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## CHAPTER 2



**European Non-Invasive Trisomy  
Evaluation (EU-NITE) study: a  
multicenter prospective cohort study  
for non-invasive fetal trisomy 21 testing**

# **ABSTRACT**

## **OBJECTIVE**

To evaluate the performance of a directed non-invasive prenatal testing method of cell-free DNA analysis for fetal trisomy 21 (T21) by shipping the whole blood samples from Europe to a laboratory in the USA.

## **METHODS**

A European multicenter prospective, consecutive cohort study was performed enrolling pregnant women from Sweden and the Netherlands. Blood samples were drawn just prior to a planned of invasive diagnostic procedure in a population at increased risk for fetal T21 and then shipped to the USA without any blood processing.

Chromosome-selective sequencing was carried out on chromosome 21 with reporting high risk or low risk of T21. Karyotyping or rapid aneuploidy detection was used as the clinical reference standard.

## **RESULTS**

Of the 520 eligible study subjects, a T21 test result was obtained in 504/520 (96.9%). Risk assessment was accurate in 503/504 subjects (99.8%). There was one false negative result for T21 (sensitivity 17/18, 94.4%, and specificity 100%).

## **CONCLUSION**

This is the first prospective European multicenter study showing that non-invasive prenatal testing using directed sequencing of cell-free DNA applied to blood samples shipped across the Atlantic Ocean, is highly accurate for assessing risk of fetal T21.



## INTRODUCTION

In most European countries, pregnant women are offered screening for trisomy 21 (T21), regardless of age, using the first trimester combined screening (FCT), consisting of maternal serum markers and nuchal translucency measurement.<sup>1</sup> Screen-positive women are offered invasive testing using chorionic villus sampling (CVS) or amniocentesis. These invasive tests are highly accurate. However, they are associated with an iatrogenic miscarriage rate up to 1%.<sup>2</sup> The majority of invasive tests (>90%) are carried out in pregnancies with a healthy fetus. The prevalence rate of T21 in the group requesting screening is around one in 500, whereas one in 20 women undergoing FCT are categorised as high risk. Many women fear the risks of invasive testing, and a significant proportion therefore refrains from testing or even from screening.<sup>3</sup> In addition, FCT in 'real world' clinical practice has a false negative rate of 10–25%.<sup>4–8</sup> Lastly, a serious limitation of FCT is its restricted time-window of 11–13 completed weeks, in particular for populations reporting late for their first clinic visit. After a decade of research, a clinically applicable technology was developed for non-invasive testing of fetal chromosomal anomalies, using sequencing of cell-free DNA (cfDNA) fragments in maternal plasma.<sup>9</sup> This method promises to eliminate iatrogenic miscarriages caused by invasive diagnostic procedures, and the fear of many women have for invasive testing. Recently, non-invasive prenatal testing (NIPT) for fetal trisomy was introduced in clinical practice in the USA, China, and Hong Kong. The first test used in these countries was based on massively parallel DNA shotgun sequencing (MPSS)<sup>10,11</sup> an apparently accurate but complex and expensive method. An alternative and more efficient approach using more directed evaluation of cfDNA fragments has been developed and shown to have similar accuracy as compared with MPSS.<sup>12–14</sup> A recent large cohort study showed a sensitivity of 100% and a false positive rate of 0.03% for the prediction of T21 using digital analysis of selected regions (DANSR), combined with an analysis algorithm, the fetal-fraction optimized risk of trisomy evaluation (FORTE).<sup>15</sup> This directed cfDNA method has also been recently evaluated in a general screening population and shown to be highly accurate.<sup>16</sup>

In several European countries, NIPT for fetal sex determination and Rh type are now standard practice. However, until recently, no laboratories were able to clinically provide NIPT for fetal trisomy testing. In August 2012, a laboratory in Germany (Lifecodexx AG, Konstanz, Germany) started to offer testing for T21 using MPSS for women from German-speaking countries, at relatively high cost as expected with this method. Several European clinical research sites participated in the large non-invasive chromosomal evaluation study, by Norton et al., on the accuracy of the DANSR and FORTE methods.<sup>15</sup> From this experience, it appeared that the logistics of shipping the whole blood samples for rapid analysis from Europe to the laboratory of Ariosa Diagnostics in San Jose, California, USA, was feasible enough to consider applying this route for introduction of NIPT in European countries without the need, for now,



to perform the testing itself in Europe. In this European multicenter prospective cohort study, our primary aim was to evaluate the performance of a directed method of a non-invasive prenatal test for fetal T21 by shipping the whole blood samples from Europe to a laboratory in the USA.

## METHODS

### Study population

In this prospective, consecutive cohort study, pregnant women scheduled to undergo an invasive diagnostic test (CVS or amniocentesis) for fetal genotyping were asked to participate by donating a blood sample just prior to the invasive procedure. Women could be included in the study, after informed consent, when  $\geq 18$  years old and carrying a singleton pregnancy with a gestational age of at least 10 weeks. Two groups of indications for the invasive test were identified: I: Women with an increased risk for T21 based on first trimester screening (serum screening, nuchal translucency measurement, and/or maternal age) and II: Women choosing to undergo invasive testing after the detection of fetal anomalies on ultrasound examination. Women requesting invasive testing for psychosocial or anxiety reasons, without abnormalities on ultrasound, were included in group I. Exclusion criteria were pregnant women with  $>1$  fetus, an invasive procedure performed prior to the blood sampling, history or active significant malignancy requiring major surgery or systemic chemotherapy, or language restriction with failure to understand the study information. Women were prospectively enrolled at different sites in the Netherlands (Leiden University Medical Center, Leiden and satellite hospitals) and Sweden (Karolinska University Hospital, Stockholm and Sahlgrenska University Hospital, Gothenburg). The study protocol was approved by the respective Institutional Review Boards of the participating centers.

### Sample collection

Eligible subjects were asked to participate in the study after the counselling session for the invasive diagnostic test. Approximately 20mL of whole blood was collected in two Cell-Free DNA BCTM tubes (Streck, Omaha, and NE) from each subject just prior to the invasive procedure. After blood collection, samples were placed into ambient shipping polystyrene containers with two room temperature gel bricks inside the container. Samples were sent the same day to the laboratory of Ariosa Diagnostics, (San Jose, California, USA) without processing. Upon receipt, blood was processed immediately into plasma, and then plasma was stored at 20 °C until all subjects had been enrolled. At time of sample analysis, only those samples received within 5 days of blood collection were deemed eligible. All samples from the invasive diagnostic tests were analysed at the respective certified genetic laboratories

of the participating university medical centers, using either full karyotyping or quantitative fluorescent polymerase chain reaction.

## **Test method**

Each subject's cfDNA sample was isolated and quantified using the DANSR assay as described previously.<sup>14</sup> Briefly, this method uses ligation of locus-specific oligonucleotides to produce a sequencing template only from selected genomic loci. The FORTE algorithm, also previously described, was used to estimate the risk of T21 in each sample.<sup>13</sup> The FORTE risk score is determined by calculating the odds ratio for T21 based on chromosome 21 cfDNA counts, and fraction of fetal cfDNA in the sample, then applying this as a likelihood ratio to the a priori T21 risk based on the maternal age and gestational age. A predefined cut-off value of one in 100 (1%) was designated as the threshold for classifying a sample as high risk versus low risk. Samples that did not generate a result were classified as low (<4%) fraction of fetal cfDNA, inability to measure fraction of fetal cfDNA, unusually high variation in cfDNA counts, and failed sequencing. The laboratory personnel who performed the analyses were blinded to the clinical information.

## **Data analysis**

On the basis of national screening program data from the Netherlands, we estimated the prevalence rate of T21 in our cohort to be around 5%. The anticipated target performance of the NIPT using DANSR and FORTE was set at 98%. During the course of the European non-invasive trisomy evaluation (EU-NITE) study, we expected to see the publication of the large NICE study, which was using the same testing methodology.<sup>15</sup> Because showing the efficacy of the directed cfDNA approach using the DANSR platform, and the FORTE algorithm was the main aim of the NICE study, we set out to analyse our data to evaluate whether collecting blood samples from European pregnant women and shipping it across the Atlantic Ocean for analysis in the same laboratory would lead to comparable accuracy. Because the results of the NICE study were unknown at the start of our study, we planned to include 1000 samples, which would enable assessment of a sensitivity and specificity for the European cohort with a lower 95% confidence interval (CI) of 93 and 98%, respectively. Using the results of the NICE study and the findings of the interim analysis, the sample size was adjusted after the first 500 samples.

## **Statistical analysis**

Categorical variables were summarised by the number and percentage of subjects. Continuous variables were described as total number and the mean with standard deviation (SD) or range. Correlation between continuous variables (e.g. percent fetal fraction and gestational age at blood sampling) was analysed using linear regression analysis. Sensitivity and specificity were



calculated by standard formulas for a binominal proportion. The accuracy of the test was determined by dividing the sum of the true positive and true negative results by the total number of subjects. Wilson's interval method was used to calculate 95% CI. The comparison of the accuracy of the current study with the NICE study was based on the comparison of unpaired proportions using the Poisson test.<sup>17</sup> Analysis of samples using DANSR and FORTE included all evaluable subjects who had undergone invasive testing with fetal genotype analysis by karyotype or quantitative fluorescent polymerase chain reaction. Prior to study unblinding, all abnormal karyotypes were reviewed by a clinical geneticist and categorized as T21 or other chromosomal abnormality. Other chromosomal abnormalities included sex aneuploidy, trisomy 13 and 18, triploidy, and balanced Robertsonian translocation involving chromosomes 13 and 14. Subjects with chromosomal abnormalities other than T21 were not included in the primary analysis. Results from the DANSR assay and FORTE algorithm were provided as a T21 risk score, with the upper and lower risk value capped at 99% (99 in 100) and 0.01% (one in 10 000), respectively. Calculations for sensitivity and specificity were based on a 1% (one in 100) cut-off to designate results as high risk or low risk for T21.

## RESULTS

A total of 595 subjects were enrolled in the Netherlands and Sweden between May 2011 and March 2012. A total of 188 (31.6%) subjects were enrolled in the Netherlands, 283 (47.5%) subjects were enrolled in Stockholm, and 124 (20.8%) subjects were enrolled in Gothenburg, Sweden. There were 75 subjects ineligible for the primary study analysis because of the failure to meet the inclusion criteria (n=21, e.g. non-invasive procedure was performed, twin pregnancy, or blood draw was not successful), insufficient plasma volume (n=19), logistic problems (n=11, e.g. shipping time >5 days or incorrect labelling), or other chromosome abnormalities besides T21 (n=24). Samples were sent to the laboratory using FedEx International Priority Service, with door-to-door times of less than 36h. Of the 520 eligible subjects and corresponding blood samples, a T21 test result was obtained in 504 subjects (96.9% test result rate). Of the two blood collection tubes drawn from each subject, one tube was processed at a time. The second tube was used in 51 cases. Low (<4%) fraction fetal DNA was present in seven/520 (1.3%) samples and nine/520 (1.7%) of samples were excluded because of laboratory processing or specimen issues. Graphical representation of the subject sample flow is given in Figure 1.

### Patient characteristics

The mean maternal age of the eligible study cohort was 36.4 years (range 20–47 years). The mean gestational age at the time of blood sampling was 14.0 weeks (range 10–28 weeks).



As showed in Table 1, the vast majority of women (n=441/520, 84.8%) were of Caucasian origin, the remaining were Asian (n=17/520, 3.3%), Mediterranean (n = 31/520, 6.0%), Black (n=7/520, 1.3%), and other (n=24/520, 4.6%). In the group, 64 (12%) subjects underwent an invasive procedure because of an earlier child with a chromosomal abnormality, one of the parents with a chromosomal abnormality or close family with a chromosomal abnormality. In the group of 520 subjects analysed for T21, there was an even distribution of CVS (n=280, 54%) and amniocentesis (n=240, 46%). Cytogenetic results of these invasive tests confirmed T21 in 18 cases.

### **Fetal fraction**

In the cohort of 504 subjects with samples analysable for T21, the overall fraction of fetal cfDNA was 11.1% (SD 4.1, range 4–30), with seven samples containing less than the pre-specified cut-off of 4%. The fraction of fetal cfDNA did not vary with ethnicity or maternal age. The fraction of fetal cfDNA by gestational age showed no statistically significant difference for gestational ages between 10 and 22weeks.

### **Test performance**

A T21 test result was obtained in 504/520 (96.9%). Risk assessment was accurate in 503/504 cases (99.8%). In Figure 2, the T21 risk probability results are shown. Applying the predefined 1% cut-off to the 18 cases of T21, 17 of the T21 were classified correctly as high risk (sensitivity 94.4%, 95%; CI 72.7–99.9%). One T21 case, determined by CVS, was classified as low risk with cfDNA testing. This false negative case was a real miss with a risk for T21 calculated with NIPT of 0.01%. This individual was a 39-year-old-Caucasian woman with a gestational age of 13weeks and 5 days. The percentage fraction of fetal cfDNA was 4%. Of the euploid cases, all 485/485 were identified as low risk (specificity 100%, 95%; CI 99.4–100%). There were no false-positive cases. In comparison with the results from the larger NICE study, accuracy was 99.8% in the EU-NITE versus 99.9% ( $p=0.2790$ ).

## **DISCUSSION**

This is the first prospective European multicenter study showing that non-invasive prenatal testing using directed sequencing of cfDNA, applied to blood samples shipped across the Atlantic Ocean, accurately assesses risk of fetal T21. A T21 test result was obtained in 504/520 (96.9%), with an accuracy of 99.8%. This is comparable to the recent large predominantly US study by Norton et al. using the same technology.<sup>15</sup> These results are of great importance in current debates in many countries on how to best incorporate this new, safe, and non-invasive trisomy test in health care programs. The possibility of choosing NIPT for European women





becomes more realistic as it appears feasible to ship the whole blood samples to the USA for processing and analysis by experienced laboratories, who have proven in large studies to master this new technology. In addition, pregnant women in German-speaking countries now have access to NIPT, through shipping of blood samples to the Lifecodexx laboratory in Konstanz, Germany. This laboratory accepts also blood samples from women from other countries, however, they currently need to travel to Germany for counselling and blood draw. Until now, no scientific evaluation of tests performed by this laboratory has been published in peer reviewed scientific literature. In the NICE study by Norton et al., 1.8% of analysed samples had to be excluded because of insufficient fetal fraction of cfDNA versus 1.3% in our study, suggesting that transatlantic shipping does not negatively influence this important parameter.<sup>15</sup> Similarly, assay failure in the NICE study was 2.8% versus 1.7% in the current study.

In the NICE study, all 81 T21 cases were correctly predicted by the DANSR and FORTE methods, and one in 2888 normal cases was classified incorrectly as T21, whereas in our study there were no false positives and one false negative result. This is the first false negative result reported with the DANSR/FORTE approach. In this subject, the percentage of fetal DNA was low (4%). Combining our results with published studies using DANSR/FORTE, a total of 5421 analysed samples of which 175 were T21, the overall sensitivity for T21 is 99.4% (95% CI 97.5–99.9%) and specificity 99.98% (95% CI 99.9–100%).<sup>12,13,15,16</sup>

Although NIPT has a higher accuracy than currently used screening methods, this discordant case underlines the importance, as is with every medical test used in screening and diagnostic settings, of appropriate pre-test and post-test counselling. Women should understand the implications of the test results before actually undergoing any type of testing, including the likelihood of test failure, incorrect results, and findings of unclear significance. For this reason, the introduction of NIPT should be designed carefully and addressed thoroughly by healthcare workers and policy makers. In the current screening and diagnosis programs, women may be falsely reassured by the first line screening test, or may be put through a time of stress and anxiety, fearing both the adverse outcome of either losing their fetus because of the invasive test, or being told that their child has a chromosomal anomaly. Recently, Hill et al. published women's strong preference for tests with no risk of miscarriage, even if such a test would not be entirely accurate.<sup>18</sup> They found that consideration for safety of the fetus is paramount in decision making. As clinicians, we should facilitate in all information so a women can make an informed choice.

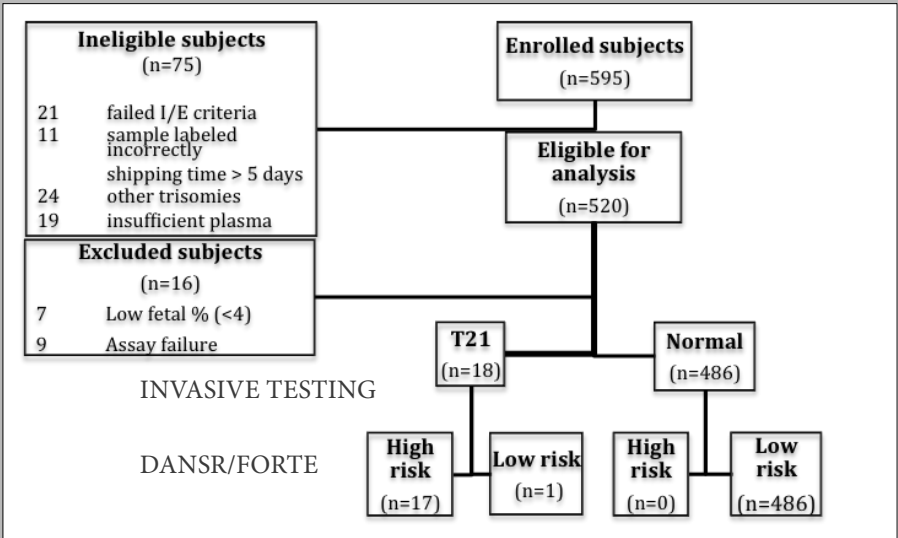
The strength of this study is that this is an international multicenter study with one of the largest European cohorts published until now. In contrast with earlier nested-case control studies, this study analysed a prospective consecutive cohort, which is more representative of actual clinical practice. Similar to all studies on NIPT thus far, our study showed that not all

subject samples were provided a test result. When introducing NIPT in clinical practice, an option not possible in published studies arises, namely a rapid redraw of a blood sample in case of test failure. In addition, careful analysis of sampling and logistics may further reduce test failure to a proportion comparable to current invasive testing or even better.

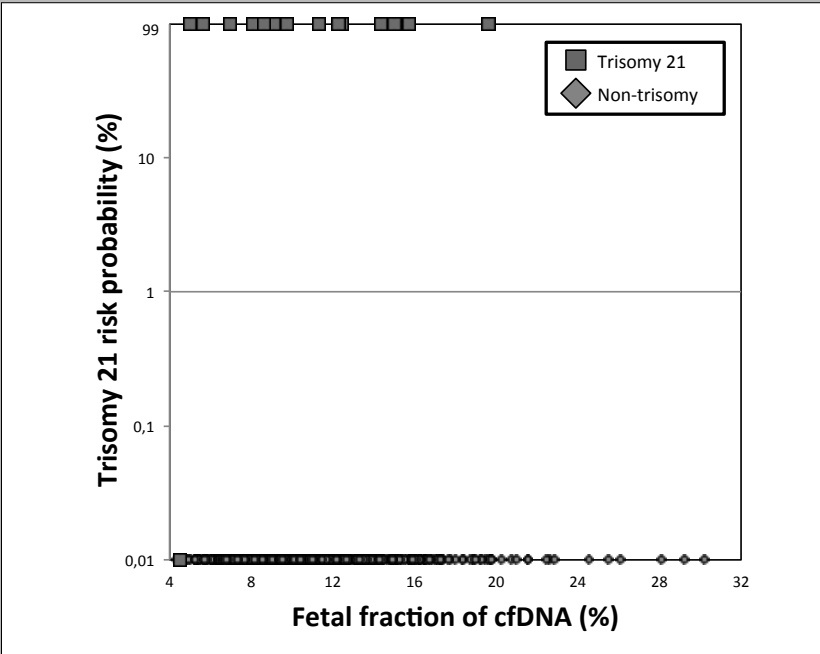
## CONCLUSION

The EU-NITE study shows that shipping the whole blood samples across the Atlantic Ocean, using a directed cfDNA approach for analysis is an accurate and feasible option. NIPT rapidly becomes a more realistic option for European women. Detailed prenatal counselling is needed to ensure that women understand the possible implications of a result. Further investigations are needed to determine the accuracy in a general population and to evaluate the causes of assay failures and how best to address them.





**Figure 1.** Graphical representation of the subject sample flow. Eligible subjects for analysis were classified into trisomy 21 and normal based on invasive testing results. DANSR, Digital Analysis of Selected Regions; FORTE, Fetal-fraction Optimized Risk of Trisomy Evaluation; I/E, inclusion/exclusion.



**Figure 2.** Trisomy 21 (T21) detection with Digital Analysis of Selected Regions and Fetal-fraction Optimized Risk of Trisomy evaluation, with cut-off line between high risk and low risk at 1%. T21 cases are squares, normal cases are diamonds.



<b>Demographic</b>	<b>Euploid (n=502)</b>	<b>T21 (n=18)</b>	<b>Total (n=520)</b>
Maternal age y, mean±SD (range)	36.4±4.6 (20-47)	36.7±4.0 (28-43)	36.4±4.6 (20-47)
≥ 35 years (%)	243 (48.4)	5 (27.8)	248 (47.7)
Gestational age, wk, mean±SD (range)	14.0±2.1 (10-28)	13.3±1.6 (11-18)	14.0±2.1 (10-28)
Maternal Ethnicity, n (%)			
<i>Caucasian</i>	425 (84.7)	16 (88.9)	441(84.8)
<i>Mediterranean</i>	31 (6.2)	-	31 (6.0)
<i>Asian</i>	17 (3.4)	-	17 (3.3)
<i>Black</i>	6 (1.2)	1 (5.6)	7 (1.3)
<i>Other</i>	23 (4.6)	1 (5.6)	24 (4.6)
Fetal DNA in sample,%, mean±SD (range)	11.2± 4.1(4-30)	10.2±4.1(4-20)	11.1±4.1(4-30)
Screening for trisomies, n (%)			
<i>First trimester</i>	174 (34.7)	12 (66.7)	186 (35.8)
<i>Second trimester</i>	2 (0.4)	0	2 (0.4)
Other risk factor (e.g. previous affected pregnancy), n (%)	63 (12.5)	0	63 (12.1)
Other (e.g. maternal anxiety), n (%)	45 (9.0)	1 (8.3)	46 (8.8)

**Table 1.** Demographic characteristics of eligible subjects



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