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The impact of the combined test on the first trimester as a method of screening for trisomies 21,18, 13 and other chromosomal abnormalities. Experience of a single Italian fetal medicine unit on 12618 consecutives pregnancies

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ABSTRACT

Introduction: scope of study is to examine the performance of the combined test at 11–13 weeks for trisomies 21, 18 , 13 and other chromosomal defects with a cut off of 1/250, as determined by italian legislative decree.

Methods: in a prospective study of 12,618 pregnant women, chorionic villous sampling (CVS) was offered for a > 1/250 risk. In the intermediate risk category (between 1/251 and 1/999) there was further risk assessment through evaluation of the nasal bone (NB) in terms of presence/absence. We compared the results obtained in terms of OAPR in 3 different groups: positive combined test, immediate CVS for maternal age, CVS for maternal age in presence of low risk (>1/1000) combined test.

Results: 1490 women (11.8%), with maternal age over 35 years at the time of delivery as the indicator, chose to undergo immediate invasive testing, as required by Italian law. 1051 women (8.3%) opted to forego chromosomal defect screening for ethical or religious reasons. 10,077 (79.86%) women chose to undergo the combined test. In the 662 patients (6.5%) with an estimated risk at the time of screening higher than 1/250, 101 fetuses with chromosomal defects (CD) were found. In the 815 (8.1%) patients with intermediate risk, we applied the two-stage approach (combined test plus NB) and found 3 CD in the 27 cases with no NB. With the application of the two-stage approach (combined test plus NB in intermediate risk), in 689 cases of high risk (6.8%) we found a total of 104 CD,

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SOMMARIO

Obiettivo: esaminare il risultato del test combinato nello screening del primo trimestre a 11-13 settimane di gravidanza per tutte le anomalie cromosomiche utilizzando un cut off di 1/250, come indicato dal decreto legislativo italiano.

Metodi: studio prospettico su 12,618 gravidanze singole: offerto il prelievo di villo coriale per un rischio al test combinato superiore a 1/250;nel rischio intermedio (tra 1/251 e 1/999) è stata effettuata una rivalutazione tramite la visualizzazione ecografica dell'osso nasale in termini di presenza/assenza.

Risultati : 1490 donne (11.8%) hanno scelto di sottoporsi direttamente all'esame invasivo avendo come unica indicazione l'età materna pari o superiore a 35 anni al momento del parto; 1051 donne (8.3%) hanno scelto di non sottoporsi ad alcun tipo di accertamento riguardante le anomalie cromosomiche fetali per motivi etici o religiosi. 10,077 (79.86%) donne hanno optato per effettuare il test combinato. Nelle 662 pazienti (6.5%) con un rischio superiore a 1/250 sono state identificati 101 difetti cromosomici fetali. Nelle 815 (8.1%) pazienti con un rischio intermedio tramite la ricerca dell'osso nasale abbiamo identificato 3 ulteriori casi di anomalia cromosomica. In totale l'applicazione del metodo in due stadi (test combinato più rivalutazione dei casi con rischio intermedio) ha identificato 104 anomalie cromosomiche su 689 casi risultati ad alto rischio (6,8%), ottenendo la identificazione di un caso patologico ogni 6,6 procedure invasive. Si sono riscontrati alla nascita due casi di falsi negativi , entrambi affetti da trisomia 21. La sensibilità del test combinato associato alla rivalutazione tramite osservazione dell'osso nasale nei casi di rischio intermedio, nonostante il cut off imposto dal decreto legislativo italiano (1/250) è stata pari al 98.1%, con una

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with the odds of being affected showing a positive result (OAPR) of 1/6.6. Two cases of trisomy 21 test were found at birth (false negatives). The sensitivity of the combined test plus NB in intermediate risk with a cut off of 1/250 was 98.1%, with FPR 5.8%, FNR 0.02%, PPV 15.2%, NPV 99.9% and OAPR 1/6.6. In the group of CVS for maternal age in presence of low risk (>1/1000) combined test no chromosomal disorders were recorded.

Discussion: the application of a screening in the first trimester with a two-stage approach (combined test plus evaluation of the nasal bone in intermediate risk) with a cut off of 1/250 has a high sensitivity and a low false positive rate not only for trisomy 21, trisomy 18 and trisomy 13, but also for other chromosomal defects. The high sensitivity of the combined test for all chromosomal defects reaffirms the validity of offering the screening test in the first trimester of pregnancy to all pregnant women. Screening by cfDNA testing improves detection of trisomies, but misses a few of the other chromosomal abnormalities detected with the combined test. The application of the model of contingent testing, using the combined test as the first step and the cfDNA as the next step for cases of intermediate risk for CD, could yield the best results. Additionally, the study encourages modification of Italian legislation, supporting the exclusion of maternal age as the only indicator for invasive diagnosis in the presence of a low risk combined test .

Keywords: Combined test, Trisomy 21, Chorionic villous sampling, nasal bone, intermediate risk, cfDNA

incidenza di falsi positivi del 5.8%, falsi negativi dello 0.02%, valore predittivo positivo 15.2%, valore predittivo negativo 99.9% e numero di procedure invasive necessarie per identificare una anomalia cromosomica fetale 6.6. Nel gruppo che ha optato per sottoporsi a villocentesi nonostante un test combinato a basso rischio (>1/1000) ma con età materna superiore a 35 anni, non sono state riscontrate anomalie cromosomiche.

L'introduzione sistematica del test combinato ha portato progressivamente ad una riduzione del numero totale di esami invasivi e alla loro collocazione nel primo trimestre di gravidanza.

Conclusioni: l'applicazione dello screening delle anomalie cromosomiche nel primo trimestre di gravidanza mediante l'approccio a due stadi (test combinato più la valutazione dell'osso nasale nei casi di rischio intermedio) permette di ottenere una elevata sensibilità con una bassa incidenza di falsi positivi non solo nei confronti della trisomia 21, 18 e 13, ma anche di altre anomalie cromosomiche, nonostante il cut off di 1/250 imposto dal decreto legislativo italiano. L'alta sensibilità del test combinato nei confronti di tutte le anomalie cromosomiche conferma la validità della offerta di tale metodo di screening come primo approccio nel primo trimestre di tutte le donne in gravidanza. Uno screening basato infatti sull'analisi del DNA circolante fetale comporterebbe un incremento nel numero di trisomie identificate, ma non sarebbe capace di identificare le altre tipologie di difetti cromosomici che invece si riscontrano nel gruppo con test combinato positivo. L'applicazione del modello di test contingente, utilizzando il test combinato come primo passaggio e l'analisi del DNA circolante fetale nei casi di rischio intermedio, potrebbe migliorare i risultati sia in termini di sensibilità che di rapporto costo beneficio. Infine questo studio incoraggia la modifica del decreto legislativo italiano, auspicando l'esclusione della età materna come unico criterio di accesso alla diagnostica invasiva prenatale.

Parole chiave: test combinato, trisomia 21, villocentesi, osso nasale, rischio intermedio, DNAcf

INTRODUCTION

In Italy access to invasive prenatal diagnosis is free of charge for mothers older than 35 at the time of delivery or in case of high risk at first trimester screening for trisomy 21 or trisomy 18 with a cut-off of 1/250 at the time of the test, as determined by legislative decree[1]. In 2007, the Tuscan Regional Government approved a screening program for Down Syndrome (based on the calculation of individual risk, achieved through the application of first trimester screening (FTS) for trisomy 21 and trisomy 18) to the entire obstetrical population in order to standardize the criteria for access to prenatal diagnosis[2].

The combined test, as indicated by the Fetal Medicine Foundation (FMF), using a combination of maternal age, fetal nuchal translucency (NT) and maternal serum markers such as free-hCG and PAPP-A at 11-14 weeks, was the method of choice[3]. This allowed for a subdivision of the obstetrical population into: high risk for trisomy 21 or trisomy 18 and 13 (greater than 1/250), and low risk (less than 1/1,000). The high risk group was offered fetal karyotyping by chorionic villus sampling (CVS), while the low risk group had no indication for further medical testing. As indicated by the two-stage approach, the patients in the intermediate risk category (between 1/251 and 1/999) had additional risk assessment with first trimester ultrasound examination to determine presence/absence of nasal bone (NB), presence/

absence of tricuspid regurgitation (TR), or normal/abnormal Doppler velocity waveform in the ductus venosus (DV). CVS was performed if adjusted risk had a value of 1/250 or greater[4]. In compliance with Italian legislation, CVS was performed at no cost to patients with a maternal age of over 35 who requested the screening, regardless of combined test results[1, 5]. Since efficacy of first-trimester screening depends on the strict application of the method, as recognized in 2011 Italian Healthcare System Guidelines for low risk pregnancy[5], this study examines the results of combined screening for all chromosomal defects in a regional setting with a cut off of 1/250, as determined by Italian law[1]. The application of a screening in the first trimester with a two-stage approach (combined test plus evaluation of additional markers in intermediate risk) with a cut off of 1/250 has a high sensitivity and a low false positive rate, not only for trisomies 21 ,18 and 13, but also for other chromosomal defects, with a moderate false positive rate (5.8%). However, replacing the combined test with cell-free DNA testing can improve detection of trisomies, but misses the other chromosomal abnormalities detected by screening with the combined test[6]. Currently new tests are available to identify not only trisomies, but also autosomes and sexual chomosomes abnormalities. Major limitations related to this new tests are prohibitive costs and an insufficient clinical validation.

The high sensitivity of the combined test for all chromosomal defects affirms the validity of offering the screening test in the first trimester of pregnancy to all pregnant women and the application of the model of contingent testing , using the combined test as the first step and the cfDNA as the next step for cases of intermediate risk for CD.

Additionally, the study encourages modification of Italian legislation , supporting the exclusion of maternal age as the only indicator for invasive diagnosis in the presence of a low risk combined test .

MATERIALS AND METHODS

From January 2010 to June 2013, 12,618 women with singleton spontaneous pregnancies and a live fetus between 9+0 and 11+6 weeks were seen in the Fetal Medicine Unit at Piero Palagi Hospital in Florence. We excluded from our study twin pregnancies and pregnancies optained from assisted reproductive technology (ART). We did not collect data about eventual use of progesterone during the first trimester of pregnancy.

Midwife-lead group counseling used audiovisual and print materials to present and explain prenatal diagnostic exam options. Patients were given a questionnaire for assessment of obstetric and genetic risk on the basis of their medical and obstetrical history (**Figure 1**).

Figure 1.
Questionnaire for assessment of obstetric and genetic risk on the basis of medical and obstetrical history.

Those at risk for genetic diseases were indicated for further genetic counseling. Patients who chose the combined test were immediately subjected to blood sampling to determine PAPP-A and free beta hCG using the Kriptor immunoassay system (Brahms GmbH, Germany). Blood samples were analyzed the same day. At the end of the consultation, a second appointment was scheduled in 12 weeks for an ultrasound examination, including measurement of fetal crow-rump length (CRL), examination of fetal anatomy for diagnosis of major fetal defects and measurement of fetal Nucal translucency (NT) thickness[7]. Scan measurement was performed by a Fetal Medicine Foundation (FMF) accredited sonographer, using a convex 3D probe RAB4-8-D (Voluson E8 Expert, GE Healthcare, Milwaukee, USA). Risk calculation was then performed using FMF software with the PIA Fetal Database program (View-Point, GE Medical System, USA). At the end of the procedure, the patient was informed of the estimated risk of trisomy 21, 18 and 13. Patients were classified as high risk if the estimated risk was ≥ 1/250, which is the cut-off established by Italian law. In these cases, the parents were advised to consider having invasive fetal karyotyping and CVS was performed if the patient consented. Patients were classified as low risk with an estimated risk less than 1/1,000 and had no indication for additional medical testing. In case of intermediate risk (1/251-1/999), as indicated by the two-stage approach, the patient was asked to give informed consent to determine additional markers. NB was sufficient

to recalculate the risk in all cases. For patients who chose to undergo direct invasive prenatal diagnosis without the combined test, the first trimester ultrasound was scheduled at the time of CVS. CVS or Amniocentesis was performed for patients over 35 years old choosing invasive prenatal diagnosis regardless of the result of the combined test, in compliance with Italian law[1]. Finally, patients who did not want to have any type of prenatal diagnosis were scheduled for the first trimester obstetric ultrasound between 11+0 and 13+6 days and excluded from the study. Results were examined in terms of odds of positive tests for chromosomal anomaly (OAPR) in 3 different groups: combined test, CVS directly for maternal age, CVS for maternal age over 35 with a low risk at screening. Follow-up was performed both through a questionnaire given to all women who had the combined test or CVS and by neonatal audit at the hospital chosen for the delivery. All patients had subsequent ultrasound examinations at our center until the end of pregnancy. The statistical software package R was used for data analyse[8].

RESULTS

During the four-year study period, 12,618 consecutive singleton spontaneous pregnancies were examined in the first trimester with a live fetus between 9+0 and 11+6 weeks.

1051 patients (8.3%) decided not to receive any type of prenatal diagnosis for ethical or religious reasons and were excluded from the study. 1490 patients (11.8%) chose to undergo direct invasive prenatal diagnosis for maternal age over 35. The combined test was performed on 10,077 (79.86%) pregnancies.

Patients characteristics are shown in **Table 1**.

Table 1.
Table 1. patients characteristics (10,077 pregnancies)

Maternal age	Ethnicity	BMI	Smoking habits
Median 33,5	White 94,8%	Median 24,6 (14,3-47,5)	No smokers 81,3%
Over 35y. 28,07%	Black 1,1%	Average 22,9	Smokers 18,7%
	Asian 1,5%	**Maternal weight**	**Number of sigarettes/day**
	East asian 1,8%	Median 60.0 Kg	Median 6 (1-40)
	Other 0,9%		Average 8,3

The median maternal age was 33.5 years, with 2829 (28.07%) women over 35 years old. Ethnicity was as follows: 94.8% white, 1.1% black, 1.5% Asian, 1.8% East Asian, and 0.9% other ethnicities. Median maternal weight was 60.0 Kg. Median maternal body mass index (BMI) was 24,6. Smoking habits was present on 18,7% of the patients and the median number of sigarettes/day was 6. Nuchal translucency measurement expressed as percentage of cases within each percentile range, and values for maternal serum biochemical measurement expressed as multiple of the expected median (MoM) corrected for gestational age, maternal weight, ethnicity, smoking habits, and type of conception are shown in **Table 2**.

Table 2.
Measurement of nuchal translucency (NT) expressed in percentiles, and of serum biochemical markers expressed as multiples of the expected median (MoM) for gestational age and maternal weight in 10,077 singleton pregnant women who underwent to first trimester screening.

	< 5th centile	< Median	> Median	> 95th centile
NT	3.9%	49.5%	50.5%	4.8%
	5%	**25%**	**Median**	**75%**
FreeBetahCG	0.3694	0.6432	0.9144	1.3286
PAPP-A	0.4370	0.7601	1.1069	1.5787

The estimated risk at the time of screening for trisomy 21,18 or 13 was higher than 1/250 in 662 (6.5%) fetuses, between 1/251 and 1/999 in 815 (8.1%) fetuses, and lower than 1/1000 in 8,600 (85.3%) fetuses. In the high risk group all patients opted for invasive diagnosis and planned chorionic villus sampling (CVS), which revealed 101 cases of CD, with an OAPR of 6,55. In the intermediate risk group, NB was evaluated and it was found to be absent in 27 cases; in this group CVS revealed 3 cases of Down syndrome. In the remaining 788 cases the NB was present and the final estimated risk of CD was lower than 1/1,000. In this group no patient opted for invasive diagnosis and the follow-up did not reveal any case of CD. In summary, the two-stage approach (combined test plus NB in intermediate risk) identified 689 (6.8%) patients in the high risk group (higher than 1/250) and 9388 (93.2%) in the low risk group (lower than 1/1000). 104 fetuses with CD were found in high-risk FTS group of 689, wich means one chromosomal defect identified for every 6.6 invasive procedure (OAPR 1/6.6), with a false positive rate of 5,8%. Two cases of false negative results were found. One was a 28-year-old woman with an estimated risk of 1/10,000, and one a 37-year- old with an estimated risk of 1/1,270 (sensitivity 98.1%, FPR 5.8%, FNR 0.02%, PPV 15.2%, NPV 99.9%). Among the 4,319 women over the age of 35, 1,490 (34.5%) of them decided not to have the combined test and opted for CVS directly. In this group, 29 cases of CD were found, which means a case of fetal CD every for 51.4 invasive tests. 147 women over 35 years of age decided to have CVS even though FTS was

negative; no CD was reported for this group. **Table 3** shows results obtained in terms of odds of positive results for chromosomal anomaly (OAPR) into 3 groups: combined test with high risk result, CVS directly for maternal age, CVS for maternal age over 35 with a low risk at screening.

Table 3.
Percentage of fetuses with CD and OAPR in relation to the indication for invasive diagnosis (n.cases 2326).

Indication	n.cases (%)	n.CD (%)	OAPR
Combined test > 1/250 (plus NB in intermediate risk)	689 (29,62)	104 (15,09)	1/6,6
Age >35 directly	1490(64,06)	29 (1,95)	1/51,4
Age > 35 plus combined test > 1/1000	147 (6,32)	0 (-)	0/147

Table 4 shows the kind of CD in relation to the indication for invasive diagnosis: chromosomal abnormalities were detected in 133 fetuses, including: 79 cases of trisomy 21, 28 cases of trisomy 18, 12 cases of trisomy 13, 6 cases of Turner syndrome, 3 cases of polyploidy, 2 cases of Klinefelter syndrome, one case of trisomy 2, one case of trisomy 16, and one case of trisomy X. In our experience, the systematic use of the combined test has identified, in addition to trisomies 21, 18 and 13, 11 cases of other chromosomal abnormalities (10.57%). Of the entire population (12,618 women), invasive diagnosis was performed in 2,326 cases (18.43%) . 649 (27.9%) used amniocentesis, and 1,677 (72.1%) used CVS.

Table 4.
Kind of chromosomal defects and indication to invasive test (n.cases 133)

Indication	n.cases	Chromosomal defects (n.cases)
Maternal age without combined test	29	trisomy 21 (20); trisomy 18(4);trisomy 13 (2); Turner syndrome (2); Klinefelter syndrome (1)
High risk combined test	104	trisomy 21 (59); trisomy 18(24);trisomy 13 (10); Turner syndrome (4); polyploidy (3);Klinefelter syndrome (1); trisomy 2 (1); trisomy 16 (1), trisomy X (1).

During the study period we observed a steady decrease in patients choosing invasive procedures, with a predominance of CVS and anticipation of the diagnosis of chromosomal disorder in the first trimester (Figure 2).

DISCUSSION

Our results indicate that the strict application of the combined test can achieve results similar to those in literature, even when complying with Italian legislation and using the cut off of 1/250 for the definition of high risk pregnancy. The combined test has gradually become an accepted method of prenatal diagnostic testing and in our region is now used by 90% of the

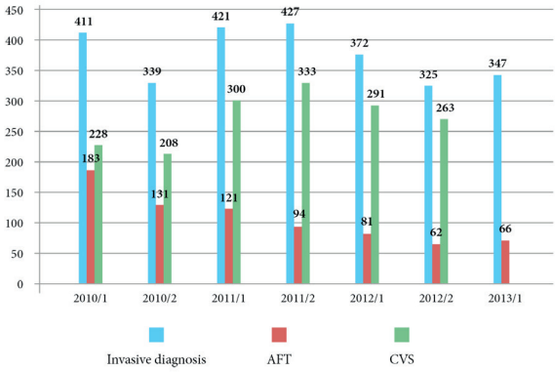

Figure 2.
Invasive procedures during the study period: progressive predominance of chorionic villous sampling (CVS) vs amniocentesis.

population, regardless of maternal age. During the study period we observed a steady decrease in patients choosing invasive procedures, with a predominance of CVS in anticipation of the diagnosis of chromosomal disorder in the first trimester (**Figure 2**) .

Despite achievements, maternal age over 35 years as the only indicator for invasive diagnosis is still the main indication for invasive testing (64%), even if only one in 51 invasive tests will identify a CD. The use of the additional risk markers has proven essential in reducing the number of invasive prenatal diagnostic procedures in women with maternal age over 35 years at the time of delivery and at the same time made it possible to diagnose 3 cases of CD in the first trimester in a population with intermediate risk. A strict application of the combined test in accordance with the directions of the FMF, with the unwinding of intermediate risk through evaluation of sonographic markers reporting, produces a very high sensitivity and a low incidence of false negatives, even when complying with Italian legislation and using the cut off 1/250 for the definition of high risk pregnancy. The validity of this method is confirmed by the absence of chromosomal defects in 147 women over 35 years of age with a risk < 1/1000 that chose to have an invasive test and by the very low false negative rate (only 2 cases) in the 9,388

patients with a negative combined test . In our experience, the systematic use of the combined test identifies, in addition to trisomies 21, 18 and 13, an additional 10.5% of CDs that would have been missed by replacing the combined test with cfDNA testing applied to the general population. There are essentially two options in the clinical implementation of screening for the major trisomies: the routine screening of the whole population by cell-free DNA, or contingent screening based on the results of first-line screening by another method, preferably the first-trimester combined test[8, 9]. cfDNA testing is not a diagnostic test when interpreting individual patient results it is necessary to know the initial risk for the given trisomy[10]. It is, therefore, conceivable that the application of the model of contingent testing using the combined test as the first step and the cfDNA as the next step for cases of intermediate risk for CD can improve the performance in terms of sensitivity not only for the detection of major trisomies , but also in the diagnosis of other chromosomal abnormalities.

The application of the combined test allows for benefit of early detection of many major fetal defects and the prediction and potential prevention of a wide range of pregnancy complications[11]. Contingent testing could also reduce the number of invasive test in women over age 35 who do not feel reassured by results of the combined test[12]. In a public health program, the need to reduce the cost reinforces the need to offer screening in the first trimester to all pregnant women, and to apply the model of contingent testing, using the combined test as the first step and the cfDNA as the next step for cases of intermediate risk for CD.

CfDNA screening should be used to optimize invasive diagnostic test uptake in pregnancies with a intermediate risk score. The selective power of the FCT diminishes beyond the 1/1001 score and cfDNA screening cannot yet be recommended routinely[13].

Currently new tests are available to identify not only trisomies, but also autosomes and sexual chomosomes abnormalities. Major limitations related to this new tests are prohibitive costs and an insufficient clinical validation[14, 15]. The benefit of additional FTS was the detection of fetal structural abnormalities and some unusual chromosomal abnormalities[16].

Finally, we also suggest the modification and enhancement of Italian legislation to exclude

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