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Clinical implementation of first trimester combined test for aneuploidies in patients aged 35 years or older

Tesi di Dottorato di Ricerca

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“Aneuploidies are major causes of perinatal death and childhood handicap. Consequently, the detection of chromosomal disorders constitutes the most frequent indication for invasive prenatal diagnosis. However…

Effective screening for all major aneuploidies can be achieved in the first trimester of pregnancy with a detection rate of about 95% and a false-positive rate of less than 3%.”

K. Nicolaides

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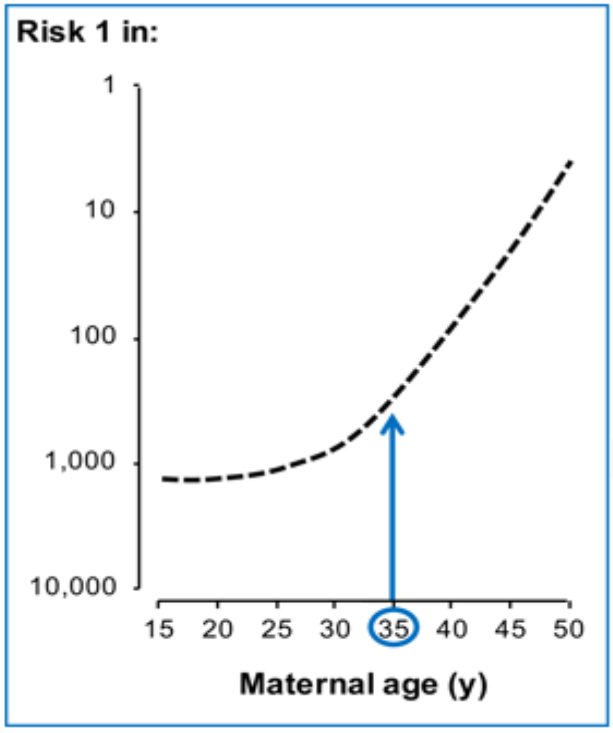
Chapter 1

INTRODUCTION

Aneuploidy, or any variation in the number of chromosomes, is the leading known cause of miscarriage, perinatal death, congenital birth defects and childhood handicap [1-2]. Down Syndrome (DS) is the most common chromosomal abnormality causing intellectual disability in western countries.

The incidence of DS relates directly to maternal age, however, because fetuses with chromosomal defects are more likely to die in uterus than euploid fetuses, the risk decreases with gestational age [3] (Fig 1).



Age (y)	Gestational age		
	12 w	20 w	40 w
20	1100	1300	1500
25	1000	1100	1400
30	650	750	900
31	550	650	800
32	450	550	650
33	400	450	550
34	300	400	450
35	250	300	350
36	200	250	300
37	150	185	220
38	120	140	160
39	90	110	130
40	70	80	100

Fig. 1 : Estimated risk for trisomies 21 (1/number given in the table), in relation to maternal age and gestation.

The detection of chromosomal disorders constitutes the most frequent indication for invasive prenatal diagnosis. Nevertheless, invasive testing, by amniocentesis or chorionic villus sampling (CVS), are associated with a 0.5-1% risk of miscarriage, and therefore these tests should be carried out only in pregnancies considered to be at high risk for chromosomal defects.

In the 1970s, healthcare screening programs all over the world used a **maternal age** above 35 years to identify the “high risk population for aneuploidies”. At that time about 5% of pregnant women were aged 35 years or more, and this group contained about 30% of the total number of fetuses with DS, with a good cost-effectiveness of the screening [4]. In the subsequent years, in developed countries there was an overall tendency for women to get pregnant at an older age, and the group of women aged 35 years or more became around 20-30% of the pregnant population [4], hence in the 1980s various fetoplacental products, including alpha fetoprotein (AFP), free beta-human chorionic gonadotropin (β-hCG), inhibin A, unconjugated estriol (uE3) and pregnancy associated plasma protein-A (PAPP-A) were studied in order to differentiate euploids from aneuploid fetuses [5-6]. **Maternal serum biochemistry** and detailed ultrasonographic examination (US) in the second trimester became the first screening method for DS in the 1980s.

In the 1990s, it was realized that the excess skin of individuals with Down’s syndrome can be visualized by US as increased nuchal translucency in the third month of intrauterine life [7]. **Fetal nuchal translucency thickness (NT)** at the 11–13+6 weeks scan has been combined with maternal age to provide an effective method of screening for DS; for an invasive testing rate of 5%, about 75% of trisomic pregnancies can be identified [7] . When β-hCG and PAPP-A at 11–13+6 weeks are also taken into account, the detection rate (DR) of chromosomal defects is about 85–90% [8-9] . This test was called **combined first trimester screening test (CT)** and it shifted emphasis of the screening from the second to the first trimester of pregnancy. In the last 20 years, several additional first-trimester sonographic markers have been described to improve the DR of aneuploidies and reduce the false-positive rate (FPR).

The most sensitive and specific **first trimester sonographic markers** of trisomy 21 are absence of the nasal bone (NB), increased impedance to flow in the ductus venosus (DV) and tricuspid regurgitation (TR). Absence of the NB, reversed a-wave in the DV and TR are observed in about 60, 66 and 55% of fetuses with trisomy 21 and in 2.5, 3.0 and 1.0%, respectively, of euploid fetuses [10].

Assessment of these new markers improves the performance of CT by increasing the detection rate and reducing the false positive rate.

Other benefits of the 11–13+6 week scan include confirmation that the fetus is alive, accurate dating of the pregnancy, early diagnosis of major fetal abnormalities, and the detection of multiple pregnancies.

In addition to its role in the assessment of risk for trisomy 21, increased NT can also identify a high proportion of other chromosomal defects and it is associated with major abnormalities of the heart and great arteries, and a wide range of genetic syndromes.

In 1997 the discovery of fetal **cell-free DNA (cfDNA)** in maternal plasma and the development of massively parallel sequencing (MPS) and counting techniques using cfDNA, led to many exciting advances in the field of non-invasive prenatal testing (NIPT) and to the launch of the first non-invasive tests for screening for fetal aneuploidies [11]. Several externally blinded cfDNA validation studies in the last 2 years have shown that it is now possible to detect more than 99% of trisomy 21 (T21), 98% of trisomy 18 (T18) and 89% of trisomy 13 (T13) cases, with false-positive rates (FPR) of about 0.1%, 0.1% and 0.4%, respectively [12-18].

However, the cost effectiveness to offer this new test to the general population is not that clear, and its expensiveness constitutes a limitation to implement the use of cfDNA in the NHS all over the world.

Consequently, to reduce the rate of invasive tests, all countries need to develop specific NHS programs to screen the high risk population at the lowest false positive rate possible, considering the available screening options and the economic resources.

BACKGROUND

In Italy invasive procedures are offered as first step approach to the aneuploidies in high risk women. The “a priori” maternal risk is based on maternal age only.

In the 1998 (D.M. 10 settembre1998 -appendix 1), the advanced maternal age (AMA) or maternal age above 35 years old, constituted the first indication for an invasive test. On 2017 the minister of Health published the new standard levels of care (DPCM 12 gennaio 2017 -appendix 2): according to this document the first indication for an invasive test in the NHS became “a positive Down syndrome screening, with a post test risk of 1/300 or greater”. Finally AMA simply disappear from the current indication to receive an invasive procedure. The new ministerial guidelines suppose a reliable screening test able to split the population in two risk group: a low risk group in which no further tests are needed; a high risk group who deserves to be offer of an invasive diagnostic test to allow definitive diagnosis. However in Italy there is no an established prenatal screening program for DS, therefore most regional health systems have not yet adopted the new ministerial guidelines.

The rate of women requiring prenatal diagnosis for DS is unknown in Italy. Often, pregnant women are not aware of the possibility to accede to national screening programs and more often they don’t know the purpose of the "screening", which leads them to seek information independently, from various sources not always reliable. In fact, most of pregnant women have some knowledge about invasive tests, in particular about amniocentesis, but they are not properly informed about the NON-invasive procedure: the screening test.

From January 2013 to December 2015, 2.474 combined tests were performed in our Unit (Prenatal Diagnosis Clinic, AOU “Policlinico-Vittorio Emanuele" Catania) , but only 257 (10.3%) were performed on women aged 35 and over. Women aged above 35, strongly preferred to have an invasive procedure: 821 amniocentesis and villocentesis were performed for AMA.

Invasive diagnostic procedures carried out a miscarriage risk of 0,5-1%. The cost for the NHS amounts to € 450.00 for each amniocentesi and € 600.00 for each villocentesis, compared to an expense of only € 89.00 for a combined test.

A particularly relevant data concerning our case series is represented by the fact that none of the 821 patients who performed an invasive procedure for AMA in the three-year period from 2013 to 2015 was previously subjected to a combined screening test in the first trimester.

The incidence of DS increases with increasing maternal age, and for a 35-year-old woman the risk is equal to 1/250. In recent decades, especially in developed countries, there is an increasingly evident trend to procrastinate the first pregnancy, so, while in the 70s the population of pregnant women over 35 years constituted about 5% of the total, today it constitutes more than 20% [4] .

Therefore, the interest to develop a research protocol based on first trimester combined test, able to implement the recourse to non-invasive prenatal diagnosis, to reduce unnecessary pregnancy loss and to decrease costs for NHS.

Chapter 2

OBJECTIVES

The objective of this study is to analyze the implementation of the first trimester combined test plus markers (CTplus) in a population of women at high risk for chromosomal defects based on maternal age with two main purposes:

- To increase the sensitivity of the screening, thanks to the introduction of the first trimester markers, according to the criteria established by the FMF (Fetal Medicine Foundation);

- To implement screening uptake in the category of women over 35 .

A secondary endpoint was to compare the rate of invasive procedure and the cost effectiveness of the screening in patients aged 35 years or older after the introduction of the “additional markers”, compared to a previous period (2013-2015), in which no additional markers where used.

MATERIALS AND METHODS

This is a prospective cohort study performed between February 2016 and September 2018. The study was approved by the Ethics Committee of AUO "Policlinico- Vittorio Emanuele" , Policlinico G. Rodolico University Hospital, Catania, Italy.

Every pregnant woman aged 35 or older and referred to the Fetal Medicine Department (Policlinico G. Rodolico, University Hospital) was enrolled on the study.

STUDY PROTOCOL

The first antenatal visit took place at 11 to 13+6 weeks' gestation according to last menstrual period (LMP). At the first visit, women received prescreening genetic counseling from a geneticist and an information booklet. Then they were informed on the difference between a screening test and invasive test from an obstetrician. During counseling patients received information about the possible screening options: the combined test, the combined test plus markers and the cfDNA test. Maternal details and previous obstetric history were entered into a database.

After counseling every patient could choose one of the following options:

- option A: to perform the combined test plus markers (CTplus) and liberally choose to have an invasive test after the results, irrespective from the risk category (group A);

- option B: to perform straight forward the invasive test (group B);

For patient wishing to perform the cfDNA test, we referred them to their obstetricians, since is not possible to offer cfDNA in public hospitals in Italy.

Informed consent was obtained after the obstetric counseling.

A ultrasound scan was carried out to determine if the pregnancy was singleton or multiple, to estimate gestational age by measurement of the fetal crown–rump length (CRL) and to rule out any major fetal abnormalities.

Inclusion criteria were : maternal age ≥ 35 years, fetal CRL within 45 and 84 mm. Exclusion criteria: ovodonation, major fetal abnormalities, maternal mental hilliness, no postpartum follow-up.

Women from Group A

The ultrasound examination took place at 11 to 13+6 weeks and was performed by operators certified by The Fetal Medicine Foundation (FMF) (2 obstetricians).

In all pregnancies a scan was performed to rule out major anomalies; crown–rump length (CRL) and NT were measured according to Fetal Medicine Foundation (FMF, London, UK) guidelines [3].

Fetuses with a CRL measurement of 45–84 mm were included in the study. Nasal bone status, tricuspid regurgitation and DV flow (qualitative assessment) were investigated according to FMF .

A clotted blood sample was obtained. The serum was separated and PAPP-A and fβ-hCG were measured on the same day using a random access immunoassay analyser (Kryptor, Brahms Diagnostica GmbH, Berlin, Germany (formerly CIS)).

The maternal serum concentrations of PAPP-A and free β-hCG, were combined with maternal age, previous history of trisomic pregnancy and the ultrasound findings to estimate the patient-specific risk for trisomies 21, 18 and 13 [4].

The Feto-maternal module of the Astraia software (version 1.18.0 88) was employed for the risk assessment according to the FMF algorithm. In twin pregnancies, risk calculation was also based on maternal age, serum biochemistry and ultrasound findings. In dichorionic twins, risk was separately calculated for each fetus, whereas in monochorionic (monoamniotic or diamniotic) twins, an average risk was generated for both fetuses.

After a week women were called back to receive the final risk. A calculated risk of “equal to” or “higher than” **1/300** was defined as **“high-risk”.** In order to facilitate patients choice and to better understand patient decision making, we create an intermediate risk group when the risk was from 1/300 and 1/1000. A post-screening counseling with a clinical geneticist and an obstetrician was then offered and every patient was asked if she wanted to perform an invasive test, irrespective for the risk results.

Women from Group B

These women declined the CT plus and they were reschedule to receive an amniocentesis at 16 weeks GA. The results from karyotype were available within 2-3 weeks from the procedure.

PREGNANCY MAMAGEMENT

The protocol for management of the pregnancies is summarized in Figure 2.

In cases in which the CT plus indicated a low risk, the parents were reassured that the fetus wass unlikely to be affected by trisomies, however they could chose to perform an invasive test if they wishes.

In the case of high risk the parents were strongly recommended to perform an invasive test.

Additional actions based on the results of the 12-week assessment include advice on the value of: first, CVS, if fetal NT>3.5mm or major fetal defects; second, follow-up scans for fetal anatomy, including fetal echocardiography, if there is increased NT>3.5mm or abnormal flow across the tricuspid valve or in the ductus venosus.

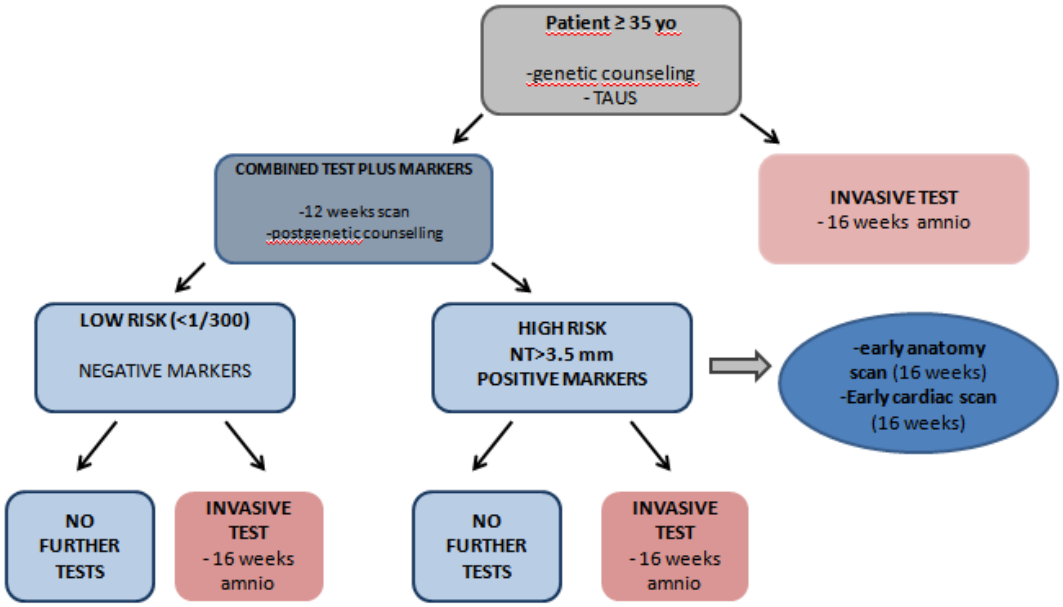


Fig. 2 Protocol for pregnancy management according to maternal choice and results of CT plus markers.

PREGNANCY OUTCOME

In order to assess the performance of the screening test, we contacted all the patients after birth, to make sure that fetal phenotype was normal. Pregnancy outcome were recorded from the labor ward for patients who delivered in our Hospital. A telephonic survey was made to receive information from the rest of the enrolled patients.

If the pregnancy outcome was not available the patient was not included in the study calculation.

STATISTICS

Statistical analyses were performed using SPSS for Windows version 18.0 (SPPSS Inc, Chicago,IL, USA) and MedCalc version 11.3.1 (MedCalc Software, Mariekerke, Belgium) statistical software. The descriptive analysis of results was performed using Microsoft Office Excel 2007. We calculated DR as the ratio of true positive cases detected in combined screening with the total

number of Down syndrome cases. FPR was calculated in a similar way as the ratio between the number of false positive cases and the total number of children born without Down syndrome.

Chapter 3

RESULTS

SUDY POPULATION

From February 2016 and September 2018 we prospectively enrolled 526 women aged 35 or older, referred to the Fetal Medicine Clinic during the first trimester of pregnancy. 33 patients were excluded because of major fetal malformations, or ovodonation, or because we were not able to obtain a postpartum follow up.

After counseling 259 (52,5%) women out of 493 chose to go straight forward for the invasive test while 234 (47,5%) underwent the CT plus, reserving the right to choose the invasive test after receiving the results.

The characteristics of the study populations are shown in table 1.

Maternal charecteristics	CT plus markers n. 234	Invasive procedure n. 259	P
Median Maternal age	36,8 (+/-1,5)	39,4 (+/2,2)	P> 0,005
Median Maternal Weight	62,0 (+/-3)	61,5 (+/-2,5)	NS
Parity	1 (+/1)	1 (+/1)	NS
Etnicity White Black Asian East asian	 98% 0,5% 1% 0,5%	 98% 1% 1% 0%	NS
Smoking			NS
Educational level Degree or higher High school others	 33,1% 50,8% 12,1%	 24,2% 53,7% 22,1%	P> 0,005
Previous aneuploids	0,9%	0,7%	NS
N. of expected T21	1,9% (4)	2,3% (6)	NS
N. Of other chromosomal defects	1,9%	2,3%	NS

Tab. 1: Maternal characteristics. Data are reported as means ± SD frequencies and percentages. Two sided p-values for continuous variables refer to Mann-Whitney or Kruskal-Wallis one-way ANOVA. Two sided p-values for categorical variables refer to Pearson or Mantel-Haenszel chi-squared.

The two group were very similar for parity, ethnicity and number of previous pregnancy with trisomy 21. We found a statistically significant difference in age

and educational levels. Women performing amniocentesis are older and have a lower educational level when compared with those choosing the combined test.

Figure 3 shows the pregnancy management according to patient's choice and CT plus results.

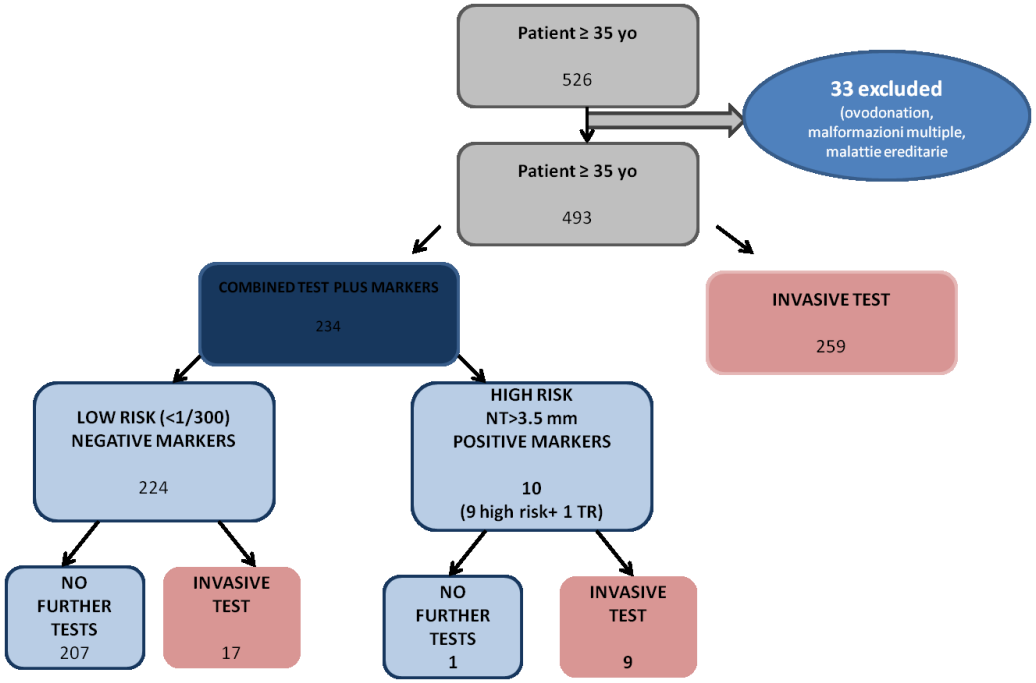


Fig. 3: Pregnancy management, according to the study protocol.

PATIENT FROM GROUP A

According to the distribution of MA we expected 2.3% cases of trisomy 21 and approximatively the same numbers of other aneuploidies. After performing the invasive tests we found 6 cases of trisomy 21 (2.3% of the total), 2 cases of trisomy 18 and 2 cases of Turner syndrome. In 249 cases the fetal karyotype was normal. We reported 2 cases of PPROM with normal full term birth and no cases of miscarriage.

PATIENT FROM GROUP B

After a week from the CT plus the 83% (n. 194) of patients from group B decide not have further tests, only the 11% (n. 26) choose to have an invasive test. A very little percentage of the patient, the 6% (14), preferred to have a cfDNA test in a private center (paying an additional cost). When we considered patients'

decision making after receiving the screening test results, we can see that among of patients choosing to have an invasive test, the 31,5% were high risk and the total of the aneuploid fetuses were included on this subgroup (Fig.3). Only 1 (0,5%) patient refusing amniocentesis after CT results was high risk, whilst the 4.5% (n8) had an intermediate risk and the vast majority (95.5%) was considered low risk (Fig. 4) .

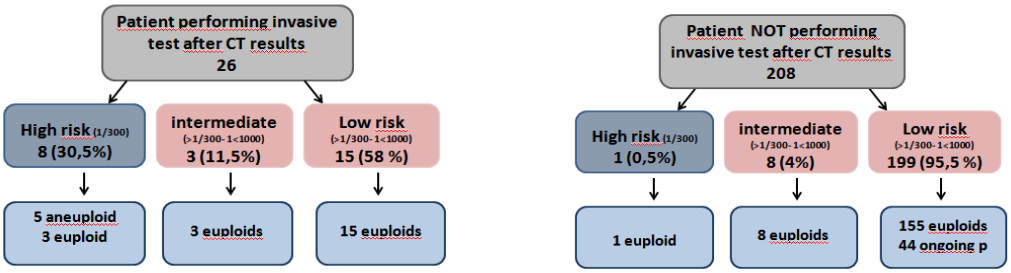


Fig. 4: Patients' decision after CT plus results. CT combined test.

Unfortunately we reported some complications of amniocentesis within this small group: **one miscarriage of a fetus with a normal karyotype** and one PPROM with a full term normal live bitrth (tab 2).

Complication of invasive procedures	Total (285)	Group A (259)	Group B (26)
miscarriage	1 (0,3%)	0	1
PPROM	3 (1%)	2	1
bleeding	0	0	0
Placental abruption	0	0	0
amnionitis	0	0	0
Maternal sepsis	0	0	0
Maternal cutaneous hematoma ta at the injection site	15 (5,3%)	14	1

Tab. 2: Complications of invasive procedures. PPROM preterm premature rupture of the membranes.

PERFORMANCE OF THE SCREENING

The performance of the screening was calculated considering only pregnancies with a postpartum outcome or a fetal karyotype. However we had to exclude from statistical analysis 43 patients because their pregnancy is still ongoing. All the consideration about detection rate (DR), false positive rate (FPR) and sensitivities, have been made on the remaining 197 patients.

On the basis of maternal age distribution it is expected that the study population of group B contains 1.9 cases of trisomy 21 and approximately the same number of other chromosomal defects. In fact among this group, there were 5 aneuploid fetuses: 4 trisomy 21 and 1 Turner syndrome .

Imagining to use maternal age (MA) as screening policy at a fixed risk cutoff of 1/300, the entire study population would be screen positive by definition, because the "a priori risk" based on age become 1/300 from the 35th years of woman's life. Therefore, MA would detect the 100 % of chromosomal defect, at FPR of 100%. That means, in our cohort, to performed invasive procedure to all women, with 192 unnecessary amniocentesis to normal fetuses. Instead, employing the same fixed cutoff for CT, and CT plus markers, DR is 100%, but FPR is acceptable for CT alone 12,5% and impressively good, 2%, when first trimester markers are added. After the CT plus the estimated risk was 1 in 300 or more just in 9 (4,5%) cases 4 euploid fetuses and 5 aneuploid ones (tab 3).

Screening policy	outcome	Fixed risk cut-offs					DR (1/300)	FPR (1/300)
		1/10	1/100	1/300	1/1000	1/2500		
MA	normals	0% (0)	16% (31)	100% (192)	100% (192)	100% (192)	100%	100%
	aneuploids	20% (1)	40 % (2)	100% (5)	100% (5)	100% (5)		
MA+NT+ biochemestry	normals	0%	3,6% (7)	12,5% (24)	27% (52)	59,9% (115)	100%	12,5%
	aneuploids	80% (4)	100% (5)	100% (5)	100% (5)	100% (5)		
MA+NT+NB+DV+TR+ biochemestry	Normals	0%	0%	2% (4)	7,8% (15)	33,8% (65)	100%	2%
	aneuploids	80% (4)	100% (5)	100% (5)	100% (5)	100% (5)		

Tab. 3: DR and FPR for fixed cutoffs, using different screening policy in a cohort of high risk patients. DR detection rate, FPR false positive rate, MA maternal age, NT nuchal transluceny, NB nasal bone, DV ductus venosus, TR tricuspid regurgitation

First trimester markers were evaluated in all patients. The mean required time for a NT scan was longer of about 8 minutes when the markers were evaluated. It was impossible to assess NB, DV and TR in 2,6%, 6%, 4,7% respectively (tab. 4).

First trimester markers	
Nasal Bone Assessed Not assesed	 228 (97,4%) 6 (2,6%)
Doctus Venosus Assessed Not assessd	 220 (94%) 14 (6%)
Tricuspid rigurgitation Assessed Not assessd	 223 (95,3%) 9 (4,7%)
Required time for a NT scan	21 minutes (+/-7)
Required time for a NT scan plus markers	29 minutes (+/-11)

Tab. 4: US evaluation of first trimester markers. NT nuchal translucency.

The DRs and FPRs were calculated in the cohort of patient with available fetal outcome (197 patients) (tab 5).

markers		total	euploids	aneuploids	DR	FPR
NT	normal	95,9% (189)	98,4% (189)	0%	100%	1,6%
	>95°	4% (8)	1,6% (3)	100%		
NB	present	96% (189)	97,9 % (188)	20% (1 turner S)	80%	0%
	absent	2% (4)	0%	80% (4 T21)		
	NA	2% (4)	2 ,1% (4)	0%		
DV	present	95,5% (188)	96,9% (186)	40% (2 T21)	60%	2%
	Absent/reverse	1,5% (3)	0%	60% (2 T21, 1turner S)		
	NA	3% (6)	3,1% (6)	0%		
TR	normal	93,4% (184)	93,7% (180)	40% (2 T21)	60%	0,5%
	abnormal	2% (4)	0,5% (1)	60% (2 T21, 1turner S)		
	NA	4,6% (9)	4,6% (9)	0%		

Tab. 5: Detection rate and false positive rates for each of the first trimester markers. NT nuchal translucency; NB nasal bone; DV ductus venosus; TR tricuspid regurgitation; DR detection rate; FPR false positive rate; NA not assessed.

The DR each first trimester marker, when isolated, is shown in table 4. The DR was 80% for NB, 60% for DV and 60% for TR, at a very low FPR for each of the markers. The NT was the most reliable isolated marker of aneuploidy with a 100% DR and 1,6% FPR.

The NT measurements are plotted on the attached reference range (fig 5). The percentage of cases within each centile range are shown in figure 6.

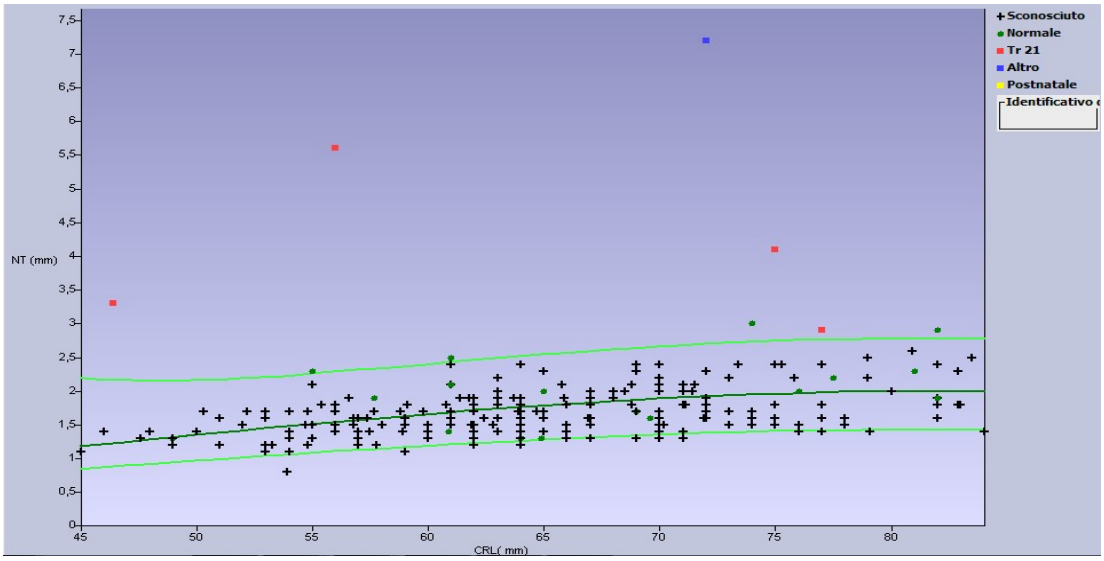


Fig. 5: NT measurements. Red dots: trisomy 21; blue dots: other abnormal karyotypes; green dots: normal karyotypes; fetus without a prenatal karyotyope.

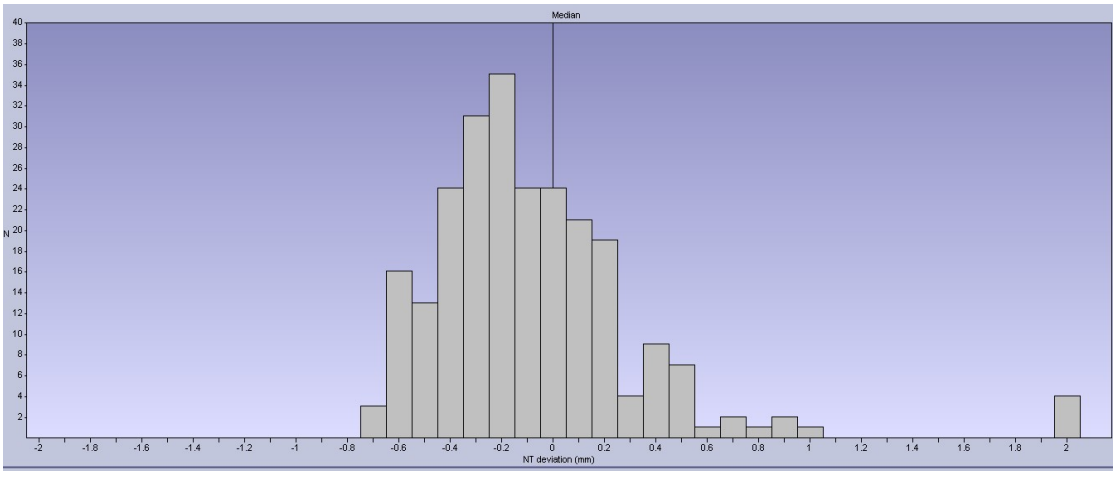


Fig. 6: NT distribution. > median 39.4%; >95° 3,7%.

Values for maternal serum biochemical measurements are expressed as multiples of the expected median for gestational age. Medians, 5th and 95th

centiles, and the percentage of cases outside the expected 5th/95th centile are shown in the following table (tab. 6).

	5° cent	median	95° cent	<5° and >95° cent
βHCG MoMs	0.445	0.976	2.669	5.2%
PAPP-A MoMs	0.429	1.024	2.281	5.2%

Tab. 6: Free βHCG MoMs and PAPP-A MoMs medians, 5th and 95th centiles, and the percentage of cases outside the expected 5th/95th centile. βHCGfree beta Human Chorionic Gonadotropin; PAPP-A Pregnancy Associated Plasma Protein A; MoMs Multipples of Median.

Figures 7 and 8 show the distributions and the measurements of free βHCG MoMs and PAPP-A MoMs

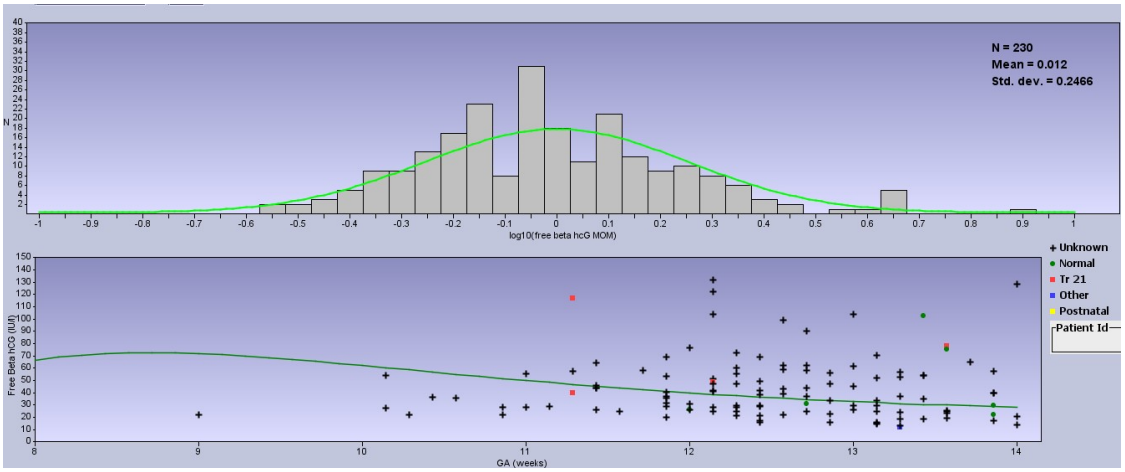


Fig. 7: Top: Free βHCG measurements. Red dots: trisomy 21; blue dots: other abnormal karytypes; green dots: normal karyotypes; fetus without a prenatal karyotyope. **Bottom:** βHCG distribution. βHCG beta Human Chorionic Gonadotropin

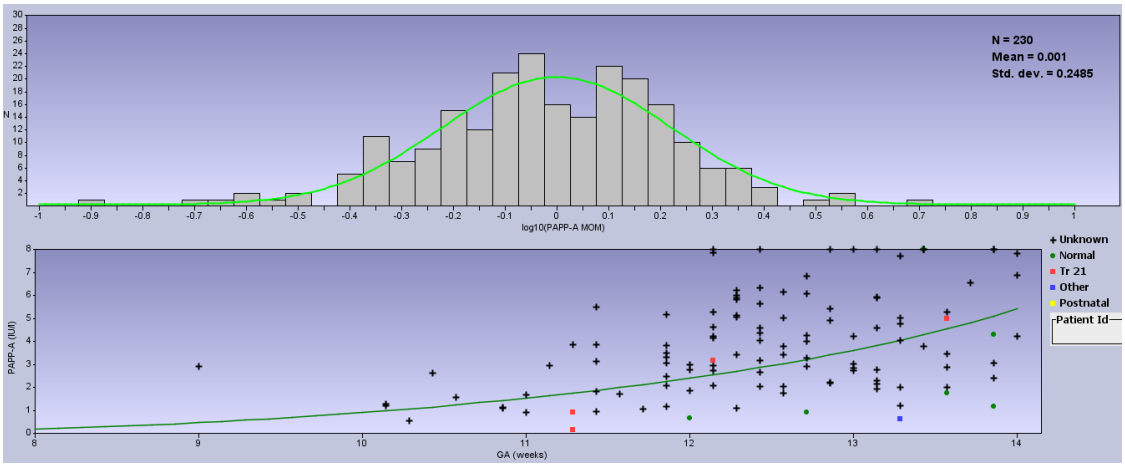


Fig. 8: Top: PAPP-A measurements. Red dots: trisomy 21; blue dots: other abnormal karytypes; green dots: normal karyotypes; fetus without a prenatal karyotyope. **Bottom:** PAPP-A distribution. PAPP-A Pregnancy Associated Plasma Protein A

The biochemistry was abnormal in the 10% of cases (n 20); 17 of these patients had a final low risk. For the 3 remaining patients with an high risk, one was a T21 with increased NT and positive markers; one was a turner syndrome with NT above the 99th centile an tricuspid regurgitation; and one with normal NT measurements and negative markers, choose to have amniocentesis which confirmed a normal 46,XX karyotpe.

ANEUPLOID FETUSES

The expected number of chromosomal abnormalities based on maternal age in the cohort of patients choosing to have the CT plus markers, was 1,9% for down syndrome and almost the same for other defects. For a fixed cutoff of 1/300, all aneuploidies were detected from the CT plus markers at FPR of 2%).

In the subgroup of aneuploid fetuses the additional use of the markers significantly improved the DR. In fact there was a combination of abnormal NT, first trimester markers and biochemistry for each fetus as shown in table 7.

The 5 aneuploids fetuses											
Age	CRL	NT	NB	DV	TR	βHCG MoMs	PAPP-A MoMs	risk by age	Risk by CT	Risk by CT plus	karyo
41	77	2.9	abnormal	normal	abnormal	2.33	0.870	1/58	1/10	1/2	47,XY+21
38	75	4.1	abnormal	abnormal	normal	0.731	0.073	1/65	1/2	1/2	47,XY+21
40	56	5.6	abnormal	abnormal	abnormal	1.108	0.998	1/100	1/5	1/2	47,XX+21
48	46.4	3.3	abnormal	normal	normal	1.995	0.455	1/8	1/2	1/2	47,XY+21
35	72.5	7.2	normal	abnormal	abnormal	1,123	0.990	1/231	1/69	1/33	45, X0

Tab 7: Fetuses with abnormal karyotype in group B. CRL crown ramp length; NT nuchal translucency, NB nasal bone, DV ductus venosus, TR tricuspid regurgitation; βHCG beta Human Chorionic Gonadotropin; PAPP-A Pregnancy Associated Plasma Protein A; MoMs Multipples of Median.

COMPARISON WITH PREVIOUS PERIOD

One of the endpoints of this study was to compare the uptake of the CT among patients aged 35 or older after the introduction of the study protocol.

Considering the total number of CT performed, only the 10% of patients aged 35 or older performed a CT in 2013-2015. This number became 16% during the study protocol period with an increase of 60%. What is more interesting is the reduction in the number of invasive procedure (fig.9).

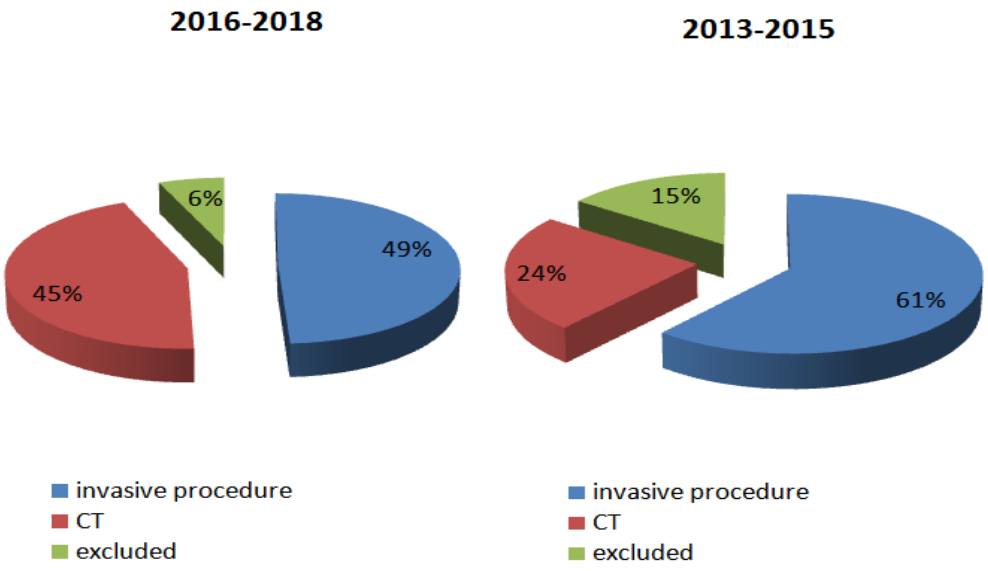


Fig. 9: Percentage of the invasive procedures and CTs in patients aged 35 or older in 2013-2015 compared with period of the study protocol. CT combined test.

The percentage of invasive procedures dropped down from 61% to 49% with reduction of 19.6%.

COST-EFFECTIVENESS OF CT PLUS MARKERS IN PATIENTS AGED 35 OR OLDER

In term of cost-effectiveness and considering that in our study CT plus has a DR of 100% with FPR of 2.0%, in women aged 35 or older; if all patients were first screened by CT plus we would be able to reduce the number of unnecessary amniocentesis at a 2% of the total. The 2% in our cohort represent a total of 10 patients and therefore 10 unnecessary invasive tests (tab 8).

Number of detected cases and unecessary invasive procedure for a fixed risk cutoff of 1/300				
Cohort of 493 patients 479 euploids 14 aneuploids	**DR**	**FPR**	**Detected**	**Unnecessary invasive procedure**
MA	100%	100%	14	479
MA+NT+ biochemestry	100%	12,5%	14	59
MA+NT+NB+DV+TR+ biochemestry	100%	2,0%	14	10

Tab. 8: Number of detected cases and unecessary invasive procedure for a fixed risk cutoff of 1/300. DR detection rate, FPR false positive rate, MA maternal age, NT nuchal transluceny, NB nasal bone, DV ductus venosus, TR tricuspid regurgitation.

Therefore if we look at the costs: we performed a total of 285 invasive procedures in our cohort (274 amniocentesis and 11 villi) with a cost of 129.900 €. The performed CT were 197, with a cost of 17.533 €. The amount of costs in our cohort considering the actual screening policies was 147.433 €. Imagining to apply the new screening policy (DPCM january 2017), as proposed from our screening protocol, we would perform CT to all patients (493) and invasive procedures only in the high risk (25 villocentesis), therefore the amount of costs for NHS would be 58.877 €, with a 73,3% reduction of the costs.

Even considering to still offer amniocentesis or cfDNA to a group with an intermediate risk (1/301 and 1/1000) the number of patients with intermediate risk after CT plus were just the 7.8% (15 patients) (tab. 3).

Chapter 4

DISCUSSION

Down Syndrome is the most common chromosomal abnormality in newborns in our population (1:540 live births) [20]. The risk of having a baby with Down Syndrome is related to maternal age, according to these data, women aged 35 or older have always been considered has a high risk population.

In March 2017 the Minister of Health abolished AMA as an indication for invasive testing and offered CT free of charge for every pregnant woman (DPCM 12 gennaio 2017- appendix 2).

Prenatal screening strategies have changed considerably over the past few years. Rising maternal age and women's demand for safety have triggered increasing requests for reliable prenatal information. Detailed information is essential for parents being offered different diagnostic alternatives. Therefore, health professionals attempt to provide their patients with informed choice through comprehensive counseling, considering that parents have to cope with hope and concern at the same time, when a prenatal test reveals more and more details about the status of the unborn offspring.

A definitive prenatal diagnosis currently requires invasive sampling followed by chromosome analysis. However, invasive tests pose an inherent risk of procedure-related complications including miscarriage. Therefore, invasive tests should be performed only in high-risk pregnancies, following appropriate counseling and reliable screening.

In Italy the rate of invasive procedures is uncertain, but they are still offered as first step approach to detect aneuploidies in high risk women based on maternal age only, according to the D.M. 10 settembre1998 (appendix 1). As much unclear is, in our country, the number of women requiring a screening test. From local data we know that 80% of women aged 35 years or older preferred

invasive procedure against screening (information from local hospitals' booking registries).

There is a lack of studies about screening programs in women aged 35 or older. Our study demonstrates the feasibility of a two step protocol to screen and diagnose aneuploidies in women aged 35 or older.

First-trimester risk assessment of common chromosomal aneuploidies is based on a combination of MA, maternal serum β-hCG, PAPP-A and NT at 11 to 13+6 weeks. In various prospective studies the detection rate ranges from 74% to 93% for a fixed false-positive rate of 5% [21-22]. Additional first trimester ultrasound markers, absent nasal bone (NB), reversed ductus venosus (DV) flow and tricuspid regurgitation (TR), have separately been found to increase the effectiveness of the screening for trisomy 21 [23-25].

In 2012 Ghaffari et al. reported detection rate of 98.0% and false-positive rate of 4.45%, when all four trimester markers were employed together [26]. These results have led to a continuing decline in invasive antenatal testing [27].

The vast majority of the studies about first trimester CT did not evaluate the screening performance among younger and older women, even if, as everybody knows, the performance of the screening depends on maternal age [28].

Peuhkurinem et al showed the DR of the CT is significantly higher (87.0%) in women aged 35 or older than in younger women (74.0%), indicating that screening favors women with advancing maternal age [29]. The higher DR in the older age group is due to two reasons: firstly, the prevalence of Down syndrome is higher in women aged 35 or older, and secondly, the algorithm of the risk calculation program emphasizes maternal age, leading to a better DR of Down syndrome overall [30].

In our population of women aged 35 or older, we showed a DR of 100% for a FPR of 2% when all first trimester markers were added to the CT. Among 197 patients performing the combined test we had 5 aneuploid fetuses and they were all detected from the test, at a very low FPR. The FPR was 12.5%, as previously reported [29], if the first trimester markers were not used for the final

risk calculation. Therefore our study demonstrated the best performance of first trimester markers in patients of AMA when also first trimester markers are used.

Assessing first trimester markers for down is feasible with a proper training. In our study we were able to assess the 97.4% of NB, the 94% of DV, the 95.3% of TR (tab. 3). The DR of each of first trimester marker, when isolated, was 80% for NB, 60% for DV and 60% for TR, at a very low FPR. All these markers are independent from the NT, that is the reason why they can be added to CT to improve DR and to reduce FPR. In our study, DR and FPR for each markers were better than in the literature [31], the reason could be that our cohort was composed from a high risk population rather than from a general one; furthermore the sample size was quite small in our study.

By definition, all the patient from our cohort was high risk, because the "a priory risk" was based on maternal age only. At a fixed cutoff of 1/300, we were detect all cases of aneuploidies present in our cohort only by MA, however the FPR was, by definition, 100%. Maintaining a fixed cutoff of 1/300 to define the high risk population post-screening, the DR was exactly the same for the CT and the CT plus markers. Nevertheless using first trimester markers we dramatically reduced the FPR from 12.5% to 2%, as stated before.

If the policy of our study protocol would be adopted from NHS, this approach would allow us to reduce the number of unnecessary amniocentesis from 60%.

All patients from our study underwent a genetic counseling followed by a second counseling by a fetal medicine obstetrician. After considering all the acquired information, patients could freely choose between having an invasive test "tout court" or pass through a screening test first.

We called group A the patient going straight for the invasive test and group B the ones choosing the screening test at first.

The percentage of woman choosing invasive procedure (group A) (n. 259) was a little greater than group B (n. 234).

There were two statistically significant differences between group A and Group B: patient from group A were older and with a lower educational level. These data leads to two different conclusions: first, patients are aware of the

correlation between maternal age and chromosomal abnormalities; second, the higher the educational level the greater is the uptake of the screening test, as previously shown [27].

A good indicator of the effectiveness of the screening is the number of invasive procedures needed to detect a single case of aneuploidies, calculated as a ratio. This ratio was 5:1 for group B and 25:1 for group A, therefore the number of procedure required using AMA as screening method was 5 time worst than CT plus markers.

After CT, 26 women choose to have an invasive procedure, 8 women were at high risk, 3 had an intermediate risk, however 15 (the 58.8%) were in the low risk group (risk lower than 1/1001). Among the high risk patients (9 out of 197) the 88.8% (8 out of 9) choose to have an amniocentesis, this percentage is a little greater than the data from general population. Previous studies report 65% opting out in a regional Dutch population [32] and 49.3% in a Canadian study [33]. The advanced maternal age in our study groups possibly explains the greater uptake of invasive test after a positive CT in our population, however, we have just a little evidence how much information is actually understood and adopted by pregnant women. Usually women tend to accept medical policies with which they are familiar, often believing what local health authorities and physicians offer them is the right thing to do and, overall, the mothers' understanding about the different prenatal screening options and their consequences are fragmentary [34-38].

During the period of the study protocol we reported one pregnancy loss in a patient that was low risk after CT plus markers. The patient asked to undergo an amniocentesis irrespective to the low risk, because she had a previous history of trisomy 21 and she felt more reassured performing a karyotype.

A part from this case, the hard work from all the health professionals and the information campaign were able to reduce the number of unnecessary invasive procedures and increase the CT in our clinic: we had a 19.6% reduction in the invasive tests, and a 60% increased in women requiring a CT among women aged 35 or older, compared to the 3 previous years.

Although a detailed cost-effectiveness analysis is beyond the scope of this study, a rough estimate learns that replacing AMA by CT would dramatically reduce the cost for NHS. The cost for one amniocentesis and villocentesis plus fetal karyotype is 450 € and 600 € respectively. Considering to offer amniocentesis to all 493 women the cost for NHS would have been 221.850 €.

The combined test plus markers cost to NHS 89 €. During the study protocol (from march 2016 to September 2018) we performed 274 amniocentesis and 11 villocentesis with a cost of 129.900 € and 197 CT plus markers with a cost of 17.533 €. The amount of costs was 147.433 €, with 33.5% reduction. Imagining to apply our screening protocol to all women aged 35 or older, as stated in the new screening policies proposed from the "DPCM 12 gennaio 2017" (appendix 2), we would perform CT to all patients (493) and invasive procedures only in the high risk ones. We can imagine the high risk group formed by the 15 aneuploid fetuses plus the FP from the CT, that is the 2% of the study population (25 high risk patients). Therefore the amount of costs for NHS would be 58.877 €, with a 73,4% reduction of the costs. The same reduction was reported from Siljee E. et al in 2014, considering women aged 35 or older in a national screening program Netherlands [30].

Even considering to offer a second and more sophisticated screening test as the cfDNA to a group with an intermediate risk, the cost for NHS would be still really good. In our population: the number of patients with an estimated risk from 1/301 and 1/1000 after CT plus were just the 7.8% (15 patients) that became 33,8% (65 patients) if we consider as "intermediate risk group", all patients with a risk from 1/301 to 1/2500 after CT plus (tab. 3). Calculating again the cost for the NHS, a cfDNA test would cost about 400 € and therefore the total amount of cost would be a maximum of 84.277€ in our cohort, offering a cfDNA test for a risk from 1/301 and 1/2500 .

A positive effect of performing CT was the detection of a series of major fetal abnormalities during first trimester. We had 12 cases of fetal major abnormalities: 1 cases of postaxial polydactyly, 6 cases of congenital heart defects, 1 case of holoprosencephaly, 1 case of acrania, 1 case of gastroschisis, 1 cases exomphalos and 1 case of severe skeletal dysplasia.

These patients were excluded from the present study, but they receive appropriate counseling and invasive procedure at a very early gestation.

The main limitation of our study was the small size of the patient enrolled, of course We need bigger numbers in order to provide more sensitive DR and FPR, nevertheless our data are consistent with the literature. Another limitation was the unavailability of the CfDNA test in the NHS. We strongly believe the introduction of cfDNA in the NHS would considerably reduce the number of unnecessary invasive procedures.

CONCLUSIONS

Invasive prenatal diagnosis based on AMA alone is still a large contributor to invasive testing. However, there are many reasons to abandon screening on the basis of AMA, given its low DR, high fetal loss rate and high costs. Therefore this indication should be abandoned and be replaced by first trimester screening free of charge for all women. The additional use of first trimester markers, NB, DV and TR, is feasible and it reduces FPR without any additional costs for NHS. Moreover the CT plus markers seems to be more affective in patients aged 35 or older. Hence first-trimester Down syndrome screening should be implemented especially in an aging population.

In spite of these findings amniocenteses can diagnose chromosome anomalies other than T21, T18, T13 and turner syndrome, including deletion, translocation and mosaicism; thus, it is essential to offer all information to the women with adequate consultation and final decisions should be made balancing risks and benefits under women's autonomy.

In conclusion this study demonstrates that maternal age is not an appropriate criterion for Down syndrome screening when CT plus is available. From the public health point of view, we also provide evidence that adequate Down syndrome screening policy are the best way to reduce the rate of invasive procedures and to improve costeffectiveness.

APPENDIX

APPENDIX 1

Decreto Ministeriale 10 settembre 1998, Allegato C

Allegato *C*

INDICAZIONI ALLA DIAGNOSI PRENATALE

(desunte dalle «Linee Guida per i test genetici» approvate dal Comitato Nazionale per la Biosicurezza e le Biotecnologie della Presidenza del Consiglio dei Ministri)

Le indicazioni per la diagnosi prenatale rientrano in due grandi categorie:

1. presenza di un rischio procreativo prevedibile a priori: età materna avanzata, genitore portatore eterozigote di anomalie cromosomiche strutturali, genitori portatori di mutazioni geniche;
2. presenza di un rischio fetale resosi evidente nel corso della gestazione: malformazioni evidenziate dall'esame ecografico, malattie infettive insorte in gravidanza, positività dei test biochimici per anomalie cromosomiche, familiarità per patologie genetiche.

Le indicazioni per le indagini citogenetiche per anomalie cromosomiche fetali sono:

- età materna avanzata (= o > 35 aa.)
- genitori con precedente figlio affetto da patologia cromosomica
- genitore portatore di riarrangiamento strutturale non associato ad effetto fenotipico
- genitore con aneuploidie dei cromosomi sessuali compatibili con la fertilità
- anomalie malformative evidenziate ecograficamente
- probabilità di 1/250 o maggiore che il feto sia affetto da Sindrome di Down (o alcune altre aneuploidie) sulla base dei parametri biochimici valutati su sangue materno o ecografici, attuati con specifici programmi regionali in centri individuati dalle singole Regioni e sottoposti a verifica continua della qualità.

APPENDIX 2

Decreto Presidente del Consiglio dei Ministri 12 gennaio 2017, Allegato 10 C

ALLEGATO 10C

CONDIZIONI DI ACCESSO ALLA DIAGNOSI PRENATALE INVASIVA, IN ESCLUSIONE DALLA QUOTA DI PARTECIPAZIONE AL COSTO

L'accesso alla diagnosi prenatale ha due principali gruppi di indicazione, che riguardano situazioni nelle quali il rischio di patologia fetale è aumentato al di sopra dei livelli medi della popolazione generale:

1) Rischio procreativo prevedibile a priori in quanto correlato ad una condizione biologica-genetica presente in uno o in entrambi i genitori o nella famiglia, da valutare in sede di consulenza genetica

2) Rischio rilevato in corso di gravidanza: difetti fetali evidenziati mediante ecografia alterazione di parametri biochimici/molecolari rilevati con sistemi validati ed erogati presso strutture appositamente individuate dalle regioni, predittivi di patologie fetali e/o cromosomiche/geniche, patologie infettive a rischio fetale.

Le condizioni per le quali è previsto l'accesso alla diagnosi prenatale invasiva sono:

1) Per le indagine citogenetiche:

- probabilità di trisomia 21, o di altre anomalie cromosomiche ≥1/300 al momento del test per la valutazione del rischio nel primo trimestre (o ≥1/250 in caso di test nel secondo trimestre) calcolata secondo i metodi indicati dalle Regioni tra quelli basati sulla età materna in combinazione con altri parametri ecografici fetali e/o di laboratorio. Tale calcolo dovrà essere effettuato utilizzando specifici protocolli nell'ambito di programmi che garantiscano uniformità di accesso in tutto il territorio regionale, in Centri individuati dalle singole regioni e sottoposti a verifica continua della qualità. L'opzione da parte delle Regioni deve essere orientata all'adozione di metodi di calcolo del rischio con una maggiore sensibilità diagnostica e un minor numero di falsi positivi tenuto conto dell'evoluzione della ricerca scientifica e tecnologica.
- genitori con precedente figlio affetto da patologia cromosomica
- genitore portatore di riarrangimento strutturale bilanciato dei cromosomi
- genitore con aneuploidia cromosomica omogenea o in mosaico
- anomalie fetali/della gravidanza evidenziate mediante ecografia

2) Per le indagini genetiche:

- genitore eterozigote per una patologia/mutazione autosomica dominante,
- genitori entrambi eterozigoti per mutazioni geniche correlate a patologie autosomiche recessive,
- madre eterozigote per mutazioni recessive legate all'X,
- madre portatrice di mutazione mitocondriale;
- segni ecografici feto-annessiali indicativi di specifiche patologie geniche
- altre condizioni di possibile rischio correlate alla storia familiare, da verificare in sede di consulenza genetica.

3) Per le indagini infettivologiche:

- condizione di rischio fetale determinato sulla base di una accertata infezione materna e/o di segni rilevati all'ecografia potenzialmente associati a patologie infettive.

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