (2001) and Shulman and Maymon (2003) show that AFP is elevated in assisted-conception pregnancies with oocyte donation. Therefore, the changes in the markers in ART pregnancies will cause an increase in the false positive rate in Down's syndrome screening in the second trimester (Maymon *et al*., 1999; Maymon and Shulman, 2001; Raty *et al*., 2002; Shulman and Maymon, 2003). In contrast to these findings, Muller *et al* (2003) and Rice *et al* (2005) reported that there were no significant differences in the second trimester markers in ART pregnancies compared with naturally conceived pregnancies and therefore, there were no changes in the false positive rate. However the report by Maymon *et al* (2006) showed that InhA levels are elevated in singleton pregnancies but not twin pregnancies conceived by ART.

In the first trimester, PAPP-A levels were decreased in IVF (Liao *et al*., 2001; Orlandi *et al*., 2002; Maymon and Shulman, 2004; Hui *et al*., 2005; Tul *et al*., 2006; Amor *et al*., 2009; Gjerris *et al*., 2009) and intracytoplasmic sperm injection (ICSI) pregnancies (Hui *et*

al., 2005; Tul *et al*., 2006; Amor *et al*., 2009; Gjerris *et al*., 2009). No significant differences in fβhCG level in ART pregnancies were reported by Orlandi *et al*., 2002; Tul *et al*., 2006; Amor *et al*., 2009; Gjerris *et al*., 2009. In contrast, Liao *et al* (2001) reported an increase in fβhCG level in IVF pregnancies and Hui *et al* (2005) reported a decrease in fβhCG level in IVF pregnancies with fresh embryos. Later studies by Tul *et al* (2006) showed no significant changes in the first trimester markers in assisted-conception pregnancies without ovarian stimulation (transfer of frozen-thawed embryo or spontaneous cycle). In 2009 Gjerris *et al* reported that there are no significant changes in the first trimester markers in the group treated by frozen embryo replacement. But Amor *et al* (2009) contradicted these findings, by reporting that PAPP-A level is decreased in frozen embryo transfer and frozen-thawed embryo transfer groups.

According to Hui *et al* (2005), NT measurement is significantly increased in pregnancies with fresh embryos from IVF, frozen-thawed embryos from IVF and fresh embryos from ICSI. However the studies by Liao *et al* (2001), Orlandi *et al* (2002), Maymon and Shulman (2002) and Tul *et al* (2006) show no significant differences in NT measurement in ART pregnancies.

1.4.7.6 OTHER FACTORS

Multiple pregnancy, fetal sex, gravidity and parity, insulin-dependent diabetes mellitus (IDDM) and vaginal bleeding are some of the other factors known to affect the level of markers in Down's syndrome screening. All serum markers levels in the first and second trimester are increased in multifetal pregnancies (Wald *et al*., 1991; Berry *et al*., 1995; Bersinger *et al*., 2003; Aitken *et al*., 2007). As per the reports by Spencer (2000c), the AFP level was significantly lowered whereas fβhCG was significantly elevated in the presence of a female fetus, compared to that of a male fetus in the second trimester. De Graaf *et al*

(2000) also reported similar findings for AFP and fβhCG. No differences in PAPP-A levels according to gender were found in the first trimester. However, the study by Spencer *et al* (2000d) showed that maternal serum fβhCG and PAPP-A were 15% and 10% higher respectively and fetal NT was 3% lower in the presence of a female fetus.

According to a report by Barkai *et al* (1996a), there is no difference in AFP level in primigravid and multigravid women. Maternal serum hCG and uE3 levels were 5.9% and 3.9% lower respectively in multigravid women than in those tested in their first pregnancy. Barkai *et al* (1996a) reported that these factors do not affect the detection and false positive rates in Down's syndrome screening. Later studies by Spencer *et al* (2000b) show that gravidity and parity is associated with a small but progressive decrease in NT measurement and a small but progressive increase in fβhCG and PAPP-A levels. However, none of these changes was statistically significant.

Second trimester Down's syndrome marker levels are decreased in women with IDDM but variations exist in studies partly due to the fact that correction on maternal weight has not been performed. According to Crossley *et al* (1996), the AFP and hCG levels in IDDM patients were 0.98 and 0.92 MoM respectively after correction for maternal weight was performed. This finding was later confirmed by Sancken and Bartels (2001) who reported no significant differences in AFP, hCG and uE3 levels in the second trimester in women with IDDM compared with women without IDDM. However, the reports by Huttly *et al* (2004) indicate that AFP and uE3 are significantly reduced in women with IDDM but no significant differences were found in hCG, fβhCG and InhA levels. The previous studies on the effect of IDDM on InhA level appear conflicting. Wallace *et al* (1997) reported that InhA was increased in women with IDDM, whereas Wald *et al* (1996) reported a decrease in the levels of InhA.

An InhA study by Aitken and Crossley (2005) for the UK NSC shows that there is no significant change in InhA level in women affected by IDDM. Pedersen *et al* (1998) reported that PAPP-A level were significantly reduced in the first trimester in women with IDDM. Spencer *et al* (2005a) reported that there are no significant differences in NT thickness, PAPP-A and fβhCG levels in women with IDDM.

A report by Cuckle *et al* in 1994b showed that the AFP level was significantly increased in women with vaginal bleeding but hCG and uE3 levels were not significantly altered. However Berry *et al* (1995) reported an increase in the AFP levels and a decrease in the fβhCG level in pregnancies with threatened abortion in the first trimester. The studies by De Biasio *et al* (2003) and Heinig *et al* (2007) indicate an increase in fβhCG level in the first trimester after early vaginal bleeding.

Table 1.5 shows a summary of the impact of various factors on first and second trimester screening marker. This summary is based on the findings from majority of the published papers. However, the impact of these factors varies from study to study.

Table 1.5: Summary of the impact of various factors on first and second trimester screening markers.

Factors	First trimester		Second trimester	
	PAPP-A	FβhCG	AFP	hCG
Smoking	↓	↓	↑	↓
Ethnic origin:				
Black	↑	↑	↑	↑
Oriental	↑	↑	↑	↑
South Asians	↑	↓		
Asians		↑		↑
ART	↓			

1.4.8 UK NATIONAL SCREENING COMMITTEE (NSC) POLICY RECOMMENDATIONS FOR DOWN'S SYNDROME SCREENING

In 2008, a report was published by the NHS Fetal Anomaly Screening Programme Committee, on the UK NSC policy recommendations for Down's syndrome screening for the period between 2007 and 2010. According to the recommendations put forward by the committee, the screening for Down's syndrome should be carried out between 10 to 20 weeks of gestation. However, it is ideal to complete the screening before the 14^{th} week of conception (NHS Fetal Anomaly Screening Programme, 2008).

As per the stipulations of the committee, a Down's syndrome detection rate of greater than 75% with a screen positive rate of less than 3% should be achieved between April 2007 and April 2010. By April 2010, a detection rate of greater than 90% with a screen positive rate of less than 2% is to be achieved. The recommended screening strategies from 2007 are the first trimester combined ultrasound and biochemical (CUB) screening, integrated testing and serum integrated testing. The Health Technology Assessment is currently reviewing two new strategies for screening, namely, repeated measure and cross trimester testing. These tests are expected to further improve the performance of Down's syndrome screening programmes in the period after 2010 (NHS Fetal Anomaly Screening Programme, 2008).

1.5 AIMS

To devise new, and refine existing approaches to the estimation of Down's syndrome risks using combinations of maternal serum marker measurements and ultrasound measurements of the fetus with the objective of maximising detection rates of Down's syndrome pregnancies and minimising false positive rates

Specific objectives:

1. To design and test, within the first trimester, a screening protocol where all women have serum marker measurements but only a proportion subsequently have ultrasound NT measurements contingent upon the results of their biochemical tests.

2. To design and test using statistical modelling tools a contingent screening protocol which incorporates repeat measures of serum markers across the first and second trimesters with and without ultrasound NT measurements.

3. To establish, through retrospective analysis of routine screening data, the effect of smoking and ethnicity on serum marker concentrations in paired first and second trimester serum samples.

4. To investigate, through retrospective analysis of routine screening data, the effects of assisted reproductive technology on serum marker concentrations and the implications for the estimation of Down's syndrome risks.

5. To investigate, through retrospective analysis of routinely collected screening data, the accuracy of self-reported maternal smoking and its effect on birth weight, duration of pregnancy and second trimester maternal serum marker concentrations, and the implications for the estimation of Down's syndrome risks.

CHAPTER 2: MATERIALS

2.1 PATIENT SAMPLES

The Biochemical Genetics department located within the Duncan Guthrie Institute of Medical Genetics, Yorkhill provides prenatal screening services for Down's syndrome and neural tube defect for the 60% of the Scottish pregnant population resident in West of Scotland. Over 20,000 women (around 70% uptake) opt for the prenatal screening test each year in the West of Scotland. Screening for Down's syndrome started in 1987 with second trimester AFP measurement and in 1991, hCG was incorporated into the screening programme. Two types of screening program are currently offered to the pregnant population; 1) first trimester CUB screening and 2) second trimester double marker screening.

First trimester CUB screening is normally performed at 9-13 weeks of gestation. At the antenatal clinic, patient's information such as age, date of last menstrual period, date of birth, weight, height, smoking status and ethnicity are collected. Maternal blood samples are collected by venepuncture in plain tubes and ultrasound scan is carried out for fetal viability, multiple pregnancy, gross abnormality and CRL or BPD measurement. Blood samples are collected from 9 weeks and 0 days of gestation to 14 weeks and 0 days of gestation and NT measurements are carried out on those women who have a fetal CRL between 40 to 84mm which equates to 10 weeks and 6 days to 14 weeks and 0 days of gestation. A portion of the serum not used for routine testing is stored at -20^{o}C.

All the ultrasound operators have receive training in the NT measurement protocol (Stenhouse *et al*., 2004) and are subjected to on-going quality assurance through a bi-monthly review of images and analyses of the distribution of NT measurements (Stenhouse

et al.,2002). The protocols for NT measurement used in CUB screening (Crossley *et al.*, 2002, Stenhouse *et al*., 2004) are similar to those described by the Fetal Medicine Foundation (FMF).

The protocol for NT measurement used by Stenhouse *et al* (2004) is summarized:

a. NT measurements are carried out on a fetus lying in the sagittal plane.
b. The ultrasound image is magnified to fill at least three-quarters of the screen.
c. The fetal skin and amnion are visualised separately by waiting for spontaneous fetal movement away from the amnion or by asking the mother to cough or by tapping the abdomen.
d. Care is taken not to include the nuchal cord in the NT measurement.
e. The maximum NT thickness is measured to the nearest 0.1mm by placing the callipers on the inner edge of the fetal skin and outer edge of the soft tissue overlying the cervical spine.
f. Measurements are made on three separately captured images and recorded.

Three measurements of NT are obtained and the mean of the three measurements are calculated. The information on NT measurement obtained, the ultrasound machine used and initials of the ultrasound operator are recorded in the CUB screening request form. A return appointment is given to those women whose gestation is less than 9 weeks to take blood samples and perform NT scan within the appropriate gestational window. The second trimester screening test is offered to those women who are too late for the first trimester screening test with CRL>84mm or BPD>28mm. These data are stored in the prenatal screening database.

Second trimester screening is offered at 15-20 weeks of pregnancy. Approximately 5 to 10 mls of maternal venous blood samples are collected in the second trimester to measure AFP and hCG levels. All the blood samples together with a standard request form providing patient's information are sent to Biochemical genetics department. At the laboratory, the clotted blood samples are given a laboratory number and centrifuged at 2000rpm for 10 minutes. An aliquot of serum is used for the assay and the remainder of the serum is stored at -20^{o}C. Patient's information and sample details are entered into a database using Lifecycle software. The results from the biochemical assay are merged with the patient's information and the risk of having a Down's syndrome or neural tube defect fetus is calculated. The first trimester database contains information on our 15,000 pregnancies. For second trimester screening, data from the current Laboratory Information Management System (Lifecycle) was used as in this new system information on ethnicity and ART were systematically recorded. This database contains information on over 50,000 pregnancies. These data and their matching serum samples were the resource accessed for the studies described in this thesis.

2.2 RETROSPECTIVE STUDY OF WITHIN-TRIMESTER CONTINGENT SCREENING

Using data from routine CUB screening, a re-analysis of the marker results using a within-trimester contingent testing model was carried out to assess the likely performance of this approach and gauge the potential for reducing the ultrasound resources required for first trimester population screening. A cohort of 10,189 pregnancies where CUB screening was performed between July 2000 and October 2005 was identified. These pregnancies had full ascertainment of Down's syndrome cases. After exclusion of twin pregnancies, there were

44 Down's syndrome and 10,145 unaffected pregnancies within this group. The median maternal age at the expected date of delivery was 33.1 years, and 36.9% of women were aged 35 years and over. The number of blood samples taken at each week of gestation is shown in Table 2.1.

Table 2.1: Number of blood samples taken at each gestational week

Gestational Week	**Number of blood samples**
9	197
10	649
11	2234
12	3987
13	2891
14	231

In the majority of pregnancies, blood samples and NT measurements were taken during the course of the same antenatal clinical appointment. In a proportion of women (28%), blood samples were not taken at the same visit as the NT measurement either because of logistic reasons or too early a presentation for NT (outside the CRL range of 40–84 mm), or inability to obtain an NT measurement at the first attempt, necessitating a return visit. Information on PAPP-A level, fβhCG level, NT measurement, gestational age based on ultrasound, maternal age risk, risk based on biochemical markers and final risk of having a Down’s syndrome fetus for these pregnancies was available in the database.

2.2.1 RETROSPECTIVE CONTINGENT TESTING BASED ON LMP ESTIMATE OF GESTATION

A requirement of the above study is the need for an accurate estimation of gestation based on ultrasound measurement of CRL. Without this, interpretation of the serum markers results is not possible. As an addition to this study the performance of the model was re-evaluated using gestational information based on LMP. This is relevant when ultrasound measurements are not available at venepuncture. Using the same data set information on last menstrual period was only available in 6895 pregnancies; 6865 unaffected and 30 Down's syndrome pregnancies. Of these pregnancies 5979 were certain with the LMP dates. All gestations were established based on LMP using the information obtained at the time of sampling. In this dataset, the median maternal age at the expected date of delivery was 33.7 years, and 39.8% of women were aged 35 years and over.

2.3 MODELLING CROSS-TRIMESTER CONTINGENT SCREENING

Statistical modelling is a reliable tool used to predict the efficacy of screening policies. In this study, S-PLUS program was used to model cross-trimester contingent screening using various combinations of markers. The medians, SD and correlation coefficients were obtained from 8 sources; Wald *et al* (2003), Glasgow dataset (as described above), Spencer *et al* (2002), Spencer *et al* (2003), Cuckle *et al* (2005), Cuckle *et al* (1995), Aitken and Crossley (2005) and Aitken *et al*., 2007. The SDs for the unaffected and Down's syndrome pregnancies was assumed to be equal for the serum markers but not for the NT measurement. The population covariance matrices for unaffected and Down's syndrome pregnancies were also assumed to be equal. This is called 'pooled covariance matrices' (personal communication from Prof. Dave Wright).

The performances of few screening policies were re-evaluated using 10% larger SDs for affected cases than for unaffected cases. Analysis using previous studies (Spencer *et al.*, 2002; Aitken and Crossley, 2005) have shown that the SDs for first trimester PAPP-A, hCG and fβhCG and second trimester AFP, hCG, fβhCG, uE3 and InhA in affected cases were approximately 10% larger compared to unaffected cases.

The first trimester PAPP-A, fβhCG and hCG medians for Down's syndrome pregnancies and SDs for unaffected pregnancies were obtained from Spencer *et al* (2002) which had a large number of unaffected and Down's syndrome cases. The medians for first trimester NT were obtained from Cuckle *et al* (2005) where the median was derived from meta-analysis of nine studies including one study using the Scottish population (Crossley *et al*., 2002). The NT SDs were obtained from Spencer *et al* (2003c) which were derived from four large prospective studies combined. The first trimester AFP, uE3 and InhA medians were obtained from the Wald *et al* (2003). Although the program required this information, first trimester AFP, uE3 and InhA were not used in the analysis in this study. All medians of second trimester markers were obtained from Aitken *et al* (2007) which were derived from meta-analysis of various studies. The SDs of second trimester AFP, hCG, uE3 and InhA were obtained from Aitken and Crossley (2005) from data obtained in a large retrospective study of InhA for the National Screening Committee (personal communication with Dr. Jenny Crossley).

Most of the correlation – coefficients were obtained from Scottish data (Aitken and Crossley, 2005; Glasgow dataset). Correlation – coefficients from Wald *et al* (2003) were only used when the information was not available from other sources. The correlation-coefficients for NT measurement were assumed to be 0 because NT has a very low correlation with other serum markers. The maternal age distribution was taken to be that of Scotland for the year 2007 (General Register Office for Scotland). The mean and SD for maternal age were obtained from Glasgow dataset. The detection and false positive rates were estimated using Monte-Carlo methods. Samples of 500 000 observations were drawn. Tables 2.2, 2.3 and 2.4 show the medians, SDs and correlation coefficient used in modeling of screening programme.

Table 2.2: Median marker levels (log_{10} MoM) for Down's syndrome pregnancies

Trimester	Markers	Week				Source
		10	11	12	13	
First	NT	-	0.363612	0.32222	0.281033	Cuckle *et al* (2005)
	AFP	-0.0655	-0.0655	-0.0655	-0.0655	Wald *et al* (2003)
	uE3	-0.0044	-0.0605	-0.1024	-0.1427	Wald *et al* (2003)
	hCG	0.0316	0.061	0.1484	0.2267	Spencer *et al* (2002)
	fβhCG	0.2549	0.2586	0.3054	0.3203	Spencer *et al* (2002)
	Inhibin A	-0.0269	0.1303	0.2380	0.3384	Wald *et al* (2003)
	PAPP-A	-0.336	-0.3269	-0.2785	-0.1883	Spencer *et al* (2002)
Second	AFP	-0.1249	-0.1249	-0.1249	-0.1249	Aitken *et al* (2007)
	uE3	-0.1427	-0.1427	-0.1427	-0.1427	Aitken *et al* (2007)
	hCG	0.316	0.316	0.316	0.316	Aitken *et al* (2007)
	fβhCG	0.3541	0.3541	0.3541	0.3541	Aitken *et al* (2007)
	Inhibin A	0.2989	0.2989	0.2989	0.2989	Aitken *et al* (2007)
	PAPP-A	0.00432	0.00432	0.00432	0.00432	Aitken *et al* (2007)

Table 2.3: Standard deviation for the screening markers in each trimester of pregnancy

Trimester	Markers	Unaffected				Affected (from papers)	Affected (used in the analysis)	Source
		Week						
		10	11	12	13			
First	NT	-	0.132	0.116	0.112	0.229	0.229	Spencer *et al* (2003c)
	AFP	0.1818	0.1818	0.1818	0.1818	0.1672	0.1818	Wald *et al* (2003)
	uE3	0.1204	0.1204	0.1204	0.1204	0.1720	0.1204	Wald *et al* (2003)
	hCG	0.2174	0.2174	0.2174	0.2174	0.2238	0.2174	Spencer *et al* (2002)
	fβhCG	0.2613	0.2613	0.2613	0.2613	0.2787	0.2613	Spencer *et al* (2002)
	Inhibin A	0.2191	0.2191	0.2191	0.2191	-	0.2191	Wald *et al* (2003)
	PAPP-A	0.2361	0.2361	0.2361	0.2361	0.2822	0.2361	Spencer *et al* (2002)
Second	AFP	0.1407	0.1407	0.1407	0.1407	0.1423	0.1407	Aitken and Crossley (2005)
	uE3	0.1187	0.1187	0.1187	0.1187	0.1385	0.1187	Aitken and Crossley (2005)
	hCG	0.2308	0.2308	0.2308	0.2308	0.2445	0.2308	Aitken and Crossley (2005)
	fβhCG	0.2613	0.2613	0.2613	0.2613	0.2787	0.2613	Spencer *et al* (2002)
	Inhibin A	0.2255	0.2255	0.2255	0.2255	0.2436	0.2255	Aitken and Crossley (2005)
	PAPP-A	0.2170	0.2170	0.2170	0.2170	-	0.2170	Glasgow dataset

Table 2.4: Correlation coefficient for serum marker

	Wald *et al* (2003)	**Glasgow dataset**	**Spencer *et al* (2002)**	**Aitken and Crossley (2005)**	**Cuckle *et al* (1995)**	**Parameters used**	**Source used**
h1 - f1	0.72	0.725				0.725	Glasgow dataset
h1 - p1	0.22	0.314	0.2382			0.2382	Spencer *et al* (2002)
h1 - a2	0.07	0.067	0.135			0.067	Glasgow dataset
h1 - u2	0.03			-0.078		-0.078	Aitken and Crossley (2005)
h1 - h2	0.72	0.667				0.667	Glasgow dataset
h1 - f2	0.72	0.632				0.632	Glasgow dataset
h1 - i2	0.32			0.329		0.329	Aitken and Crossley (2005)
h1 - p2	0.39	0.382	0.2382			0.382	Glasgow dataset
f1 - p1	0.14	0.283	0.2178			0.2178	Spencer *et al* (2002)
f1 - a2	0.02	-0.014	0.0428			0.0428	Spencer *et al* (2002)
f1 - u2	-0.03				-0.136	-0.136	Cuckle *et al* (1995)
f1 - h2	0.56	0.547				0.547	Glasgow dataset

h1: hCG in 1st trimester, f1: fβhCG in 1st trimester, p1: PAPP-A in 1st trimester, a2: AFP in 2nd trimester, u2: uE3 in 2nd trimester, h2: hCG in 2nd trimester, f2: fβhCG in 2nd trimester, i2: InhA in 2nd trimester, p2: PAPP-A in 2nd trimester

Table 2.4: Correlation coefficient for serum marker (cont)

	Wald *et al* (2003)	**Glasgow dataset**	**Spencer *et al* (2002)**	**Aitken and Crossley (2005)**	**Cuckle *et al* (1995)**	**Parameters used**	**Source used**
f1 - f2	0.76	0.753				0.753	Glasgow dataset
f1 - i2	0.29					0.29	Wald *et al* (2003)
f1 - p2	0.27	0.319	0.2178			0.319	Glasgow dataset
p1 - a2	0.12	0.124				0.124	Glasgow dataset
p1 - u2	0.12					0.12	Wald *et al* (2003)
p1 - h2	0.06	0.158	0.2382			0.2382	Spencer *et al* (2002)
p1 - f2	0.06	0.194	0.2178			0.2178	Spencer *et al* (2002)
p1 - i2	0.02					0.02	Wald *et al* (2003)
p1 - p2	0.7	0.777				0.777	Glasgow dataset
a2 - u2	0.2			0.182	0.21	0.182	Aitken and Crossley (2005)
a2 - h2	0.15	0.171	0.135	0.136	0.122	0.136	Aitken and Crossley (2005)
a2 - f2	0.1	0.065	0.0428		0.058	0.0428	Spencer *et al* (2002)

h1: hCG in 1st trimester, f1: fβhCG in 1st trimester, p1: PAPP-A in 1st trimester, a2: AFP in 2nd trimester, u2: uE3 in 2nd trimester, h2: hCG in 2nd trimester, f2: fβhCG in 2nd trimester, i2: InhA in 2nd trimester, p2: PAPP-A in 2nd trimester

Table 2.4: Correlation coefficient for serum marker (cont)

	Wald *et al* (2003)	**Glasgow dataset**	**Spencer *et al* (2002)**	**Aitken and Crossley (2005)**	**Cuckle *et al* (1995)**	**Parameters used**	**Source used**
a2 - i2	0.2			0.191		0.191	Aitken and Crossley (2005)
a2 - p2	0.2	0.175				0.175	Glasgow dataset
u2 - h2	-0.04			-0.078	-0.092	-0.078	Aitken and Crossley (2005)
u2 - f2	-0.06				-0.136	-0.136	Cuckle *et al* (1995)
u2 - i2	-0.09			-0.05		-0.05	Aitken and Crossley (2005)
u2 - p2	0.1					0.1	Wald *et al* (2003)
h2 - f2	0.87	0.86				0.86	Glasgow dataset
h2 - i2	0.43			0.329		0.329	Aitken and Crossley (2005)
h2 - p2	0.28	0.287	0.2382			0.287	Glasgow dataset
f2 - i2	0.41						Wald *et al* (2003)
f2 - p2	0.28	0.285	0.2178			0.285	Glasgow dataset
i2 - p2	0.25						Wald *et al* (2003)

h1: hCG in 1st trimester, f1: fβhCG in 1st trimester, p1: PAPP-A in 1st trimester, a2: AFP in 2nd trimester, u2: uE3 in 2nd trimester, h2: hCG in 2nd trimester, f2: fβhCG in 2nd trimester, i2: InhA in 2nd trimester, p2: PAPP-A in 2nd trimester

2.4 RETROSPECTIVE STUDY ON EFFECT OF SMOKING & ETHNICITY ON SERUM MARKER CONCENTRATION IN PAIRED FIRST AND SECOND TRIMESTER SAMPLES

The effect of smoking and ethnicity on AFP, hCG, PAPP-A and fβhCG concentrations were studied using paired first and second trimester serum samples. All normal pregnancies which were not affected by chromosomal abnormalities and which had CUB screening performed at the Queen Mother's Maternity Hospital in Glasgow were identified between August 2000 and October 2006. After exclusion of twin pregnancies, samples with insufficient serum and missing samples, 939 first trimester serum samples could be paired with a second trimester sample taken for AFP measurement at 15 to 20 weeks of gestation as a screen for neural tube defects. Information about the ethnic origin and maternal smoking habits of these women was obtained from the screening database. A recheck against the original request form and reclassification of the ethnic origin of the patients was performed to confirm the accuracy of the information. The study group consisted of 501 Caucasian, 268 South Asian, 66 Oriental, 42 Middle Eastern, 35 Black and 27 Asian women. The Caucasians were used as controls. The Caucasians were a random selection of cases matched to the non-Caucasian group. Maternal serum PAPP-A and fβhCG levels were available for all the first trimester samples and AFP and hCG levels were available for all the second trimester samples. To study the effect of smoking, paired first and second trimester serum samples from 459 Caucasian women (366 non-smokers and 93 smokers) were analysed.

2.5 RETROSPECTIVE STUDY ON EFFECT OF ASSISTED REPRODUCTIVE TECHNOLOGY ON SERUM MARKER CONCENTRATION

The level of first and second trimester biochemical markers in women conceived after various form of ART was assessed in this study. Pregnant women who had CUB screening or second trimester screening for Down's syndrome between October 2005 and January 2009 were identified from the screening database. Due to patients' confidentiality, information on ART was not requested in the screening request forms. Therefore, ART information was only available in cases where this information was volunteered.

There were 127 first trimester ART pregnancies and 129 second trimester ART pregnancies identified. A recheck against the original request form and classification of the type of ART procedure was performed. The pregnancies were classified into four categories; 1. normal pregnancy, 2. IVF or ICSI with fresh eggs; 3. IVF or ICSI with frozen embryo and 4. IVF with donor egg. Table 2.5 shows the number of pregnancies in each category of ART procedure.

Table 2.5: Number of pregnancies in each ART procedure category

ART	**1st trimester**	**2nd trimester**
1. Normal	10891	61448
2. IVF or ICSI with fresh eggs	91	105
3. IVF or ICSI with frozen embryo	29	15
4. IVF with donor's egg	7	9

2.6 RETROSPECTIVE STUDY ON BIRTH WEIGHT, DURATION OF PREGNANCY AND SECOND TRIMESTER MATERNAL SERUM SCREENING MARKERS IN NON-SMOKERS AND SMOKERS

The maternal serum AFP and hCG levels, birth weight and gestation at delivery in a large cohort of self-reported non-smokers and smokers were studied to establish the modifying effect of smoking on these pregnancy and birth parameters. A cohort of 21,029 pregnant women who had second trimester screening for Down's syndrome and neural tube defects in the West of Scotland between May 2003 and July 2004 were identified. The records of those women who had second trimester prenatal screening were matched with their obstetric records (Scottish Morbidity Records (SMR02), NHS Information Services Division). The SMR02 dataset contains self-reported smoking information at booking appointment, baby's date-of-birth, mother's date-of-birth, maternal deprivation category of residence, date of booking, birth weight and gestation at delivery. The second trimester screening records contain self-reported smoking information at screening appointment and gestation at sampling. After data linkage, the final dataset contained maternal weight, AFP MoM, hCG MoM, self-reported smoking information at both booking and screening appointment, birth weight and gestation at delivery. The screening request form was used to record information on smoking status at screening appointment. Smoking information at booking appointment was recorded as one of three options: current smoker, former smoker and never smoker. At screening, four options were offered: non-smoker, smoker, stopped smoking during pregnancy and stopped smoking prior to pregnancy. The smoking status was recorded as 'not available' for those women who did not respond to the question or where smoking information was not recorded on the form.

2.6.1 ACCURACY OF SELF-REPORTED SMOKING INFORMATION AT BOOKING AND SCREENING APPOINTMENTS

In this study, the reliability of self-reported smoking information at booking and screening appointments were validated using cotinine analysis. From the database 3550 serum samples were randomly selected for cotinine analysis. After excluding samples with insufficient serum, cotinine testing was carried out on 3475 thawed serum samples using the Cozart STD Micro-Plate Cotinine EIA (Cozart UK Ltd). Selection of study sample for cotinine analysis is illustrated in Figure 2.1.

2.6.2 EVALUATION ON THE EFFECTIVENESS OF TWO SCREENING FORMS USED TO COLLECT SELF-REPORTED SCREENING INFORMATION AT ANTENATAL CLINICS

A small study was performed to compare two different screening forms used for collecting self-reported smoking information. Two datasets (March 2006 and March 2008) were used in this study. The self-reported smoking information in March 2006 dataset was collected using the screening form where women were given four options; non-smoker, smoker, stopped smoking during pregnancy and stopped smoking prior to pregnancy. The self-reported smoking information in March 2008 dataset was collected using the screening form where women were given only two options; non-smoker or smoker. Those women who stopped smoking during pregnancy and stopped smoking prior to pregnancy were classified as ‘non-smoker’. The smoking status information was also included in the screening report allowing antenatal clinic staff to contact the West of Scotland Regional Genetics Service department if there was any mistake in the smoking information as this could affect the interpretation of results. From each dataset maternal serum samples from 100 self-reported non-smokers and 100 self-reported smokers were randomly selected for cotinine testing. The accuracy of self-reported smoking information was calculated.

2.6.3 BIRTH WEIGHT AND GESTATION AT DELIVERY

The associations between birth weight, gestation at delivery and second trimester markers in self-reported smokers and non-smokers were investigated using data from the routine second trimester prenatal screening programme in Scotland. Of 21,029 second trimester records 15,973 singleton pregnancies which had full information on birth weight, gestation at delivery, AFP level, hCG level and self-report as smoker or non-smoker were selected for this analysis. Those who responded with stopped during or prior to pregnancy were excluded from further analysis. The pregnancy was classified as 'low birth weight' if the infant was under 2500g (Wilcox and Johnson, 1992).

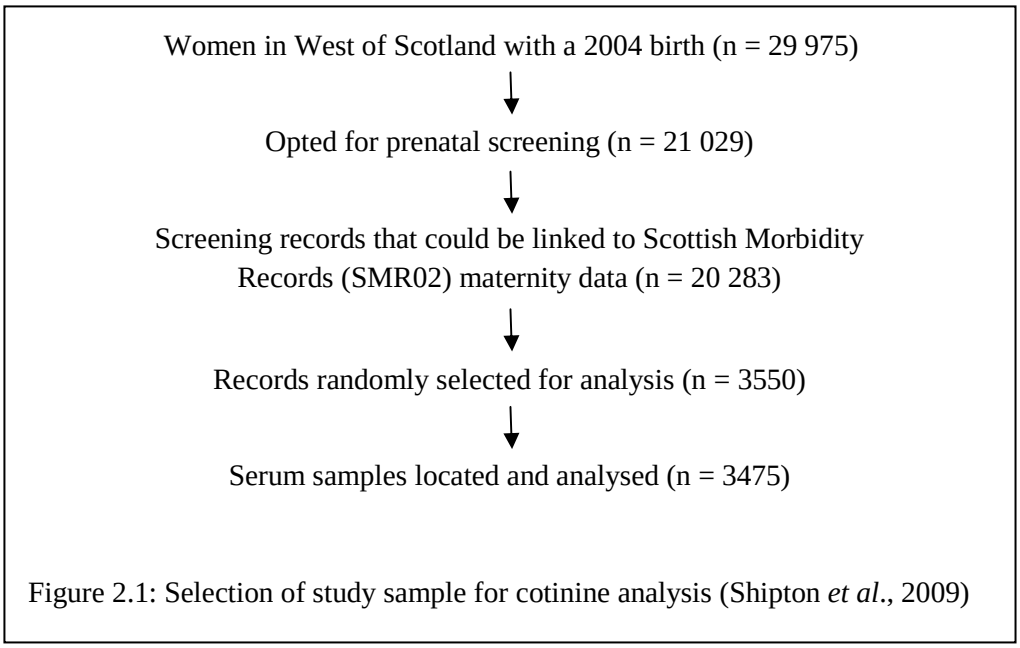


Figure 2.1: Selection of study sample for cotinine analysis (Shipton *et al*., 2009)

CHAPTER 3: METHODS

3.1 RETROSPECTIVE STUDY OF WITHIN - TRIMESTER CONTINGENT SCREENING

Retrospectively, the performance of the full CUB screening test was compared with that of the two-stage contingent protocol of Christiansen and Larsen (2002) but using the same final cut-off risk as the CUB screening programme. In this screening protocol, women would be offered NT measurement based on their first trimester biochemical test (Figure 3.1). Initially, a risk at term was calculated from the PAPP-A and fβhCG results combined with the maternal age risk for all women. A high risk cut-off of 1:42 and a low-risk cut-off of 1:1000 were defined using the statistical approach described by Christiansen and Larsen (2002). The high-risk cut-off is dependent on the final risk and the low-risk cut-off is chosen empirically to adjust the proportion of women requiring NT measurement.

Women were divided into three groups according to their initial biochemistry and maternal age risk. For those with intermediate risks between 1:42 and 1:1000, the likelihood ratio derived from the NT measurement in MoM was then combined with the biochemistry and maternal age risk and the composite risk assessed against a final cut-off risk of 1:250 at term. Those women with a final risk ≥1:250 were classified as screen positive and added to the initial high-risk group. Those with final risks of <1:250 were classified as screen negative and added to the initial low-risk group. The final risk cut-off of 1:250 was chosen based on the current first trimester CUB screening cut-off. From the distribution of risks in Down syndrome and unaffected pregnancies the detection rate and false positive rate of the contingent screening model was calculated.

The performance of contingent screening using LMP based gestational age at the first stage of screening was also evaluated. Multiple of the appropriate gestation medians (MoM)

(with maternal weight correction for PAPP-A and fβhCG and smoking correction for PAPP-A) were calculated for the biochemical markers using LMP based gestational age. The correlation co-efficient between markers, medians and standard deviation values of all the markers for the unaffected and Down's syndrome pregnancies were taken from the literature (Spencer *et al*., 1999b). Maternal age risk was calculated using the equation as described by Cuckle *et al* (1987). The likelihood ratio was calculated based on the double test (PAPP-A and fβhCG) and maternal age risk at the first stage of screening. Of those who were offered NT measurement, the MoM values of the biochemical markers were re-calculated using CRL/BPD based gestational age. The likelihood ratio derived from NT measurement, PAPP-A and fβhCG in MoMs was then combined with maternal age risk. The detection rate and false positive rate of the contingent screening model was calculated.

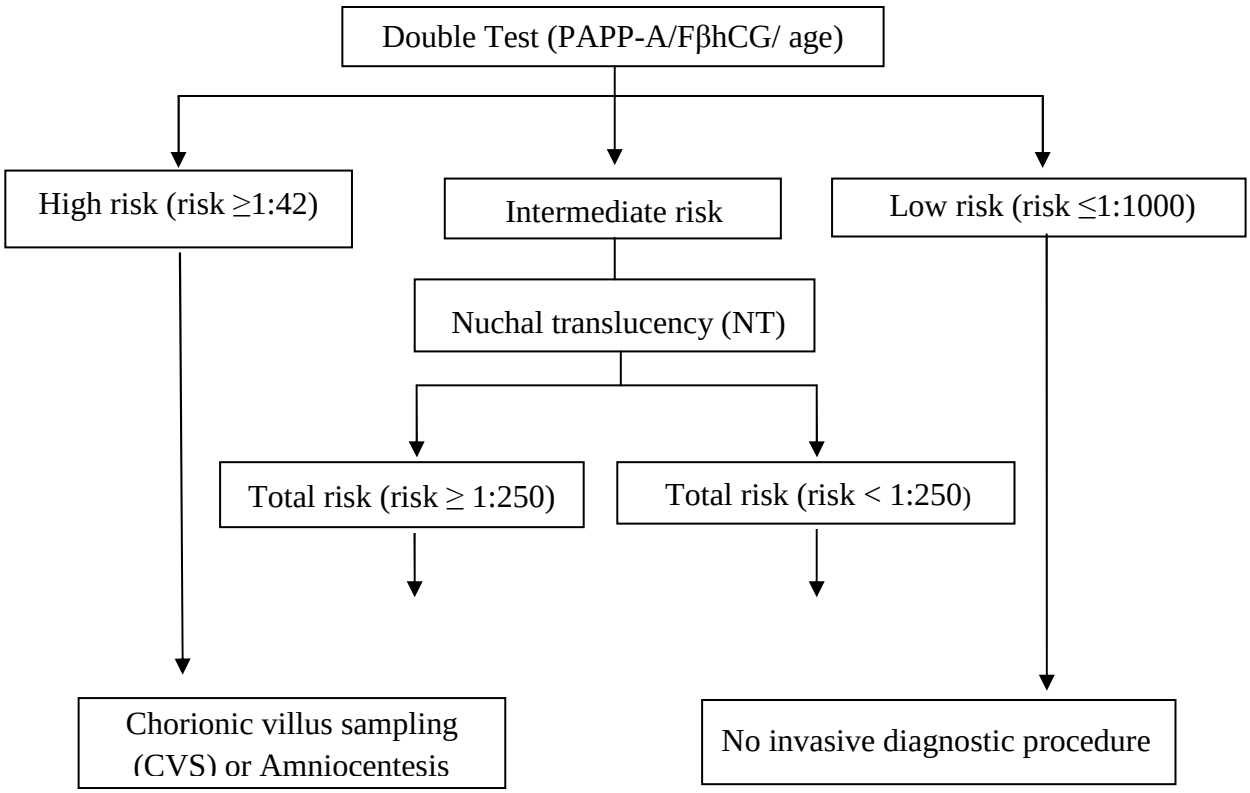


Figure 3.1 - Within-trimester contingent screening protocol. Risks derived from serum markers and maternal age were estimated for all women and used to triage the population into high, low and intermediate risk groups. Women whose risk is equal or greater than 1:42 would be offered a diagnostic test and those women whose risk is equal or lower than 1:1000 would not be offered any further testing. Women with an intermediate risk would be offered NT measurement and those with a composite risk equal or greater than the cut-off of 1:250 would be offered diagnostic testing.

3.1.1 STATISTICAL CALCULATION TO DETERMINE THE CUT-OFFS

The method used in this study was based on the statistical calculations used in a study by Christiansen and Larsen (2002). The final risk for a particular pregnancy is based on the serological test, *a*, and NT measurement, *r*. Therefore, the final risk is, *a x r*. If the final risk is >1:250 it follows that:

$$ar > 1:250$$

$$r > 0.004/a$$

The likelihood ratio of NT measurement was established using the published NT distribution (Cuckle and van Lith, 1999) and the formulae for the distribution of NT log MoM in normal and DS pregnancies.

$$Log_{10} MoM\ NT = -0.1076 + 0.2995 \times \sqrt{(0.7863 + log_{10} r)}.$$

$$Log_{10} r \geq -0.7863\ (\ log_{10} r \text{ can not be } < -0.7863)$$

$$r \geq 0.164$$

Therefore, if the serologically defined risk, *a*, is > 0.024, then no NT measurement can reduce the final risk to a value <1:250. Such calculations were performed to determine the initial high risk cut-offs for different final risk cut offs (Christiansen and Larsen, 2002)

3.2 MODELLING CROSS-TRIMESTER CONTINGENT SCREENING

Using the S-PLUS statistical programme, the performances of various types of cross-trimester contingent screening policies were evaluated. Protocols were designed in which all women would receive a first trimester screening test and those with intermediate risks would receive a follow up second trimester screening test (Figure 3.2).

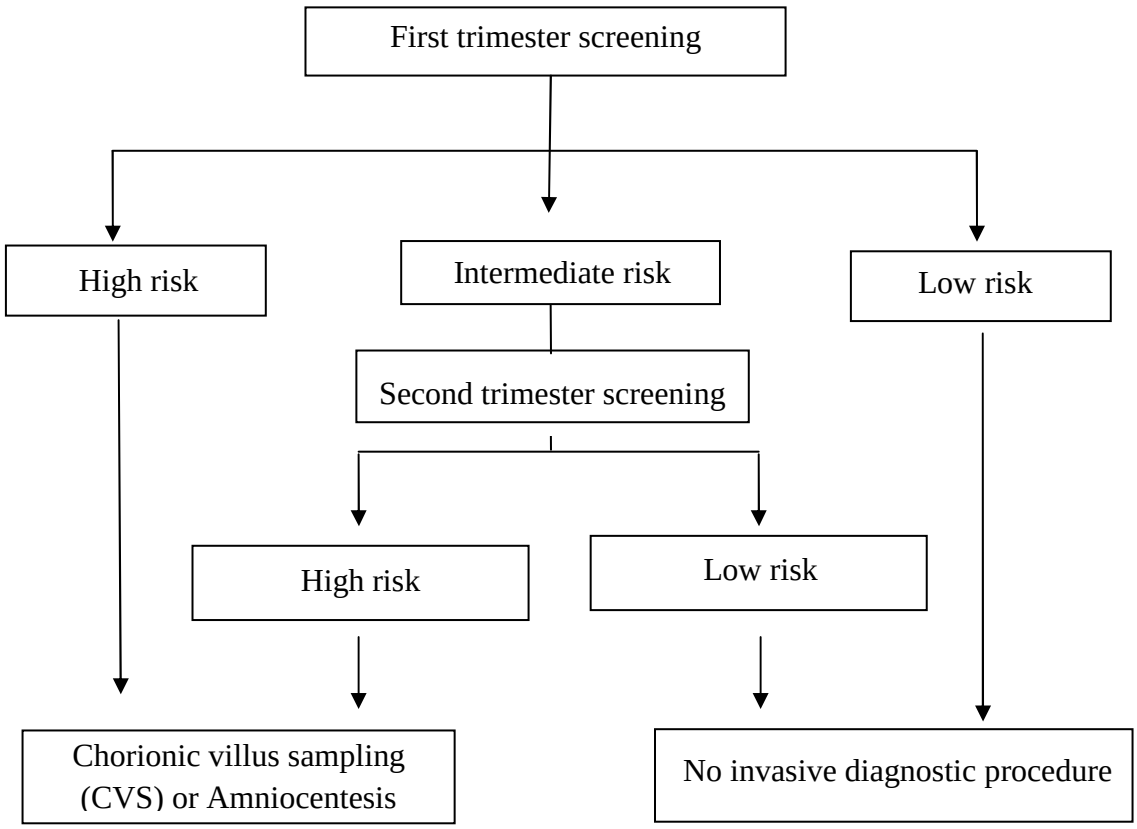


Figure 3.2: Cross- trimester contingent screening protocol.

Risks derived from first trimester screening were estimated for all women and used to triage the population into high, low and intermediate risk groups. Women with high risk would be offered a diagnostic test and those women with low risk would not be offered any further testing. Women with an intermediate risk would be offered second trimester screening and those with composite risk greater than the cut-off would be offered diagnostic testing.

3.2.1 MODELLING

The performance of cross-trimester contingent screening using various combinations of markers was evaluated using S-PLUS statistical software. The $\log_{10}$ transformed marker values were assumed to follow multivariate Gaussian distributions for both unaffected and Down's syndrome pregnancies. Truncation limits from Wald et al (2003) (SURUSS) were applied in the risk calculation. These were: first trimester: NT (0.5–2.5), AFP (0.4–3.0), uE3 (0.4–2.0), total hCG (0.3–3.0), fβhCG (0.3–5.0), InhA (0.3–5.0), PAPP-A (0.2–3.0); second trimester: AFP (0.4–3.0), uE3 (0.4–2.0), total hCG (0.4–5.0), fβhCG (0.3–5.0), InhA (0.3–5.0), PAPP-A (0.2–3.0).

Before analysis was performed, the SDs, medians and correlation-coefficients for each week of gestation for unaffected and Down's syndrome pregnancies were entered into the database in the S-PLUS software programme (Figures 3.3 and 3.4). The maternal age distributions (12 to 50 years), the mean and SD of maternal age were also entered into the database in the software (Figure 3.5).

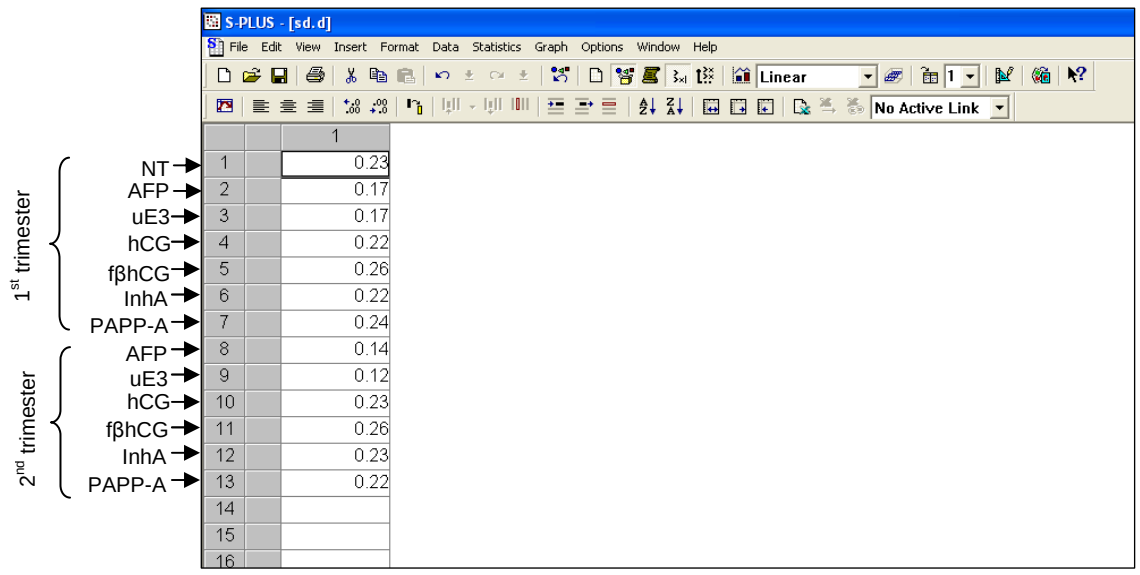
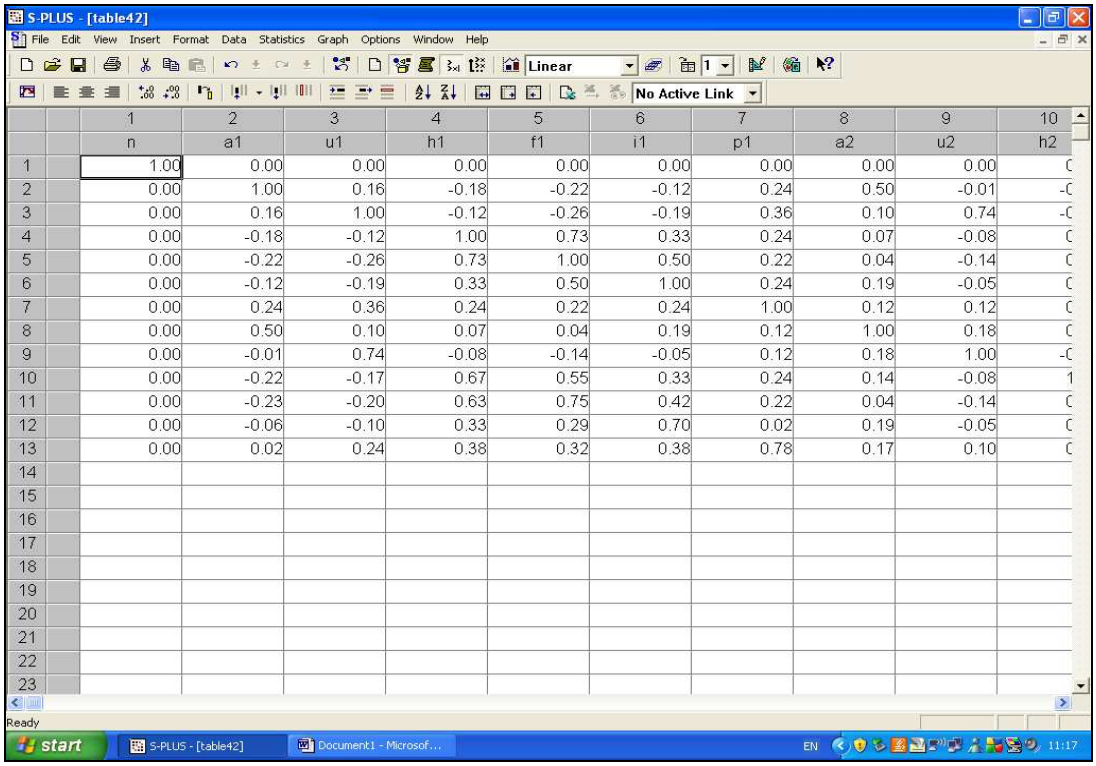
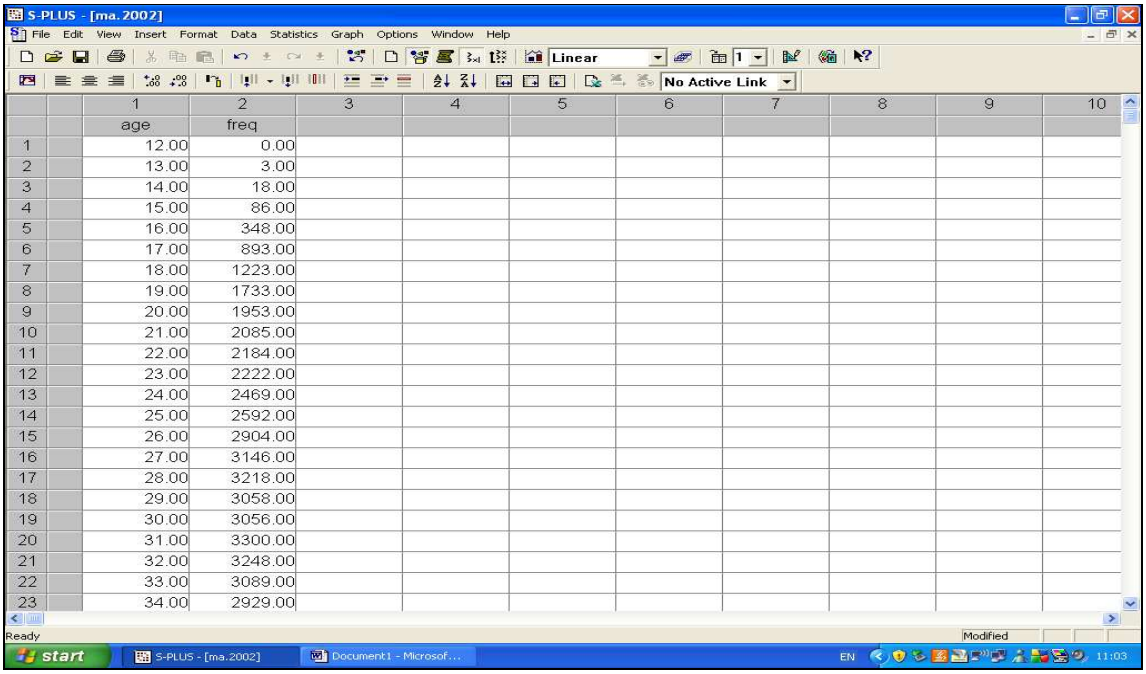


Figure 3.3: Screen shot of the database where the standard deviations for all the markers are recorded



		1	2	3	4	5	6	7	8	9	10
		n	a1	u1	h1	f1	i1	p1	a2	u2	h2
1		1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0
2		0.00	1.00	0.16	-0.18	-0.22	-0.12	0.24	0.50	-0.01	-0
3		0.00	0.16	1.00	-0.12	-0.26	-0.19	0.36	0.10	0.74	-0
4		0.00	-0.18	-0.12	1.00	0.73	0.33	0.24	0.07	-0.08	0
5		0.00	-0.22	-0.26	0.73	1.00	0.50	0.22	0.04	-0.14	0
6		0.00	-0.12	-0.19	0.33	0.50	1.00	0.24	0.19	-0.05	0
7		0.00	0.24	0.36	0.24	0.22	0.24	1.00	0.12	0.12	0
8		0.00	0.50	0.10	0.07	0.04	0.19	0.12	1.00	0.18	0
9		0.00	-0.01	0.74	-0.08	-0.14	-0.05	0.12	0.18	1.00	-0
10		0.00	-0.22	-0.17	0.67	0.55	0.33	0.24	0.14	-0.08	1
11		0.00	-0.23	-0.20	0.63	0.75	0.42	0.22	0.04	-0.14	0
12		0.00	-0.06	-0.10	0.33	0.29	0.70	0.02	0.19	-0.05	0
13		0.00	0.02	0.24	0.38	0.32	0.38	0.78	0.17	0.10	0
14											
15											
16											
17											
18											
19											
20											
21											
22											
23											

Figure 3.4: Screen-shot of the database where the correlation coefficients between markers are recorded



		1	2	3	4	5	6	7	8	9	10
		age	freq								
1		12.00	0.00								
2		13.00	3.00								
3		14.00	18.00								
4		15.00	86.00								
5		16.00	348.00								
6		17.00	893.00								
7		18.00	1223.00								
8		19.00	1733.00								
9		20.00	1953.00								
10		21.00	2085.00								
11		22.00	2184.00								
12		23.00	2222.00								
13		24.00	2469.00								
14		25.00	2592.00								
15		26.00	2904.00								
16		27.00	3146.00								
17		28.00	3218.00								
18		29.00	3058.00								
19		30.00	3056.00								
20		31.00	3300.00								
21		32.00	3248.00								
22		33.00	3089.00								
23		34.00	2929.00								

Figure 3.5: Screen-shot of the database where the maternal age distributions are recorded

Two functions; gen2.lr and rep2f, written by Prof. Dave Wright (Plymouth) were used in the statistical modelling. The markers used in the analysis, the number of observation and the gestational week when the screening was performed were entered in the first function, gen2.lr (Figure 3.6). In this study, samples of 1, 000,000 observations were drawn (500, 000 were taken as Down's syndrome pregnancies and 500, 000 as unaffected pregnancies).

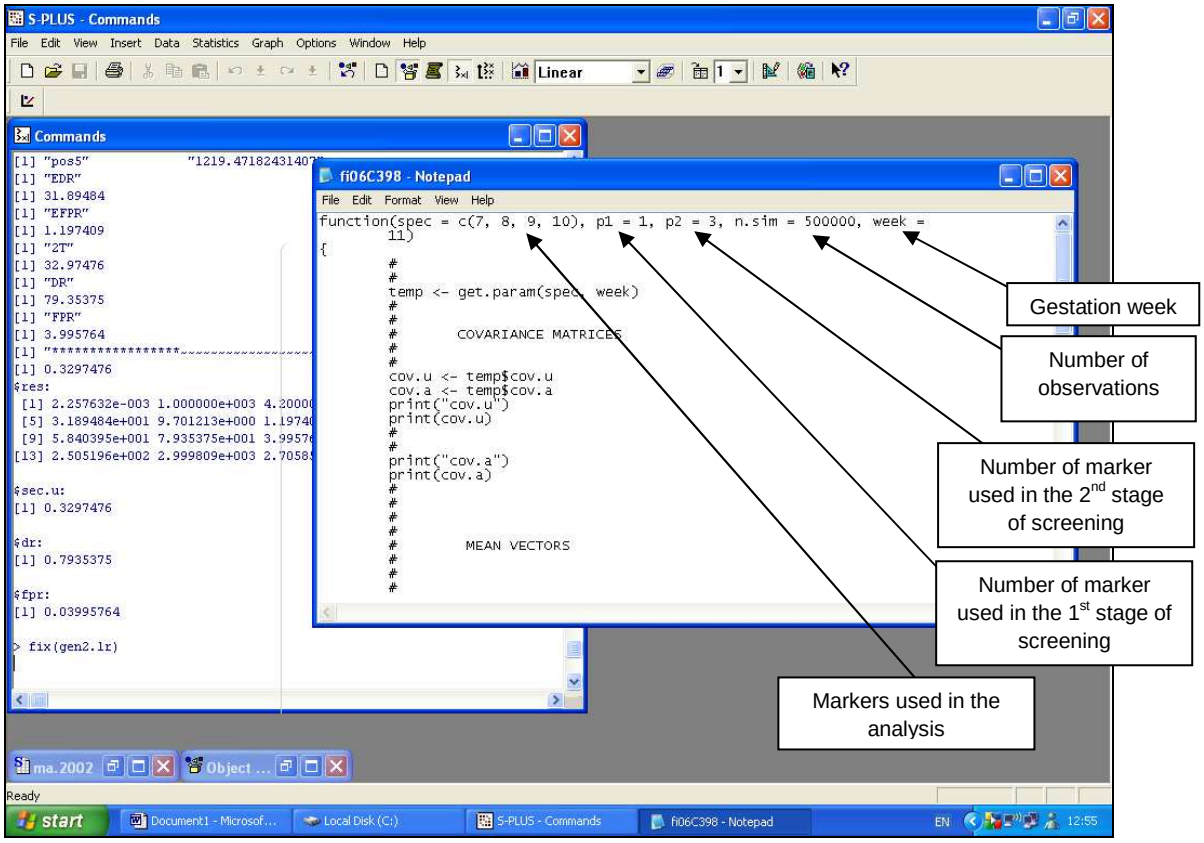


Figure 3.6: Screen-shot of the gen2.lr function

When the gen2.lr function was executed, for each observation, the likelihood ratio was computed for each set of markers at each stage of screening (Figure 3.7).

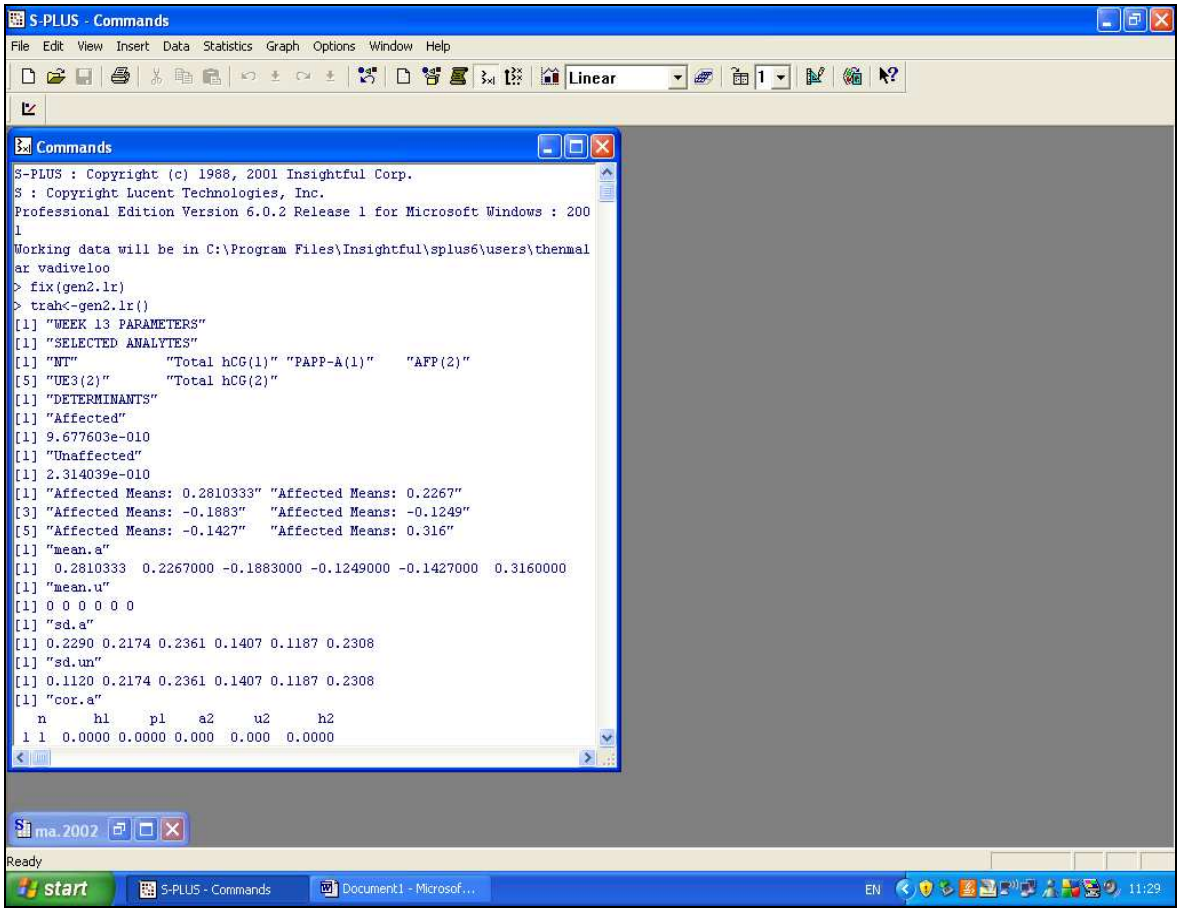


Figure 3.7: Screen-shot of S-PLUS programme

Once all the likelihood ratios were computed, the rep2f function was executed. Before the function was executed, the high, low and final cut-off risks were entered into the function. In this study, a high risk cut-off of 1:42 and a low-risk cut-off of 1:1000 were used, similar to the one used in the within-trimester contingent screening policy (see Figure 3.1). A final cut-off risk at term of 1:150 was chosen based on the current UK NSC policy. Apart from the above information, the gestation week when screening was performed, the range of maternal age and the name of the database where the maternal age distributions were recorded were also entered in the function (Figure 3.8).

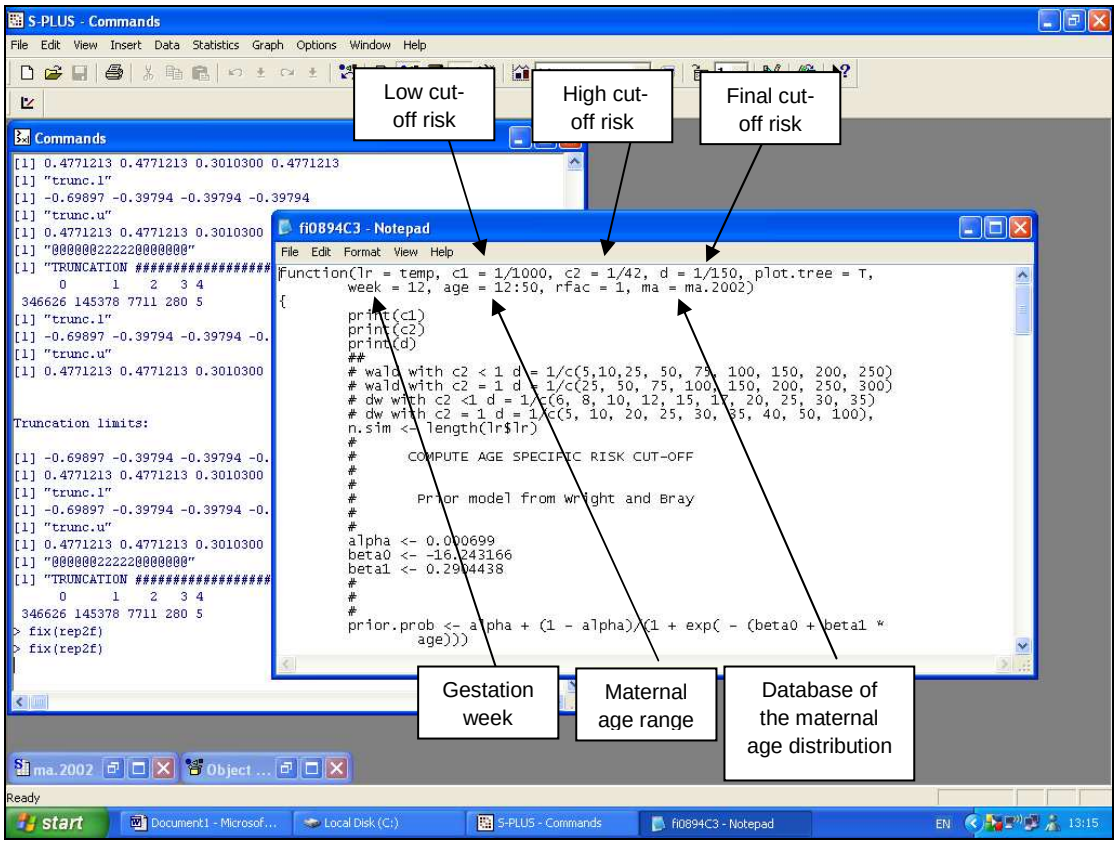
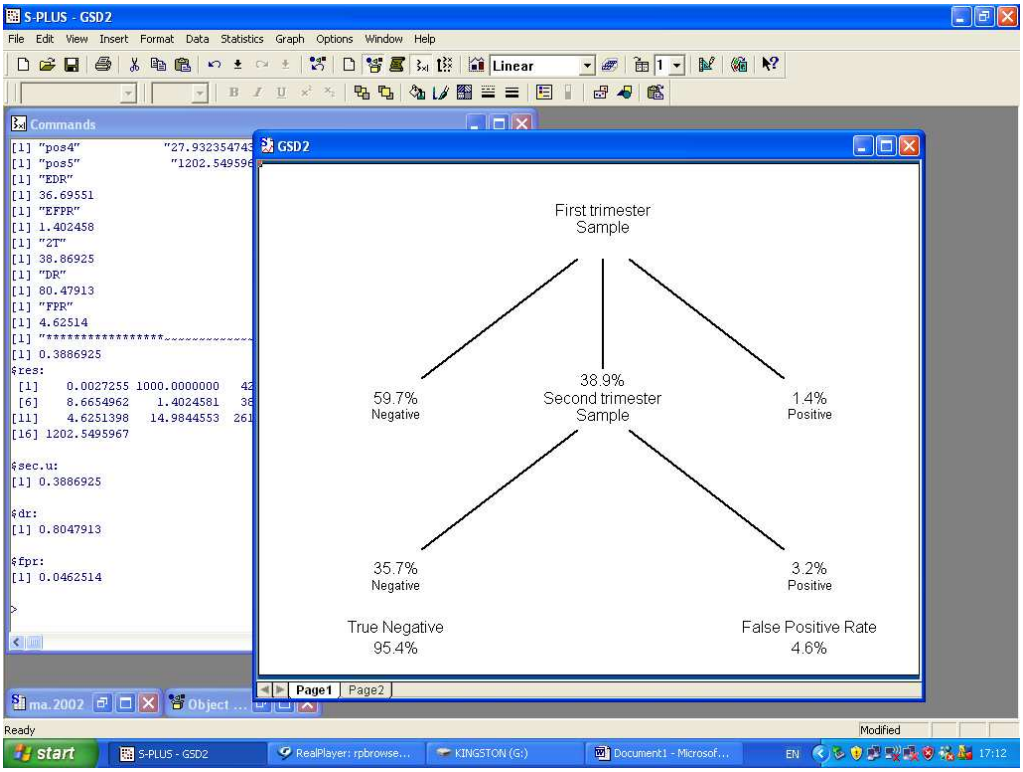


Figure 3.8: Screen-shot of the rep2f function

When the rep2f function was executed, the maternal age specific detection and false positive rates were derived from the likelihood ratios computed earlier and the maternal age distribution of Down's syndrome and unaffected pregnancies. The early completion rates were computed based on those women who were offered diagnostic test after the first stage of screening and those who were not offered any further screening after the first stage of screening. Figure 3.9 shows an example of the output once the analysis was completed.



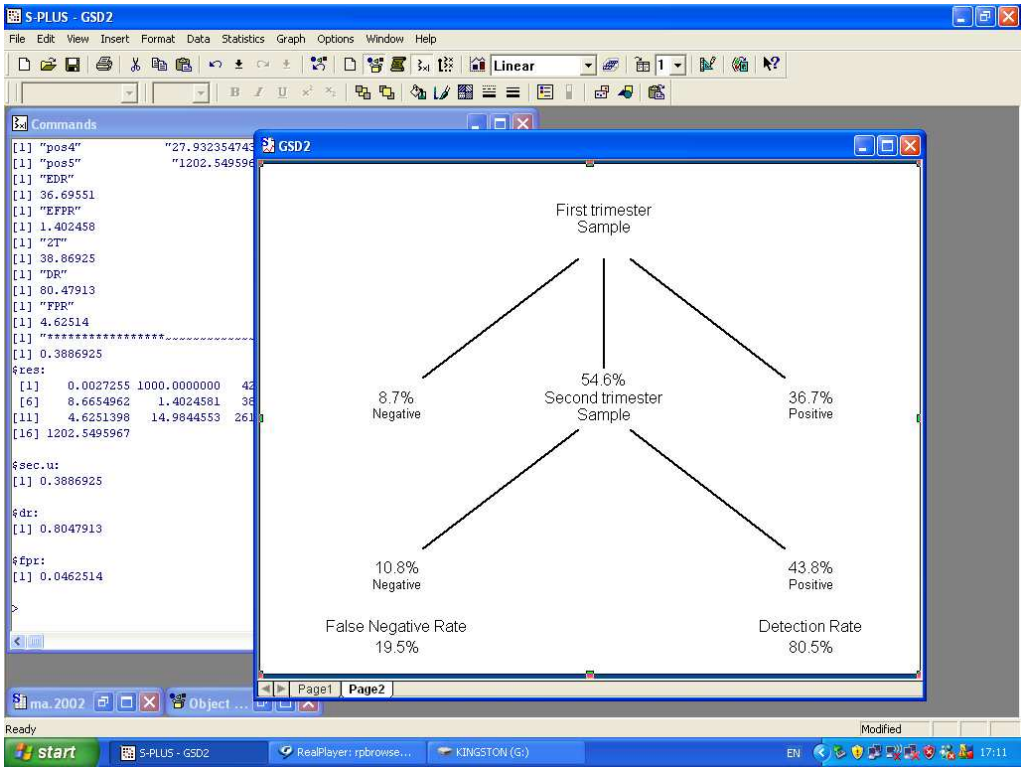


Figure 3.9: Screen-shot of the example of output once analysis was completed

In summary, SDs, medians of markers, correlation-coefficients, maternal age distributions, gestation when the screening was performed, number of observations, markers used and cut-off risks are the variables which can be configured based on local circumstances when using S-PLUS statistical software.

3.2.2 CROSS-TRIMESTER CONTINGENT SCREENING PROTOCOL WITH AND WITHOUT REPEAT MEASURES

The effectiveness of cross trimester contingent screening policies with various combinations of markers was evaluated. An initial risk at term was calculated from the first trimester screening results combined with the maternal age risk for all women. A high risk cut-off of 1:42 and a low-risk cut-off of 1:1000 were used in this screening policy. Women were divided into three groups according to their initial first trimester screening test results and maternal age risk. For those with intermediate risks between 1:42 and 1:1000, the likelihood ratio derived from the second trimester screening test was then combined with the first trimester screening test and maternal age risk and the composite risk assessed against a final cut-off risk of 1:150. Those women with a final risk ≥1:150 were classified as screen positive and added to the initial high-risk group. Those with final risks of <1:150 were classified as screen negative and added to the initial low-risk group (Figure 3.10). From the distribution of risks in Down syndrome and unaffected pregnancies the detection rate and false positive rate of the cross-trimester contingent screening model was calculated. The combinations of markers examined in this study are shown in Table 3.1.

Table 3.1: Cross-trimester contingent screening protocols evaluated in this study

Screening protocol
1. Cross-trimester contingent screening with second trimester double, triple or quadruple test (with and without NT measurement)
2. Cross-trimester contingent screening with repeat measure of FβhCG (with and without NT measurement)
3. Cross-trimester contingent screening with repeat measure of PAPP-A (with and without NT measurement)
4. Cross-trimester contingent screening with repeat measure of hCG and PAPP-A (with and without NT measurement)
5. Cross-trimester contingent screening with repeat measure of FβhCG and PAPP-A (with and without NT measurement)

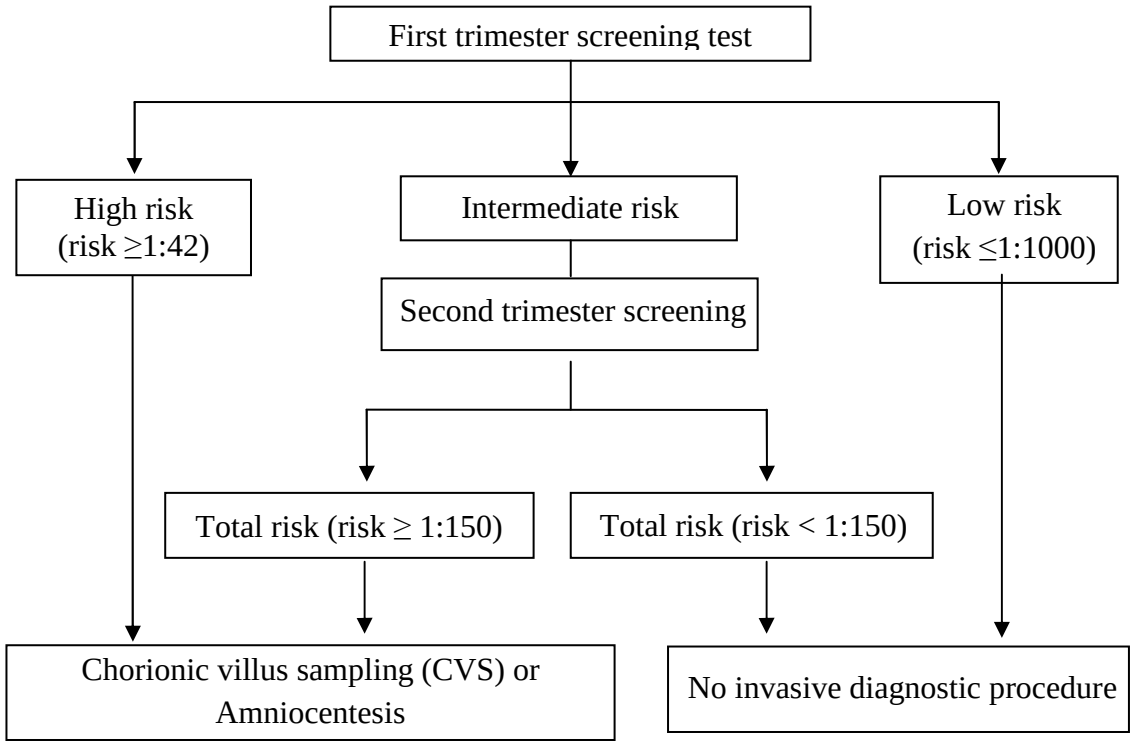


Figure 3.10 - Cross-trimester contingent screening protocol. Risks derived from first trimester screening test and maternal age were estimated for all women and used to triage the population into high, low and intermediate risk groups. Women whose risk is equal or greater than 1 in 42 would be offered a diagnostic test and those women whose risk is equal or lower than 1 in 1000 would not be offered any further testing. Women with an intermediate risk would be offered second trimester screening test and those with a composite risk equal or greater than the cut-off of 1 in 150 would be offered diagnostic testing.

3.3 RETROSPECTIVE STUDY ON EFFECT OF SMOKING & ETHNICITY ON SERUM MARKER CONCENTRATION IN PAIRED FIRST AND SECOND TRIMESTER SAMPLES

Maternal serum PAPP-A and fβhCG levels were available for all the first trimester samples and AFP and hCG levels were available for all the second trimester samples. The first trimester AFP and hCG levels and second trimester PAPP-A and fβhCG levels were measured in 939 paired first and second trimester serum samples using the DELFIA fluoroimmunoassay system (Perkin Elmer LAS, UK) according to the manufacturer's instructions. All samples were coded before analysis such that their origin was unknown to the assay operator.

3.3.1 FLUOROIMMUNOASSAY - AutoDELFIA

AutoDELFIA is an automatic immunoassay system used in diagnostic or screening laboratories. In the DELFIA assay, the labels employed are chelates of europium or other lanthanide metals. The AutoDELFIA uses time resolved fluorometry (TRF) to measure the signal. Extreme sensitivity combined with a wide dynamic measuring range is obtained due to the large Stokes' shift and long decay times of europium. Furthermore, several different lanthanides have unique fluorescence emission profiles. This allows multiple assays to be performed using AutoDELFIA system where dual label kits utilizing europium and samarium allow simultaneous measurement of analytes that are commonly required at the same time.

The system consists of a sample processor where automatic dilution and pipetting of serum samples are performed and a plate processor where reagent handling and all assay stages

including measurement are performed. AutoDELFIA is controlled by the Windows- based AutoDELFIA workstation software.

3.3.1.1 PREGNANCY ASSOCIATED PLASMA PROTEIN A (PAPP-A)

PAPP-A, a glycoprotein, is produced by trophoblastic tissues in the placenta of pregnant women. PAPP-A is secreted into maternal circulation as a heterotetrameric complex of two PAPP-A subunits disulfide-bonded to two molecules of proMBP. Maternal serum PAPP-A level is found to be significantly decreased in the first trimester in Down's syndrome pregnancies.

The PAPP-A concentration in maternal serum was measured using a solid phase two-site fluorometric assay based on the indirect sandwich technique (DELFIA). Biotin labeled capture antibodies, added in the first incubation period, reacts with the microtitration strips coated with streptavidin. The strips are washed before adding the standards, controls and samples in the second incubation. PAPP-A molecules in the serum samples react with the tracer antibodies labeled with chelates of europium. The strips are washed and enhancement solution is added to dissociate europium ion from the labeled antibody. The europium ion and components of the enhancement solution forms highly fluorescent chelates, and the fluorescent counts are measured by the AutoDelfia machine.

3.3.1.2 FREE β HUMAN CHORIONIC GONADOTROPIN (FβhCG)

FβhCG, a glycoprotein, is one of the two subunits of hCG. FβhCG is expressed in the placenta and found to be significantly elevated in maternal serum of Down's syndrome pregnancies. The fβhCG concentration in maternal serum was measured using a solid phase two-site fluorometric assay based on the direct sandwich technique (DELFIA). The fβhCG molecules in maternal serum are reacted with immobilized fβhCG specific

monoclonal antibodies and samarium-labeled monoclonal antibodies at different antigen sites. The enhancement solution is added to dissociate samarium ion from the labeled antibody. The samarium ion and components of the enhancement solution forms highly fluorescent chelates, and the fluorescent counts are measured by the AutoDelfia machine.

3.3.1.3 ALPHA FETOPROTEIN (AFP)

AFP, a glycoprotein of fetal origin, is produced by the embryonic yolk sac in the early stage of pregnancy and later by the fetal liver. AFP diffuses into the maternal blood circulation through the amniotic membrane. AFP level is found to be decreased significantly in the second trimester in Down's syndrome pregnancies. The AFP concentration in maternal serum was measured using a solid phase two-site fluoroimmunometric assay based on the direct sandwich technique (DELFIA). In the one incubation period protocol, the AFP molecules in maternal serum are reacted simultaneously with immobilized AFP specific monoclonal antibodies and europium-labeled monoclonal antibodies at different antigen sites on the same AFP molecules. The enhancement solution is added to dissociate europium ion from the labeled antibody. The europium ion and components of the enhancement solution forms highly fluorescent chelates, and the fluorescent counts are measured by the AutoDelfia machine.

3.3.1.4 HUMAN CHORIONIC GONADOTROPIN (hCG)

Human chorionic gonadotropin, a glycoprotein hormone, is produced by the trophoblastic cells of the fertilized ovum in the early stage of pregnancy and later by the placental tissue. hCG diffuses into the maternal blood circulation through the placenta. hCG level is found to be elevated significantly in the second trimester in Down's syndrome pregnancies. The hCG concentration in maternal serum was measured using a solid phase two-site

fluoroimmunometric assay based on the direct sandwich technique (DELFIA). The hCG molecules in maternal serum are firstly reacted with immobilized monoclonal antibodies directed against a specific antigen site on the β subunit of hCG and then with europium-labeled antibodies directed against a specific antigen site on the α subunit. The enhancement solution is added to dissociate europium ion from the labeled antibody. The europium ion and components of the enhancement solution forms highly fluorescent chelates, and the fluorescent counts are measured by the AutoDelfia machine.

3.3.2 PROTOCOL OF THE ASSAY

All the samples retrieved from the freezer were left to thaw slowly at 4^{o}C. The samples were then vortexed and given barcodes. The quality controls for the first trimester (PAPP-A and fβhCG) and second trimester (AFP and hCG) assays were commercially produced by Brahms Kryptor and Biorad respectively**.** The quality control samples have three different levels and are composed of pooled, lyophilised human serum. Information about the samples and controls were entered in the AutoDelfia software. Samples, controls and standard were placed in the vials according to the information given in the software and then loaded into the machine. The reagents; wash solution, buffer, enhancement solution were placed into the reagent cassette. The plates were loaded into the machine and then the assays were started. After the assays were completed, all the samples, controls, standards and plates were discarded. The results were automatically calculated by WIACALC programme on Multicalc 2000. Dilution (1 in 10) was performed on those samples which had biochemical marker concentrations above the assay top standard and the samples were reanalysed. All the results were entered into SPSS software.

3.3.3 ASSAY PARAMETERS

All the results were converted to multiple of median (MoM) of the appropriate gestation. Three quality control samples were assayed twice in each batch of samples. Table 3.2 shows the mean and intra- and inter- assay coefficient of variations (CVs) of the quality control samples.

Table 3.2: The mean and intra- and inter-assay CVs of the quality control samples

Biochemical Markers	**Parameters**	**Quality Control 1**	**Quality Control 2**	**Quality Control 3**
AFP	Mean	8.5 U/ml	26.1 U/ml	72.1 U/ml
	Intra-assay CV	1.7%	2.2%	1.5%
	Inter-assay CV	1.7%	2.8%	2.4%
hCG	Mean	13.2 U/ml	38.3 U/ml	77.2 U/ml
	Intra-assay CV	2.6%	2.8%	3.2%
	Inter-assay CV	3.0%	3.5%	3.6%
PAPP-A	Mean	265.1 mU/L	1489.5 mU/L	4386.7 mU/L
	Intra-assay CV	4.6%	4.0%	3.1%
	Inter-assay CV	4.8%	5.0%	3.7%
fβhCG	Mean	69.4 ng/ml	17.2 ng/ml	6.9 ng/ml
	Intra-assay CV	2.2%	2.0%	3.9%
	Inter-assay CV	3.1%	3.1%	4.2%

3.3.4. STATISTICAL ANALYSIS

AFP, hCG, fβhCG and PAPP-A levels were measured and regressed medians were calculated for each gestational week (from week 9 to week 20) using the data from Caucasian women with normal singleton pregnancies. The gestational ages were calculated either from CRL or the time since the first day of the LMP. The MoM for each marker at each gestation was calculated using the regression equation from the best fitted model for each marker. This was done by using the curve estimation routine in SPSS. To check whether the simple regression chosen was appropriate, the regression curves were compared with the regression curve normally used in routine screening at Institute of Medical Genetics, Glasgow. All MoM values were corrected for maternal weight and smoking status by dividing the observed MoM value by the expected MoM value. These formulas were derived solely from Caucasian women. The Mann Whitney test was used to compare the median values of the serum markers in the smoking group with the non-smoking group among the Caucasians and the median values of the serum markers in each ethnic group with the Caucasian group. Results were classified as significant when $p<0.05$.

3.4 RETROSPECTIVE STUDY ON THE EFFECT OF ASSISTED REPRODUCTIVE TECHNOLOGY ON SERUM MARKER CONCENTRATION

The Down's syndrome screening marker levels in 127 first trimester and 129 second trimester pregnancies conceived after ART were compared with the marker levels in naturally conceived pregnancies. The pregnancies were classified into four categories; 1. normal pregnancy, 2. IVF or ICSI with fresh eggs, 3. IVF or ICSI with frozen embryo and 4. IVF with donor egg. The Mann Whitney test was used to compare the median values of

AFP and hCG between the controls and ART groups in the second trimester and fβhCG and PAPP-A in the first trimester. Marker measurements were carried out using DELFIA assays as described in Section 3.3. Results were classified as significant when $p<0.05$.

3.5 RETROSPECTIVE STUDY ON BIRTH WEIGHT, DURATION OF PREGNANCY AND SECOND TRIMESTER MATERNAL SERUM SCREENING MARKERS IN NON-SMOKERS AND SMOKERS

3.5.1 COTININE ANALYSIS

The accuracy of the self-reported smoking information on the screening form was established using cotinine analysis. Cotinine is the major metabolite of nicotine and can be detected in the biological fluids of both active and passive smokers. Due to its high specificity for tobacco smoke, long half-life of 15 to 19 hours in different body fluids and easy detection with sensitive analytical techniques, cotinine has become the biochemical marker of choice to detect smokers.

From the database of 21,029 pregnant women, 3550 serum samples were randomly selected for cotinine analysis. After excluding samples with insufficient serum, cotinine testing was carried out on 3475 thawed serum samples using the Cozart STD Micro-Plate Cotinine EIA (Cozart UK Ltd). All samples were assayed without knowledge of smoking status and in singleton. Those women who had cotinine levels above 13.7ng/ml were classified as smokers (Jarvis *et al.,* 1987). Those samples with cotinine levels between 10 and 30ng/ml (close to the chosen cut off of 13.7 ng/ml) were re-assayed and the final cotinine concentration was taken from the mean of the two values.

3.5.1.1 PRINCIPLE OF THE COTININE ASSAY

Cotinine in maternal serum was detected using a semi quantitative assay; Cozart STD Micro-Plate Cotinine EIA (Cozart UK Ltd). Aliquots of maternal serum are added to the wells of the microtitre strips which are coated with anti-cotinine antibody. Horseradish peroxide (HRP)-labelled cotinine competes with the free cotinine in the serum samples for the anti-cotinine antibody binding sites on the microtitre strips during the first incubation. Tetramethylbenzidine substrate solution is added after the wells are washed to remove any excess enzyme material. Stop solution terminates the reaction and the absorbance is read spectrophotometrically at 450nm using the Wallac Victor multilabel counter.

3.5.1.2 PROTOCOL OF THE COTININE ASSAY

All the samples retrieved from the freezer were left to thaw slowly at 4^{o}C and were then vortexed. Two quality control samples; positive and negative (smokers and non-smokers), were used and they were composed of pooled human serum from the routine screening programme. Forty-two serum samples from self-reported smokers and forty-three samples from self-reported non-smokers were pooled together for the positive and negative controls respectively. The positive controls had values above the top positive standard (50ng/mL) and the negative controls had values below the bottom positive standard (5ng/mL). Each Cozart Cotinine EIA Serum kit contained each of the following components and reagents.

1. Anti-Cotinine Coated Plate – 12 x 8 well strips in break-apart format. Anti-cotinine polyclonal antibody immobilised on a polystyrene plate supplied in dry form.
2. Enzyme Conjugate – Cotinine derivative labelled with horseradish peroxidase and diluted in a protein matrix with stabilisers.
3. Wash buffer – Each vial is diluted to 1500mL with distilled water.
4. Substrate solution – Each bottle containing <0.05% 3,3’,5,5’-tetramethylbenzidine.

5. Stop solution – Each bottle containing 1mol/L sulphuric acid.
6. Negative calibrator – Protein matrix negative for cotinine.
7. Positive calibrator – Protein matrix containing 10ng/mL, 25ng/mL and 50ng/mL cotinine.

An additional positive calibrator was required in order to improve the fit of the standard curve. A 5ng/mL calibrator solution was prepared by a 1/10 dilution of 50ng/mL calibrator solution. All samples were assayed anonymously and in singleton. 10µL of controls, samples or calibrator was added to each well within 25 minutes. 100µL of enzyme conjugate was then added to each well and the plate was incubated for 30 minutes. After the incubation, the plate was washed four times with wash buffer (which was diluted by 1:30 dilution with distilled water) using the DELFIA® Platewasher. 100µL of substrate solution was added to the well and the plate was incubated for 30 minutes. 100µL of stop solution was added after the incubation and the absorbance was measured at 450nm using Wallac Victor 1420 Multilabel Counter.

3.5.1.3 WALLAC VICTOR 1420 MULTILABEL COUNTER

Wallac 1420 is a multi-task, multi-label plate counter which is used for quantitative detection of light emitting or light absorption markers. The Victor measures all commonly used florescent labels and time-resolved florescence labels. After measurement of a plate, the results were automatically calculated by MultiCalc.

3.5.1.4 DATA ANALYSIS

All the results were entered into SPSS software. Those samples with cotinine levels between 10 and 30ng/ml (close to the chosen cut off of 13.7 ng/ml) were re-assayed and the final cotinine concentration was taken from the mean of the two values. Those women

who had cotinine levels above 13.7ng/ml were classified as smokers (Shipton *et al*., 2009, Jarvis *et al.*, 1987). The accuracy of self-reported smoking information at booking and screening were calculated.

3.5.2 BIRTH WEIGHT AND GESTATION AT DELIVERY

The mean birth weight in self-reported non-smokers and smokers was stratified according to maternal serum AFP and hCG levels (in MoM) in the second trimester. The pregnancy was classified as 'low birth weight' if the infant was under 2500g (Wilcox and Johnson, 1992). The Mann Whitney test was used to compare the mean birth weight for the smoking group with the non-smoking group. Results were classified as significant when $p<0.05$. Regression was performed to test the trend in birth weight with AFP and hCG levels in smokers and non-smokers. The median gestation at delivery for non-smokers and smokers was calculated according to maternal serum AFP and hCG levels (in MoM). The percentage of pregnancies delivered at 38 weeks and earlier were calculated for each AFP and hCG group.

3.6 STATISTICAL METHODS

3.6.1 MEDIANS

The median is the middle value when the data are sorted in ascending order. The median measures the central tendency and is not sensitive to extreme values. It is usually used when the distribution is skewed. Medians were calculated using the SPSS 12.0.1 program.

3.6.2 MEANS

The mean is a measure of central tendency but greatly influenced by outliers. The mean value is calculated by dividing the sum of all the data by the number of data. Means were calculated using the SPSS 12.0.1 program.

3.6.3 PERCENTILES

Percentile is the value below which a certain percentage of observations fall. For example the 90^{th} percentile is the value below which 90% of the cases fall. Percentiles were calculated using the SPSS 12.0.1 program.

3.6.4 STANDARD DEVIATION

The standard deviation (SD) measures the amount of variation or spread of the data. A low standard deviation indicates that all the values in the dataset are close to the mean while a high standard deviation indicates that the values in the dataset are spread out over a large range of values. Standard deviation was calculated using the following equations:

$$SD = \frac{\sqrt{\sum (x - \bar{x})}}{n-1} \text{ or } \frac{\log_{10}(90^{th}\, centile) - \log_{10}(10^{th}\, centile)}{2.56}$$

where $\bar{x}$ is the mean and n is the number of cases. Standard deviations were calculated using the SPSS 12.0.1 program.

3.6.5 STANDARD ERROR OF MEAN (SEM)

The standard error of mean indicates the variability of the mean among many samples taken from the same distribution. SEM is calculated using the following equation:

$$SEM = \frac{SD}{\sqrt{n}}$$

where *n* is the number of cases.

3.6.6 CONFIDENCE INTERVALS (CI)

A confidence interval is a range of values derived from a sample, which represents where the true population value is likely to fall. In this study, 95% CI were used and this is interpreted as a range of which contains the true population mean with probability of 0.95%.

3.6.7 COEFFICIENT OF VARIANCE (CV)

Assay reproducibility is measured using coefficient of variance (CV). CV indicates the ratio of standard deviation (SD) to the mean ($\bar{x}$) expressed as percentage. The inter- and intra-assay CV was calculated using the following equation.

$$CV = 100 \times \left(\frac{SD}{\bar{x}} \right)$$

3.6.8 CORRELATION COEFFICIENT (*r*)

Correlation coefficient indicates the level of association between two variables; *X* and *Y*. The *r* value has a range between -1 and 1. A positive value indicates positive correlation and a negative value indicates negative correlation. If there is no association between the two variables, the r value would be close to 0. The formula to calculate *r* value for two variables is:

$$r = \frac{\sum (x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum (x_i - \bar{x})^2 \sum (y_i - \bar{y})^2}}$$

where x_i and y_i are the values of *X* and *Y* for the i^{th} individual. A simple box-plot was used to check for outliers. Outliers between the ranges of ± 3SD were accepted. Correlation coefficients were calculated using the SPSS 12.0.1 program.

3.6.9 COVARIANCE MATRIX

The covariance matrix is derived from the standard deviations and correlation coefficients. The covariance matrix of variable *x* and *y* was calculated using the following equation.

$$Cov_{x,y} = SD(x) \times SD(y) \times r(x, y)$$

where *r* is the correlation coefficient between *x* and *y*.

3.6.10 REGRESSION ANALYSIS

Regression analysis is performed to estimate the relationship between two variables. In this study, regression was used to determine the relationship between 1) marker levels and gestational week and 2) marker levels with maternal weight. Various models such as quadratic, cubic and inverse were used to estimate the relationship between two variables. Regression coefficient, r^2, was taken into consideration when choosing the best fitted

model. The MoM of the appropriate gestation was calculated using the regression equation from the best fitted model for each marker. This was done by using the curve estimation routine in SPSS.

3.6.11 MULTIPLE OF MEDIAN OF THE APPROPRIATE GESTATION

All the Down's syndrome screening marker levels were converted to a multiple of the control median (MoM) at the appropriate gestational week. This allows changes of marker levels with the gestational age to be compared. The equation used to calculate the MoM value is as follows.

$$MoM = \frac{\text{Marker concentration}}{\text{Regressed median concentration at appropriate gestation}}$$

3.6.12 CORRECTION FACTORS

The biochemical marker levels were corrected for maternal weight and smoking. For correcting the maternal weight, an equation is derived using regression analysis. In this study, Caucasian women who were non-smokers were used to derive this equation. Correcting for smoking was done by dividing the observed MoM value in smokers by the expected MoM value in non-smokers. The expected MoM values were derived from the Caucasian women who were non-smokers. The equations used to for correcting these factors are as follows:

Maternal weight:

$$MoM = \frac{\text{Marker concentration}}{\text{Regressed median concentration at appropriate maternal weight}}$$

Maternal smoking

$$MoM_2 = \frac{\text{MoM}_1}{\text{Expected MoM}}$$

where MoM_1 is the multiple of median marker level of the appropriate gestation.

3.6.13 DETECTION RATE, FALSE POSITIVE RATE AND SCREEN POSITIVE RATE

Detection rate is the ratio of the number of affected cases which are correctly identified to the total number of affected cases. This is sometimes referred to as the sensitivity of screening. False positive rate is the ratio of the number of unaffected pregnancies with a screen positive test result to the total number of unaffected cases. Both detection and false positive rate are normally expressed in percentages (%). Screen positive rate is the percentage of pregnancies reported to have an increased risk of having an affected pregnancy.

3.6.14 MANN-WHITNEY TEST

Mann-Whitney test is a non-parametric test based on ranking and ordering of data. This test compares the medians of two independent groups by combining and ordering the data from the two groups from lowest to highest. Mann-Whitney test was used to compare the medians values for biochemical markers in various ethnic groups with Caucasians, smokers with non-smokers and ART treated pregnancies with normal pregnancies. *P* value less than 0.05 were considered as significant. The SPSS 12.0.1 program was used to perform the Mann-Whitney test.

3.7 RISK CALCULATION FOR DOWN'S SYNDROME

The risk of having a Down's syndrome pregnancy was calculated using the following equations.

Gestational age

For the ultrasound based gestational age, the CRL measurement was used. If there was only BPD measurement, BPD was converted to CRL using the following formula:

$$CRL = BPD \times 3 \text{ (Crossley et al., 2002)}$$

$$Gestational\ age = (\sqrt{((crl+1)\times 1.037)}\times 8.052) + 23.73$$

For the LMP based gestational age, the gestational age was calculated using the following equation:

$$Gestational\ age = the\ date\ of\ sampling - the\ date\ of\ LMP$$

Age at estimated date of delivery (EDD)

To calculate the age at EDD, firstly the age at NT scan was calculated.

Age at NT scan = (date of NT scan – date of birth)/365.25

Age at EDD = Age at NT scan + ((280 – gestational age)/365)

Maternal age risk

The maternal age risk at term was calculated as described by Cuckle *et al* (1987), where

$$p = 0.000627 + e^{(-16.2395+0.286*(\text{age at EDD}-0.5))}$$

and the risk of having a Down's syndrome pregnancy was

$$\text{Term risk} = 1: (1-p)/p.$$

A correction factor of 0.5 is used when the maternal age is recorded in fractions of years.

Screening marker levels

Firstly, an average NT measurement was calculated if more than one measurement was taken.

For example, if three measurements were taken:

$$\text{Average NT} = (nt1 + nt2 + nt3)/3$$

Then, NT MoM was calculated. The equation was obtained from the regression analysis using the curve estimation routine in SPSS.

$$NTMoM = \frac{\text{Average NT}}{\text{Regressed median levels at appropriate gestation}}$$

The fβhCG and PAPP-A MoMs were also calculated using the same method.

Correcting for maternal weight and smoking

The MoM values of the screening markers were corrected for maternal weight and smoking (refer to section 3.6.12).

Likelihood ratio for Down's syndrome from NT measurement

Truncation of the NT risk at 0.8 MoM was applied. This is done because the risks start to increase again below 0.8 MoM due to the shapes of the Gaussian distributions.

Likelihood ratio:

$$a = ((\log_{10}(NT\ MoM) - Mean_x) / SD_x)^2$$

$$b = (\log_{10}(NT\ MoM) / SD_y)^2$$

$$Likelihood\ ratio\ from\ NT = (SD_y / SD_x) * e^{(-0.5 \times (a-b))}$$

where *x* is Down's syndrome pregnancies and *y* is unaffected pregnancies

Likelihood ratio for Down's syndrome from fbhCG and PAPP-A

The equations below were used to calculate the likelihood ratio from fβhCG and PAPP-A.

$$c = log_{10}(f\beta hCG\ MoM) / SD_y^q$$

$$d = (log_{10}(f\beta hCG\ MoM) - Mean_x^q) / SD_x^q$$

$$e = log_{10}(PAPP\text{-}A\ MoM) / SD_y^p$$

$$f = (log_{10}(PAPP\text{-}A\ MoM) - Mean_x^p) / SD_x^p$$

$$g = (c^2 - (2 * r_y * c * e) + e^2) / (1 - r_y^2)$$

$$h = (d^2 - (2 * r_x * d * f) + f^2) / (1 - r_x^2)$$

Likelihood ratio from fβhCG and PAPP-A =

$$((SD_y^q * SD_y^p) / (SD_x^q * SD_x^p)) * \sqrt{((1 - r_y^2) / (1 - r_x^2))} * e^{((g - h) / 2)}$$

where: *x* - Down's syndrome pregnancies, *y* - unaffected pregnancies, *q* - fβhCG, *p* - PAPP-A and *r* - correlation coefficient between PAPP-A and fβhCG

Truncation limits for PAPP-A (0.1 – 5.0) and fβhCG (0.2 – 5.0) applied in the risk calculation were based on the truncation limits used in routine screening in Glasgow.

Combined likelihood ratio for Down's syndrome from NT, fβhCG and PAPP-A

Combined likelihood = likelihood ratio from NT x likelihood ratio from fβhCG and PAPP-A

Risk for Down's syndrome

Risk for Down's syndrome = Maternal age risk / combined likelihood ratio

CHAPTER 4: RESULTS

4.1 RETROSPECTIVE STUDY ON WITHIN-TRIMESTER CONTINGENT SCREENING

The performance of a two-stage contingent screening protocol for Down's syndrome based on initial serum marker analysis for all women and NT measurement only in women with intermediate risks was assessed. Biochemical marker and NT data in 10189 women who had CUB screening, were re-analysed using the contingent model (refer to section 2.2). A risk was calculated from the results of the PAPP-A and fβhCG measurements and maternal age. For risks between 1:42 and 1:1000, the likelihood ratio from the NT measurement was incorporated and assessed against a final cut-off risk at term of 1:250.

Figure 4.1 illustrates the performance of the contingent screening model in this study group using initial high and low cut-offs of 1:42 and 1:1000 respectively and a final cut off of 1:250. There were 313 (3.1%) unaffected and 27 (61.4%) Down's syndrome pregnancies with initial risks ≥1:42 and these were classified as high risk. In this approach to screening these women would be offered a diagnostic test (CVS/amniocentesis) at this stage. NT measurement would not be offered to these women because their initial risk is so high that a subsequent NT measurement would be unlikely to bring the risk down below the final threshold risk of 1:250.

Within the low risk group with risks ≤1:1000, there were 6887 (67.9%) unaffected and 2 (4.5%) Down's syndrome pregnancies. According to the protocol, these women would be counselled that they would not be offered any further test because the initial risk is low. The remaining 2960 (29%) women fell within the intermediate risk category and would be offered NT measurement. Of these, when the risk from the NT measurement was

combined with the initial risk, 276 (2.7%) unaffected pregnancies and 12 (27.3%) Down's syndrome pregnancies had final risk of ≥1:250 and would be offered a diagnostic test.

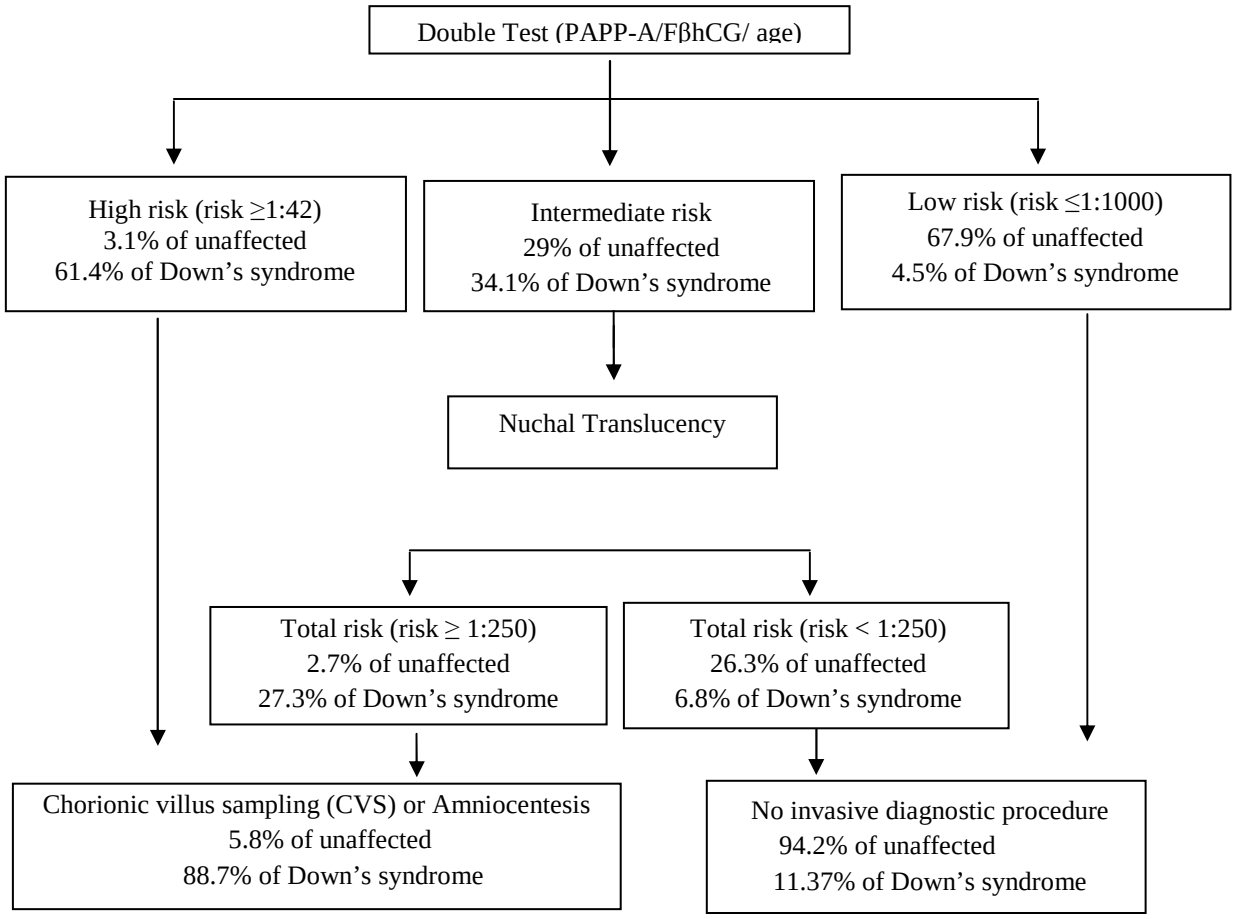


Figure 4.1 – The performance of within-trimester contingent screening in this study. There were 313 (3.1%) unaffected and 27 (61.4%) Down's syndrome pregnancies with initial risks ≥1:42. These pregnancies were classified as high risk and offered a diagnostic test. Within the low risk group with risks ≤1:1000, there were 6887 (67.9%) unaffected and 2 (4.5%) Down's syndrome pregnancies. The remaining 2960 (29%) women fell within the intermediate risk category and would be offered NT measurement. Of these, when the risk from the NT measurement was combined with the initial risk, 276 (2.7%) unaffected pregnancies and 12 (27.3%) Down's syndrome pregnancies had final risk of ≥1:250 and would be offered a diagnostic test. This contingent screening protocol would have achieved a detection rate of 88.7% at a false positive rate of 5.8% but with only 29% of women requiring an NT measurement.

Therefore in the CUB screened population in the West of Scotland, adopting the above contingent screening protocol would have achieved a detection rate of 88.7% at a false positive rate of 5.8% (compared with 90.9% detection at a 6.4% false positive rate for the full CUB screen) but with only 29% of women requiring an NT measurement. By changing the initial and final cut-off risks the detection rate, false positive rate and NT measurement rate can be varied (Table 4.1). This would allow individual centres to develop protocols best suited to local circumstances. If, for example, it was desired to keep the false positive rate low, an initial high risk cut-off of 1:24 and final risk cut off of 1:150 gives a false positive rate of 3.7% for only a small reduction in detection to 84.1%.

Table 4.1: The frequency of nuchal translucency (NT) measurement and overall screening performance in contingent testing with different risk cut-off values.

Final risk cut offs (at term)	**High risk cut offs (at term)**	**Low risk cut offs (at term)**	**NT frequency (%)**	**Detection rate (%)**	**False positive rate (%)**
1:250	1:42	1:1000	29.1	88.7	5.8
		1:800	25.1	86.4	5.8
		1:600	20.7	84.1	5.6
		1:400	15.3	81.9	5.5
1:200	1:33	1:1000	29.7	86.4	4.8
		1:800	25.8	84.1	4.8
		1:600	21.3	81.8	4.6
		1:400	16.0	79.6	4.5
1:150	1:24	1:1000	30.3	84.1	3.7
		1:800	26.4	81.8	3.7
		1:600	21.9	79.5	3.6
		1:400	16.6	77.3	3.5

4.1.1 RETROSPECTIVE CONTINGENT TESTING BASED ON LMP ESTIMATE OF GESTATION

As the performance of this contingent screening model is very dependent on an accurate interpretation of biochemical marker results, accurate assessment of gestation is essential. However, due to limited availability of ultrasound resources in some areas, gestational age is often determine by relying on LMP. Using the same data-set, the performance of contingent testing model was re-evaluated by using LMP based gestational age.

For all women, a risk was calculated from the maternal age and the results of the PAPP-A and fβhCG measurements using LMP based gestational age. For women with risks between 1:42 and 1:1000, the biochemical marker measurements in MoM were re-calculated using ultrasound based gestational age (this being available at the NT measurement appointment) and the likelihood ratio from the NT measurement was incorporated. The composite risk was assessed against a final cut-off risk at term of 1:250. Information on LMP was only available in 6895 pregnancies; 6865 unaffected and 30 Down's syndrome pregnancies. Of the 6895 pregnancies, 5979 pregnancies had certain LMP dates.

Figures 4.2 and 4.3 illustrate the performance of the contingent screening model in all pregnancies (uncertain and certain LMP dates) and pregnancies with certain LMP dates using initial high and low cut-offs of 1:42 and 1:1000 respectively and a final cut off of 1:250. When analysis was performed on all the pregnancies, there were 275 (4.0%) unaffected and 18 (60.0%) Down's syndrome pregnancies with initial risks ≥1:42 and these were classified as high risk. Within the low risk group with risks ≤1:1000, there were 4814 (70.1%) unaffected and 3 (10.0%) Down's syndrome pregnancies. The remaining 1785 (25.9%) women fell within the intermediate risk category and would be offered NT

measurement. Of these, when the risk from the NT measurement was combined with the initial risk, 230 (3.4%) unaffected pregnancies and 7 (23.3%) Down's syndrome pregnancies had final risk of ≥1:250 and would be offered a diagnostic test.

When analysis was performed only on pregnancies with certain LMP dates, there were 217 (3.6%) unaffected and 17 (63.0%) Down's syndrome pregnancies in the high risk group with risks initial ≥1:42. Within the low risk group with risks ≤1:1000, there were 4231 (71.1%) unaffected and 2 (7.4%) Down's syndrome pregnancies. The remaining 1512 (25.3%) women fell within the intermediate risk category and of these, when the risk from the NT measurement was combined with the initial risk, 197 (3.3%) unaffected pregnancies and 7 (25.9%) Down's syndrome pregnancies had final risk of ≥1:250.

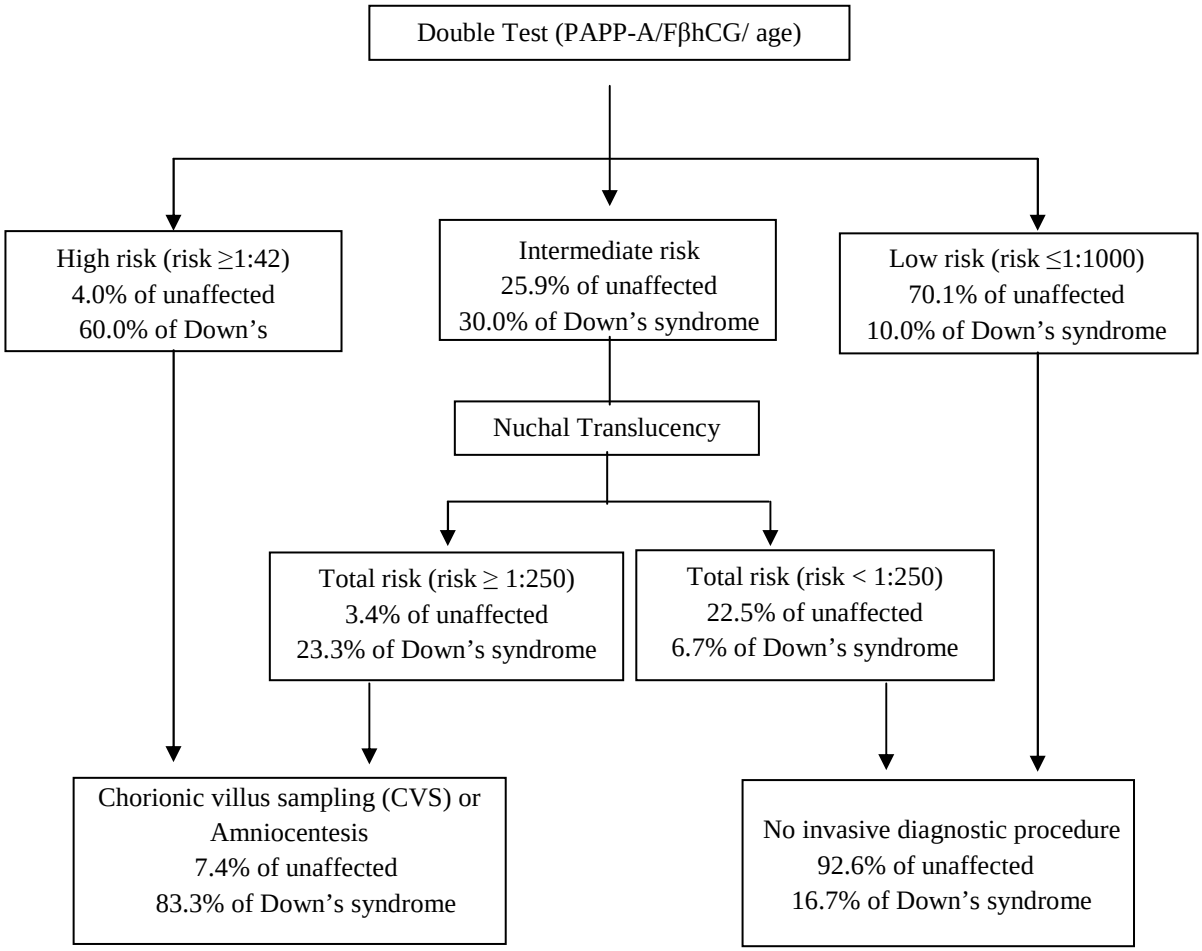


Figure 4.2 – The performance of within-trimester contingent screening using LMP based gestation in pregnancies with certain and uncertain LMP dates. There were 275 (4.0%) unaffected and 18 (60.0%) Down's syndrome pregnancies with initial risks ≥1:42. These pregnancies were classified as high risk and would be offered a diagnostic test. Within the low risk group with risks ≤1:1000, there were 4814 (70.1%) unaffected and 3 (10.0%) Down's syndrome pregnancies. The remaining 1785 (25.9%) women fell within the intermediate risk category and would be offered NT measurement. Of these, when the risk from the NT measurement was combined with the initial risk, 230 (3.4%) unaffected pregnancies and 7 (23.3%) Down's syndrome pregnancies had final risk of ≥1:250 and would be offered a diagnostic test. Using LMP based gestation, this screening protocol would have achieved a detection rate of 83.3% at a false positive rate of 7.4% with 25.9% of women requiring an NT measurement.

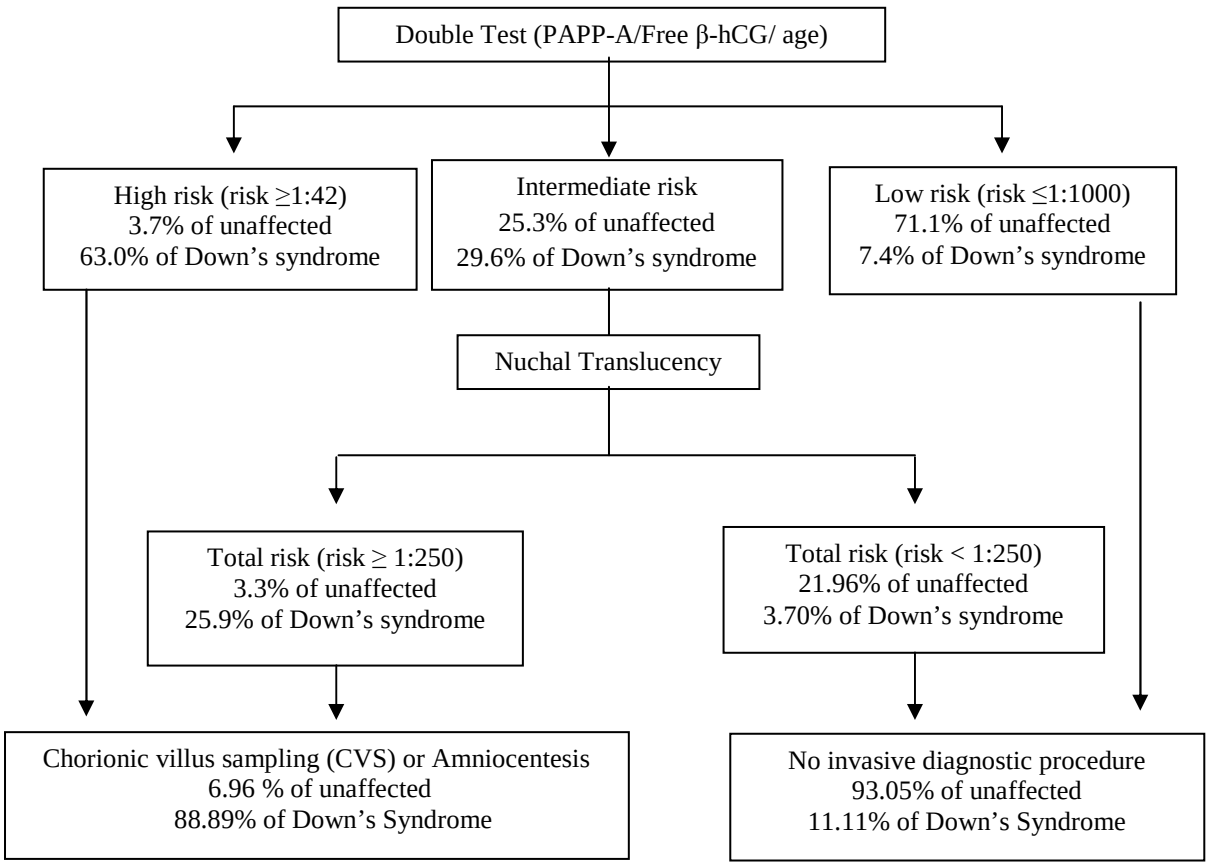


Figure 4.3 – The performance of within-trimester contingent screening using LMP based gestation in pregnancies with certain LMP dates. There were 217 (3.6%) unaffected and 17 (63.0%) Down's syndrome pregnancies in the high risk group with risks initial ≥1:42. Within the low risk group with risks ≤1:1000, there were 4231 (71.1%) unaffected and 2 (7.4%) Down's syndrome pregnancies. The remaining 1512 (25.3%) women fell within the intermediate risk category and of these, when the risk from the NT measurement was combined with the initial risk, 197 (3.3%) unaffected pregnancies and 7 (25.9%) Down's syndrome pregnancies had final risk of ≥1:250. This contingent screening protocol would have achieved a detection rate of 88.9% at a false positive rate of 7.0% with 25.3% of women requiring an NT measurement.

Therefore using LMP based gestational age, contingent screening protocol would have achieved a detection rate of 83.3% at a false positive rate of 7.4% with 25.9% of women requiring an NT measurement. When analysis was performed only on pregnancies with certain LMP dates, contingent screening protocol would have achieved a detection rate of 88.9% at a false positive rate of 7.0% with 25.3% of women requiring an NT measurement. By changing the initial and final cut-off risks the detection rate, false positive rate and NT measurement rate can be varied (Tables 4.2 and 4.3).

Table 4.2: The frequency of nuchal translucency (NT) measurement and overall screening performance in contingent testing with different risk cut-off values in pregnancies with certain and uncertain LMP dates.

Final risk cut offs (at term)	**High risk cut offs (at term)**	**Low risk cut offs (at term)**	**NT frequency (%)**	**Detection rate (%)**	**False positive rate (%)**
1:250	1:42	1:1000	25.9	83.3	7.4
		1:800	22.5	83.3	7.3
		1:600	18.5	80.0	7.1
		1:400	13.3	80.0	6.9
1:200	1:33	1:1000	26.5	80.0	6.3
		1:800	23.1	80.0	6.2
		1:600	19.1	76.7	6.1
		1:400	13.9	76.7	5.9
1:150	1:24	1:1000	27.3	80.0	5.0
		1:800	23.9	80.0	5.0
		1:600	19.9	76.7	4.9
		1:400	14.6	76.7	4.7

Table 4.3: The frequency of nuchal translucency (NT) measurement and overall screening performance in contingent testing with different risk cut-off values in pregnancies with certain LMP dates.

Final risk cut offs (at term)	**High risk cut offs (at term)**	**Low risk cut offs (at term)**	**NT frequency (%)**	**Detection rate (%)**	**False positive rate (%)**
1:250	1:42	1:1000	25.3	88.9	7.0
		1:800	22.0	88.9	6.9
		1:600	18.0	85.2	6.8
		1:400	12.9	85.2	6.5
1:200	1:33	1:1000	25.9	85.2	6.1
		1:800	22.6	85.2	6.0
		1:600	18.6	81.5	5.9
		1:400	13.4	81.5	5.7
1:150	1:24	1:1000	26.5	85.2	4.8
		1:800	23.2	85.2	4.8
		1:600	19.3	81.5	4.7
		1:400	14.1	81.5	4.5

Table 4.4 shows the summary of NT measurement frequency and overall screening performance of contingent screening according to type of gestational estimate. The use of LMP based gestation leads to increased false positive rate compared with using ultrasound based gestation. There is a decrease in detection rate in the LMP dating (certain and uncertain) group compared with the ultrasound scan group. Although there was no significant difference in the detection rate between the ultrasound scan group and certain LMP dating group, the false positive rate was higher in the certain LMP dating group. For the LMP dating groups, ultrasound scan is not required in the first stage of screening. The

NT frequency in the second stage of screening was also lower in the LMP dating groups compared with the ultrasound scan group. Although contingent screening using LMP based gestational age significantly reduces the ultrasound workload, the false positive rate increases from 5.8% to 7.0%.

Table 4.4: Summary of NT measurement frequency and overall screening performance of contingent screening according to method of gestational estimate.

Method of gestational estimate	**NT frequency (%)**	**Detection rate (%)**	**False positive rate (%)**
Ultrasound scan	29.1	88.7	5.8
LMP dating (certain and uncertain LMP)	25.9	83.3	7.4
Certain LMP dating	25.3	88.9	7.0

Final risk cut-off: 1:250; high risk cut-off: 1:42; low risk cut-off: 1:1000

4.2 CROSS - TRIMESTER CONTINGENT SCREENING

Using S-PLUS programme, various types of cross-trimester contingent screening were modelled (refer to sections 2.3 and 3.2.1). Protocols were designed in which all women would receive a first trimester screening test and those with intermediate risks would receive follow up second trimester screening test (refer to Figure 3.2).

4.2.1 CROSS-TRIMESTER CONTINGENT SCREENING WITH SECOND TRIMESTER DOUBLE, TRIPLE OR QUADRUPLE TEST

In this screening policy, all women would be offered first trimester screening (PAPP-A, fβhCG and NT measurement) and those with intermediate risk would be offered a second trimester double serum marker test. To demonstrate the performance of this screening policy, a theoretical population of 500,000 pregnant women comprising 714 Down's syndrome pregnancies and 499,286 unaffected pregnancies were used (refer to section 3.2.1). A high risk cut-off of 1:42, a low-risk cut-off of 1:1000 and a final cut-off risk of 1:150 were used in this study.

Using the S-PLUS statistical software programme the model identified 542 (75.9%) Down's syndrome and 3495 (0.7%) unaffected pregnancies with initial risks ≥1:42, and these were classified as high risk. In this approach to screening these women would be offered a diagnostic test (CVS/amniocentesis) at this stage. Within the low-risk group with risks ≤1:1000, there were 35 (4.9%) Down's syndrome and 447,360 (89.6%) unaffected pregnancies. According to the protocol, these women would be counselled that they would not be offered any further test because the initial risk was low. The remaining 48,568 (9.7%) women fell within the intermediate risk category and would be offered second trimester double test. Of these, when the risk from the second trimester double test was

combined with the initial risk, 99 (13.8%) Down's syndrome and 5492 (1.1%) unaffected pregnancies had final risk of ≥1:150 and would be offered a diagnostic test. Therefore, this model suggests that this screening policy can achieve a detection rate of 89.7% with a false positive rate of 1.8% but with only 9.7% requiring second trimester screening. Figure 4.4 illustrates the performance of this cross-trimester contingent screening policy for final risk cut-off of 1:150.

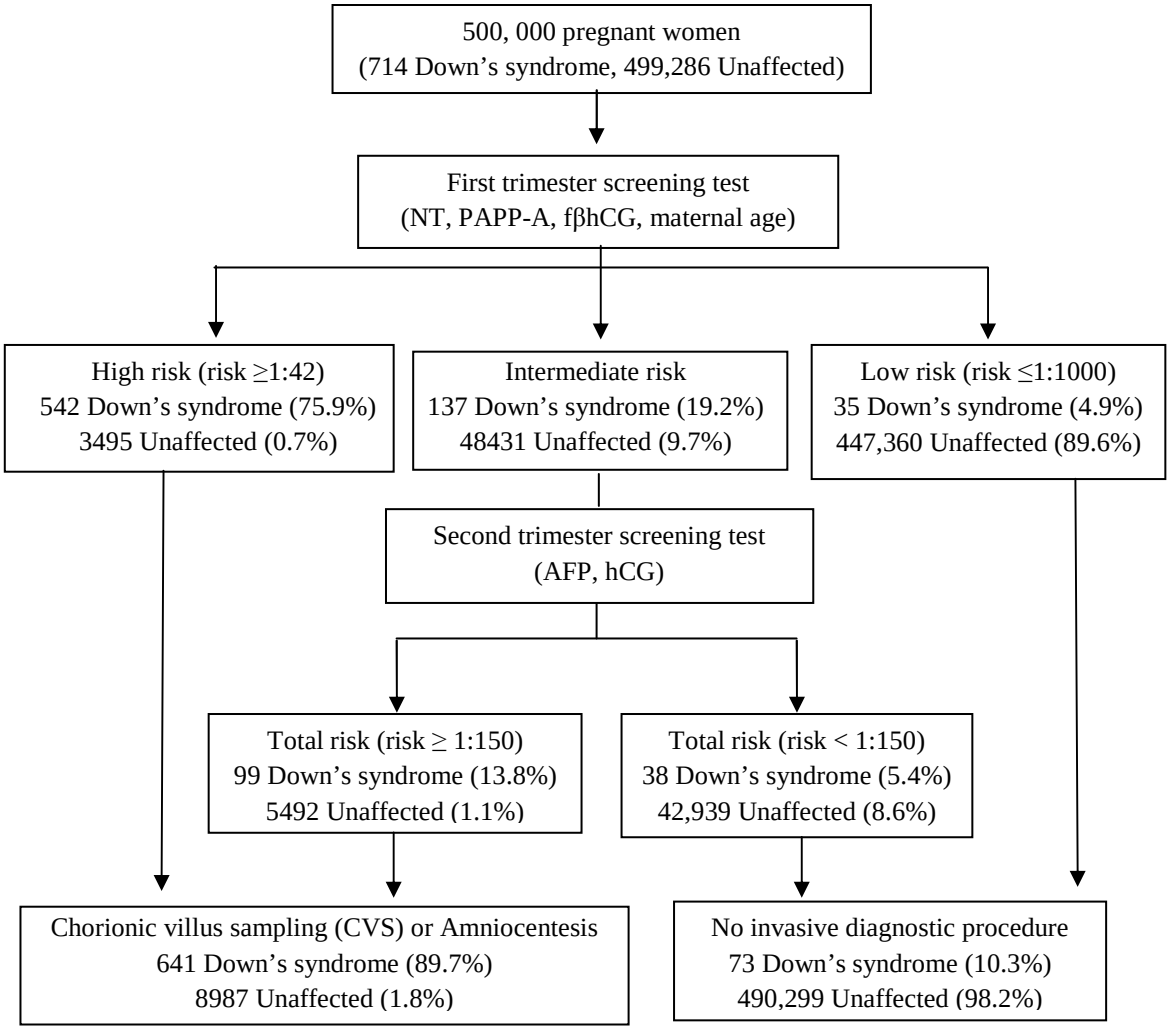


Figure 4.4: The modelled performance of cross-trimester screening with a second trimester test. There were 542 (75.9%) Down's syndrome and 3495 (0.7%) unaffected pregnancies with initial risks ≥1:42, and these were classified as high risk. Within the low-risk group with risks ≤1:1000, there were 35 (4.9%) Down's syndrome and 447,360 (89.6%) unaffected pregnancies. The remaining 48,568 (9.7%) women fell within the intermediate risk category and would be offered second trimester double test. Of these, when the risk from the second trimester double test was combined with the initial risk, 99 (13.8%) Down's syndrome and 5492 (1.1%) unaffected pregnancies had final risk of ≥ 1:150 and would be offered a diagnostic test. This screening policy can achieve a detection rate of 89.7% with a false positive rate of 1.8% but with only 9.7% requiring second trimester screening.

Tables 4.5 (a-f) shows the performance of contingent screening with second trimester double, triple or quadruple serum marker tests using various combinations of screening markers. Addition of NT measurement to the serum markers in the first trimester improved the overall screening performances. Table 4.5a shows the performance of the screening policy based on first trimester NT and PAPP-A followed by selective use of a second trimester screening test. Addition of fβhCG in the first trimester further increased the detection rate and early completion rate, decreased the second trimester testing frequency and gave less fall off of screening performance as gestation increased. There were no significant changes in the false positive rate. The detection rate and early completion rate decreased and second trimester frequency increased when fβhCG was replaced with hCG in the first trimester. For those screening policies with NT measurement, there were no changes in the false positive rates. For the screening policies without NT measurement, when fβhCG was replaced with hCG in the first trimester, the false positive rate generally decreased when screening was performed at 10 or 11 weeks of gestation. There were no changes in the false positive rates when screening was performed at 12 or 13 weeks of gestation.

The screening policy with second trimester quadruple test (AFP, hCG, InhA and uE3) had the highest detection rate and the lowest false positive rate compared with the second trimester double and triple marker tests. For the second trimester triple test, addition of InhA to the base test comprising AFP and hCG/fβhCG had a higher detection rate and lower false positive rate compared to addition of uE3 to the double test (Tables 4.5a, 4.5b and 4.5c). Therefore, InhA and not uE3 was used as part of the triple test in the subsequent analysis. The detection rate decreased and false positive rate increased as gestation advanced in all screening policies. The early completion rate decreased and second

trimester frequency increased as gestation advanced in all but one screening policy. In the screening policy in which all women would be tested for PAPP-A and hCG level in the first trimester and those with intermediate risks would be tested in the second trimester for double, triple or quadruple test, the early completion rate increased and second trimester testing frequency decreased from week 10 to week 12. At week 13, the early completion rate decreased and second trimester testing frequency increased.

When hCG in the second trimester was replaced with fβhCG, there was a decrease in the detection rate and an increase in the false positive rate (Table 4.6a and 4.6b). This suggests that fβhCG is a better marker in the first trimester compared to hCG but hCG is the better marker in the second trimester.

Table 4.5a: Performance of contingent screening in which all women would receive first trimester serum biochemical test (PAPP-A) with NT measurement and those with intermediate risk would be offered second trimester double, triple or quadruple test.

Biochemical markers (First stage / Second stage)	Week	Overall				1st trimester		2nd trimester	
		DR (%)	FPR (%)	ECR (%)	2nd trimester frequency (%)	DR (%)	FPR (%)	DR (%)	FPR (%)
NT, PAPP-A / AFP, hCG	**11**	88.5	1.9	86.9	13.1	69.5	0.7	19.0	1.2
	12	86.2	2.0	84.6	15.4	66.7	0.7	19.5	1.3
	13	81.9	2.4	79.9	20.1	58.9	0.7	23.0	1.7
NT, PAPP-A / AFP, hCG, uE3	**11**	89.0	1.8	87.0	13.0	69.5	0.7	19.5	1.1
	12	86.7	1.9	84.7	15.3	66.7	0.7	20.0	1.2
	13	82.7	2.3	79.9	20.1	58.8	0.7	23.9	1.6
NT, PAPP-A / AFP, hCG, InA	**11**	89.2	1.7	87.0	13.0	69.4	0.8	19.8	0.9
	12	87.2	1.9	84.6	15.4	66.7	0.7	20.5	1.2
	13	83.3	2.2	80.0	20.0	58.6	0.7	24.7	1.5
NT, PAPP-A / AFP, hCG, InA, uE3	**11**	89.6	1.7	87.0	13.0	69.3	0.7	20.3	1.0
	12	87.6	1.8	84.7	15.3	66.7	0.7	20.9	1.1
	13	83.9	2.0	80.0	20.0	58.7	0.7	25.2	1.3

DR: detection rate; FPR: False positive rate; ECR: Early completion rate

Table 4.5b: Performance of contingent screening in which all women would receive first trimester serum biochemical test (PAPP-A and fβhCG) with NT measurement and those with intermediate risk would be offered second trimester double, triple or quadruple test.

Biochemical markers (First stage / Second stage)	**Week**	**Overall**				**1st trimester**		**2nd trimester**	
		DR (%)	**FPR (%)**	**ECR (%)**	**2nd trimester frequency (%)**	**DR (%)**	**FPR (%)**	**DR (%)**	**FPR (%)**
NT, PAPP-A, fβhCG / AFP, hCG	**11**	89.7	1.8	90.3	9.7	75.9	0.7	13.8	1.1
	12	88.4	2.0	89.3	10.7	74.9	0.7	13.5	1.3
	13	84.9	2.4	85.9	14.1	68.2	0.8	16.7	1.6
NT, PAPP-A, fβhCG / AFP, hCG, uE3	**11**	90.2	1.7	90.3	9.7	75.9	0.7	14.3	1.0
	12	89.0	1.9	89.4	10.6	74.9	0.7	14.1	1.2
	13	85.9	2.3	85.9	14.1	68.4	0.8	17.5	1.5
NT, PAPP-A, fβhCG / AFP, hCG, InA	**11**	90.7	1.7	90.3	9.7	75.9	0.7	14.8	1.0
	12	89.5	1.8	89.3	10.7	74.8	0.7	14.7	1.1
	13	86.6	2.2	85.9	14.1	68.3	0.7	18.3	1.5
NT, PAPP-A, fβhCG / AFP, hCG, InA, uE3	**11**	91.1	1.6	90.3	9.7	76.0	0.7	15.1	0.9
	12	90.0	1.7	89.3	10.7	74.8	0.7	15.2	1.0
	13	87.2	2.1	85.9	14.1	68.3	0.8	18.9	1.3

DR: detection rate; FPR: False positive rate; ECR: Early completion rate

Table 4.5c: Performance of contingent screening in which all women would receive first trimester serum biochemical test (PAPP-A and hCG) with NT measurement and those with intermediate risk would be offered second trimester double, triple or quadruple test.

Biochemical markers (First stage / Second stage)	**Week**	**Overall**				**1st trimester**		**2nd trimester**	
		DR (%)	**FPR (%)**	**ECR (%)**	**2nd trimester frequency (%)**	**DR (%)**	**FPR (%)**	**DR (%)**	**FPR (%)**
NT, PAPP-A, hCG / AFP, hCG	**11**	88.9	1.8	87.9	12.1	70.9	0.7	18.0	1.1
	12	86.9	2.0	87.1	12.9	70.6	0.7	16.3	1.3
	13	83.6	2.5	84.8	15.2	65.9	0.7	17.7	1.8
NT, PAPP-A, hCG / AFP, hCG, uE3	**11**	89.5	1.7	87.9	12.1	71.0	0.7	18.5	1.0
	12	87.6	1.9	87.1	12.9	70.5	0.7	17.1	1.2
	13	84.8	2.3	84.9	15.1	65.8	0.7	19.0	1.6
NT, PAPP-A, hCG / AFP, hCG, InA	**11**	90.0	1.6	87.9	12.1	70.9	0.7	19.1	0.9
	12	88.4	1.8	87.0	13.0	70.6	0.7	17.8	1.1
	13	85.6	2.2	84.8	15.2	65.9	0.7	19.7	1.5
NT, PAPP-A, hCG / AFP, hCG, InA, uE3	**11**	90.3	1.5	87.9	12.1	70.9	0.7	19.4	0.8
	12	88.8	1.8	87.0	13.0	70.6	0.7	18.2	1.1
	13	86.4	2.1	84.9	15.1	65.9	0.8	20.5	1.3

DR: detection rate; FPR: False positive rate; ECR: Early completion rate

Table 4.5d: Performance of contingent screening in which all women would receive first trimester serum biochemical test (PAPP-A) without NT measurement and those with intermediate risk would be offered second trimester double, triple or quadruple test.

Biochemical markers (First stage / Second stage)	**Week**	**Overall**				**1st trimester**		**2nd trimester**	
		DR (%)	**FPR (%)**	**ECR (%)**	**2nd trimester frequency (%)**	**DR (%)**	**FPR (%)**	**DR (%)**	**FPR (%)**
PAPP-A / AFP, hCG	**10**	78.3	4.2	67.0	33.0	31.9	1.2	46.4	3.0
	11	77.8	4.2	66.1	33.9	30.6	1.2	47.2	3.0
	12	75.1	4.5	61.0	39.0	24.3	1.0	50.8	3.5
	13	69.7	4.9	51.4	48.6	14.8	0.7	54.9	4.2
PAPP-A / AFP, hCG, InA	**10**	80.4	3.8	67.0	33.0	31.9	1.2	48.5	2.6
	11	80.0	3.8	66.1	33.9	30.7	1.2	49.3	2.6
	12	77.6	4.0	61.1	38.9	24.3	1.0	53.3	3.0
	13	73.4	4.3	51.3	48.7	14.8	0.7	58.6	3.6
PAPP-A / AFP, hCG, InA, uE3	**10**	81.2	3.6	67.0	33.0	32.0	1.2	49.2	2.4
	11	80.8	3.7	66.1	33.9	30.6	1.2	50.2	2.5
	12	78.7	3.8	61.0	39.0	24.4	1.0	54.3	2.8
	13	74.9	4.0	51.3	48.7	14.8	0.7	60.1	3.3

DR: detection rate; FPR: False positive rate; ECR: Early completion rate

Table 4.5e: Performance of contingent screening in which all women would receive first trimester serum biochemical test (PAPP-A and fβhCG) without NT measurement and those with intermediate risk would be offered second trimester double, triple or quadruple test.

Biochemical markers (First stage / Second stage)	**Week**	**Overall**				**1st trimester**		**2nd trimester**	
		DR (%)	**FPR (%)**	**ECR (%)**	**2nd trimester frequency (%)**	**DR (%)**	**FPR (%)**	**DR (%)**	**FPR (%)**
PAPP-A, fβhCG / AFP, hCG	**10**	80.7	4.1	77.8	22.2	49.9	1.4	30.8	2.7
	11	80.3	4.1	77.3	22.7	49.1	1.4	31.2	2.7
	12	78.9	4.4	76.6	23.4	47.7	1.4	31.2	3.0
	13	74.9	4.8	71.8	28.2	39.4	1.3	35.5	3.5
PAPP-A, fβhCG / AFP, hCG, InA	**10**	82.8	3.7	77.8	22.2	49.8	1.4	33.0	2.3
	11	82.5	3.8	77.3	22.7	49.1	1.4	33.4	2.4
	12	81.3	4.0	76.6	23.4	47.6	1.4	33.7	2.6
	13	77.9	4.4	71.8	28.2	39.2	1.3	38.7	3.1
PAPP-A, fβhCG / AFP, hCG, InA, uE3	**10**	83.6	3.6	77.8	22.2	50.0	1.4	33.6	2.2
	11	83.3	3.6	77.3	22.7	49.1	1.4	34.2	2.2
	12	82.1	3.8	76.7	23.3	47.6	1.3	34.5	2.5
	13	79.3	4.2	71.8	28.2	39.4	1.3	39.9	2.9

DR: detection rate; FPR: False positive rate; ECR: Early completion rate

Table 4.5f: Performance of contingent screening in which all women would receive first trimester serum biochemical test (PAPP-A and hCG) without NT measurement and those with intermediate risk would be offered second trimester double, triple or quadruple test.

Biochemical markers (First stage / Second stage)	Week	Overall				1st trimester		2nd trimester	
		DR (%)	FPR (%)	ECR (%)	2nd trimester frequency (%)	DR (%)	FPR (%)	DR (%)	FPR (%)
PAPP-A, hCG / AFP, hCG	**10**	79.5	3.8	69.0	31.0	34.9	1.3	44.6	2.5
	11	78.8	4.0	69.2	30.8	35.3	1.3	43.5	2.7
	12	76.0	4.5	69.8	30.2	35.8	1.2	40.2	3.3
	13	72.5	5.0	68.6	31.4	33.3	1.2	39.2	3.8
PAPP-A, hCG / AFP, hCG, InA	**10**	82.1	3.3	69.0	31.0	34.9	1.3	47.2	2.0
	11	81.6	3.5	69.3	30.7	35.3	1.3	46.3	2.2
	12	79.2	4.0	69.7	30.3	35.7	1.3	43.5	2.7
	13	76.3	4.5	68.7	31.3	33.3	1.2	43.0	3.3
PAPP-A, hCG / AFP, hCG, InA, uE3	**10**	82.9	3.2	69.0	31.0	34.9	1.2	48.0	2.0
	11	82.3	3.3	69.2	30.8	35.3	1.3	47.0	2.0
	12	80.3	3.8	69.8	30.2	35.8	1.3	44.5	2.5
	13	77.9	4.2	68.7	31.3	33.3	1.2	44.6	3.0

DR: detection rate; FPR: False positive rate; ECR: Early completion rate

Table 4.6a: Performance of contingent screening in which all women would receive first trimester serum biochemical test (PAPP-A) with NT measurement and those with intermediate risk would be offered second trimester double, triple or quadruple test (with fβhCG in the second trimester).

Biochemical markers (First stage / Second stage)	**Week**	**Overall**				**1st trimester**		**2nd trimester**	
		DR (%)	**FPR (%)**	**ECR (%)**	**2nd trimester frequency (%)**	**DR (%)**	**FPR (%)**	**DR (%)**	**FPR (%)**
NT, PAPP-A / AFP, fβhCG	**11**	88.1	2.0	87.0	13.0	69.4	0.7	18.7	1.3
	12	85.8	2.2	84.7	15.3	66.6	0.7	19.2	1.5
	13	81.4	2.6	79.9	20.1	58.8	0.7	22.6	1.9
NT, PAPP-A / AFP, fβhCG , InhA	**11**	88.9	1.8	87.0	13.0	69.4	0.7	19.5	1.1
	12	86.7	2.0	84.7	15.3	66.8	0.7	19.9	1.3
	13	82.7	2.3	79.9	20.1	58.7	0.7	24.0	1.6
NT, PAPP-A / AFP, fβhCG , InhA, uE3	**11**	89.1	1.8	87.0	13.0	69.3	0.7	19.8	1.1
	12	87.1	1.9	84.7	15.3	66.7	0.7	20.4	1.2
	13	83.3	2.2	80.0	20.0	58.7	0.7	24.6	1.5

DR: detection rate; FPR: False positive rate; ECR: Early completion rate

Table 4.6b: Performance of contingent screening in which all women would receive first trimester serum biochemical test (PAPP-A) without NT measurement and those with intermediate risk would be offered second trimester double, triple or quadruple test (with fβhCG in the second trimester).

Biochemical markers (First stage / Second stage)	**Week**	**Overall**				**1st trimester**		**2nd trimester**	
		DR (%)	**FPR (%)**	**ECR (%)**	**2nd trimester frequency (%)**	**DR (%)**	**FPR (%)**	**DR (%)**	**FPR (%)**
PAPP-A / AFP, fβhCG	**10**	77.4	4.5	67.0	33.0	31.9	1.2	45.5	3.3
	11	77.0	4.6	66.0	34.0	30.7	1.2	46.3	3.4
	12	74.1	4.8	61.1	38.9	24.4	1.0	49.7	3.8
	13	68.7	5.2	51.4	48.6	14.8	0.7	53.9	4.5
PAPP-A / AFP, fβhCG , InhA	**10**	79.3	4.1	67.0	33.0	32.0	1.2	47.3	2.9
	11	78.9	4.1	66.2	33.8	30.6	1.2	48.3	2.9
	12	76.3	4.3	61.1	38.9	24.3	1.0	52.0	3.3
	13	72.1	4.6	51.3	48.7	14.8	0.7	57.3	3.9
PAPP-A / AFP, fβhCG , InhA, uE3	**10**	80.1	3.9	66.9	33.1	32.0	1.2	48.1	2.7
	11	79.7	3.9	66.1	33.9	30.7	1.2	49.0	2.7
	12	77.4	4.2	61.0	39.0	24.3	1.0	53.1	3.2
	13	73.6	4.3	51.4	48.6	14.7	0.7	58.9	3.6

DR: detection rate; FPR: False positive rate; ECR: Early completion rate

4.2.2 CROSS-TRIMESTER CONTINGENT SCREENING WITH REPEAT MEASURE

The performance of the cross-trimester contingent model described in section 4.2.1 was re-evaluated by repeating the measurement of each of the first trimester markers in the second trimester. Table 4.7 (4.7a and 4.7b) shows the performance of contingent screening with repeat measure of fβhCG. In this screening policy, a repeat sampling and testing for maternal serum fβhCG is carried out in those with intermediate risks in the second trimester. The performance of this screening policy was compared with the performance of screening policy in which women are selected for second trimester double, triple or quadruple test (with hCG) based on initial first trimester PAPP-A and fβhCG measurement (with or without NT) (Tables 4.5b and 4.5d). This screening policy with repeat measure of fβhCG had lower detection rate and higher false positive rate than contingent screening with second trimester double, triple or quadruple test.

The performance of contingent screening with repeat measure of PAPP-A was also evaluated (Table 4.8 a-d). Addition of repeat measure of PAPP-A to the double, triple or quadruple test in the second trimester increased the detection rate and decreased the false positive rate. Repeat measure of hCG and PAPP-A (Table 4.9a and 4.9b) had lower detection rate and early completion rate and higher second trimester frequency compared with repeat measure of PAPP-A alone. This again showed that fβhCG is a better marker in the first trimester compared to hCG.

At week 11, repeat measure of PAPP-A and fβhCG (with NT measurement) achieved a detection rate of 90.8% with a false positive rate of 1.7% (Table 4.10a and 4.10b). This screening policy also had an early completion rate of 90.3% and second trimester testing frequency of 9.7%. The screening policies with repeat measure of PAPP-A and fβhCG had higher detection rates, false positive rates and early completion rates and lower second trimester frequencies compared to the screening policies with repeat measure of PAPP-A and hCG.

Table 4.7a: Performance of contingent screening (with NT measurement) with repeat measure of fβhCG

Biochemical markers (First stage / Second stage)	**Week**	**Overall**				**1st trimester**		**2nd trimester**	
		DR (%)	**FPR (%)**	**ECR (%)**	**2nd trimester frequency (%)**	**DR (%)**	**FPR (%)**	**DR (%)**	**FPR (%)**
NT, PAPP-A, fβhCG / AFP, fβhCG	**11**	89.2	2.0	90.3	9.7	76.0	0.7	13.2	1.3
	12	87.7	2.1	89.3	10.7	74.9	0.7	12.8	1.4
	13	83.9	2.6	85.9	14.1	68.3	0.8	15.6	1.8
NT, PAPP-A, fβhCG / AFP, fβhCG , InhA	**11**	90.2	1.8	90.4	9.6	75.9	0.7	14.3	1.1
	12	89.0	2.0	89.4	10.6	75.0	0.7	14.0	1.3
	13	85.9	2.4	85.8	14.2	68.3	0.8	17.6	1.6
NT, PAPP-A, fβhCG / AFP, fβhCG , InhA, uE3	**11**	90.6	1.8	90.3	9.7	75.9	0.7	14.7	1.1
	12	89.5	1.9	89.3	10.7	74.9	0.7	14.6	1.2
	13	86.6	2.3	85.9	14.1	68.3	0.7	18.3	1.6

DR: detection rate; FPR: False positive rate; ECR: Early completion rate

Table 4.7b: Performance of contingent screening (without NT measurement) with repeat measure of fβhCG

Biochemical markers (First stage / Second stage)	Week	Overall				1st trimester		2nd trimester	
		DR (%)	FPR (%)	ECR (%)	2nd trimester frequency (%)	DR (%)	FPR (%)	DR (%)	FPR (%)
PAPP-A, fβhCG / AFP, fβhCG	**10**	79.6	4.5	77.7	22.3	49.9	1.4	29.7	3.1
	11	79.1	4.5	77.3	22.7	49.1	1.4	30.0	3.1
	12	77.1	4.8	76.6	23.4	47.6	1.4	29.5	3.4
	13	72.6	5.3	71.8	28.2	39.3	1.3	33.3	4.0
PAPP-A, fβhCG / AFP, fβhCG , InhA	**10**	81.7	4.1	77.8	22.2	49.9	1.4	31.8	2.7
	11	81.3	4.1	77.4	22.3	49.0	1.4	32.3	2.7
	12	80.0	4.4	76.6	23.4	47.7	1.4	32.3	3.0
	13	76.5	4.8	71.8	28.2	39.4	1.3	37.1	3.5
PAPP-A, fβhCG / AFP, fβhCG , InhA, uE3	**10**	82.5	3.9	77.8	22.2	49.9	1.4	32.6	2.5
	11	82.3	4.0	77.4	22.6	49.2	1.4	33.1	2.6
	12	81.1	4.2	76.7	23.3	47.7	1.4	33.4	2.8
	13	78.0	4.6	71.7	28.3	39.3	1.3	38.7	3.3

DR: detection rate; FPR: False positive rate; ECR: Early completion rate

Table 4.8a: Performance of contingent screening (with PAPP-A and NT measurement in the first trimester) with repeat measure of PAPP-A

Biochemical markers (First stage / Second stage)	Week	Overall				1st trimester		2nd trimester	
		DR (%)	FPR (%)	ECR (%)	2nd trimester frequency (%)	DR (%)	FPR (%)	DR (%)	FPR (%)
NT, PAPP-A / AFP, hCG, PAPP-A	**11**	90.1	1.5	87.0	13.0	69.3	0.7	20.8	0.8
	12	87.8	1.7	84.7	15.3	66.8	0.7	21.0	1.0
	13	82.9	2.2	79.9	20.1	58.7	0.7	24.2	1.5
NT, PAPP-A / AFP, hCG, InhA, PAPP-A	**11**	90.2	1.5	87.1	12.9	69.2	0.7	21.0	0.8
	12	88.0	1.7	84.7	15.3	66.7	0.7	21.3	1.0
	13	83.6	2.1	79.9	20.1	58.7	0.7	24.9	1.4
NT, PAPP-A / AFP, hCG, InhA, uE3, PAPP-A	**11**	90.5	1.4	87.0	13.0	69.3	0.7	21.2	0.7
	12	88.4	1.6	84.7	15.3	66.7	0.7	21.7	0.9
	13	84.2	2.0	80.0	20.0	58.8	0.7	25.4	1.3

DR: detection rate; FPR: False positive rate; ECR: Early completion rate

Table 4.8b: Performance of contingent screening (with PAPP-A, fβhCG and NT measurement in the first trimester) with repeat measure of PAPP-A

Biochemical markers (First stage / Second stage)	Week	Overall				1st trimester		2nd trimester	
		DR (%)	FPR (%)	ECR (%)	2nd trimester frequency (%)	DR (%)	FPR (%)	DR (%)	FPR (%)
NT, PAPP-A, fβhCG / AFP, hCG, PAPP-A	**11**	91.7	1.5	90.3	9.7	75.8	0.7	15.9	0.8
	12	90.1	1.7	89.3	10.7	74.9	0.7	15.2	1.0
	13	86.0	2.2	85.9	14.1	68.2	0.7	17.8	1.5
NT, PAPP-A, fβhCG / AFP, hCG, InhA, PAPP-A	**11**	91.9	1.5	90.3	9.7	76.0	0.7	15.9	0.8
	12	90.4	1.6	89.3	10.7	74.9	0.7	15.5	0.9
	13	86.8	2.2	85.9	14.1	68.2	0.8	18.6	1.4
NT, PAPP-A, fβhCG / AFP, hCG, InhA, uE3, PAPP-A	**11**	92.2	1.4	90.3	9.7	75.8	0.7	16.4	0.7
	12	90.9	1.6	89.4	10.6	74.9	0.7	16.0	0.9
	13	87.5	2.1	85.9	14.1	68.3	0.8	19.2	1.3

DR: detection rate; FPR: False positive rate; ECR: Early completion rate

Table 4.8c: Performance of contingent screening (with PAPP-A in the first trimester) with repeat measure of PAPP-A

Biochemical markers (First stage / Second stage)	Week	Overall				1st trimester		2nd trimester	
		DR (%)	FPR (%)	ECR (%)	2nd trimester frequency (%)	DR (%)	FPR (%)	DR (%)	FPR (%)
PAPP-A / AFP, hCG, PAPP-A	**10**	82.9	3.2	67.1	32.9	32.0	1.2	50.9	2.0
	11	82.4	3.2	66.1	33.9	30.6	1.2	51.8	2.0
	12	79.1	3.7	61.0	39.0	24.3	1.0	54.8	2.7
	13	72.4	4.4	51.3	48.7	14.7	0.7	57.7	3.7
PAPP-A / AFP, hCG, InhA, PAPP-A	**10**	83.2	3.1	67.1	32.9	32.0	1.2	51.2	1.9
	11	82.7	3.2	66.1	33.9	30.6	1.2	52.1	2.0
	12	79.7	3.5	61.0	39.0	24.3	1.0	55.4	2.5
	13	74.3	4.1	51.4	48.6	14.8	0.7	59.5	3.4
PAPP-A / AFP, hCG, InhA, uE3, PAPP-A	**10**	83.9	2.9	67.0	33.0	32.0	1.2	51.9	1.7
	11	83.3	3.0	66.0	34.0	30.6	1.2	52.7	1.8
	12	80.6	3.4	61.0	39.0	24.3	1.0	56.3	2.4
	13	75.6	3.8	51.3	48.7	14.8	0.7	60.8	3.1

DR: detection rate; FPR: False positive rate; ECR: Early completion rate

Table 4.8d: Performance of contingent screening (with PAPP-A and fβhCG in the first trimester) with repeat measure of PAPP-A

Biochemical markers (First stage / Second stage)	Week	Overall				1st trimester		2nd trimester	
		DR (%)	FPR (%)	ECR (%)	2nd trimester frequency (%)	DR (%)	FPR (%)	DR (%)	FPR (%)
PAPP-A, fβhCG / AFP, hCG, PAPP-A	**10**	85.3	3.2	77.7	22.3	49.9	1.4	35.4	1.8
	11	84.8	3.2	77.3	22.7	49.1	1.4	35.7	1.8
	12	82.6	3.7	76.6	23.4	47.7	1.4	34.9	2.3
	13	76.8	4.5	71.8	28.2	39.3	1.3	37.5	3.2
PAPP-A, fβhCG / AFP, hCG, InhA, PAPP-A	**10**	85.6	3.1	77.8	22.2	49.9	1.4	35.7	1.7
	11	85.3	3.2	77.3	22.7	49.2	1.4	36.1	1.8
	12	83.2	3.6	76.6	23.4	47.7	1.4	35.5	2.2
	13	78.6	4.3	71.8	28.2	39.3	1.3	39.3	3.0
PAPP-A, fβhCG / AFP, hCG, InhA, uE3, PAPP-A	**10**	86.2	3.0	77.7	22.3	49.9	1.4	36.3	1.6
	11	85.8	3.0	77.3	22.7	49.0	1.4	36.8	1.6
	12	84.0	3.4	76.6	23.4	47.7	1.4	36.3	2.0
	13	79.8	4.1	71.9	28.1	39.4	1.3	40.4	2.8

DR: detection rate; FPR: False positive rate; ECR: Early completion rate

Table 4.9a: Performance of contingent screening (with NT measurement) with repeat measure of PAPP-A and hCG

Biochemical markers (First stage / Second stage)	Week	Overall				1st trimester		2nd trimester	
		DR (%)	FPR (%)	ECR (%)	2nd trimester frequency (%)	DR (%)	FPR (%)	DR (%)	FPR (%)
NT, PAPP-A, hCG / AFP, hCG, PAPP-A	**11**	91.1	1.3	87.9	12.1	70.9	0.7	20.2	0.6
	12	89.1	1.7	87.1	12.9	70.5	0.7	18.6	1.0
	13	85.0	2.3	84.8	15.2	65.9	0.7	19.1	1.6
NT, PAPP-A, hCG / AFP, hCG, InhA, PAPP-A	**11**	91.3	1.3	87.8	12.2	70.8	0.7	20.5	0.6
	12	89.4	1.6	87.1	12.9	70.5	0.7	18.9	0.9
	13	86.0	2.2	84.8	15.2	65.8	0.7	20.2	1.5
NT, PAPP-A, hCG / AFP, hCG, InhA, uE3, PAPP-A	**11**	91.5	1.2	87.9	12.1	70.8	0.7	20.7	0.5
	12	89.8	1.5	87.1	12.9	70.5	0.7	19.3	0.8
	13	86.7	2.1	84.8	15.2	65.9	0.7	20.8	1.4

DR: detection rate; FPR: False positive rate; ECR: Early completion rate

Table 4.9b: Performance of contingent screening (without NT measurement) with repeat measure of PAPP-A and hCG

Biochemical markers (First stage / Second stage)	Week	Overall				1st trimester		2nd trimester	
		DR (%)	FPR (%)	ECR (%)	2nd trimester frequency (%)	DR (%)	FPR (%)	DR (%)	FPR (%)
PAPP-A, hCG / AFP, hCG, PAPP-A	**10**	84.9	2.7	69.0	31.0	34.9	1.3	50.0	1.4
	11	84.3	2.8	69.3	30.7	35.4	1.3	48.9	1.5
	12	80.9	3.6	69.8	30.2	35.7	1.2	45.2	2.4
	13	75.1	4.6	68.7	31.3	33.3	1.2	41.8	3.4
PAPP-A, hCG / AFP, hCG, InhA, PAPP-A	**10**	85.4	2.6	69.0	31.0	34.9	1.3	50.5	1.3
	11	84.7	2.8	69.3	30.7	35.3	1.3	49.4	1.5
	12	81.7	3.5	69.9	30.1	35.7	1.3	46.0	2.2
	13	77.2	4.3	68.7	31.3	33.4	1.2	43.8	3.1
PAPP-A, hCG / AFP, hCG, InhA, uE3, PAPP-A	**10**	85.9	2.5	69.0	31.0	34.9	1.3	51.0	1.2
	11	85.4	2.6	69.3	30.7	35.4	1.3	50.0	1.3
	12	82.7	3.2	69.8	30.2	35.7	1.3	47.0	1.9
	13	78.8	4.0	68.7	31.3	33.3	1.2	45.5	2.8

DR: detection rate; FPR: False positive rate; ECR: Early completion rate

Table 4.10a: Performance of contingent screening (with NT measurement) with repeat measure of PAPP-A and fβhCG

Biochemical markers (First stage / Second stage)	Week	Overall				1st trimester		2nd trimester	
		DR (%)	FPR (%)	ECR (%)	2nd trimester frequency (%)	DR (%)	FPR (%)	DR (%)	FPR (%)
NT, PAPP-A, fβhCG / PAPP-A, fβhCG	**11**	90.8	1.7	90.3	9.7	75.8	0.7	15.0	1.0
	12	88.7	2.0	89.3	10.7	74.9	0.7	13.8	1.3
	13	83.4	2.7	85.9	14.1	68.4	0.8	15.0	1.9
NT, PAPP-A, fβhCG / AFP, fβhCG, PAPP-A	**11**	91.5	1.6	90.3	9.7	75.8	0.7	15.7	0.9
	12	89.7	1.8	89.3	10.7	74.9	0.7	14.8	1.1
	13	85.3	2.5	85.9	14.1	68.3	0.8	17.0	1.7
NT, PAPP-A, fβhCG / AFP, PAPP-A, fβhCG, InhA	**11**	91.6	1.5	90.3	9.7	75.9	0.7	15.7	0.8
	12	90.1	1.8	89.3	10.7	74.9	0.7	15.2	1.1
	13	86.2	2.4	85.8	14.1	68.2	0.8	18.0	1.6
NT, PAPP-A, fβhCG / AFP, PAPP-A, fβhCG, InhA, uE3	**11**	91.9	1.5	90.3	9.7	75.9	0.7	16.0	0.8
	12	90.5	1.7	89.3	10.7	74.9	0.7	15.6	1.0
	13	86.9	2.2	85.9	14.1	68.2	0.8	18.7	1.4

DR: detection rate; FPR: False positive rate; ECR: Early completion rate

Table 4.10b: Performance of contingent screening (without NT measurement) with repeat measure of PAPP-A and fβhCG

Biochemical markers (First stage / Second stage)	Week	Overall				1st trimester		2nd trimester	
		DR (%)	FPR (%)	ECR (%)	2nd trimester frequency (%)	DR (%)	FPR (%)	DR (%)	FPR (%)
PAPP-A, fβhCG / PAPP-A, fβhCG	**10**	83.4	3.7	77.8	22.2	49.9	1.4	33.5	2.3
	11	82.8	3.8	77.4	22.6	49.1	1.4	33.7	2.4
	12	79.5	4.5	76.5	23.5	47.8	1.4	31.7	3.1
	13	71.5	5.5	71.8	28.2	39.3	1.3	32.2	4.2
PAPP-A, fβhCG / AFP, fβhCG, PAPP-A	**10**	84.8	3.4	77.8	22.2	49.8	1.4	35.0	2.0
	11	84.3	3.5	77.4	22.6	49.1	1.4	35.2	2.1
	12	81.6	4.0	76.6	23.4	47.6	1.4	34.0	2.6
	13	75.3	5.0	71.8	28.2	39.3	1.3	36.0	3.7
PAPP-A, fβhCG / AFP, PAPP-A, fβhCG, InhA	**10**	85.0	3.4	77.8	22.2	49.8	1.4	35.2	2.0
	11	84.7	3.4	77.3	22.7	49.1	1.4	35.6	2.0
	12	82.2	3.9	76.6	23.4	47.6	1.4	34.6	3.9
	13	77.3	4.7	71.8	28.2	39.3	1.3	38.0	3.4
PAPP-A, fβhCG / AFP, PAPP-A, fβhCG, InhA, uE3	**10**	85.7	3.2	77.7	22.3	49.9	1.4	35.8	1.8
	11	85.3	3.3	77.3	22.7	49.1	1.4	36.2	1.9
	12	83.3	3.7	76.7	23.3	47.8	1.4	35.5	2.3
	13	78.8	4.4	71.9	28.1	39.3	1.3	39.5	3.1

DR: detection rate; FPR: False positive rate; ECR: Early completion rate

Tables 4.11a and 4.11b show the summary of the performance of all the screening policies at week 12 with and without NT measurement. Addition of fβhCG to PAPP-A and NT measurement in the first trimester screening increased the detection rate and early completion rate and decreased the second trimester testing frequency. There were no significant changes in the screening performance when hCG was added to PAPP-A and NT measurement in the first trimester screening. When hCG measurement in the second trimester was replaced with fβhCG, there was a slight decrease in the detection rate. Among all the cross-trimester contingent screening with repeat measure policies, the screening policy with repeat measure of PAPP-A had the highest detection rate of 91.7% with a false positive rate of 1.5%. The screening policy with repeat measure of PAPP-A and hCG had the lowest false positive rate of 1.3% with a detection rate of 91.1%. The early completion rate and second trimester frequency was the highest and lowest respectively in the screening policies with repeat measure of fβhCG, repeat measure of PAPP-A and repeat measure of fβhCG and PAPP-A.

Without NT measurement in the first trimester the detection rates and early completion rates were decreased and false positive rates and second trimester frequencies were increased (Table 4.11b). Therefore adopting the contingent screening protocol with repeat measure of PAPP-A (NT, PAPP-A, fβhCG / AFP, hCG, InhA, uE3, PAPP-A) would have achieved a detection rate of 92.2% at a false positive rate of 1.4% (compared with 91.8% detection at a 1.5% false positive rate for the complete integrated test with quadruple test in the second trimester) but with only 9.7% of women requiring a second trimester screening test (Figure 4.5). Without NT measurement, this screening policy would have achieved a detection rate of 86.2% at a false positive rate of 3.0% (compared with 84.7% detection at a 3.3% false positive rate for the full serum integrated test with quadruple test in the

second trimester) but with only 22.3% of women requiring a second trimester screening test.

The performances of screening policies in tables 4.11a and 4.11b were re-evaluated using larger SDs for affected cases (Table 4.11c and 4.11d). The SDs used for the affected cases were calculated by inflating the SDs for unaffected cases by 10%. Although there was deterioration in the screening performance by using larger SDs for affected cases, the differences were small. For example, screening policy using NT, fβhCG and PAPP-A in the first trimester and AFP, fβhCG and PAPP-A in second trimester would have achieved a detection rate of 89.7% with a false positive rate of 1.8% using the same SDs for unaffected and Down's syndrome cases. A detection rate of 87.6% with a false positive rate of 1.9% would have been achieved when larger SDs was used for the affected cases.

Table 4.11a: Summary of the performance of all the screening policies at week 12 with NT measurement

Biochemical markers (First stage / Second stage)	Overall				1st trimester		2nd trimester	
	DR (%)	FPR (%)	ECR (%)	2nd trimester frequency (%)	DR (%)	FPR (%)	DR (%)	FPR (%)
NT, PAPP-A / AFP, hCG	86.2	2.0	84.6	15.4	66.7	0.7	19.5	1.3
NT, PAPP-A, fβhCG / AFP, hCG	88.4	2.0	89.3	10.7	74.9	0.7	13.5	1.3
NT, PAPP-A, hCG /AFP, hCG	86.9	2.0	87.1	12.9	70.6	0.7	16.3	1.3
NT, PAPP-A / AFP, fβhCG	85.8	2.2	84.7	15.3	66.6	0.7	19.2	1.5
NT, PAPP-A, fβhCG / AFP, fβhCG	87.7	2.1	89.3	10.7	74.9	0.7	12.8	1.4
NT, PAPP-A, fβhCG / AFP, hCG, PAPP-A	90.1	1.7	89.3	10.7	74.9	0.7	15.2	1.0
NT, PAPP-A, hCG / AFP, hCG, PAPP-A	89.1	1.7	87.1	12.9	70.5	0.7	18.6	1.0
NT, PAPP-A, fβhCG / AFP, fβhCG, PAPP-A	89.7	1.8	89.3	10.7	74.9	0.7	14.8	1.1

DR: detection rate; FPR: False positive rate; ECR: Early completion rate

Table 4.11b: Summary of the performance of all the screening policies at week 12 without NT measurement

Biochemical markers (First stage / Second stage)	Overall				1st trimester		2nd trimester	
	DR (%)	FPR (%)	ECR (%)	2nd trimester frequency (%)	DR (%)	FPR (%)	DR (%)	FPR (%)
PAPP-A / AFP, hCG	75.1	4.5	61.0	39.0	24.3	1.0	50.8	3.5
PAPP-A, fβhCG / AFP, hCG	78.9	4.4	76.6	23.4	47.7	1.4	31.2	3.0
PAPP-A, hCG /AFP, hCG	76.0	4.5	69.8	30.2	35.8	1.2	40.2	3.3
PAPP-A / AFP, fβhCG	74.1	4.8	61.1	38.9	24.4	1.0	49.7	3.8
PAPP-A, fβhCG / AFP, fβhCG	77.1	4.8	76.6	23.4	47.6	1.4	29.5	3.4
PAPP-A, fβhCG / AFP, hCG, PAPP-A	82.6	3.7	76.6	23.4	47.7	1.4	34.9	2.3
PAPP-A, hCG / AFP, hCG, PAPP-A	80.9	3.6	69.8	30.2	35.7	1.2	45.2	2.4
PAPP-A, fβhCG / AFP, fβhCG, PAPP-A	81.6	4.0	76.6	23.4	47.6	1.4	34.0	2.6

DR: detection rate; FPR: False positive rate; ECR: Early completion rate

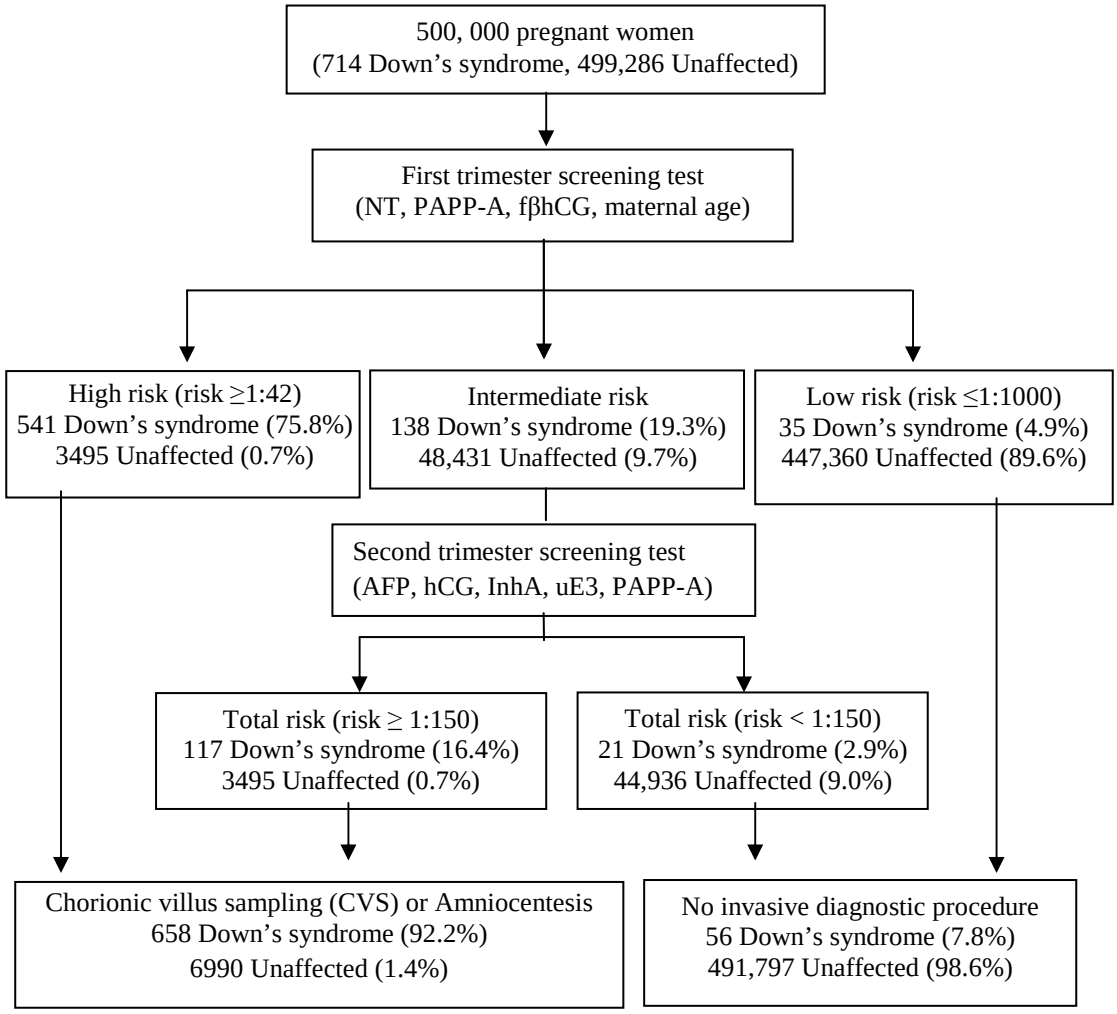


Figure 4.5: The performance of cross-trimester screening with repeat measure of PAPP-A. There were 541 (75.8%) Down's syndrome and 3495 (0.7%) unaffected pregnancies with initial risks ≥1:42, and these were classified as high risk. Within the low-risk group with risks ≤1:1000, there were 35 (4.9%) Down's syndrome and 447,360 (89.6%) unaffected pregnancies. The remaining 48,568 (9.7%) women fell within the intermediate risk category and would be offered second trimester double test. Of these, when the risk from the second trimester double test and PAPP-A was combined with the initial risk, 117 (16.4%) Down's syndrome and 3495 (0.7%) unaffected pregnancies had final risk of ≥ 1:150 and would be offered a diagnostic test. This screening policy can achieve a detection rate of 92.2% with a false positive rate of 1.4% but with only 9.7% requiring second trimester screening.

Table 4.11c: Summary of the performance of all the screening policies at week 12 with NT measurement (SDs for affected cases 10% larger than for unaffected)

Biochemical markers (First stage / Second stage)	Overall				1st trimester		2nd trimester	
	DR (%)	FPR (%)	ECR (%)	2nd trimester frequency (%)	DR (%)	FPR (%)	DR (%)	FPR (%)
NT, PAPP-A / AFP, hCG	83.8	2.2	82.8	17.2	62.7	0.7	21.1	1.5
NT, PAPP-A, fβhCG / AFP, hCG	86.1	2.1	87.9	12.1	71.5	0.7	14.6	1.4
NT, PAPP-A, hCG /AFP, hCG	84.5	2.1	85.4	14.6	66.9	0.7	17.6	1.4
NT, PAPP-A / AFP, fβhCG	83.5	2.3	82.7	17.3	62.7	0.7	20.8	1.6
NT, PAPP-A, fβhCG / AFP, fβhCG	85.4	2.2	87.9	12.1	71.6	0.7	13.8	1.5
NT, PAPP-A, fβhCG / AFP, hCG, PAPP-A	88.0	1.8	87.9	12.1	71.6	0.7	16.4	1.1
NT, PAPP-A, hCG / AFP, hCG, PAPP-A	86.8	1.8	85.5	14.5	66.8	0.7	20.0	1.1
NT, PAPP-A, fβhCG / AFP, fβhCG, PAPP-A	87.6	1.9	87.9	12.1	71.5	0.7	16.1	1.2

DR: detection rate; FPR: False positive rate; ECR: Early completion rate

Table 4.11d: Summary of the performance of all the screening policies at week 12 without NT measurement (SDs for affected cases 10% larger for than unaffected)

Biochemical markers (First stage / Second stage)	**Overall**				**1st trimester**		**2nd trimester**	
	DR (%)	**FPR (%)**	**ECR (%)**	**2nd trimester frequency (%)**	**DR (%)**	**FPR (%)**	**DR (%)**	**FPR (%)**
PAPP-A / AFP, hCG	72.4	4.2	60.8	39.2	24.6	1.0	47.8	3.2
PAPP-A, fβhCG / AFP, hCG	76.2	4.0	75.5	24.5	46.4	1.3	29.8	2.7
PAPP-A, hCG /AFP, hCG	73.0	4.2	69.1	30.9	35.2	1.2	37.8	3.0
PAPP-A / AFP, fβhCG	71.6	4.5	60.7	39.3	24.6	1.0	47.0	3.5
PAPP-A, fβhCG / AFP, fβhCG	74.7	4.4	75.6	24.4	46.4	1.2	28.3	3.2
PAPP-A, fβhCG / AFP, hCG, PAPP-A	80.1	3.5	75.6	24.4	46.4	1.3	33.7	2.2
PAPP-A, hCG / AFP, hCG, PAPP-A	78.3	3.4	69.0	31.0	35.2	1.1	43.1	2.3
PAPP-A, fβhCG / AFP, fβhCG, PAPP-A	79.2	3.8	75.6	24.4	46.4	1.2	32.8	2.3

DR: detection rate; FPR: False positive rate; ECR: Early completion rate

4.3 EFFECT OF SMOKING & ETHNICITY ON FIRST AND SECOND TRIMESTER SERUM MARKERS

Maternal smoking habit and ethnic origin are two factors known to affect the biochemical marker levels in Down's syndrome screening. However, there is little information on whether correction factors for ethnicity and maternal smoking status vary between trimesters for AFP, hCG, fβhCG and PAPP-A. Of the CUB screening cohort between August 2000 and October 2006, 939 paired first and second trimester serum samples were identified, recovered from frozen storage and assayed for all serum markers where the information was not available routinely (refer to section 2.4). The description of the study population is shown in Table 4.12.

Table 4.12: Description of the study population

Ethnicity	Number of women	Median age (years)	Median weight (kg) (1^{st}trimester)	Median weight (kg) (2^{nd}trimester)	% Smokers
Caucasian	501	31.0	65.0	65.0	18.56%
South Asian	268	**28.0** ***(p=0.000)***	**59.2** ***(p=0.000)***	**58.8** ***(p=0.000)***	3.36%
Oriental	66	30.0	**54.8** ***(p=0.000)***	**55.0** ***(p=0.000)***	3.03%
Middle Easterners	42	29.5	62.4	63.6	2.38%
Black population	35	**29.0** ***(p=0.023)***	68.0	68.0	11.43%
Asians	27	32.0	**58.0** ***(p=0.003)***	**58.0** ***(p=0.002)***	0%

The Mann-Whitney test was used to compare the median values. The median values (shown in bold) are significantly different ($p<0.05$) when compared with the median values of Caucasian women.

The median age of South Asian and Black women at the time of screening was found to be significantly younger compared to Caucasian women. Although Oriental and Middle East women were found to be younger than Caucasian women, the differences in median age were not significant. The information on maternal weight in first and second trimester was collected by weighing pregnant women in each trimester. The median weights of Caucasian women in their first and second trimester were 65.0kg. In all the ethnic groups, there was no significant difference between the first and second trimester median weight. The South Asian, Oriental and Asian women were found to be significantly lighter ($p<0.05$) compared to the Caucasian women. There was a higher percentage of smokers amongst the Caucasian (18.56%) and the Black (11.43%) women compared to the other ethnic groups. There were no smokers found among the Asian women.

4.3.1 SMOKING

To study the effect of smoking, paired 1st and 2nd trimester serum samples from 459 Caucasian women who had provided smoking information (366 non-smokers and 93 smokers) were analysed. Apart from Caucasians, the number of smokers in individual ethnic groups was too small to examine the effect of smoking in these groups (Table 4.12).

The AFP level in smokers was increased significantly in the first trimester by 16.3% ($p=0.001$) but not in the second trimester ($p=0.077$) when compared with the non-smokers. This change between trimesters was significant ($p=0.024$). The hCG level in smokers was significantly decreased by 27.6% and 30.5% in the first and second trimesters respectively ($p<0.05$), with no significant trend between trimesters ($p=0.407$). The fβhCG level was significantly decreased in smokers in the second trimester by 17.1% ($p=0.007$) but not in the first trimester ($p=0.998$) when compared with non-smokers. There was a significant

trend (*p=0.027*) between trimesters. The PAPP-A level was significantly decreased by 14% and 22.8% in first and second trimesters respectively (*p<0.05*) when compared with non-smokers, with no significant trend between trimesters (*p=0.661*) (Figure 4.6).

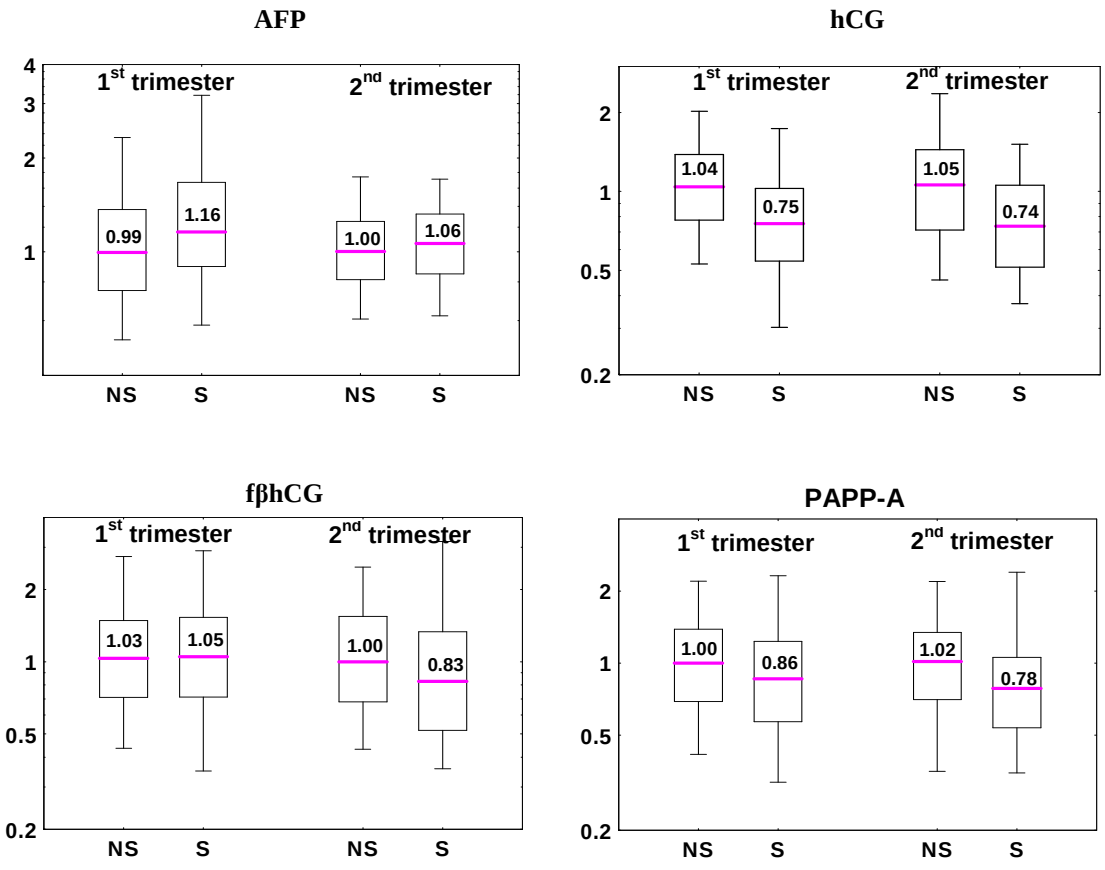


Figure 4.6: Multiple of median levels for AFP, hCG, fβhCG and PAPP-A in non-smokers and smokers in first and second trimester

Middle line represents median level, the box represents the 25th and 75th percentiles, the lines represent the 5th and 95th percentiles

4.3.2 ETHNICITY

To study the effect of ethnicity, 939 paired first and second trimester serum samples were analysed for AFP, hCG, fβhCG and PAPP-A levels routinely or retrospectively. The Caucasians were used as the reference population. Median marker levels for different ethnic groups are shown in Table 4.13. Results are corrected for maternal weight and smoking. South Asian women had significantly higher hCG levels in the first trimester ($p=0.020$) but not in the second trimester ($p=0.759$) compared with Caucasian women, with a significant trend between trimesters ($p<0.001$). They also had significantly lower fβhCG and PAPP-A in the second trimester (MoM=0.87, $p=0.006$ and MoM=0.93, $p=0.018$ respectively) when compared with Caucasian women, with a significant trend between trimesters ($p<0.001$). Oriental women had significantly higher first and second trimester hCG levels, with median MoMs of 1.41 ($p<0.001$) and 1.19 ($p=0.001$) respectively when compared with Caucasian women, with a significant trend between trimesters ($p=0.022$). They also had significantly higher fβhCG and PAPP-A levels in the first trimester (MoM=1.08, $p=0.037$ and MoM=1.20, $p=0.044$ respectively).

Middle East women had significantly lower first trimester AFP with a median MoM of 0.88 ($p=0.036$) when compared with Caucasian women, but no other significant changes. There was also no significant trend between trimesters for all markers. Black women had significantly higher hCG in the first trimester ($p=0.029$) but not in the second trimester when compared with Caucasian women, with a significant trend ($p=0.004$) between trimesters. In Black women, the median PAPP-A level was also significantly elevated in both trimesters (1.43 MoM, $p<0.001$ and 1.62 MoM, $p<0.001$ respectively) when compared with Caucasian women, with no significant trend between trimesters.

Table 4.13: Median marker levels in different ethnic groups

Ethnic groups	Median AFP(MoM)		Median hCG (MoM)		Median fβhCG (MoM)		Median PAPP-A (MoM)	
	1st trimester	2nd trimester	1st trimester	2nd trimester	1st trimester	2nd trimester	1st trimester	2nd trimester
Caucasian	0.99	1.01	1.02	1.01	1.02	1.00	1.00	1.01
South Asians	0.99	0.98	***1.09 (p=0.020)***	0.98	0.91	***0.87 (p=0.006)***	0.97	***0.93 (p=0.018)***
Orientals	0.98	0.98	***1.41 (p<0.001)***	***1.19 (p=0.001)***	***1.08 (p=0.034)***	1.07	***1.20 (p=0.044)***	1.14
Middle Easterners	***0.88 (p=0.036)***	0.96	1.12	1.09	1.02	1.00	0.92	0.98
Black women	1.07	1.01	***1.26 (p=0.029)***	0.91	0.98	0.90	***1.43 (p<0.001)***	***1.62 (p=0.001)***

The Mann-Whitney test was used to compare the median values. The median values (shown in bold) are significantly different ($p<0.05$) when compared with the median values of Caucasian women.

4.4 RETROSPECTIVE STUDY ON THE EFFECT OF ASSISTED REPRODUCTIVE TECHNOLOGY ON SERUM MARKER CONCENTRATION

The effect of ART on first and second trimester biochemical markers in Down's syndrome screening requires clarification. In this study, the level of first and second trimester biochemical markers in women pregnant after various form of ART was assessed (refer to section 2.5).

4.4.1 FIRST TRIMESTER

From the CUB screening cohort between October 2005 and January 2009, 127 ART pregnancies were identified. The control group consisted of 10891 pregnancies. The pregnancies were grouped into 4 categories; 1. normal pregnancy, 2. IVF or ICSI with fresh eggs, 3. IVF or ICSI with frozen embryo and 4. IVF with donor's egg. Table 4.14

shows the baseline parameters of the controls and ART pregnancies. The women pregnant after ART with a median maternal age of 38.6 years (range, 29.8 – 47.0) were significantly older (*p<0.05*) compared with women who had conceived spontaneously. The proportion of women of advanced maternal age (maternal age ≥ 35 years) was higher in the ART group compared with the controls, at 82.5% vs. 41.3%. When the median maternal age of each ART treatment groups were compared with the control group, women pregnant after IVF or ICSI with fresh eggs,with frozen embryos and donor's egg were significantly older (*p<0.05*).

Table 4.14: Baseline parameters of the ART pregnancies in the first trimester

Parameters	**Controls (n = 10891)**	**ART pregnancies**			
		All (n = 127)	**1 (n = 91)**	**2 (n = 29)**	**3 (n = 7)**
Maternal age (years)	33.5	***38.6 (p<0.05)***	***38.7 (p<0.05)***	***36.8 (p<0.05)***	***44.7 (p<0.05)***
GA at Blood sampling (days)	88.0	***89.0 (p=0.002)***	***89.0 (p=0.022)***	89.0 (p=0.075)	91.0 (p=0.161)
GA at NT measurement (days)	89.0	89.0 (p=0.545)	89.0 (p=0.872)	88.5 (p=0.908)	93.0 (p=0.084)

1. IVF or ICSI with fresh eggs, 2. IVF or ICSI with frozen embryo and 3. IVF with donor's egg
The Mann-Whitney test was used to compare the median values. The median values (shown in bold) are significantly different (*p<0.05*) when compared with the median values of controls.

Women pregnant after ART had their blood samples taken for PAPP-A and fβhCG analysis significantly later (*p=0.002*) in pregnancy compared with the control group, at a median gestational age of 89 days (range, 81-97) vs. 88 days (range, 63-101). When the median gestational age at sampling of each ART treatment groups was compared with the controls, women pregnant after IVF or ICSI with fresh eggs had blood taken significantly

later *(p=0.022)* in pregnancy. There was no significant difference in the gestational age at NT measurement between the women pregnant after ART and women who had conceived spontaneously.

The median MoM levels of PAPP-A, fβhCG and NT together with the 95% CI is shown in Table 4.15. There were no significant differences in PAPP-A and fβhCG levels found when the ART group overall was compared with the controls. The women pregnant after ART had significantly higher (*p=0.016*) NT measurement compared with women who had conceived spontaneously with a median MoM of 1.1041. The median PAPP-A was significantly lower *(p = 0.035)* in the IVF or ICSI with fresh eggs group when compared with the controls. Among the ART treatment groups, the NT was significantly higher *(p = 0.006)* in the IVF or ICSI with fresh eggs group when compared with the controls. There were no significant differences in the fβhCG concentrations in all the different ART treatment groups when compared with the controls.

Table 4.15: Median multiples of the median (MoM) levels of PAPP-A, fβhCG and NT measurement in ART and control pregnancies.

Markers	Controls	ART pregnancies			
		All	1	2	3
PAPP-A	1.0164 (1.0058, 1.0271)	0.9240 (0.8177, 1.0441) (p=0.083)	***0.8901 (0.7856, 1.0085) (p=0.035)***	1.0210 (0.7814, 1.3331) (p=0.734)	1.2448 (0.6923, 2.2382) (p=0.396)
FβhCG	0.9811 (0.9702, 0.9922)	1.0564 (0.9485, 1.1764) (p=0.109)	1.0821 (0.9439, 1.2405) (p=0.119)	0.9841 (0.7906, 1.2209) (p=0.717)	0.9498 (0.5140, 1.7554) (p=0.631)
NT	1.0088 (1.0038, 1.0139)	***1.1041 (1.0538, 1.1568) (p=0.016)***	***1.1296 (0.9945, 1.2832) (p=0.006)***	1.0417 (0.9295, 1.1674) (p=0.983)	1.1041 (0.9034, 1.3493) (p=0.622)

1. IVF or ICSI with fresh eggs, 2. IVF or ICSI with frozen embryo and 3. IVF with donor egg
The Mann-Whitney test was used to compare the median values. The median values (shown in bold) are significantly different ($p<0.05$) when compared with the median values of controls.

4.4.2 SECOND TRIMESTER

A cohort of 129 ART pregnancies where second trimester screening was performed between October 2005 and January 2009 was identified from the routine screening database. The control group consisted of 61,448 pregnancies. The baseline parameters of the ART pregnancies and controls are shown in Table 4.16. The women pregnant after ART were significantly older ($p<0.05$) compared with those who had conceived spontaneously with a median maternal age of 35.5 years (range, 20.2 – 43.5). The proportion of women of advanced maternal age (maternal age ≥ 35 years) at screening was 53.2% in the ART group and 19.8% in the control group. When the median maternal age of each ART treatment groups was compared with the controls, the median maternal age was significantly higher ($p<0.05$) in groups 1, 2 and 3.

Table 4.16: Baseline parameters of the ART pregnancies in the second trimester

Parameters	**Controls (n=61448)**	**ART pregnancies**			
		All (n = 129)	**1 (n = 105)**	**2 (n = 15)**	**3 (n = 9)**
Maternal age (years)	29.5	***35.5 (p<0.05)***	***35.7 (p<0.05)***	***35.2 (p=0.001)***	**38.6 (*p=0.002*)**
GA at Blood sampling (days)	113	***112 (p<0.05)***	***111 (p=0.001)***	114 (p=0.699)	112 (p=0.671)

1. IVF or ICSI with fresh eggs, 2. IVF or ICSI with frozen embryo and 3. IVF with donor's egg
The Mann-Whitney test was used to compare the median values. The median values (shown in bold) are significantly different (*p<0.05*) when compared with the median values of controls.
GA: Gestational age

Women pregnant after ART had blood taken for AFP and hCG analysis significantly earlier (*p=0.003*) in pregnancy compared to those who had conceived spontaneously at the median gestational age of 112 days (range, 105-144) vs. 113 days (range, 105-146). When the median gestational age at sampling of each ART treatment groups was compared with the controls, women pregnant after IVF or ICSI with fresh eggs had blood taken significantly earlier *(p=0.001)* in pregnancy.

The median MoM levels of AFP and hCG together with the 95% CI are shown in Table 4.17. There was no significant difference found in the AFP levels when the ART group overall was compared with the controls. The AFP level was significantly higher (*p=0.011*) in the IVF with donor's egg group when compared with the controls. There were no significant differences in the AFP levels between the other ART treatment groups and controls. The hCG level was significantly higher (*p<0.05*) in the ART group overall when compared with the controls. When the median hCG MoM level of each ART treatment

groups was compared with the controls, women pregnant after IVF or ICSI with fresh eggs (Group 1) and after IVF or ICSI with frozen embryo (Group 2) had significantly higher ($p<0.05$) levels of hCG. There was no significant difference in hCG levels in the IVF with donor egg (Group 3) when compared with the controls.

Table 4.17: Median multiples of the median (MoM) levels of AFP and hCG in ART and control pregnancies.

Markers	**Controls**	**ART pregnancies**			
		All	**1**	**2**	**3**
AFP	1.0037 (1.0011, 1.0064)	0.9962 (0.9347, 1.0616) (p=0.681)	0.9631 (0.9254, 1.0200) (p=0.677)	1.0225 (0.8294, 1.2606) (p=0.676)	***1.1909 (0.8960, 1.5796) (p=0.011)***
hCG	1.0189 (1.0145, 1.0232)	***1.2203 (1.1201, 1.3292) (p<0.05)***	***1.1967 (1.0850, 1.3199) (p=0.005)***	***1.3014 (0.9712, 1.7430) (p=0.034)***	1.2149 (0.8175, 1.8049) (p=0.128)

1. IVF or ICSI with fresh eggs, 2. IVF or ICSI with frozen embryo and 3. IVF with donor's egg
The Mann-Whitney test was used to compare the median values. The median values (shown in bold) are significantly different ($p<0.05$) when compared with the median values of controls

4.5 RETROSPECTIVE STUDY ON BIRTH WEIGHT, DURATION OF PREGNANCY AND SECOND TRIMESTER MATERNAL SERUM SCREENING MARKERS IN NON-SMOKERS AND SMOKERS

4.5.1 ACCURACY OF SELF-REPORTED SMOKING INFORMATION AT BOOKING AND SCREENING APPOINTMENTS

In antenatal care, self-reported smoking is commonly used to determine the smoking status of pregnant women. The accuracy of this information is still questionable. Inaccurate self report during pregnancy can result in inaccurate risk calculation for Down's syndrome. In this study, the accuracy of self-reported smoking information at booking and screening appointments in West of Scotland was assessed (refer to section 2.6). The smoking information at booking was obtained from the SMR02 records and the smoking information at screening from the second trimester screening records. Of the 29975 women in the West of Scotland who gave birth in 2004 21,029 pregnant women opted for second trimester screening. Of these cotinine testing was performed on 3475 randomly selected maternal serum samples. The cotinine cut-off concentration used to distinguished smokers and non-smokers was 13.7ng/ml. Re-testing of cotinine was performed on 71 samples with cotinine values between 10-30ng/ml (close to the cut-off of 13.7ng/ml) and the average concentration taken as the final result. Table 4.18 shows the characteristics of the study population.

Table 4.18: Description of the study population.

Characteristic	Whole sample (n=21029)	Cotinine-validated sample (n=3475)
Maternal age (years), median	29.8	29.9
Infant birth weight (g), median	3420	3430
Gestation at delivery (weeks), median	40	40
Gestation at screening (weeks), median	16	16
Self-reported smoking status at booking appointment (%):		
Non – smokers	54.0	56.7
Current smokers	23.3	24.1
Former smokers	9.9	10.6
Unknown	9.3	8.6
Self-reported smoking status at screening appointment (%):		
Non – smokers	57.2	57.2
Current smokers	22.0	21.4
STDUR*	4.8	4.9
STPR*	3.5	3.8
Unknown	12.5	12.7

*STDUR: Stopped smoking during pregnancy, STPR: Stopped smoking prior to pregnancy

From the smoking status at booking information, obtained from the SMR02 records (section 2.6), 1971 (56.7%) women self-reported as non-smokers, 839 (24.1%) as smokers and 367 (10.6%) as former smokers. The self-reported smoking information was not available for 298 (8.6%) women. The percentage of self-reported smokers (24.1%) at booking was significantly lower than the cotinine-validated estimate of 30.1%. At booking, 4.9% and 25.6% of women who self-reported as non-smoker and former smoker respectively had cotinine level ≥13.7ng/ml (Table 4.19). Sixty-one (7.3%) women who self-reported as smokers had cotinine level below the cut-off. These women could have quit smoking between booking and screening appointment, be light smokers or this might be due to recording errors.

Table 4.19: Number of women with cotinine levels above and below the cotinine cut-off of 13.7 ng/ml in each self-reported smoking category at booking appointment

		Self-reported smoking status at booking				**Total**
		Non-smokers	**Smokers**	**Former smokers**	**Unknown**	
Cotinine (ng/ml)	**<13.7**	1875	61	273	220	2429
	≥13.7	96	778	94	78	1046
Total		1971	839	367	298	3475
Misclassification		4.9%	7.3%	25.6%	-	

At the screening appointment, 1985 (57.2%) women self-reported as non-smokers, 745 (21.4%) as smokers, 172 (4.9%) as stopped smoking during pregnancy and 131 (3.8%) as stopped smoking prior to pregnancy. The self-reported smoking information was not available for 442 (12.7%) women. The percentage of self-reported smokers (21.4%) at screening was significantly lower than the cotinine-validated estimate of 30.1%. One-hundred and thirteen (5.7%) women who self-reported as non-smokers had cotinine level ≥13.7ng/ml (Table 4.20). Among those who self-reported as stopped smoking during or prior to pregnancy, 32.6% and 21.4% of these women had a cotinine level ≥13.7ng/ml respectively. Twenty-eight (3.8%) women who self-reported as smokers had cotinine levels below the cut-off.

Table 4.20: Number of women with cotinine levels above and below the cotinine cut-off of 13.7 ng/ml in each self-reported smoking category at screening appointment

		Self-reported smoking status at screening					**Total**
		Non-smokers	**Smokers**	**STDUR**	**STPR**	**Unknown**	
Cotinine (ng/ml)	**<13.7**	1872	28	116	103	310	2429
	≥13.7	113	717	56	28	132	1046
Total		1985	745	172	131	442	3475
Misclassification		5.7%	3.8%	32.6%	21.4%	-	-

*STDUR: Stopped smoking during pregnancy, STPR: Stopped smoking prior to pregnancy

This study shows that, 25.6% and 31.5% cotinine-validated smokers were not detected by self-report at booking and screening appointment respectively. The highest proportion of inaccurate reporting was amongst women who stated that they were former smokers (25.6% of former smokers at booking, 32.6% of those who stated at screening that they had stopped smoking during pregnancy and 21.4% of those who stated at screening that they had stopped smoking prior to pregnancy). In women who stated that they were smokers or non-smokers the level of accuracy was much higher.

Since the cut-off used here was derived from a different assay method, the impact of using different cotinine cut-offs on the percentage of misclassification of self-reported non-smokers and smokers at booking and screening appointments was evaluated (Table 4.21). The data from this study showed that there is very little variation in the findings when any cut-off between 10 and 30ng/ml is used.

Table 4.21: The percentage of misclassification of non-smokers and smokers for various cut-off based on self-reported smoking status at booking and screening appointments

Cut-off (ng/ml)	Misclassification			
	Booking appointment		Screening appointment	
	Non-smokers (%)	Smokers (%)	Non-smokers (%)	Smokers (%)
10	5.1	6.2	5.9	2.8
11	4.9	6.3	5.8	3.0
12	4.9	6.9	5.8	3.2
13	4.9	7.3	5.8	3.8
14	4.9	7.4	5.7	3.9
15	4.8	7.7	5.6	3.9
16	4.8	8.0	5.6	4.0
17	4.7	8.1	5.5	4.2
18	4.7	8.1	5.5	4.2
19	4.6	8.3	5.5	4.6
20	4.5	8.3	5.4	4.6
21	4.5	8.3	5.4	4.6
22	4.5	8.5	5.3	4.7
23	4.5	8.5	5.3	4.7
24	4.5	8.6	5.3	5.0
25	4.5	8.7	5.2	5.1
26	4.4	8.7	5.1	5.1
27	4.4	8.8	5.1	5.1
28	4.3	8.8	5.1	5.1
29	4.3	8.8	5.1	5.1
30	4.3	8.9	5.1	5.1
13.7 (used in this study)	*4.9*	*7.3*	*5.7*	*3.8*

4.5.2 FORMS USED FOR COLLECTING SELF - REPORTED SMOKING INFORMATION AT SCREENING

The form used to collect self-reported smoking information was replaced with a new form in 2007. An analysis was performed to compare the efficiency between the old and new forms used to collect self-reported smoking information. Two datasets (March 2006 and March 2008) were used in this study (refer to section 2.6.2). The self-reported smoking information in March 2006 dataset was collected using the screening form where women were given four options; non-smoker, smoker, stopped smoking during pregnancy and stopped smoking prior to pregnancy. The self-reported smoking information in March 2008 dataset was collected using the screening form where women were given only two

options; non-smoker or smoker. Those women who stopped smoking during pregnancy and stopped smoking prior to pregnancy were classified as 'non-smoker'. For both data-sets, the smoking status information was also included in the screening report allowing the antenatal clinic to contact the laboratory if there was any mistake in the smoking information. From each dataset maternal serum of 100 self-reported non-smokers and 100 self-reported smokers were randomly selected for cotinine testing. The description of the study population is shown in Table 4.22.

Table 4.22: Description of the study population.

Characteristic	March 2006 (n=1676)	Cotinine-validated samples for March 2006 (n=200)	March 2008 (n=1507)	Cotinine-validated samples for March 2008 (n=200)
Maternal age (years), median	29.3	27.7	29.0	28.7
Gestation at screening (weeks), median	16	16	16	16
Self reported smoking status at screening appointment (%):				
Non-smoker	72.1	50.0	78.4	50.0
Smoker	21.7	50.0	21.1	50.0
Not answered	6.2	-	0.5	-

The accuracy of self-reported smoking information where women were given four options; non-smoker, smoker, stopped smoking during pregnancy and stopped smoking prior to pregnancy on the screening form was 95.5% (Table 4.23). The accuracy of smoking information where women were given two options; non-smoker and smoker on the screening form and were allowed to correct their smoking status once they receive their screening report was 96%. Therefore, those women who stopped smoking during pregnancy and stopped smoking prior to pregnancy can be classified as 'non-smoker'.

Allowing smoking status to be corrected after the issue of the screening report improves the accuracy of self-reported smoking information. Making women aware that mis-reporting of smoking status may affect the accuracy of the risks that they are given from the screening test may also improve the quality of smoking status information at the time of screening.

Table 4.23: Number of women with cotinine levels above and below the cotinine cut-off of 13.7 ng/ml in each self-reported smoking category

		March 2006			March 2008		
		Non-smokers	**Smokers**	**Total**	**Non-smokers**	**Smokers**	**Total**
Cotinine (ng/ml)	**<13.7**	95	4	99	96	4	100
	≥13.7	5	96	101	4	96	100
Total		100	100	200	100	100	200

4.5.3 BIRTHWEIGHT, DURATION OF PREGNANCY AND SECOND TRIMESTER MARKERS

From the 21,029 second trimester screening cohort, 15,973 singleton pregnancies which had full information on birth weight, gestation at delivery, AFP level, hCG level and self-report as smoker or non-smoker were selected for this analysis. Those who responded with stopped during or prior to pregnancy were excluded from further analysis.

4.5.3.1 BIRTH WEIGHT AND SECOND TRIMESTER MARKERS

Table 4.24 shows the mean birth weight of all the infants according to AFP level for non-smokers and smokers. As the AFP level increased from <0.5 MoM to ≥2.0 MoM there was an overall reduction of 448.3g *($p<0.05$)* in the mean birth weight in non-smokers and by 619.2g *($p<0.05$)* in smokers. For AFP levels less than 0.5 MoM, the percentage of infants born weighing less than 2500g was 5.8% for non-smokers and 11% for smokers. As the AFP MoM increased from 0.5 to ≥2.0, the percentage of infants born weighing less than 2500g increased gradually from 2.9% to 18.3% for non-smokers and 8.7% to 39.8% for smokers (Table 4.24).

As hCG levels increased from < 0.5 to 1.99 MoM there was an increase (50.9g) in the mean birth weight in non smokers but this failed to reach statistical significant ($p=0.068$). The group of women with hCG ≥2.0 MoM for both non-smokers and smokers had the lowest mean birth weight (3385.4g in non smokers and 3068.7g in smokers) and the greatest percentage of infants born weighing less than 2500g (7.9% and 17.5% respectively) (Table 4.25).

In pregnant women who reported smoking there was a significant ($p<0.05$) reduction in mean birth weight of infants (average 270g) across all the AFP MoM and hCG MoM groups when compared to birth weight in non-smoking pregnant women. Regression analysis showed that the trends in birth weight for non-smokers and smokers according to AFP and hCG levels were significant, with the most marked changes associated with AFP (Figure 4.7).

Since low birth weight can be associated with earlier delivery (Wilcox and Johnson, 1992), and smokers tend to have earlier deliveries (McCowan *et al.*, 2009) the data were re-

analysed in those pregnancies delivered at 39 to 41 weeks of gestation. . Table 4.26 shows the mean birth weights of all the infants according to AFP level for these pregnancies. Women with high AFP levels at screening had lower birth weight babies and were more likely to have low birth weight (<2500g) babies than those with lower AFP but this was less marked than that seen with the whole dataset when all gestations at delivery were included. Table 4.27 shows the equivalent data for hCG. Unlike AFP levels and birth weight, there was no clear association between birth weight and hCG either in non-smokers or smokers (Figure 4.8).

Table 4.24: Birth weight according to maternal serum AFP level for non-smokers and smokers in the study group of 15973 cases

AFP level (MoM)	No. of non-smokers	Birth weight (NS) (g)		Infants born weighting 2500g or less (NS)		No. of smokers	Birth weight (S) (g)		Infants born weighting 2500g or less (S)	
		Mean	95% CI	No.	%		Mean	95% CI	No.	%
<0.50	189	3566.4	3454.9, 3677.9	11	5.8	73	3254.0	3125.4, 3382.7	8	11.0
0.50 - 0.74	2102	3522.0	3499.4, 3544.5	60	2.9	733	3259.0	3209.9, 3308.0	64	8.7
0.75 - 0.99	3837	3509.8	3491.8, 3527.8	135	3.5	1423	3195.7	3165.0, 3226.3	142	10.0
1.00 - 1.49	4358	3461.0	3443.5, 3478.4	193	4.4	1781	3110.3	3081.7, 3138.9	246	13.8
1.50 - 1.99	782	3337.1	3291.9, 3382.2	62	7.9	358	3028.9	2962.8, 3094.9	67	18.7
>=2.00	229	3118.1	3008.9, 3227.4	42	18.3	108	2634.8	2482.3, 2787.4	43	39.8

* NS – non-smokers, S – smokers

Table 4.25: Birth weight according to maternal serum hCG level for non-smokers and smokers in the study group of 15973 cases.

hCG level (MoM)	No. of non-smokers	Birth weight (NS) (g)		Infants born weighting 2500g or less (NS)		No. of smokers	Birth weight (S) (g)		Infants born weighting 2500g or less (S)	
		Mean	95% CI	No.	%		Mean	95% CI	No.	%
<0.50	807	3457.9	3419.0, 3496.8	30	3.7	850	3119.8	3077.9, 3161.8	112	13.2
0.50 – 0.74	1906	3465.4	3439.7, 3491.1	73	3.8	1272	3153.8	3119.9, 3187.7	153	12.0
0.75 – 0.99	2283	3482.7	3459.6, 3505.8	84	3.7	967	3185.8	3149.0, 3222.6	104	10.8
1.00 – 1.49	3595	3493.4	3474.7, 3512.1	141	3.9	970	3132.6	3092.2, 3173.0	132	13.6
1.50 – 1.99	1684	3508.8	3478.7, 3538.9	78	4.6	263	3143.5	3045.5, 3241.4	42	16.0
>=2.00	1222	3385.4	3348.4, 3422.3	97	7.9	154	3068.7	2958.4, 3179.0	27	17.5

* NS – non-smokers, S – smoker

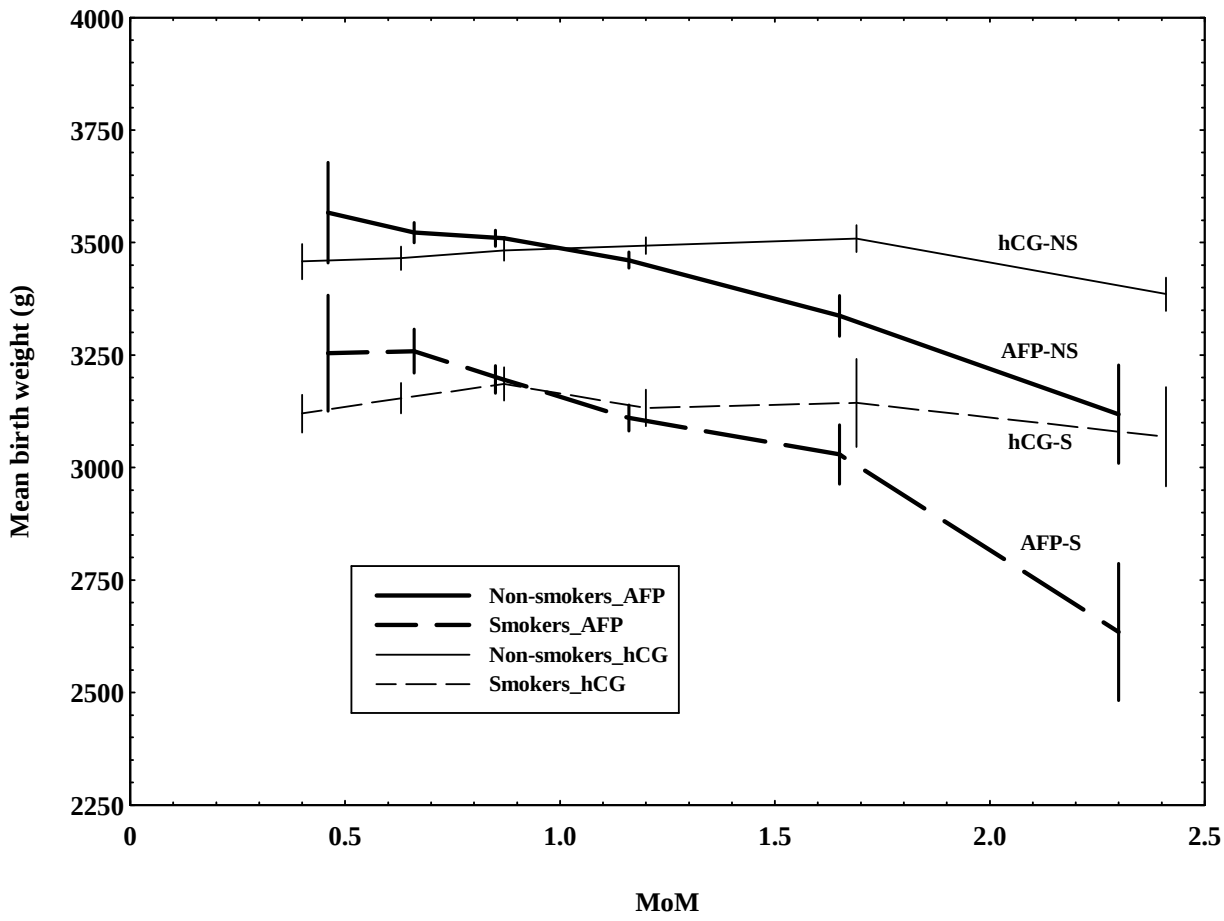


Figure 4.7 - Trend of birth weight for non-smokers and smokers according to AFP and hCG levels in the study group of 15973 cases. The mean birth weight decreased as the AFP level increased from < 0.5 to ≥ 2.00 MoM in non-smokers and smokers. The mean birth weight in smokers was lower compared to non-smokers in all AFPMoM groups. As for the hCG levels, there were insignificant increase in the mean birth weight in non-smokers as hCG levels increased from <0.5 to 1.99MoM. The mean birth weight was reduced in the group of women with hCG MoM ≥ 2.0 for non-smokers and smokers

Table 4.26: Birth weight according to maternal serum AFP level for non-smokers and smokers for the cases delivered at 39 – 41 weeks.

AFP level (MoM)	No. of non-smokers	Birth weight (NS) (g)		Infants born weighting 2500g or less (NS)		No. of smokers	Birth weight (S) (g)		Infants born weighting 2500g or less (S)	
		Mean	95% CI	No.	%		Mean	95% CI	No.	%
<0.50	136	3663.3	3575.4, 3751.2	2	1.5	58	3356.1	3234.3, 3477.9	2	3.4
0.50 – 0.74	1627	3607.5	3586.0, 3629.0	8	0.5	547	3389.3	3343.4, 3435.3	14	2.6
0.75 – 0.99	2888	3615.9	3599.6, 3632.3	10	0.3	1010	3344.8	3317.1, 3372.5	26	2.3
1.00 – 1.49	3137	3595.5	3579.1, 3611.9	18	0.6	1176	3300.0	3273.4, 3326.6	48	4.1
1.50 – 1.99	499	3543.7	3499.4, 3588.1	11	2.2	213	3315.7	3254.8, 3376.6	10	4.7
>=2.00	129	3483.9	3397.2, 3570.6	5	3.9	48	3154.9	3002.3, 3307.5	9	18.8

* NS – non-smokers, S – smokers

Table 4.27: Birth weight according to maternal serum hCG level for non-smokers and smokers for the cases delivered at 39 – 41 weeks.

hCG level (MoM)	No. of non-smokers	Birth weight (NS) (g)		Infants born weighting 2500g or less (NS)		No. of smokers	Birth weight (S) (g)		Infants born weighting 2500g or less (S)	
		Mean	95% CI	No.	%		Mean	95% CI	No.	%
<0.50	591	3581.3	3546.1, 3616.6	1	0.2	575	3275.9	3238.5, 3313.2	23	4.0
0.50 – 0.74	1434	3566.2	3542.8, 3589.7	10	0.7	887	3324.3	3293.1, 3355.5	34	3.8
0.75 – 0.99	1673	3595.0	3573.5, 3616.6	13	0.8	692	3347.1	3313.6, 3380.6	20	2.9
1.00 – 1.49	2676	3614.6	3596.9, 3632.3	12	0.4	634	3343.8	3308.6, 3379.0	22	3.5
1.50 – 1.99	1217	3635.4	3608.8, 3662.0	9	0.7	164	3433.5	3326.3, 3540.6	7	4.3
>=2.00	825	3594.3	3562.2, 3626.4	9	1.1	100	3338.7	3239.2, 3438.1	3	3.0

* NS – non-smokers, S – smokers

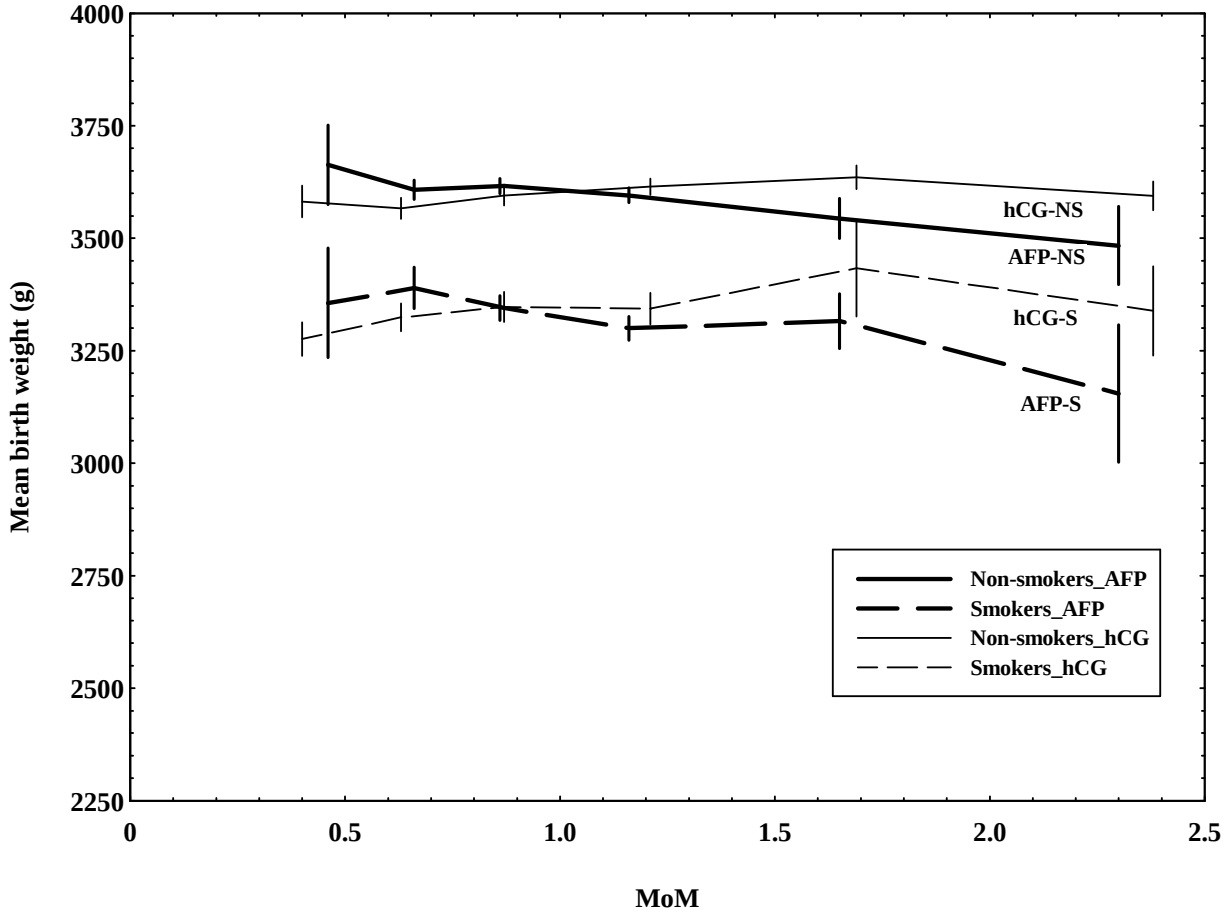


Figure 4.8 - Trend of birth weight for non-smokers and smokers according to AFP and hCG levels for the cases delivered at 39 – 41 weeks. There was a general trend of declining birth weight as AFP MoM increased from < 0.5 to ≥ 2.0 in non-smokers and smokers but there was no clear association between birth weight and hCG level either in non-smokers or smokers.

4.5.3.2 GESTATION AT DELIVERY AND SECOND TRIMESTER MARKERS

The median gestation at delivery was 40 weeks for non smokers and 39 weeks for smokers. Table 4.28 shows the median gestation at delivery and percentage of pregnancies delivered at 38 weeks and less according to AFP level for non-smokers and smokers. The percentage of pregnancies delivered at 38 weeks or less increased as AFP MoM increased in non-smokers and smokers. In smokers with AFP levels greater than 2.0 MoM over half (55.6%) delivered at 38 weeks or earlier compared to 40.2% in non-smokers. There was no clear association between hCG level and the percentage of pregnancies delivered at 38 weeks or earlier in the non-smoking and smoking groups (table 4.29). As might be expected, given the known association between smoking and low birth weight and premature delivery, the percentage of pregnancies delivered at 38 weeks and earlier was higher in the smoking group compared to the non-smoking group in all but one of the AFP MoM groups and all hCG MoM groups (Figure 4.9).

Table 4.28: Percentage of pregnancies delivered at 38 weeks or less for non-smokers and smokers according to AFP levels

AFP level (MoM)	No. of non-smokers	Median gestation at delivery (NS)	Pregnancies delivered at 38 weeks and less (NS)		No. of smokers	Median gestation at delivery (S)	Pregnancies delivered at 38 weeks and less (S)	
			No	%			No.	%
<0.50	189	40	47	24.9	73	40	12	16.4
0.50 – 0.74	2102	40	415	19.7	733	40	168	22.9
0.75 – 0.99	3837	40	833	21.7	1423	39	385	27.1
1.00 – 1.49	4358	40	1082	24.8	1781	39	565	31.7
1.50 – 1.99	782	39	260	33.2	358	39	136	38.0
>=2.00	229	39	92	40.2	108	38	60	55.6

* NS – non-smokers, S – smokers

Table 4.29: Percentage of pregnancies delivered at 38 weeks or less for non-smokers and smokers according to hCG levels

hCG level (MoM)	No. of non-smokers	Median gestation at delivery (NS)	Pregnancies delivered at 38 weeks and less (NS)		No. of smokers	Median gestation at delivery (S)	Pregnancies delivered at 38 weeks and less (S)	
			No	%			No.	%
<0.50	807	39	192	23.8	850	39	267	31.4
0.50 – 0.74	1906	40	425	22.3	1272	39	357	28.1
0.75 – 0.99	2283	40	543	23.8	967	40	253	26.2
1.00 – 1.49	3595	40	805	22.4	970	39	312	32.2
1.50 – 1.99	1684	40	401	23.8	263	39	90	34.2
>=2.00	1222	39	363	29.7	154	39	47	30.5

* NS – non-smokers, S – smokes

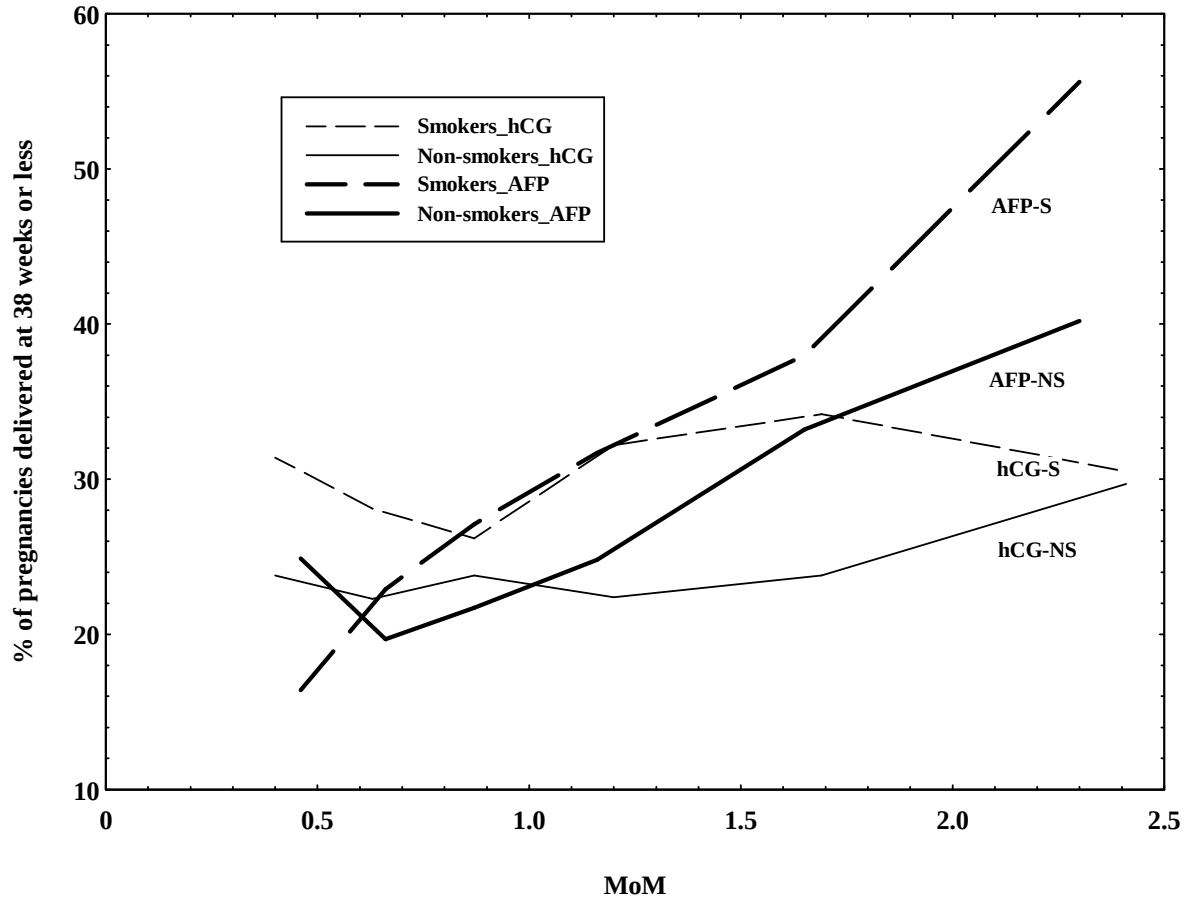


Figure 4.9 – The percentage of pregnancies delivered at 38 weeks or less according to AFP and hCG levels for non-smokers and smokers. As AFP levels increased from 0.75 to ≥ 2.0 MoM there was an increase in the percentage of pregnancies delivered at 38 weeks or less in non-smokers and smokers. There was no clear association between hCG levels and the percentage of pregnancies delivered at 38 weeks or less either in non-smokers or smokers. The percentage of pregnancies delivered at 38 weeks or less was higher in the smoking group compared to the non-smoking group

CHAPTER 5: DISCUSSION

5.1 RETROSPECTIVE STUDY ON WITHIN-TRIMESTER CONTINGENT SCREENING

CUB screening using PAPP-A, fβhCG and NT measurement is proven to be an effective method of detecting Down syndrome pregnancies in the first trimester, with a detection rates of 85-91% at a 4-5% screen positive rate (Spencer *et al*., 2000a; Stenhouse *et al*., 2004; Perni *et al*., 2006). However, this screening policy requires considerable investment in ultrasound equipment and operator training to maintain the required standard when screening large numbers of pregnant women.

In this model of first-trimester contingent screening, women who are found to have a high risk based on the initial biochemical test and maternal age are not offered NT measurement because these women will end up with a final risk ≥1:250 irrespective of the NT measurement. Women with a low risk will also not be offered NT measurement as their risk is unlikely to be modified sufficiently to reach the final cut-off. Thus, this form of contingent screening allows those centres with limited resources to target the group of pregnant women whose screening results can be most usefully modified by information from an NT measurement. This study showed that this contingent screening protocol would have achieved a detection rate of 88.7% at a false positive rate of 5.8% but with only 29% of women requiring an NT measurement. The results of the within-trimester contingent screening study are presented in Section 4.1.

This form of within-trimester contingent testing has other advantages. Unlike across-trimester integrated testing (Wald *et al*., 1999a) where there is no disclosure of results after the first stage of testing, by the contingent method described here all women receive a risk result following their initial PAPP-A/fβhCG test. Decisions on whether to proceed to NT

measurement or not are therefore based on the woman's awareness of clearly defined criteria, which aid counselling and are likely to help ensure that women do not default on their NT appointment. Further, all women complete screening before the end of the first trimester and more than two-thirds (which also includes 60% of the Down syndrome pregnancies) complete screening at an even earlier stage when there is no requirement to carry out an NT measurement.

To maximise the efficiency of this screening protocol it is important that women attend the initial blood sampling test as early as possible to allow time for those women requiring an NT scan to return no later than 14 weeks + 0 days of gestation. Although the number of women requiring an NT scan is reduced, ultrasound assessment of gestation is indispensable in all cases as this information is essential for accurate interpretation of the serum marker results. Therefore, ultrasound assessment of gestation is essential in order to maintain the sensitivity and specificity of the screening policy. However a dating scan is generally less time consuming than an NT scan, and can be carried out by staff without specific training in NT measurement.

Due to limited availability of ultrasound resources in some centres, gestational age is often determined by relying on LMP. The accuracy of LMP based gestational age is affected by the variation in menstrual cycle duration, non-menstrual vaginal bleeding, maternal recall error and clerical error (Wier *et al*., 2007). However, in areas with limited ultrasound resources, LMP is the most practical method of determining gestational age. In this study, the performance of this screening policy was re-evaluated by using LMP based gestational age at the first stage of screening (refer to section 4.1). In pregnancies with certain LMP dates, this screening policy would have achieved a similar detection rate of 88.9% at the

cost of an increase in the false positive rate from 5.8% to 7.0%. Thus, using LMP based gestation in the first stage of screening for pregnancies with certain LMP dates degrades the performance of screening achievable by reliance on ultrasound estimation of gestation. However, performance remains acceptable and allows those centres with limited resources of ultrasound to provide risks for the group of pregnant women who are uncertain of their LMP dates.

Although the performance of within-trimester contingent screening using LMP based gestational age is acceptable, multiple pregnany will not be identified. Therefore, ultrasound scan in the first trimester remains essential to assess accurate gestational age and identify multiple pregnany.

This form of contingent testing could be modified through the use of alternative serum markers measured earlier in pregnancy. It is well known that PAPP-A has better discriminatory power earlier in pregnancy at 8 weeks of gestation (Spencer *et al*., 2002) while other markers, notably total or intact hCG (Spencer *et al*., 2002), InhA (Christiansen and Nørgaard-Pederson, 2005) and ADAM12 (Laigaard *et al*., 2006b) may also perform better than fβhCG at this early stage. However, in routine CUB screening, PAPP-A and fβhCG are usually measured at the same gestation as NT at 11–13 weeks, and at that stage the reduced power of PAPP-A is compensated by the increased power of fβhCG. In this contingent model, a detection rate of 61.4% at a 3.1% false positive rate was predicted using serum markers plus maternal age alone (i.e. without NT measurement). This is similar to that estimated by Cuckle (2000) of 64.9% detection with a false positive rate of 5%, and by Spencer *et al*. (2003d) of 64.7% detection with a false positive rate of 5%. It is likely that additional serum markers assayed in early pregnancy may improve the primary

screen detection rate even further. However, the selective addition of NT measurements as shown here, adds to screening performance and should be used wherever resources allow. Ultrasound based gestational age is also essential in order to meet the standard screening recommendations.

5.2 CROSS-TRIMESTER CONTINGENT SCREENING

The SURUSS trial reported that integrated testing is the most effective screening method for Down's syndrome with detection rates of 93% at a 5% false positive rate (Wald *et al*., 2003). However, this screening policy has many disadvantages. The results from the first trimester test will not be interpreted or informed to the patients until the second trimester test is performed. This could deprive many pregnant women the chance of getting early diagnostic tests and increases the anxiety due to the long wait for the test results until the second trimester.

One possible compromise solution is cross-trimester contingent screening. In this screening policy all women receive an initial estimate of risk but only women with intermediate risks are offered a second trimester screening test. Women who are found to have a high risk based on the initial first trimester screening test and maternal age are not offered a second trimester screening test because these women will end up with a final risk ≥1:150 irrespective of the second trimester screening test results. Women with a low risk will also not be offered a second trimester screening test as their risk is unlikely to be modified sufficiently to reach the final cut-off. This study has shown that this screening policy with repeat measure of PAPP-A could achieve a detection rate of 92.2% at a false positive rate of 1.4% but with only 9.7% of women requiring a second trimester screening test (refer to section 4.2.2). In this study, an early detection rate of 75.9% at a false positive

rate of 0.7% was estimated using NT, fβhCG and PAPP-A and maternal age in the first trimester. This is similar to that estimated by Cuckle *et al* (2005) using statistical modelling of 70.0% early detection at a false positive rate of 0.7%, and by Cuckle *et al* (2008) of 60% detection with a false positive rate of 1.2% using FASTER trial data. Table 5.1 shows the performance of cross-trimester contingent screening from various studies for the full cross-trimester screen and also after the initial (first trimester) test.

Table 5.1: Performance of cross-trimester contingent screening from various studies.

Studies	Marker combination	Overall			1st trimester	
		DR (%)	FPR (%)	2nd trimester frequency (%)*	DR (%)	FPR (%)
Benn *et al* (2005)	NT, fβhCG, PAPP-A/ AFP, fβhCG, uE3, InhA	90.4	2.3	20.7	60.4	0.3
Cuckle *et al* (2005)	NT, fβhCG, PAPP-A/ AFP, fβhCG, uE3, InhA	92.0	3.0	15.0	70.0	0.7
Wald *et al* (2006)	NT, fβhCG, PAPP-A/ AFP, fβhCG, uE3, InhA	89.8	2.4	21.4	66.0	0.5
Cuckle *et al* (2008)	NT, fβhCG, PAPP-A/ AFP, hCG, uE3, InhA	91.0	4.5	23.0	60.0	1.2
This study	NT, fβhCG, PAPP-A/ AFP, fβhCG, uE3, InhA	90.6	1.8	9.7	75.9	0.7
This study	NT, fβhCG, PAPP-A/ AFP, fβhCG, uE3, InhA, PAPP-A	91.9	1.5	9.7	75.9	0.7

*Proportion of women requiring a second trimester test.

The cross-trimester contingent screening policy could not only achieve a screening performance similar to the full integrated test, but also permits clinicians to disclose the first trimester screening results to the patient. This screening policy also allows women with an extremely high risk of carrying Down's syndrome fetus to have an early diagnosis. Furthermore, more than two-third of women with unaffected pregnancies can avoid the second trimester screening test and thus, have early completion of screening. However, due to the complexity of this screening policy, it needs to be explained to women through counselling sessions why different risk cut-offs are used at each stage of screening. The acceptability or otherwise of this screening policy to pregnant women is unknown.

The potential value of using highly correlated repeated measures of serum markers taken in the first and second trimester of pregnancy was first demonstrated by Wright and Bradbury (2005). The statistical modelling in this study shows that, in the cross-trimester contingent screening, there is a substantial benefit of adding repeated measurement of PAPP-A in the second trimester. At 11 weeks, adding repeated measurement of PAPP-A to a base test comprising NT, PAPP-A and fβhCG in the first trimester and AFP, hCG, uE3 and InhA in the second increases the detection rate by 1.1% from 91.1% to 92.2% and decreases the false positive rate by 0.2% from 1.6% to 1.4%.

This study has also shown that the performance of this screening policy deteriorates when a larger SD is used for affected cases (Tables 4.11c and 4.11d). However, the differences in the overall screening performance when same SDs were used for unaffected and affected cases compared to using a larger SD for affected cases were small.

Most of the marker parameters used in this analysis are considered to be unbiased as these parameters were taken from a meta-analysis of several studies comprising several hundred Down's syndrome cases (Aitken *et al*, 2007, Spencer *et al*., 2002, Cuckle *et al*., 2005). Although statistical modelling is a useful tool to evaluate the efficacy of Down's syndrome screening policy, modelling is based on assumptions which may cause overestimation of the screening performance. For example, in this analysis, it is assumed that a Gaussian fit is reasonable in the tails of a multivariate distribution whereas in practice this will rarely be the case. Therefore, it is important to carry out prospective intervention studies in order to confirm the performance of testing and the practicality and acceptability of this cross-trimester contingent screening policy.

5.3 EFFECT OF SMOKING AND ETHNICITY ON BIOCHEMICAL MARKERS

This study (refer to section 3.3) confirmed previous findings that maternal smoking habits and ethnic origin affect the biochemical marker levels in Down's syndrome screening. A unique aspect of this study was the use of paired first and second trimester serum samples in 939 women allowing for an assessment of the trends in marker levels between gestations. This study has showed that the pattern of change caused by smoking and ethnicity on biochemical markers varies from marker to marker and trimester to trimester. The results of the effect of smoking and ethnicity are presented in Section 4.3.

In the data presented here markedly higher levels of AFP in the first trimester but only slightly higher in the second trimester were found in smokers with unaffected singleton pregnancies. A significant reduction in hCG and PAPP-A levels was also found in both the first and second trimesters in smokers. The fβhCG level was significantly decreased in

smokers in the second trimester but not in first trimester. Similar patterns of change have been reported in previous studies. Table 5.2 shows the summary of first and second trimester marker levels in non-smokers and smokers in this and previous studies.

Overall, in this study, there was a 14.0% reduction in PAPP-A, a 1.6% reduction in fβhCG, a 16.3% elevation in AFP and a 27.6% reduction in hCG in the first trimester. In the second trimester, there was a 6.1% elevation in AFP, a 30.5% reduction in hCG, a 17.1% reduction in fβhCG and a 22.8% reduction in PAPP-A. Comparing with other studies which show reduced fβhCG levels ranging from 3.0% to 13.0%, this study showed a smaller reduction in fβhCG level in the first trimester in smokers but confirms the trend to larger reduction (20% to 30%) in the second trimester. This study has also shown that in smokers, with the exception of AFP, marker levels tend to show larger changes in the second trimester than in the first trimester. PAPP-A, hCG and fβhCG are produced by the placenta whereas AFP is of fetal origin and is transported across the placenta to the maternal circulation.

Table 5.2: Summary of first and second trimester marker levels in non-smokers and smokers in this and previous studies

Studies	First trimester				Second trimester			
	PAPP-A	**FβhCG**	**AFP**	**hCG**	**PAPP-A**	**FβhCG**	**AFP**	**hCG**
Bartels *et al* (1993)							↑ 4.5%	↓ 20.1
Spencer (1998)						↓ 13.9%	↑ 3.0%	
de Graaf *et al* (2000)	↓ 24.3%	↓ 11%	↑ 3.1%					
Crossley *et al* (2002b)							↑ 5.1%	↓ 29.2
Rudnicka *et al* (2002)						↓ 20.0%	↑ 5.0%	
Spencer *et al* (2004)	↓ 17.6%	↓ 3.0%						
Kagan *et al* (2007)	↓ 19.6%	↓ 3.1%						
Miron *et al* (2008)	↓ 16.5%	↓ 13.0%						
Kagan et al (2009)	↓ 17.0%	↓ 4.0%						
Present study	↓ 14.0%	↓ 1.6%	↑ 16.3%	↓ 27.6%	↓ 22.8%	↓ 17.1%	↑ 6.1%	↓ 30.5%
Weighted average	↓ 18.3%	↓ 5.6%	↑ 6.4%	↓ 27.6%	↓ 22.8%	↓ 15.3%	↑ 3.7%	↓ 23.3%
Correction factor	0.82	0.94	1.06	0.72	0.77	0.85	1.04	0.77

The reason behind these changes in the marker concentration is not clearly understood. Jauniaux and Burton (1992) demonstrated that smoking causes morphological changes in the trophoblast which might explain the disturbance in hCG production or increased permeability of the placenta promoting increased transfer of AFP across the placental barrier. PAPP-A is another placental protein produced by syncytiotrophoblast and studies have shown that there is an increase in syncytiotrophoblast necrosis in smokers (Jauniaux and Burton, 1992; Zdravkovic *et al*., 2005). The further decrease in hCG, PAPP-A and fβhCG levels evident in the second trimester suggest that there is an increased effect on the placenta in women who continued smoking during pregnancy which causes further reduction in production of these markers. Therefore, it is important to derive appropriate correction factors for smoking for each trimester for individual biochemical markers.

Those who are smokers among the pregnant population tend to be younger than those who are non-smokers. Due to the marked difference in the age distribution of those pregnant women who smoke compared with those who do not, the expected rate of Down's syndrome in pregnant women who smoke will be lower (Spencer *et al.*, 1998, Crossley *et al.*, 2002b, Spencer *et al.*, 2004). Therefore, it is important to take into account the maternal age effect when studying the incidence of Down's syndrome in women who smoke.

Ethnic origin has an impact on the biochemical marker levels, which cannot be explained by differences in maternal weight. Results from this study based on 939 pairs of first and second trimester samples along with the results from other studies are summarised in Table 5.3. In this study (see section 3.3), after maternal weight adjustment, South Asian women had a significantly higher level of first trimester hCG compared to Caucasians. In the first

trimester, although the fβhCG and PAPP-A levels were slightly decreased compared to Caucasian, the differences were not significant. This finding is in contrast to Spencer *et al* (2005b) who reported that South Asian women had higher PAPP-A and lower fβhCG levels compared to Caucasians. This difference might be due to a larger South Asian data in Spencer et al (2005b) compared to this study.

In this study the Oriental women had higher levels of first trimester hCG, fβhCG and PAPP-A compared to Caucasian. This finding is similar to that of Spencer *et al* (2005b) that Oriental women had higher levels of PAPP-A and fβhCG compared to Caucasians. In the data presented in this study, after weight correction, Black women had higher levels of first trimester hCG and PAPP-A but slightly lower fβhCG levels compared to Caucasian. These findings are in contrast to Krantz *et al* (2005), Spencer *et al* (2005b) and Spencer *et al* (2000e) who found that fβhCG level was higher in Black women compared to Caucasian. In this study a cohort of Middle East women were identified who had lower levels of AFP in the first trimester after weight adjustment compared to Caucasian.

South Asian women had significantly lower levels of second trimester fβhCG and PAPPA but similar AFP and hCG levels compared to Caucasian. These findings were in contrast to Watt *et al* (1996) who found higher hCG and lower AFP levels in South Asian women compared to Caucasian. However, Watt *et al* (1996) also found similar decrease in fβhCG levels in South Asian women compared to Caucasian.

Oriental women had higher levels of second trimester hCG compared to Caucasians. The AFP levels were slightly lower and fβhCG were higher in Oriental women compared to Caucasian but the difference was not significant. Hseih *et al* (1995) reported 2.9% higher fβhCG and 10% lower AFP in the Taiwan population compared to Caucasians. Black

women had significantly higher PAPP-A levels in the second trimester but similar levels of AFP, hCG and fβhCG compared to Caucasian women. These finding are in contrast to previous studies which reported higher AFP, hCG and fβhCG levels in Black women compared to Caucasian (Benn *et al*., 1997, Watt *et al*., 1996, Kulch *et al*., 1993).

It is possible that some of the findings from this study which are at odds with those reported in other studies may be due to the small number of women in each ethnic group (especially the Black population) and the difficulty of ensuring that ethnic categories are the same between studies. However, the use of paired first and second trimester serum samples allows an assessment of the relative change in marker levels between trimesters and the results suggest that the changes are more marked for PAPP-A and fβhCG in the second trimester, but greater for hCG in the first trimester. Table 5.3 shows the summary of first and second trimester marker levels in different ethnic origins in this and previous studies.

Table 5.3: Summary of first and second trimester marker levels in different ethnic groups in this and previous studies.

Studies	Ethnic origin	First trimester				Second trimester			
		PAPP-A	FβhCG	AFP	hCG	PAPP-A	FβhCG	AFP	hCG
Kulch *et al* (1993)	Black (n = 134)								↑ 16.0%
Watt *et al* (1996)	Black (n = 4215)						↑ 12.0%	↑ 22.0%	↑ 19.0%
	South Asian (n = 4392)						↓ 9.0%	↓ 6.0%	↑ 6.0%
Spencer *et al* (2000e)	Black (n = 752)	↑ 57.0%	↑ 21.0%						
	Asian (n = 170)	↑ 17.0%	↑ 4.0%						
Spencer *et al* (2005b)	Black (n = 2943)	↑ 55.0%	↑ 11.0%						
	South Asian (n = 4835)	↑ 8.0%	↓ 7.5%						
	Oriental (n = 3925)	↑ 9.0%	↑ 6.0%						
Krantz *et al* (2005)	African Americans (n = 2682)	↑ 35.0%	↑ 16.0%						
	Asians (n = 2228)		↑ 6.0%						
	Hispanic (n = 2795)		↓ 9.0%						
Kagan et al (2009)	Black (n = 2144)	↑ 57.0%	↑ 12.0%						
Present study	South Asian (n=268)	↓ 3.0%	↓ 10.7	↓ 0.7%	↑ 6.7%	↓ 8.1%	↓ 13.6%	↓ 3.5%	↓ 2.2%
	Oriental (n=66)	↑ 20.9%	↑ 5.9%	↓ 1.6%	↑ 37.8%	↑ 12.4%	↑ 6.9%	↓ 3.2%	↑ 18.4%
	Middle Easterner (n=42)	↓ 7.2%	↓ 0.1%	↓ 11.6%	↑ 10.0%	↓ 3.6%	↓ 0.6%	↓ 5.4%	↑ 8.2%
	Black (n=35)	↑ 43.2%	↓ 4.0%	↑ 7.3%	↑ 23.1%	↑ 60.1%	↓ 10.0%	↓ 0.2%	↓ 9.2%
Weighted average	Black	↑ 49.4%	↑ 13.6%	↑ 7.3%	↑ 23.1%	↑ 60.1%	↑11.8%	↑21.8%	↑18.7%
	South Asian	↑ 7.4%	↓ 7.7%	↓ 0.7%	↑ 6.7%	↓ 8.1%	↓ 9.3%	↓ 5.9%	↑ 5.5%
	Oriental	↑ 9.2%	↑ 6.0%	↓ 1.6%	↑ 37.8%	↑ 12.4%	↑ 6.9%	↓ 3.2%	↑ 18.4%
Correction factors	Black	1.49	1.14	1.07	1.23	1.60	1.12	1.22	1.19
	South Asian	1.07	0.92	0.99	1.07	0.92	0.91	0.94	1.06
	Oriental	1.09	1.06	0.98	1.38	1.12	1.07	0.97	1.18

Watt *et al* (1996) proposed a method to derive the median MoM of second trimester serum markers in a multiethnic population. In this approach, the ratios of median MoM in an ethnic group (e.g Black population) to that in the main ethnic group (e.g. Caucasian) were used to correct the marker concentration in different ethnic groups. Such an approach is useful if there is insufficient data for a particular ethnic group. If there is an adequate number of women in each ethnic group, separate MoM equations can be derived for each ethnic group.

In terms of screening for Down's syndrome by first and second trimester markers, it is important to take into account maternal smoking habit and ethnic origin when a risk is calculated. Correcting for smoking and ethnicity can be performed by dividing the appropriate MoM by the correction factors given in Tables 5.2 and 5.3. Although correcting for smoking and ethnicity has little impact on the overall Down's syndrome screening performance, it will provide individual women with more accurate risks and contribute to reduction in the screen positive rate. Reducing the number of women requiring diagnostic testing is generally desirable and particularly so in ART pregnancies where there is increased reluctance to expose the pregnancy to the risk of procedure-related miscarriage. More data are required to explore the combined effect of smoking and ethnicity on marker levels.

5.4 EFFECT OF ASSISTED REPRODUCTIVE TECHNOLOGY ON FIRST AND SECOND TRIMESTER SERUM MARKERS

In this screened population, approximately 50% of women conceived by ART are aged 35 years or more. Due to their age-related risk, these women are often classified as 'at risk' following a Down's syndrome screening. The effect of ART on Down's syndrome markers still remains to be clarified. In this study, in the overall ART group, PAPP-A and fβhCG levels were not significantly different from the levels found in naturally conceived pregnancies. The first trimester screening marker PAPP-A was significantly decreased in pregnancies conceived through IVF or ICSI with fresh eggs compared with pregnancies which were conceived spontaneously. This is in line with previous findings (Gjerris *et al*., 2009, Anckaert *et al*., 2008, Tul and Novak-Antolic, 2006, Hui *et al*., 2005, Liao *et al*., 2001). In pregnancies conceived after IVF or ICSI with frozen embryo, there were no significant differences in the PAPP-A level. Gjerris *et al* (2009) and Anckaert *et al* (2008) also reported similar findings that the median PAPP-A MoM was not significantly different in the pregnancies conceived after frozen embryo transfer from that in naturally conceived pregnancies. The results are presented in section 4.4 and summarised along with the results of other studies in tables 5.4 and 5.5.

In this study no significant difference in the concentration of fβhCG between ART and normally conceived pregnancies was found. This is in agreement with most of previous studies (Gjerris *et al*., 2009, Anckaert *et al*., 2008, Tul and Novak-Antolic, 2006, Lambert-Messerlian *et al*., 2006) although a few papers have reported an increase in the fβhCG concentration (Ghisoni *et al*., 2003, Wojdemann *et al*., 2001).

In this study in the overall ART group, NT measurements were significantly increased over the measurements found in naturally conceived pregnancies. NT measurement was significantly increased in the pregnancies conceived after IVF with fresh eggs. However, the majority of previous studies found no significant difference in the NT measurement in ART pregnancies compared with spontaneously conceived pregnancies (Liao *et al*., 2001, Orlandi *et al*., 2002, Maymon and Shulman, 2002 and Tul and Novak-Antolic, 2006). But Maymon and Shulman (2004) and Hui *et al* (2005) reported that NT measurement was significantly increased in pregnancies with fresh embryos from IVF, frozen-thawed embryos from IVF and fresh embryos from ICSI.

In 2009, Amor and co-workers conducted one of the largest and comprehensive studies on the effect of ART on first trimester Down's syndrome markers. This study, which comprised more than 1,700 ART pregnancies, showed that PAPP-A levels were significantly lower in ART pregnancies compared with non-ART pregnancies. There were no significant differences in NT measurement and fβhCG levels between ART and non-ART pregnancies. Another prospective study of 1000 ART pregnancies by Gjerris et al (2009) showed that PAPP-A levels were significantly decreased in IVF and ICSI pregnancies compared to naturally conceived pregnancies.

In this study, the concentration of second trimester AFP was significantly increased but not the concentration of hCG in pregnancies conceived after IVF with donor's egg compared with naturally conceived pregnancies. There was no significance differences in the AFP levels in pregnancies conceived after IVF with fresh eggs or frozen embryos. This is in agreement with previous studies (Lambert-Messerlian *et al*., 2006, Shulman and Maymon, 2003, Perheentupa *et al*., 2002; Maymon and Shulman, 2001). In this study the

concentration of total hCG was significantly increased in pregnancies treated with IVF with fresh and frozen eggs compared with naturally conceived pregnancies, in line with most previous studies (Lambert-Messerlian *et al*., 2006, Maymon and Shulman, 2001). In contrast to these findings, Muller *et al* (2003) and Rice *et al* (2005) reported that there were no significant differences in the second trimester markers in ART pregnancies compared with naturally conceived pregnancies. Tables 5.4 and 5.5 show the overview of studies on the first and second trimester markers levels in ART pregnancies.

The changes in the marker level in ART pregnancies might have an effect on the false positive rate. Numerous studies have confirmed that ART increases the second trimester serum marker false positive rate (Barkai *et al*., 1996b, Ribbert *et al*., 1996, Heinonen *et al*., 1996, Frishman *et al*., 1997, Maymon *et al*., 1999, Raty *et al*., 2002). Lambert-Messerlian *et al* (2006) reported that the decrease in uE3 levels and increase in hCG and InhA levels in IVF pregnancies causes significant increase in the second trimester screen positive rate. But in the first trimester, Lambert-Messerlian *et al* (2006) reported that the differences in the first trimester serum marker levels were not sufficient to affect the screen positive rate. This is in agreement with previous other studies (Tul and Novak-Antolic, 2006; Bellver *et al*., 2005). However two other studies (Gjerris et al., 2009; Orlandi et al., 2002) have reported higher false positive rate in ART pregnancies when compared with naturally conceived pregnancies in the first trimester.

Table 5.4: Comparison of studies on the first trimester Down's syndrome markers in pregnancies achieved naturally and by assisted reproduction

Studies	Natural conception	ART pregnancies	PAPP-A	FβhCG	NT measurement
Liao et al (2001)	1233	220 (IVF)	↓	↑	=
		30 (ICSI)	↓	=	=
Orlandi *et al* (2002)	370	32 (IVF)	↓	=	=
		42 (ICSI)	=	=	=
Ghisoni *et al* (2003)	426	50 (IVF)	=	↑	=
		92 (ICSI)	=	↑	=
Maymon and Shulman (2004)	1781	99 (IVF)	↓	N/A	↑
Tul and Novak-Antolic (2006)	914	130 IVF	↓	=	=
		54 ICSI	↓	=	=
Lambert-Messerlian *et al*. (2006)	37,070	277 IVF	=	=	=
		56 (IVF with egg donation)	=	=	
Anckaert *et al*. (2008)	4088	59 IVF	↓	=	N/A
		163 ICSI	↓	=	N/A
Gjerris *et al* (2009)	2532	512 (IVF)	↓	=	=
		396 (ICSI)	↓	=	=
Kagan *et al*., (2009)	18829	784 (IVF)	↓	↑	N/A
This study	10891	91 (IVF or ICSI with fresh eggs)	↓	=	↑
		29 (IVF or ICSI with frozen embryos)	=	=	=
		7 (IVF with donor's eggs)	=	=	=

Table 5.5: Comparison of studies on the second trimester Down's syndrome markers in pregnancies achieved naturally and by assisted reproduction

Studies	Natural conception	ART pregnancies	AFP	hCG
Maymon and Shulman (2002)	285	71 (IVF)	↑	=
Muller *et al* (2003)	21014	970 (IVF)	=	=
		545 (ICSI + IVF)	=	=
Maymon and Shulman (2004)	1781	99 (IVF)	=	=
Rice *et al* (2005)	596	88 (IVF)	=	=
Lambert-Messerlian *et al* (2006)	37,070	277 IVF	=	↑
		56 (IVF with egg donation)	↑	=
This study	61448	105 (IVF or ICSI with fresh eggs)	=	↑
		15 (IVF or ICSI with frozen embryos)	=	↑
		9 (IVF with donor's eggs)	↑	=

The contradictory results from previous studies are possibly due to different underlying causes of infertility and different treatment methods. Tul and Novak-Antolic (2006) reported that with an increasing number of retrieved oocytes, the concentration of PAPP-A was significantly decreasing and InhA was increasing but not statistically significantly. Based on their finding that InhA, which is secreted by the corpus luteum, was increased with decreasing PAPP-A, the authors hypothesized that the number of oocytes retrieved reflected the number of corpora lutea in pregnancy. The authors proposed that the secretion of PAPP-A is hampered by InhA. Hui *et al* (2005) suggested that a delay in placental maturation causes decreased PAPP-A level. The author also suggested that ICSI itself as well as the freezing and thawing procedure produce different effects on placental development, supported by their finding that additional ICSI procedures cause the largest reduction in PAPP-A levels especially after freezing and thawing of embryos. In this study, PAPP-A were close to normal levels in pregnancies after IVF with frozen eggs but was decreased in pregnancies after IVF with fresh eggs. Hui *et al* (2005) also reported that there was a negative correlation between the number of transferred embryos and PAPP-A. With the increasing number of embryo transferred, the concentration of PAPP-A was decreasing.

Several theories have been proposed to explain the elevated hCG levels in ART pregnancies. An earlier study by Wald *et al* (1999b) suggested that increased hCG in ART pregnancies is not due to the administration of hCG as part of the IVF protocol but due to the continuing high progesterone concentration in IVF pregnancies. In IVF pregnancies, multiple follicle development causes the formation of multiple corpora lutea. This would lead to further production of progesterone and thus increase the production of hCG from the developing placenta (Wald *et al*., 1999b). This theory seemed unlikely when Raty *et al*.

(2002) reported increased hCG levels in frozen embryo transfer (FET) pregnancies. In FET and oocyte donation pregnancies, there is no excessive follicles or corpora lutea. However, this study showed that the levels of hCG were not elevated in IVF pregnancies with donor egg. Perheentupa *et al*. (2002) showed that the second trimester hCG levels were similar in pregnancies following stimulated and un-stimulated cycles and therefore, super-ovulation therapy is unlikely to be the cause of the elevated hCG levels in ART pregnancies.

Therefore, although many theories have been proposed, the biological basis of altered screening markers levels in pregnancies conceived after ART remains unknown. The treatments or drugs used in ART protocols or infertility conditions might be the cause of the altered marker concentrations (Maymon and Jauniaux, 2002; Raty *et al*., 2002; Hui *et al*., 2003). Whatever the biologic basis, the effect of ART on Down's syndrome screening markers must not be overlooked. Correcting for ART would provide women with more accurate individual risks and reduces the increased screen positive rate.

Some of the findings from this study were not consistent with previous studies. This might be due to the small number of cases in each ART group especially in the IVF with donor's egg group. Further research need to be conducted using a larger database to investigate the effect of current IVF procedure on Down's syndrome screening markers.

5.5 RETROSPECTIVE STUDY ON BIRTH WEIGHT, DURATION OF PREGNANCY AND SECOND TRIMESTER MATERNAL SERUM SCREENING MARKERS IN NON-SMOKERS AND SMOKERS

5.5.1 ACCURACY OF SELF-REPORTED SMOKING STATUS AT BOOKING AND SCREENING APPOINTMENT

At most prenatal screening centres, self-reported smoking information is usually used to correct the biochemical marker levels for maternal smoking habit. Using cotinine-validation, this study has estimated the prevalence of smoking among pregnant women as 30.1%. This figure is 24.9% and 40.6% higher than figures based on self-report at booking and screening appointment respectively. In 2008, Usmani *et al* reported that at least 10% of pregnant women in Glasgow population likely not telling the truth about their smoking habits which causing under estimation of smoking prevalence in the Scottish population. In this study, approximately one-quarter of validated smoking pregnant women were undetected through self-report at booking and screening. This finding is similar to that is seen in previous studies (Lindqvist *et al*., 2002, Klebanoff *et al*., 2001, Ford *et al*., 1997). Webb *et al* (2003) reported over 50% of cotinine-validated smokers were undetected by self report in the US.

This study has also found that there is no change in the sensitivity and specificity of the self-reported smoking information when the 'former smokers' are classified as 'non-smokers'. However, allowing pregnant women to correct their smoking status once they receive their screening report improves the accuracy of self-reported smoking information.

Previous studies have shown that the concentration of cotinine, whether measured in serum, plasma, saliva or urine, is the best biomarker for measuring smoking status due to its long half life and optimised sensitivity and specificity (Russell *et al*., 2004, Dempsey *et al*., 2002, Jarvis *et al*., 1987). The cotinine cut-off used between current smokers and non-smokers is arbitrary. This is because there is an over-lap between non-smokers who are highly exposed to ETC with occasional smokers or those inhale very little. There is little variation in the cotinine cut-off used in different previous studies. Some studies used 10ng/ml (Klebanoff *et al*., 2001, McDonald *et al*., 2005) as cotinine cut-off in pregnant women where as some other studies used 24ng/ml (Lindqvist *et al*., 2002, Boyd *et al*., 1998). The cotinine cut-off of 13.7ng/ml used in this study was based on a previous study by Jarvis *et al* (1987) who used gas chromatography to measure cotinine concentration. The data from this study showed that there is very little variation in the findings when any cut-off between 10 and 30ng/ml is used (Table 4.21).

Both nicotinine replacement therapy (NRT) and exposure to environmental tobacco smoke (ETS) is known to increase the cotinine levels. However, the median cotinine level measuring the impact for ETS exposure was reported as 4ng/ml and 8ng/ml for office staff and bar staff respectively (Hammond *et al*., 1995, Jarvis *et al*., 1992).Therefore, the chosen cut-off of 13.7ng/ml in this study would unlikely misclassify women exposed to ETS as smokers. Furthermore, in the dataset used in this study, 69.0% of pregnant women had cotinine levels below 10ng/ml and 29.0% of women had cotinine level 30ng/ml and above. Therefore, any cut-off between 10 and 30ng/ml would not make much difference to the findings in this study as 98% of pregnant women had cotinine level either below 10ng/ml or above 30ng/ml. The pregnant women in this study were not routinely recommended NRT. Community Action on Tobacco for Children's Health (CATCH) (Bryce *et al*., 2008)

was the only service offering NRT during the time the study women were pregnant which was in 2003/4. However, NRT was offered to only 65 women. Therefore, it is unlikely to bias the finding in this study due to the small number of women involved in NRT.

The findings in this study are based on assumptions that the screened population represents the West of Scotland population and the differences between the West of Scotland and the Scottish population are accounted for in generating the projected figures. The random selection of the sample from the screened population is successful as all characteristics tested in the study sample are similar to that of the screened samples. The high screening rate (70% of all women are screened) in this population reduces the possibilities for differences between the screened population and target population.

As anticipated, there were some errors in the recording or transcribing of the self-reported smoking information at booking and screening appointment. For an example, when the duration between the booking date and screening date was calculated, for 182 pregnant women the booking date was after the screening date (some of them were more than 3 months after the screening date) and for 9 pregnant women the screening date was more than 84 days after the booking date. In order to check if there was an error in the booking date or screening date, the days between gestation at screening and gestation at birth were compared with the days between date of screening and baby's date of birth. For 93.7% of these women, their gestation at screening and gestation at birth matched with date of screening and baby's date of birth. Therefore, the date of booking was not accurate in these cases. In the remaining cases, one of the other dates (DOB or screening date) was not correct. However, such errors are unlikely to bias the findings in this study as the recording error would not be systematic (e.g. by smoking status).

The self-report smoking information collected at the maternity booking and screening visit is usually used to refer smoking pregnant women to specialist smoking cessation services and for refining the estimation of women's individual risks of Down's syndrome by prenatal screening, since maternal smoking causes changes in the levels of the biochemical markers used in the screening test. Therefore, accurate self-report smoking information is important. However, this study and other previous studies (O'Gorman, 2008) have demonstrated poor quality of the routinely collected self-report smoking data. Better methods of routinely identifying smokers during pregnancy are required to improve the quality of smoking information. Currently in Glasgow, all women attending antenatal clinic have to provide both self-report smoking status and undergo carbon monoxide breath test. Usmani *et al* (2008) reported that the use of both self-report smoking information and carbon monoxide validated measurement would be able to identify 95.8% of pregnant smokers.

In summary, the use of self-report to collect smoking information among pregnant women significantly underestimates the number of pregnant smokers in Scotland. Therefore, a more reliable method is required to accurately identify pregnant smokers in Scotland.

5.5.2 BIRTHWEIGHT, DURATION OF PREGNANCY AND SECOND TRIMESTER MARKERS

Although the association between birth weight, early delivery and AFP level has been previously reported, this study shows the impact of smoking on these variables. In this study, women who smoke and have AFP levels greater than 2.0 MoM have a 39.8% chance of delivering a low birth weight infant and a 55.6% chance of delivery prior to 39 weeks. This compares to a 4.4% chance of delivering a low birth weight infant and a

23.8% chance of delivery prior to 39 weeks in non-smokers with AFP levels less than 2.0 MoM. This study shows that women who smoke and have an elevated AFP level (≥2.0 MoM) give birth to babies which are on average around 900g lighter than those born to non-smoking women with AFP values <1.00 MoM.

Although smoking has a significantly greater effect on maternal serum hCG levels than on AFP levels, in this study, there was little association with high or low hCG levels and either birth weight or early delivery. The association between birth weight, delivery prior to 39 weeks and second trimester markers is presented in section 4.5. The findings from this study are consistent with previous studies showing that pregnant women who smoke tend to deliver low birth weight infants (Brooke *et al*., 1989, May, 2007, Schell and Hodges, 1985). In this study, the birth weights of infants born to women who smoke and had deliveries at 39 to 41 weeks were, on average 270g less than infants born to non-smoking women.

The reasons of decreased birth weight in smoking mothers are still debatable. Some studies have suggested that carbon monoxide from smoking cause placental hypoxia and limits oxygen-carrying capacity of haemoglobin (Longo, 1970, Cole *et al*., 1972 and Astrup, 1972). Pathological placental hypoxia leads to decrease in cytotrophoblast proliferation and abnormal differentiation during the cell cycle in the placenta which causes restricted fetal growth (Zdravkovic *et al*., 2005, Albuquerque *et al.,* 2004). One study proposed that nicotine causes vasoconstriction of uterine arteries and uteroplacental arteries which subsequently leads to restricted fetal growth (Andrews and McGarry, 1972). van der Velde *et al* (1983) suggested that the structural changes in the placenta of smoking pregnant women which causes restricted fetal growth is due to cadmium from tobacco smoke.

Cadmium content has been shown to be higher in smokers' blood circulation compare to non-smokers (De Voogt *et al*., 1980) and causes reduction in birth weight (Sutou *et al.*, 1980).

While this and other studies show that there is a clear association between elevated AFP level and low birth weight, AFP is a poor screening test for low birth weight in the whole pregnant population due to its low sensitivity and specificity (Smith, 1980). Chard *et al.* (1986), in a prospective study on 887 randomly selected pregnant women, found that if elevated AFP is used as a predictor of low birth weight, five out of every six cases will be missed and for every case correctly identified there would be nine false-positives. This study shows that if AFP is used as a screening test in smokers its predictive value is doubled over that in non-smokers but remains poor: maternal serum AFP levels ≥2.0 MoM can predict only around 7.5% of low birth weight (<2500g) pregnancies at a false positive rate of 2.4%.

Part of the association between AFP level and birth weight can be due to preterm delivery. The association between delivery prior to 39 weeks and AFP level has also been shown in this study. In this study, women who smoke tend to have deliveries prior to 39 weeks with a median gestation at delivery of 40 weeks in non smokers and 39 weeks in smokers. Although there is a clear association between early delivery and AFP level, part of this association might be due to bias. Abnormally high AFP level and early delivery can be also due to under-estimation of gestational week at the time of screening (Brock *et al.*, 1980). A study by Wald *et al* (1977) showed that by using gestational age based on ultrasound, some of the association between high AFP level and early delivery was eliminated.

Gitlin (1975) reported that AFP, a fetal protein produced by the yolk sac and fetal liver, is transported from the fetus to mother mainly across the placenta. The amount of AFP transported from the fetus to the mother via the transplacental route depends on the permeability of the placenta, the villous surface area and the fetal AFP concentration (Gitlin, 1975, Boyd and Keeling, 1986, Boyd, 1992). Boyd and Keeling (1986) also reported that increase in the amount of AFP transported from the fetus to the mother causing elevated maternal serum AFP level can be associated with infarcted placental tissue and feto-maternal haemorrhage.

The birth weight of a fetus depends on the functionality of different mechanism in the placenta. Any biological relevant stress on the fetoplacenta can cause changes in the birth weight (Salafia *et al*., 2008). Ferguson-Smith *et al* (1979) suggested that the association of elevated AFP level and low birth weight can be explained by fetal haemorrhage due to placental lesion causing increase transport of AFP from fetus to mother.

Although hCG level was thought to reflect the early placental pathology, in this study there was no any association between low birth weight, delivery prior to 39 weeks and hCG level. HCG, a placental protein produced by cytotrophoblast and excreted directly into maternal circulation, reflects placental function. Elevated second trimester hCG level is normally associated with preeclampsia, Down's syndrome, still birth and spontaneous abortion (Onderoglu and Kabukcu, 1997, Duric *et al*., 2003).

In summary, although AFP is a poor screening test for low birth weight, pregnant women who have high AFP levels and who smoke should be monitored more carefully than non-smoking pregnant women with normal AFP levels.

5.6 GENERAL DISCUSSION

The UK NSC advises that first trimester CUB screening should be the preferred screening policy for Down's syndrome. This is because in this screening policy, pregnant women only need to visit the antenatal clinic once and a risk for Down's syndrome will be provided before 14 weeks of gestation allowing earlier decision making for the parents. Although CUB screening is proven to be an effective method of detecting Down syndrome pregnancies in the first trimester, with a detection rates of 85-91% at a 4-5% screen positive rate (Spencer *et al*., 2000; Stenhouse *et al*., 2004, Perni *et al*., 2006), one of the critical factors in maintaining the performance of CUB screening is consistent and accurate NT measurement. This requires ultrasonographers with specific training and a system of on-going monitoring within a quality assured programme. This has hampered the adoption of CUB screening in some screening centres which lack the ultrasound resources to provide high quality NT measurements to the entire booking population. Therefore, an alternative screening policy was proposed by Christiansen and Larsen (2002) where women were selected for NT measurement based on PAPP-A and FβhCG measurements. This study showed that within-trimester contingent screening policy offers the prospect of reducing the NT measurement workload to around 25–30% whilst maintaining high sensitivity and specificity. Therefore, this screening policy allows those centres with limited resources to target the group of pregnant women whose screening results can be most usefully modified by information from an NT measurement.

Although CUB screening is an efficient screening method to detect Down's syndrome pregnancy, this screening policy would not be able to achieve the mission of the UK NSC; detection rate of greater than 90% with a screen positive rate of less than 2%. Repeat

measure testing, one of the strategies currently being reviewed by the Health Technology Assessment programme (Wright *et al*., 2010), is expected to further improve the performance of Down's syndrome screening programmes if implemented in the period after 2010. In this study, the performance of cross-trimester contingent screening with repeat measure was assessed. Contingent screening policy with repeat measure of PAPP-A in the second trimester (NT, PAPP-A, fβhCG / AFP, hCG, InhA, uE3, PAPP-A) could potentially meet the 2010 recommended outcome with a detection rate of 92.2% at a false positive rate of 1.4% but with only 9.7% of women requiring a second trimester screening test. In screening centres where there is lack of ultrasound resources to provide NT measurements, this screening policy without NT measurement could achieve a detection rate of 86.2% at a false positive rate of 3.0% but with only 22.3% of women requiring a second trimester screening test. The cost of the additional marker (PAPP-A) to be added to the second trimester quadruple test has to be evaluated. However, only a slight increase in the screening cost would be expected as 90.0% (without NT measurement - 78.0%) of women would complete their screening in the first trimester without the need for a second trimester screening test.

These findings using statistical modelling are based on assumption that a Gaussian fit is reasonable in the tails of a multivariate distribution which might cause overestimation of the screening performance. Further prospective intervention studies need to be carried out in order to confirm these findings and the practicality of cross-trimester contingent screening policy. The evaluation of this screening policy would require large number of blood samples both from affected and unaffected pregnancies collected at two different stages of pregnancy. Therefore, a multi-centre prospective study would be recommended to confirm the results from this study.

Although previous studies have reported that correcting for factors such as maternal smoking habits, ethnic origin and ART has a little impact on the overall Down's syndrome screening performance, the effect of these factors on the biochemical markers used in Down's syndrome screening should not be overlooked. This study on the effect of ethnicity and smoking on Down's syndrome biochemical markers is unique as paired first and second trimester serum samples were used to assess the trends in marker levels between gestations. The findings from this study have shown that the pattern of change caused by smoking and ethnicity on biochemical markers vary depending on the trimester of screening and marker used. Therefore, the correction factors also vary between trimesters for certain biochemical markers. Further studies on larger numbers of women in each ethnic group are indicated to refine the correction factor found in this study and these may need to be specific for individual weeks of gestation. Correcting for these factors would provide women with more accurate individual risks and reduces the increased screen positive rate. This would certainly reduce the number of women requiring diagnostic testing.

In Glasgow, PAPP-A levels are corrected for smoking before the risk of Down's syndrome is calculated. The self-reported smoking information collected during the screening appointment is usually used to determine maternal smoking habit. Using cotinine-validation, this study has estimated the prevalence of smoking among pregnant women as 30.1% which is 40.6% higher than figures based on self-report at screening appointment. Therefore, approximately 30.0% cotinine-validated smokers were not detected by self-report at screening appointment. The individual risk for Down's syndrome calculated for these women would not be accurate. This calls for a better method of collecting smoking information at antenatal clinics. Therefore, it is important that detailed and accurate

information on maternal smoking status, ethnic origin and the type of ART used are recorded at the antenatal clinic. Appropriate MoM adjustment for these factors should be included in the screening software.

In summary, this study has shown that it is possible to meet the UK NSC mission to achieve a detection rate of 90% with a screen positive rate of less than 2% by April 2010. The contingent screening policy with repeat measure appears to hold much promise to meet the 2010 recommended Down's syndrome screening outcome. Correcting for factors such as maternal smoking habits, ethnicity and ART would further improve the Down's syndrome screening programme in the UK.

CHAPTER 6: CONCLUSION

In the past decade, there have been great developments in Down's syndrome screening. Much research is still being carried out to further improve detection at lower false positive rates and meet the UK NSC goal to achieve a detection rate of 90% with a screen positive rate of less than 2% by April 2010. Considerable emphasis has been placed on screening in the first trimester, driven in part by women expressing a preference for early testing. Early screening for Down's syndrome allows early reassurance or diagnosis and elective termination of affected pregnancies, which is simpler, safer and less traumatic than at a later stage.

However, although this study and others have demonstrated that within-trimester contingent screening can deliver useful benefits through minimising the proportion of women requiring an NT scan, the scope to increase detection rates and reduce false positive rates in the first trimester is limited.

Great potential for better screening performance seems possible through the use of samples collected at two different stages of pregnancy – cross trimester testing. As shown in this study, these policies can be designed to allow a proportion of women a proportion of women to complete screening early, in the first trimester, but give overall higher detection rates and lower false positive rates when repeat measures are incorporated into the model.

The studies in this thesis have shown that a contingent screening policy with repeat measures appear to meet the UK NSC performance goal. Correcting for factors such as maternal smoking habits, ethnicity and ART would further improve the Down's syndrome screening programme in the UK.

The results on cross-trimester contingent screening presented in this thesis are based on statistical modelling. Therefore, prospective intervention studies need to be carried out in order to confirm these findings and the practicality of a cross-trimester contingent screening policy. The evaluation of this screening policy would require a large series of blood samples both from affected and unaffected pregnancies collected at two different stages of pregnancy, first and second trimester. Therefore, a multi-centre prospective study would be recommended for further research on cross-trimester contingent screening.

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APPENDICES

APPENDIX A

PRENATAL DIAGNOSIS
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First-trimester contingent screening for Down syndrome can reduce the number of nuchal translucency measurements required†

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Background To assess the performance of a two-stage screening protocol for Down syndrome based on initial serum marker analysis for all women and nuchal translucency (NT) measurement only in women with intermediate risks.

Methods Biochemical marker and NT data in 10 189 women who had had combined ultrasound and biochemical (CUB) screening, were re-analysed using the contingent model. A risk was calculated from the results of the pregnancy-associated plasma protein A (PAPP-A) and free β human chorionic gonadotrophin (FβhCG) measurements and maternal age. For risks between 1 in 42 and 1 in 1000, the likelihood ratio from the NT measurement was incorporated and assessed against a final cut-off risk of 1 in 250.

Results A total of 3.1% unaffected and 61.4% Down syndrome pregnancies had risks $\geq$1: 42. In women with risks $<$1 in 42 and $>$1 in 1000 (29%), a further 2.7% unaffected pregnancies and 27.3% Down syndrome pregnancies had risks above 1 in 250 when NT was incorporated. Overall detection rate was 88.6%, and false positive rate 5.8% (compared with 90.9% and 6.4% for CUB screening). NT measurements were required in 29% of women.

Conclusions Within first-trimester, contingent screening provides good sensitivity and specificity with the potential for considerable saving in ultrasound resources. Copyright © 2008 John Wiley & Sons, Ltd.

KEY WORDS: prenatal screening; Down syndrome; serum markers; nuchal translucency; first trimester

INTRODUCTION

Combined ultrasound and biochemical (CUB) screening for Down syndrome using nuchal translucency (NT) measurements, maternal serum pregnancy-associated plasma protein A (PAPP-A) and free β human chorionic gonadotrophin (FβhCG) is offered routinely in many centres. The effectiveness of CUB screening in clinical practice is well documented with detection rates of 85–90% at a 4–5% screen-positive rate being typically reported (Spencer *et al*., 2000; Stenhouse *et al*., 2004). Although CUB screening has a better detection rate at a lower screen-positive rate than second-trimester screening, a critical factor in maintaining this performance is consistent and accurate NT measurement. This requires ultrasonographers with specific training and a system of on-going monitoring within a quality assured programme. This has hampered the adoption of CUB screening in some centres which lack the ultrasound resources to provide high-quality NT measurements to the entire booking population.

A possible solution to this problem was proposed by Christiansen and Larsen (2002) who suggested a within-trimester contingent testing approach in the first trimester. In this protocol, women first have biochemical testing (PAPP-A and FβhCG) and then go on to have NT measurement only if the risk calculated from maternal age and serum markers falls within an intermediate risk range. Women who fall within the high-risk group are offered diagnostic testing whilst those in the low-risk group are not offered any further testing. Based on mathematical modelling, and with initial high and low cut-off risks of 1 in 65 and 1 in 1000, respectively, and a final risk cut-off of 1 in 400, Christiansen and Larsen (2002) estimated that only 19.4% of women would require an NT scan to yield a detection rate of 78.9% for a 4% false positive rate. This small reduction in detection rate compared to full CUB screening in all women is offset by an increase in the cost-effectiveness of CUB screening due to a significant decrease in the number of NT measurements required.

Using data from routine CUB screening of over 10 000 women in Scotland screened between 2000 and 2005, we have carried out a re-analysis of the marker results using a contingent testing model similar to that described by Christiansen and Larsen (2002) to assess the likely performance of this approach and gauge the potential for reducing the ultrasound resources required for first-trimester population screening.

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METHODS

Study population

A cohort of 10 189 pregnancies where CUB screening was performed in Scotland between July 2000 and October 2005 was identified. The screening protocol is described more fully in Stenhouse *et al.* (2004). There were 44 Down syndrome and 10 145 unaffected pregnancies within this group. The median maternal age at the expected date of delivery was 33.1 years, and 36.9% of women were aged 35 years and over. The number of blood samples taken at each week of gestation is shown in Table 1. In the majority of pregnancies, blood samples and NT measurements were taken during the course of the same antenatal clinical appointment. In a proportion of women (28%), blood samples were not taken at the same visit as the NT measurement either because of logistic reasons or too early a presentation for NT (outside the CRL range of 40–84 mm), or inability to obtain an NT measurement at the first attempt, necessitating a return visit.

The concentrations of PAPP-A and FβhCG in maternal serum were measured using the DELFIA fluoroimmunoassay method (Perkin Elmer LAS, Finland). Ultrasound operators had all received training in the NT measurement protocol (Stenhouse *et al.*, 2004) and were subjected to on-going quality assurance through a bimonthly review of images and analyses of the distribution of NT measurements (Stenhouse *et al.*, 2002). All biochemical marker and NT measurements were converted to multiples of the appropriate gestational medians (with maternal weight correction for PAPP-A and FβhCG, and smoking correction for PAPP-A) and a combined risk calculated for each pregnancy.

Within the study group, where all women had biochemical and NT measurements and using a 1 in 250 cut-off risk at term, the detection rate for Down syndrome was 90.9% at a false positive rate of 6.4% (equivalent to a detection rate of 88.6% at a 5.0% false positive rate).

Contingent screening strategy

The objective was to compare retrospectively the performance of the full CUB screening test with that of the contingent protocol of Christiansen and Larsen (2002) but using the same final cut-off risk as the CUB screening programme. A two-stage protocol was used. Initially, a risk at term was calculated from the PAPP-A and FβhCG results combined with the maternal age risk for all women. A high-risk cut-off of 1 in 42 and a low-risk cut-off of 1 in 1000 were defined using the statistical approach described by Christiansen and Larsen (2002). The high-risk cut-off is dependent on the final risk and the low-risk cut-off is chosen empirically to adjust the proportion of women requiring NT measurement. Women were divided into three groups according to their initial biochemistry/age risk. For those with intermediate risks between 1 in 42 and 1 in 1000, the likelihood ratio derived from the NT measurement in multiples of the median (MoM) was then combined with the biochemistry/age risk and the composite risk assessed against a final cut-off risk of 1 in 250. Those women with a final risk $\geq$1 in 250 were classified as screen positive and added to the initial high-risk group. Those with final risks of $<$1 in 250 were classified as screen negative and added to the initial low-risk group. From the distribution of risks in Down syndrome and unaffected pregnancies the sensitivity and specificity of the contingent screening model was calculated.

Table 1—Number of blood samples taken at each gestational week

Gestational week	Number of blood samples
9	197
10	649
11	2234
12	3987
13	2891
14	231

RESULTS

Figure 1 illustrates the performance of the contingent screening model in our study group using initial high and low cut-offs of 1 in 42 and 1 in 1000, respectively, and a final cut off of 1 in 250. There were 313 (3.1%) unaffected and 27 (61.4%) Down syndrome pregnancies with initial risks $\geq$1 in 42, and these were classified as high risk. In this approach to screening these women would be offered a diagnostic test (CVS/amniocentesis) at this stage. NT measurement would not be offered to these women because their initial risk is so high that a subsequent NT measurement would be unlikely to bring the risk down below the final threshold risk of 1 in 250. Within the low-risk group with risks $\leq$1 in 1000, there were 6887 (67.9%) unaffected and 2 (4.5%) Down syndrome pregnancies. According to the protocol, these women would be counselled that they would not be offered any further test because the initial risk was low. The remaining 2960 (29%) women fell within the intermediate risk category and would be offered NT measurement. Of these, when the risk from the NT measurement was combined with the initial risk, 276 (2.7%) unaffected pregnancies and 12 (27.3%) Down syndrome pregnancies had risks above the final cut-off risk of 1 in 250 and would be offered a diagnostic test.

Therefore, in our CUB-screened population, adopting the above contingent screening protocol would have achieved a detection rate of 88.6% at a false positive rate of 5.8% (compared with 90.9% detection at a 6.4% false positive rate for the full CUB screen) but with only 29% of women requiring an NT measurement. For over 10 000 unaffected and 44 affected pregnancies the 95% confidence intervals for the false positive rates and detection rates are $\pm$0.6% and $\pm$10% respectively. By changing the initial and final cut-off risks, the detection rate, false positive rate and NT measurement rate can

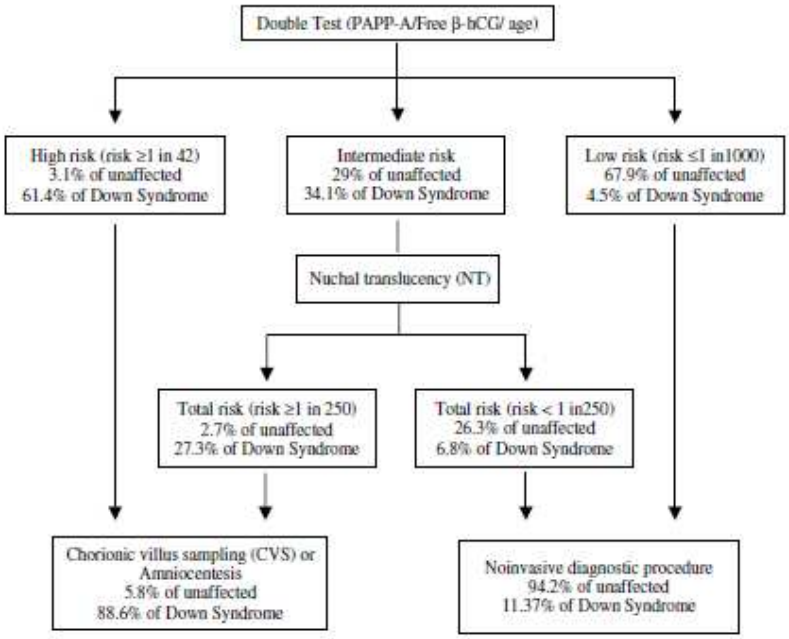

Figure 1—Within-trimester contingent screening protocol. Risks derived from serum markers and maternal age were estimated for all women and used to triage the population into high, low and intermediate risk groups. Women whose risk is greater that 1 in 42 would be offered a diagnostic test, and those women whose risk is lower than 1 in 1000 would not be offered any further testing. Women with an intermediate risk would be offered NT measurement, and those with a composite risk greater than the cut-off of 1 in 250 would be offered diagnostic testing

be varied (Table 2). This would allow individual centres to develop protocols best suited to local circumstances. If, for example, it was desired to keep the false positive rate low, an initial high-risk cut-off of 1 in 24 and final risk cut off of 1 in 150 gives a false positive rate of 3.7% for only a small reduction in detection to 84.1%.

DISCUSSION

While CUB screening is a proven, effective method of detecting Down syndrome pregnancies in the first trimester, it requires considerable investment in ultrasound equipment and operator training to maintain the required standard when screening large numbers of pregnant women. In this model of first-trimester contingent screening, women who are found to have a high risk based on the initial biochemical test and maternal age are not offered NT measurement because these women will end up with a final risk ≥1 in 250 irrespective of the NT measurement. Women with a low risk will also not be offered NT measurement as their risk is unlikely to be modified sufficiently to reach the final cut-off. Thus,

Table 2—The frequency of NT measurement and overall screening performance in contingent testing with different risk cut-off values

Final risk cut offs (at term)	High-risk cut-offs (at term)	Low-risk cut-offs (at term)	NT frequency (%)	Detection rate (%)	False positive rate (%)
1 in 250	1 in 42	1 in 1000	29.1	88.6	5.8
		1 in 800	25.1	86.4	5.8
		1 in 600	20.7	84.1	5.6
		1 in 400	15.3	81.9	5.5
1 in 200	1 in 33	1 in 1000	29.7	86.4	4.8
		1 in 800	25.8	84.1	4.8
		1 in 600	21.3	81.8	4.6
		1 in 400	16.0	79.6	4.5
1 in 150	1 in 24	1 in 1000	30.3	84.1	3.7
		1 in 800	26.4	81.8	3.7
		1 in 600	21.9	79.5	3.6
		1 in 400	16.6	77.3	3.5

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this form of contingent screening allows those centres with limited resources to target the group of pregnant women whose screening results can be most usefully modified by information from an NT measurement. The method offers the prospect of reducing the NT measurement workload to around 25–30% whilst maintaining high sensitivity and specificity.

This form of within-trimester contingent testing has other advantages. Unlike across-trimester integrated testing (Wald *et al*., 1999) where there is no disclosure of results after the first stage of testing, by the contingent method described here all women receive a risk result following their initial PAPP-A/FβhCG test. Decisions on whether to proceed to NT measurement or not are therefore based on the woman's awareness of clearly defined criteria, which aid counselling and are likely to help ensure that women do not default on their NT appointment. Further, all women complete screening before the end of the first trimester and more than two-thirds (which also includes 60% of the Down syndrome pregnancies) complete screening at an even earlier stage when there is no requirement to carry out an NT measurement.

To maximise the efficiency of this screening protocol it is important that women attend the initial blood sampling test as early as possible to allow time for those women requiring an NT scan to return no later than 14 weeks + 0 days of gestation. Although the number of women requiring an NT scan is reduced, ultrasound assessment of gestation is indispensable in all cases as this information is essential for accurate interpretation of the serum marker results. However a dating scan is generally less time consuming than an NT scan, and can be carried out by staff without specific training in NT measurement.

This form of contingent testing could be modified through the use of alternative serum markers measured earlier in pregnancy. It is well known that PAPP-A has better discriminatory power earlier in pregnancy at 8 weeks of gestation (Spencer *et al*., 2002) while other markers, notably total or intact hCG (Spencer *et al*., 2002), Inhibin A (Christiansen and Nørgaard-Pederson, 2005) and ADAM12 (Laigaard *et al*., 2006) may also perform better than FβhCG at this early stage. However, in routine CUB screening, PAPP-A and FβhCG are usually measured at the same gestation as NT at 11–13 weeks, and at that stage the reduced power of PAPP-A is compensated by the increased power of FβhCG. In this contingent model, a detection rate of 61.4% at a 3.1% false positive rate was predicted using serum markers plus maternal age alone. This is similar to that estimated by Cuckle (2000) of 64.9% detection with a false positive rate of 5%, and by Spencer *et al*. (2003) of 64.7% detection with a false positive rate of 5%. It is likely that additional serum markers assayed in early pregnancy may improve the primary screen detection rate even further. However, the selective addition of NT measurements as shown here, adds to screening performance and should be used wherever resources allow.

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APPENDIX B

BMJ

RESEARCH

Reliability of self reported smoking status by pregnant women for estimating smoking prevalence: a retrospective, cross sectional study

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ABSTRACT

Objective To determine what impact reliance on self reported smoking status during pregnancy has on both the accuracy of smoking prevalence figures and access to smoking cessation services for pregnant women in Scotland.

Design Retrospective, cross sectional study of cotinine measurements in stored blood samples.

Participants Random sample (n=3475) of the 21029 pregnant women in the West of Scotland who opted for second trimester prenatal screening over a one year period.

Main outcome measure Smoking status validated with cotinine measurement by maternal area deprivation category (Scottish Index of Multiple Deprivation).

Results Reliance on self reported smoking status underestimated true smoking by 25% (1046/3475 (30%) from cotinine measurement *v* 839/3475 (24%) from self reporting, *z* score 8.27, P<0.001). Projected figures suggest that in Scotland more than 2400 pregnant smokers go undetected each year. A greater proportion of smokers in the least deprived areas (deprivation categories 1+2) did not report their smoking (39%) compared with women in the most deprived areas (22% in deprivation categories 4+5), but, because smoking was far more common in the most deprived areas (706 (40%) in deprived areas compared with 142 (14%) in affluent areas), projected figures for Scotland suggest that twice as many women in the most deprived areas are undetected (n=1196) than in the least deprived areas (n=642).

Conclusion Reliance on self reporting to identify pregnant smokers significantly underestimates the number of pregnant smokers in Scotland and results in a failure to detect over 2400 smokers each year who are therefore not offered smoking cessation services.

INTRODUCTION

Although the risks of smoking during pregnancy for both mother and child are well established,[1] smoking during pregnancy is still common, with smoking rates varying from 24% in Scotland[2] to 17% in England.[3] Smoking prevalence generally increases with deprivation, and this is certainly true of Scotland, where 41% of women in the most deprived areas report smoking compared with only 14% in the least deprived areas.[4] Alarmingly, the gap in smoking prevalence between deprivation areas is larger in the pregnant population, where smoking is reduced to 8% in the least deprived areas but to only 38% in the most deprived area (data from the Scottish Morbidity Record (SMR02) for 2005, provided by NHS Information Services Division, www.isdscotland.org).

Self reported smoking is commonly used in antenatal clinics to determine the smoking status of pregnant women. However, this is an inaccurate method of identifying smokers,[5] with studies suggesting up to a quarter of pregnant smokers are missed when self reporting is relied on.[6-8] The accuracy of self report varies by the setting in which the questions are asked. Routinely collected data, such as in antenatal care, is often less accurate than data collected in settings perceived as more neutral, such as in research interviews.[9] With mounting social and medical pressure on women to quit smoking during pregnancy, there is greater likelihood that pregnant women will not report their smoking. Such inaccuracy can affect the reliability of smoking prevalence figures and access to smoking cessation services.

National targets to improve the nation's health generally include targets to reduce smoking during pregnancy,[10 11] often with the explicit aim of reducing inequalities related to deprivation. The Scottish government uses self reported smoking at maternity booking to construct targets and to measure the success of services in reaching such targets. These measures need to be robust in order to assess if services are achieving their aim and if money is being well spent in trying to achieve these targets.

Smoking cessation interventions have been shown to be moderately effective in reducing continued smoking into late pregnancy, with a risk of smoking for the intervention group relative to the control group of 0.94.[12] Perhaps more importantly intervention is effective at reducing the proportion of babies

of low birth weight (relative risk 0.81 (95% CI 0.70 to 0.94)) and of preterm births (0.84 (0.72 to 0.98)), and at increasing birth weight (33 g (11 g to 55 g) increase in weight).[12] These findings have helped promote the development of smoking cessation programmes directed at pregnant women.[13] Entry to such specialist cessation services in Scotland, however, usually depends on self reporting of smoking at the maternity booking visit.[14] Therefore, unless a woman admits to being a current smoker at maternity booking she will not be referred to specialist smoking cessation services and will not receive appropriate support, putting her own health and the health of her unborn child at risk. This study aimed to assess the robustness of self reporting at maternity booking as a method to set targets and to identify pregnant smokers.

Few studies have looked at the variation in accuracy of self reported smoking by deprivation, although the education level of pregnant women has been linked to the accuracy of self reporting.[15] We hypothesised that self reporting is less effective at identifying smokers in the most deprived areas compared with identifying those in more affluent areas, and that this further compounds existing health disparities during pregnancy.[16]

We compared the routinely collected self reported smoking status of pregnant women with their smoking status validated by means of serum cotinine measurement in order to: use the validated smoking prevalence in the study population to estimate the true smoking prevalence in pregnant women in Scotland; to identify the number of pregnant women in the study population who smoked but had no access to smoking cessation services in order to estimate the number of such women in Scotland; and to identify any variation in the level of under-reporting of smoking by area deprivation and determine what impact this will have on existing health inequalities in pregnancy.

METHODS

Sample

The records of all women in the West of Scotland who opted for prenatal screening in the second trimester for Down's syndrome and neural tube defects between May 2003 and July 2004 (that is, likely to result in a 2004 birth) were matched with their obstetric records from the Scottish Morbidity Records (SMR02) (data for 2004, provided by NHS Information Services Division, www.isdscotland.org). The prenatal screening process involves measuring maternal serum α fetoprotein and human chorionic gonadotrophin. Excess serum is stored at −20°C. At the time of linking, 2004 was the most recent complete year in the SMR02 dataset. The SMR02 data contain self reported smoking information collected by the midwife at the maternity booking appointment, usually carried out at 8-12 weeks of gestation, for all women delivering in an NHS facility. Women were asked for their smoking status and were recorded as current, former, or never smokers (or unknown if the response was not recorded). Information on the baby's date of birth, mother's date of birth, maternal area deprivation (Scottish Index of Multiple Deprivation, which is based on postcode of residence reflecting material deprivation)[17] and date of booking was also available in the SMR02 data. Second trimester prenatal screening was usually carried out at 15-16 weeks of gestation. The records were matched using the mother's surname, forename, date of birth, and hospital number using probability matching techniques.[18] After data linkage, the database was returned with all patient-identifiable information removed and samples selected for cotinine measurement.

A simple random sample of linked records was selected for cotinine analysis from the births in the 2004 calendar year.

Sample size

A sample size of 3200 records allowed a 3% difference in the proportion of cotinine validated smoking and self reported smoking to be detected in the sample as a whole and a difference of 3% to be detected when comparing the combined deprivation categories 1 and 2 with the combined categories 4 and 5. We made the a priori decision to collapse the two highest and two lowest deprivation categories to give a greater power with the chosen sample size. To allow for technical difficulties (such as insufficient serum, etc), 3550 women were randomly selected from the linked dataset for cotinine analysis.

Cotinine analysis

As cotinine is derived only from nicotine metabolism, its measurement in serum is a good indicator of recent nicotine exposure. Cotinine testing was carried out on thawed serum samples at the West of Scotland Regional Genetics Service laboratories using commercially available kits (Cozart STD Micro-Plate Cotinine EIA), and those performing the tests were blind to the women's reported smoking status. Samples were assayed in singletons only. Samples with cotinine concentrations between 10 and 30 ng/ml (close to the cut-off value) were re-assayed and the mean of the two values taken as the final cotinine concentration. Cotinine concentrations ≥13.7 ng/ml were taken to indicate current smoking.[19]

Statistical analysis

The prevalences of cotinine validated current smoking and self reported current smoking were determined for the whole sample, and by area deprivation categories, and statistically compared (one-sample test of equality of proportions) using STATA (version 8).

The number of cotinine validated smokers not captured by self reporting (referred to as undetected smokers) was determined by identifying women with never, former, or unknown smoking status who had cotinine values ≥13.7 ng/ml, for the whole sample and by deprivation category.

The degree to which the study sample represents the population from which the sample was drawn and the population of pregnant women in Scotland was

Table 1| Basic characteristics of 3475 pregnant women in the West of Scotland and their subsequent babies. (Values are numbers (percentages) unless stated otherwise)

Characteristic	Value
Self reported smoking status in pregnancy:	
Current smoker	839 (24.1)
Former smoker	367 (10.6)
Never smoker	1971 (56.7)
Unknown	298 (8.6)
Mean (SD) maternal age (years)*	29.4 (6.0)
Mean (SD) gestation at delivery (weeks)	39.2 (2.0)
Mean (SD) birth weight of baby (g)	3390.8 (600.0)
Deprivation category†:	
1 (least deprived)	440 (12.7)
2	545 (15.7)
3	733 (21.1)
4	730 (21.0)
5 (most deprived)	1023 (29.5)

*Data missing for one record.
†Based on Scottish Index of Multiple Deprivation. Data missing for four records.

determined by comparing the distribution of maternal age, deprivation category, and self reported smoking status in the study sample with the population of pregnant women in the West of Scotland who opted to be screened, the total pregnant population in the West of Scotland (including those who did not opt for screening), and the population of pregnant women in Scotland. Cotinine validated smoking prevalence in the study sample was used to estimate the true smoking prevalence in the population of pregnant women in Scotland, accounting for differences in the distribution of deprivation, maternal age, and self reported smoking status using standardisation techniques. For example, the estimated number of cotinine validated pregnant smokers in Scotland aged <20 in deprivation categories 1 and 2 who reported never smoking would be calculated by multiplying the proportion of such women in the study sample by the number of pregnant women aged <20 in deprivation categories 1 and 2 reporting never smoking in the Scottish pregnant population. Scottish Morbidity Records (SMR02) data were used to project figures for Scotland.

The number of undetected smokers was estimated by subtracting the number of smokers identified by self reporting from the estimated number of true smokers. Fifty women with no data on deprivation category or age were excluded, leaving 52591 pregnant women in Scotland with a 2004 birth. In generating the projected estimates for Scotland, we collapsed the maternal age categories (to <24, 25-29, 30-34 and ≥35 years) and the highest and lowest deprivation categories (1+2 and 4+5) because of small numbers in the sample population.

Statistical significance of differences between categorical variables was determined with Pearson's χ^2 test. All analyses were performed with SPSS (version 15) or STATA (version 8). To explore the sensitivity of the findings to the cotinine concentration we used in this study to denote a current smoker, we repeated the analyses using alternative cotinine levels found in the literature.

Ethical approval and data protection

We contacted the Central Office for Research Ethics Committees (corec.org.uk), which advised that ethical approval was not needed for the study. Data protection issues were discussed with the data protection officer at the University of Glasgow and a confidentiality statement for use of NHS patient data was completed. The Privacy Advisory Committee of the Information Services Division (ISD) of NHS National Services approved the study for linkage with routinely collected data held by ISD.

RESULTS

Of the 29975 women in the West of Scotland who gave birth in 2004, 21029 (70%) opted for second trimester screening. Of these, 97% could be linked to their obstetric SMR02 data and 3550 were randomly selected for cotinine analysis; 98% of samples were located and assayed (fig 1). Seventy one serum samples with cotinine concentration of 10-30 ng/ml (close to the cut-off value) were re-analysed.

Over half of the 3475 women in the sample reported never smoking, and just less than a quarter reported being current smokers. The self reported smoking status was unknown for 9% of the study population (table 1). The profile of maternal age, baby's birth weight, and gestation at delivery were all typical of that seen in Scotland.[20] Women in deprived areas were over-represented in this sample, as in the West of Scotland.[17]

Under-reporting of smoking in the sample population

According to the figures for self reported smoking status, 839 (24.1%) of the pregnant women were current smokers. This value is significantly lower than the cotinine validated estimate of 1046 (30.1%) who were current smokers (table 2). As expected, the prevalence of smoking in the most deprived categories was greater than in the least deprived categories, with both the self reported and cotinine validated estimates. However, the difference between the cotinine validated and self reported smoking estimates is greater in the

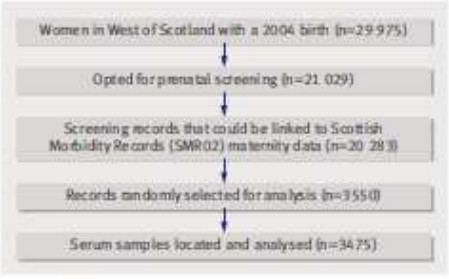

Fig 1| Selection of study sample for cotinine analysis

Table 2 | Prevalence of current smoking among 3475 pregnant women in the West of Scotland according to self reported smoking and cotinine validated smoking

	Self reported smoking*		Cotinine validated smoking†		Difference		
	No of women	Percentage (95% CI)	No of women	Percentage (95% CI)	Percentage points	z score	P value
Total sample (n=3475)	839	24.1 (22.7 to 25.6)	1046	30.1 (28.6 to 31.6)	6.0	8.27	<0.001
Deprivation category‡:							
1+2 (n=985)	101	10.3 (8.5 to 12.3)	142	14.4 (12.3 to 16.7)	4.1	4.23	<0.001
4+5 (n=1753)	587	33.5 (31.3 to 35.7)	706	40.3 (38.0 to 42.6)	6.8	6.03	<0.001

*Measured at booking appointment (8-12 weeks of gestation).
†Measured at about 15 weeks of gestation. Cotinine concentrations ≥13.7 ng/ml were taken to indicate current smoking.
‡Based on Scottish Index of Multiple Deprivation (categories 1+2 are least deprived, 4+5 are most deprived).

most deprived groups than in the least deprived groups (table 2).

Sixty one (7%) of the self reported smokers had cotinine concentrations below the cut-off value for current smoking, and were therefore classified as non-smokers (table 3). These women could have quit between their booking and screening appointments, have been light smokers, or be recording errors.

The proportion of cotinine validated current smokers who were correctly identified by self reporting was 74.4%. This means that 25.6% (n=268) of cotinine validated smokers were not detected by self reporting and were therefore not offered smoking cessation services (table 4). The number of such undetected smokers was about three times greater in the most deprived areas than in the least deprived areas. However, this represented a smaller proportion of the total number of cotinine validated smokers in the deprived areas than in the affluent areas; it seems that a greater proportion of women in affluent areas failed to report their smoking compared with women in deprived areas.

Although not the focus of this study, the proportion of cotinine validated smokers in the "unknown" category for self reported smoking broadly represented the distribution across the whole study population.

Projected smoking figures for Scotland

We determined the degree to which our sample was representative of the screened population, the West of Scotland population (from which the sample was drawn), and the Scottish population of pregnant women (table 5). We found no differences between the sample population and the screened population in any of the characteristics examined. Comparing the sampled population with the West of Scotland population, there were no significant differences in the distribution of self reported smoking or maternal age, but there were small but significant differences by area deprivation. The distribution of maternal age within deprivation categories was also compared across populations and no differences were seen between the sampled and West of Scotland populations. Comparing the sampled population with the Scottish population, there was a significant difference in area deprivation and self reported smoking but not maternal age. These data showed the need to adjust at least for differences in self reported smoking and area deprivation when generating projected figures for Scotland. Although no differences in the maternal age distribution were detected, projected figures were also adjusted for maternal age to account for any residual confounding that might arise between age categories.

After adjustment for the distribution of area deprivation, self reported smoking, and maternal age, we estimated the number of pregnant smokers in Scotland to be 14521 (that is, a smoking prevalence of 27.6%), and 2400 of these would be undetected by self report, representing 4.6% of the total population of pregnant women in Scotland. The number of undetected smokers in the most deprived areas (deprivation categories 4+5, n=1196) is nearly twice that in the least deprived areas (categories 1+2, n=642). Using the projected figures for Scotland, the prevalence of smoking in the most deprived areas is 39% compared with 14% in the least deprived areas.

The distribution of cotinine values produced two quite distinct populations (see figure in the extra material on bmj.com), suggesting that the findings were robust to the chosen cotinine cut-off value of 13.7 ng/ml. Using an alternative cut-off of 24 ng/ml[21] produced a prevalence of cotinine validated smoking for the study population of 29.3% (n=1018), very similar to the 30.1% produced using the original cut-off (see extra material on bmj.com for all other data using the alterative cut-off value).

DISCUSSION

Using cotinine validation to identify smokers, we estimate that 1046 of the 3475 women in the study population smoked during pregnancy, which is 25% higher than figures based on self reported smoking (839/

Table 3 | Proportion of pregnant women with serum cotinine concentration above and below the cut-off value for current smoking by their self reported smoking status. (Values are numbers (percentages))

	Serum cotinine concentration	
Self reported smoking status	<13.7 ng/ml	≥13.7 ng/ml
Current (n=839)	61 (7)	778 (93)
Former (n=367)	273 (74)	94 (26)
Never (n=1971)	1875 (95)	96 (5)
Unknown (n=298)	220 (74)	78 (26)

Table 4 | Cotinine validated current smoking among pregnant women that was undetected (not captured by self reported smoking status)

	No of cotinine validated smokers	No (%) of cotinine validated smokers not captured by self reporting	Undetected smokers as % of all pregnant women
Total sample (n=3475)	1046	268 (25.6)	7.7
Deprivation category*:			
1+2 (n=985)	142	56 (39.4)	5.7†
4+5 (n=1753)	706	155 (22.0)	8.8

*Based on Scottish Index of Multiple Deprivation (categories 1+2 are least deprived, 4+5 are most deprived).
†z=0.293, P=0.003 for difference in distribution of undetected smokers across deprivation categories 1+2 and 4+5.

3475). The projected true smoking prevalence for pregnant women in Scotland (after adjusting for area deprivation, maternal age, and self reported smoking) is 28%, notably higher than the 23% based on self reporting.[2] Projected figures suggest that in Scotland each year more than 17% of pregnant smokers (n=2400) are not identified as such and are therefore not offered smoking cessation services.

There was a striking difference in smoking prevalence in pregnant women between area deprivation categories, reflecting that seen elsewhere.[22] Nearly 40% of smokers in the least deprived areas (deprivation categories 1+2) did not report their smoking status compared with only 22% of smokers in the most deprived areas (categories 4+5). This possibly reflects a greater expectation in more affluent areas that women will quit smoking during pregnancy. However, because of the larger number of smokers in deprived areas, in absolute terms, there were three times as many undetected smokers in the most deprived areas compared with the least deprived areas in the sample population.

In our sample, about a quarter of validated smokers went undetected. This proportion is similar to that seen in other studies of pregnant women in the UK[10] and elsewhere.[6-8 23] Higher proportions of undetected smokers have been seen: in one US study more than 50% of cotinine validated smokers were undetected by self reporting.[24] High proportions of undetected smokers (the proportion of smokers who do not report their smoking) are commonly seen in pregnant populations involved in cessation programmes,[25 26] as might be expected. Other studies have reported lower proportions of undetected smokers,[12 27] some as low as 1%.[28] The variation in proportion of undetected smokers can be largely explained by different populations and settings in which smoking status was reported. The study that reported only 1% undetected smokers was based on a Swedish population in which only 8% of the pregnant population were smokers,[28] whereas the study reporting over 50% undetected smokers examined a population in a deprived area with a smoking prevalence of 35%.

There is general agreement that serum cotinine is ideal for measuring smoking status because of its long half life (9 hours in pregnant women)[29] and the procedure's optimised sensitivity (94%) and specificity (81%).[4 29] There is little variation in the cotinine cut-off level used to define a pregnant smoker, with some studies using the lower value of 10 ng/ml[17 27] and others using values up to 24 ng/ml.[8 21] The results of our study were robust to the chosen cut-off value. Cotinine is specific for nicotine, but not necessarily for smoking; both nicotine replacement therapy and exposure to environmental tobacco smoke have been shown to elevate serum cotinine levels. The median cotinine level recorded in studies measuring the impact of exposure to environmental tobacco smoke is well below our chosen cut-off value of 13.7 ng/ml—a median cotinine concentration of 4 ng/ml has been reported for office staff[30] and 8 ng/ml for bar staff.[31] Therefore, women exposed to environmental tobacco smoke were unlikely to be miscoded as smokers in our study. The women in this study were pregnant in 2003-4, and nicotine replacement therapy was not routinely recommended in the West of Scotland until two specialist smoking cessation programmes began offering it in 2002 (CATCH)[32] and 2004 (BREATHE).[33] The only service offering nicotine replacement therapy during the time the study women were pregnant was CATCH, which offered it to 65 women. Even if all these 65 women used nicotine replacement products the small number of women involved would be unlikely to bias our findings greatly. In addition, a smoking cessation study based in Glasgow during 2001-3 showed that only 0.8% (6/718) of women reported taking nicotine replacement therapy,[34] again suggesting that any effects of nicotine replacement therapy in the study population would be minimal.

The recording of self reported smoking status at the antenatal clinic booking visit usually took place about three weeks before the collection of blood used for cotinine analysis. It was possible, therefore, for women to have quit smoking and correctly report being a former smoker at the booking appointment but to resume smoking before the blood was collected. Therefore, it should be acknowledged that some of the unreported smokers may have been former smokers who relapsed after reporting their smoking status and not smokers deliberately denying their smoking status. This distinction may not be so important for the objective of this study—determining the true prevalence of smoking among pregnant women. It may be that, within reason, the later in pregnancy that smoking is measured the more accurate it will be in terms of recording the true number of pregnant smokers—in that it will capture more of those that relapse.[23] As a result, these data will slightly overestimate the number of current

Table 5 | Distribution of maternal age, area deprivation, and self reported smoking status among pregnant women in the study sample, the screened sample, the West of Scotland, and Scotland. (Values are numbers (percentages) unless stated otherwise)

	Pregnant women (2004 birth)*			
All	Study sample (n=3475)	Screened population (n=20283†)	West of Scotland‡ (n=29 975)	Scotland (n=52862)
Self reported smoking status§				
Current	839 (24)	4896 (24)	7303 (25)	12 366 (24)
Former	367 (11)	2072 (10)	2770 (9)	4712 (9)
Never	1971 (57)	11 353 (56)	16 980 (57)	30 680 (59)
Unknown	298 (9)	1962 (10)	2614 (9)	4074 (8)
Difference from study sample	—	χ^2=4.4, P=0.2	χ^2=5.5, P=0.1	χ^2=13.3, P=0.004
Deprivation category (Scottish Index of Multiple Deprivation)				
1+2	985 (28)	5683 (28)	8738 (29)	19621 (37)
3	733 (21)	4028 (20)	5601 (19)	9825 (19)
4+5	1753 (51)	10 531 (52)	15 636 (52)	23 145 (44)
Difference from study sample¶	—	χ^2=4.5, P=0.3	χ^2=17.4, P<0.002	χ^2=130, P<0.001
Maternal age (years) by deprivation category				
Categories 1+2:				
<20	21 (2)	140 (3)	235 (3)	528 (3)
20-24	74 (8)	505 (9)	770 (9)	1753 (9)
25-29	220 (22)	1243 (22)	1836 (21)	4360 (22)
30-34	390 (40)	2246 (40)	3455 (40)	7744 (40)
35-39	244 (25)	1354 (24)	2085 (24)	4395 (22)
≥40	36 (4)	193 (3)	357 (4)	764 (4)
Difference from study sample	—	χ^2=2.8, P=0.7	χ^2=4.2, P=0.5	χ^2=5.7, P=0.3
Category 3:				
<20	38 (5)	188 (5)	303 (5)	586 (6)
20-24	117 (16)	642 (16)	882 (16)	1629 (17)
25-29	188 (26)	1084 (27)	1452 (26)	2568 (27)
30-34	230 (31)	1270 (32)	1835 (33)	3068 (32)
35-39	138 (19)	741 (18)	959 (17)	1529 (16)
≥40	22 (3)	101 (3)	170 (3)	306 (3)
Difference from study sample	—	χ^2=1.4, P=0.9	χ^2=1.6, P=0.9	χ^2=5.4, P=0.4
Categories 4+5:				
<20	215 (12)	1205 (12)	1907 (12)	3026 (13)
20-24	430 (25)	2672 (25)	4052 (26)	6318 (27)
25-29	464 (27)	2774 (26)	4018 (26)	5922 (25)
30-34	411 (24)	2434 (23)	3575 (23)	5211 (22)
35-39	197 (11)	1258 (12)	1763 (11)	2444 (10)
≥40	35 (2)	177 (2)	321 (2)	450 (2)
Difference from study sample	—	χ2=2.9, P=0.7	χ^2=1.8, P=0.9	χ^2=7.2, P=0.2
All categories combined:				
Difference from study sample	—	χ^2=3.1, P=0.7	χ^2=4.4, P=0.5	χ^2=3.6, P=0.6

*Excluding home births and births at non-NHS hospitals.
†Refers to those screening records that could be linked with their obstetric records from the Scottish Morbidity Records (SMR02).
‡Made up of Argyll and Clyde, Ayrshire and Arran, Dumfries and Galloway, Forth Valley, Greater Glasgow, Highland, Lanarkshire, and Western Isles health boards.
§Smoking figures for West of Scotland are for 2005.
¶Collapsed categories are used for χ^2 test.
Missing data: Sample population—4 records with no deprivation data, 1 record with no maternal age; screened population—18 records with no maternal age, 787 (3.7%) with no deprivation data, 746 with no self reported smoking data (that is, not linked records); West of Scotland—38 with no region data; Scottish data—163 with no deprivation data.

smokers who were not offered smoking cessation services because, at the time of asking, the to-be relapsers were in fact former smokers, although they are arguably in need of such services.

Extrapolation of the sample results to all pregnant smokers in Scotland is based on two assumptions, the first being that the study population accurately represents the population from which it was drawn (West of Scotland). The study sample is similar to the screened sample in all characteristics tested, which suggests successful random selection of the sample from the screened population. Differences between the study population and the West of Scotland will therefore reflect differences between the screened and non-screened women. Only if such differences are also related to the accuracy of self reported smoking will there be potential to introduce bias; differences that differentiate screened and non-screened women that relate only to self reported smoking status but not the accuracy of self reporting will not introduce bias. The data suggest that neither self reported smoking status nor maternal age were related to the decision to opt for screening in this setting (table 5), as the study sample and the population of the West of Scotland have similar profiles. A major strength of these data is the high screening rate (70% of all women were screened), reducing the potential for differences between the screened and target population. Indeed the lack of a maternal age effect is consistent with the continued monitoring of this screened population. The sample population had a slightly different area deprivation profile compared with the population of the West of Scotland (52% in the West of Scotland from deprivation categories 4+5 compared with 51% of the sample population). However, there is little difference in the percentages; the significance of the result is related to the large sample size rather than an important difference in deprivation profile. Other factors that may be related to a decision to opt for screening include ethnicity and religion. Ethnicity is unlikely to have a large impact on the data as there was very little representation of black and ethnic minority populations in Scotland at the time of data collection (2% non-white people in the 2001 census, General Register Office for Scotland). Catholic women are likely to be underrepresented in the screened population. Although there are substantial numbers of Catholics in Scotland (about 16% at the 2001 census), it is unlikely that the accuracy of self reported smoking status provided by pregnant Catholic women would be substantially different from that of the rest of the population and therefore these factors are unlikely to introduce bias. There may be yet other differences between the population of screened and non-screened women that we were not able to investigate that also relate to the accuracy of self reported smoking. However, given the high screening rate (70%), these differences are unlikely to bias the findings significantly.

The second assumption made is that differences between the West of Scotland and the Scottish population that also relate to the accuracy of self report are accounted for in generating the projected figures. Differences between the pregnant women from these two populations are expected because the West of Scotland includes some of the more deprived areas of the

WHAT IS ALREADY KNOWN ON THIS TOPIC

Self reported smoking during pregnancy is known to be an inaccurate method of identifying smokers

Self reported smoking in Scotland is used to generate smoking prevalence and, largely, to target smoking cessation services

WHAT THIS STUDY ADDS

Reliance on self reported smoking during pregnancy underestimates the true smoking prevalence in Scotland by 17%

Each year in Scotland twice as many pregnant smokers from more deprived areas go undetected compared with pregnant smokers in the least deprived areas

Reliance on self reporting results in a failure to detect over 2400 pregnant smokers each year in Scotland who are therefore not offered smoking cessation services

country. The projected figures take account of area deprivation, smoking status, and maternal age, which probably accounts for the major differences between east and west Scotland. Again religion (Catholicism in the West of Scotland) is unlikely to be related to the accuracy of self report.

Some errors in the recording or transcribing of the self reported smoking status at the booking appointment will have happened. It is not likely that these recording errors would be systematic (that is, by deprivation or smoking status), and such errors are therefore unlikely to bias these finding away from the null hypothesis. The recording of self reported smokers with a serum cotinine concentration below the cut-off value for non-smokers may slightly underestimate the true prevalence of smokers.

For 9% of the routinely collected data used for this study there was no valid information about self reported smoking status (the information was either not requested or not recorded), compounding the problem of inaccurate self reported data. There is some evidence that the issue of smoking is not necessarily given high priority in the relationship between midwife and expectant mother,[34] possibly explaining cases of missing data. Similar problems with data quality have also been highlighted with routinely collected self reported data in other regions.[35] The poor quality and accuracy of routinely collected self reported smoking data in pregnant women shown in this study and elsewhere[28] call for better methods of routinely identifying smokers during pregnancy. The current policy in Glasgow is for all women to provide self reported smoking status and undergo a carbon monoxide breath test at maternity booking. If implemented fully, this could increase the identification of pregnant smokers from 75% to about 95%.[33,36] Further studies are required to determine if offering routine biochemical validation (such as carbon monoxide breath tests) to all pregnant women at maternity booking would be the most cost effective method to identify smokers, to monitor targets, and to increase the reach of specialist smoking cessation support. Accurate smoking information also refines the estimation of individual fetal risks of Down's syndrome by prenatal screening, since maternal smoking causes changes in the levels of the biochemical markers used in the screening test[37] (discussed further in a subsequent paper).

In conclusion, reliance on self reporting to measure smoking during pregnancy significantly underestimates the number of pregnant smokers in Scotland, with more than 2400 unrecognised pregnant smokers a year who will not be offered smoking cessation services. Reliance on self reporting resulted in twice as many undetected smokers in the most deprived areas compared with the least deprived areas. Overall, these figures call for more accurate methods of identifying pregnant smokers, especially when such data are used to inform policy and provide patient care.

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