

# Noninvasive detection of fetal trisomy 21: systematic review and report of quality and outcomes of diagnostic accuracy studies performed between 1997 and 2012

**E. Mersy<sup>1,2</sup>, L.J.M. Smits<sup>3</sup>, L.A.A.P. van Winden<sup>4</sup>,  
C.E.M. de Die-Smulders<sup>1,2</sup>, The South-East Netherlands NIPT  
Consortium<sup>5†</sup>, A.D.C. Paulussen<sup>1,2</sup>, M.V.E. Macville<sup>1,2</sup>,  
A.B.C. Coumans<sup>2,4</sup>, and S.G.M. Frints<sup>1,2,\*</sup>**

<sup>1</sup>Department of Clinical Genetics, Reproductive Genetics, Maastricht University Medical Center<sup>+</sup>, PO Box 5800, 6202 AZ Maastricht, The Netherlands <sup>2</sup>GROW School for Oncology and Developmental Biology, Maastricht University Medical Center<sup>+</sup>, PO Box 616, 6200 MD Maastricht, The Netherlands <sup>3</sup>Department of Epidemiology, Faculty of Health Medicine and Life Sciences, Maastricht University, PO Box 616, 6200 MD Maastricht, The Netherlands <sup>4</sup>Department of Obstetrics & Gynaecology, Prenatal Diagnosis, Screening and Therapy, Maastricht University Medical Center<sup>+</sup>, PO Box 5800, 6202 AZ Maastricht, The Netherlands <sup>5</sup>South-East Netherlands NIPT Consortium, Maastricht University Medical Center<sup>+</sup>, PO Box 5800, 6202 AZ Maastricht, The Netherlands

Correspondence address. Tel: +31-4-33-87-78-55; Fax: +31-4-33-87-58-00; E-mail: s.frints@mumc.nl

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<sup>†</sup> The complete member list is supplied in the Supplementary data.

**BACKGROUND:** Research on noninvasive prenatal testing (NIPT) of fetal trisomy 21 is developing fast. Commercial tests have become available. To provide an up-to-date overview of NIPT of trisomy 21, an evaluation of the methodological quality and outcomes of diagnostic accuracy studies was made.

**METHODS:** We undertook a systematic review of the literature published between 1997 and 2012 after searching PubMed, using MeSH terms 'RNA', 'DNA' and 'Down Syndrome' in combination with 'cell-free fetal (cff) RNA', 'cffDNA', 'trisomy 21' and 'noninvasive prenatal diagnosis' and searching reference lists of reported literature. From 79 abstracts, 16 studies were included as they evaluated the diagnostic accuracy of a molecular technique for NIPT of trisomy 21, and the test sensitivity and specificity were reported. Meta-analysis could not be performed due to the use of six different molecular techniques and different cutoff points. Diagnostic parameters were derived or calculated, and possible bias and applicability were evaluated utilizing the revised tool for Quality Assessment of Diagnostic Accuracy (QUADAS-2).

**RESULTS:** Seven of the included studies were recently published in large cohort studies that examined massively parallel sequencing (MPS), with or without pre-selection of chromosomes, and reported sensitivities between 98.58% [95% confidence interval (CI) 95.9–99.5%] and 100% (95% CI 96–100%) and specificities between 97.95% (95% CI 94.1–99.3%) and 100% (95% CI 99.1–100%). None of these seven large studies had an overall low risk of bias and low concerns regarding applicability. MPS with or without pre-selection of chromosomes exhibits an excellent negative predictive value (100%) in conditions with disease odds from 1:1500 to 1:200. However, positive predictive values were lower, even in high-risk pregnancies (19.7–100%). The other nine cohort studies were too small to give precise estimates (number of trisomy 21 cases:  $\leq 25$ ) and were not included in the discussion.

**CONCLUSIONS:** NIPT of trisomy 21 by MPS with or without pre-selection of chromosomes is promising and likely to replace the prenatal serum screening test that is currently combined with nuchal translucency measurement in the first trimester of pregnancy. Before NIPT can be introduced as a screening test in a social insurance health-care system, more evidence is needed from large prospective diagnostic accuracy studies in first trimester pregnancies. Moreover, we believe further assessment, of whether NIPT can be provided in a cost-effective, timely and equitable manner for every pregnant woman, is required.

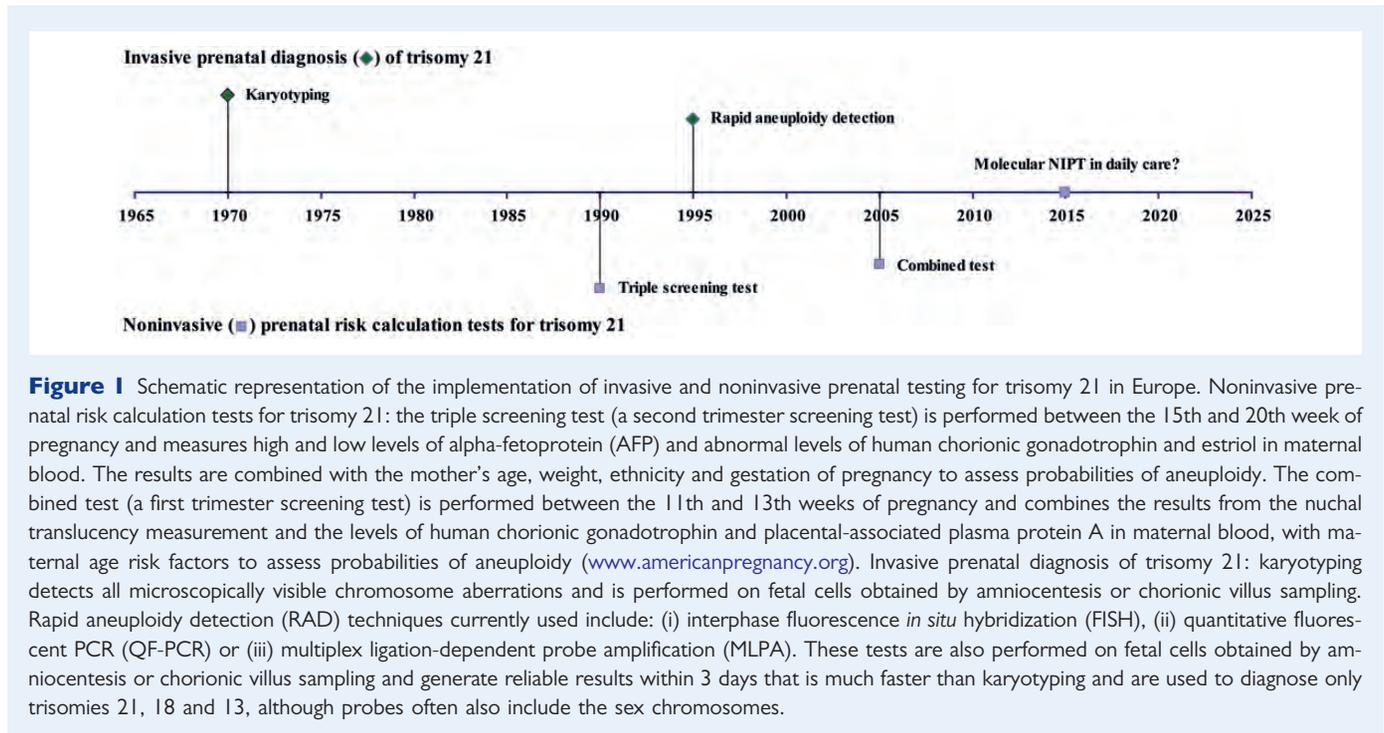
**Key words:** noninvasive prenatal testing / trisomy 21 / cell-free fetal DNA/RNA / QUADAS-2

## Introduction

In developed countries, the two-step prenatal care system includes a noninvasive risk assessment for the most common aneuploidies, before invasive prenatal procedures are offered. In this prenatal risk assessment, along with nuchal translucency measurement by ultrasound, different maternal serum biochemical screening tests are offered in the first or second trimester. At the moment, the most used noninvasive screening test for individual trisomy 21 (Down syndrome) risk calculation worldwide is the combined test. The combined test includes a serum screening test containing two blood markers, namely pregnancy-associated plasma protein-A and free beta human chorionic gonadotrophin, combined with nuchal translucency measurement (Fig. 1). In the case of an *a priori* high-risk or a positive individual risk assessment of trisomy 21 (Down syndrome), 18 (Edwards syndrome) or 13 (Patau syndrome), invasive prenatal diagnosis by fetal karyotyping or rapid aneuploidy detection is performed after preservation of fetal cells by amniocentesis or chorionic villus sampling (de Jong *et al.*, 2011a; Faas *et al.*, 2011). These invasive procedures carry a risk of miscarriage ranging from 0.6%, within 14 days of the procedure, to 2% for total pregnancy loss (Mujezinovic and Alfirovic, 2007). To avert this risk of miscarriage, there is an increasing demand for a reliable and safe noninvasive prenatal test that is applicable as early in pregnancy as possible. Since the discovery of the presence of cell-free fetal (cff) DNA and cff placental-specific mRNA (cffRNA) in maternal plasma, the possibility of using this as the target for noninvasive prenatal testing (NIPT) of fetal genetic conditions is being explored widely (Lo *et al.*, 1997; Poon *et al.*, 2000; Chiu and Lo, 2011; de Jong *et al.*, 2011b). Maternal and fetal cell-free (cf) DNA consist of nuclease-fractionated histone-bound DNA sequences, with the most abundant sequence length (predominantly

maternal) being between 133 and 166 base pairs (Lo *et al.*, 2010). The cffDNA represents a subfraction of 6–10% of the total cfDNA in first and second trimester pregnancies and rises up to 10–20% in third trimester pregnancies (Lun *et al.*, 2008; Lo *et al.*, 2010).

The small-sized fragments and the subfraction of cffDNA/RNA next to abundantly present maternal disomic genome are bottlenecks in developing NIPT techniques for aneuploidy detection. Nevertheless, several molecular techniques have been proposed for the detection of trisomy 21, 18 and 13. Early studies concerning NIPT of aneuploidy focused on the quantification of male cffDNA sequences in maternal plasma samples by real-time PCR (qPCR) (Lo *et al.*, 1999; Zhong *et al.*, 2000; Ohashi *et al.*, 2001; Lee *et al.*, 2002; Hromadnikova *et al.*, 2003; Spencer *et al.*, 2003; Wataganara *et al.*, 2003; Gerovassili *et al.*, 2007). Later, qPCR was used to examine a possible elevation of specific genes, e.g. 'hemoglobin beta (*HBB*)' and 'chromosome 21 open reading frame 105 (*C21orf105*)' (Ng *et al.*, 2004; Jorgez *et al.*, 2006, 2007; Go *et al.*, 2007). Another approach for noninvasive detection of trisomy 21 is determining the dosage of chromosome 21 cffRNA single nucleotide polymorphism (SNP) alleles of genes that are not expressed in maternal blood cells, but only in placental tissue such as 'placenta-specific 4 (*PLAC4*)' or 'serpin peptidase inhibitor, clade B (ovalbumin), member 2 (*SERPINB2*)' (Lo *et al.*, 2007a; Tsui *et al.*, 2009, 2010; Deng *et al.*, 2011). The ratio of SNP alleles using cffDNA instead of cffRNA is also examined for NIPT of trisomy 21 (Dhallan *et al.*, 2007; Ghanta *et al.*, 2010). A limitation of the SNP-dependent approaches is that they are only applicable for fetuses that are heterozygous for the studied SNP (Lo and Chiu, 2012). Furthermore, additional conditions are sometimes required, e.g. a heterozygous or a homozygous mother. To overcome this, several researchers focus on single molecule counting techniques, including digital PCR, that



**Figure 1** Schematic representation of the implementation of invasive and noninvasive prenatal testing for trisomy 21 in Europe. Noninvasive prenatal risk calculation tests for trisomy 21: the triple screening test (a second trimester screening test) is performed between the 15th and 20th week of pregnancy and measures high and low levels of alpha-fetoprotein (AFP) and abnormal levels of human chorionic gonadotrophin and estriol in maternal blood. The results are combined with the mother's age, weight, ethnicity and gestation of pregnancy to assess probabilities of aneuploidy. The combined test (a first trimester screening test) is performed between the 11th and 13th weeks of pregnancy and combines the results from the nuchal translucency measurement and the levels of human chorionic gonadotrophin and placental-associated plasma protein A in maternal blood, with maternal age risk factors to assess probabilities of aneuploidy ([www.americanpregnancy.org](http://www.americanpregnancy.org)). Invasive prenatal diagnosis of trisomy 21: karyotyping detects all microscopically visible chromosome aberrations and is performed on fetal cells obtained by amniocentesis or chorionic villus sampling. Rapid aneuploidy detection (RAD) techniques currently used include: (i) interphase fluorescence *in situ* hybridization (FISH), (ii) quantitative fluorescent PCR (QF-PCR) or (iii) multiplex ligation-dependent probe amplification (MLPA). These tests are also performed on fetal cells obtained by amniocentesis or chorionic villus sampling and generate reliable results within 3 days that is much faster than karyotyping and are used to diagnose only trisomies 21, 18 and 13, although probes often also include the sex chromosomes.

has not been applied in real plasma samples yet (Fan and Quake, 2007; Lo et al., 2007b; Chiu et al., 2009). Recently, massively parallel sequencing (MPS) of maternal plasma DNA has been introduced (Chiu et al., 2008, 2010, 2011; Fan et al., 2008; Chu et al., 2009; Ehrich et al., 2011; Lau et al., 2011; Palomaki et al., 2011; Sehnert et al., 2011; Bianchi et al., 2012; Dan et al., 2012). MPS can identify the chromosomal origin of each sequenced plasma DNA molecule and can detect the over- or underrepresentation of any chromosome in maternal plasma.

Recently, two MPS techniques, in which only the chromosomes of clinical interest are sequenced, have been described. In these targeted MPS techniques, pre-selection of chromosomes leads to less unutilized sequencing data. Moreover, rapid next-generation sequencing devices can be used, altering the costs, turnaround time and the number of patients who can be tested per week. One of these techniques is digital analysis of selected regions (DANSR), in which selected nonpolymorphic loci on chromosomes of clinical interest are simultaneously quantified. (Ashoor et al., 2012; Nicolaides et al., 2012; Norton et al., 2012; Sparks et al., 2012a, b). The other technique is parental support, in which the observed allele distribution after sequencing of polymorphic loci on the chromosomes of clinical interest is compared with the expected allele distribution based on parental genotypes (Zimmerman et al., 2012). Next to these techniques, fetal chromosome dosage determination has been studied based on the well-known epigenetic differences between maternal and fetal DNA, an approach that also can be used for all fetuses (Chim et al., 2005; Tong and Lo, 2006; Papageorgiou et al., 2009, 2011; Tong et al., 2010a, b; Lim et al., 2011; Zhang et al., 2011; Tsaliki et al., 2012).

At the moment, commercial NIPT of trisomy 21 by MPS has become available for high-risk pregnant women and includes the MaterniT21™ PLUS test from Sequenom (<http://www.sequenomcmm.com>), the Praena-Test® from their European partner LifeCodexx (<http://www.lifecodexx.com>), the verifi® test from Verinata (<http://www.verinata.com>) and the Harmony™ prenatal test from Ariosa (<http://www.ariosadx.com>).

The International Society of Prenatal Diagnosis (ISPD) formulated, on 24 October 2011 in a rapid response statement, its considerations and recommendations for the clinical use of the commercial MPS tests in women at high risk and at lower risk (Benn et al., 2012). A final position statement on screening for fetal aneuploidy of ISPD is in the process of being updated and will soon be released. Nowadays, the option of NIPT of aneuploidy has reached pregnant women through the Internet, social media and gynecologists. Interest in large-scale validation studies grows. Molecular NIPT of trisomy 21 will play an important role in prenatal diagnosis and possibly in prenatal screening in the coming years (Fig. 1).

In this article, we provide an up-to-date overview of all studies that have evaluated the diagnostic accuracy of one or more molecular techniques for NIPT of trisomy 21 in a clinical setting and that have reported test sensitivity and specificity. Firstly, we compared the characteristics of the different studies, including the molecular genetic technique used for NIPT, the size, the inclusion criteria, the reference standard test and the timing of testing in pregnancy. Secondly, we derived or calculated diagnostic parameters and evaluated potential bias and applicability of the evidence using the revised tool for Quality Assessment of Diagnostic Accuracy (QUADAS-2) (Whiting et al., 2011).

## Methods

### Search strategy and selection criteria

We undertook a systematic review of the literature and selected relevant studies published between 1 January 1997 and 15 December 2012 in the online database PubMed. The search strategy used was ('RNA'[Mesh]) or ('DNA'[Mesh]) or (cff RNA) or (cffDNA) and (trisomy 21) or ('Down Syndrome'[Mesh]) and (noninvasive prenatal diagnosis). The last search

was performed on 15 December 2012. No limits were used. In addition, reference lists were checked for further published studies, and studies suggested by colleagues and reviewers were also assessed for eligibility. Studies were included, if they evaluated the diagnostic accuracy of one or more molecular techniques for NIPT of trisomy 21 and mentioned test sensitivity and specificity. We excluded studies that examined NIPT of other disorders, reviews, bioethical or other comments, studies that did not use cfDNA/RNA for the detection of trisomy 21 and proof of concept studies.

## Comparison of the included studies and calculation of predictive values

We compared the characteristics of the different studies, including the molecular genetic technique used for NIPT, the size, the inclusion criteria, the reference standard test and the timing of testing in pregnancy. If a study mentioned more than one sensitivity and specificity, e.g. because the authors tested different ways of multiplexing, we included the best result in the comparison. If we believed that sensitivity and/or specificity should have been calculated differently than done by the authors, we recalculated and explained our motives in the comments of the table generated. In addition, we calculated the positive and negative predictive values (PPVs and NPVs, respectively NPVs) of the tests for different prevalence conditions because they are dependent on the prevalence. Firstly, we calculated them for a prevalence of trisomy 21 of 1:200 (which is equal to the *a priori* risk of a 38-year-old pregnant woman and which is the cutoff value for a high-risk pregnancy in the Netherlands), secondly, for a prevalence of 1:380 (the *a priori* risk of a 35-year-old pregnant

woman and an estimated trisomy 21 risk based on the European population) and thirdly, for a prevalence of 1:1500 (the *a priori* risk of a 20-year-old pregnant woman).

## Quality assessment using the QUADAS-2 tool

The quality of the diagnostic studies was evaluated by three independent reviewers (E.M., L.A.A.P.v.W. and L.J.M.S.) using the QUADAS-2 tool (Whiting *et al.*, 2011). In this redesigned tool, studies are scored as 'high risk of bias', 'low risk of bias' or 'unclear risk of bias' and 'high concerns regarding applicability', 'low concerns regarding applicability' or 'unclear concerns regarding applicability' for four key domains: patient selection (domain 1), index test (domain 2), reference standard test (domain 3) and flow and timing (domain 4). The 'unclear' category is used when insufficient data are reported to permit judgment.

We set up a list of characteristics of the most ideal study on NIPT of trisomy 21 (Table 1). To tailor the QUADAS-2 tool to studies about NIPT of trisomy 21, we adjusted the original signaling questions of the tool according to this list and formulated extra signaling questions to check applicability (Supplementary data).

## Results

### Inclusion and exclusion strategy

Fig. 2 shows the process of study selection. We identified 79 studies, of which 45 described 1 or more molecular NIPT technique(s) of

**Table 1** The 'ideal study' on NIPT of trisomy 21.

#### Low risk of bias

A consecutive or random sample of pregnant women is enrolled.

A case-control design is avoided.

Inappropriate exclusions based on the reference standard test results are avoided (e.g. another aneuploidy).<sup>a</sup>

NIPT results are interpreted without knowledge of the reference standard test results.

Reference standard test results are interpreted without knowledge of the NIPT results.

Cutoff values or the threshold is explained before data analysis.

The reference standard test used is karyotyping after amniocentesis or chorionic villus sampling or post-natal karyotyping, and all patients receive this reference standard test.

All patients are included in the analysis.

#### High applicability

Pregnant women are considered as having high risk of trisomy 21 offspring, if the goal of the study is to compare the accuracy of NIPT with karyotyping after amniocentesis or chorionic villus sampling during pregnancy.<sup>b</sup>

NIPT is performed before invasive testing.<sup>c</sup>

One threshold is used, or in case the results can fall in between two thresholds (and is inconclusive), this is the case in maximum 5% of tested women.

Fetal trisomy 21 is detected early in the pregnancy by the used reference standard test.

The manuscripts include samples until 20 weeks of gestation.<sup>d</sup>

The window of gestational age is narrow enough (<4 weeks).<sup>e</sup>

NIPT, noninvasive prenatal testing.

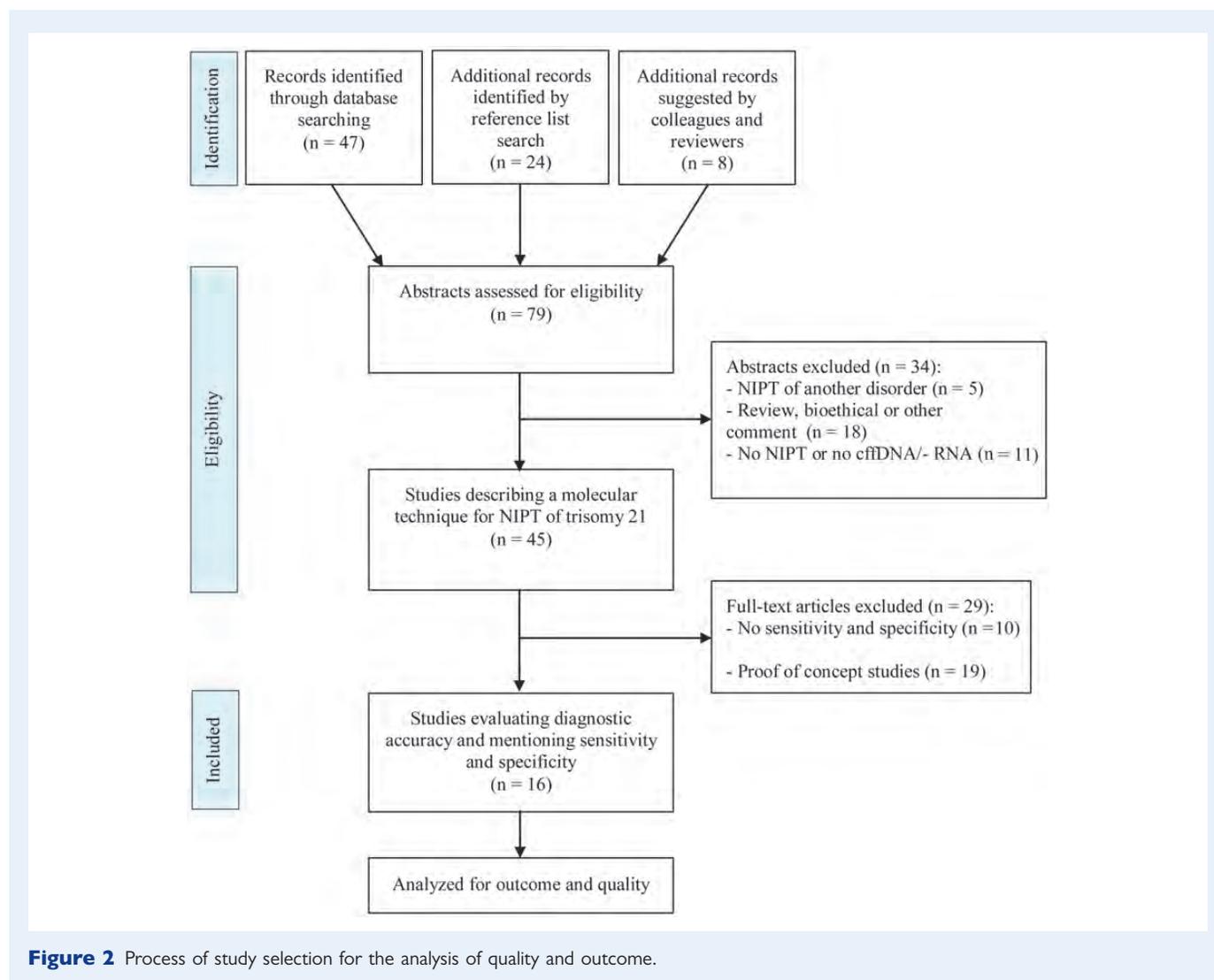
<sup>a</sup>The test has to be able to exclude all non-trisomy 21 samples, including samples with other genetic abnormalities.

<sup>b</sup>A woman can be at high risk of trisomy 21 offspring when there is a positive family or obstetric history, when she is aged above 35 years or when other diagnostic and screening procedures without cfDNA/RNA, such as ultrasound abnormalities, indicate a high risk of aneuploidy.

<sup>c</sup>The performance of an invasive procedure before the NIPT can increase the concentration of cfDNA/RNA in maternal plasma samples that can lead to an easier detection of the target condition by the index test (Bussani *et al.*, 2011).

<sup>d</sup>Concerns rise when NIPT has been performed late in the second trimester or even in the third trimester. To permit a consequence to the result of NIPT in countries, where by law a limit of gestational age for termination is set, the result of NIPT should be known before 22 weeks of gestation (United Nations Population Division, 2002). Taking into account the run time of NIPT itself, the studies should only include samples until 20 weeks of gestation (domain 4A).

<sup>e</sup>There are concerns about the derivation of a preferable time point in pregnancy for NIPT when a too broad window of gestational age is examined (>4 weeks).



trisomy 21 detection during pregnancy. From these, 29 studies were further excluded, mainly because they were proof of concept studies. Only 16 of these 45 studies evaluated the diagnostic accuracy of 1 or more molecular NIPT technique(s) and mentioned test sensitivity and specificity and could, therefore, be included in the review (Fig. 2).

### Characteristics of the included studies

The 16 included studies, dating from 2007 to 2012, applied 6 different molecular genetic techniques for NIPT of trisomy 21 in a high-risk population (Table 2). Five studies investigated the diagnostic accuracy of NIPT of trisomy 21 by MPS without pre-selection of chromosomes, and three studies examined one of the MPS methods with pre-selection of chromosomes, the DANSR method (Chiu et al., 2011; Ehrich et al., 2011; Palomaki et al., 2011; Ashoor et al., 2012; Bianchi et al., 2012; Dan et al., 2012; Nicolaidis et al., 2012; Norton et al., 2012). Two of the 16 studies examined fetal chromosome 21 dosage by analysis of different epigenetic regions between maternal and fetal cfDNA by 2 different molecular techniques: the unmethylation index of the

phosphodiesterases gene 9A (*PDE9A*) and methylated DNA immunoprecipitation and qPCR (Lim et al., 2011; Papageorgiou et al., 2011). Three of the included studies calculated the ratio of cfRNA SNP alleles of *PLAC4*, a gene that is not expressed in maternal blood cells, but only in placental tissue (Lo et al., 2007a; Tsui et al., 2010; Deng et al., 2011). Two studies calculated the ratio of chromosome 21 cfDNA SNP alleles (Dhallan et al., 2007; Ghanta et al., 2010). Finally, one study examined the amplification of the *HBB* gene by qPCR in fetal trisomy 21 cases (Jorgez et al., 2007).

The majority of the studies (9 out of 16) evaluated diagnostic accuracy in remarkably small cohorts ( $\leq 25$  trisomy 21 cases). Four studies included less than ten trisomy 21 cases, and the lowest number of controls included was 20 (Dhallan et al., 2007; Ghanta et al., 2010; Tsui et al., 2010; Nicolaidis et al., 2012). Seven recently published larger studies (7 out of 16) evaluated the diagnostic accuracy of NIPT of trisomy 21 by MPS with or without pre-selection of chromosomes in larger cohorts (Table 2, studies 2–5, 7, 8 and 11) (Chiu et al., 2011; Ehrich et al., 2011; Palomaki et al., 2011; Ashoor et al., 2012; Bianchi et al., 2012; Dan et al., 2012; Norton et al., 2012). In the majority of the studies, high-risk pregnancies were included,

**Table II** Characteristics of the 16 included studies examining NIPT of trisomy 21.

No.	Study	Molecular genetic NIPT technique	T21 cases <sup>a</sup> (n)	Negative controls <sup>a</sup> (n)	Gestational age [weeks, range or mean ( $\pm$ SD)]	Inclusion criteria	Consecutive sample or matched T21:euploid	Blood sample before/after AC or CVS	Reference standard test
1	Nicolaides <i>et al.</i> (2012)	DANSR	8	1941	11–13	First trimester and undergoing combined test	Consecutive	Before	KT or birth outcome
2	Dan <i>et al.</i> (2012)	MPS	139	2820	9–28	High risk regardless of prior risk assessment	Consecutive	Before	KT
3	Norton <i>et al.</i> (2012)	DANSR	81	2888	10–38.7	Planned for invasive procedure	Consecutive	Before	KT, FISH or QF-PCR after AC or CVS
4	Ashoor <i>et al.</i> (2012)	DANSR	50	297	11–13	High risk <sup>b</sup>	Matched 1:3	Before	KT after CVS
5	Bianchi <i>et al.</i> (2012)	MPS 6-plex	92	404	10–23	High risk <sup>c</sup>	Matched 1:4 and selection of samples	Before	KT or FISH
6	Lim <i>et al.</i> (2011)	qMSP UI	18	90	? <sup>d</sup>	Later T21 detected by AC or CVS	Matched 1:5	Before	KT or infant records
7	Palomaki <i>et al.</i> (2011)	MPS 4-plex	212	1471	11–20	High risk <sup>e</sup>	Matched 1:7	Before	KT
8	Ehrich <i>et al.</i> (2011)	MPS 4 plex	39	449	8–36	High risk <sup>f</sup>	Matched 1:11	Before	KT or QF-PCR after AC or CVS
9	Papageorgiou <i>et al.</i> (2011)	MeDiP	14	26	11.1–14.4	?	?	?	KT after AC or CVS
10	Deng <i>et al.</i> (2011)	cffRNA SNP ratio	25	85	9–20	Unknown <sup>g</sup> and high risk <sup>h</sup>	Case control	Before	KT after AC or CVS/ newborn reports
11	Chiu <i>et al.</i> (2011)	MPS 2-plex	86	146	13.1 $\pm$ ?	Indication of AC or CVS	Matched 1:5	Before	KT after AC or CVS
12	Ghanta <i>et al.</i> (2010)	cffDNA SNP ratio	7	20	9–36.1	Indication of AC or CVS	?	Before and after	KT after AC or CVS
13	Tsui <i>et al.</i> (2010)	cffRNA SNP ratio	4	58	T21: 12.9 $\pm$ 0.5 Co: 12.8 $\pm$ 0.7	High risk <sup>i</sup>	Consecutive	Before	KT after CVS

Continued

Table 11 Continued

No.	Study	Molecular genetic NIPT technique	T21 cases <sup>a</sup> (n)	Negative controls <sup>a</sup> (n)	Gestational age [weeks, range or mean ( $\pm$ SD)]	Inclusion criteria	Consecutive sample or matched T21: euploid	Blood sample before/after AC or CVS	Reference standard test
14	Jorgez et al. (2007)	qPCR	17	30	12.23 $\pm$ 0.77	?	Matched 1: 1 or 2	?	KT after AC or CVS
15	Dhallan et al. (2007)	cffDNA SNP ratio	3	57	8–38	?	?	?	KT after AC/newborn reports

although the inclusion criteria were often not clearly described, and different definitions of 'high risk of trisomy 21' were applied. Blood sampling took place throughout pregnancy, including sampling in the third trimester. The diagnostic accuracy of NIPT was compared with karyotyping, although in some studies in combination with another reference standard test.

### Diagnostic performance

NIPT of trisomy 21 shows variable levels of sensitivity (58.82–100.00%) with, in general, higher levels of specificity (83.33–100.00%) (Table 3). Pooling of results could not be performed due to the use of different molecular techniques and different cutoff points. The larger studies on NIPT by MPS or DANSR (Table 3, studies 2–5, 7, 8 and 11) showed higher sensitivities (98.58–100.00%) and specificities (97.95–100.00%), combined with narrower 95%-confidence intervals (CIs) (Chiu et al., 2011; Ehrich et al., 2011; Palomaki et al., 2011; Ashoor et al., 2012; Bianchi et al., 2012; Dan et al., 2012; Norton et al., 2012). Three smaller studies (1, 9 and 12) report a sensitivity and specificity of 100.00%, with, however, broad 95%-CIs (Ghanta et al., 2010; Papageorgiou et al., 2011; Nicolaides et al., 2012). Study 1 included 1949 subjects to examine sensitivity and specificity in a low-risk population. In this group, only a small number of subjects will have a child with trisomy 21, leading to a poor estimation of the sensitivity in particular (Nicolaides et al., 2012).

We calculated the positive and negative predictive values (PPVs and NPVs, respectively) of the tests for different prevalence conditions: a high-risk group (odds 1:200) and two low-risk groups (odds 1:380 and 1:1500) (Table 3). In the larger studies, due to the low likelihood of trisomy 21 on the one hand, and good sensitivity of NIPT on the other hand, NPVs were excellent (100%). However, PPVs showed large variation, ranging from 19.3% to 100% for the group with a high risk of trisomy 21 offspring (1:200), from 11.2% to 100% for a risk of 1:380 and from 3.1% to 100% for a risk of 1:1500 (Chiu et al., 2011; Ehrich et al., 2011; Palomaki et al., 2011; Ashoor et al., 2012; Bianchi et al., 2012; Dan et al., 2012; Norton et al., 2012).

### Analysis of quality by the QUADAS-2 assessment

The QUADAS-2 assessment (Table 4) demonstrates that none of the 16 included studies had a low risk of bias or low concerns regarding applicability in all 4 domains evaluated by the reviewers. In general, most studies had a high risk of bias in domain 1 of patient selection and had high concerns regarding applicability, in domains 4A and 4B, the domains concerning the timing of NIPT in pregnancy. This was also the case for the seven larger studies (Table 4, studies 2–5, 7, 8, and 11) examining NIPT of trisomy 21 by MPS with or without pre-selection of chromosomes (Chiu et al., 2011; Ehrich et al., 2011; Palomaki et al., 2011; Ashoor et al., 2012; Bianchi et al., 2012; Dan et al., 2012; Norton et al., 2012). Five out of seven studies (studies 4, 5, 7, 8 and 11) had a risk of bias in patient selection (Chiu et al., 2011; Ehrich et al., 2011; Palomaki et al., 2011; Ashoor et al., 2012; Bianchi et al., 2012), and five out of seven (studies 2, 3, 5, 7 and 8) had high concerns regarding applicability in domains 4A and/or 4B because a too broad window of gestational age was included or blood sampling took place too late in pregnancy (Ehrich et al., 2011; Palomaki et al., 2011; Bianchi et al., 2012; Dan et al.,

**Table III** Comparison of the diagnostic parameters of the evaluated NIPT techniques.

No.	Study	Molecular genetic NIPT technique	T21 cases <sup>a</sup> (n)	Negative controls <sup>a</sup> (n)	True positive (n)	True negative (n)	Not testable/not in analysis (n)	Sensitivity <sup>a,b</sup> (95% CI)	Specificity <sup>a,b</sup> (95% CI)	PPV for 1:200	NPV for 1:200	PPV for 1:380	NPV for 1:380	PPV for 1:1500	NPV for 1:1500
1	Nicolaides <i>et al.</i> (2012)	DANSR	8	1941	8	1941	100 out of 2049	100.00% <sup>c</sup> (67.5–100%)	100.00% <sup>c</sup> (99.8–100%)	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%
2	Dan <i>et al.</i> (2012)	MPS	139	2820	139	2819	8176 out of 11 105	100.00% (97.3–100%)	99.96% (99.8–100%)	93.4%	100.0%	88.2%	100.0%	65.3%	100.0%
3	Norton <i>et al.</i> (2012)	DANSR	81	2888	81	2887	370 out of 3228	100.00% (95.5–100%)	99.97% (99.8–100%)	93.6%	100.0%	88.4%	100.0%	65.8%	100.0%
4	Ashoor <i>et al.</i> (2012)	DANSR	50	297	50	297	3 out of 350	100.00% (92.9–100%)	100.00% (98.7–100%)	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%
5	Bianchi <i>et al.</i> (2012)	MPS 6-plex	92	404	92 <sup>b</sup>	404	36 out of 532	100.00% (96–100%) <sup>d</sup>	100.00% (99.1–100%) <sup>d</sup>	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%
6	Lim <i>et al.</i> (2011)	qMSP UI	18	90	15	85	0 out of 108	83.33% (60.8–94.2%)	94.44% (87.6–97.6%)	7.0%	99.9%	3.8%	100.0%	1.0%	100.0%
7	Palomaki <i>et al.</i> (2011)	MPS 4-plex	212	1471	209	1468	13 out of 1696	98.58% (95.9–99.5%)	99.80% (99.4–99.9%)	70.8%	100.0%	56.1%	100.0%	24.4%	100.0%
8	Ehrich <i>et al.</i> (2011)	MPS 4 plex	39	410	39	409	31 out of 480	100.00% (91–100%) <sup>e</sup>	99.76% (98.6–100%) <sup>e</sup>	67.3%	100.0%	52.0%	100.0%	21.5%	100.0%
9	Papageorgiou <i>et al.</i> (2011)	MeDiP	14	26	14	26	0 out of 40	100.00% (78.5–100%)	100.00% (87.1–100%)	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%
10	Deng <i>et al.</i> (2011)	cffRNA SNP ratio	24	87	23 <sup>d</sup>	87	10 out of 121	95.83% (79.8–99.3%) <sup>f</sup>	100.00% (95.8–100%) <sup>f</sup>	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%
11	Chiu <i>et al.</i> (2011)	MPS 2-plex	86	146	86	143	11 out of 764	100.00% (95.7–100%)	97.95% (94.1–99.3%)	19.7%	100.0%	11.4%	100.0%	3.1%	100.0%
12	Ghanta <i>et al.</i> (2010)	cffDNA SNP ratio	7	20	7	20	13 out of 40	100.00% (64.6–100%)	100.00% (83.9–100%)	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%
13	Tsui <i>et al.</i> (2010)	cffRNA SNP ratio	4	58	4	52	91 out of 153	100.00% (51–100%) <sup>g</sup>	89.66% (79.2–95.2%) <sup>g</sup>	4.6%	100.0%	2.5%	100.0%	0.6%	100.0%
14	Jorgez <i>et al.</i> (2007)	qPCR	17	30	10	25	0 out of 47	58.82% (36%–78.4%)	83.33% (66.4–92.7%)	1.7%	99.8%	0.9%	99.9%	0.2%	100.0%
15	Dhallan <i>et al.</i> (2007)	cffDNA SNP ratio	3	57	2	56	0 out of 60	66.67% (20.8–93.9%)	98.25% (90.7–99.7%)	16.0%	99.8%	9.1%	99.9%	2.5%	100.0%
16	Lo <i>et al.</i> (2007a)	cffRNA SNP ratio	10	57	9	55	0 out of 67 heterozygous samples	90.00% (59.6–98.2%)	96.49% (88.1–99%)	11.4%	99.9%	6.3%	100.0%	1.7%	100.0%

NIPT, noninvasive prenatal testing; T21, trisomy 21; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value; DANSR, digital analysis of selected regions; MPS, massively parallel sequencing; qMSP, quantitative methylation-specific polymerase chain reaction; UI, unmethylation index; MeDiP, methylated DNA immunoprecipitation; cffRNA, cell-free fetal RNA; SNP, single nucleotide polymorphism; cffDNA, cell-free fetal DNA; qPCR, real-time PCR.

<sup>a</sup>If a study mentions more than one sensitivity and specificity, e.g. because of different multiplex, we report the best result in this table and report the number of trisomy 21 cases and controls that were examined for that sensitivity and specificity.

<sup>b</sup>Depending on the scope of this article, a positive test result can mean diagnosis of trisomy (studies 2 and 5–16) or reporting high risk of trisomy (studies 1, 3 and 4). Negative result of the test means diagnosis of euploidy or reporting low risk of trisomy.

<sup>c</sup>Subcalculation for only trisomy 21 because this is the scope of this article.

<sup>d</sup>Bianchi *et al.* found a positive trisomy 21 NIPT results in 92 of the selected cases, 3 of which were mosaic cases and excluded from the sensitivity and specificity calculation in their manuscript. We included the mosaic cases in our calculation.

<sup>e</sup>Recalculated sensitivity and specificity based on the results reported by Ehrich *et al.* It is unclear for us why some numbers differ from our numbers.

<sup>f</sup>Deng *et al.* reported a sensitivity of 92%. They detected 23 trisomy 21 samples with their test. Two more 21 samples were present in the study population, but only 1 was testable with their method and the other one had only 1 heterozygous SNP locus and could, therefore, not be classified by this NIPT method. Analogous with the other studies, we excluded non-testable samples from the sensitivity and specificity analysis.

<sup>g</sup>Recalculated 95% CIs based on the numbers reported by Tsui *et al.* (2010). It is unclear for us why our CI differs from the reported CI.

**Table IV** QUADAS-2 assessment of the 16 included studies examining NIPT of trisomy 21.

No.	Study	Molecular genetic NIPT technique	Risk of bias				Applicability concerns					
			1	2	3	4	1	2 Index test		3	4 Flow and timing	
			Patient selection	Index test	Reference standard	Flow and timing	Patient selection	A	B	Reference standard	A Timing	B Window
1	Nicolaidis <i>et al.</i> (2012)	DANSR	H	L	H	H	N/A	L	L	H	L	L
2	Dan <i>et al.</i> (2012)	MPS	L	?	?	L	N/A	L	L	L	H	H
3	Norton <i>et al.</i> (2012)	DANSR	L	L	L	H	H	L	L	L	H	H
4	Ashoor <i>et al.</i> (2012)	DANSR	H	L	L	H	L	L	L	L	L	L
5	Bianchi <i>et al.</i> (2012)	MPS 6-plex	H	L	L	H	L	L	L	L	L	H
6	Lim <i>et al.</i> (2011)	qMSP UI	H	H	H	H	H	L	L	H	L	L
7	Palomaki <i>et al.</i> (2011)	MPS 4-plex	H	L	L	L	L	L	L	L	L	H
8	Ehrich <i>et al.</i> (2011)	MPS 4 plex	H	L	L	H	L	L	L	L	H	H
9	Papageorgiou <i>et al.</i> (2011)	MeDiP	H	L	?	L	?	?	L	L	L	L
10	Deng <i>et al.</i> (2011)	cffRNA SNP ratio	H	L	H	H	H	L	L	H	L	H
11	Chiu <i>et al.</i> (2011)	MPS 2-plex	H	L	?	L	L	L	L	L	?	?
12	Ghanta <i>et al.</i> (2010)	cffDNA SNP ratio	?	L	L	L	L	H	L	L	H	H
13	Tsui <i>et al.</i> (2010)	cffRNA SNP ratio	H	H	?	L	L	L	L	L	L	L
14	Jorgez <i>et al.</i> (2007)	qPCR	H	?	L	L	H	?	L	L	L	L
15	Dhallan <i>et al.</i> (2007)	cffDNA SNP ratio	?	L	H	H	?	H	L	H	H	H
16	Lo <i>et al.</i> (2007a)	cffRNA SNP ratio	H	?	?	L	H	L	L	L	L	H

QUADAS-2, revised tool for Quality Assessment of Diagnostic Accuracy; NIPT, noninvasive prenatal testing; DANSR, digital analysis of selected regions; H, high risk of bias/high concerns regarding applicability; L, low risk of bias/low concerns regarding applicability; N/A, not applicable; MPS, massively parallel sequencing; ?, unclear risk of bias/unclear concerns regarding applicability; qMSP, quantitative methylation-specific polymerase chain reaction; UI, unmethylation index; MeDiP, methylated DNA immunoprecipitation; cffRNA, cell-free fetal RNA; SNP, single nucleotide polymorphism; cffDNA, cell-free fetal DNA; qPCR, real-time PCR.

2012; Norton *et al.*, 2012). Moreover, studies 3–5 and 8 had also high risk of bias in domain 4, flow and timing because not all samples were included in the analysis (Ehrich *et al.*, 2011; Ashoor *et al.*, 2012; Bianchi *et al.*, 2012; Norton *et al.*, 2012).

## Discussion

### Implementation of NIPT of trisomy 21 in high-risk pregnancies

The diagnostic accuracy of NIPT was compared with karyotyping in high-risk pregnant women, in 14 out of 79 studies assessed for eligibility in this systematic review (Table 2, studies 3–16). Our study demonstrates that only a small number of studies were conducted on the topic and that the ideal study on NIPT has not been performed yet in high-risk pregnancies. In general, strikingly, small cohorts of pregnant women were examined. Eight cohort studies were too small to give precise estimates (number of trisomy 21 cases:  $\leq 25$ ). Therefore, conclusions were drawn from six large cohort studies that are currently all the studies evaluating the diagnostic accuracy of NIPT of trisomy 21 by MPS with or without pre-selection of chromosomes (Table 2, studies 3–5, 7, 8 and 11).

The outcome analysis illustrates variable levels of sensitivity (58.82–100.00%) with, in general, higher levels of specificity (83.33–100.00%) throughout the 14 articles. The large cohort studies on NIPT by MPS with and without pre-selection of chromosomes have a higher sensitivity, ranging from 98.58% to 100.00%, as well a high specificity, ranging from 97.95% to 100.00%. These NIPT techniques exhibit an excellent NPV in natural conditions with disease odds up to 1:200. However, PPVs were often very low, even in high-risk pregnancies. A PPV of 19.7% as can be calculated from the study of Chiu *et al.* in 2011 (Table 2, study 11) indicates that, even in a high-risk population (odds 1:200), a positive test result is false in  $>80\%$  of the cases.

None of these six large cohort studies had an optimal quality. The QUADAS-2 results depict high concerns of sampling time in pregnancy in most of these studies, which was often too late in pregnancy or in a too broad window of gestational age. Only the DANSR technique was evaluated in a small window of gestational age in the first trimester (Table 2 study 4) (Ashoor *et al.*, 2012). At the moment, this technique can be performed more rapidly than MPS without pre-selection of chromosomes. Although the perfect study has not been performed yet, three studies, studies 4, 5 and 7, were of better quality (Palomaki *et al.*, 2011; Ashoor *et al.*, 2012; Bianchi *et al.*, 2012). In the future, NIPT by MPS with and without pre-selection of chromosomes should be further explored, focusing on the inclusion of a consecutive sample early in the first trimester of pregnancy and the incorporation of all samples in the analysis. Large prospective studies will give more certainty about the predictive values in the high-risk group.

### NIPT of trisomy 21 as a screening test in low-risk pregnancies

The sensitivity and specificity of NIPT were examined in a population of low-risk pregnant women and in a mixed population in 2 out of 16 studies included in the QUADAS-2 assessment (Table 2, studies 1 and 2) (Dan *et al.*, 2012; Nicolaidis *et al.*, 2012). Furthermore, we

calculated the PPVs and NPVs in an average-risk population (1:380) and a low-risk population (1:1500) for these 2 studies and for the remaining 14 studies. In this systematic review, we demonstrate that the diagnostic parameters of NIPT are better than those of the current first trimester prenatal screening risk assessment for fetal trisomy 21. Therefore, NIPT is likely to replace the prenatal serum screening test that is currently combined with nuchal translucency measurement in the first trimester of pregnancy. The replacement of the current two-step triage testing procedure by NIPT of trisomy 21 would maximize the benefits of NIPT at an early stage of pregnancy: the drawbacks of false-positive and false-negative results generated by the current risk assessment and the miscarriage risk attached to current invasive diagnostic methods would, thereby, be reduced (de Jong *et al.*, 2011b). In that case, less invasive procedures would be needed, only to verify a positive NIPT result and to confirm non-inheritable or inheritable forms of Down syndrome, using the gold standard that is still karyotyping. Most likely, more women will opt for NIPT in pregnancy because of its proven diagnostic accuracy. However, there is still more evidence needed before NIPT of trisomy 21 can be introduced in routine prenatal care, e.g. as routine screening NIPT in the first trimester of pregnancy. Preferably, large prospective diagnostic accuracy studies, including low-risk pregnant women recruited in a clinical setting early in pregnancy, will have to be performed before this will become a reality in the public, social insurance health-care systems in Europe. One study included in this systematic review examined diagnostic accuracy in low-risk pregnant women (Table 2, study 1) (Nicolaidis *et al.*, 2012). However, a 5–10 times larger sample size than the 1949 samples analyzed in this study is needed for a reliable estimation of the sensitivity in *a priori* low-risk first trimester pregnancies. Additional accuracy studies are currently designed and ongoing. Moreover, we believe that NIPT should be provided in a cost-effective, timely and equitable manner. Finally, further ethical exploration and evaluation of the current opinion of pregnant women and the formulation of proper informed consent information are needed (de Jong *et al.*, 2011b). We estimate that it will take a few years until NIPT of trisomy 21 can be integrated as a part of routine prenatal care for every pregnant woman in the public, social insurance health-care system (Fig. 1) We speculate that NIPT by MPS will replace the current serum screening test after legal licenses are arranged and informed consent information is drawn.

## Conclusion

The 16 NIPT studies included in the QUADAS-2 assessment illustrate that the results are promising. Nevertheless, considering the limited size and quality of the studies, additional large prospective studies will allow more precise estimates about sensitivity, specificity and predictive values in high-risk and low-risk pregnancies. We demonstrate that the diagnostic parameters of NIPT of trisomy 21 are better than those of the current first trimester risk assessment. Therefore, NIPT is likely to be implemented in this risk assessment, as a replacement for the current serum screening test. Due to the reduction in false-positive and false-negative results, fewer trisomy cases would be missed at the first screening step and fewer invasive procedures would be needed, only to verify a positive NIPT result and to confirm non-inheritable or inheritable forms of Down syndrome,

using the gold standard karyotyping. Before NIPT of trisomy 21 can be introduced as a replacement for the current serum screening test in a public, social insurance health-care system, still more evidence is needed from large prospective diagnostic accuracy studies, including low-risk pregnant women early in pregnancy. Moreover, we believe that NIPT should be provided in a cost-effective, timely and equitable manner. Finally, further ethical exploration and evaluation of the current opinion of pregnant women and the formulation of proper informed consent information are needed.

## Supplementary data

Supplementary data are available at <http://humupd.oxfordjournals.org/>.

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## Authors' roles

S.G.M.F., C.E.M.d.D.-S. and L.J.M.S. designed this study. E.M., L.A.A.P.v.W. and L.J.M.S. selected the studies for inclusion and evaluated the quality. E.M. and L.J.M.S. derived or calculated the diagnostic parameters. All authors and the other members of The South-East Netherlands NIPT consortium made substantial contributions to the interpretation of the results. E.M. wrote the first draft of the manuscript. All authors and the spokeswomen of The South-East Netherlands NIPT consortium, S.G.M.F. and A.B.C.C. revised the manuscript critically and approved the final version for publication.

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