First-trimester Screening for Down Syndrome and Other Aneuploidies: Methodological Issues

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Cover : Lionfish (Pterois Volitans), the perfect invader. Due to interference of men, this fish is introduced from the Indo-Pasific into the southern part of the US Atlantic, Mexican Gulf and Caribbean Sea, where it has hardly any natural enemies and where it is destroying the coral reefs and its natural inhabitants. A small mistake with large consequences.
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Eerste-trimesterscreening voor downsyndroom en andere chromosomale afwijkingen: methodologische facetten

Proefschrift

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“It always seems impossible, until it is done.”

(Nelson Mandela 1918-2013)

Aan mijn broer Xander van Heesch. Strength of the will and mind.

Contents

Chapter 1

Introduction and outline of this thesis

1.1 Down syndrome

Down syndrome, which is synonymous with trisomy 21 (47, +21), is the most common chromosomal anomaly in live born children. In 1866 John Langdon Down first described children with common phenotypically features distinct from other children with mental retardation [1]. These children were referred by Down as 'mongoloids', based on the typical facial characteristics of individuals with Down syndrome. Specific characteristics of newborns with Down syndrome include a flat nasal bridge, epicanthic folds, small ears, a protruding tongue, a short neck and hypotonia [2]. Beside the visual features, newborns with Down syndrome have an increased risk of congenital structural anomalies such as heart defects, gastrointestinal defects, hearing and ophthalmic problems, hypothyroidism and leukemia [3-7]. In 1956 Joe Hin Tjio and Albert Levan [8] reported that the total number of human chromosomes in 'normal subject' is 46, rather than 48 as was supposedly established some three decades earlier. The importance of this finding was not the total number of chromosomes itself, but rather the ability to distinguish the number of 46 chromosomes from numerical chromosomal abnormalities. In fact, it was three years later (1959) when Lejeune et al [9] demonstrated that Down syndrome is associated with the presence of an additional chromosome 21. Due to this extra copy of chromosome 21, the clinical condition Down syndrome is also known as trisomy 21. As a result of the technical advances in chromosomal analysis of human amniotic-fluid cells demonstrated by Steele and Breg [10], the first prenatal diagnosis of Down syndrome by amniocentesis was reported in 1968 [11]. Because of the development of the new obstetric techniques together with the advances in direct analysis of spontaneous mitoses in fetal tissue [12] it was possible from 1989 to carry out prenatal chromosome diagnosis in the first trimester of pregnancy. Chorionic villi, which typically have the same genotype as the fetus, were obtained by gentle suction under constant real-time ultrasound guidance. These techniques enabled pregnant women to choose between chorionic villus sampling (CVS) at the 9th to 11th week of gestation and amniocentesis at the 16th to 18th week of gestation. These invasive procedures are associated with an iatrogenic miscarriage rate of 0.3- 0.5% [13-18].

For The Netherlands nowadays the estimated birth prevalence of Down syndrome is 14 per 10.000 with around 322 total annual births [19,20]. The relatively high prevalence of this condition and the association with perinatal morbidity and mortality has been one of the main reasons for the implementation of prenatal screening for Down syndrome.

1.2 Screening

The outline for a successful screening programme for any disease was formulated by Wilson and Jungner in 1968 [21]. They argued that the concept of screening is different from diagnosis. Screening tests help to identify a specified disease or condition among asymptomatic individuals while diagnostic tests are carried out among individuals with signs or symptoms of the condition of interest [22]. They claimed that a successful screening programme should need the following requirements (Table 1).

Table 1 **Desirable characteristics for a successful screening programme**

1. Is the condition being screened for an important health problem?
2. Is the screening test and its consequences in terms of further diagnostic testing and subsequent treatment acceptable to the population?
3. Does the target condition have a recognizable latent and early symptomatic phase?
4. How valid and reliable is the screening test?
5. Are there adequate facilities for confirming the diagnosis and for adequate treatment?
6. Is the screening programme a continuing process and not just a one-off activity?
7. Is early treatment of the target condition effective?
8. Do the objectives of the screening programme justify the costs?

In fact, the target condition should be severe and frequent enough to justify screening. Morbidity, mortality and quality of life of those affected should be considered in judgement of the severity. The group of individuals eligible for screening is often selected by certain

demographic risk factors associated with the disease, e.g., age of onset of the condition of interest. For the purpose of screening the usefulness of a screening test is determined by test performance which includes sensitivity (the % of all affected individuals detected by a positive test) and specificity (the % of unaffected individuals with a negative test). Besides the advantages of screening in terms of timely – pre-symptomatic - detection of an adverse health condition, there are also associated problems, such as false-negative test results and undue anxiety following false-positive test results. A good screening programme is based on objective information on the potential benefits, as well as on the limitations of screening.

From the early 70s Down syndrome screening by means of invasive prenatal tests was offered to women of advanced maternal age, i.e., from 36 years and above. With respect to Down syndrome, the Dutch Health Council committee concluded in 2007 in their report that on the basis of relatively good test properties, the presumed acceptance by the target group and feasibility of testing, the combined test was considered the best option for prenatal screening for Down syndrome. The combined test involves a blood test and nuchal translucency (NT) measurement by means of ultrasound, both conducted in the first trimester of pregnancy [23]. The committee recommended the Minister of Health, Welfare and Sports to implement prenatal screening for Down syndrome in the Netherlands, thereby taking into account most criteria of Wilson and Jungner. In 2007, the Minister of health, Welfare and Sports granted formal permission for prenatal screening for Down syndrome and neutral tube defects (See also paragraph 1.5). Currently screening tests for aneuploidy and fetal malformations have been incorporated in the day to day obstetric practice in the Netherlands and other high-income countries [24]. Before the test is conducted women need informed about the opportunity of testing and, where indicated, counselled about potential implications of testing including the advantages and disadvantages of screening, the likelihood of false positive and false negative results, invasive procedures and the subsequent consequences of abnormal findings. The cut-off point for increased risk is set at 1 in 200 [25]. In 2011 the first-trimester screening test was expanded with the screening for trisomy 13 and 18 with the

same cut-off point off 1 in 200. Since April 2014 women at increased risk of trisomy 21, 13 or 18 in the Netherlands are offered the Non-Invasive Prenatal Test (NIPT) next to the golden standard of invasive testing (chorionic villus sampling or amniocentesis) [26]. In January 2015, the criterion of maternal age for prenatal testing has been abandoned. In the screening setting, only women at increased risk from the findings of the combined test, will be eligible for further testing.

1.3 Ultrasound screening

The use of ultrasound in the evaluation of pregnancy for both diagnostic and screening purposes is well established. Detection and assessment of a specific characteristic of the fetus and its association with an anomaly can be used as a feature for prenatal screening. The nuchal translucency is one of these features. It is a fluid layer in the posterior neck region between the fetal skin and extends for a variable distance over the head and neck. The thickness of this translucent area increases with the fetal crown-rump length (CRL) and can be visualized by ultrasound in the short time frame between the 11th and 14th week of gestation. An enlarged NT is defined as an NT above the 99th centile (3.5 mm or more) and is associated with an increased prevalence of aneuploidy [27], particularly trisomy 21, but also with other abnormalities such as genetic syndromes and cardiac defects [28,29] .These observations and the term 'nuchal translucency' were published by Nicolaïdes et al. in 1992 [30]. The assessment of risk of trisomy 21 by maternal age and NT thickness was described in 1998 by Snijders et al. and the Fetal Medicine Foundation First-trimester Screening Group in a large multicenter study [31]. About 80% of the affected pregnancies could thus be detected. Reliable screening performance is essential and depends first and foremost on the quality and reproducibility of the NT measurement [32-37]. If an enlarged NT is detected the risk of other chromosomal anomalies is also increased. Of all the fetuses with an enlarged NT about 20% will have a chromosomal abnormality. There is a clear interrelation between prevalence of chromosomal abnormalities and the increase in NT above the 99th centile [27]. Trisomies 21,

18, 13 and Turner syndrome (45 X0) are the most frequently associated chromosomal anomalies found [38].

To achieve higher rates of detection of Down syndrome and to further reduce the need for invasive testing, several strategies involving both first-trimester and second-trimester screening tests have been introduced [39]. These include biochemical and sonographic markers for chromosomal abnormalities.

Improvement of the performance of the combined test (see paragraph 1.5) may be achieved by the inclusion of other first-trimester sonographic markers, such as absence of the nasal bone, abnormal flow in the ductus venosus and across the tricuspid valve, and abnormal fronto-maxillary facial angel, in the algorithm for risk assessment [38,40-42]. These makers, however, are not incorporated in the national screening programme for Down syndrome in the Netherlands. In some tertiary clinics these markers are used for women at intermediate risk who are reluctant to have invasive testing because of the inherent risk of miscarriage following amniocentesis or chorionic villus sampling. These markers could modify the risk derived from the combined test, potentially resulting in a lower risk of Down syndrome [39]. This strategy has an effect on the numbers of false positive test results from the combined test, presumably without jeopardizing the detection rate of Down syndrome. Two of these markers, Doppler of the ductus venosus en tricuspid valve, have proven to be more reliable in screening for congenital hearts defects. A normal sonographic examination, however, may provide false reassurance to women with a risk that is increased on the basis of first trimester combined test. Since April 2014 the Health Council of the Netherlands has approved the use of NIPT in a study setting in case of an increased risk for aneuploidy (trisomies 21, 13 or 18) of 1 in 200 or more [26]. Second-trimester ultrasound examinations i.e. the so-called 20-weeks fetal anomaly scan, are often routinely performed to detect fetal anatomical abnormalities. The finding of a major anomaly or two or more minor structural malformations increases the likelihood of aneuploidy. A number of fetal structural abnormalities and isolated sonographic markers detectable on second-trimester sonographic examination have been associated with Down syndrome [39,43,44]. These include intracardiac echogenic focus, ventriculomegaly,

increased nuchal fold, hyper echogenic bowel, mild hydronephrosis, short femur, short humerus, absent or hypoplastic nasal bone and abnormal Doppler flow of the ductus venosus and tricuspid valve.

In the Netherlands, combined first and second trimester screening tests are not used for the individual risk assessment since such approach is not included in the national screening programme for Down syndrome. However, in a euploid fetus with an NT above the 99th centile there is considerable evidence that shows an increased risk for structural anomalies, as especially with congenital heart defects [45-50]. A detailed ultrasound examination at 14 weeks and at 20 weeks however, is recommended in a contingently screening and diagnostic protocol [29].

1.4 Biochemical screening

In 1988, the value of maternal serum screening for Down syndrome was shown by Wald [51]. Combinations of second-trimester maternal serum biochemical markers, such as alpha fetoprotein (AFP), human chorionic gonadotropin (hCG) and unconjugated estriol (uE3) in combination with a priori maternal age-specific risk of fetal aneuploidy, were used in the so called triple-test to refine patient-specific risk of trisomy 21. Limitations of this kind of serum screening include the limited detection rate (60%) for a 5% false positive rate and timing of the test, i.e., early second trimester.

The value of the use of serum markers pregnancy-associated plasma protein-A (PAPP-A) and free ß-human chorionic gonadotropin (free β-hCG) in maternal serum screening in the first-trimester between 8-14 weeks was determined in a large study in which seven potential serum markers were compared. The test performance of free β-hCG and PAPP-A at 10 weeks of pregnancy were similar to that of the triple-test at 15-22 weeks for a given maternal age [52-54]. The levels of these two potential markers differed between affected and unaffected pregnancies. In affected pregnancies the median level of free beta-human chorionic gonadotropin (free β-hCG) was 1.79 times the median level for unaffected pregnancies. The other marker, PAPP-A, was 0.43 times the normal median [55-58]. In a screening program for

Down syndrome, in which these serum markers were combined with maternal age, and with a cut-off level of 1:300, about 63% of the trisomy 21 affected pregnancies could be detected. Additionally low levels of first-trimester maternal serum PAPP-A are also shown to be predictive of other fetal chromosomal abnormalities, such as trisomy 13 and 18, triploidy and sex chromosome aneuploidy. Moreover, abnormal PAPP-A levels are associated with several adverse pregnancy outcomes including pre-eclampsia, fetal growth restriction, fetal demise and preterm birth among others [59]. These issues are beyond the scope of the thesis.

1.5 First-trimester combined screening.

According the Dutch Health Council the most favorable screening test for Down syndrome in the first-trimester of pregnancy is the combination of the ultrasound measurement of NT with the placentally derived biochemical markers PAPP-A and free β-hCG [60]. The so-called combined test is used as part of screening programme for trisomy 21, but also for other aneuploidies, of which approximately 90% can be identified. The combined test provides an individual risk estimate which is based on the combination of maternal age, NT and maternal serum free β-hCG and PAPP-A concentration at 11+0 to 13+6 weeks' gestation. By this method it was shown that for a false positive rate of 5% the detection rate of trisomy 21 is about 76-90% [61-64], which is superior to the 44% achieved by maternal age alone and 60% by the second-trimester serum screening [54,60]. In many countries world-wide an ever rising number of pregnant women have NT measurements, combined with biochemical tests, for the assessment of risk of Down syndrome [65-67].

The test performance is dependent on a consistent and standardized determination of all parameters and is only applicable in a small time frame. The use of ultrasound to estimate gestational age improves the sensitivity and specificity of maternal serum screening [68,69]. To allow for systematic changes in serum levels of PAPP-A and free β-hCG with changing gestational age, serum concentrations are converted into multiple of the normal median (MoM) at a given gestational age for both affected and non-affected pregnancies. By using the observed estimates of likelihood ratios (LRs) derived from maternal serum levels of the

markers, the individual risk of Down syndrome can be calculated using a mathematical model thereby taking into account the maternal age-related a priori risk of the woman. In fact, using Bayes theorem, the individual risk is obtained from the age-specific prevalence of Down syndrome and the various LRs derived from the observed estimates of the combined test [22].

The overall prevalence of Down syndrome in a given population depends on the distribution of the maternal age, as the probability of trisomy 21 increases with the age of the pregnant woman [70]. If the results of the first-trimester screening show an increased risk for Down syndrome an invasive diagnostic procedure is offered (chorionic villus sampling or amniocentesis) [25].

In January 2007 the national program for prenatal screening for Down Syndrome has been implemented in the Netherlands [71]. First-trimester screening by means of the combined test for the detection of Down syndrome) is made available to all pregnant women (Health Council of the Netherlands) [23]. In the Netherlands the first-trimester screening test was in 2010 extended with a risk assessment for Edward syndrome (trisomy 18) and Patau syndrome (trisomy 13) [72].

Methodological issues of first trimester screening

In both screening and diagnostic settings, the reliability of a test is a very important asset. Reliability is synonymous to reproducibility, repeatability, transferability, precision and consistency. These terms refer the degree of stability when a measurement is repeated under the same conditions. The extent to which a test is reliable is affected by the variation arising from three main sources (1) the examination (laboratory facilities or equipment, including software packages with the various algorithms used for risk calculation; the ultrasound machine), (2) the examiner (skills, concentration and time taken for the examination) and (3) the examined (characteristics). Lack of reliability may result in measurement errors [22].

In order to improve test performance of the combined test, several suggestions have been made, including adjusting the algorithm for ethnicity, mode of conception, smoking and obesity [73-75].

Since two different software approaches are used for risk calculation (i.e., Fetal Medicine Foundation (FMF) and Perkin Elmer) the reference laboratory for The Netherlands, the RIVM lab introduced harmonization of test results in 2008. The first step in harmonization was informing the pregnant woman of her risk of down syndrome at the time of testing rather than her risk at term [76]. This had minor implications for the cut-off level for invasive testing. The second step was reporting risks based on MoMs rather than biochemical findings. This had implications for FMF software-users, who needed upgraded software for risk assessment. Other issues for methodological quality include the required skills for measuring an accurate NT and the quality of ultrasound equipment, in terms of contrast and minimal spacial resolution including image processing algorithms derived from CRL for the calculation of gestational age, display and grey scale settings, the prognostic value of jugular sacs among others.

1.6 Aim of this thesis

In this thesis we present several studies which describe some methodological issues of first-trimester screening for Down syndrome and two other chromosomal abnormalities (trisomies 18 and 13) by using ultrasound markers and maternal serum markers and combinations of these markers in order to provide more insight in the day to day use of the first-trimester screening test. The overall objectives include the study of the effectiveness of first-trimester testing for Down syndrome in daily practice and to determine factors associated with the variation of test performance.

The specific aims of this thesis include the answers to the following questions:

1. Is there a difference in first trimester risk estimates for trisomy 21, as calculated by two different software packages used in the Netherlands?

2. Do the different methods of estimating gestational age affect reliability of first-trimester screening for Down syndrome?
3. What is the impact of laboratory manufacturing errors of the concentration of the pregnancy-associated plasma protein A (PAPP-A) on the test performance of first-trimester screening (FTS) for Down syndrome?
4. What is the prevalence of detectable jugular lymphatic sacs in a setting for first trimester screening of Down syndrome, and is there an influence of jugular? lymphatic sacs on the screening performance for chromosomal abnormalities?
5. Is there an added value in incorporating additional first trimester markers in extensive risk assessment for aneuploidy screening in the first trimester?
6. What is the prognostic value of differences in growth patterns of aneuploid fetuses (trisomy 21, 18, 13 and X0) during the late first trimester when compared euploid and aneuploid fetuses using 3D Virtual Reality (VR)?

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Part 1

The biochemical issues in first-trimester screening

Chapter 2

Estimating gestational age affects test performance of combined first-trimester screening for Down syndrome: a preliminary study

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Abstract

Objective: To establish how different methods of estimating gestational age (GA) affect reliability of first-trimester screening for Down syndrome.

Methods: Retrospective single-center study of 100 women with a viable singleton pregnancy, who had had first-trimester screening. We calculated multiples of the median (MoM) for maternal-serum free beta human chorionic gonadotropin (free β-hCG) and pregnancy associated plasma protein-A (PAPP-A), derived from either last menstrual period (LMP) or ultrasound-dating scans.

Results: In women whit a regular cycle, LMP-derived estimates of GA were two days longer (range -11 to18), than crown-rump length (CRL)-derived estimates of GA whereas this discrepancy was more pronounced in women who reported an irregular cycle, i.e., six days (range -7 to 32). Except for PAPP-A in the regular-cycle group, all differences were significant. Consequently, risk estimates are affected by the mode of estimating GA. In fact, LMP-based estimates revealed ten 'screen-positive' cases compared to five 'screen-positive' cases where GA was derived from dating-scans.

Conclusion: Provided fixed values for nuchal translucency are applied, dating-scans reduce the number of false screen-positive findings on the basis of biochemical screening. We recommend implementation of guidelines for Down syndrome screening based on CRL-dependent parameters of GA.

Introduction

It is widely acknowledged that first-trimester screening is currently a sensitive method of screening for Down syndrome and other chromosomal abnormalities[1-2]. The combined first-trimester screening test is typically based on information of maternal age, the sonographic findings of the nuchal translucency (NT) thickness and by the laboratory findings of two biochemical markers in maternal serum, i.e. free beta human chorionic gonadotropin (free β-hCG) and pregnancy associated plasma protein-A (PAPP-A). The test performance of the NT-measurement is optimal in the short time frame from 11+0 to 14 weeks' gestation, which corresponds with a crown-rump length (CRL) of 45 to 84 mm[1].

The Fetal Medicine Foundation (FMF) introduced the one step clinic for assessment of risk (OSCAR) approach for the detection of Down syndrome and other chromosomal abnormalities by combined biochemical maternal serum and fetal ultrasound testing at a single visit[3-6]. This approach involves a 1-h visit, which includes the testing of the biochemical markers in maternal serum, the NT-examination and subsequent counseling about the individual risk for Down syndrome. An alternative strategy used for the combined first-trimester screening test for Down syndrome is the so-called two-step approach or consecutive combined test which requires two separate visits. For the same false-positive rates, the reported detection rates of the two-step approach are seemingly better than those of the OSCAR approach[7-9]. These studies have demonstrated that the combined first-trimester screening test performs best when the maternal blood sample is taken at 10 weeks[10] and the measurement of NT is performed at 12 weeks' gestation[11]. With the introduction of enhanced sonographic technology it is nowadays also possible to detect of a number of major structural malformations during the first-trimester screening scan[12-13].

Since all three markers in the first-trimester screening test for Down syndrome vary with GA, the estimates of free β-hCG and PAPP-A are converted to a multiple of the expected normal median (MoM) to adjust for the effect of GA, while delta-values for NT are derived from information of CRL at the time of NT measurement. In the two-step approach the ultrasonically derived CRL measurement (dating scan) is not necessarily available at the time

of maternal blood sampling. In fact, the blood sample is sent to the laboratory with information on dating based either on the last menstrual period (LMP) or on sonographic measurement (CRL) at the first or booking visit. The NT is measured at a subsequent visit. This is done simultaneously with a CRL-measurement. The GA thus derived does not necessarily corroborate with that derived from LMP or the dating scan at the booking visit. The aims of this preliminary study are: (1) to determine the difference between the GA estimate on the basis of available information of the LMP and the GA estimate on the basis of the ultrasound CRL-findings at booking, and (2) to assess the impact of these two modes of estimating GA on risk estimates for Down syndrome which are derived from the two-step approach by the combined first-trimester screening test.

Materials and Methods

From November 2005 to January 2006, each eligible woman attending the out-patient clinic at the Division of Obstetrics and Prenatal Medicine at Erasmus University Medical Center, Rotterdam, the Netherlands, was informed about our study. Women were excluded from the study if their index pregnancy was complicated by multiple-pregnancy, diabetes or high blood-pressure. They were also excluded if they had a history of pre-eclampsia, preterm birth or intra-uterine growth restriction. All participants in this study gave informed consent.

At the booking visit, which typically takes place between 8+4 to 13+6 weeks' gestation based on LMP dating, background information of the study participants was collected. The menstrual cycle of 28 (± 4) days was considered regular.

At the booking visit, a blood sample was taken and an ultrasound scan was done to determine CRL. This so-called dating scan was carried out either abdominally (C5-2 probe) or transvaginally (C8-4V probe), using a Philips Envisor C-HD (Philips Medical Division, Eindhoven, the Netherlands). GA based on CRL was determined using the formula obtained from Robinson and Flemming[14]. The NT-measurement, and additional fetal-biometry, was performed at the subsequent visit for the first-trimester screening for Down syndrome, which typically takes place between 11+0 and 14 weeks (CRL 45-84 mm.). NT-measurements were

carried out in accordance with the FMF protocol[15-17], either abdominally (C5-2 probe) or transvaginally (C8-4V probe), using a Philips iU22 (Philips Medical Division, Eindhoven, the Netherlands).

The maternal serum samples were analyzed with the Auto-DELFIA analyzer (Perkin Elmer Life Science, Boston, MA, USA) and commercially available kits. Analysis was done at the laboratory of Star Medical Diagnostic Center in Rotterdam, one of the six regional laboratories for Down syndrome screening in the Netherlands. Maternal-weight-corrected MoM values of the biochemical markers free β-hCG and PAPP-A were derived from the concentrations adjusted for GA at sampling date; this was on the basis of the available information on dating (LMP or ultrasound) and was related to the medians provided by the Dutch reference institute for first-trimester serum screening, Diagnostic Laboratory for Infectious Diseases and Perinatal Screening, National Institute for Public Health and Environment (RIVM), Bilthoven, the Netherlands.

Adjusted for maternal weight, two different MoM-values of the biochemical parameters were calculated. One was calculated from information of the GA derived from the LMP at sampling date, whereas the other was calculated from information of the GA on the basis of CRL-finding at booking. The calculations of the maternal-weight-corrected MoMs of the biochemical markers were done using the LifeCycle-Elips software (Perkin Elmer Life Science, Boston, MA, USA). For the final risk estimation, the ultrasound data and the results of the maternal serum analysis of the free β-hCG and

PAPP-A were combined in the FMF-module in an obstetrical software database (Astraia version 1.17.69, Astraia Software GmbH, München, Germany). The NT was measured at a second visit. The delta-values for NT were derived from information on CRL-findings at the time of NT measurement. The software (Astraia version 1.17.69) used in this study, does not allow input of LMP data to calculate the risk on the basis of NT.

In the Netherlands, the threshold for ‘screen positive’ and for the subsequent offer of invasive diagnostic testing, i.e. chorionic villus sampling (CVS) or amniocentesis is set at 1 in 200 at the time of the risk assessment [18]. Since all participants were recruited from the obstetric

Table 1: General characteristics grouped by women who reported a regular and those who reported an irregular cycle. Differences between group medians are tested for statistical significance by applying the Mann-Whitney U test.

	Regular cycle N=70 Median (min, max)	Irregular cycle N=30 Median (min, max)	M W-U P value
Maternal age (years)	36 (27, 42)	35.5 (25, 42)	0.123
GA at intake (days)	74 (53, 99)	76.5 (63, 96)	0.149
GA intake [CRL] (days)	72 (53, 97)	71.5 (55, 91)	0.491
CRL (dating scan) (mm)	36.4 (14.0, 82.0)	35.5 (15.8, 69.4)	0.480
GA at NT CRL (days)	87 (77, 97)	88 (79, 97)	0.363
CRL at NT (mm)	60.8 (41.3, 81.1)	63.4 (46.5, 82.3)	0.417
NT (mm)	1.6 (0.9, 2.9)	1.7 (1.2, 3.5)	0.335
fβ-*h*CG (ng/ml)	44.7 (7.9, 199.9)	55.5 (18.5, 272.5)	0.118
MoM fβ-*h*CG [LMP]	1.0 (0.2, 4.6)	1.5 (0.4, 9.0)	0.002*
MoM fβ-*h*CG [CRL]	0.9 (0.2, 4.3)	1.2 (0.4, 9.4)	0.020*
PAPP-A (mU/l)	866 (125, 4319)	889 (224, 4591)	0.285
MoM PAPP-A [LMP]	1.0 (0.1, 5.1)	0.9 (0.2, 3.4)	0.545
MoM PAPP-A [CRL]	1.0 (0.2, 3.4	1.1 (0.3, 3.2)	0.323

* Statistically significant at the level $p < 0.05$, M-W U; Mann-Whitney U test, GA; Gestational Age, CRL; crown-rump length, NT; Nuchal Translucency, β-*h*CG free beta-human chorionic gonadotropin, PAPP-A; Pregnancy associated plasma protein-A, [LMP]; dating on the basis of last menstrual period, [CRL]; dating on the basis of crown-rump length measurement, MoM; Multiple of the Median.

outpatient-clinic of our university hospital, a complete follow-up of the pregnancy and delivery was available.

A distinction was made between women who were reported to have a regular cycle and those reported to have an irregular menstrual cycle. To assess whether the observations from these two groups came from the same distribution, the Mann-Whitney U test was applied. Like the paired differences between dating on the basis of LMP and CRL, the biochemical-variables expressed as MoM-values on the basis of these two dating methods are presented as medians and ranges.

The statistical analyses were performed using the SPSS statistical package for Windows release 15.1 (SPSS Inc. Chicago, IL). Statistical significance was defined as a p value < 0.05.

Results

In our preliminary study we included a total of 100 consecutive women with a viable singleton pregnancy. Median maternal age of all the women in the study population was 36 years (range 18 to 43 years). The study population was mainly white, i.e. 85% Caucasian, 8% Asian and 7% Afro-Caribbean. Seventy percent of them reported having a regular cycle, whereas the remainder had an irregular cycle. The background characteristics of the two groups are shown in Table 1. Except for free β-hCG there were no statistically significant differences in background characteristics between the two groups. The median GA at delivery was 279 days (range 238-297 days) or 39+6 weeks (range 34+0 – 42+3 weeks) and the median birth weight was 3568 grams (range 2010-5080 grams). None of the pregnancies was complicated by pre-eclampsia or diabetes. There was one case of prenatal diagnosed placental confined trisomy 16[19] and one case of a postnatal diagnosis of atresia of the pulmonary artery.

Table 2 presents the paired differences resulting from the two dating methods for GA, and the MoM-values for the free β-hCG and PAPP-A. The differences are summarized as medians and ranges for both regular and irregular menstrual-cycle groups.

Table 2

The paired differences of gestational age and the MoM values for the free β-hCG and PAPP-A as determined by dating on the basis of LMP and CRL (dating scan). They are presented as medians and range in the regular and irregular menstrual cycle groups.

	Regular cycle N=70		Irregular cycle N=30	
	Median (min, max)	P value	Median (min, max)	P value
GA[CRL]– GA[CRL] (days)	2 (-11, 18)	0.006*	6 (-7, 32)	0.001*
Free β-*h*CG MoM[LMP] – MOM[CRL[	0.06 (-0.86, 1.08)	0.008*	0.18 (-0.51, 2.23)	0.003*
PAPP-A MoM[LMP] – MOM[CRL]	-0.14 (-0.93, 3.32)	0.141	-0.23 (-2.18, 0.76)	0.006*

* Statistically significant at the level of $p < 0.05$ as determined by the Wilcoxon signed rank test. GA; Gestational Age, MoM; Multiple Of the Median, free β-*h*CG free beta-human chorionic gonadotropin, PAPP-A; Pregnancy Associated Plasma Protein-A, [LMP]; dating on the basis of last menstrual period, [CRL]; dating on the basis of crown-rump length.

In women who reported to have a regular cycle, there was a small but statistically significant difference of two days (range -11 to 18); women who reported an irregular cycle had a more pronounced difference of six days (range -7 to 37). The scatter plot showing GA on the basis of LMP vs. the GA on the basis of CRL is presented in Figure 1. As the expected normal-medians for the biochemical markers are specific to GA, the differences resulting from the two dating modes obviously influence the estimates of the MoM-values and the subsequent risk estimates. In the irregular-cycle group the MoM of the free β-hCG is statistically significantly higher for both LMP-dating and CRL-dating compared to the regular cycle group.

Table 3.

Agreement between test results for the risk to detect a fetus with Down syndrome in a group of 100 pregnant women on the basis of dating from LMP and CRL.

Test results*		
CRL based	LMP based	N
-	-	89
-	+	6
+	-	1
+	+	4

* The test is considered positive if the calculated risk is greater than 1 in 200.

Table 3 shows the potential impact on risk-estimation for Down syndrome. Four women were 'screen positive', independent of the method used.

Calculations of the MoM-values for free β-hCG and PAPP-A on the basis of ultrasound dating by CRL at booking showed that five women were 'screen positive'. If dating was on the basis of LMP, ten women were 'screen positive'. This result indicates that the method of dating has a considerable effect on the test result of the combined first-trimester screening test for Down syndrome.

Figure 1.

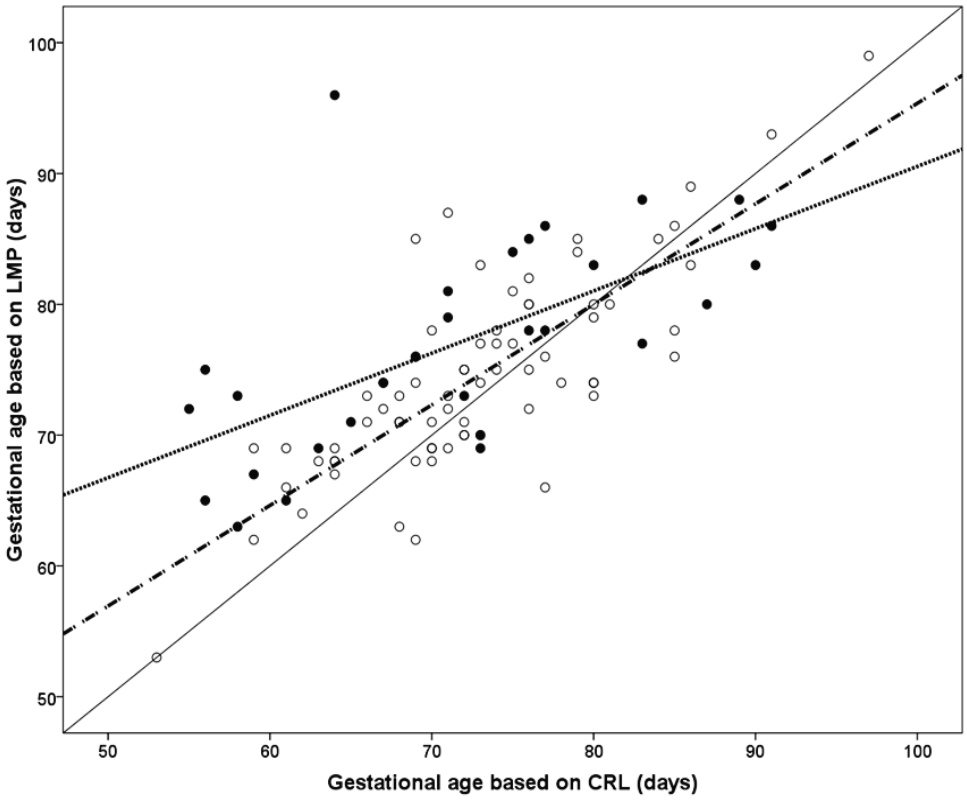

Figure 1: Scatter plot is showing gestational age (GA) on the basis of last menstrual period (LMP) versus gestational age on the basis of crown-rump length (CRL) using the formula obtained from Robinson and Flemming 1975. The diagonal line represents the line of equality. Open circles (○) and dotted linear regression line (----) represent the group that reported an irregular menstrual cycle: GA = 0.769 · GA-CRL (days) + 18.5 (R2 = 0.622, p<0.001) and filled circles (●) and dotted /dashed linear regression line (▪—▪) represent the group that reported a regular menstrual cycle: GA = 0.476 · GA-CRL (days) + 43.9 (R2 = 0,406, p<0.001).

Discussion

This preliminary study was undertaken to evaluate the optimal mode of GA assessment for the calculation Down syndrome risk by first-trimester screening using a two-step approach. This study shows that correct dating of the pregnancy is crucial. If the booking visit is not accompanied by an ultrasound dating-scan, erroneous risk assessment might result, undermining the test performance of the two-step approach. Considerable differences in pregnancy dating, ranging from -11 to 18 days, were found in our study population (Figure 1), even in women who reported a regular menstrual cycle.

Because the missing or the lack of reliable information, the LMP cannot be used in about 40% of the women [20]. Hence, dating by means of ultrasound in early pregnancy has become an accepted way of estimating GA. In fact, recent results from studies involving large cohorts of pregnant women support the hypothesis that early dating scan provide more reliable information on GA than the first day of the LMP [21-22]. The CRL has been proven to be a reliable measurement for determining the GA of a chromosomal normal fetus [23-24].

In the Netherlands first-trimester screening is the prevailing risk assessment strategy for Down syndrome [25] where likelihood ratios are obtained from delta-NT findings which are derived from CRL-dating at the time of NT measurement. As CRL does not necessarily reflects true GA, the risk calculation derived from CRL at NT measurement, might be wrong. Theoretically, this is applicable for fetuses with chromosomal abnormalities, as they are typically growth restricted, even in the first trimester of pregnancy [19, 26]. Given an absolute value of NT-thickness, the smaller CRL in fetuses with chromosomal abnormalities will generate potentially a higher risk if gestational length is spuriously adjusted for the CRL-finding. In fact spuriously adjusting the GA on the findings of a “small” CRL, a fixed NT will have a positive impact on the detection rate. It is well established that the interpretation of absolute values of NT is CRL dependent.

As the values of NT, free β-hCG and PAPP-A are GA-specific, accurate assessment of GA is essential for the proper interpretation of the combined first-trimester screening test. In this context, national guidelines should be set for the choice of the reference curve and for the

accurate measurement of CRL, irrespective of GA. Even in a small highly developed country, such as the Netherlands, there is neither standardization in the software used to calculate risk for Down syndrome [27] nor in CRL reference curves [28]. From the findings of our study we recommend the implementation of guidelines for a Down syndrome screening that is based on CRL rather than LMP for GA [28]. We postulate that reduction in 'screen positive' test results might considerably decrease unnecessary invasive procedures, and hence in iatrogenic loss of pregnancy when first-trimester screening for Down syndrome is determined explicitly on information of CRL. However, our study sample was too small to substantiate this notion. For this reason, it is important to estimate the test performance of this screening policy with large population-based data.

In conclusion, provided fixed values for NT are applied, dating scans reduce the number of 'screen-positive' findings on the basis of biochemical screening. We recommend implementation of guidelines for Down syndrome screening-policy, which is based on CRL-dependent rather than LMP-dependent parameters of GA.

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Chapter 3

Combined first trimester screening for trisomy 21:

lack of agreement between risk calculation methods

Peter N.A.C.M. van Heesch, Peter C.J.I. Schielen, Mark F. Wildhagen, Karin den Hollander, Eric A.P. Steegers, Hajo I.J. Wildschut

J Perinat Med. 2006; 34(2): 162-165

Abstract

Objective: To call attention to differences in first trimester risk estimates for trisomy 21, as calculated by two different software packages.

Methods: A total of ninety-four pregnant women who had a first trimester risk assessment for trisomy 21 that was based on maternal age, biochemical analysis and a nuchal translucency (NT) measurement. Two commonly used software packages were used for the estimation of individual risks (i.e. Wallac-Perkin-Elmer®software and Fetal Medicine Foundation® software).

Results: Risk estimates derived from each software programme were strikingly different. In each case the discrepancy in reported magnitude of risk resulted from disparities between the two calculation methods for the assessment of the individual risk for trisomy 21. The disparities in risk estimates can be explained by significant differences in reported likelihood ratio's for biochemical analyses ($p=0.01$), NT measurements ($p<0.0001$) and both screening parameters combined ($p=0.003$).

Conclusion: It is illustrated that the lack of agreement between these risk calculation methods could give rise to major counselling problems. In order to avoid confusion, there is a need for estimating individual risks of trisomy 21 in a standardized way. It is proposed to select a set of parameters that have a proven track record as judged by detection rates and false positive rates and then use that set exclusively, while simultaneously monitoring its performance.

Introduction

Worldwide an ever-increasing number of women have nuchal translucency (NT) measurements in their pregnancy, often together with biochemical tests, for the assessment of risk of trisomy 21 [1-4]. While policymakers, insurance companies and medical organizations in many countries are still reluctant to endorse screening of all pregnant women, patient autonomy and self-determination has led to the situation where almost every pregnant woman in Europe will have the opportunity to be screened for trisomy 21, either because it is offered or because she asks for it. Women who decide to take up non-invasive testing will be informed about their individual risk of trisomy 21. Information on the risk estimate could help them, and their partners, to make decisions about the need for invasive diagnostic testing (amniocentesis or chorionic villus sampling) in order to obtain the definite fetal karyotype. Women's decisions to take up invasive testing depend on the magnitude of the reported risk [3]. In fact, there is a complete trust among caregivers and the public in the reliability of risk calculations that are produced on the basis of the various parameters of non-invasive testing, such as maternal age, gestational age, nuchal translucency (NT) measurements and biochemical serum markers. However, the individual perception of what may constitute 'high risk' of 'low risk' varies greatly since the individuals' assessment of risks is mostly determined by emotions rather than facts [5]. In the Netherlands, a screening-derived risk estimate of $\geq$1 in 200 [6] (i.e., odds for an infant with trisomy 21 born alive at term) is used as an objective criterion for classifying women as 'high risk'; invasive testing for fetal karyotyping is subsequently offered. This cut-off level, however, could have a great emotional impact on the woman as was illustrated recently by French authorities who reported that women have been known to seek pregnancy termination without waiting for the confirmation of the possible fetal - chromosomal - defect, a practice called "precautionary eugenics" (eugénisme de précaution) [7]. Alternatively, when the combined first trimester screening test result is compatible with a low risk of trisomy 21, women may decide to refrain from invasive testing, thereby avoiding iatrogenic fetal loss.

For individual risk estimations several software programs are commercially available. In our department, risk estimates derived from biochemical parameters are given - for logistic reasons - by the National Institute for Public Health and the Environment (RIVM), using 1T-risks version 1.7 Wallac-Perkin-Elmer® software, while risk estimates derived from NT findings are given by Fetal Medicine Foundation (FMF®) software, which is directly accessible in our department. Individual numerical first trimester risk estimates, however, are not always unequivocal as we have witnessed several times in our department. At times, the combined test results reported by the RIVM yielded a combined risk estimate that was below the cut-off value for invasive testing, while that derived from the FMF®software was above this threshold value or vice versa. Both caregivers and couples felt at loss with these discrepant test results. Concern about the lack of agreement between the risk estimation methods has led us to analyse differences between the reported findings derived from the two software packages.

Methods

To investigate the differences in risk estimates derived from two widely used software packages, risk of trisomy 21 was assessed in a total of 94 women who consecutively visited the out-patient department of obstetrics and prenatal diagnosis at our University Hospital. Each woman had a viable, singleton pregnancy. Risk estimates were derived in the first trimester of pregnancy from the woman's age at the time of testing, levels of first trimester biochemical serum markers (i.e., maternal serum free beta-human chorionic gonadotropin (fßhCG) and pregnancy-associated plasma protein-A (PAPP-A)), NT measurements, gestational age and relevant medical history. Blood sampling was done from 8 to 14 week's gestation, in almost all cases prior to NT measurement in order to be able to discuss the results together with the findings of the NT measurement. Trained sonographers who all were certified by the FMF conducted NT measurements on ATL 3000 machines. All NT measurements were performed between 11 and 14 weeks of gestation.

The compared software programmes included 1T-risks version 1.7 Wallac-Perkin-Elmer®, that is based on an algorithm derived from data published by Cuckle & Van Lith [8] and Wald and Hackshaw [9], and the FMF® software that is based on an algorithm derived from data published by Spencer et al [10] and Snijders et al. [11]. The Wilcoxon signed-rank sum test was used to compare the interrelations between the two sets of observations (SPSS inc. version 12.01, Chicago, Ill. USA). P-values below 0.05 were considered statistically significant.

Results

Figure 1 illustrates that the maternal age specific a priori risks derived from both software packages are similar. However, as is also demonstrated in Figure 1, risk estimates based on the combination of maternal age, biochemical findings and NT measurements were strikingly different. In fact, the combined risks derived from Wallac-Perkin-Elmer®software are in general lower than those derived from the FMF®software. This is especially true when the estimated risks are between 1 in 1000 and 1 in 100.000, thus not in the vicinity of any commonly used risk threshold value for invasive testing. To investigate the source of these differences, the contributions of the biochemical analyses and the NT measurements were investigated by calculating the likelihood ratios (LRs) with both software programmes. Figure 2 shows that the LRs based on the NT measurements using the Wallac-Perkin-Elmer®software are significantly higher than the LRs derived from the FMF®software ($p<0.001$). Moreover, it is demonstrated that the FMF® software truncates LRs based on NT at 0.12, a point where the Wallac-Perkin-Elmer®software gives higher LRs. In our study, the lowest LR based on NT as derived from the Wallac-Perkin-Elmer®software is 0.16. Figure 3 shows that the LRs based on the biochemical parameters using Wallac-Perkin-Elmer®software are statistically significantly lower than those derived on FMF® software ($p=0.01$).

Figure 1

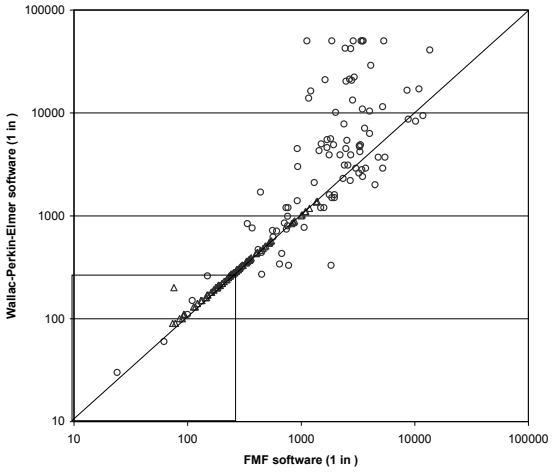

Figure 1: Comparison of the a priori risks (Δ) and the combined risks (○) derived from the FMF and Wallac-Perkin-Elmer software packages (n=94). The solid line represents points of equal risk. The box illustrates a risk of 1 in 250 (the threshold value for invasive testing).

Figure 2

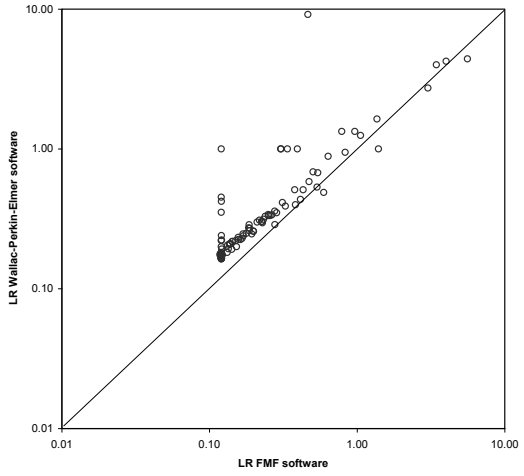

Figure 2: Comparison of LRs based on NT measurements as calculated with the FMF and Wallac-Perkin-Elmer software packages (n=94). The solid line represents points of equal LRs.

Figure 3

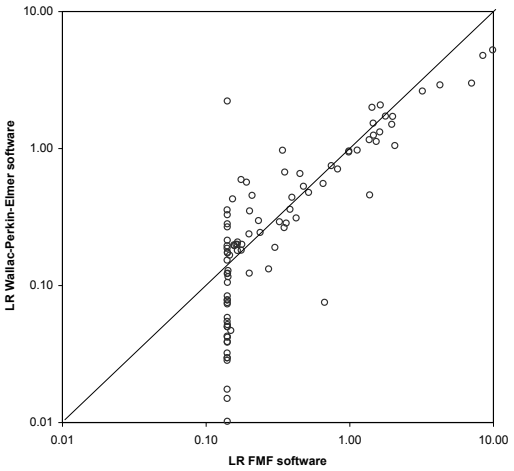

Figure 3: Comparison of LRs based on biochemical analysis of PAPP-A and fßhCG as calculated with the FMF and Wallac-Perkin-Elmer software packages (n=94). The FML truncates LRs based on NT at 0.12. The solid line represents points of equal LRs

Figure 4

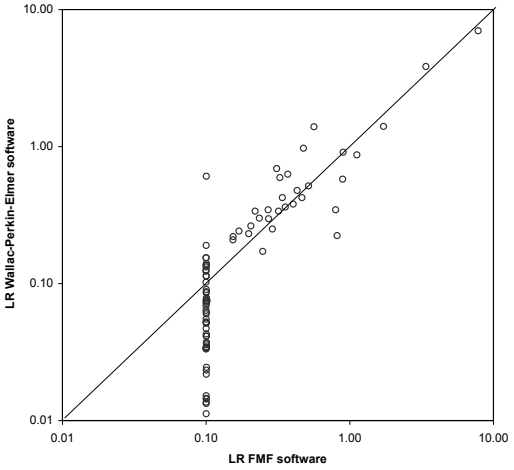

Figure 4: Comparison of LRs based on the combined (i.e., multiplied) biochemical parameters and NT (n=94). Unlike the Wallac-Perkin-Elmer software, the FMF software applies an extra truncation at 0.1 for the multiplied LRs. The solid line represents points of equal LRs.

In fact, the FMF®software truncates LRs based on the biochemical parameters at 0.14. At this truncation point the Wallac-Perkin-Elmer®software shows considerably lower LRs values. In Figure 4 the LRs based on the combined (i.e., multiplied) biochemical serum markers and NT as derived from each software package are compared. Again, there are statistically significant differences between Wallac-Perkin-Elmer®software and FMF®software (p=0.003), whereby LRs derived from the Wallac-Perkin-Elmer®software are generally lower than those derived from The FMF®software. This may be due to an extra truncation at 0.1 for the multiplied LRs, as applied by the FMF® software and not by the Wallac-Perkin-Elmer®software.

Discussion

Much time is spent by caregivers on counselling women about their individual risk for trisomy 21 as derived from non-invasive testing in the first trimester of pregnancy. Counsellors, thereby, typically rely on the reported numerical risk estimates. This information is communicated to their parents often in a sterile and 'matter-of-fact' way [12]. Most caregivers and parents, however, have difficulty in appreciating the true magnitude of the woman's risk of having a child with trisomy. Conflicting or poorly presented statistical information may cause erroneous communication of risks, with serious consequences such as undue parental anxiety or unwarranted invasive procedures [5, 12-13]. This is not restricted to the first trimester of pregnancy. Discordances in individual risk estimates were also observed in women screened for trisomy 21 by second trimester markers [14]. Software designs, and its underlying algorithms, have a major impact on test performance in terms of sensitivity, specificity, positive and negative predictive values. Most centres, however, are unsure of what method for calculating risks of trisomy 21 is used in their computer software packages [15]. As a result, information on the test performance is often lacking.

This study shows that with the same screening parameters, marked disparities were observed between numerical risk estimates derived from the FMF software package and those derived from Wallac-Perkin-Elmer®software. These disparities are mainly explained by

the truncation limits. While the discussion on risk estimation methods is lively and ongoing [16-19], none of the published truncation limits have been seriously challenged [20]. Truncation limits on both the MoM-values and the combined LRs may cause considerable disparities in the reported risk estimates, especially when the screening parameters move into the tails of the distributions. Since truncation can have such an important influence on the risk calculation, the software companies should have a transparent policy regarding information on truncation limits and reasons for their application. The means and standard deviations of the screening parameters for the normal and trisomy 21 populations, the correlation coefficients between screening parameters and weight correction equations can also have a considerable effect on LRs and subsequent risk estimations. Currently, manufactures constantly update software packages without fully informing caregivers who are involved in prenatal counselling about the changes made in their new releases. This is also true for FMF® and Wallac-Perkin-Elmer®. Recently, both manufacturers issued updates of their first trimester software packages without providing clear information about underlying algorithms and subsequent test performance. Hence, both the consumer and the caregiver rely on the diligence of the software developers.

As stated by Muller et al. [14], it is important that the different manufacturers provide detailed information about the test performance of their software packages, in terms of detection rates and false positive rates, preferably as a function of maternal age. For both psychological and legal reasons, caregivers should incorporate such information when counselling pregnant women who consider non-invasive testing for trisomy 21. From the public health point of view, non-invasive testing for trisomy 21 should be preferably done in a standardized way, thereby incorporating a set of parameters that have a proven track record in terms of detection rates and false positive rates, and then use that set exclusively, while simultaneously monitoring its performance.

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Chapter 4

Erroneous production of PAPP-A kits: the impact of a downward shift in PAPP-A concentration on the test performance of first-trimester combined screening for Down syndrome

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Hajo I.J. Wildschut

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Abstract

Objective: to evaluate a 20% downward shift in the pregnancy-associated plasma protein A (PAPP-A) concentration on the test performance of first-trimester screening (FTS) for Down syndrome (DS) following a flaw in the production of PAPP-A kits on FTS for DS.

Methods: a retrospective re-evaluation of PAPP-A in stored sera. Including criteria were a maternal-weight-corrected PAPP-A MoM value $\leq$ 0.9 and a biochemical risk of DS $\geq$ 1: 200 at the time of testing.

Results: Of the 3100 women, 473 (15%) fulfilled the inclusion criteria. After combining the biochemical risk based on the incorrect PAPP-A values with nuchal translucency findings, an increased risk for DS was initially found in 107 women [false positive rate (FPR): 3.1]. Eighty-two (77%) out of 107 women opted for invasive testing. Following re-analysis of PAPP-A, the biochemical risk and the combined risk were statistically significantly different from the initial risk estimates ($p< 0.001$.). We noticed that 25 women (30%) had invasive testing while this was unjustified given the re-analysed PAPP-A.

Conclusion: Erroneous PAPP-A kits resulted in an increase of the FPR by 1.2%. There were no reports of iatrogenic miscarriage. The occurrence of this problem reaffirms the importance of continuous monitoring of quality in FTS.

Introduction

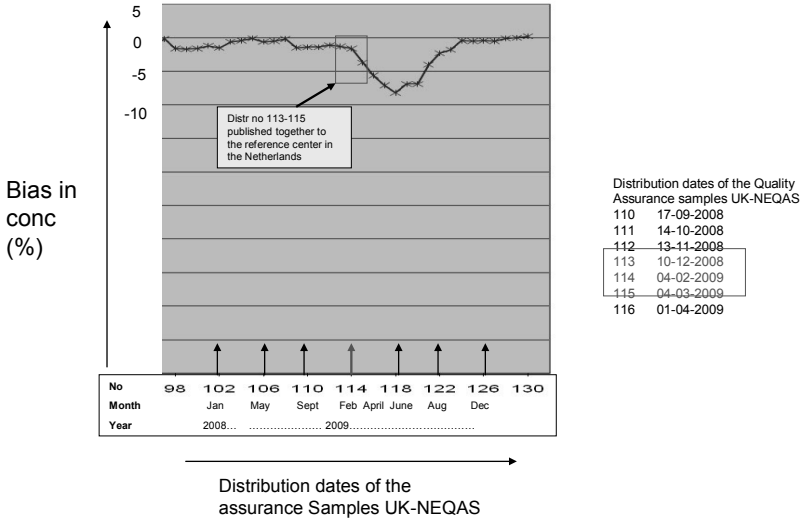
In January 2007 the national program for prenatal screening for Down Syndrome (DS) has been implemented in the Netherlands [1]. For this purpose, the first-trimester combined screening (FTS) test was advocated. This test encompasses the assessment of two biochemical markers in maternal serum, i.e. free beta human chorionic gonadotropin (free β-hCG) and pregnancy-associated plasma protein A (PAPP-A), and the sonographic assessment of the fetal nuchal translucency (NT). Combining these three markers, together with maternal age, 76-91 % of the pregnancies with trisomy 21 can be detected with a 3-7% false positive rate [2-5]. 'Screen positive' is defined as an increased risk of DS at the time of testing (≥ 1:200) [6].

The seven screening laboratories in the Netherlands participate in the UK National External Quality Assessment Service (UK-NEQAS Edinburgh, United Kingdom) first trimester combined test quality assurance scheme. During early April 2009 the UK-NEQAS reported a downward shift in concentrations of PAPP-A over the previous months (Figures 1 and 2). In the same period health care providers from two sonographic prenatal screening centres in the region expressed their concern about the increase in number of screen-positive findings following combined risk calculation they encountered in the last months.

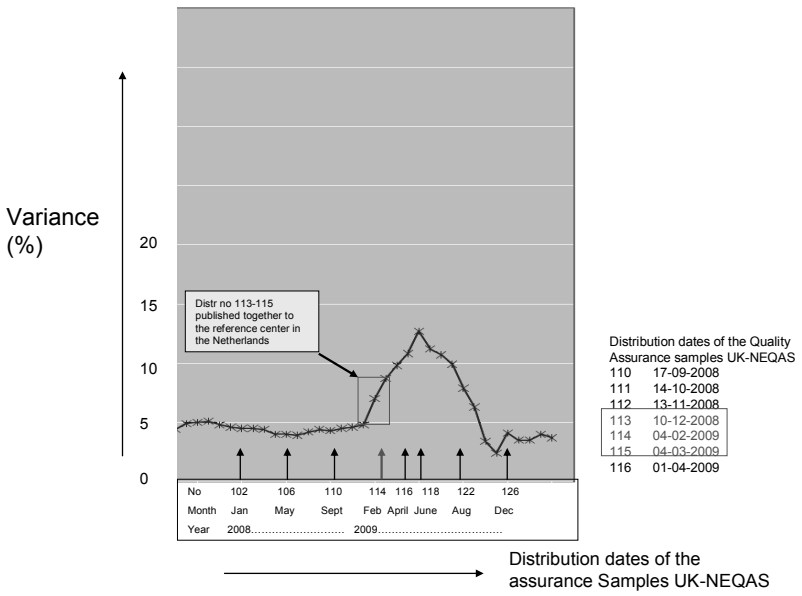
Our regional laboratory, Star Medical Diagnostic Centre (Star-MDC), could confirm the downward shift in concentrations with an increase in percentage of monthly positive findings (18.0% compared to 13.7% over 2008). The multiple of the median (MoM) in de study period was 0.85 as compared to 1.01 in 2008. Because of a 20% difference in concentration, a retrospective study was performed.

In the present retrospective study, we report the impact of the downward shift of the PAPP-A concentration on the test performance of the FTS test. The use of the erroneous PAPP-A kits in the study period (December 2008-April 2009) resulted in lower MoM values for maternal weight-corrected PAPP-A and, subsequently, to an unduly increased risk of DS in the subset of women. In the Netherlands invasive diagnostic testing, that is chorionic villus sampling or amniocentesis, is typically offered following a 'screen-positive' test result of the FTS test [6].

Figure 1: PAPP-A accuracy in the period 2008 – 2010
(UK-NEQAS international data of the Delfia Xpress)



Firgure 2: PAPP-A precision in the period 2008 – 2010
(UK-NEQAS international data of the Delfia Xpress)



Invasive diagnostic testing, however, is associated with an increased risk of miscarriage [7-12]. In this context, in the present study, the number of miscarriages as a result of (unjustified) invasive diagnostic testing was also analysed.

Materials and Methods

The study period was identified as the period from 26 November 2008 to 14 April 2009. During the present evaluation period, 3100 stored maternal serum samples were analysed by the laboratory of Star-MDC using the DELFIA Xpress® analyzer (PerkinElmer, Turku, Finland) in May and June 2009.

The study group was defined on the basis of a biochemical risk of Down syndrome $\geq$ 1: 200 at the time of testing [6] and a maternal-weight-corrected PAPP-A MoM value $\leq$ 0.9. Only first-trimester sera that were included in this study group during the described evaluation period were re-analysed.

Back-up procedure in case of a reagent recall

In the Netherlands there are seven officially registered laboratories for first-trimester biochemical screening, including the laboratory of Star-MDC, which is responsible for the southwest region of the Netherlands. In case of a reagent recall, there is an adequate back-up situation in the Netherlands. Half of the screening laboratories uses the AutoDELFIA® analyser (PerkinElmer, Turku, Finland), while the other half uses the DELFIA Xpress® analyzer (PerkinElmer, Turku, Finland) [13]. No downward shift had been observed for the PAPP-A kits used on the AutoDELFIA® analyzer. The Research & Development department of PerkinElmer (Turku, Finland) released a new PAPP-A kits in the second half of April 2009.

Re-analysis process

The re-analysis of maternal serum samples of the study group were performed in 2 rounds: Firstly, between 14 and 28 of April 2009, the Dutch reference institute for first-trimester serum screening, Diagnostic Laboratory for Infectious Diseases and Perinatal Screening,

National Institute for Public Health and Environment (RIVM) re-analysed the concentrations of PAPP-A in the most recently (from 1 March to 14 April: <6 weeks old) stored maternal serum samples. Secondly, in May and June 2009 the Star-MDC laboratory re-analysed the samples from 26 November 2008 to 1 March 2009 with the new released and qualified PAPP-A kits.

Recalculated MoM values for PAPP-A and the corrected biochemical risk-assessment (based on PAPP-A and original free β-hCG) were reported to the collaborating sonographic centres for prenatal screening. These centres were asked to recalculate the FTS risk of Down syndrome with the original findings of the NT and the corrected biochemical risk of DS. The quality of the re-calculation process was assessed on a daily and monthly basis by calculation of the medians of the MoM PAPP-A.

Information process

All women in the study period initially having an increased risk (≥1:200) for DS based on a (probably falsely) increased biochemical risk were informed by regular mail about their re-calculated combined risk by the sonographic screening centres. In close cooperation with Star-MDC, the Foundation for Prenatal Screening in the southwest of the Netherlands made two templates for letters of information (The Foundation for Prenatal Screening in the southwest of the Netherlands is the regional centre of quality assurance, evaluating and monitoring of the national prenatal screening program.). One letter was intended for women who had received an increased risk after the FTS test and who remained 'screen positive' after re-analysis and re-calculation. The other letter was intended for those who initially had an increased risk, but who became 'screen negative' (<1:200) after re-analysis and re-calculation. In theory, the latter group could have had an unnecessary invasive procedure. Complementary and extensive counselling was offered to women in both groups. This plan of action was carried out by Star-MDC under supervision of the Foundation for Prenatal Screening in the southwest of the Netherlands, and in cooperation with the Division of

Obstetrics and Prenatal Medicine at the Department of Obstetrics and Gynaecology, and the board of directors of the Erasmus University Medical Center in Rotterdam.

Statistical analysis

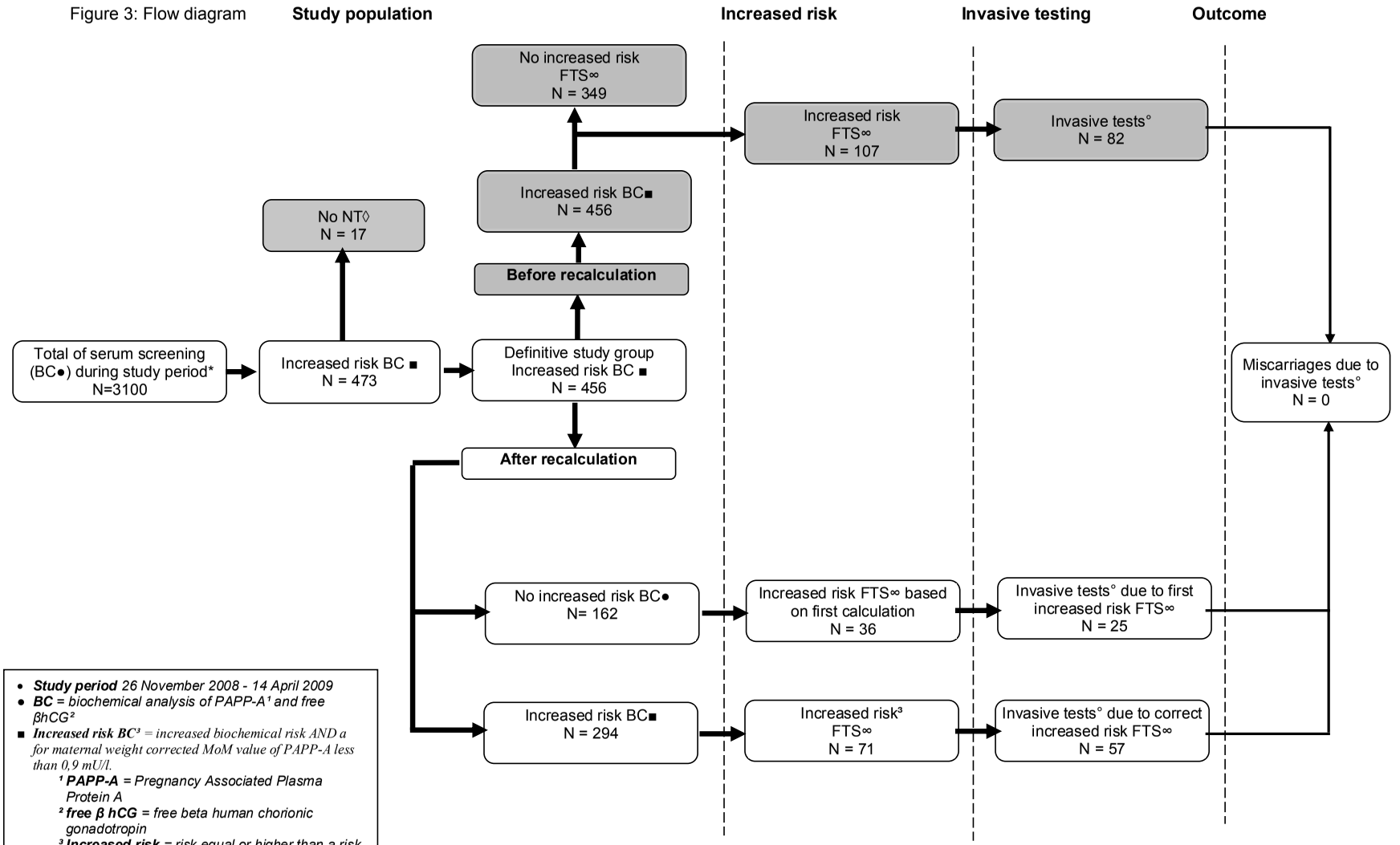
The paired t-test was applied to test whether the characteristics of these two groups were from the same population. The biochemical variables expressed as MoM-values are presented as medians and ranges. Statistical analyses were performed using the SPSS statistical package for Windows release 15.1 (SPSS Inc. Chicago, IL). Statistical significance was defined as a *p*-value < 0.05.

Results

With the rejected lot numbers of PAPP-A kits, we found a downward shift in PAPP-A concentration of -21.5% [95% confidence interval (CI): -43.2% to 0.3%], while with adequate lot numbers of the PAPP-A kits, we found a downward shift in PAPP-A concentration of -3.6% (95% CI: -4.9 to -1.8%). This shift was confirmed by the reported results of the UK-NEQAS. Under normal conditions, the ratio of PAPP-A concentration reported by Star-MDC / RIVM was 0.93 ± 0.05, while in the evaluation period, December 2008 to April 2009, the ratio was 0.73 ± 0.04.

In the study period 473 (15%) out of 3100 test results were identified for re-analysis, re-calculation and evaluation (Figure 3). In this group 17 women did not have an NT measurement and thus did not have the complete FTS test. Nine women had a miscarriage before NT measurement and seven women declined the option of an NT measurement during the ultrasound investigation and the FTS test was not done. In one case NT measurement was not performed because a serious fetal anomaly (Siamese twins) was diagnosed. Hence, a total of 456 women were included in our study. The study population was mainly white, that is 85% Caucasian, 8% Asian and 7% Afro-Caribbean. Median maternal age was 35.4 years (range 21 to 45.5 years). Of the 456 included women having had an FTS test during the study period, 107 (FPR 3.1) had an increased risk of DS after

Figure 3: Flow diagram



- ***Study period*** *26 November 2008 - 14 April 2009*
- ***BC*** *= biochemical analysis of PAPP-A¹ and free βhCG²*
- ■ ***Increased risk BC***[3] *= increased biochemical risk AND a for maternal weight corrected MoM value of PAPP-A less than 0,9 mU/l.*
  - [1] ***PAPP-A*** *= Pregnancy Associated Plasma Protein A*
  - [2] ***free β hCG*** *= free beta human chorionic gonadotropin*
  - [3] ***Increased risk*** *= risk equal or higher than a risk of 1:200*
- ◊ ***NT*** *= Nuchal translucency*
- ∞ ***FTS*** *= First-trimester combined screening test*
- ° ***Invasieve test*** *= chorionic villus sampling or amniocentesis*

combining biochemical screening findings with the findings of NT and maternal age. Eighty-two (77%) out of 107 women opted for an invasive procedure, that is, chorionic villus sampling or amniocentesis. Apart from the 82 invasive procedures on the basis of the 'increased risk' (≥1:200) after the FTS test, another 17 invasive procedures were done in this group for other reasons, such as advanced maternal age (≥ 36 years) and abnormal fetal sonographic findings. After the PAPP-A re-analysis and the risk re-calculation, statistically significant differences were found between the PAPP-A MoM values, biochemical risk and the corrected risk after the FTS test (Table 1). The group of the initially increased combined risk of DS (n = 107) (FPR 3.1) was reduced to 71 (FPR 1.9) women with an increased risk based on the corrected measurements. All chromosome abnormalities (n=18) were confined to the group that remained having an increased risk or had a sonographic detected fetal malformation. With the re-calculated combined risk for DS, probably fewer women would have opted for an invasive procedure. So the effect of the erroneous PAPP-A shift was to increase the FPR by 1.2% (95% CI: 0.82 to 1.61) or 38% (95% CI: 28.1% to 48.4%) that is, an extra 12 women in every 1000 with an unaffected pregnancy were offered an invasive test. In the study period we did not encountered fetal loss due to an invasive procedure.

Table 1. Results of the re-analysis and recalculation

	Before reanalysis / recalculation **Median** (min-max) (P25-P75)	**After reanalysis / recalculation** **Median** (min-max) (P25-P75)	***p* value** paired t-test
PAPP-A MoM	**0.35** (0.04-0.90) (0.28-0.60)	**0.57** (0.05-1.20) (0.38-0.75)	**< 0.001***
BC risk 1:	**97** (5-200) (50-140)	**173** (5-700) (85-240)	**< 0.001***
FTS risk 1:	**541** (2-2401) (227-831)	**982** (2-4448) (373-1371)	**< 0.001***

Study period: *26 November 2008 - 14 April 2009*
Study group: *defined as PAPP-A ≤ 0.9 MoM and biochemical screening risk of ≥ 1:200*
PAPP-A: *pregnancy-associated plasma protein A*
MoM: *multiple of the median*
BC: *Biochemical screening of maternal serum (free β-hCG and PAPP-A).*
NT: *nuchal translucency.*
FTS: *First-trimester combined screening test (BC and NT)*
* Statistically significant at the level p < 0.05.

Discussion

In this article, we describe the impact on FTS performance of an approximately 20% downward shift in the PAPP-A concentrations due to the erroneously produced PAPP-A kits used for the FTS test for DS. We evaluated the potential clinical consequences of this impact. Low PAPP-A MoM values may result in more 'screen-positive' test results after FTS test. Such an increase in screen-positive test results was noticed by the prenatal screening centres and the UK-NEQAS reports were indicative for the quality assessment by the laboratory in detecting the down shift in PAPP-A concentration. Counteractive actions were taken by the laboratory in re-analysis of stored blood samples, which were identified with a PAPP-A concentration that derived 10% of the standard of 1.0 MoM and with a biochemical-based risk that could influence the combined risk in a negative way. The first round of re-analysis was performed by the RIVM. The second round of reanalysis was carried out by the Star-MDC laboratory as soon as the production problem of adequate PAPP-A reagents was resolved by PerkinElmer. Three weeks after reporting the shift to the head office of PerkinElmer information about the new lot number was available.

In May 2009, the quality assurance activities of PerkinElmer formally stated in a letter that the FPR was increased due to a level shift downward in the measurement of the PAPP-A concentration. PerkinElmer confirmed that this level shift was not observed in the Maternal Health Early Controls, which have been used with the PAPP-A reagent.

During corrective and preventative action activities, PerkinElmer identified the PAPP-A concentrations to be 7 to 24% lower than normal, the shift being more pronounced at lower concentrations. The primary cause was identified to be the DX-coated wells and one lot of a raw material used in the coating process. As a consequence PerkinElmer has taken measures to prevent recurrence by intensified testing of the raw material before taking new lot numbers of PAPP-A kits into use, and continue to develop incoming inspection of the raw material. Since then, PerkinElmer uses additional controls to ensure that the PAPP-A kits currently released do not have this problem. After this incident, during five days, four times a day, a sample from a pool of high levels and from a pool of low levels of PAPP-A and free β-

HCG were testedeven as the daily routine control samples (three levels). The results were compared with the results of the previous lot number that has been proven to be reliable. New reagent kit lots are tested in the laboratory first, using pools of both high and low level of PAPP-A and free β-HCG, before using them in screening.

This study underscores that a substantial delay in the interpretation of the internal and external quality assessment data may have serious consequences for the women who have to decide whether they opt for an invasive test or not. Both laboratories and manufacturers do need to evaluate their own performance critically and take all possible measures to ensure that they are providing high-quality risk estimates based on maximum precision [14-15]. Very important are the UK-NEQAS reports in this quality assessment. However, health care professionals in the field of prenatal screening of DS are still accountable for the implementation of the FTS. A frequent audit of the distribution of the biochemical markers free β-hCG and PAPP-A alongside with the distribution of NT is advocated. We conclude that the consequences of erroneous low PAPP-A and thereby an increased biochemical risk calculation were considerable in terms of an increase of 30% (25/82) in unjustified invasive diagnostic testing from the group who opted for this procedure based on an increased combined risk estimate. Of the total study group 5% (25/456) had an invasive procedure that was based on an incorrect increased combined risk estimate. Fortunately, this has not resulted in iatrogenic miscarriages. Although the estimated risk of miscarriage as a result of invasive diagnostic testing is small (0.3-1.0%) [7-12], this decision should be taken on basis of an adequate FTS test result. It is plausible that the risk among the samples that were not re-assayed for PAPP-A (i.e. the original 3100 minus 473), might also have changed and hence also could have influenced the screen-positive rate and FPR, but this cannot be shown in our analysis. However, any effect of this is considered small.

Despite the absence of negative side effects, the need for continuous and vigorous monitoring and verification of quality in FTS test is of paramount importance.

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Part 2

Ultrasound issues in first-trimester screening

Chapter 5

Jugular lymphatic sacs in the first trimester of pregnancy: the prevalence and the potential value in screening for chromosomal abnormalities

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Abstract

Objective: To investigate the prevalence of detectable jugular lymphatic sacs in a setting for first-trimester screening for Down syndrome, and to evaluate the influence of jugular lymphatic sacs on the screening performance for chromosomal abnormalities.

Methods: A prospective single centre study (Erasmus University Medical Center, Rotterdam, The Netherlands) over a period of 1 year (January 2003 to February 2004). First-trimester nuchal translucency measurement was performed in a study population of 415 fetuses. Additionally, the transversal plane with the spine and mandible was visualized to verify the presents of jugular lymphatic sacs. The jugular lymphatic sacs were measured anterior-posterior. The association between nuchal translucency and jugular lymphatic sacs was tested statistically.

Results: Follow up was completed in 406 cases (97.8%). Jugular lymphatic sacs could be visualized in 98 out of 415 (23.5 %). The nuchal translucency thickness and the mean of the left and right jugular lymphatic sac were significantly correlated.

Conclusion: The sonographic visualization of jugular lymphatic sacs significantly predicts chromosomal abnormalities, although nuchal translucency is a better predictor. Nuchal translucency and jugular lymphatic sacs are strongly correlated and therefore not applicable in a combination test.

Introduction

First trimester measurement of the nuchal translucency (NT) with maternal blood sampling is standard antenatal care in the Netherlands. This screening strategy is a sensitive method for the identification of fetuses at risk of aneuploidy [1-2] and is associated with increased risk of genetic syndromes and fetal structural malformations [3-4]. Although many theories have been put forward a common morphogenesis explaining the interrelationship between the complete spectrum of fetal malformations and enlarged nuchal translucency is still lacking. Previous studies demonstrated abnormal developed jugular lymphatic sacs (JLS) in combination with an enlarged NT [5-7]. Since a disturbance in the lymph-angiogenesis precedes the development of an increased NT [6-7], it is hypothesized that JLS size could be an earlier and better predictor of chromosomal abnormalities.

We conducted an observational study to investigate the prevalence of detectable JLS and to evaluate the test performance by adding this parameter to first-trimester screening (FTS). This study was integrated in a setting for FTS for Down syndrome (DS).

Methods

During a period of 13 months (January 9, 2003-February 26, 20040, an observational study was performed. All women with singleton pregnancies who attended the department of Obstetrics and Prenatal Medicine of the Erasmus University Medical Center for first-trimester screening were informed about the study. Patients were included in this study after giving oral informed consent. Ultrasound examinations of 415 fetuses were carried out between the 11 and 14 weeks' of gestation.

Scans were performed on an ATL HDI-3000 ultrasound system (Advanced Technology Laboratories, Seattle, WA, USA) transvaginally, using a 7-MHz probe or abdominally using a 5-MHz probe. Gestational age was derived on the basis of the first day of the last menstrual period (LMP) and confirmed by ultrasound assessment of fetal biometry. The ultrasound examinations were carried out following the strict methodological criteria set by the Fetal Medicine Foundation (FMF)® [8-9]. An experienced and special trained FMF-certified

sonographer (PH) conducted all first-trimester ultrasound examinations. The NT was measured in the mid-sagittal section of the fetus as the maximum thickness of the subcutaneous translucency between the soft tissue overlaying the cervical spine and the skin. The presence and size of the jugular lymphatic sacs were investigated in a transversal plane through the mandible and spinal cord and confirmed in sagittal and coronal planes, where feasible. Measurements of the anterior-posterior size of the JLS were performed in the transversal plane as shown in Figure 1.

Figure 1.

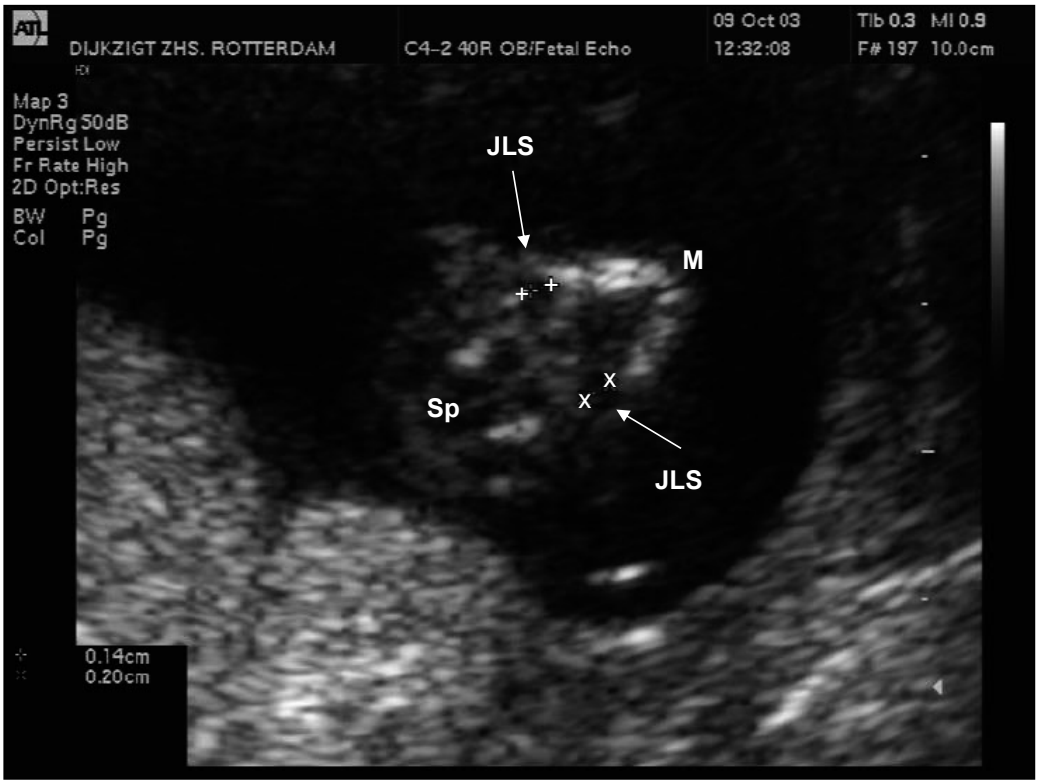

Figure 1. transversal plane through the mandible (M) and spinal cord (Sp).
Bilateral normal size jugular lymphatic sac (JLS). Measurement of JLS is donein a anterior-posterior direction.

Digital recordings of the first-trimester screening and a transverse plane of the neck, including mandible and spine cord and the presence or absence of the jugular lymphatic sacs, were collected for further analysis. In all women, first-trimester screening with additional fetal biometry and visualization, measurement and documentation of jugular lymphatic sacs were completed within a time frame of 30 minutes. Invasive procedures, i.e., transabdominal chorionic villus sampling at 11-14 weeks' gestation or amniocentesis at 15-19 weeks' gestation, were performed, where indicated. Follow-up was obtained by means of questionnaires, which were returned to us by the obstetrician or midwife supervising the delivery.

Statistical analysis

If JLS could not be visualized the size was set to zero. The nonparametric Kendall's Tau-b test, that takes ties into account, was done to measure the association between NT and JLS size. In the subset where JLS size is larger than zero, the Pearson correlation coefficient between NT and the logarithm of JLS size was determined.

Statistical significance was defined as a p-value < 0.05. In the study group the risk of chromosomal abnormalities was modelled using binary logistic regression. Statistically significant effects and interactions were identified by backward stepwise elimination using the likelihood ratio test. The probability criterion for stepwise entry was set to 0.05 and for removal from the equation to 0.1. The statistical analyses were performed using the SPSS® statistical package for Windows release 11.1 (SPSS Inc. Chicago, IL).

Results

In the study period, a total of 415 women with a viable singleton pregnancy were evaluated. Median maternal age in the total study population was 36 years (range 18 to 43 years). The study population was mainly white, i.e. 95% Caucasian, 1.2% Asian and 3.8% Afro-American. As a result of various pleas and reminders, complete follow-up was obtained in 406 women (study group).

Table 1. Chromosomal abnormalities.

	N	gest.age	NT	JLS left	JLS right	Test NT	Test JLS
Trisomy 21	N=7	12+5	3.1	2.1	2.9	+	+
		12+1	4.5	1.4	1.7	+	-
		12+4	4.6	4.3	6.4	+	+
		13+0	4.8	1.9	2.4	+	+
		13+6	5.2	15.5	14.6	+	+
		13+2	5.7	0.0	0.0	+	-
		13+6	8.5	4.6	4.5	+	+
Trisomy 18	N=3	11+2	5.3	6.6	6.1	+	+
		12+3	6.0	3.4	2.8	+	+
		13+4	4.3	1.7	1.3	+	-
Trisomy 13	N=1	12+4	1.6	0.0	0.0	-	-
Monosomy X	N=1	11+0	9.2	12.9	9.1	+	+
47 XYY	N=1	12+2	2.0	1.9	1.7	-	-

Table 2. Adverse pregnancy outcome.

	N	gest.age	NT	JLS left	JLS right	Test NT	Test JLS
Early fetal loss (<20 wks)	N=5	11+3	2.0	0.0	0.0	-	-
		13+5	1.0	0.0	0.0	-	-
		13+1	0.8	0.0	0.0	-	-
		13+6	1.0	0.0	0.0	-	-
		13+3	1.0	0.0	0.0	-	-
Intra uterine fetal death	N=2	13+5*	3.8	0.0	0.0	+	-
		13+6	1.9	0.0	0.0	-	-
Neonatal death	N=2	12+1	1.5	0.0	0.0	-	-
		13+5°	2.3	2.8	2.9	-	+

* Second pregnancy. First pregnancy hydrops fetalis, TOP at 19 wks.

° complex cor vitium.

In the latter group 13 chromosomal abnormalities (3.2 %) were detected, while adverse pregnancy outcome (fetal loss, fetal death and neonatal death) was documented in nine other cases (2.2%), as summarized in Tables 1 & 2.

NT was successfully examined in all fetuses. There were no differences between the groups with regard to maternal age, ethnicity and gestational age at examination. Median gestational age was 12 weeks 3 days (range, 11 wks 0 days-13 wks 6 days) and median CRL was 61.7mm (range, 45-84mm). After excluding the chromosomal abnormalities and adverse pregnancy outcome a subset group of 384 (94.6%) women with an uncomplicated pregnancy were included for final analyses.

In this subset the 95th percentile for NT size was 2.5 mm. Women were therefore considered 'test-positive' if the NT size was above this level. The JLS were detectable in 23% (97/406) women of the study group. In the same subset of 384 uncomplicated pregnancies the 95th percentile for the mean left and right JLS size was 2.0 mm. Women were therefore considered 'test-positive' if the JLS size was above this level. Median size of detectable JLS size was 1.7 mm, with a range of 0.7 mm up to 15.5 mm. In Table 1 shows that in the group of chromosomal abnormalities, all seven fetuses with trisomy 21 tested positive for NT and 5/7 for JLS. For trisomy 18, all fetuses also tested positive for NT and 2/3 for JLS. The fetus with monosomy X (Turner syndrome, TS) tested positive for both tests. Because of hypoplasia of the lymphatic system in fetuses with TS [10], it is questionable whether these should be included in our study. However, during the ultrasound investigation, the karyotype was not yet known to the investigator and the septated hygroma colli was identified as an enlarged NT accompanied with bilateral enlarged JLS. Although NT can identify about 80% of the fetuses with trisomy 13 in a normal first trimester screening program [11] in our study one fetus with Patau syndrome was tested negative by NT and JLS (Table 1.). On ultrasound, however, dextro cardia was observed, which classified the fetus as at increased risk of chromosomal abnormality. An abnormal karyotype of 47 XYY is not associated with enlarged NT and was tested negative by both NT and JLS. In the group of adverse pregnancy outcome (Table 2.) post mortem reports on the early fetal loss were not available. In the

study group of 406 fetuses a significant non-parametric correlation (Kendall's Tau-b, R=0.129) was found between NT and JLS (*p*-value < 0.01). As shown in Figure 2, the subset of fetuses, in which the mean left and right JLS size was larger than zero (N=97), showed a strong correlation between NT and the logarithm of JLS.

Figure 2

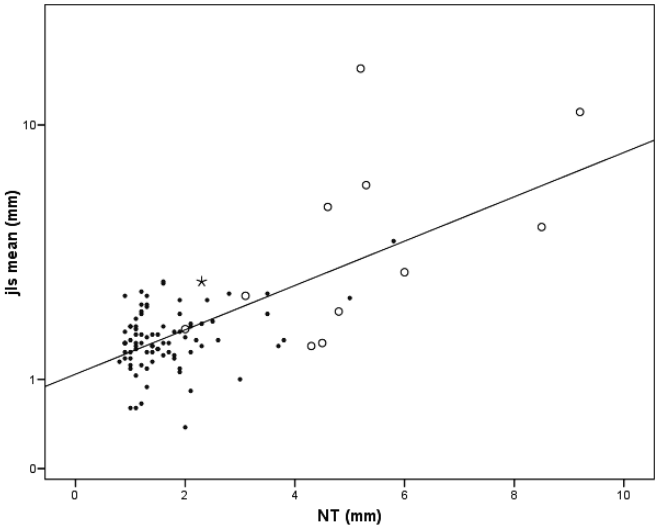

Figure 2

. Correlation between NT and the mean JLS in the subset of the study group, where measured JLS size is larger than zero: * adverse pregnancy outcome • uncomplicated pregnancy outcome ° chromosomal abnormality

The Pearson correlation coefficient found was 0.636 and the *p*-value was highly significant (*p*-value < 0.001). The binary regression results are shown in Table 3. In this study group, NT in combination with maternal age and JLS did not contribute to a statistically better prediction of chromosomal abnormalities than NT alone. Moreover, as enlarged NT and JLS are strongly interrelated, these parameters cannot be considered independent and therefore not applicable in a multiple parameter binary logistic regression analysis. Although both NT and

JLS parameters are statistically significant predictors, the sensitivity for NT was better than for JLS.

Table 3. Binary logistic regression results for chromosomal abnormalities

	Threshold◆ (mm)	Sensitivity (%)	Specificity (%)	Odds ratio	95% confidence interval	
JLS	2.0	62	95	3.81°	2.19	6.64
NT	2.5	85	95	5.53°	3.12	9.82

◆Women were considered screened positive if the distance was above this level.

°Statistically significant at the level P<0.001.

Discussion

This study was carried out to evaluate the prevalence of JLS and was integrated in a setting of FTS for DS to evaluate its test performance in the screening for chromosomal abnormalities. The association between NT, JLS and fetal outcome were investigated. In this context, the prerequisite for adding JLS to first trimester measurement of the nuchal translucency is its independent association with chromosomal abnormalities.

Nuchal translucency is a subcutaneous fluid accumulation in the neck region of unknown origin. However, Haak et al. [7, 12] showed that in mouse embryos with trisomy 16 the development of the JLS preceded the NT and that the JLS showed distension just prior to the occurrence of the increased NT. This notion was supported by other investigators [5-6, 13-14].

Trisomy 16 mouse embryos are considered to be the animal model of the trisomy 21 in human fetuses [15-18]. The delayed organization and connection of these JLS to the venous circulation might explain the transient nature of the NT. Human studies on NT and JLS [5, 7, 12-14, 19] were not able to give conclusive evidence on the question if JLS precedes NT or visa versa. It merely suggests that there seems to be a fetus-specific pattern in the development of the jugular lymphatic system, and therefore a unique expanding phase of the jugular lymphatic sacs.

If the development of JLS in human fetuses is similar to the animal model of the T 16 mouse embryo [5-7, 12-14], further analysis might determine its possibility as an early marker for chromosomal abnormalities before the 11th week of gestation. Considering the large number of attrition of chromosomal abnormal pregnancies and pregnancies with major anomalies during the first trimester, this additional value is still to be discussed. The absence of JLS in early pregnancy could perhaps be used as a sonographic marker for ruling out chromosomal abnormalities (negative predictive value). In our study, the smallest

JLS size detectable by ultrasound was 0.7 mm, which approximated the spatial resolution of the ultrasound equipment in combination with the 7 MHz transvaginal and 5 MHz (broadband) abdominal transducers. In fact, the ability to detect JLS is limited by the spatial resolution of ultrasound machines. Although one could hypothesize that improvement of ultrasound imaging performance might improve the visualization of small JLS, their potential additional value for the early detection of fetal chromosomal abnormalities merits further scientific research. Nevertheless, the sonographic visualization of JLS smaller than.2, 0 mm is time consuming and requires specially trained and highly skilled ultrasound operators.

From our study, we conclude that clearly visualized JLS significantly predict chromosomal abnormalities, although NT is a better predictor. In terms of test performance, however, the additional value of combined testing is limited as both predictors are interrelated.

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Chapter 6

Second-tier risk assessment after first-trimester trisomy 21, 18 and 13 screening using selected sonographic markers among women at intermediate risk

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Submitted

Abstract

Objectives: To evaluate the additional value of specific sonographic markers to further assess the risk of trisomy 21, 18 and 13 in women with an intermediate increased risk of trisomy 21, 18 and/or 13 after the first-trimester combined test.

Methods: During a 30 months period the combined test (CT) 'plus' was evaluated among women at intermediate risk (i.e., > 1: 200 and < 1: 50). The CT-plus was based on the assessment of sonographic makers, including the nasal bone, the Doppler velocity waveform of the ductus venosus, tricuspid regurgitation and the fronto-maxillary facial angle.

Results: In 190 women the CT-plus was performed. A low sensitivity (0.25; 95% CI: 0.01 – 0.78) and high specificity (0.80; 95% CI: 0.73 – 0.85) were found, resulting in a decrease of the false positive rate coinciding with a decrease in the detection rate of fetal aneuploidy.

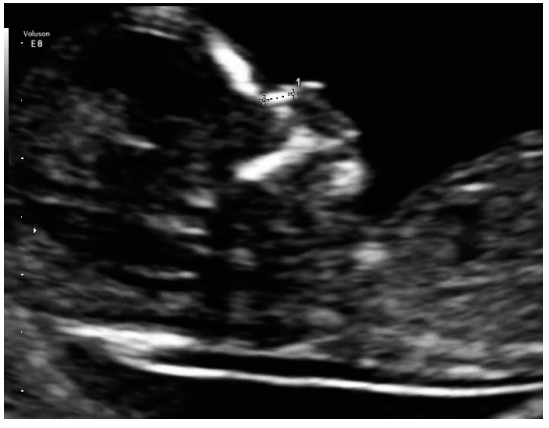
Conclusions: The CT-plus is a tool that can be used for decreasing the false-positive rate after the first-trimester combined test. Decrease in screen-positive rates leads to fewer invasive procedures and, subsequently, prevents potential iatrogenic miscarriages. However, the assessment of specific sonomarkers among this specific category of women at intermediate risk of trisomy 21 and 18 is of limited value, since a relatively large proportion of fetal aneuploidy will be missed.

Introduction

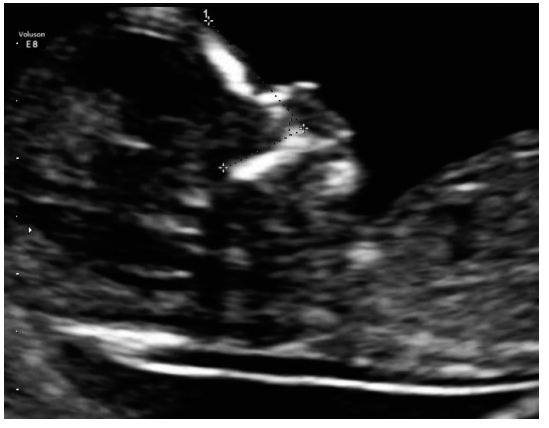
Since January 2007 in the Netherlands the first trimester combined test has officially been introduced as a screening test for the detection of trisomy 21. In June 2010 this test has been extended to the detection of trisomy 13 and 18. This test is offered to every pregnant woman in the first trimester of pregnancy. First-trimester screening (FTS) by the combined test provides an individual risk estimation, based on the combination of maternal age, fetal nuchal translucency (NT) and two biochemical markers in maternal blood, i.e., free ß-human chorionic gonadotropin (ß -hCG) and pregnancy-associated plasma protein-A (PAPP-A). By combining these markers 76-91 % of the pregnancies with trisomy 21 can be detected with a 3-7% false positive rate [1-5].

Women are considered 'screen positive' when the test result indicates an increased risk of trisomy 21, 18 and/or 13, i.e. a risk of ≥ 1:200. To these women an invasive diagnostic test such as chorionic villus biopsy or amniocentesis is offered. Some of them, however, are reluctant to have invasive testing because these tests are associated with a risk of iatrogenic miscarriage (0.3-0.5%) [6-11]. Additional sonographic markers have been identified to assess the risk of trisomy 21, 18 and 13 in the first trimester of pregnancy. These markers include the nasal bone (sensitivity 60-70%, specificity 98%)[12,13], fronto-maxillary facial angle [14], ductus venosus (sensitivity 80%, specificity 95%)[15], and tricuspid valve Doppler evaluation (sensitivity 70% and specificity 95%)[16]. This approach, the so-called combined test 'plus' (CT-plus) has been used as a second-tier test following FTS. It is hypothesized that CT-plus may achieve higher detection rates than screening by the combined test alone (detection rate by using two markers 94%, three markers 95%, and four markers 96%) and a decrease in false positive test results (false positive rate of 2% for trisomy 21 [17]. A decrease in iatrogenic miscarriages due to invasive procedures can be expected if less invasive procedures are performed.

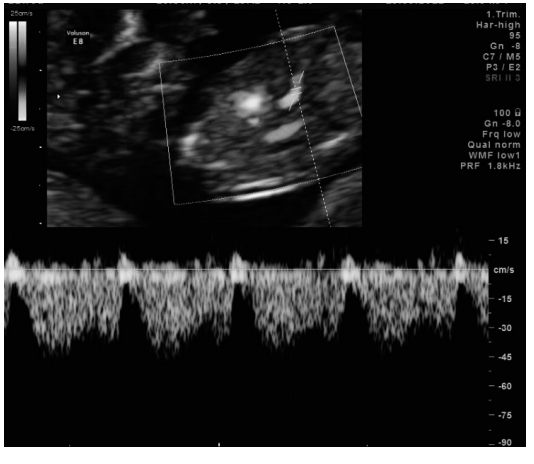
In this study we evaluate the additional value of CT-plus examination in women with an intermediate risk of trisomy 21, 18 and/or 13 after the first trimester combined test with respect to outcomes (karyotyping) and number of invasive procedures after the CT-plus.



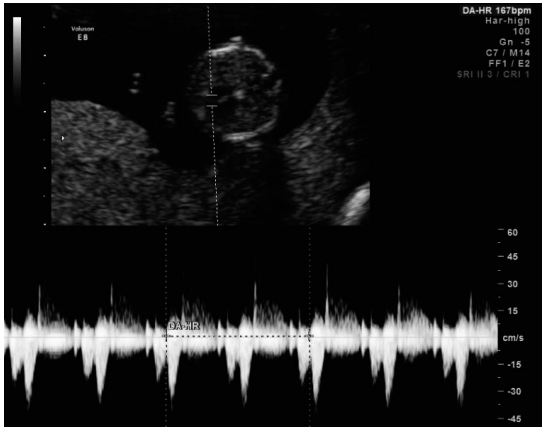
Nasal bone



Facial angle



Ductus venosus flow



Tricuspid flow

Methods

From July 2010 to December 2012 CT-plus was performed among all eligible women with singleton pregnancies who were referred to the department of Obstetrics and Prenatal Medicine of the Erasmus University Medical Center, Rotterdam, The Netherlands. Women were potentially eligible if they tested 'screen positive' for trisomy 21, 18 and/or 13 after the first-trimester combined screening test (NT, maternal age and serum markers). 'Screen positive' was defined as an increased risk at the time of testing of ≥ 1 in 200 [18]. In case of a NT ≥ 3.5mm or a risk greater than 1 in 50, women were excluded and only an invasive test was offered. Hence, women at intermediate risk (i.e., ≥ 1 in 200 and ≤ 1 in 50) were counselled about the options for subsequent testing, i.e., sonographic assessment of 4 markers to recalculate the risk of trisomy 21, 18 and 13 (the so-called CT-plus approach), and/or invasive testing or wait and see. The use of the CT-plus test was always counselled as being assessed in a clinical trial. Regardless of the result of the CT-plus test, the option of invasive testing was always available. In case of an abnormal marker women were offered an advanced fetal anomaly scan at 20 weeks of gestation.

The CT-plus ultrasound examinations were performed following the strict methodological criteria set by the Fetal Medicine Foundation (FMF®). The presence or absence of the nasal bone, the Doppler velocity waveform of the ductus venosus, tricuspid regurgitation and the fronto-maxillary facial angle were evaluated additional to the NT. All ultrasound examinations were conducted by four FMF-certified sonographers (PH, KH, EG and AR). Scans were performed on a GE Voluson E8 system (GE, Zipf, Austria) either transvaginally, using a 5-9 MHz probe or abdominally using a 2-7 MHz probe.

The maternal serum samples were analysed with the AutoDELFIA analyser (Perkin Elmer Life Science, Boston, MA, USA) and commercially available kits. Analysis was carried out at the laboratory of Star Medical Diagnostic Center in Rotterdam, one of the six regional laboratories for FTS in the Netherlands. The calculations of the maternal-weight-corrected Multiple of the Mean (MoM) of the biochemical markers were done using the LifeCycle-Elips software (PerkinElmer Life Science, Boston, MA, USA). For the final CT-plus risk estimation,

the ultrasound data and the results of the maternal serum analysis of the free β-hCG and PAPP-A were combined in the FMF-module in an obstetrical software database (Astraia®, version 1.21.7, Astraia Software GmbH, München, Germany).

Follow-up was obtained by means of questionnaires, which were returned to us by the obstetrician or midwife supervising the delivery. The sensitivity (detection rate) was calculated by dividing the number of true positives by the number of true positives plus the number of false negatives. The specificity (true negative rate) was calculated by dividing the number of true negatives by the number of true negatives plus the number of false positives.

Table 1**:** Study group characteristics (N = 190)

Parameter	Median	Range
Maternal age (years)	35	22 – 44
Risk trisomy 21 CT	1:130	1 :52 – 1:4471
Risk trisomy 18 CT	1:2646	1:53 – 1:72334
Risk trisomy 13 CT	1:2590	1:108 – 1:126128
β-hCG (MoM)	1.46	0.22 – 10.66
PAPP-A (MoM)	0.43	0.09 – 2.55
NT (mm)	1.8	1.1 – 3.5
Risk trisomy 21 CT-plus	1:1398	1:2 – 1:19206
Risk trisomy 18 CT-plus	1:6579	1:6 – 1:50302
Risk trisomy 13 CT-plus	1:13746	1:15 – 1:157443
GA at birth (weeks)	39 3/7	14 6/7 – 41 6/7
Birth weight (gram)	3350	135 – 4755

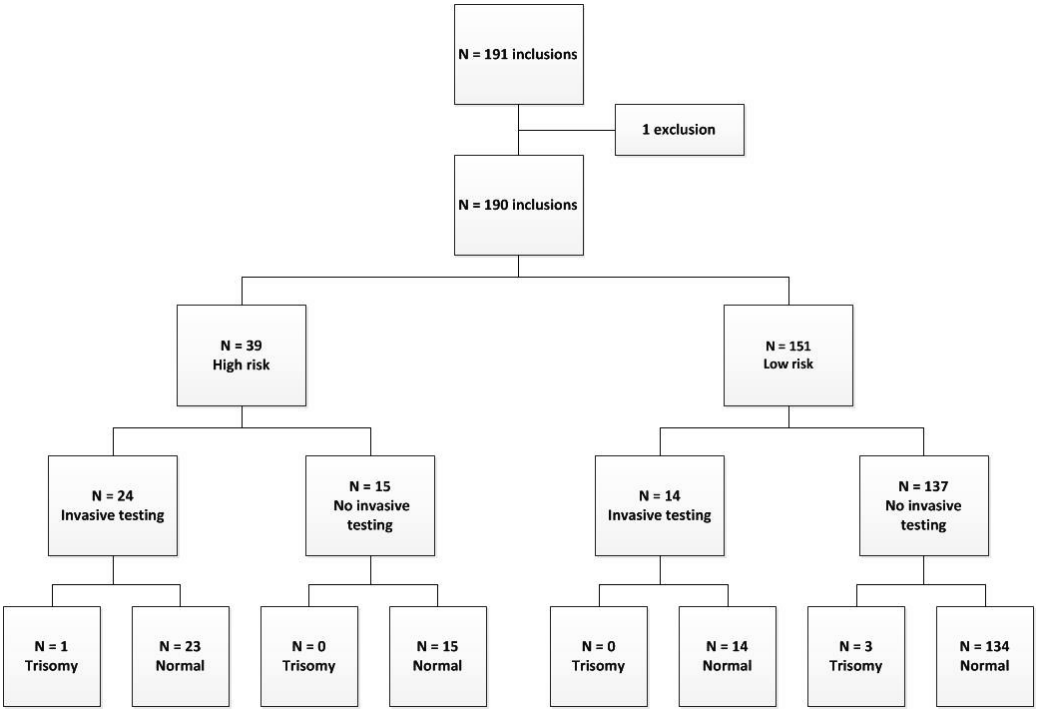
CT = first trimester combined test. MoM = Multiple of the Mean. NT = Nuchal translucency.

CT-plus = Combined test plus. GA = gestational age.

Results

In the 30 months period 191 women at intermediate risk were included in the study. One woman was excluded due to maternal obesity and unfavourable position of the fetus. In this woman a chorionic villi biopsy was performed, showing normal karyogram. Baseline characteristics and neonatal outcome are shown in Table 1. In three women the risk of trisomy 13 and 18 was not calculated in the first trimester combined test. In 9 women the GA at time of birth was unknown, and in 12 women information of birth weight was missing.

Figure 1: Flowchart showing the results and outcome after CT-plus test.



In 160 women there was an intermediate risk of trisomy 21 based on the first trimester combined test. 16 women had an intermediate risk of trisomy 18 and one had an intermediate risk of trisomy 13. In 13 women, an intermediate risk existed for more than one aneuploidy. Seven women had an intermediate risk of both trisomy 21 and trisomy 18, while one woman had an increased risk of trisomy 18 and 13, two women of trisomy 21 and 13. Three women had an intermediate risk of trisomy 21, 18 as well as trisomy 13.

After CT-plus 39 (21%) out of the 190 women remained at increased risk of trisomy 21, 18 and/or 13. Of these 39 women 24 chose invasive testing while 15 women decided to wait and see. Of the 151 women at low risk after CT-plus 14 women chose invasive testing and 137 opted for wait and see (Figure 1).

In three women CT-plus gave a false negative result. In two of them CT-plus showed a low risk of trisomy 21 (1 in 5861 and 1 in 3460); in one of these two women the fronto-maxillary facial angle could not be measured, all other markers were normal. Post-partum the both neonates were diagnosed with trisomy 21. In the third woman CT-plus showed a low risk of trisomy 21, as well as 18 and 13 (1:352 vs 1:647 vs 1:2951), all markers were normal. At the 20-weeks' fetal anomaly scan multiple congenital anomalies were seen; trisomy 18 was diagnosed by subsequent amniocentesis.

The detection and false positive rates of the CT-plus are shown in Table 2. In 38 women the CT-plus gave a false positive result, meaning the CT-plus indicated an increased risk while the child was born without a trisomy 21, 18 or 13. In one woman there was a true positive result, the CT-plus showed an increased risk of trisomy 21 (1 in 3) and trisomy 21 was diagnosed by chorionic villus biopsy. The odds of being affected given a positive result (affected positive: unaffected positive) was found to be 1 in 38.

In 148 women the test gave a true negative result, meaning the CT-plus indicated a low risk and the child was born without trisomy 21, 18 or 13. In this study there were 10 unexpected findings. In one woman who had an increased risk after CT-plus examination, was diagnosed with trisomy 20 mosaicism by amniocentesis. The other unexpected findings included an unbalanced translocation of chromosome 6; 16, two intra-uterine fetal deaths, a right

diaphragmatic hernia, hypospadias, hydrocephalus, arachnoid cyst, a skeletal dysplasia and hypoplastic right heart syndrome. Only in the trisomy 20 mosaicism an abnormal Doppler velocity waveform of the ductus venosus was found; all other markers were normal in the group with unexpected findings. The characteristics of these women are shown in Table 3.

Table 2: evaluation of the CT-plus for detection of trisomy 21, 18 and 13.

	Trisomy 21, 18 or 13	No trisomy 21, 18 or 13	Total
CT-plus Positive	1	38	39
CT-plus Negative	3	148	151
Total	4	186	190
		95% CI	
Sensitivity	0.25	0.01 – 0.78	
Specificity	0.80	0.73 – 0.85	

CI = Confidence Interval

Discussion

In this study we evaluated the additional value of specific sonographic markers to assess the risks of trisomy 21, 18 and 13 in women who were at intermediate risk of these chromosomal abnormalities after the first trimester combined test. We found a low sensitivity and high specificity, resulting in a relatively low false positive rate which coincides with a low detection rate of fetal aneuploidy.

FTS is widely used and acknowledged as valuable screening test for fetal aneuploidy. Women who tested 'screen positive' are reluctant to have subsequent invasive testing because of the risk of iatrogenic miscarriage. For that reason, further non-invasive assessment of the fetus is of interest since this may reduce the false positive rate following FTS. The added value of the additional first trimester ultrasound markers was summarised in a review by Sonak and Nicolaïdes [17] who concluded that the addition of the sonomarkers for

Table 3: showing the characteristics of the women with false negative results, true positive result and the unexpected findings

	Age	T21 CT	T18 CT	T13 CT	NT	β-hCG	PAPP-A	NB	FMF	TCV	DV	T21 CT+	T18 CT+	T13 CT+	Chromosome result	Outcome
False negative results																
Case 1	36	119	1290	4982	1.6	2.94	0.49	nl	nm	nl	nl	3460	7900	24925	No invasive test after CT+	Post-partum trisomy 21 diagnosed
Case 2	42	85	1417	363	2.1	0.58	0.29	nl	nl	nl	nl	5861	8910	2587	No invasive test after CT+	Pregnant by oocyte donation performed in Spain. Contractures of legs at 20 weeks scan, reluctant to have amniocentesis. Post-partum trisomy 21 diagnosed
Case 3	43	87	189	860	1.7	0.55	0.39	nl	nl	nl	nl	352	647	2951	No invasive test after CT+	MCA at 20weeks scan, amniocentesis →T18
True positive result																
Case 1	34	115	12167	38434	2.7	8.85	0.67	nm	nm	nl	nl	3	11956	32622	Trisomy 21	TOP
Unexpected findings																
Case 1	25	71	126	483	1.9	0.43	0.15	nl	nl	nl	anl	23	15	201	Trisomy 20 mosaicism	Birth at 26 weeks → neonatal death
Case 2	25	1882	118	418	1.7	0.24	0.27	nl	nl	nl	nl	18663	385	3360	No invasive test after CT+	MCA at 20weeks scan, amniocentesis → unbalanced translocation chromosome 6;16
Case 3	36	73	1632	7409	1.2	2.1	0.28	nl	nl	nl	nl	500	5046	19317	No invasive test after CT+	IUFD at 34 weeks, no anomalies. No chromosome results known.
Case 4	39	70	676	3067	1.5	0.93	0.18	nl	nl	nl	nl	1300	2185	14310	No invasive test after CT+	Growth restriction at 20 weeks, amniocentesis → normal chromosome results. IUFD at 26 weeks, placental dysfunction
Case 5	40	96	3199	8356	1.6	2.58	0.64	nl	nl	nl	nl	1408	3542	11083	No invasive test after CT+	Right diaphragmatic hernia at 20weeks scan, amniocentesis → normal chromosome results
Case 6	27	78	476	465	1.8	2.19	0.17	nl	nl	nl	nl	2116	1508	5168	No invasive test after CT+	Hypospadias at birth
Case 7	36	190	9849	15514	2.0	2.38	0.57	nl	nl	nl	nl	3961	9849	30849	No invasive test after CT+	Hydrocephalus caused by bleed diagnosed at 32 weeks
Case 8	26	2677	122	521	1.3	0.23	0.27	nl	nl	nl	nl	18120	417	1778	No invasive test after CT+	Arachnoid cyst diagnosed at 20 weeks scan, amniocentesis → normal chromosome results
Case 9	32	123	2838	3988	3.2	3.59	2.55	nl	nl	nl	nl	6250	8120	11475	No invasive test after CT+	Suspicion skeletal dysplasia at CT+ confirmed by amniocentesis, TOP at 17 weeks
Case 10	28	123	10594	3931	1.9	1.52	0.45	nl	nl	nl	nl	15579	37962	64192	No invasive test after CT+	Hypoplastic right heart syndrome at 20weeks scan, amniocentesis → normal chromosome results, TOP

Table 3: showing the characteristics of the false negative results, true positive result and the unexpected findings.
T = trisomy, CT = first trimester combined test, NT fetal nuchal translucency, ß –HCG = free ß-human chorionic gonadotropin, PAPP-A = pregnancy-associated plasma protein-A, NB = nasal bone, FMF = fronto-maxillary facial angle, TCV = tricuspid valve Doppler evaluation, DV = ductus venosus Doppler evaluation, nl = normal, nm = not measured, anl = abnormal, CT+ = combined test plus. TOP = Termination of pregnancy. MCA = multiple congenital anomalies, IUFD = intra-uterine fetal death. The risk on trisomy 21, 18 and 13after CT and CT+ are 1 in (the given number).

aneuploidy increases the detection rate for Down syndrome while decreasing the false-positive rate. The authors, however, had a different definition of 'intermediate' risk. Their definition represented a risk of 1 in 51 to 1 in 999. This will increase the burden of the ultrasound department as women who are considered low risk after FTS (< one in 200) will be advised to have further sonographic testing. Moreover, this will create more anxiety among the pregnant women involved. Recently, Ghaffari et al. [19] conducted a prospective study among 13.476 women in Tehran who were screened for chromosomal abnormalities in the first trimester of pregnancy. They followed the ALARA (as low as reasonably achievable) principle, where nasal bone status, tricuspid valve regurgitation and ductus venosus Doppler flow were measured, where feasible. In 34.4% they succeeded to assess these additional sonomarkers, with subsequent increase in detection rates for trisomy 21 and decrease of false-positive rates at a cut-off level of < 1 in 300 for subsequent invasive testing. Such approach, however, is time consuming and requires high-skilled sonographers. For this reason, this approach does not seem appropriate in a screening setting.

Although the FTS is primarily and most effectively used for trisomy 21 screening, a positive side effect is the early identification of fetus with trisomy 18 or trisomy 13, which are the second and third most frequent chromosomal abnormalities. Kagan et al. concluded that ~ 95% of trisomy 18 and 13 fetus can be detected with a 0.1 % increase in the false positive rate [20]. A large proportion (38%) of the women having an increased risk following CT-plus did not change their mind about invasive testing since they did not opt for invasive testing despite their increased risk of trisomy 21, 18 or 13. Especially with the recent introduction of the non-invasive prenatal diagnosis [21,22] of trisomy 21, 18 and 13 the question arises which role remains the ultrasound in the follow-up assessment of those who screen positive after FTS. It is known that an abnormal flow in the ductus venosus, an abnormal flow over the tricuspid valve and an increased NT thickness are associated with cardiac defects [23-29], and that the majority of significant structural defects are already detectable at this point in pregnancy [17,30]. It is likely that in the near future the first trimester scan becomes a fetal anomaly scan rather than a screening tool for aneuploidy. In this study we found ten

unexpected findings indicating that pregnancies with an increased risk of aneuploidy are also at risk for other congenital anomalies, this should be kept in mind when monitoring these pregnancies. We conclude that the CT-plus is a tool that can be used for decreasing the false-positive rate after the FTS. The decrease in screen-positive rates leads to fewer invasive procedures and in that way prevents iatrogenic miscarriages. However, among this specific category of women at intermediate risk examination of specific sonomarkers missed 3 out of 4 fetuses with fetal aneuploidy.

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Chapter 7

First trimester crown-rump length and embryonic volume of aneuploid fetuses measured in 3D-Virtual Reality

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Abstract

Objectives: To examine whether embryonic volume (EV), as measured using three-dimensional (3D) ultrasound and a virtual reality approach, is a better measure of growth restriction than is crown-rump length (CRL) in aneuploid fetuses.

Methods: We retrospectively measured CRL and EV in prospectively collected 3D ultrasound volumes of 55 aneuploid fetuses using the Barco I-Space VR system. The gestational age ranged from 11^{+2} to 14^{+4} weeks. We compared our measured data with previous published reference curves of euploid fetuses. Delta-values were calculated by subtracting the expected mean of euploid fetuses of the same gestational age from observed values. The one-sample t-test was used to test significance of differences observed.

Results: The CRL measurements of fetuses with trisomy 21 (n=26), trisomy 13 (n=5) and monosomy X (n=5) were comparable with those of euploid fetuses, but in fetuses with trisomy 18 (n=19) the CRL was on average 14.5% smaller ($p<0.001$). The EV in fetuses with trisomy 21, 18, 13 and monosomy X was smaller than euploid fetuses (-27.8%, $p<0.001$; -39.4% p=<0.001; -40.9%, p=0,004 and -27.3% p=0.055, respectively).

Conclusion: When relying on CRL measurements alone, first-trimester growth restriction especially manifest in trisomy 18. Using EV, growth restriction is also evident in trisomy 21, 13 and monosomy X. EV seems to be an effective measurement for assessment of first-trimester growth restriction in aneuploid fetuses.

Introduction

It has been known for a long time that fetal growth restriction may be a marker for aneuploidy [1-3]. Typically, growth restriction in aneuploid pregnancies is of early onset, and is evident from the first trimester onwards. In trisomy 21, however, crown-rump length (CRL) measurements are similar to chromosomally normal fetuses of the same gestational age (GA) [1, 3].

Traditionally, first trimester fetal growth has been documented by two-dimensional (2D) CRL measurements. With the introduction of three-dimensional (3D) ultrasound (US) it became possible to measure embryonic and fetal volumes. Earlier studies show that the relative increment of fetal volume is much larger than the increment of CRL during the same period [4]. Rousian et al.[4] demonstrated that, when the CRL doubles, the embryonic volume (EV) increases 6.5-fold. Volume measurement might therefore enable earlier detection of fetal growth restriction in pregnancy.

Several other studies have been performed measuring fetal volumes using 3D US [5-8]. To estimate the embryonic or fetal volume in these studies 2D contours were defined manually in several different planes. As various methods have been used and different normal values for EV have been reported there is a need for standardization [9-10].

The introduction of the virtual reality (VR) visualization technique enables us to use all three dimensions of these 3D US scans. The Erasmus MC operates a BARCO I-Space VR system (Barco NV, Kortrijk, Belgium). This is a four-walled CAVE^{TM} -like VR system in which investigators are surrounded by stereoscopic images [11]. A hologram is created by the V-Scope volume rendering application and polarized glasses enable the viewer to perceive depth and to interact with 3D volumes in an intuitive manner [12]. Using V-Scope it is possible to perform precise EV calculations semi-automatically while benefitting from true 3D depth perception [4].

The aim of this study was to examine fetal growth pattern in aneuploid fetuses (trisomy 21, 18, 13 and monosomy X) during the late first trimester and to compare EV and CRL between euploid and aneuploid pregnancies.

Methods

Between 2008 and 2010 we collected 3D ultrasound volumes of singleton pregnancies in which an increased nuchal translucency (NT) was measured (>3.5 mm) during routine ultrasound examination. Ultrasound scans were performed using the Voluson 730 Expert ultrasound machine (GE Medical Systems, Zipf, Austria). Later, following invasive prenatal diagnosis, aneuploid pregnancies were identified (N=63). The GA was calculated based on the first day of the last menstrual period or, when assisted reproductive technology had been carried out, on the day of conception; GA ranged from 11^{+2} to 14^{+4} weeks.

The 3D volumes were converted to Cartesian volumes, using 3D software (4D View, GE Medical Systems), and transferred to the BARCO I-Space. In the I-Space all volumes were evaluated and the best volume for each case was selected based on image quality and completeness of the volume. We excluded eight cases from the study because of poor image quality (n=5), incompleteness of the volume (n=1) or because of absence of fetal heart activity at the time of ultrasound scan (n=2). Of these eight cases three were diagnosed with trisomy 21, three with trisomy 18 and two with monosomy X (45, XO). There were four 3D volumes (two cases with trisomy 21 and two cases with trisomy 18) in which it was not possible to measure EV because of poor image quality, but in which it was possible to measure CRL. Following exclusions, 26 pregnancies trisomy 21, 19 with trisomy 18, five with trisomy 13 and five with monosomy X were available for analysis. A fetus affected with trisomy 18 as visualized in the I-Space VR-system is shown in Figure 1.

CRL and EV were measured using the V-Scope software. The V-Scope application includes a region-growing segmentation algorithm for semi-automatic volume calculation in selected structures [11-12]. The innovative VR technique has already successfully been applied in prenatal medicine [13-15].

The procedure for measuring EV is described in detail by Rousian et al.[4] Both physiological and pathological omphalocele were included in the embryonic volume. Hydrops, frequently present in fetuses with chromosomal abnormalities, was also included in the embryonic volume calculations. This is relatively easy to archive y performing a second segmentation of

the anechonic fluid layer of the hydrops, aside from the segmentation of the body volume. If necessary this fluid layer can also be segmented manually. All measurements in the I-Space were performed by the same investigator (LB). The accuracy and reproducibility of length and volume measurements has been proven by previous studies in which the growth trajectories of euploid pregnancies for CRL and EV have also been determined and reference curves established [4, 16-18]. The collected data in the present study were compared to the results of these previous studies on euploid fetuses.

Figure 1.

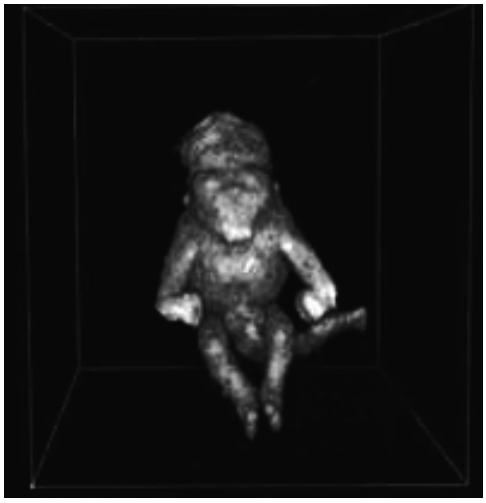


Figure 1: Fetus affected by trisomy 18 as visualized in the I-Space virtual reality system. Multiple congenital abnormalities can be seen: Exencephaly, radial aplasia and omphalocele. Spina bifida and polydactyly were also present.

Statistical analysis

The expected mean EV of the euploid fetuses at the same GA was subtracted from the observed EV of aneuploid fetuses. This expected value was obtained from equations published in earlier studies [4, 17-18], and the difference was expressed as a proportion of the

mean EV of euploid fetuses. The same analysis was performed to investigate the possible association of EV and CRL, and for CRL and GA. We furthermore determined the difference in GA, comparing observed GA and expected GA (expressed in days) according to the observed EV and CRL. The one-sample t-test was used to test for statistically significant differences between observed values of aneuploid pregnancies and expected values of euploid pregnancies. Statistical analysis was performed using SPSS v.17.0.2 (SPSS Inc., Chicago, IL, USA) and *p*-value <0.05 was considered statistically significant.

Results

Of the 26 trisomy 21 cases three presented with hydrops and/or hygroma colli. In the trisomy 18 group nine were diagnosed with a pathological omphalocele and four with hydrops fetalis. Three of the 19 trisomy 18 cases had multiple congenital malformations (exencephaly, holoprosencephaly, spina bifida, skeletal abnormalities, and nephron-urinary abnormalities). Two cases of holoprosencephaly, three cases with hydrops and/or hygroma colli, one omphalocele and one hypoplastic left heart syndrome were diagnosed in the trisomy 13 group. Three of the five cases with monosomy X presented with hydrops. Other congenital abnormalities diagnosed in this group were hydronephrosis and cardiac abnormalities. Fetuses diagnosed with trisomy 18 showed a 14.53% smaller CRL than expected (p<0.001), corresponding to a differences in GA of -4.78 days. The other groups of aneuploid fetuses, trisomy 21 and 13 and monosomy X, showed a non-significant smaller CRL than did euploid fetuses (Table 1).

In all groups o aneuploid fetuses the EV was smaller than expected for gestational age: -27.76% for trisomy 21 (p<0.001), -39.37% for trisomy 18 (p<0.001), -40.87% for trisomy 13 p=0.004, although the difference was not quite statistically for monosomy X (-27.29%, p=0.055). In terms of days' GA, these differences ranged from -3.45 to -5.14.

In Figure 2, the CRL and EV of fetuses with trisomy 21, 18 and 13 and monosomy X are plotted gestational-age based reference ranges for euploid fetuses.

Table 1: Mean percentage difference in crown-rump length and embryonic volume in aneuploid fetuses as a percentage of normal mean according to gestational age (GA) in euploid fetuses, with corresponding mean differences in GA.

Karyotype	*n*	*Mean difference %* *(95% CI for mean)*	*p**	*Mean difference* *days GA (SD)*	***p****
Crown-rump length					
Trisomy 21	26	-1.29 (-4.97 to 2.38)	0.475	-0.55 (-1.79 to 0.69)	0.369
Trisomy 18	19	-14.53 (-19.94 to -9.12)	<0.001	-4.78 (-6.77 to -2.78)	<0.001
Trisomy 13	5	-5.44 (-14.39 to 3.52)	0.167	-1.75 (-4.55 to 1.06)	0.159
Monosomy X	5	-3.65 (-18.72 to 11.43)	0.539	-1.43 (-6.18 to 3.32)	0.450
Embryonic volume					
Trisomy 21	24	-27.76 (-35.80 to -19.72)	<0.001	-3.45 (-4.56 to -2.34)	<0.001
Trisomy 18	17	-39.37 (-48.23 to -30.50)	<0.001	-5.14 (-7.04 to -3.23)	<0.001
Trisomy 13	5	-40.87 (-59.75 to -22.00)	0.004	-5.25 (-8.63 to -1.88)	0.012
Monosomy X	5	-27.29 (-55.56 to 0.0097)	0.055	-3.63 (-8.33 to -1.07)	0.097

* For observed mean difference vs. 0.

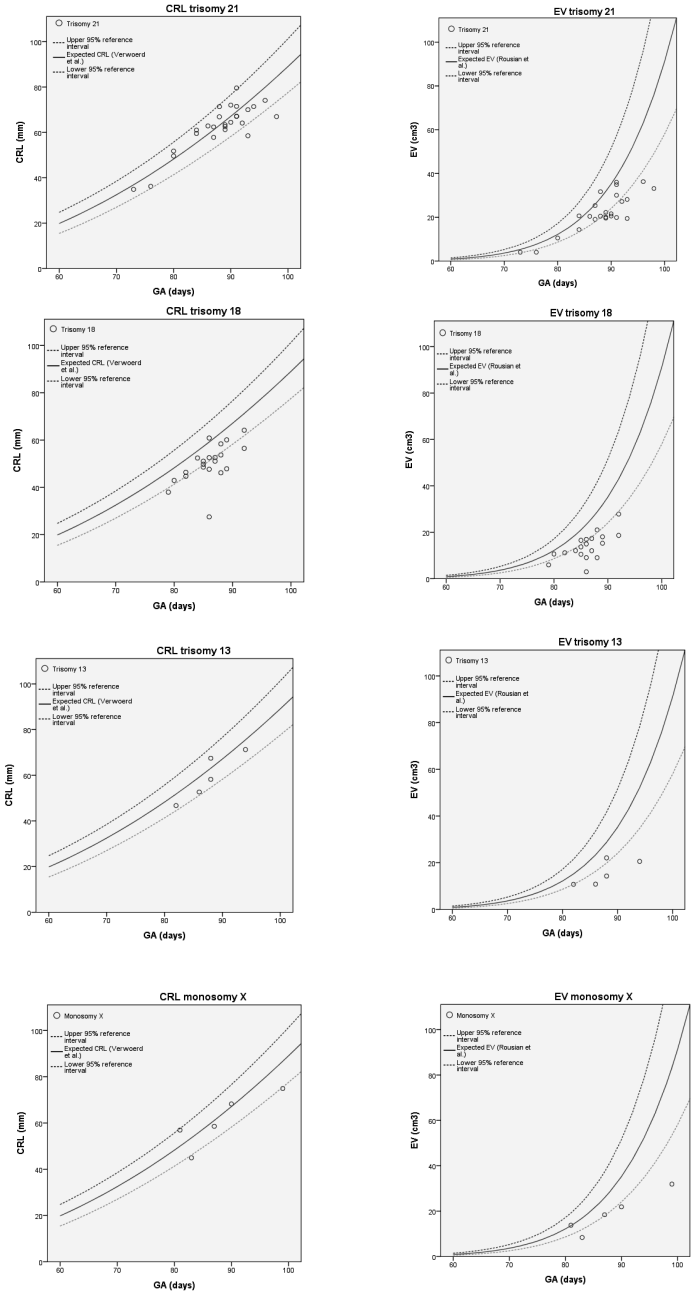
Table 2: Mean percentage difference for embryonic volume (EV) in aneuploid fetuses as a percentage of normal mean according to crown-rump length in euploid fetuses.

Karyotype	*n*	*Mean difference %* *(95% CI for mean)*	***p****
Trisomy 21	24	-0.37 (-8.56 to 7.83)	0.927
Trisomy 18	17	-17.37 (-28.04 to -6.68)	0.003
Trisomy 13	5	-9.75 (-31.78 to 12.28)	0.287
Monosomy X	5	5.48 (-20.63 to 31.60)	0.591

* For observed mean difference vs. 0.

The difference between observed EV for the aneuploid fetuses and expected according to their CRL is presented in Table 2. Significant differences were found for fetuses affected by trisomy 18 which had on average a 17.37% smaller EV than the normal mean for CRL

Figure 2: First trimester measurements of crown-rump length (CRL) and embryonic volume (EV) according to gestational age in fetuses affected by trisomy 21 (first row) , trisomy 18 (second row) , trisomy 13 (third row) and monosomy X (fourth row) , plotted on reference curves (mean solid line) and 95% reference interval (dashed line) for CRL and EV of euploid fetuses by Verwoerd-Dikkeboom et al.[18] and Rousian et al.[4], respectively.



(p=0.003). Smaller measurements of EV than expected for CRL were also found for trisomy 13, although not statistically significant. For trisomy 21 and monosomy X, no difference was observed.

Discussion

The results of this study are in line with previous research demonstrating that chromosomal abnormalities are often accompanied by growth restriction. The data show that, based on CRL, growth restriction can be observed in trisomy 18 at 11-14 weeks' gestation (-14.53%, p<0.001). Small, non-significant, differences in CRL were found for trisomy 13, monosomy X, and trisomy 21. In contrast to CRL, EV was significantly smaller than expected in trisomy 21, 18 and 13, with a large but marginally non-significant difference in the small sample of fetuses with monosomy X. The mean percentage difference in EV was also more evident than the mean percentage difference in CRL, and was as high as -40.87% for trisomy 13. These findings show the same trend as the reported birth weight in these conditions: infants with trisomy 18 are most likely and those with trisomy 21 least likely to be small for GA [19].
The EV is fetuses affected by trisomy 21 and 13 and monosomy X was found to be in proportion to their CRL, as no significant difference from that expected was found when EV was corrected for the observed CRL. However, disproportionate growth restriction was found in the fetuses with trisomy 18, when corrected for CRL, EV was significantly decreased (by 17% on average). This disproportionality points to an asymmetric growth disturbance that affects the internal organs more than the skeleton, which is associated with more severe growth restriction. To further examine aspects of disproportionality, future research will be performed on the head-to-body volume ratio in both euploid and aneuploid fetuses in order to evaluate the type of growth restriction (symmetric or asymmetric).
Structural congenital abnormalities are frequently present in chromosomally abnormal fetuses. As explained, we accounted for omphalocele and hydrops fetalis in the EV calculations. Holoprosencephaly, associated with trisomies 13 and 18, might have a small influence on EV. However, holoprosencephaly can be accompanied by both microcephaly

and hydrocephaly, each contributing to EV in a different direction. Moreover, it is unlikely that a 30-40% smaller EV is caused by structural abnormalities exclusively. Abnormal EV in aneuploid fetuses can be explained by an increased duration of the cell-cycle, due to checkpoint control genes, resulting in a significantly reduced numerical cell count compared to euploid fetuses [20].

At present, it is only possible to speculate regarding the clinical importance of first-trimester growth restriction in aneuploid fetuses. It is as yet unclear as to whether first-trimester growth restriction is helpful in identifying fetuses with chromosomal abnormalities during the first trimester of pregnancy. It may, however, be hypothesized that markedly growth-restricted aneuploid fetuses are more prone to intrauterine fetal death in the second and third trimesters of pregnancy, and these pregnancies may be identified earlier by measuring EV. This hypothesis should be the subject of further research on EV and pregnancy outcome.

Limitations of the study include the low number of cases for both trisomy 13 and monosomy X; the groups with the lowest incidence of the aneuploidies investigated in this study. However, the fact that we found a statistically significant difference in EV for trisomy 13 and only marginally non-significant difference for monosomy X ($p=0.004$ and $p=0.055$, respectively) suggests that there is a strong relationship between aneuploidy and decreased EV. It seems likely that analysis of additional cases in these groups would confirm the relationship. Another limitation of our study is that at this time the BARCO I-Space is too large (requiring a separate $40m^2$/400 sq. ft.) and too expensive for 3D VR becoming a routine diagnostic procedure, which limits routine practice. However, a desktop version of this 3D VR system is currently being developed, which will make this new and innovative technique more accessible to hospitals in the near future. A prototype is already being evaluated at our department for use in both research and daily clinical practice.

In conclusion, evaluation of growth in the first trimester is typically performed by measuring CRL using 2D ultrasound. CRL can only be used as a reliable indicator of growth restriction in aneuploid fetuses in the first trimester for pregnancies with trisomy 18. Using EV, growth restriction is also evident in trisomy 21 and trisomy 13 and monosomy X. This study shows

that in aneuploid fetuses, EV measurements can be used to diagnose abnormal first trimester growth.

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Chapter 8

Embryonic delay in growth and development related to confined placental trisomy 16 mosaicism, diagnosed by I-space Virtual Reality

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Objective: To demonstrate the use of a novel three-dimensional (3D) virtual reality (VR) system in the visualization of first trimester growth and development in a case of confined placental trisomy 16 mosaicism (CPM+16).

Design: Case report.

Setting: Prospective study on first trimester growth using a 3D VR system.

Patient(s): A 34-year-old gravida 1, para 0 was seen weekly in the first trimester for 3D ultrasound examinations.

Intervention(s): Chorionic villus sampling was performed because of an enlarged nuchal translucency (NT) measurement and low pregnancy-associated plasma protein-A levels, followed by amniocentesis.

Results(s): Amniocentesis revealed a CPM+16. On two-dimensional (2D) and 3D ultrasound no structural anomalies were found with normal fetal Doppler's. Growth remained below the 2.3 percentile. At 37 weeks, a female child of 2010 g (<2.5 percentile) was born. After birth, growth climbed to the 50^{th} percentile in the first 2 months.

Conclusion(s): The I-Space VR system provided information about phenotypes not obtainable by standard 2D ultrasound. In this case, the delay in growth and development could be observed very early in pregnancy. Since first-trimester screening programs are still improving and becoming even more important, systems such as I-Space open a new era for in vivo studies on the physiologic processes involved in embryogenesis.

Introduction

In this paper we describe a case of confined placental trisomy 16 mosaicism that was documented in detail in a prospective study on first trimester growth using three-dimensional (3D) Virtual Reality (VR)[1]. The aim of this paper was to demonstrate that by using this imaging technique, delay in both growth and development can be depicted, very early in the first trimester of the pregnancy.

It is expected that as many as 1% [2] to 1.5% [3] of all (clinically recognized) conceptions may have trisomy 16, which is the most frequent chromosome abnormality at conception [4]. In trisomy 16 an early embryonic arrest usually results in a miscarriage between 8 and 15 weeks of gestational age. Trisomy 16 miscarriages show either empty sacs, disorganized embryos or minimal embryonic development [2]. Almost all cases of trisomy 16 surviving in the second trimester of pregnancy are found to be mosaic (meaning that the cell lines contain both euploid and trisomic cells) [5]. To survive, the mosaic trisomic 16 cell lines must be completely or at least predominantly confined to the placenta and this phenomenon is referred to as confined placental trisomy 16 mosaicism (CPM+16) [5]. Robinson et al [6] found that most cases of CPM+16 originate during maternal meiosis I. The “rescue” means that a chromosome 16 is lost in one of the cells of the trisomic conceptus, resulting in an euploid cell line. This can be either one of the two maternal chromosomes 16, resulting in biparental disomy 16 (BPD 16) or the paternal chromosome 16, resulting in uniparental disomy 16 (UPD 16) [7].

Besides an increased risk for (severe) fetal malformations, CPM+16 is associated with intra-uterine growth restriction, which is described in both BPD and UPD 16 cases [8,9]. Early detection of placental confined trisomy 16 is important since patients are at increased risk for several maternal obstetrical complications, such as severe preeclampsia [10]. The aim of this paper is to present a case of mosaicism trisomy 16 that caused an apparent delay in embryonic growth very early in pregnancy.

Case report

A 34-year old gravida 1, para 0 participated in a prospective study to determine the beneficiary aspects of a novel imaging technique for optimizing first trimester visualization. Women enrolled in this study early in pregnancy, and a 3D ultrasound scan was made weekly from about 5 to 6 weeks of gestation till 13 to 14 weeks. This patient had an accurately documented first day of last menstrual period and a positive pregnancy test on the 29^{th} day of her cycle. On the first ultrasound examination, gestational sac, yolk sac and an indication of embryonic structures were visualized. At eleven weeks, an increased nuchal fold was seen, possibly fetal hydrops. A sonographer licensed by the Fetal Medicine Foundation (FMF; Certificate of Competence in the 11^{+0} – 13^{+6} – week scan) carried out a nuchal translucency measurement 6 days later. The nuchal fold was 2.9 mm. The free β-HCG level was 70.70 IU/ l (1.323MoM), and the pregnancy-associated plasma protein-A (PAPP-A) level was 0.013 IU/l (0,150 MoM; AutoDELFIA™ analyzer and LifeCycle™ Elips software-package, PerkinsElmer®, Wallac, Turku, Finland). The crown-rump length (CRL) was only 41.3 mm (the expected range for 12 weeks of gestation is between 46 and 63 mm). The corrected risk for trisomy 21, 13 &18 was 1 in 5. Following these results, a chorionic villus biopsy was performed. Five milligrams of chorionic villi were obtained, and in short-term cultured villi an additional chromosome, most likely chromosome 16, was seen in all analyzed cells. To discriminate between CPM+16 and true fetal mosaicism of trisomy 16, amniocentesis was performed. Using fluorescence in situ hybridization with chromosome 16-specific probes, normal signal distributions were noted in 100 uncultured amniotic fluid cells, and a normal female karyotype was seen in 37 colonies of cultured amniotic fluid cells. UPD 16 was excluded. Since there still remains a residual risk on fetal congenital anomalies due to somatic mosaicism, the pregnancy was carefully monitored with two-dimensional (2D) and 3D ultrasound, which revealed no structural anomalies; fetal Doppler remained normal. Growth of the fetus remained below the percentile 2.3 birth centile throughout the pregnancy. At 36 weeks, the patient was admitted into hospital for pregnancy-induced hypertension. At 37 weeks, a caesarean section was performed for failed induction of labor. A female infant

was born with Apgar scores of 6 and 8 after 1 and 5 minutes. The infant had a birth weight of 2010 grams (< 2.5 percentile). The placenta weighed 735 grams after fixation. Besides localized chorangiomatosis in one slice of the placenta, no abnormalities were found. The infant was monitored on the pediatric ward and was discharged 10 days after birth.
The following year, the girl developed normally and her growth climbed to the 50th percentile in the first 2 months and remained there. No congenital abnormalities were found.

Materials and Methods

Two-dimensional and 3D ultrasound scanning was performed on a GE Voluson 730 Expert system (GE, Zipf, Austria). The 3D volumes were transferred to a personal computer for off-line evaluation using specialized 3D software (4D view, GE Medical Systems). These data were transferred to the BARCO I-Space at the department of Bioinformatics of the Erasmus MC. This four-walled CAVE-like [11] VR system has been described in detail elsewhere [1,12-14]. Using this system, we measured standard biometry such as CRL, biparietal diameter (BPD), occipito-frontal diameter (OFD), and calculated the head circumference (HC). We also established the Carnegie Stage of the embryo. The embryo was staged according to the description of the external morphological features, mainly limb development, of the Carnegie Stages illustrated and described by O'Rahilly and Müller [15]. This method is described in detail in Verwoerd-Dikkeboom et al [1].

Results

The results of the biometry measurement (mean of three measurements) in the I-Space and the assigned Carnegie stages are displayed in table 1.

The Carnegie Staging system ends at day 57 post conception, therefore when the patient was seen at gestational age 11 weeks + 1 day, assignment of Carnegie stages is no longer possible.

Table 1

AGE (WEEKS)	CARNEGIE STAGE (A)	CRL(B)	BPD	OFD	HC
7+1	14	5.2	3.1	6.9	16.4
8+1	16	9.6	6.5	11.1	28.1
9+4	19	19.2	8	11.8	32
11+1	--	32.9	11.5	12.7	38
12+0	--	41.3	13.5	16.4	47

(age is gestational age in weeks)

Table 1

Age is gestational age in weeks.

(A) The expected Carnegie Stage for that gestational age is given according to O'Rahilly and Müller [15], calculated as gestational age – 14 days to obtain the postovulatory age.

(B) The normal 5th-95th percentiles for that age are given for the different parameters. For CRL, the Robinson chart [23] was used, for BPD and HC the Kustermann charts [24] were used.

Discussion

Growth restriction

Most case reports on CPM+16 (or other chromosomes) start with the result of the chorionic villus sampling (CVS) or amniocentesis. Therefore, little is known about embryonic and / or placental phenotypes in the first trimester of these pregnancies. This patient demonstrated growth retardation already very early in pregnancy. Very early growth retardation can be mistaken for gestational age discrepancy. Adjusting the gestational age could then have serious consequences. We encountered another issue related to very early growth aberrations: problems with performing proper first trimester screening. For first trimester screening most research describe both a gestational age period and CRL lengths. In first reports, this period was 10^{+0} - 14^{+0} weeks [16]. Nowadays the FMF uses a 11^{+0} – 13^{+6} week period [17], corresponding with a CRL between 45 and 84mm. The First and Second Trimester Evaluation of Risk for Aneuploidy (FASTER) trial [18] used pregnancies with CRLs between 36

and 79 mm, corresponding with 10^{+3} – 13^{+6} weeks. At 11 weeks of gestation, our patient had a CRL of only 33 mm; at 12 weeks this was 42 mm, indicating that in the desired period of first trimester screening (11-13+6 weeks) the minimal CRL requirement according to the FMF is still not met. This implies that the effect of any delay in embryonic growth and development on the reliability of the results of combined first-trimester screening is unclear.

PAPP-A

The combined first-trimester screening for Down syndrome revealed that serum PAPP-A level in this patient was extremely low. Several studies have indicated the association between low levels of serum PAPP-A, with a number of adverse pregnancy outcomes, such as an increased risk of pre-eclampsia [17,18], gestational hypertension [18] and intra-uterine growth restriction[17-19]. Smith et al [20] stated in their study that this predictive value of PAPP-A implies a fundamental role of this system in the development of the placenta in early pregnancy. This patient is a good example of the presumption that impaired placentation is reflected by low serum PAPP-A. To our knowledge, this is the first report that describes a low serum PAPP-A level in association with a placental confined trisomy 16. Groli et al [21] described five cases of trisomy 16 confined to the placenta that were found after high-risk results in a second trimester maternal serum screening program for Down syndrome. Amniocentesis and CVS was performed. All five pregnancies displayed unusually high levels of hCG and four out of five had raised alpha-fetoprotein (AFP) values. All five pregnancies were complicated by fetal growth retardation. Other studies have also shown extremely high levels of hCG [2,22]. Our patient, however, displayed an hCG level within the normal range on first-trimester screening; AFP level (26 IU/ml, measured at 16 weeks in amniotic fluid, normal range 15-30 IU/ml).

I-Space implementation

We analyzed the 3D volumes of this patient in the I-Space, and we were able to easily measure and calculate CRL, BPD, OFD and HC using this system. We also established the Carnegie Stage of the embryo. The Carnegie Stage we assigned to this embryo corresponded very well with the measured CRL compared with the original data of the Carnegie Collection described by O'Rahilly and Muller [15], indicating that growth and development were still in concordance. Delay in either growth or development would have meant that the assigned Carnegie Stages did not correspond with CRL measurements, for instance, a CRL measurement of a stage 21 embryo with morphological features of a stage 19 embryo or the exact opposite, morphological features of stage 21 with CRL of stage 19. Age, however, did not correspond with CRL: the age discrepancy was more than 8 days in general. CRL parallels the Carnegie Stages in the discrepancy between CRL and gestational age. Since gestational age is not questioned in this patient, the only conclusion can be that both growth and development were delayed very early in pregnancy. The question is whether this can all be attributed to the placental confined mosaicism. If it is, it implies that placentation is already of vital importance in the earliest stages of pregnancy.

We believe that it is essential to combine biometry measurements with evaluation of morphological features. The I-Space VR system provides us with information about phenotypes not obtainable by standard 2D ultrasound. In this case, the delay in growth and development could be observed very early in pregnancy. Since first-trimester screening programs are still improving and becoming even more important, we believe that systems such as the I-Space open a new era to study embryonic growth and development in vivo. This will eventually lead to better understanding of both physiologic and pathologic processes involved in embryogenesis.

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Chapter 9

General discussion

The aim of this thesis is to study the overall effectiveness of the combined first-trimester screening test for Down syndrome and other aneuploidies in the Dutch clinical setting and to determine the factors which are associated with the variation of the test performance. During the period in which parts of this research was conducted, the first-trimester screening test in The Netherland was extended in June 2010 by additional screening for trisomies 13 and 18. Furthermore, the no-invasive prenatal test (NIPT) was introduced in April 2014.

We focused on the components of combined first-trimester screening, i.e., the biochemical and the sonographic components. In Part 1 the biochemical issues of first-trimester maternal serum screening are discussed. These are followed in Part 2 by a summary of the sonographic markers involved and related innovations in first-trimester screening for Down.

In this thesis we concentrated on the following questions:

1. Do the different methods of estimating gestational age affect reliability of first-trimester screening for Down syndrome and other aneuploidies?
2. Is there a difference in first trimester risk estimates for trisomy 21 and other aneuploidies, as calculated by two different software packages used in the Netherlands?
3. What is the impact of laboratory manufacturing differences in of the concentration of the pregnancy-associated plasma protein A (PAPP-A) on the test performance of first-trimester screening (FTS) for Down syndrome and other aneuploidies?
4. What is the prevalence of detectable jugular lymphatic sacs in a setting for first trimester screening of Down syndrome, and is there an effect of jugular lymphatic sacs on the screening performance for chromosomal abnormalities?
5. Is there an added value of incorporating additional first trimester markers to an extensive risk assessment for aneuploidy screening in the first trimester?
6. What is the difference in growth patterns of aneuploid fetuses (trisomy 21, 18, 13 and X0) during the late first trimester using a 3D Virtual Reality (VR) system?

All studies were done on prospectively collected data of our first-trimester screening population, attending the out-patient clinic of the division of Obstetrics and Prenatal Medicine at Erasmus University Medical Center, Rotterdam, The Netherlands. Research questions were formulated based upon findings that were encountered during the first-trimester screening program and known from the relevant literature at the time of the studies.
The topics being elaborated in this discussion are the influence of the biochemical screening, ultrasound screening and the combination of those two in the first-trimester screening program. Furthermore the future of the first-trimester screening program for Down syndrome and other aneuploidies will be discussed.

In January 2007 the national program for prenatal screening for Down Syndrome has been implemented in the Netherlands[1]. For this purpose, the first-trimester combined screening (FTS) test was advocated. First-trimester screening by means of the combined test (CT) for the detection of Down syndrome (trisomy 21) is offered to all pregnant women. This test encompasses the assessment of two biochemical markers in maternal serum, i.e. free beta human chorionic gonadotropin (free β-hCG) and pregnancy-associated plasma protein A (PAPP-A), and the sonographic assessment of the fetal nuchal translucency (NT). Combining these three markers, together with maternal age, 76-91 % of the pregnancies with trisomy 21 can be detected with a 3-7% false positive rate [2-5]. First-trimester screening in the Netherlands was expanded to screening for trisomies 13 and 18 in June 2010.

The value of maternal serum markers, i.e., pregnancy-associated plasma protein-A (PAPP-A) and free ß-human chorionic gonadotropin (free β-hCG) has been determined in large studies. In affected pregnancies the median level of free beta-human chorionic gonadotropin (free β-hCG) was 1.79 times the median level for unaffected pregnancies. The other marker, PAPP-A, was 0.43 times the normal median [6-9]. Additionally low levels of first-trimester maternal serum PAPP-A are also shown to be predictive of other fetal chromosomal abnormalities, such as trisomy 13 and 18, triploidy and sex chromosome aneuploidy.

The blood sample for the maternal serum screening has to be taken between 8+4 to 13+6 weeks' gestation. Studies have demonstrated that the combined FTS test performs best when the maternal blood sample is taken at 10 weeks gestation [10] and the measurement of NT is performed at 12 weeks [11]. All maternal serum samples were analyzed with the AutoDELFIA analyzer (Perkin Elmer Life Science, Boston, MA, USA) and commercially available kits. The calculations of the maternal-weight-corrected MoMs of the biochemical markers were done using the LifeCycle-Elips software (Perkin Elmer Life Science, Boston, MA, USA).

As demonstrated in **Part 1** of this thesis the biochemical screening in the first-trimester is an area in which small differences may have large consequences. Considerable differences in pregnancy dating can be found, even in women who reported a regular menstrual cycle. Because the missing - or the lack of reliable - information, LMP cannot be used in an estimated 40% of the eligible women [12].

Hence, dating by means of ultrasound in early pregnancy has become a valid way of estimating gestational age. The test performance is dependent on a consistent and standardized determination of all parameters and is only applicable in a relatively small time frame. To allow for systematic changes in serum levels of PAPP-A and free β-hCG with changing gestational age, serum concentrations are converted into multiple of the normal median (MoM) at a given gestational age. Calculations of the maternal-weight-corrected MoMs of the biochemical markers depend on reliable determination of gestational age. Different methods of estimating gestational age do affect reliability of first-trimester screening for Down syndrome. The use of ultrasound to estimate gestational age in normal pregnancies improves the sensitivity and specificity of maternal serum screening [13,14]. However, as in measurement of NT there should be a reliable measurement of the CRL as both NT and biochemical screening algorithms are CRL depended [15,16]. Typically, growth restriction in aneuploid pregnancies is of early onset, and is evident from the first trimester onwards. In trisomy 21, however, crown-rump length (CRL) measurements are similar to chromosomally normal fetuses of the same gestational age (GA) [17-19]. Growth restriction can

be observed in trisomy 18 at 11-14 weeks' gestation. Though small, non-significant, differences in CRL are observed in trisomy 13, monosomy X, and trisomy 21 [20]. For the final risk estimation, the ultrasound data and the results of the maternal serum analysis of the free β-hCG and PAPP-A can be combined in the Fetal Medicine Foundation® (FMF) module in an obstetrical software database (always using the latest available version of Astraia ®, Astraia Software GmbH, München, Germany). However if the FMF module or stand-alone FMF software is not available for sonographers these calculations can be made by the laboratory using the Wallac-Perkin-Elmer®software in LifeCycle-Elips (Perkin Elmer Life Science, Boston, MA, USA). Risk estimates derived from each software program appeared to be strikingly different. There is a discrepancy in reported size of risk resulted from disparities between the two calculation methods for the assessment of the individual risk for trisomy 21. The disparities in risk estimates can be explained by significant differences in reported likelihood ratio's for biochemical analyses ($p=0.01$), NT measurements ($p<0.0001$) and both screening parameters combined ($p=0.003$). Manufactures constantly update software packages without fully informing those who are involved in prenatal counselling and screening about the changes made in their new releases. From the public health point of view, first-trimester screening for trisomy 21 and other aneuploidies should be preferably done in a standardized way. For this purpose 'harmonization' was introduced in the Netherlands. However, this is only applicable on biochemical analyses of the maternal serum. There is no check on applied gestational age and the calculation of the combined risk is done in the LifeCycle or in the FMF software.

The fact that even fully implemented harmonization is not always reliable, is shown by the fact that between the 7 laboratories, which perform the biochemical testing in the Netherlands, differ in their methodological approach. According to the national reference laboratory of the RIVM, half of the laboratories use the DELFIA Xpress® analyzer (PerkinElmer, Turku, Finland) and the other half makes use of the AutoDELFIA® analyzer (PerkinElmer, Turku, Finland). However, all are dependent on commercially available kits, produced by the same manufacturer PerkinElmer (Turku, Finland). The fact that this small

difference in harmonization was a turn for the better, is demonstrated when an erroneous PAPP-A kit for the DELFIA Xpress® analyzer was produced and a 20% downward shift in concentrations with a five percent increase in monthly screen-positive findings was found. Corrections and re-assessment of the maternal serum samples could be performed by the national reference laboratory of the RIVM, which uses the AutoDELFIA® analyzer. Strangely enough, no other laboratory with the DELFIA Xpress® analyzer reported similar findings in a downward shift of PAPP-A concentrations or an increase in false positive findings in the described period.

In **Part 2** we described different ways of introducing different ultrasound techniques into the FTS-test and their additional value. First and foremost, these sonomarkers or techniques should be independent, i.e., not be interrelated. Nuchal translucency (NT) is portrayed as subcutaneous fluid accumulation in the neck region of unknown origin. Although many theories have been put forward a common morphogenesis explaining the interrelationship between the complete spectrum of fetal malformations and enlarged NT is still lacking. Earlier studies [21] described the development of the Jugular Lymphatic Sacs (JLS) preceded the NT and that the JLS showed distension just prior to the occurrence of the increased NT [22-24]. However they were not able to give conclusive evidence on the question if JLS precedes NT or visa-versa. In this thesis we concluded that visual JLS significantly predict chromosomal abnormalities, although NT is a better predictor. In terms of test performance, however, the additional value of combined testing is limited as both predictors are interrelated.

The assessment of the fetal NT in combination with maternal blood sampling has shown to be an accurate and sensitive screening test for trisomy 21 and other aneuploidies with a sensitivity of 90% and a specificity of 95% [4,19,25,26].

The sensitivity of this way of screening has shown to be superior to that achieved by maternal age alone (44%) and the second trimester serum biochemistry (60%). Studies from specialist centers have demonstrated that, in addition to NT, absence of nasal bone [27,28]

(sensitivity 60-70% and specificity 98%), increased impedance to flow in the ductus venosus [29] (sensitivity 80% and specificity 95%) and tricuspid regurgitation [30] (sensitivity 70% and specificity 95%) are other highly sensitive and specific first trimester sonographic markers of trisomy 21 [31]. This innovative first-trimester screening by adding multiple so called sonomarkers may achieve higher detection rates among women at intermediate risk (>1 in 999 and <1 in 50) than screening by combination test alone (Sonek and Nicolaïdes reported detection rates by using two markers 94%, three markers 95%, and four markers 96% at a false positive rate of 2%) [32].

In this thesis we tested these four sonomarkers in a clinical trial of 30 months with an intermediate risk after first-trimester combined screening, i.e., a risk of > 1:200 to <1:50. All women included in our study population were offered invasive diagnostic testing such as chorionic villus biopsy or amniocentesis. Some of these women, however, were reluctant to have invasive testing because these tests are associated with a risk of iatrogenic miscarriage (0.3-0.5%) [33-38]. Additional sonographic markers have been identified to assess the risk of trisomy 21, 18 and 13 in the first trimester of pregnancy. This approach, the so-called combined test 'plus' (CT-plus) has been used as a second-tier test following FTS. In this study we evaluate the additional value of CT-plus examination in patients with an intermediate risk of trisomy 21, 18 and/or 13 after the first-trimester combined test with respect to outcomes (karyotyping). The CT-plus ultrasound examinations were performed following the strict methodological criteria set by the Fetal Medicine Foundation (FMF®). CT-plus is a tool that can be used for decreasing the false-positive rate after the FTS. The decrease in screen-positive rates may lead to fewer invasive procedures and in that way prevents iatrogenic miscarriages. However, the additional value of the assessment of sonomarkers is limited, since it is associated with a low detection rate of fetal aneuploidy (sensitivity 25%).

In addition to the first trimester sonomarkers new ultrasound techniques have been introduced in the last decade. Because aneuploidy is associated with fetal growth restriction [39-44], already present from the first trimester of pregnancy onwards, three-dimensional (3D)

ultrasound can be used to measure Embryonic Volume (EV) in addition to crown-rump length (CRL). EV seems to be a more accurate parameter to assess first trimester growth restriction in aneuploid fetuses. The introduction of the Virtual Reality (VR) visualization technique enables us to use all three dimensions of these 3D US scans [45]. CRL growth restriction may be observed in trisomy 18 although this is less clear in fetuses with trisomy 13, monosomy X and trisomy 21 [39,40,42]. In contrast to CRL, EV is significant smaller in trisomy 21, trisomy 18 and trisomy 13. Therefor EV measurements may be used to diagnose abnormal first trimester growth. This is also shown in the case report on a placental confined trisomy 16 we presented. Early fetal growth restriction was detected by 2D, 3D and EV measurements. Chorionic villus sampling (CVS) and amniocenteses will remain the gold standard for detecting fetuses with chromosomal anomalies. Invasive diagnostic testing, however, is associated with an increased risk of miscarriage [33-37]. Screening by means of the FTS- test has been a proven and reliable alternative, providing that the test is executed in standard way. Since April 2014 NIPT is accessible in case of increased risk for aneuploidies [46].
The use of the sonographic markers requires accurate examination by highly skilled operators and is time consuming and should be restricted to specialist centers.
The use of 3D and VR is a useful tool for detection of structural anomalies in early pregnancy (6-12 weeks). Especially in case of X-linked, dominant or recessive inherited anomalies of the brain (for instants X-linked hydrocephaly or Meckel-Gruber syndrome), anomalies of the skull (for instants Adams-Oliver syndrome, cranio-fronto-nasal dysplasia, Carpenter syndrome) and anomalies of the limbs (such as TAR syndrome, SHSF syndrome, oral-facial digital syndrome and many others). EV however could be a real asset in first-trimester screening for Down syndrome and other chromosomal or structural anomalies, although this technique needs more research. It has proven its value in diagnosing early growth restricted fetuses, which is associated with chromosomal abnormalities and pregnancy complications such as early fetal demise and IUGR. The addition of the VR technique is already being developed into an application that can be used as a desktop [45] and the value of 3D ultrasound and EV are shown in the last chapter in which we describe a case diagnosing of

trisomy 16. In this case very early growth restriction was found by 3D and EV next to an enlarged NT and abnormal results of maternal serum sampling. Throughout the pregnancy the fetus was assessed for structural anomalies by 2D and 3D ultrasound. None were diagnosed.

The future of the FTS-test in screening for aneuploidies is to be discussed because of new developments in Non-Invasive Prenatal Tests (NIPT). Since the end of the nineteen nineties the research in cell-free fetal DNA in maternal serum has taken a flight [47]. In the Netherlands is has already been used for genotyping fetal rhesus-D and early pregnancy gender typing, in case of gender confined recessive genetic anomalies. In the area of detecting trisomy 21 and 18 much progress has been made. NIPT is a highly sensitive test for trisomy 21 in comparison with the first-trimester screening test. False positive results of NIPT are estimated less than 0.3%, which gives a test specificity of 99,7% [48]. The positive predicting value of this test, however, is reliant on the a priori risk of the pregnant woman. The higher the a priori risk, the higher the positive predicting value. However, in case of an unfavorable NIPT outcome, confirmation by invasive testing is currently required for validation purposes. NIPT has a failure rate of 3%, i.e. no definitive result is possible. This is more likely in women with a high body mass index (BMI). Furthermore the validation in multiple pregnancies is still controversial [49]. International studies have proven the reliability of NIPT [48] even in a low-risk population [50]. In the Netherlands NIPT is since April 2014 available, but only in case of an increased risk for chromosomal anomalies after FTS. In the future NIPT may be available for other genetic and congenital anomalies [51].

Even though the screening for aneuploidies by means of the first-trimester screening test could slowly become replaced by non-invasive karyotyping [52-57], the assessment of early pregnancy ultrasound screening by 2D and 3D ultrasound and use of Doppler, in combination with EV techniques, will be valuable in early screening for structural malformations. It is known that an abnormal flow in the ductus venosus, an abnormal flow over the tricuspid valve and an increased NT thickness are associated with cardiac defects, other structural anomalies and poor pregnancy outcome (miscarriage or intrauterine demise)

[58-68]. Recently, the measurement of low-resistance flow in the hepatic artery in addition to the tricuspid regurgitation and abnormal ductus venosus flow, has become an added screening instrument for chromosomal abnormalities, genetic syndromes and structural anomalies [69,70]. The majority of significant structural defects are already detectable at this point in pregnancy [32,71,72]. With the use of additional biomarkers, like placenta growth factor (PLGF) and alpha-fetoprotein (AFP), not only increase the detection rate (DR) (87.9%) and lowers the false positive rate (FPR) (1.8%) in first-trimester screening for aneuploidies [73,74], but are shown useful in first-trimester screening for pre-eclampsia, fetal growth restriction and preterm birth [75-78]. Nowadays a great deal of research is being done on risk assessment for pre-eclampsia, preterm labour, intrauterine growth restriction, macrosomia and gestational diabetes.

It is not likely that in the near future the first trimester screening will be replaced by NIPT. The ultrasound scan at 11-14 weeks is not just for measurement of CRL and NT. It can be and is more frequently used for meticulous check of fetal structures and early diagnosis of major fetal abnormalities [79]. The use of NIPT should be used complementary to the existing strategy of first-trimester ultrasound and biochemistry [74].

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Chapter 10

Addendum

- **Summary in English**
- **Samenvatting in Nederlands**
- **Authors and Affiliations**
- **Publications**
- **Word of thanks/Dankwoord**

Summary

The overall aim of the thesis is to describe the first trimester screening test for Down syndrome and several methodological issues. This first-trimester screening test combines biochemical screening of maternal serum and ultrasound screening. In this way both subsets of combined screening depend on interpreting measurements for the individual's risk of Down syndrome, thereby taking into account maternal age. The test performance is highly dependent on a consistent and standardized determination of parameters and is only applicable in a relatively small time frame. Reliability is an important characteristic of a screening test. Reliability is synonymous to reproducibility, repeatability, transferability, precision and consistency. These terms refer to the degree of stability when a measurement is repeated under the same conditions. In this thesis we present several studies which describe some methodological issues of the first trimester screening for Down syndrome by using ultrasound markers and maternal serum markers and the combination of these markers in order to provide more insight in the day to day use of the first trimester screening test.

Chapter 1 describes how the first trimester combined test consists of the measurement of the fetal nuchal translucency measurement, or NT, and combination of this ultrasound marker with the maternal serum component, which involves the determination in the maternal serum of two placentally derived biochemical markers. By a mathematical algorithm, these measurements adjust the maternal age related risk of Down syndrome at the time of testing. This approach of testing is used in a screening setting. Screening is aimed at asymptomatic (low risk) populations and helps to distinguish between high risk and low risk. The success of the test depends on the reliability of the test itself, the sensitivity and specificity of the test and the execution of the test.

Part 1: Biochemical issues in the first-trimester screening test

Chapter 2 deals with the dating of a pregnancy. The first-trimester screening test combines the woman's a priori risk of Down syndrome with the NT and the findings of maternal serum

screening. The test performance of the combined first-trimester screening test is optimal in the relatively short time frame from 11+0 to 14 weeks' gestation, which corresponds with a crown-rump length (CRL) of 45 to 84 mm. Since all markers of the first-trimester screening test for Down syndrome vary with gestational age, the estimates of free β-hCG and PAPP-A are converted to a multiple of the expected normal median (MoM) to adjust for the effect of gestational age, while values for NT are derived from information of CRL at the time of NT-measurement. Therefore correct dating of the pregnancy is crucial for the assessment of the individual risk of Down syndrome. Dating by means of ultrasound in early pregnancy has become an accepted way of estimating gestational age. Provided fixed values for NT are applied, dating-scans reduce the number of screen-positive findings on the basis of biochemical screening. For the implementation of policy guidelines for Down syndrome screening it is recommended to use CRL-dependent parameters rather than LMP-dependent parameters of gestational age.

In **Chapter 3** our study shows the outcome of individual risk assessment for Down syndrome by using the commercially available software packages. The result of the first trimester combined screening is calculated by a mathematical model, thereby taking in account the maternal age-related a priori risk of the eligible women, the observed estimation of likelihood ratios (LRs) derived from maternal serum levels of the markers, free β hCG and PAPP-A, and the NT. This study shows that with the same screening parameters, marked disparities were observed between numerical risk estimates derived from the Fetal Medicine Foundation (FMF) [software] package and those derived from Wallac-Perkin-Elmer®software (Lifecycle). The different software packages with their underlying algorithms have an impact on the quality of testing by influencing sensitivity, specificity, and the positive and negative predicting values. Risk estimates based on the combination of maternal age, biochemical findings and NT measurements were for most women strikingly different. However, major disparities arise with outliners, which are affected by disparate use of truncation limits. These resulting differences in risk estimates could have a major emotional impact on the woman. In fact, both caregivers and couples felt at loss with these discrepant test results. Software

manufacturers are not transparent with regard to the truncation limits used. It is illustrated that the lack of agreement between these risk calculation methods could give rise to major counselling problems.

In **Chapter 4** we describe the impact on FTS performance of an approximately 20% downward shift in the PAPP-A concentrations due to the erroneously produced PAPP-A kits used for the FTS test for Down syndrome. In the Netherland seven laboratories are responsible for the assessment of the biochemical first-trimester serum markers. For a short period of time in 2009 a downward shift in concentrations of PAPP-A was observed by the laboratory quality institute UK-NEQAS and prenatal screening centres in the region expressed their concern about the increase in number of screen positive findings following combined risk calculation they encountered in the last months. Our study evaluated the potential clinical consequences of this impact and concluded that the consequences of erroneous low PAPP-A and thereby an increased biochemical risk calculation were considerable. Of the total study group 5% had an invasive procedure that was based on an incorrect increased combined risk estimate. Fortunately this has not resulted in reported iatrogenic miscarriages. Both laboratories and manufacturers need to evaluate their own performance critically and take all possible measures to ensure that they are providing high-quality risk estimates based on maximum precision. Very important are the UK-NEQAS reports in this quality assessment. However, health care professionals in the field of prenatal screening of Down syndrome are still accountable for the implementation of the FTS. A frequent audit of the distribution of the biochemical markers free β-hCG and PAPP-A alongside with the distribution of NT is advocated.

Part 2: ultrasound issues in first-trimester screening for Down syndrome

Chapter 5. Measurement of the NT and the combination with maternal serum sampling in the first trimester screening for Down syndrome has become standard in Dutch antenatal care. The explanation of the relationship between the complete range of fetal malformations and an enlarged NT is still lacking. The entity of an enlarged NT and the presences of

abnormal development of Jugular Lymphatic Sacs (JLS) have been described in many studies, hypothesizing that a disturbance in lymphatic growth precedes an enlarged NT and could be an early prognostic sign. In an observational study the prevalence and detectability of JLS were investigated. Furthermore, the potential of JLS as an early marker for Down syndrome was tested. In this context, the prerequisite for adding JLS to first trimester measurement of the nuchal translucency is its independent association with chromosomal abnormalities. The absence of JLS in early pregnancy could perhaps be used as a sonographic marker for ruling out chromosomal abnormalities (negative predictive value). However the ability to detect JLS is limited by the spatial resolution of ultrasound machines. The sonographic visualization of JLS smaller than 2.0 mm is time consuming and requires specially trained and highly skilled ultrasound operators. The additional value of combined testing is limited as both predictors are interrelated.

Chapter 6. The first-trimester screening test is widely used and acknowledged method for the assessment of the risk of fetal aneuploidy. Women are considered "screen positive" when the test result indicates an increased risk of trisomy 21, 18 and/or 13 i.e. risk $\geq$ 1 in 200. These patients are offered an invasive diagnostic test such as chorionic villus biopsy or amniocentesis. Some of these women, however, are reluctant to have invasive testing because these tests are associated with a risk of iatrogenic miscarriage (0.3-0.5%). In this chapter we evaluate the additional value of CT-plus examination in patients with an intermediate risk ($\geq$1 in 200 and <1 in 50) of trisomy 21, 18 and/or 13 after the first-trimester combined test. These markers include the nasal bone, fronto-maxillary facial angle, ductus venosus, and tricuspid valve Doppler evaluation. This approach, the so-called combined test 'plus' (CT-plus) has been used as a second-tier test following first-trimester screening. The CT plus is a tool that can be used for decreasing the false-positive rate after the first trimester combined test, The decrease in screen-positive rates may lead to fewer invasive procedures and in that way prevents iatrogenic miscarriages. However, the assessment of these specific sonomarkers coincided with a decreased detection rate of trisomy 21 and 18, making its additional value quite limited among this specific category of women.

Chapter 7. Aneuploidy is associated with fetal growth restriction already present from the first trimester of pregnancy onwards. With three-dimensional (3D) ultrasound it is possible to measure Embryonic Volume (EV) in addition to crown-rump length (CRL). In this chapter we examined whether EV is a better predictor of growth restriction in aneuploid fetuses. Traditionally, first trimester fetal growth has been documented by two-dimensional (2D) CRL measurements. With the introduction of three-dimensional (3D) ultrasound (US) it became possible to measure embryonic and fetal volumes. The relative increment of fetal volume is much larger than the increment of CRL during the same period. Using V-Scope it is possible to perform several biometric measurements, like CRL and EV, in the I-Space benefiting from the true 3D depth perception. CRL, the golden standard, can only be used as a reliable indicator of growth restriction in aneuploid fetuses in the first trimester for pregnancies with trisomy 18 (-42.5%). Using EV, growth restriction is also evident in trisomy 21 (-28%) and trisomy 13 (-43.4%). In monosomy X a non-significant smaller (-35.5%) EV was found. The atypical volume of aneuploid fetuses may be explained by an extended cell cycle under influence of so called checkpoint control gens, resulting in a significant smaller number of cells compared to an euploid fetus. This study shows that in aneuploid fetuses EV measurements may be used to diagnose abnormal first-trimester growth.

Chapter 8 The additional value of 3D sonography and I-Space innovation is discussed in a well-documented case of “confined placental trisomy 16 mosaicism”. The patient enrolled in a study early in pregnancy and a 3D ultrasound scan was made weekly from about 5 to 6 weeks of gestation till 13 to 14 weeks in which measurements were taken and anatomical futures (particularly the development of the limbs) were compared to embryonic development described according the Carnegie Stages. In this case there was a discrepancy in gestational age based on a certain last period, sonographic measurements and development. Besides the growth restriction there was an enlarged NT and a low PAPP-A concentration. Additional invasive diagnostic tests (CVS and amniocenteses) revealed a placental confined trisomy 16 mosaicism. Growth and development were strictly followed up by 2D and 3D ultrasound. No anomalies were found and growth stayed symmetrical small for gestational age. The 3D

ultrasound and assessment in I-Space clearly contributed to the early detection of intra uterine growth restriction and development in a fetus with an uncommon chromosomal anomaly. We believe that systems such as the I-Space open a new era to study embryonic growth and development in vivo.

In the general discussion, the last part and **chapter 9** of this thesis, the results of all studies are discussed and combined in a broader perspective. Our goal was to discuss the methodological issues of first-trimester screening (FTS) and the impact of small changes within the biochemical and ultrasound components, and the application in the day to day practice. Alongside the new ultrasound techniques of 3D and Virtual Reality are discussed. With the introduction of the non-invasive prenatal test (NIPT) the importance of FTS has hardly changed. If the result of FTS shows an increased risk for trisomy 21, 13 and/or 18 of 1 in 200 of more, there is now the possibility to choose for the NIPT, besides the known invasive diagnostic testing (chorionic villus sampling or amniocenteses) without the risk of a miscarriage due to the invasive techniques.

The ultrasound scan between the 11^{th} and 14^{th} week of gestation is not just for measurement of the CRL and NT. It can and has become more and more a first structural scan of fetal structures and a tool for early diagnosis of major fetal anomalies. The application of fetal Doppler sonomarkers has proven its value in early detection for congenital heart defects.

The possibility of NIPT should be used complementary to the existing strategy of first-trimester ultrasound and biochemistry.

Samenvatting

Dit proefschrift heeft als doel het beschrijven van de eerste-trimesterscreeningtest voor downsyndroom en een aantal methodologische facetten. Deze test, ook wel combinatietest genoemd, bestaat uit twee verschillende onderdelen: een biochemisch deel en een echoscopisch deel. Beide delen, en daarmee ook de combinatie ervan, zijn afhankelijk van het op een juiste manier hanteren van bepalingen en berekeningen van de individuele kans op downsyndroom. Bij de berekening wordt de leeftijd van de betrokkene verdisconteerd. De testeigenschappen berusten op een consequente en gestandaardiseerde meting van variabelen die alleen binnen een relatief kort tijdsbestek worden uitgevoerd. De betrouwbaarheid van een screeningstest, en daarmee de sensitiviteit en specificiteit, is verwant met reproduceerbaarheid, herhaalbaarheid, overdraagbaarheid, precisie en consistentie. Deze termen verwijzen naar de mate van stabiliteit als een meting wordt herhaald onder dezelfde omstandigheden. In dit proefschrift presenteren we studies die een aantal methodologische facetten van de eerste-trimesterscreening voor downsyndroom met behulp van echoscopische markers en maternale serummarkers, en de combinatie van deze markers, evalueert om meer inzicht te krijgen in de dagelijkse toepasbaarheid van de eerst-trimesterscreeningtest.

In **hoofdstuk1** wordt beschreven hoe de eerste-trimestercombinatietest is opgebouwd uit de meting van de foetale nekplooi, of wel nuchal translucency (NT), en combinatie van deze echoscopische bepaling met de biochemische component, bestaande uit de bepaling in het maternale serum van twee eiwitten. Deze metingen zetten de leeftijdsspecifieke kans voor downsyndroom om in een zwangerschap specifieke ofwel gecorrigeerde kans. Deze testen worden in screeningssetting gebruikt. Screening heeft betrekking op asymptomatische (laagrisico) populaties en helpt een onderscheid te maken tussen hoog risico en laag risico. Het succes van de test is afhankelijk van de betrouwbaarheid van de test zelf, de sensitiviteit en specificiteit en de uitvoering van de test.

Deel 1: Biochemische kwesties in de combinatietest

In **hoofdstuk 2** wordt de invloed van het dateren van een zwangerschap op de betrouwbaarheid van de eerste-trimesterscreeningstest voor downsyndroom of combinatietest besproken. De screeningtest moet worden uitgevoerd tussen de zwangerschapsduur van 11 tot 14 weken die overeenkomt met een kruin-stuitlengte of Crown Rump Lengh (CRL) van 45-84 mm. Aangezien alle markers van de combinatietest variëren met de zwangerschapsduur, worden de waardes van free β-hCG en PAPP-A omgezet naar een zgn. 'multiple of the expected normal median (MoM)' om te corrigeren voor zwangerschapsduur, terwijl de NT-meting afgezet wordt tegen de CRL ten tijde van de meting. Het correct dateren van de zwangerschap is daarom van cruciaal belang voor het berekenen van de zwangerschap specifieke kans op downsyndroom. Het is aan getoond dat echoscopie in de vroege zwangerschap meer betrouwbare informatie oplevert om de zwangerschapsduur vast te stellen. Bij gebruik van vaste NT-waardes kunnen termijnbepalingen het aantal foutpositieve uitslagen reduceren. Implementatie van een richtlijn om bij de screening op downsyndroom de zwangerschapsduur op basis van CRL in plaats van LMP te gebruiken, wordt aanbevolen.

Hoofdstuk 3 laat zien dat de commerciële softwarepakketten die worden gebruikt voor de berekening van de kans op downsyndroom tijdens het eerste trimester van de zwangerschap niet altijd leiden tot hetzelfde resultaat. De kans op een kind met downsyndroom wordt berekend op basis van de uitgangkans (a priori kans) passend bij een maternale leeftijd, in combinatie met de echoscopisch gemeten foetale nekplooidikte en de verhoudingen van twee door de placenta geproduceerde eiwitten, free β hCG en PAPP-A, in het moederlijk serum. In dit hoofdstuk wordt aangetoond dat ondanks gelijke screeningparameters de berekenende kansen bij het softwarepakketten van de Fetal Medicine Foundation (FMF) en die van Wallac-Perkin-Elmer®software (LifeCycle) verschillende uitkomsten opleveren. De verschillende software pakketten, met hun onderliggende algoritmes, hebben een impact op de testeigenschappen in de vorm van sensitiviteit, specificiteit, positieve en negatieve voorspellende waarden. Voor de grootste groep van de bepalingen zal er geen uitgesproken

verschil te detecteren zijn. Maar bij de uiterste, de zgn. ‘uiteinden of afkappunten (truncaties)’ van de spreiding van de bepaalde waarden zijn de verschillen soms aanzienlijk. Dit kan, bij diegene die mogelijk onterecht in de hoog risico zone zijn gekomen, lijden tot onnodige ongerustheid bij de aanstaande ouders. De fabrikanten van de softwarepakketten zijn niet transparant over de limieten en redenen van de gehanteerde afkappunten. Verschillen in risicoberekeningen kunnen worden teruggevoerd op significante verschillen in gehanteerde likelihood ratio’s van de biochemische analyse, NT meting en de combinatie van beide parameters. Het is illustratief voor het gebrek aan overeenkomst tussen de risicoberekening methoden en kan voor grote problemen zorgen bij de counseling omdat het niet duidelijk is wat de feitelijke individuele kans is.

In **Hoofdstuk 4** wordt de impact van een 20% daling van de PAPP-A concentraties ten gevolge van een productiefout in de PAPP-A bepalingsets op de prestatie van de combinatietest beschreven. In Nederland zijn zeven laboratoria belast met de bepaling van de eerste-trimester serum screening. Gedurende een korte periode in 2009 werden door aangesloten centra voor prenatale screening en het UK-NEQAS een toename van het aantal foutpositieve bevindingen en een verlaging van de mediaan in de PAPP-A waardes waargenomen. De studie evalueerde de potentiële consequenties van de impact en concludeerde dat de consequenties van de foutief lage PAPP-A waarden en daarmee de verhoogde biochemische risicoberekening aanzienlijk. Op de hele studiegroep had 5% een invasieve ingreep op basis van een niet correct gecombineerde risicoberekening. Gelukkig waren er geen iatrogene miskramen na deze ingrepen gemeld. Deze studie toont aan dat continue en strenge controle van de kwaliteit van de eerste-trimsterscreeningstest van groot belang is.

Deel 2: Echoscopische kwesties in de combinatietest

Hoofdstuk 5. Er zijn veel theorieën over het ontstaan van de verdikte nekplooi. In studies werd aangetoond dat een verstoring van de ontwikkeling van het lymfvatenstelsel en daarmee een verdikking van de lymfvaatzakjes (Jugular Lymphatic Sacs of JLS), voorlopers

van de lymfknopen, in de nek regio, vooraf gaat aan een verdikte nekplooi. Voortbordurend op die hypothese zou het kunnen zijn dat verdikte JLS in de vroege zwangerschap een aanwijzing zijn voor het ontwikkelen van een verdikte NT. In een observationele studie werd de aanwezigheid en meetbaarheid van JLS getest om te beoordelen of het een geschikte vroege marker is. In deze context was de toevoeging van de JLS meting onafhankelijkheid van de gemeten NT en de associatie met chromosomale afwijkingen een voorwaarde. De afwezigheid van JLS zou mogelijk gebruikt kunnen worden om chromosomale afwijkingen uit te sluiten (negatief voorspellende waarde). De detecteerbaarheid van JLS is echter afhankelijk van de resolutie van de echomachine. Het echoscopisch in beeld brengen van JLS kleiner dan 2,0 mm is een tijdrovende bezigheid en vereist speciaal getrainde en bekwame echoscopisten. Er kon geen meerwaarde voor JLS in de eerste-trimesterscreening aangetoond worden en er bleek afhankelijkheid van JLS in combinatie met een verdikte NT.
Hoofdstuk 6. De eerste-trimestercombinatietest is een veel gebruikte en erkende test voor screening op trisomie 21, 13 en 18. Als er een gecorrigeerd individuele kans van 1 op 200 of hoger wordt gevonden, is er sprake van een verhoogde kans en komt de zwangere in aanmerking voor invasieve diagnostiek (vlokkentest of vruchtwaterpunctie). Sommige zwangere vrouwen zien op tegen een dergelijk invasieve test omdat deze geassocieerd is met een kans op een miskraam (0,3-0,5%). In dit hoofdstuk is onderzoek gedaan naar de toegevoegde waarde van bepaling van zogenaamde specifieke echomarkers. Deze echomarkers zijn het neusbotje (nasal bone), de hoek van het aangezicht met de bovenkaak (fronto-maxillary facial angle), het stroomprofiel van de ductus venosus en de functie van de rechter hartklep (tricuspid regurgitatie). De beoordeling en resultaten van deze echomarkers werden gecombineerd met NT en biochemie. Hieruit werd opnieuw een individueel kansberekening gemaakt. Deze benadering werd de combinatietest-plus (CT-plus) genoemd. Het CT-plus onderzoek werd aangeboden aan vrouwen met een verhoogd individuele kans tussen 1 op 200 tot 1 op 50. Na het CT-plus onderzoek werd altijd de invasieve diagnostiek alsnog aangeboden.

De CT-plus test scoorde relatief goed op de correctie van de zogenaamde foutpositieve testuitslagen. CT-plus kan worden ingezet om het aantal foutpositieve uitslagen, ofwel het hoge aantal onterechte hoog-risico uitslagen omlaag te brengen en daarmee het aantal invasieve ingrepen met kans op miskraam t.g.v. die ingreep te verminderen. CT-plus onderzoek scoort echter onder de maat als het gaat om de detectie van chromosoomafwijkingen (lage sensitiviteit) heeft niet bijgedragen aan een betere identificatie van chromosomale afwijkingen. De toegevoegde waarde van het CT-plus onderzoek bij deze categorie vrouwen is om die reden beperkt

Hoofdstuk 7. Chromosomale afwijkingen worden vaak geassocieerd met groeivertraging. Vooral trisomie 18 is bekend om de vroege uiting van een verstoring in groei. Dit verschil is minder zichtbaar bij trisomie 13 en monosomie X. Bij trisomie 21 verloopt de groei, uitgedrukt in de CRL meting, echter ongeveer gelijk aan die van normale foetus. De CRL metingen worden traditioneel uitgevoerd in 2 dimensionale (2D) echoscopie. Met de introductie van 3 dimensionale (3D) echoscopie werd het mogelijk om embryonale en foetale volumes te meten. Met de komst van Virtual Reality kunnen 3D volumes in een holografische projectie (I-Space) worden omgezet en kunnen volumes nauwkeurig worden gerekend. Eerdere studies hebben aangetoond dat terwijl de CRL verdubbelde, het volume 5 tot 6 maal toenam. Met deze technieken en eerdere studies werden de volumes van chromosomaal afwijkende foetus vergeleken met die van normale foetus. Bij meting van de volumes bleken respectievelijk de verschillen van foetus met trisomie 21 met -28%, foetus met trisomie 18 met -42,5%, foetus met trisomie 13 met -43,4% en monosomie X met -35,5% kleiner dan de volumes van normale foetus. Het abnormale volume van chromosomaal afwijkende foetus kan worden verklaard door langere duur van de celcyclus ten gevolge van zogenaamde ‘checkpoint controlegenen’ met als resultaat een significant verlaagd aantal cellen in vergelijking met normale foetus. Het meten van het foetale volume kan een toegevoegde waarde hebben in de vroege screening en diagnose van chromosomaal afwijkende foetus.

Hoofdstuk 8. De toegevoegde waarde van 3D echoscopie en de I-Space komen ook tot uiting in de bespreking van een goed gedocumenteerde registratie van een foetus met een

specifieke chromosomale afwijking die alleen in de placenta kon worden aangetoond (mozaïek trisomie 16) In het kader van onderzoek met 3D en I-Space werden wekelijkse metingen werden verricht in het eerste trimester van de zwangerschap (van week 5-6 tot en met week 13-14) en daarnaast werd de zichtbare anatomische ontwikkeling (vooral ontwikkeling van de ledematen) van de foetus vergeleken met de ontwikkelingsstadia van de foetus beschreven aan de hand van de Carnegie Stadia. In deze casus bleek een discrepantie tussen de zwangerschapsduur gebaseerd op een zekere laatste menstruatie met regulaire cyclus en de echoscopische termijnbepalingen op basis van de CRL. Daarnaast werd een verdikte nekplooi waargenomen. Aanvullende invasieve diagnostiek (vlokkentest en vruchtwaterpunctie) werd aansluitend verricht. Hieruit bleek dat er sprake was van een placentaire trisomie 16. De zwangerschap werd verder nauwkeurig gevolgd met 2D en 3D echoscopie. Er werden geen structurele afwijkingen gezien. De groei bleef symmetrisch te klein voor de verwachte zwangerschapsduur. De 3D echoscopie en beoordeling in de I-Space hebben bijgedragen aan de vroege detectie van de groei- en ontwikkelingsvertraging bij een chromosomale afwijking.

In de algemene discussie, het laatste deel en **hoofdstuk 9** van dit proefschrift, worden de resultaten uit alle studies besproken en waar mogelijk gecombineerd. Het doel was het bediscussiëren van de methodologische aspecten van de eerste-trimestercombinatietest (CT) en de invloed van kleine veranderingen binnen de biochemische en echoscopische onderdelen van de test en de toepasbaarheid daarvan in de dagelijkse praktijk. Daarnaast zijn nieuwe echotechnieken belicht. Met de intrede van de non-invasieve prenatale test (NIPT) lijkt de rol van CT niet veranderd. Wanneer de uitslag van de CT een verhoogde kans voor trisomie 21, 13 en/of 18 aangeeft van 1 op 200 of hoger, kan van april 2014 in een testfase gekozen worden voor verdere diagnostiek met NIPT, naast de reeds bekende invasieve diagnostische technieken (vlokkentest en vruchtwaterpunctie) met kans op een miskraam. t.g.v. de punctie.

Het echo-onderzoek tussen de 11de en 14de week van de zwangerschap is niet alleen voor het meten van de CRL en de NT. Het kan en wordt ook steeds vaker gebruikt als een vroege

structurele scan van de foetale structuren en vroege diagnostiek van ernstige foetale afwijkingen. Hierin kunnen, vooral bij hartafwijkingen, de eerder beschreven Doppler sonomarkers een grote rol in spelen. De mogelijkheden van NIPT moeten gezien worden als toegevoegde waarde in plaats van een vervanging van de combinatietest.

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