

ORIGINAL ARTICLE

**ANTENATAL DETECTION OF CHROMOSOMAL ABNORMALITIES
COMBINING QF-PCR AND CYTOGENETIC ANALYSIS**

Ana Vicic^{1,2}, Vedrana Skaro^{3,4}, Petar Projic^{3,4}, Petra Korac⁵, Romana Gjergja-Juraski^{4,6}, Feodora Stipoljev^{1,4}

Abstract

Aim: To compare the diagnostic values and limitations of quantitative fluorescent polymerase chain reaction (QF-PCR) and conventional cytogenetic analysis in prenatal diagnosis of chromosomal abnormalities.

Methods: A prospective study included simultaneous QF-PCR and cytogenetic analysis of 133 prenatal samples routinely obtained by amniocentesis or chorionic villus sampling (CVS). Additionally, QF-PCR analysis was performed on 14 tissue samples collected after termination of pregnancy (TOP) for which karyotyping could not be performed due to culture failure.

Results: Among 133 analyzed prenatal samples, chromosomal abnormalities were diagnosed in 12 cases (9%), including 10 cases of numerical chromosomal aberrations and two cases with unbalanced structural rearrangements. Nine out of 12 chromosomal abnormalities were also detected with QF-PCR. However, all cases of major aneuploidies were successfully disclosed with QF-PCR, resulting in 100% detection rate for chromosomes 21, 18, 13, X and Y. Using a set of markers specific for chromosomes 21, 18 and 13, QF-PCR analysis of tissues collected after TOP revealed chromosomopathy in 21.4% of cases (two cases of trisomy 18 and one triploidy). A comparison of STR markers confirmed monozygosity in two monochorionic/diamniotic twin pregnancies.

Conclusion: QF-PCR has been shown as a rapid and reliable method for prenatal diagnosis of the most common chromosomal aneuploidies, and as an adequate alternative to conventional karyotyping in cases where cytogenetic analysis is not possible due to failure of culturing process. However, conventional cytogenetics still presents a gold standard for the detection of structural aberrations and rare aneuploidies.

¹*Cytogenetic Laboratory, Department of Obstetrics and Gynecology, Clinical Hospital "Sveti Duh", Zagreb, Croatia*

²*Univesity of Applied Health Sciences, Zagreb, Croatia*

³*Genos Ltd., DNA Laboratory, Zagreb, Croatia*

⁴*Faculty of Medicine, University J.J. Strossmayer of Osijek, Osijek, Croatia*

⁵*Division of Molecular Biology, Department of Biology, Faculty of Science, University of Zagreb, Zagreb, Croatia*

⁶*Children's Hospital Srebrnjak, Zagreb, Croatia*

Corresponding author:

Ana Vicic
Cytogenetic Laboratory, Department of Obstetrics and Gynecology,
Clinical Hospital "Sveti Duh", Sveti Duh 64, 10000 Zagreb, Croatia
Tel: +385 1 3712 273
Fax: +385 1 3745 534
E-mail: vicic.ana@gmail.com

Submitted: January, 2020

Accepted: April, 2020

Key words: chromosomal abnormalities, cytogenetic analysis, miscarriage, prenatal diagnosis, QF-PCR

INTRODUCTION

Chromosomal abnormalities have been ascertained as the most common cause of intellectual disability¹ and are associated with approximately 15% of major congenital anomalies in the human population.² According to EUROCAT data, a total prevalence of unbalanced chromosomal aberrations of 43.8 per 10000 births was recorded in Europe for the period 2000-2006, while trisomies 21, 18 and 13 together with triploidy and sex chromosome aneuploidies accounted for 86% of all registered chromosomopathies.³

Over the last few decades, prenatal diagnosis of chromosomal abnormalities has been routinely performed as part of obstetric management of high-risk pregnancies, assessed by noninvasive screening methods. Conventional cytogenetic analysis of cultured fetal cells obtained by one of the invasive procedures, i.e. amniocentesis or chorionic villus sampling (CVS), is still considered the gold standard as a method that enables the detection of numerical aberrations, as well as unbalanced and balanced rearrangements of all chromosomes. However, the method is labor-intensive, expensive and, most importantly, time-consuming, with an average reporting time of 10 to 14 days. Parental anxiety due to the extended wait for results and the necessity of rapid diagnosis in advanced pregnancies and those with a particularly high risk of chromosomal abnormalities have prompted the implementation of techniques that enable faster diagnosis assessment. Quantitative fluorescent

polymerase chain reaction (QF-PCR) was firstly introduced in the early 1990s⁴, and it has been proven as an economic, simple and reliable method with the high specificity and sensitivity for detection of the most common aneuploidies. The technique is based on the amplification of highly polymorphic DNA sequences (short tandem repeats, STR) located on target chromosomes, followed by relative quantification of amplified markers. As a result of the avoidance of culturing process, results are mostly observed within 24 hours.⁵⁻⁷

A number of recent studies and guidelines have been directed towards the ascertainment of the most appropriate approach regarding the effectiveness and cost efficiency of methods used for prenatal diagnosis of chromosomal abnormalities. Since its implementation, QF-PCR has been performed in combination with conventional karyotyping as a rapid test for the detection of major aneuploidies. Soon after, the question of whether it could be used as a stand-alone method was raised. Therefore, two different strategies have been proposed. In one, the choice between QF-PCR and full karyotype analysis is given to women with no increased risk of a structural chromosomal abnormality, while, according to more commonly reported guidelines, rapid testing is performed on all prenatal samples, followed by conventional cytogenetics in those with observed fetal ultrasound anomalies or a familial history of chromosomal rearrangements.^{8,9}

The aim of this study was to compare the diagnostic values and limitations of both QF-PCR and conventional cytogenetic analysis in prenatal diagnosis of chromosomal abnormalities.

MATERIAL AND METHODS

A prospective study encompasses the analysis of 133 prenatal samples from 128 women (five twin pregnancies), routinely referred for prenatal diagnosis due to advanced maternal age (≥ 35 years), the presence of the abnormal ultrasound finding, a positive maternal serum screening test, or other factors associated with an increased risk of chromosomal abnormalities (personal/family history of chromosomal abnormality, recurrent miscarriages). Only those cases analyzed by both QF-PCR and conventional karyotyping were included in the study. Abnormal ultrasound findings detected at the first-/ second-trimester examination included various major abnormalities and minor/soft markers associated with aneuploidies. All patients received genetic counseling, including information about advantages and limitations of the invasive procedure and methods used for chromosomal analysis; routine written consent was obtained prior to the invasive procedure. Ethical approval was acquired from institutional Ethics committee of Clinical Hospital "Sveti Duh", Zagreb.

Between 15 and 20 ml of amniotic fluid was obtained by amniocentesis; 2-3 ml was designated for the QF-PCR analysis and the rest was used for a routine cell culturing. CVS was performed transabdominally, and at least 15-20 mg of chorionic villi was acquired. A short-term cytotrophoblast and mesenchymal stroma cultures were set up, and the remainder of the sample was used for DNA isolation.

The study also included 14 tissue samples obtained after termination of pregnancy (TOP) for which routine cytogenetic analysis could not be performed due to culture failure, and solely the QF-PCR analysis was carried out on the DNA isolated from fetal skin samples. The indications for chromosomal analysis were presence of the fetal malformations, recurrent miscarriage (three or more consecutive miscarriages) or fetal loss in the second trimester of pregnancy.

QF-PCR analysis

Chelex 100 method (Bio-Rad Laboratories Inc, Hercules, CA) was used to extract DNA from all samples, following the protocol described in the user's manual.¹⁰ In very few cases when slightly bloodstained pellet was observed after amniotic-fluid centrifugation, two-step red cell lysis and water wash was performed followed by centrifugation for 5 minutes at 13.000 rpm. In these cases, as additional precautionary step, maternal buccal swab sample was tested in parallel with the amniotic fluid samples.

For the first 115 samples, QF-PCR analysis was performed as described in the previously published article.¹¹ Details of primers used in the QF-PCR multiplex are shown in Table 1.

Lyophilized primers (10 nM) were ordered from Applied Biosystems (Cheshire, UK) and 100 μ M stock primer solution was prepared by adding 100 μ L of TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0) to each forward and reverse primer. Primer mix for the full assay was prepared in the total volume of 50 μ L, and for each of the back-up assays the total volume of primer mix was 25 μ L. Primer concentrations are given in Table 1. Primer mix was tested, aliquoted and stored at 20°C. To prepare the PCR reaction mix, 2x QIAGEN Multiplex PCR Master Mix (Hilden, Germany) was used. The PCR reaction mix was prepared in the total volume of 15 μ L, respecting the primer mix ratio as noted in QIAGEN Multiplex PCR Handbook 10/2010¹². As recommended in the previously published article¹¹, but proportionally adjusted to the reduced volume of PCR reaction, 6 μ L of DNA template was added to the PCR reaction mix.

Microsatellite loci on chromosomes 13, 18 and 21 were amplified in a single-tube assay with the following PCR reaction conditions: initial denaturation was set up at 95°C for 15 minutes, followed by 25 cycles of denaturation at 94°C for 30 seconds, annealing step at 57°C for 1 minute 30 seconds, and elongation at 72°C for 1 minute 30 seconds. The final elongation step was

Table 1. Details of primers used in the in-house QF-PCR multiplex reaction.

Marker	Location	Size range (bp)	Primer sequence 5' - 3'	Concentration / primer mix (µM)		Final concentration / PCR reaction (µM)	
				Full assay (50 µl)	Back-up assay (25 µl)	Full assay (50 µl)	Back-up assay (25 µl)
D13S305	13q13.3	430-465	(F) HEX-GCCTGTTTGAGGACCTGTCGTTA (R) TGGTTATAGAGCAGTTAAGGCAC	3	2	0.30*	0.20
D13S628	13q31.1	425-470	(F) NED-TAACATTCATTGTCCCTTACAGAT (R) GCAAGGCTATCTAACGATAATTCA	8	2	0.80*	0.20
D13S634	13q21.33	385-440	(F) 6-FAM-GGCAGATTCAATAGGATAAAATAGA (R) GTAACCCCTCAGGTTCTCAAGTC	1.5	2	0.15*	0.20
D13S742	13q12.12	235-315	(F) HEX-ATAACTGGGCTAGGAATGGAAATA (R) GACTTCCCAATTCAGGAGGACT	2	NA	0.20	NA
D18S978	18q12.3	180-220	(F) NED-GTAGATCTTGGGCTATTGTCAGA (R) GTCTCCCATGGTCACAATGCT	4	2	0.40*	0.20
D18S386	18q22.1	330-400	(F) HEX-TGAGTCAGGAGAATCACTTGGAAC (R) CTCTCCATGAAGTAGCTAAGCAG	2	2	0.20	0.20
D18S499	18q22.1	390-410	(F) NED-AGATTACCCAGAAATGAGATCAG (R) GAAAATGTAGAAGTGAGTCACCT	6	2	0.60*	0.20
D18S391	18p11.31	140-180	(F) HEX-GGACTTACCACAGGCAATGTGACT (R) CTGGCTAATTGAGTTAGATTACAA	1	2	0.10*	0.20
D18S535	18q12.3	455-500	(F) 6-FAM-CAGCAAACCTTCATGTGACAAAAGC (R) CAATGGTAACCTACTATTACGTC	1.5	2	0.15*	0.20
D21S11	21q21.1	225-280	(F) 6-FAM-TTTCTCAGTCTCCATAAATATGTG (R) GATGTTGTATTAGTCAATGTTCTC	2	2	0.20	0.20
D21S1270	21q22.11	285-340	(F) 6-FAM-CTATCCCCTGATTATTACAGGGC (R) TGAGTCTCCAGGTTGCAGGTGACA	2	2	0.20	0.20
D21S1411	21q22.3	256-340	(F) ATAGGTAGATACATAAATATGATGA (R) NED-TATTAATGTGTGCTCTCCAGGC	4	2	0.40*	0.20
D21S1435	21q21.3	160-200	(F) 6-FAM-CCCTCTCAATTGTTGTCTACC (R) ACAAAGGAAAGCAAGAGATTCA	8	2	0.80*	0.20
D13S252	13q12.1	270-320	(F) 6-FAM-GCAGATGTACTGTTTTCCTACCAA (R) AGATGGTATATTGTGGGACCTTGT	NA	2	NA	0.20
D13S762	13q31-q32	270-320	(F) HEX-AATGAGATTGCTGGGTCAGA (R) HEX-AAT GAG ATT GCT GGG TCA GA	NA	2	NA	0.20
D18S1002	18q11-q11	340-370	(F) 6-FAM-GTT TGA TGG GAG GAA GCT ATC TAT (R) GTG AAG TAG CGG AAG GCT GTA AT	NA	2	NA	0.20
IFNAR	21q22.1	370-410	(F) NED-CATTTGATCTTAGCCATCTATTGC (R) ACTATGCAGCCATTGAAAGACTA	NA	2	NA	0.20
D21S226	21q22.1	440-470	(F) 6-FAM-GCAAATTTGTGGATGGGATTAACAG (R) AAGCTAAATGTCTGTAGTTATTCT	NA	2	NA	0.20

Legend: The markers are amplified in three chromosome-specific assays. Marker and primer information are given in Mann K, Donaghue C, Fox SP, Docherty Z, Ogilvie CM. Strategies for the rapid prenatal diagnosis of chromosome aneuploidy. *European Journal of Human Genetics*. 2004;12:907-915. Primer mix for full assay was prepared in total volume of 50 µl while the primer mix of each back-up assay was 25 µl. Total volume of PCR reaction was 15 µl.

*For each back-up assay, final concentration of each primer in 15 µl PCR reaction volume was 0.2 µM.

NA - not applicable

set up at 72°C for 20 minutes. A total of 13 STR loci were amplified simultaneously when the Full Assay was used. Markers used for the detection of chromosome 21 are located in the Down syndrome critical region (21q22.1–21q22.3), except of D21S11 which is located near the centromere. Four markers on chromosome 13 and five on chromosome 18 are distributed along each chromosome in order to increase the likelihood of detecting unbalanced chromosome rearrangements. In cases where two or more of the Full Assay markers on any one of these chromosomes were found to be uninformative (homozygous), the back-up sets were used. The chromosome-specific back-up sets were also used to confirm any abnormal results, as well as in cases when maternal sample was tested in parallel. This was done in order to compare STR profiles and confirm or exclude the fetal origin of the predominant cell population if a slightly bloodstained pellet was observed in amniotic fluid sample. In each

chromosome-specific set, almost all markers amplified in Full Assay were repeated in order to confirm sample identity. There were no cases when additional markers were mostly homozygous. Since there were no cases in which, of total markers tested for each chromosome, more than two markers were uninformative, all detected chromosomal abnormalities were confirmed. Otherwise, such results would be considered inconclusive. All markers are shown in Table 2.

For 32 samples, DNA fragments were amplified using the Aneufast™ QF-PCR Kit (Genomed AG, Wollerau Switzerland), as described in the user's manual.¹² These samples were received at the point when the *in-house* kit was no longer in use because we had already switched to the abovementioned commercially available kit. Two sets of markers multiplexes S1 and S2 enabled the simultaneous amplification of five STR loci on each of the autosomes 13, 18 and 21; in addition to three pseudoautosomal (DXYS267, X22

Table 2. In-house set of markers used for rapid diagnosis of trisomies 13, 18 and 21 used for analysis of 115 samples.

Full Assay marker set	Chromosome-specific back-up marker sets		
	Assay 13	Assay 18	Assay 21
D13S305	D13S305	D18S978	D21S11
D13S628	D13S628	D18S386	D21S1270
D13S634	D13S634	D18S499	D21S1411
D13S742	D13S252*	D18S391	D21S1435
D18S978	D13S762*	D18S535	IFNAR*
D18S386		D18S1002*	D21S226*
D18S499			
D18S391			
D18S535			
D21S11			
D21S1270			
D21S1411			
D21S1435			

Legend: * - extra markers not included in Full Assay

and DXYS218) and one X-linked STRs, as well as two non-polymorphic sequences, Amelogenin (AMXY) and SRY (amplified for sex determination). The chromosome-specific back-up sets were also used in cases as described earlier. All markers are shown in Table 3.

Amplified fragments were separated by capillary electrophoresis on genetic analyzer 3130 (Applied Biosystems, Foster City, CA, USA). Fragment size was determined by internal size standard by GeneMapper®ID-X software (Applied Biosystems, Foster City, CA, USA). Results were interpreted in accordance to the user's manual.¹³ STR marker

analysis was used for determination of zygosity in twin pregnancies.

Cytogenetic analysis and FISH

Cytogenetic analysis of prenatal samples was performed on cultured amniocytes or short-term cytotrophoblast/mesenchymal stroma cultures following European Cytogeneticists Association guidelines.¹⁴ Clinically significant chromosomal abnormalities were considered to be those associated with high or uncertain risk of adverse clinical outcome, including aneuploidies of all chromosomes, unbalanced structural aberrations and marker chromosomes.¹⁵ Inherited balanced translocations and inversions were considered as chromosomal aberrations with no or low risk of adverse outcome. For parental karyotyping, a short-term phytohemagglutinin-stimulated whole blood culturing was used. Fluorescence *in situ* hybridization (FISH) was carried out with commercially available DiGeorge region probes (N25, TUPLE, TBX1), satellite enumeration probe D14Z1/D22Z1, SHANK3 (Kreatech FISH probes, Leica Biosystems, Nussloch, Germany), and *BCR* locus specific probe (Vysis, Abbott Laboratories, Illinois, U.S.A.), according to manufacturer's protocols.

Statistical analysis

Comparisons for categorical variables were made using Fischer's exact test, and for continuous variables between two groups using the Mann-Whitney *U*-test. $P \leq 0.01$ was considered statistically significant. Statistical analysis was carried out using R programming language (version 3.2.0).

Table 3. Markers amplified with the Aneufast™ QF-PCR Kit used for analysis of additional 32 samples.

S1	S2	MXY	M21	M18	M13
AMXY	SRY	SRY	D21S1411	D18S386	D13S631
DXYS267	X22	AMXY	D21S1435	D18S391	D13S634
D21S1414	DXYS218	HPRT	D21S1437*	D18S858*	D13S742*
D21S1446	HPRT	TAF9L*	D21S1412*	D18S499*	D13S628*
D21S1442	D21S1411	DXYS156*	D21S1809*	D18S1002*	
D18S535	D21S1435	SBMA*			
D18S391	D13S634	DXS6803*			
D18S976	D13S258	DXS6809*			
D13S797	D18S386	DXS8377*			
D13S631	D18S390				
D13S305					

Legend: * - extra markers not included in S1 and S2

RESULTS

Indications for referrals and maternal age distribution

A total of 147 samples were analyzed, while simultaneous QF-PCR and cytogenetic analysis were performed for 131 amniotic fluid and two chorionic villus samples, and QF-PCR only was performed for all 14 tissue samples collected after TOP. The indications for invasive prenatal diagnostic procedures of all 128 pregnant women (including five twin pregnancies) are given in Table 4. QF-PCR analysis of fetal tissue samples collected after TOP was performed due to the presence of the fetal malformations in 57.2% of cases, recurrent miscarriages in 21.4% or because of fetal loss in the second trimester of pregnancy in 21.4% of cases. Among all cases, an abnormal first-/second-trimester ultrasound scan was observed in 52 fetuses (33.1%). The mean maternal age was 34.9 years (range 18 - 47 years), without a statistically significant difference between the group of women who underwent invasive procedure and those who suffered spontaneous loss of pregnancy (mean maternal age of 35.1 years vs. 33 years) ($P=0.1$, Mann-Whitney U -test). Furthermore, mean maternal age of 35.5 years (range 26 - 46 years) was observed within the group with diagnosed chromosomal abnormality, similarly to those receiving normal reports (mean 34.8 years, range 18 - 47). The mean gestational age at the time of diagnosis was 17.3 and 17 weeks for samples obtained by invasive procedure and those collected after TOP, respectively.

Karyotyping and QF-PCR results

A total of 12 clinically significant chromosomal aberrations were revealed by cytogenetic analysis of 133 prenatal samples, including 10 cases of numerical chromosomal abnormalities and two cases with unbalanced structural rearrangement (Table 5). Using an *in-house* designed set of STR markers for chromosomes 21, 13 and 18 for QF-PCR analysis of the first 101 cases, and the Aneufast commercial kit for the following 32 samples, four cases of trisomy 21 and one triploidy (Figure 1) were detected. Since an *in-house* method was used, four cases of sex chromosome aneuploidies remained undisclosed; however, all four cases were subsequently evaluated and confirmed with the Aneufast kit. Therefore, a QF-PCR detection rate of 100% was recorded for the most common aneuploidies. There was no difference in the detection rate between the two sets of markers used, other than the detection of sex chromosome abnormalities. In case of 45,X/46,XX mosaicism, a QF-PCR analysis also indicated abnormal karyotype, since the percentage of 45,X cell line was 90%. Overall, nine out of 12 chromosomal abnormalities (75%) were detected with QF-PCR.

Table 4. Indications for invasive diagnostic procedure in a group of women with diagnosed chromosomopathy and those with normal results.

Indication	Karyotype		
	Normal N (%)	Abnormal N (%)	Total N (%)
Maternal age alone	36 (31.0)	1 (8.3)	37 (28.9)
Ultrasound anomaly*	29 (25.0)	8 (66.7)	37 (28.9)
Maternal serum screening*	27 (23.3)	1 (8.3)	28 (21.9)
Maternal serum screening and ultrasound anomaly*	4 (3.4)	2 (16.7)	6 (4.7)
Other	20 (17.3)	0	20 (15.6)
Total	116 (100)	12 (100)	128 (100)

Legend: N – number of cases

Two cases of unbalanced structural rearrangements and one autosomal trisomy, i.e. clinically significant chromosomal aberrations, have remained undisclosed by QF-PCR analysis (Table 5). In the first case a 41-year-old woman, G2P1, underwent amniocentesis at 16 weeks of pregnancy due to advanced maternal age alone. Fetal ultrasound was unremarkable, and she had one healthy child. A mosaicism for supernumerary marker chromosome (SMC) was diagnosed by conventional karyotyping. FISH analysis showed a

Table 5. Chromosomal abnormalities detected by cytogenetic and QF-PCR analysis of 133 prenatal samples (amniotic fluid and chorionic villi samples) and 14 tissue samples collected after TOP.

	Cytogenetics	QF-PCR
CHA detected among prenatal samples	12	9
Trisomy 21	4	4
Triploidy	1	1
45,X	2	2
45,X (90%)/46,XX (10%)	1	1
47,XXX	1	1
47,XX,+mar.ish der(22)(D22Z1+,N25-,TUPLE-,TBX1-,BCR-,SHANK-)/46,XX	1	-
46,XY,der(4),t(1;4)(q23;p15.2)mat	1	-
47,XX,+9	1	-
CHA detected among tissue samples collected after TOP*	-	3
Trisomy 18	-	2
Triploidy	-	1

Legend: CHA - chromosomal abnormalities; TOP - termination of pregnancy; * - Cytogenetic analysis could not be performed due to culture growth failure

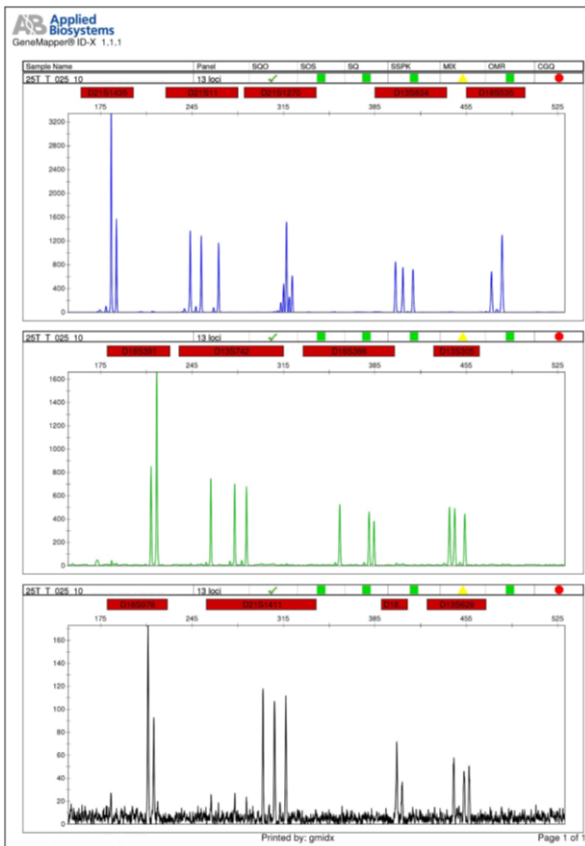


Figure 1. QF-PCR detection of triploidy using Full assay with chromosome 21, 18 and 13 specific markers. All 13 markers used were informative and showed either diallelic or triallelic trisomic patterns.

chromosome 22 origin of SMC, comprising only the short arm and pericentromeric region, whilst it was negative for the DiGeorge region (3 probes), the BCR region and the control probe region 22q13 (Figure 2). A mos 47,XX,+mar.ish der(22)(D22Z1+,N25-,TUPLE, TBX1-,BCR-,SHANK3-)/46,XX karyotype was disclosed, with the percentage of cell line with SMC of 90%. Parental karyotyping revealed the maternal origin of SMC, and a healthy newborn was delivered at term. In the second case, a 19-year-old woman, G2P1, was referred to our Clinic at 14 weeks of pregnancy due to an ultrasound finding of fetal cystic hygroma and a combined test showing risk for T21 of 1:20, and T13/T18 of 1:5. A derivative chromosome 4 was detected by cytogenetic analysis of cultured chorionic villi. In order to ascertain the origin of the derivative chromosome, parental karyotypes were performed, disclosing a balanced reciprocal translocation 1q23;4p15.2 in the mother. Thus, the fetal karyotype was designated as 46,XY,der(4),t(1;4)(q23;p15.2)mat. After genetic counseling, the parents opted for the termination of pregnancy. The third case, a 41-old-women G2P1, was referred because of a high risk for T13/T18 (1:15) obtained by combined screening. CVS was performed at 13+4 weeks of gestation, and non-mosaic trisomy 9 (47,XX,+9) was disclosed. The pregnancy, however, ended in a spontaneous abortion.

In addition, cytogenetic analysis detected one case of balanced Robertsonian translocation of maternal origin (45,XX,dic(13;14)(p11.2;q11.2)mat).

Furthermore, QF-PCR analysis of tissues collected after TOP revealed two cases of trisomy 18 and one triploidy, which would otherwise not be detected since the karyotyping could not be performed due to culture growth failure. In summary, a total of 15 chromosomal abnormalities were revealed during the study, 12 detected by conventional karyotyping, and three additionally with QF-PCR. Aneuploidies involving chromosomes 21, 18, 13, X and Y accounted for 80% of all determined aberrations. Out of 133 samples obtained by invasive procedures chromosomopathies were detected in 9% of cases, in comparison with the detection rate of 21.4% revealed within samples collected after TOP ($P=0.01$). Furthermore, a significantly higher proportion of trisomy 18 cases were detected within samples collected after TOP (14.3% vs. 0% within invasive procedures, $P=0.01$).

Amniocentesis was performed in five twin pregnancies; in four cases the indication for prenatal diagnosis was ultrasound abnormality present in a single or in both twins, and one patient was referred because of a previous pregnancy with chromosomal abnormality. Normal karyotypes were obtained in all cases, while the comparison of STR markers confirmed monozygosity in two monochorionic/diamniotic cases.

DISCUSSION

As a rapid, cost-effective and reliable test for the detection of the most common aneuploidies, QF-PCR has been widely established as a part of routine prenatal diagnosis of chromosomal abnormalities, mainly performed together with conventional karyotyping. By combining QF-PCR and cytogenetic analysis, a total of 15 chromosomopathies were revealed in the present study, while aneuploidies of chromosomes 21, 18, 13, X and Y, together with triploidy accounted for 80% of detected aberrations, which is in concordance with the proportion of major aneuploidies observed in the general population.³

To date, a number of studies and reviews referred toward the determination of accuracy of QF-PCR have reported the detection rates for the non-mosaic major aneuploidies in a range of 98.6% to 100%, and the specificity of 100%.^{6, 16, 17} Comparably, all cases involving aneuploidies of chromosomes 21, 18, 13, X and Y were detected in our study, without false positive results. In contrast, the reported proportion of detected mosaics was approximately 60%, including only those with the percentage of abnormal cell line above 20-30%. The present study included only one case of mosaicism, which was also indicated by QF-PCR, since cell lines with monosomy X accounted for 90%. Although the inability of detection of low-level mosaics is stated as one of the main disadvantages of QF-PCR, a survey of Donaghue et al.¹⁸ showed a

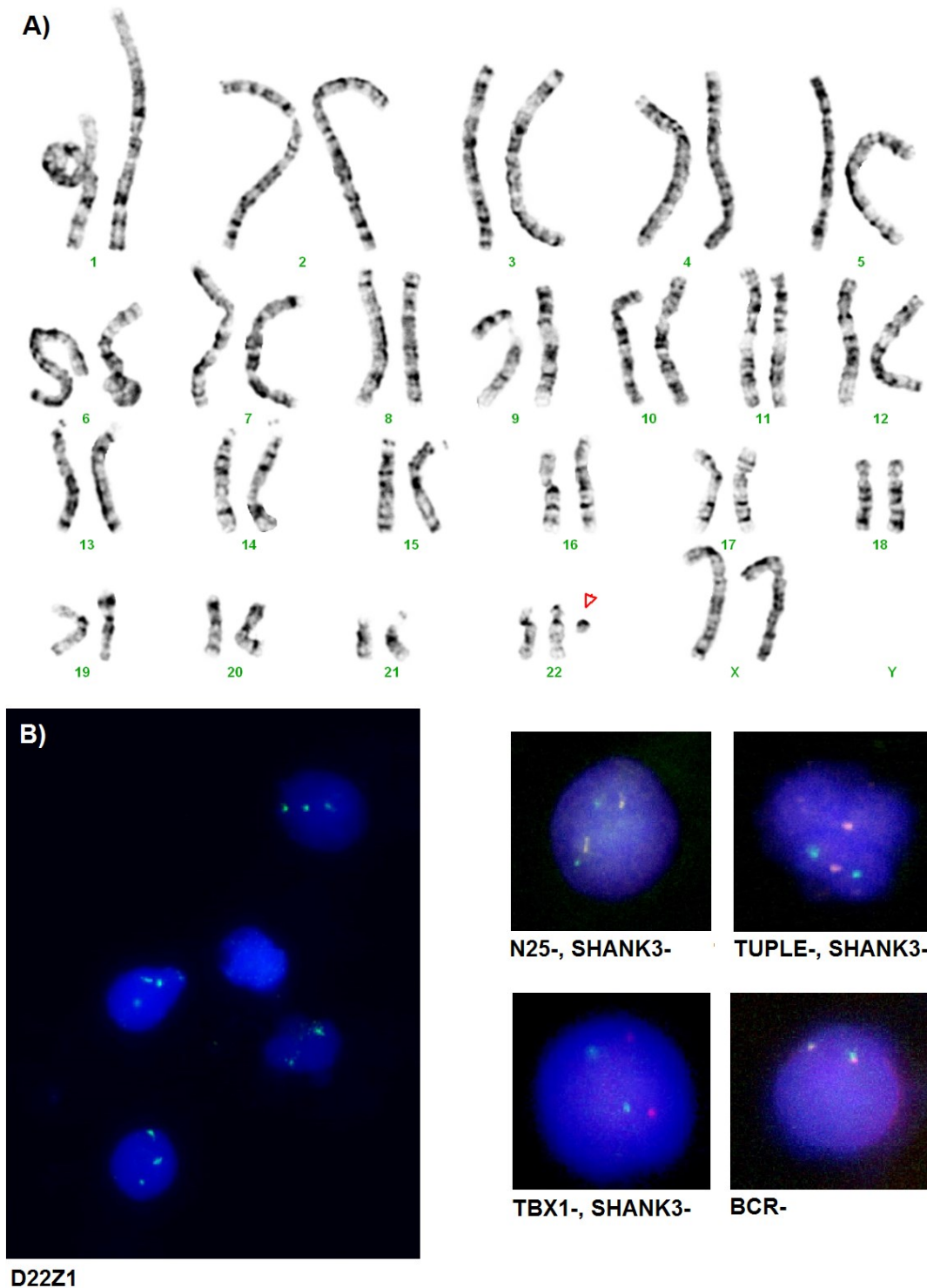


Figure 2. A) Karyogram of the fetus with supernumerary marker chromosome derived from chromosome 22. B) FISH analysis of SMC using different probes specific for chromosome 22: SE D22Z1 probe, DiGeorge specific region probes (N25, TUPLE, TBX1), BCR, and SHANK3 region probes.

discrepancy in mosaicism detection between QF-PCR and cytogenetic analysis of uncultured and cultured CVS and amniotic fluid samples. Out of 18 mosaic cases, 12 were revealed by QF-PCR and eight by cytogenetics. Although the finding of mosaicism in CVS could be confined only to placenta, mosaicisms detected in uncultured amniotic fluid samples are very

likely to represent a real fetal genotype. If we take into account that the culturing process could result in the overgrowth of a single cell line, QF-PCR analysis of uncultured samples may reflect more similar proportions with those present *in vivo*. Thus, a combined approach using both QF-PCR and cytogenetic analysis presents the best strategy for the

detection of mosaicism and the interpretation of the obtained results.

Of the 12 chromosomopathies observed among prenatal samples, three (25%) were undisclosed by QF-PCR. Undetected aberrations included trisomy 9, karyotype with supernumerary marker chromosome resulting in the normal phenotype, and derivative chromosome 4. In cases with poor prognosis, i.e. trisomy 9 and derivative chromosome 4, the indications for prenatal diagnosis were a positive combined screening test and abnormal ultrasound finding, respectively, highlighting the necessity of conventional karyotyping especially in pregnancies associated with a high risk of chromosomal abnormalities. In both cases with the structural aberrations, abnormalities were inherited from parents. These cases emphasize the value of classical cytogenetic analysis, not only for the disclosure of pathological karyotypes in the current pregnancy, but also providing genetic information to evaluate the risk of having offspring with chromosome aberrations in subsequent pregnancies.

Using an *in-house* set of markers specific for chromosomes 21, 18 and 13, sex chromosome aneuploidies could not be detected, and therefore three cases with the Turner syndrome (two non-mosaics and 45,X/46,XX mosaicism), and one case of 47,XXX remained undisclosed (Table 5). However, subsequent evaluation with Anefast kit indicated the existence of sex chromosome aneuploidies in all four cases. In some genetic laboratories, QF-PCR sex chromosome testing is performed only in pregnancies with evidenced ultrasound anomalies suggestive for the Turner syndrome. As stated, the diagnosis of aneuploidies such as 47,XXY, 47,XXX or 47,XYY is of debatable value anyway, since these are associated mainly with a normal or mild clinical phenotype.^{8, 19} However, a routine application of sex chromosome aneuploidy testing enables an accurate and rapid assessment of diagnosis, providing useful information for further pregnancy management and allowing parents more time to make a decision.

Several studies regarding the comparison of QF-PCR and conventional cytogenetics in the detection of clinically significant chromosomopathies, and potential usage of QF-PCR as a stand-alone method have been reported. Although according to the majority of those reports >90% of the clinically significant chromosomal abnormalities have been detected by QF-PCR^{6, 16, 17, 19-22}, some authors have reported that approximately 30% of such abnormalities stayed undisclosed with QF-PCR analysis.^{23, 24} The variability in reported results is primarily obtained due to differences in the indications for prenatal testing and determination of clinical relevance of diagnosed chromosomopathies. A proportion of 25% of clinically significant aberrations undetected by QF-PCR in our study could be explained by the high percentage of women undergoing the invasive procedure due to abnormal ultrasound findings (28.9%) or positive maternal serum screening test (26.6%) (Table 4). Considering the indication for

referrals, it has been reported that less than 7% of clinically significant abnormalities undetected by QF-PCR are observed within the group of women referred because of advanced maternal age alone^{17, 21, 25}, which was expected since those women have lower risk of chromosomopathies in comparison with cases when ultrasound anomaly or positive biochemical screening is found. In contrast, when ultrasound abnormality or positive biochemical screening are present, false negative results obtained by QF-PCR increase to an approximately 30%.^{17, 25, 26} The presence of ultrasound anomaly or higher risk obtained by biochemical screening are indicative for higher risk of chromosomopathy, and not only for the most common aneuploidies but also other chromosomal aberrations. For example, an ultrasound finding of cystic hygroma carries a risk of chromosomal abnormalities of approximately 50%, in comparison with the maternal age of 35 years in which the risk of Down syndrome is 1 in 246.^{27, 28} Thus, our study likewise emphasizes the necessity of karyotype analysis in cases with abnormal ultrasound findings and maternal serum screening tests. Although the possibility of replacement of the conventional karyotyping with QF-PCR for certain referrals has been investigated by a number of studies, conciliated conclusions have not yet been designated. To date, QF-PCR has been proven as a reliable, fast and cost-effective method for the detection of the most common aneuploidies; nevertheless, a proportion of clinically significant chromosomopathies could not be disclosed, as well as balanced rearrangements and some aberrations with no adverse outcome for the current pregnancy, raising a number of ethical questions. However, in some countries strategies based on rapid testing performed for all prenatal samples, followed by conventional cytogenetics in those cases with observed fetal ultrasound anomalies or a familial history of chromosomal rearrangements are implemented in the routine praxis.^{6, 8, 9} Using this approach, Hills et al.⁸ reported a proportion of 0,3% of chromosomal abnormalities misdiagnosed by QF-PCR, while cases with poor clinical outcome accounted for 0,069%. In contrast, in Croatia rapid QF-PCR testing is still not routinely performed as a part of prenatal diagnostics, and the guidelines toward its implementation have not yet been established. Therefore, this study could implicate the assessment of its utility as a standard procedure. Furthermore, misdiagnosed cases observed in the present study highlight the importance of full karyotyping in cases with a substantial risk of chromosomal abnormalities, but also the necessity of counseling parents about the advantages and limitations of chosen diagnostic procedures.

About 10-15% of all recognized pregnancies end in a spontaneous abortion²⁹, while chromosomal abnormalities are considered as the most common cause, found in approximately 60% of first-trimester miscarriages.³⁰ Chromosomal analysis of products of conception is routinely performed by conventional

karyotyping of cultured chorionic villi or fetal skin samples. However, difficulties such as culture failure due to microbial contamination or samples obtained from nonviable fetuses, poor quality samples, or maternal cell contamination, lead to the inability of diagnosis ascertainment in up to 30% of investigations. As a method which does not require the culturing process, QF-PCR has been shown as an adequate alternative to conventional karyotyping, with a success rate of over 95%.³¹ Maternal cell contamination can also be an issue for the QF-PCR method. In our experience, two-step red cell lysis and water wash followed by centrifugation is worthwhile since no extra STR alleles in the QF-PCR profiles that might be doubtful for the interpretation of results were observed. For amniotic fluids, it is possible to analyze samples containing about 20% of visible blood in the cell pellets without noticing extra STR alleles in the QF-PCR profiles. If special precautions are undertaken to identify the source of the blood contamination, either maternal or fetal, it is possible to undertake lysis/wash step for heavily bloodstained amniotic fluids as well.¹³ It is not likely that this can completely resolve potential doubts, but it might be possible to draw certain conclusions based on the maternal / fetal allele ratio and comparison of their STR profiles if the maternal sample is tested as well. However, the results should not be reported officially, and severely bloodstained amniotic fluids should be considered as unsuitable for QF-PCR diagnosis.

According to recent reports, by using QF-PCR analysis with a set of markers for chromosomes 21, 13 and 18, chromosomal abnormalities are detected in a range of 13% to 17% of analyzed samples.^{31,32} A slightly higher proportion was observed in our study, where QF-PCR analysis with an *in-house* set of markers revealed a proportion of 21.4% of abnormal karyotypes. Although structural chromosomal aberrations could not be detected using QF-PCR, valuable information regarding the determination of the cause of pregnancy loss, recurrence risk in next pregnancies and the management of following pregnancies are obtained.

Twin pregnancies always require special attention during obstetric management due to the increased risk of preterm delivery, fetal growth restriction and higher rate of perinatal mortality and morbidity. Furthermore, monozygotic twins are even more frequently associated with complications, mainly because of twin-twin-transfusion-syndrome (TTTS) appearance, while a higher rate of intrauterine infections have been observed among dizygotic pregnancies.³³ The incidence of twinning has been estimated to approximately 1 in 65 live births, with the proportion of dizygotic twins being settled to 70%, and monozygotic to 30%.³⁴ Assessment of zygosity is routinely based on sonographic identification of chorionicity and fetal gender determination, while in cases when the invasive procedure was conducted, a genetic determination using STR markers could be performed. However, the establishment of an accurate

assertion using the sonographic approach is possible only in a case of dichorionic twins of differing sex. In the presence of the same gender twins an accuracy of 67.7% for the determination of monozygotic twins, and 88.9% for dizygotic twins has been observed.³³ Thus, STR analysis should be considered as a method of choice in cases of dichorionic like-sex twins, in cases when chorionicity could not be determined, or when discordance in fetal malformations is observed. In the present study, all three cases of BC/BA were proved to be dizygotic, and both cases of MC/BA were determined as monozygotic. The differentiation of zygosity is important in planning obstetric management of twin pregnancies, as well as counseling parents regarding the possible outcome of pregnancy. However, it should be considered that monozygosity not necessarily denotes the same genetic or chromosomal constitution, since discrepancies were recorded for a number of chromosomal and genetic disorders.³⁵ Furthermore, in both monozygotic and dizygotic twins, the possibility of blood chimerism should be taken into account.³⁶

In conclusion, QF-PCR has been showed as a rapid and reliable test for detection of major chromosomal aneuploidies, providing rapid results in affected pregnancies, and decreasing parental anxiety in cases with normal results. However, conventional cytogenetics still presents a gold standard for the detection of structural aberrations and rare aneuploidies in prenatal diagnosis. A combined approach using both QF-PCR and cytogenetics is recommended, since it provides information important not only for the management of the current pregnancy, but also for genetic counseling of parents and their families.

Acknowledgments

The authors are deeply grateful to Lucija Klaric (Genos Ltd, Zagreb) for a valuable assistance with the statistical analysis.

Funding

QF-PCR analysis was performed as a part of the project "Development of the molecular diagnostic methods"; cofounded by the Business Innovation Center Croatia (BICRO), IRCRO Programme.

REFERENCES

1. Vissers LE, de Vries BB, Veltman JA. Genomic microarrays in mental retardation: from copy number variation to gene, from research to diagnosis. *J Med Genet.* 2010;47:289-297.
2. Dolk H, Loane M, Garne E. The prevalence of congenital anomalies in Europe. *Adv Exp Med Biol.* 2010;686:349-364.
3. Wellesley D, Dolk H, Boyd PA, Greenlees R, Haeusler M, Nelen V, Garne E, Khoshnood B, Doray B, Rissmann A, Mullaney C, Calzolari E, Bakker M, Salvador J, Addor

- MC, Draper E, Rankin J, Tucker D. Rare chromosome abnormalities, prevalence and prenatal diagnosis rates from population-based congenital anomaly registers in Europe. *Eur J Hum Genet.* 2012;20:521-526.
4. Mansfield ES. Diagnosis of Down syndrome and other aneuploidies using quantitative polymerase chain reaction and small tandem repeat polymorphisms. *Hum Mol Genet.* 1993;2:43-50.
 5. Pavlinić D, Džijan S, Stipoljev F, Wagner J, Ćurić G, Lauc G. Quantitative Fluorescent PCR - A Rapid Approach to Prenatal Diagnostics of Common Autosomal Aneuploidies. *CCACAA.* 2008;81:219-222.
 6. Mann K, Ogilvie CM. QF-PCR: application, overview and review of the literature. *Prenat Diagn.* 2012;32:309-314.
 7. Pan M, Han J, Zhen L, Yang X, Li R, Liao C, Li DZ. Prenatal diagnosis of fetuses with increased nuchal translucency using an approach based on quantitative fluorescent polymerase chain reaction and genomic microarray. *Eur J Obstet Gynecol Reprod Biol.* 2016;197:164-167.
 8. Hills A, Donaghue C, Waters J, Waters K, Sullivan C, Kulkarni A, Docherty Z, Mann K, Ogilvie CM. QF-PCR as a stand-alone test for prenatal samples: the first 2 years' experience in the London region. *Prenat Diagn.* 2010;30:509-517.
 9. Langlois S, Duncan A. Use of a DNA method, QF-PCR, in the prenatal diagnosis of fetal aneuploidies. *J Obstet Gynaecol Can.* 2011;33:955-960.
 10. Bio-Rad Laboratories. 2006. Chelex® 100 product insert (2006). (http://www.bio-rad.com/webmaster/pdfs/9184_Chelex.PDF. Accessed: September 6, 2007.)
 11. Mann K, Donaghue C, Fox SP, Docherty Z, Ogilvie CM. Strategies for the rapid prenatal diagnosis of chromosome aneuploidy. *European Journal of Human Genetics.* 2004;12:907-915.
 12. QIAGEN Multiplex PCR Handbook 10/2010 <https://www.qiagen.com/us/resources/resourcedetail?id=a541a49c-cd06-40ca-b1d2-563d0324ad6c&lang=en> Accessed: October 2, 2010.
 13. Aneufast User's Manual (Revised Aug 2011 V2). (<http://www.aneufast.com/files/ANF%20User%20Manual%20v2%20Aug%202011.pdf>. Accessed: September 24, 2011.)
 14. Hastings R, Howell R, Bricarelli FD, Kristofferson U, Cavani S. General Guidelines and Quality Assurance for Cytogenetics. E.C.A. - European Cytogeneticists Association Newsletter 2012; 29. (http://www.e-ca.eu/files/downloads/Guidelines/E.C.A._General_Guidelines_Version-2.0.pdf. Accessed: April 15, 2014.)
 15. Liehr T, Claussen U, Starke H. Small supernumerary marker chromosomes (sSMC) in humans. *Cytogenet Genome Res.* 2004;107:55-67.
 16. Cirigliano V, Voglino G, Ordoñez E, Marongiu A, Paz Cañadas M, Ejarque M, Rueda L, Lloveras E, Fuster C, Adinolfi M. Rapid prenatal diagnosis of common chromosome aneuploidies by QF-PCR, results of 9 years of clinical experience. *Prenat Diagn.* 2009;29(1):40-49.
 17. Papoulidis I, Siomou E, Sotiriadis A, Efstathiou G, Psara A, Sevastopoulou E, Anastasakis E, Sifakis S, Tsiligianni T, Kontodiou M, Malamaki C, Tzimina M, Petersen MB, Manolagos E, Athanasiadis A. Dual testing with QF-PCR and karyotype analysis for prenatal diagnosis of chromosomal abnormalities. Evaluation of 13,500 cases with consideration of using QF-PCR as a stand-alone test according to referral indications. *Prenat Diagn.* 2012;32:680-685.
 18. Donaghue CI, Mann K, Docherty Z, Ogilvie CM. Detection of mosaicism for primary trisomies in prenatal samples by QF-PCR and karyotype analysis. *Prenat Diagn.* 2005;25:65-72.
 19. Ogilvie CM, Donaghue C, Fox SP, Docherty Z, Mann K. Rapid prenatal diagnosis of aneuploidy using quantitative fluorescence-PCR (QF-PCR). *J Histochem Cytochem.* 2005;53(3):285-258.
 20. Putzova M1, Soldatova I, Pecnova L, Dvorakova L, Jencikova N, Goetz P, Stejskal D. QF-PCR-based prenatal detection of common aneuploidies in the Czech population: five years of experience. *Eur J Med Genet.* 2008;51(3):209-218.
 21. Comas C, Echevarria M, Carrera M, Serra B. Rapid aneuploidy testing traditional karyotyping in amniocentesis for certain referral indications. *J Matern Fetal Med.* 2010;23(9):949-955.
 22. de la Paz-Gallardo MJ, Garcia FS, de Haro-Muñoz T, Padilla-Vinuesa MC, Zafra-Ceres M, Gomez-Capilla JA, Gomez-Llorente C. Quantitative-fluorescent-PCR versus full karyotyping in prenatal diagnosis of common chromosome aneuploidies in southern Spain. *Clin Chem Lab Med.* 2015;53(9):1333-1338.
 23. Caine A, Maltby AE, Parkin CA, Waters JJ, Crolla JA; UK Association of Clinical Cytogeneticists (ACC). Prenatal detection of Down's syndrome by rapid aneuploidy testing for chromosomes 13, 18, 21 by FISH or PCR without a full karyotype: a cytogenetic risk assessment. *Lancet.* 2005;366:123-128.
 24. Kozłowski P, Grund I, Hickmann G, Stressig R, Knippel AJ. Quantitative fluorescent polymerase chain reaction versus cytogenetics: risk-related indication and clinical implication of nondetected chromosomal disorders. *Fetal Diagn Ther.* 2006;21(2):217-223.
 25. Sparkes RL1, Bernier FP, Chernos JE, Johnson JA. Suitability of rapid aneuploidy detection for prenatal diagnosis. *J Obstet Gynaecol Can.* 2008;30(9):781-787.
 26. Leung WC1, Waters JJ, Chitty L. Prenatal diagnosis by rapid aneuploidy detection and karyotyping: a prospective study of the role of ultrasound in 1589 second-trimester amniocenteses. *Prenat Diagn.* 2004;24(10):790-795.
 27. Malone FD, Ball RH, Nyberg DA, Comstock CH, Saade GR, Berkowitz RL, Gross SJ, Dugoff L, Craigo SD, Timor-Tritsch IE, Carr SR, Wolfe HM, Dukes K, Canick JA, Bianchi DW, D'Alton ME; FASTER Trial Research Consortium. First-trimester septated cystic hygroma: prevalence, natural history, and pediatric outcome. *Obstet Gynecol.* 2005;106(2):288-294.
 28. Nicolaides KH. Screening for chromosomal defects. *Ultrasound Obstet Gynecol.* 2003;21:313-321.
 29. Nybo Andersen AM, Wohlfahrt J, Christens P, Olsen J, Melbye M. Maternal age and fetal loss: Population based register linkage study. *BMJ.* 2000;320:1708-1712.
 30. Ljungner E, Cnattingius S, Lundin C, Annerén G. Chromosomal anomalies in first-trimester miscarriages. *Acta Obstet Gynecol Scand.* 2005;84:1103-1107.
 31. Wou K, Hyun Y, Chitayat D, Vlasschaert M, Chong K, Wasim S, Keating S, Shannon P, Kolomietz E. Analysis of tissue from products of conception and perinatal losses using QF-PCR and microarray: A three-year retrospective study resulting in an efficient protocol. *Eur J Med Genet.* 2016;59(8):417-424.
 32. Teles TM, Paula CM, Ramos MG, Costa HB, Andrade CR, Coxir SA, Penna ML. Frequency of Chromosomal Abnormalities in Products of Conception. *Rev Bras Ginecol Obstet.* 2017;39(3):110-114.
 33. Spiegler J, Härtel C, Schulz L, von Wurmb-Schwark N, Hoehn T, Kribs A, Küster H, Siegel J, Wieg C, Weichert J, Herting E, Göpel W; German Neonatal Network (GNN). Causes of delivery and outcomes of very preterm twins stratified to zygosity. *Twin Res Hum Genet.* 2012;15(4):532-536.
 34. Hoekstra C, Zhao ZZ, Lambalk CB, Willemsen G, Martin NG, Boomsma DI, Montgomery GW. Dizygotic twinning. *Hum Reprod Update.* 2008;14(1):37-47.
 35. Zwijnenburg PJ, Meijers-Heijboer H, Boomsma DI. Identical but not the same: the value of discordant monozygotic twins in genetic research. *Am J Med Genet B Neuropsychiatr Genet.* 2010;153B(6):1134-1149.
 36. Fiddler M, Frederickson MC, Chen PX, Pergament E. Assessment of fetal status in multiple gestation pregnancies using interphase FISH. *Prenat Diagn.* 2001;21:196-199.