First Trimester Screening for Common Trisomies and Microdeletion 22q11.2 Syndrome Using Cell-Free DNA: A Prospective Clinical Study

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Keywords

Microdeletion 22q11.2 · Aneuploidy · Screening · Genetics · Pregnancy

Abstract

Objectives: The aims of the study were to assess the false-positive and uninformative test rate with first trimester cell-free DNA (cfDNA) screening for common trisomies and microdeletion 22q11.2 (22q11.2DS) and to examine women's attitudes toward such an approach. **Methods:** This is a prospective study at the Prenatal Medicine Department of the University of Tübingen, Germany, at 11–13 weeks. In all pregnancies, a detailed ultrasound examination was carried out, followed by a cfDNA analysis for common trisomies and 22q11.2DS. In cases where the cfDNA analysis indicated 22q11.2DS, invasive prenatal diagnostic testing and parental testing were performed. After delivery, a detailed neonatal clinical examination was carried out including further genetic testing. Prior to counselling about the study, we asked the pregnant women who were potentially eligible for the study to anonymously report on their knowledge about 22q11.2DS. **Results:** A total of 1,127 pregnancies were included in the final analysis of the study. The first cfDNA test was uninformative in 15 (1.33%) pregnancies. In 10 (0.89%)

cases, the test remained uninformative, even after the second blood sample. There were 3 (0.27%) cases with a positive cfDNA test for 22q11.2DS. In all, 983 women returned the anonymous questionnaire prior to study participation. Only 80 (8.1%) women responded that they felt familiar or very familiar with 22q11.2DS. **Conclusion:** The addition of 22q11.2DS in first trimester cfDNA screening for common trisomies is feasible. The uninformative test rate for common trisomies and 22q11.2DS is 0.9%, and the false-positive rate for 22q11.2DS is 0.3%. Awareness and education around 22q11.2DS should be improved.

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Introduction

For the last 2 decades, first trimester combined screening (FTCS) has been considered the gold standard in screening for trisomies 21, 18, and 13 [1]. However, this approach has been challenged by the introduction of cell-free DNA (cfDNA) screening. It is likely that this method will replace classical FTS as the primary test for detection

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Fig. 1. Schematic representation of the structure of the 22q11.2DS critical region. White bars represent proximal deletions and gray bars, central deletions. The percentage represents the proportion of 22q11.2DS patients with each specific type of deletion. The black bar represents the microarray probe coverage of the DANSR targeted assay. The position of the FISH probes (D22S75 and RP11-801O20) are also shown. DANSR, Digital ANalysis of Selected Regions; FISH, fluorescence in situ hybridization; LCR, low-copy repeat.

of common trisomies [2]. The detection rate for each of the common trisomies is about 99% for a false-positive rate of <0.1% [3].

Current research in cfDNA technology focuses on the detection of other chromosomal conditions, especially the 22q11.2 microdeletion syndrome (22q11.2DS; MIM#188400) [4]. This autosomal dominant disease is caused by different microdeletions that are mediated by low-copy repeats (LCRs; LCR22A→LCR22H) mapping to the q11.2 region of chromosome 22 (Fig. 1). The first 4 LCRs (LCR-A→LCR-D) account for the majority of 22q11.2DS cases. Over 90% of patients have “proximal” deletions encompassing the LCR22A-D (A-D, A-C, and A-B). About 5% of patients have “central” deletions encompassing B-D LCRs. Distal D-H deletions are rare [5, 6].

The gold standard test for prenatal diagnosis of detecting 22q11.2DS is chromosomal microarray (CMA) performed on genomic DNA from invasively obtained samples (chorionic villi and amniotic fluid) [4]. There are also targeted assays available (i.e., Prenatal BACs-on-Beads, fluorescence in situ hybridization [FISH], and multiplex ligation-dependent probe amplification [MLPA]), which are often utilized in familial cases where the microdeletion has already been identified with these technologies in the proband [4].

Both genome-wide and targeted cfDNA test methodologies can be used to screen for 22q11.2DS [4]. Targeted technologies are designed to interrogate the proximal deletions (A-B, A-C, A-D, A-E, or A-F) at a high analysis depth. Nontargeted genome-wide technologies based on

massively parallel shotgun sequencing (MPSS) by a shallow NGS of maternal and fetal circulating cfDNA fragments include the whole chromosome 22. Screening for the entire A-H region is technically feasible through a bioinformatics post hoc process extrapolating from chromosome 22 only the selected fragments of interest from the q11.2 region [4].

Microdeletion 22q11.2 has a heterogeneous clinical presentation with multi-organ dysfunction. Common postnatal phenotypic findings include growth and developmental delay, cardiac defects, cleft palate, recognizable facial features, learning disabilities, and immunodeficiency [7–10]. Overall, it is the second most common cause of developmental delay and major congenital heart disease after Down syndrome [11]. Despite the full penetrance, it shows a highly variable expressivity so that there are affected individuals with milder phenotypes that may remain unrecognized, even by healthcare professionals. Antenatally, the diagnosis is generally prompted in cases with a fetal anomaly, especially a cardiac defect [12].

In prenatal samples, the prevalence of the disease is dependent on the ultrasound findings. Fetuses with congenital heart defects show the highest prevalence, while in anatomically normal fetuses with a normal karyotype, it is in the range of 1:800–1:1,000. The prevalence is independent on maternal age [4,13–16].

So far, most studies on cfDNA screening for 22q11.2DS have either used artificial or biobank samples or, when testing clinical populations, provide incomplete outcome data [17, 18]. In a large retrospective study from Martin et al. [17], the authors reviewed the 22q11.2DS

results with the targeted SNP-based cfDNA technology in a general population. The positive predictive value (PPV) was 15.7%. Only one large prospective study of general population screening has been published so far [19]. Using an MPSS-based cfDNA test applied in 94,085 second trimester pregnancies, they reported a false-positive rate of 0.001% (0–0.006), a PPV of 92.9% (68.5–98.7), and a sensitivity of 86.7% (62.2–96.3) based on the clinical examination at birth and molecular follow-up of low-risk cases with fetal/newborn anomalies. Due to the lack of prospective screening studies and clinical validation, most scientific societies do not endorse routine cfDNA screening for 22q11.2DS. Herein, we present a prospective clinical study, aimed at assessing the uninformative test rate and the false-positive rate with first trimester cfDNA screening for common trisomies and 22q11.2DS and examining women's attitudes toward such an approach.

Methods

This is a prospective study, which was performed at the Prenatal Medicine Department of the University of Tübingen, Germany. The subjects were pregnant women who were referred for first trimester risk assessment. In our department, first trimester risk assessment is routinely done at 11–13 weeks' gestation. In addition to crown-rump length and nuchal translucency (NT) measurements, it also includes a detailed ultrasound examination based on ISUOG guidelines [20]. All operators who perform this examination are certified by the FMF UK.

In general, we do not calculate a specific risk for aneuploidy if the NT measurement is >3.5 mm or if a fetal anomaly is identified. These cases are deemed to be at a very high risk for the whole spectrum of chromosomal abnormalities and are offered invasive testing.

If the NT thickness is 3.5 mm or less, we offer cfDNA testing to assess the risk for trisomies 21, 18, and 13. Assessment of sex chromosome aneuploidy is not offered.

For the purpose of this study, the cfDNA analysis was performed by TOMA Advanced Biomedical Assays S.p.A., Impact Lab Group (Busto Arsizio, VA, Italy) using Harmony® Prenatal Test (Roche, Inc., San Jose, CA, USA) as previously described [21]. In brief, this is a targeted cfDNA analysis performed using microarray quantitation of DANSR (Digital ANalysis of Selected Regions) assays of non-polymorphic (chromosomes 13, 18, 21, X, and Y) and polymorphic loci (chromosomes 1–12) to estimate chromosome proportion and fetal fraction, respectively [22]. The FORTE (Fetal-fraction Optimized Risk of Trisomy Evaluation) algorithm is used to provide patient-specific risk assessments for trisomy [23].

Approximately 500 additional DANSR assays were designed against targets uniformly distributed within a 3.0-Mb region of 22q11.2 between LCR-A and D (Fig. 1). Custom DNA microarrays were manufactured by Affymetrix Inc. (Santa Clara, CA, USA) to specifically quantify all DANSR assay products. Each sample was analyzed on a single custom microarray [18].

In all cases where a cfDNA analysis is performed, an additional tube of blood is collected for backup FTCS in case of an uninformative cfDNA testing result, which is expected in a certain proportion of cases. This serum sample is centrifuged at 4,000 rpm for 5 min; the serum is aliquoted and stored at –80°C. In any case where no result is obtained by cfDNA analysis, the serum sample is gently thawed and free beta-hCG and PAPP-A levels are measured. The risk for trisomy 21 is then computed using FTCS according to the algorithm of the FMF UK [1].

All women with pregnancies who were referred to us during the study period were considered eligible for this study. The only exclusion criteria were maternal age <18 years, CRL measurement of >84 or <45 mm, and multiple pregnancy, including vanishing twins.

We included women who were referred to us for routine FTCS and those who were sent with abnormal FTCS results. In the latter group, the risk was predominantly increased due to increased NT. As increased NT is also a marker for cardiac defects, we suspected a higher risk for fetuses with 22q11.2DS in this group. Therefore, for the purpose of this study, cfDNA screening for 22q11.2DS was performed in all cases, irrespective of the NT thickness and of whether there was a fetal defect or not.

In cases where the cfDNA analysis indicated a high probability of 22q11.2DS, invasive diagnostic testing by amniocentesis or chorionic villus sampling (CVS) was carried out. Parental testing was carried out on peripheral T-lymphocytes. Standard karyotyping was performed following national cytogenetic and molecular cytogenetic guidelines [24]. In general, chromosomes were prepared from cultured cells according to standard procedures and characterized by molecular karyotyping and/or standard banding. The number of analyzed metaphases was a minimum of 15 per sample at a 400 GTG-band resolution level or higher and at least 10 metaphases per FISH probe. For CMA analysis, genomic DNA was isolated by routine procedures.

For 22q11.2DS assessment in cases with a positive cfDNA test result, the analysis was conducted by CMA on DNA from fresh CVS biopsy or long-term culture in combination with FISH analysis on spontaneous metaphases from cytotrophoblasts using BAC probes mapping in the proximal (A-B LCRs; D22S75 [Abbott/Vysis]) and in the central critical regions (C-D LCRs; RP11-801O20 provided by Thomas Liehr, Jena, Germany). FISH analysis was performed when CMA provided a normal result to exclude the presence of rare confined placental mosaicism for 22q11.2DS in cytotrophoblasts [25]. Parental testing was carried out by FISH analysis. After delivery, a detailed neonatal clinical examination was performed, including further genetic testing on cord blood or placenta by FISH and/or microarray.

High-resolution chromosome analysis (SNP array analysis) was performed using the CytoScan™ Optima Array (Thermo Fisher Scientific, Waltham, MA USA) according to the manufacturer's instructions. Genotypes were called with Chromosome Analysis Software (version 3.0.0.139). The minimum detection limit for copy number variants was 50 kb with at least 5 markers included. All nucleotide positions refer to the Human Genome February 2009 assembly (GRCh37/hg19). We filtered for quality parameters (median absolute pairwise difference ≤0.25 and waviness standard deviation ≤0.12).

In pregnancies with a normal cfDNA test result for 22q11.2DS, further genetic examinations were not routinely carried out. However, in Germany, all children are examined directly after birth and at least 6 times by a pediatrician within the first year of life. At the

Table 1. Summary of the study characteristics

	High risk, <i>n</i> = 65	Low risk, <i>n</i> = 1,062
Maternal age, years, median (25–75th centile)	35.8 (30.4–38.3)	33.9 (31.0–36.7)
Gestational age, weeks, median (25–75th centile)	12.9 (12.4–13.2)	12.9 (12.5–13.3)
Maternal weight, kg, median (25–75th centile)	66.2 (60.0–73.7)	65.6 (59.0–74.5)
Cigarette smoking, <i>n</i> (%)	2 (3.1)	21 (2.0)
Caucasian origin, <i>n</i> (%)	64 (98.5)	1,027 (97.3)
Assisted reproduction, <i>n</i> (%)	4 (6.2)	35 (3.3)
Crown-rump length, mm, median (25–75th centile)	67.8 (58.5–73.8)	68.5 (63.6–73.3)
NT thickness, median (25–75th centile)	3.7 (1.9–5.0)	1.8 (1.6–2.1)
Fetal defects, <i>n</i> (%)	44 (67.9%)	0

NT, nuchal translucency.

Fig. 2. Description of the study population.

time of the manuscript preparation, the median age of the children was 354 days (range 198–542 days).

Clinically relevant ultrasound and screening information was recorded in the Viewpoint database (GE Healthcare, Munich, Germany). Information on maternal weight and height was extracted from the patient records. Ethnicity, smoking status, and mode of conception were assessed using a questionnaire, and this information was also recorded in the Viewpoint database. Outcome data were added as soon as it became available. The study was accompanied by a questionnaire to assess the women's knowledge about 22q11.2DS and their attitude toward screening.

Prior to counselling about the study and, in particular, about the spectrum of physical and mental development of children with 22q11.2DS, we asked the pregnant women who were potentially eligible for the study to anonymously report on their knowledge about 22q11.2DS. For comparison, they were also asked about the common trisomies; toxoplasmosis, parvovirus B19, and cytomegalovirus infection; and common pregnancy complications, namely, preeclampsia, gestational diabetes, and fetal growth restriction. The following answers were allowed: don't know at all, rather unknown, familiar, or very familiar. Four weeks after the final results of the cfDNA test were available, we asked the pregnant women (including those with false-positive results) again whether they

find that an expansion of cfDNA testing to include 22q11.2DS is useful (possible responses: not useful, rather not useful, useful, or very useful) and if they would recommend cfDNA screening in general and screening for 22q11.2DS in particular to their best friend (possible responses: no, rather no, rather yes, or yes). Approval for the study was obtained from the local Ethics Committee (University Hospital of Tübingen, Germany; October 18, 2017; No. 644/2017BO1). The study was registered in the clinicaltrials.gov registry (NCT03375359).

Statistical Analysis

The study cohort was divided into a high- and a low-risk group according to the presence of an increased NT of >3.5 mm or of a fetal defect that requires postnatal follow-up. The additional ultrasound risk markers nasal bone as well as tricuspid and ductus venosus flow were not considered as anomalies.

Primary endpoints were the proportion of false-positive and uninformative cfDNA tests for 22q11.2DS. These results were stratified according to the 2 risk groups. The results of the questionnaires are given as proportion with 1 of the 2 positive answers. Results are given as median with the 25 and 75th interquartile range or as proportion with the 95th confidence interval using the Clopper-Pearson method.

Table 2. Characteristics of the 3 cases with a false-positive cfDNA test result for microdeletion 22q11.2

Characteristic	Case 1	Case 2	Case 3
Maternal age, years	35.2	41.3	29.7
Gestational age, weeks	12.4	13.4	12.9
Maternal weight, kg	63.5	129.0	56.8
Ethnicity	Caucasian	Caucasian	Afro-Caribbean
Cigarette smoking	No	No	No
Assisted reproduction	No	No	No
Medical history	None	None	Sickle cell anemia
Crown-rump length, mm	61.5	76.1	67.7
NT, mm	1.5	2.0	1.9
NB/TF/DVF	n/n/n	n/n/n	n/n/n
Fetal defects	None	None	None
Free beta hCG in MoM	1.19	1.74	0.60
PAPP-A in MoM	1.82	1.38	0.80
Group	Low risk	Low risk	Low risk
Fetal fraction, %	9.0	7.0	6.0
Pregnancy complications	Premature rupture of membranes at 36 weeks	None	Sickle cell crisis
Gestational age at delivery, weeks	36.3	37.3	37.0
Birth weight, g	3,265	3,250	2,560

NT, nuchal translucency; cfDNA, cell-free DNA.

Results

Between January and December 2018, 1,188 women with singleton pregnancies were referred for first trimester risk assessment. Of those, 43 (3.6%) women declined study participation. Thus, 1,145 women initially participated in this study. However, 9 (0.9%) pregnancies were lost to follow-up, and 9 (0.9%) women miscarried the fetuses during the subsequent course of the pregnancy. In these pregnancies, the first trimester risk assessment or the cfDNA test was low risk and none opted for an autopsy or genetic testing. They were excluded from further analysis. Thus, 1,127 pregnancies were included in the final analysis (Fig. 2).

These pregnancies were divided into 2 groups according to the result of the ultrasound examination. The high-risk group consisted of 65 fetuses, either due to a fetal defect ($n = 44$) or due to an isolated increased NT thickness of 3.5 mm or more ($n = 21$). Within this group, 20 fetuses had trisomy 21, 18, or 13.

The remaining 1,062 cases were classified as low-risk pregnancies. The characteristics of the study population are given in Table 1.

In the study population, there were 26 fetuses with trisomies 21, 18, and 13. The results of the ultrasound examination are shown in online suppl. Table 1 (see www.karger.com/doi/10.1159/000510069 for all online suppl. material) as are the results of serum and additional ultra-

sound markers. The cfDNA test was uninformative in 2 of these cases; both fetuses had trisomy 18. In all abnormal cases with a test result, the cfDNA test indicated a high-risk result. For trisomies 21, 18, and 13, there were no false-positive results.

In the whole study population, the first cfDNA test was uninformative in 15 (1.33%, 95% CI 0.75–2.19%) pregnancies. In 10 (0.89%) cases, the test remained uninformative, even after the second blood sample. Seven (0.66%) of these pregnancies were in the low-risk group, and 3 (4.6%) were in the high-risk group. In both groups, the median fetal fraction was 11.0%. We did not observe cases that were partially uninformative. The median maternal weight of the women with a final uninformative test was 68.7 kg. In this group, there was one neonate with a birth weight below the 5th centile.

There were 3 (0.27%, 95% CI 0–0.78%) cases with a positive cfDNA test for 22q11.2DS. All belonged to the low-risk group. The fetal fractions were 6.0, 7.0, and 9.0%. The maternal and pregnancy characteristics of these 3 cases are highlighted in Table 2.

In all 3 cases, neither the pre- and postnatal testing (prenatal: 2 CVS, 1 amniotic fluid; postnatal: 1 fetal cord blood, 2 placental analyses) nor the parental testing indicated 22q11.2DS. Clinical examination by the pediatrician was also normal. In none of the children of the whole study population was there a suspicion of 22q11.2DS.

Fig. 3. Awareness of pregnancy complications.

Prior to the cfDNA study participation and the associated counselling, the pregnant women were asked if they knew anything about 22q11.2DS. For comparison, we also asked them about their knowledge of trisomies 21, 18, and 13 as well as in utero infections and common pregnancy complications. A total of 983 women returned the anonymous questionnaire. Only 80 (8.1%, 95% CI 6.5–10.3%) women responded that they felt familiar or very familiar with 22q11.2DS. Their responses about the other pregnancy complications are shown in Figure 3. For none of the conditions was the maternal knowledge as low as for 22q11.2DS. The posttest questionnaire was returned by 1,057 pregnant women. In all, 1,013 (95.8%, 95% CI 94.5–97.0%) women found that the expansion of cfDNA screening to include 22q11.2DS was useful or very useful; 1,046 (99.0%, 95% CI 98.2–99.5%) women would recommend cfDNA screening to their best friend and 1,006 (95.2%, 95% CI 93.7–96.4%) of them with the inclusion of 22q11.2DS. All 3 women with false-positive results for 22q11.2DS would still recommend cfDNA testing to their best friend and 2 women even with the inclusion of 22q11.2DS.

Discussion

In this prospective clinical study, we have demonstrated that cfDNA screening for common trisomies and 22q11.2DS results in an uninformative test rate of 0.9% (none gave a partial uninformative result for 22q11.2DS

only) and a false-positive rate for 22q11.2DS of 0.3%. In addition, we observed that the disease is rather unknown, as <10% of the pregnant women feel familiar or very familiar with 22q11.2DS. However, after screening, about 95% of the women would recommend cfDNA screening with the inclusion of 22q11.2DS to their best friend. Our study was not designed to comment on the detection rate.

In a previous prospective study, we examined the test performance of cfDNA screening for trisomies 21, 18, and 13 without 22q11.2DS in fetuses without structural defects and a NT thickness of <3.5 mm. There were no false-positive cases, and the uninformative test rate was 1.5% using the same procedure as in this study [2]. Compared to our previous study with a similar design, the overall false-positive rate and uninformative test rate in this study were not increased by the additional screening for 22q11.2DS.

Screening for 22q11.2DS by cfDNA can be carried out by targeted and genome-wide technologies [4]. Still, for none of the technologies are enough clinical prospective data available so far. Table 3 summarizes the findings of the recent clinical studies.

Wapner et al. [26] used a SNP-based platform and examined 43 and 65 artificial samples from affected and unaffected pregnancies. They reported that all cases were correctly identified, and among the 3 real samples, 2 were detected [26]. Ravi et al. [27] also used a SNP-based technology with a revised protocol and detected 9 of 10 affected samples. There was one false-positive case

Table 3. cfDNA test studies reporting 22q11.2 deletions [17–19, 26–31]

Type of test (22q 11.2 region covered by test design)	Study	Type of technology	Study type	Type of prenatal population	Total analyzed cases	Sample type	Performance specifications, %	Comments
Targeted (A–D region)	Wapner et al. [26]	SNP based	Analytical validation	Controlled plasma and artificial samples	443	43 artificial samples with 22q11.2DS	Analytical detection: 100 (91.8–100)	Performance referred to 3 Mb deletion
						65 artificial samples without 22q11.2DS	Specificity: 100 (94.4–100)	
						3 plasma samples with 22q11.2DS	DR: 66.7 (20.8–93.9)	
						332 plasma samples without 22q11.2DS	Specificity: 99.1 (97.4–99.7)	
	Ravi et al. [27]	SNP-based enhanced ^a	Analytical validation	Controlled plasma samples	419	10 plasma samples with 22q11.2DS	DR: 90 (55.5–99.8)	Performance referred to 3 Mb deletion In 99 unaffected cases with the fetal fraction of 2.8–6.5%, risk evaluation was performed only for the presence or absence of the paternally inherited haplotype Specificity does not include samples reported as “risk unchanged” or “no- call” (4.6%)
						409 plasma samples without 22q11.2DS	Specificity: 99.74 (98.6–99.99) Projected PPV: 19.6 (assuming a prevalence of 1/1,442 of A-D deletion in the general population)	
	Martin et al. [17] (including Gross et al. [31])	SNP based	Retrospective, registry-based clinical study	General (high + low risk)	80,449	Plasma samples	General population: FPR: 0.3 (0.2–0.4) PPV: 15.7 (8.5–54.4)	No result or result of “risk unchanged” rate: 6.85 and 3.75%, respectively; not structured to calculate the DR NPV
							Low-risk population: PPV: 6.4	
							High-risk population: PPV: 82.6	
	Schmid et al. [18]	DANSR ^c	Analytical validation	Controlled plasma and artificial samples	1,953	122 artificial samples with 22q11.2DS 1,614 plasma samples from presumed unaffected pregnancies	Analytic detection: 75.4 (67.1–82.2)	159/289 (55%) cases with a high-risk result had a follow-up with molecular testing: 24 were fetal and 6 were maternal in origin 1.96–3.25 Mb deletions tested
		Clinical verification				7 plasma samples with 22q11.2DS 210 plasma samples without 22q11.2DS	DR: 71.4 (35.9–91.8)	Assessment of 22q11.2 del performed with FISH or microarray

Table 3 (continued)

Type of test (22q 11.2 region covered by test design)	Study	Type of technology	Study type	Type of prenatal population	Total analyzed cases	Sample type	Performance specifications, %	Comments
Targeted (A–C region)	Neofytou et al. [28]	TACS ^d	Analytical validation	Artificial samples 53	3 artificial samples with 22q11.2DS 50 artificial samples without 22q11.2DS	3 artificial samples with 22q11.2DS	Analytical detection: 66.7 (20.8–93.9) Specificity: 100 (92.9–100)	Performances referred to 3 Mb deletion
	Koumbarris et al. [29]		Clinical verification	Controlled plasma samples	2,033	3 plasma samples with 22q11.2DS 2,030 plasma samples without 22q11.2DS	DR: 100 (43.8–100) Specificity: 100 (99.8–100)	Assessment of 22q11.2 del performed by microarray
	Helgeson et al. [30]	MPSS ^f	Retrospective, registry-based clinical study	High risk (malformed)	175,393	Plasma samples	PPV: 96.9–100 (82.0–100)	Unknown no result rate; false negatives of 2.2 Mb in size; 23/32 (72%) cases with high-risk result had a follow-up with molecular testing: 2 were maternal, 9 fetal, and 12 both maternal and fetal
Liang et al. [19]								
		MPSS enhanced ^g	Prospective clinical study	General second trimester (high + low risk)	94,085	Plasma samples	PPV: 92.9 (68.5–98.7) FPR: 0.001 (0–0.006) DR: 86.7 (62.2–96.3)	Clinical examination of all newborns and molecular follow-up of low-risk cases with fetal/newborn anomalies; 1 high-risk case confirmed as maternal only not included in the PPV/FPR calculation; 1 high-risk case without clinical manifestations at birth and without molecular follow-up considered as TP; 4/13 TP cases with US scan anomalies, for the remainders, US scan information is not available, but 8/9 opted for TOP

cfDNA, cell-free DNA; DANSR, Digital Analysis of Selected Regions; TACS, Target Capture Sequences; MPSS, massively parallel shotgun sequencing; PPV, positive predictive value; FISH, fluorescence in situ hybridization. ^a Increased #SNPs in the 22q11.2 A–D region, increased positive confidence call threshold, and reflex sequencing at a higher depth of read (~14 million reads/sample) of high-risk cases. ^b Increased positive confidence call threshold and standard depth of read of ≥3.2 million reads/sample and reflex sequencing at a higher depth of read (≥6 million reads/sample) of high-risk cases. ^c DANSR. ^d Targeted capture enrichment by TACS. ^e Whole chromosome 22. ^f MPSS. ^g Increased read depth with 10–19 million uniquely mapped reads.

among 390 unaffected pregnancies [27]. In a large retrospective study including >80,000 samples, the false-positive rate of a SNP-based test was 0.1–0.3%. In this study, reflex testing of high-risk 22q11.2DS cases with an increased sequencing depth decreased the false-positive rate to 0.07%. Unfortunately, in the high-risk group, in about half of the cases (159/289), no outcome was available [17]. Within the latter group, there were 24 affected fetuses and 6 affected mothers [17]. Schmid et al. [18] used the DANSR assay probe design and examined 1,736 artificial and 217 real samples including 122 and 7 cases with 22q11.2DS and 1,824 presumed or known unaffected samples and reported on a combined detection rate of 75.2% (67.1–81.8) and a specificity of 99.6% (99.1–99.8). Neofytou et al. [28] used a TACS-based assay and examined 3 and 50 artificial samples from affected and unaffected pregnancies. They reported that 2 of the 3 cases were correctly identified and no false-positive results. Koumbaris et al. [29] described the clinical verification with the same technology including 3 and 2,030 controlled plasma samples with and without 22q11.2DS, respectively. They reported a sensitivity and a specificity of 100% (43.8–100; 99.8–100, respectively). Helgeson et al. [30] retrospectively reviewed the results of >175,000 genome-wide examinations in high-risk pregnancies. There were 32 abnormal examinations for 22q11.2DS, and in 23 and 8 cases, the results were either confirmed in the mother or the fetus or at least clinically suspected. The incidence of fetal 22q11.2DS was threefold lower than with a SNP-based targeted technology (0.01%, 24/80 449 vs. 0.03%, 21/175 393), strongly suggesting under-ascertainment by MPSS [4]. Liang et al. [19] prospectively analyzed >94,000 second trimester pregnancies from the general population with a genome-wide test. They performed a clinical examination of all newborns, and low-risk cases with fetal/newborn anomalies underwent molecular follow-up. They reported a high sensitivity of 86.7% (62.2–96.3). The incidence of fetal 22q11.2DS is 1/7,200 (13/94,000), which is far from the 1/1,000 prevalence in an unselected prenatal population [16]. This supports the evidence that the high sensitivity might be inflated by undetected cases with subtle/absent clinical signs at birth. They also reported a false-positive rate of 0.001% (0–0.006) and a high PPV of 92.9 (68.5–98.7). In this population, pregnant women underwent a routine fetal ultrasound scan at 18 weeks' gestation. Four of 13 TP cases showed malformations compatible with 22q11.2DS; therefore, the inclusion of cases with US scan anomalies might have inflated the PPV.

In summary, the results of previous studies are in line with our results. However, our study is the first to report on the false-positive rate and the test failure rate at 11–13 weeks in a prospective clinical study design with a detailed clinical follow-up of the entire study population, which included at least 6 pediatric examinations to investigate features consistent with 22q11.2DS.

We can only speculate about the origin of the 3 false-positive results. We have used FISH and microarray analysis for pre- and postnatal follow-up testing and, in all cases, found normal results. In general, potential reasons for discordant results between the cfDNA analysis and the fetal genetic examination may be technical issues, maternal 22q11.2DS, feto-placental mosaicism, absence of heterozygosity, and a dichorionic twin pregnancy with a vanishing twin [4,9,32]. A recent study performing a systematic analysis of copy number variations associated with early pregnancy loss showed that 22q11.2DS was the submicroscopic imbalance most frequent in miscarriage. Therefore, it was considered to be associated with early pregnancy loss, possibly due to major cardiac malformations incompatible with pregnancy progression and development [33]. While vanishing twin was a reason for exclusion in our study, cases may be missed if the gestational sac can no longer be identified. Still, they can be the origin of discordant results [25].

Although from a technical standpoint it would be easy to add further conditions to the spectrum of cfDNA screening, scientific societies do not endorse antenatal cfDNA screening for 22q11.2DS so far. The International Society for Prenatal Diagnosis (ISPD) recommends that cfDNA screening should be limited to clinically significant disorders or well-defined severe conditions where the test performance of the screening test is well established [34]. Because 22q11.2DS shows variable expressivity, one could argue that the prenatal diagnosis may produce more harm than good as the severity of the phenotype in the newborn cannot be predicted. However, there are several potential benefits of antenatal screening for this condition. Despite the variable expressivity, the syndrome shows full penetrance, meaning that all 22q11.2DS patients have clinical manifestations. Currently, the majority of fetuses with 22q11.2DS are identified by genetic diagnosis after the detection of fetal anomalies, usually cardiac defects, in the second trimester or later. Screening for 22q11.2DS in the first trimester would provide the possibility of anticipatory care and early diagnosis for neonatal complications [7,9,35,36]. The spectrum of 22q11.2DS involves more than antenatally detectable de-

fects. For example, hypocalcemia and subsequent neonatal seizures, dysphagia, and immune defects will certainly remain undetected before birth and may require neonatal intervention [36,37]. As such, perinatal management and the clinical outcome can be significantly improved with the knowledge of the microdeletion and transition of care to a pediatric specialist. Although, to the best of our knowledge, there is no study comparing the outcome of children with 22q11.2DS with and without antenatal diagnosis, one can speculate that the knowledge of 22q11.2DS before birth would reduce the associated morbidity and mortality [9].

Clear strengths of our study are the prospective design, its initiation by a prenatal medicine team and not by a laboratory, the detailed follow-up examination of the screen-positive pregnancies, the high outcome rate of all screen-negative pregnancies, and the detailed ultrasound examination that was carried out prior to the screening test. The monocentric setup of our study can be considered as the most relevant weakness. Also, for the purpose of this study, we included high-risk pregnancies but grouped them separately.

Conclusion

In summary, the addition of 22q11.2DS to first trimester cfDNA screening for common trisomies with a targeted cfDNA approach is feasible and does not substantially increase the false-positive rate of the test. Awareness and education around the clinical relevance of 22q11.2DS should be improved.

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Statement of Ethics

The research presented in the article was conducted ethically in accordance with the World Medical Association Declaration of Helsinki and the appropriate guidelines for human studies, as well as according to animal welfare regulations, including the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines, and was approved by the Ethics Committee at the University Hospital of Tübingen, Germany (October 18, 2017; No. 644/2017BO1). The study was registered in the clinicaltrials.gov registry (NCT03375359). Written consent was obtained from all patients who participated in the study.

Conflict of Interest Statement

Maximilian Schmid is an employee of Roche Sequencing Solutions Inc. Francesca Romana Grati, Livia Marcato, and Barbara Malvestiti are full-time employees of TOMA Laboratory without ownership shares. F.R. Grati is an expert panel member for Roche and Menarini Biomarkers. The unnamed authors have no disclosures.

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Author Contributions

K.O.K.: conception, planning, carrying out, analyzing, and writing. M.H.: carrying out. T.P.: carrying out and analyzing. N.P.: carrying out. P.W.: carrying out. M.S.: planning. A.D.: planning, carrying out, and writing. U.M.H.: carrying out. S.B.: planning. L.M.: carrying out. B.M.: carrying out. F.R.G.: planning, carrying out, analyzing, and writing.

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