ORIGINAL ARTICLE

High-resolution chromosomal microarrays in prenatal diagnosis significantly increase diagnostic power

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ABSTRACT

Objective The objective of this study was to determine for the first time the reliability and the diagnostic power of high-resolution microarray testing in routine prenatal diagnostics.

Methods We applied high-resolution chromosomal microarray testing in 464 cytogenetically normal prenatal samples with any indication for invasive testing.

Results High-resolution testing revealed a diagnostic yield of 6.9% and 1.6% in cases of fetal ultrasound anomalies and cases of advanced maternal age (AMA), respectively, which is similar to previous studies using low-resolution microarrays. In three (0.6%) additional cases with an indication of AMA, an aberration in susceptibility risk loci was detected. Moreover, one case (0.2%) showed an X-linked aberration in a female fetus, a finding relevant for future family planning. We found the rate of cases, in which the parents had to be tested for interpretation of unreported copy number variants (3.7%), and the rate of remaining variants of unknown significance (0.4%) acceptably low. Of note, these findings did not cause termination of pregnancy after expert genetic counseling. The 0.4% rate of confined placental mosaicism was similar to that observed by conventional karyotyping and notably involved a case of placental microdeletion.

Conclusion High-resolution prenatal microarray testing is a reliable technique that increases diagnostic yield by at least 17.3% when compared with conventional karyotyping, without an increase in the frequency of variants of uncertain significance.

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INTRODUCTION

Chromosomal microarray (CMA) analysis is now widely used for the clinical evaluation of pediatric patients with congenital anomalies, cognitive deficits, developmental delay, growth abnormalities, or behavior problems.[1,2] Although conventional microscopic karyotyping reveals a disease causing aberration in 4%, CMA is capable of detecting clinically significant submicroscopic aberrations in an additional 14–18% of such cases.[3] In the last few years, several studies using low-resolution microarrays have been performed to explore the usefulness of CMA in prenatal diagnosis.[4] Despite the potential advantages of CMA with superior sensitivity compared with conventional G-banding and faster turnaround time when using uncultured material, there are potential limitations: Balanced aberrations are not detectable, and the increased detection of variants of unknown clinical significance (VOUS) may cause counseling problems, parental distress, and unwarranted terminations of pregnancies (TOP). Therefore, several professional societies (American College of Obstetricians and Gynecologists, Canadian College of Medical Genetics, and Italian Society of Human Genetics) do not encourage to replace prenatal G-band karyotyping with CMA but recommend it as an adjunct test in specific cases such as ultrasound (US) fetal anomalies, apparently balanced chromosomal rearrangements,

or supernumerary marker chromosomes.[5–7] Recent studies on larger series showed that after exclusion of conventionally visible aberrations, low-resolution CMA additionally detects a clinically significant aberration in about 7% of cases with US abnormalities, without an appreciable increase in the detection of VOUS, whereas the overall additional detection rate for all indications was 1.4–2.5%.[8–14] We have now introduced high-resolution CMA in routine prenatal diagnostics in samples with normal conventional karyotyping and investigated the added diagnostic value of our platform in a cohort of 464 patients.

MATERIALS AND METHODS

Patients and clinical indications

CMA analysis was performed in addition to conventional karyotyping on specimens obtained from couples who chose an invasive prenatal genetic testing procedure. The indications for invasive prenatal testing included increased risk of fetal aneuploidy associated with advanced maternal age (AMA; 35 years or older at the time of conception), abnormal results of maternal serum screening (MSS) tests, abnormal US findings, nuchal translucency (NT) ≥ 3.0 mm, family history (FH) of a genetic condition or chromosome abnormality, or parental anxiety (PA).

Prenatal samples

Samples included in this data set were received between August 2010 and April 2013 from healthcare providers in Switzerland. Specimen types included amniotic fluid (AF), chorionic villi samples (CVS), fetal pleural effusion (FPE), and fetal blood (FB). From August 2010 to February 2012, samples were included only randomly for organizational reasons ($n = 71$). From February 2012 to April 2013, CMA analysis was performed for all CVS, which were normal in Q-banding after short-term culture ($n = 323$) and for AF ($n = 67$), FPE ($n = 2$), and FB ($n = 1$) samples with normal G-banding, in which the analysis was deemed indicated (US or NT). Parental blood samples were requested in case of unclear CMA results.

Cell culture and DNA extraction

DNA was extracted from native or cultivated CVS (after accurate microscopic separation of maternal and fetal tissues), native AF (only in case of sufficient amount of native cells and in the absence of contaminating maternal blood), or cultivated AF, FB, and FPE. Typically, 1–2 mg native CVS tissue at a gestational age of 12–13 weeks was used for direct DNA extraction and 5–10 mg for cell culture. With respect to AF, FB, and FPE, 3–6 mL each was used for direct DNA extraction and for cell culture.

DNA was extracted using the QIAamp DNA Mini kit (Qiagen, Hombrechtikon, Switzerland).

In each case, cell cultures were set up with the remainder of the fetal samples for conventional karyotyping (Q-banding in CVS after short-term culture and G-banding for AF, FB, FPE, and CVS after long-term culture).

DNA from parental peripheral blood was extracted using a Chemagen automated device according to the manufacturer's instruction (Perkin Elmer, Baesweiler, Germany).

Chromosomal microarray studies

DNA was analyzed with the Affymetrix cytogenetics Whole Genome 2.7M array (containing about 400 000 single-nucleotide polymorphisms (SNPs) and 2.3 million non-polymorphic probes) or with the newer Cytoscan HD Array (containing about 750 000 genotype-able SNPs and 1.9 million non-polymorphic probes) (Affymetrix, Inc., Santa Clara, CA, USA) at a genome-wide resolution of 20–100 kb. Array hybridization was performed according to the manufacturer's protocols. Data were analyzed with the Chromosome Analysis Suite software (Affymetrix) for changes of relative intensities and SNP genotype patterns. The copy number variant (CNV) analyses were based on the annotations NetAffx build 30 for the 2.7 arrays and on build 32–32.1 for the Cytoscan HD arrays (Affymetrix). Genomic coordinates are based on GRCh37/hg19. In order to exclude common benign CNVs, we used the Database of Genomic Variants from the Centre for Applied Genomics (February 2009, hg19) in combination with a reference set of 820 in-house controls and 450 Affymetrix controls. CNVs were called using the following filter settings: size 20–100 kb, marker count 5, and confidence level 86%, for both gains and losses. CNVs not present in the control population were assessed for clinical significance by comparison with regions of known syndromes and analysis of gene content using the following databases: DECIPHER v4.1 (Database of Chromosome Imbalance and Phenotype in Humans using Ensemble Resources, http://decipher.sanger.ac.uk/), ISCA consortium (https://www.iscaconsortium.org/), OMIM (Online Mendelian Inheritance in Man, http://www.omim.org/), UCSC genome browser (http://genome.ucsc.edu/), and PubMed (http://www.ncbi.nlm.nih.gov/pubmed).

Confirmatory analysis

Pathologic CNVs and CNVs of unclear significance were further investigated in parental blood specimens, by CMA, fluorescence *in situ* hybridization (FISH), or multiplex ligation-dependent probe amplification (MLPA). FISH analysis was performed using commercial bacterial artificial chromosomes (BAC) probes (BlueGnome Ltd, Cambridge, UK) according to standard protocols on metaphase preparations, and MLPA was performed using the MRC-Holland protocol. In case of parental FISH or MLPA testing, fetal material was investigated in parallel as positive controls, and probes were also used in the respective aborted material to confirm the aberrations.

Classification of results

We classified our results as follows: clinically significant CNV (which we termed 'pathologic'), likely benign ('normal'), relevant for future pregnancies ('future pregnancy'), variants in susceptibility risk loci ('VISL') or variants of unknown clinical significance ('VOUS').

An imbalance was considered pathologic if it overlapped with the critical region of a known genetic syndrome or was previously reported to cause a specific phenotype in the databases mentioned earlier. Benign CNVs were defined as those that are common or observed in the general population without known phenotypic signs or inherited from a healthy parent. CNVs relevant for future pregnancies were those that are not normal according to the aforementioned definition

and those that are located on the X chromosome, and thus, the phenotype depends on the sex of the fetus. VISL were defined as *de novo* or inherited CNVs with variable phenotypic expression and incomplete penetrance, for which literature is available. VOUS was defined as *de novo* CNVs with significant gene content, for which phenotypic consequences are difficult to predict because of lack of scientific information.[15]

Statistical analysis

Statistical analysis to compare the different data sets was performed using the STATA software, applying a chi-square test.

RESULTS

Samples and clinical indications

A total of 464 prenatal samples with normal results on conventional karyotyping were processed and only in one case (AF cultivated) the analysis did not succeed even after three replicates. The sample was therefore not included in the statistics (1/464, 0.2%). Of the successfully hybridized samples, 372 (80.3%) were CVS (354 native and 18 cultivated), 88 (19%) AF (13 native and 75 cultivated), 2 cultivated FB (0.4%) and 1 cultivated FPE (0.2%). Hence, overall, analysis was performed on uncultured material in 367 (79.3%) and on cultured material in 96 (20.7%) samples (Table 1). A total of 57 (12.3%) samples were processed with the Affymetrix 2.7 array, and 406 (87.7%) with the Cytoscan HD array.

Indications to perform invasive prenatal testing were AMA ($n=187$; 40.4%), MSS ($n=86$; 18.6%), US ($n=91$; 19.6%), NT ($n=53$; 11.4%), FH ($n=36$; 7.8%), and PA ($n=10$; 2.2%) (Table 1).

Detection of microaberrations using high-resolution CMA karyotyping

The majority of prenatal samples ($n=426$; 92%) showed only benign CNVs and were classified as normal.

In 17/463 samples (3.7%), we identified CNVs, which were neither overlapping with reported pathologic CNVs nor present in our controls. Of those, 15/17 (88.2%; mean size 973 kb; range 27 kb to 2.4 Mb) were found to be inherited from a healthy parent and therefore, after having reported them to the parents, classified as likely benign (normal) (Table 2). Of note, one of these deletions contained the *CNGB3* gene and thus represented carriership for autosomal recessive macular degeneration but was benign with reference to childhood developmental disorders. Therefore, only two cases remained, in which a rare *de novo* CNV was found after parental testing (0.4% VOUS in the total cohort) (Table 2). In one of these cases, we performed *PTPN11* gene sequencing because of increased NT and detected a known Noonan mutation. This finding rendered the concomitant VOUS clinically not relevant. In the other case, the detected deletion was overlapping with two DECIPHER entries; however, we did not classify it as pathologic because personal correspondence revealed that after family studies, the significance of these deletions remained questionable, since they were each found inherited from healthy family members.

Clinically significant chromosome aberrations were identified in 17/463 (3.7%) samples (six cultivated AF, ten native and one cultivated CVS). However, all pathologic CNVs that were found on native CNV material were double checked on long-term cultivated material and could be confirmed in all cases, except for two: in one sample, we first found a pathologic CNV (arr 2p23.1p22.3(32031898-33010594)x1 dn, 979 kb) in native CVS, which was confirmed neither on a second DNA extraction from another native villus nor later in the cultivated CVS sample. We then recommended an AF analysis, in which we could not find the initial aberration, either. After exclusion of a sample mix-up by microsatellite testing and confirmation of all the array results by MLPA, we concluded that the deletion was a mosaic confined to the placenta and the sample was therefore classified as normal. The second sample was normal in conventional karyotyping (short-term culture, Q-banding, 15 cells analyzed); however, CMA on native material (CVS) identified a trisomy 8 in mosaic form (about 40%). We therefore re-analyzed the direct preparation using FISH (200 nuclei) and could confirm the mosaic in 25% of the cells. Because this trisomy 8 was absent in long-term culture (FISH), as well as in the AF analysis performed additionally (FISH on native AF), we concluded that it represents a confined placental mosaic and classified the sample as normal.

In addition, in 3/463 samples (0.6%; native CVS), all with indication AMA, we found microduplications in susceptibility risk loci (VISL) known to be associated with various degrees of developmental delay and speech disturbances[16,17] (Table 3). Of note, because one of these cases of microduplication (case

Table 1 **Overview of all the prenatal samples processed, with the respective indications for testing**

	Material						
Indications	CVS native	CVS cultivated	AF native	AF cultivated	FB cultivated	FPE cultivated	Total
AMA	178	6	1	2	0	0	187
MSS	79	4	0	3	0	0	86
US	15	0	9	64	1	2	91
NT	44	6	0	3	0	0	53
FH	30	1	2	3	0	0	36
PA	8	1	1	0	0	0	10
Total	354	18	13	75	1	2	463

CVS, chorionic villi sample; AF, amniotic fluid; FB, fetal blood; FPE, fetal pleural effusion; AMA, advanced maternal age; MSS, maternal serum screening; US, ultrasound abnormality; NT, nuchal translucency > 3.0 mm; FH, family history; PA, parental anxiety.

Table 2 **Overview of rare variants of unknown significance in which parental analysis was needed**

Case	Indication	Material	Microarray abnormality	Size	VOUS	Number of gene involved	OMIM gene involved
1-34606	AMA	CVS cultivated	arr 8q22.3(103918403-104907126)x1 pat	969 kb		8	RIMS2
2-35810	US	AF cultivated	arr 8q21.3(86778228-88369887)x1 dn	1.6 Mb	X	10	WWP1, FAM82B, CPNE3, CNGB3
3-36048	NT	CVS native	arr 7p21.2p21.1(15989516-17068190)x3 mat	1.1 Mb		9	ISPD, SOSTDC1, TSPAN13, AGR2, AGR3
4-36585	MSS	CVS native	arr 8q21.2p21.3(86247488-88246946)x1 pat	2.0 Mb		13	CA1, CA3, CA2, WWP1, FAM82B, CPNE3, CNGB3
5-35277	US	AF cultivated	arr 16q23.1(78080329-78706360)x3 pat	629 kb		1	WWOX
6-37098	NT	AF cultivated	arr 17p13.2(3626734-4525069)x3 dn	898 kb	X[a]	15	ITGAE, GSG2, CAMKK1, P2RX1, ATP2A3, ANKFY1, UBE2G1, SPNS3, SPNS2, MYBBP1A, GGT6
7-37399	MSS	CVS native	arr 6q25.3(158640495-160992068)x3 pat	2.4 Mb		29	TMEM181, DYNLT1, EZR, TAGAP, FNDC1, SOD2, WTAP, ACAT2, TCP1, MRPL18, MAS1, IGF2R, AIRN, SLC22A1, SLC22A2, SLC22A3, LPAL2, LPA
8-35432	MSS	CVS native	arr Xp22.31(6091260-6434800)x3 pat	343 kb		1	NLGN4X
9-37552	NT	CVS native	arr 11q14.1(84459458-84688062)x1 mat	229 kb		1	DLG2
10-37651	AMA	CVS native	arr Xp22.31(8430919-8628505)x3 pat	198 kb		2	KAL1
11-37685	AMA	CVS native	arr Xp22.2(11246199-12008045)x3 mat	762 kb		3	ARHGAP6, AMELX, MSL3
12-37603	MSS	CVS cultivated	arr 4q35.1q35.2(186950636-187201211)x1 pat	251 kb		6	TLR3, CYP4V2, KLKB1, F11
13-37824	AMA	CVS native	arr 20p12.2(10183782-11587181)x3 pat	1.4 Mb		6	SNAP25, MKKS, JAG1
14-38203	NT	CVS native	arr Xp22.31(6052606-6895311)x2 mat	843 kb		5	NLGN4X, VCX3A, HDHD1
15-38215	US	AF cultivated	arr 18p11.31(6990579-7017647)x1 mat	27 kb		1	LAMA1
16-38672	FH	CVS native	arr 5q23.1(115816906-117412944)x3 mat	1.6 Mb		2	SEMA6A
17-38851	US	FPE cultivated	arr 20p13(3539291-4881416)x1 mat	1.3 Mb		23	ATRN, ADAM33, SIGLEC1, HSPA12B, SPEF1, CENPB, CDC25B, AP5S1, MAVS, PANK2, MIR103A2, RNF24, ADRA1D, PRNP, PRND, RASSF2, SLC23A2

VOUS, variants of unknown significance; OMIM, Online Mendelian Inheritance in Man; AMA, advanced maternal age; CVS, chorionic villi samples; AF, amniotic fluid; NT, nuchal translucency; MSS, maternal serum screening.
[a]Noonan screening revealed a known PTPN11 mutation as the likely cause for the increased NT.

Table 3 **Overview of the variants in susceptibility risk loci (VISL)**

Case	Indication	Material	Microarray abnormality	Size (Mb)	Outcome
1-36807	AMA	CVS native	arr 22q11.23(23690387-25079586)x3 mat	1.4	ni (mother normal, highly educated, learning disability in her brother)
2-38354	AMA	CVS native	arr 16p13.11p12.3(14892880-18191725)x3 mat	3.3	ni (mother normal, highly educated)
3-38831	AMA	CVS native	arr 22q11.23(23690387-25039163)x3 pat	1.3	ni (father learning and behavioral problems)

AMA, advanced maternal age; CVS, chorionic villi samples; ni, no pregnancy interruption.

3-38831, arr 22q11.23(23690387-25039163)x3 pat) was inherited from a father with learning and behavioral problems, it is possible that a neurodevelopmental disorder will later manifest in the child as well. In the other two cases (case 1-36807 arr 22q11.23(23690387-25079586)x3 mat and case 2-38354 arr 16p13.11p12.3(14892880-18191725)x3 mat), the microduplications were inherited from the healthy mother. However, the FH of case 1 is striking: the maternal brother suffers from undiagnosed learning disability. Moreover, in 1/463 samples (0.2%; native CVS), we found an X-linked aberration in a female fetus, which was hence relevant for potential future pregnancies (Table 4). Interestingly, this family showed evidence of previously undiagnosed X-linked disorders within their pedigree, the cause of which might be the CNV observed in the fetus. However, this hypothesis could not be tested because of lack of material.

Thus, a total of 15/463 cases (3.2%) showed aberrations with clear clinical significance for the actual fetus. The indications to perform a prenatal test in these cases were as follows: US in seven cases, AMA in three, NT in three, FH in one, and PA in one (Table 5). Notably, 12 (80%) of the 15 pathologic aberrations were truly submicroscopic (≤9.2 Mb) and would have remained unequivocally undetected if only conventional karyotyping had been performed. Three of the pathologic aberrations sizing of more than 10 Mb were not found in the actual conventional analysis but may have been detected under luckier circumstances. In one of these cases, CMA from directly extracted DNA indicated a marker chromosome of 29 Mb, which was present in 46% of the cultivated CVS cells but absent in the 15 metaphases that had been analyzed after the direct preparation. The second one was a deletion of 14 Mb terminally on chromosome 4, which was not visible on the Q-banding performed on the direct CVS preparation at a resolution of 250 bands and could not been identified retrospectively, either. The third case was an unbalanced translocation in mosaic form affecting chromosomes 17 and X, leading to a partial trisomy 17 and a partial monosomy X, absent in the 15 AF clones analyzed in the culture preparation at a resolution of 450 bands. This mosaic was confirmed retrospectively by FISH analysis on the cultivated material and observed in three out of 16 additional clones (19%), in accordance with the array ratio result. For the pregnancies that were terminated ($n=11$) and for which abort material was kindly provided ($n=5$), we performed a control test and the pathologic CNVs were confirmed in all cases.

To assess the increase in diagnostic power, we compared the CMA detection rate with the yield of conventional karyotyping in our institute. We focused on the second inclusion period only (February 2012 to April 2013) to reduce potential inclusion biases. In this period, our CMA detection rate in cytogenetically normal samples was comparable with the one in the entire study period (3.3%; 13/393 compared with 3.2%; 15/463). In the very same period, we analyzed 423 cases of AF and 373 cases of CVS by conventional cytogenetics. In the first group, 25 (5.9%) were found to be pathologic, and in the second group 50 (13.4%). In total, we detected 75 abnormalities in 796 cases (9.4%) using conventional karyotyping. Therefore, applying CMA in all cytogenetically normal CVS samples and in cytogenetically normal AF samples with abnormal US in this series resulted in an increased diagnostic yield of 17.3% (13/75). If we included the three VISL cases in this calculation, as well, CMA analysis would result in an increased diagnostic yield of 21.3% (16/75).

Table 4 **Overview of the case, in which the aberrations are relevant for the next pregnancies**

Case	Indication	Material	Microarray abnormality	Size (kb)
1-34719	AMA	CVS native	arr Xp22.32p22.31 (5845377-6447196)x1 mat	602

AMA, advanced maternal age; CVS, chorionic villi samples.

Ten of the 15 identified pathologic samples were *de novo* aberrations as confirmed in parental analyses. In two cases, the parents did not consent to have their blood tested; however, both of them were clearly pathologic, as described in the literature or in online databases. However, in one of these two cases (case 8-37259), the deletion of the X-linked *ichthyosis* locus did not explain intrauterine growth retardation in a male fetus. In the remaining three cases that were all parentally inherited, the aberration ranged in size from 2.6 to 4.58 Mb. One of these aberrations represented an X-linked recessive condition inherited from the healthy mother (case 3-36175) (Table 5). TOP was performed in that case. In a further case (case 14-38134) with a paternally inherited 4.58 Mb deletion, the father was mildly affected. The parents initially decided to continue the pregnancy but eventually terminated after the later detection of a severe fetal neural tube defect. In the third case (case 4-36248), pregnancy was continued; however, the baby died within 12 h after birth (Table 5). No aberrations were found in the 86 cases referred for abnormal MSS (Table 6). We also found no evidence for uniparental isodisomy in any of the samples.

DISCUSSION

In the present study, we investigated the reliability, feasibility, and benefits of the highly sensitive SNP array technology in invasive prenatal diagnostics. To the best of our knowledge, this is the first study that used high-resolution SNP arrays exclusively (as opposed to lower-resolution BAC, oligo, or customized arrays or a combination of different platforms) in a cohort that was not restricted to the indications US or NT.

Our data show that CMA with high-resolution SNP arrays reliably works on conventional prenatal samples with only 0.2% of technical failures. All results were reproducible by alternative methods. However, a microdeletion and a mosaic trisomy 8 were found to be confined to the placenta. The rate of confined placental mosaicism of whole chromosome aneuploidies using conventional karyotyping has been estimated to be roughly 1%.[18,19] However, to the best of our knowledge, very few microaberrations confined to the placenta have been reported so far.[14] Because our findings confirm that CMA is capable of detecting confined placental mosaicism, we recommend that pathogenic results from direct CVS preparations be confirmed on long-term cultured material.

Table 5 **Overview of the pathologic cases**

Case	Indication	Material	Microarray abnormality	Size	Outcome	Confirmation in abort material
1-33963	US: VSD, kidney-agenesy	AF cultivated	arr 20q11.22q11.23(32305562-35518605)x3 dn arr 4q32.3(165149890-167850894)x3 dn arr Xp22.11(22299941-24857546)x3 dn	3.1 Mb 2.7 Mb 2.5 Mb	TOP	Yes
2-35123	PA	CVS cultivated	arr 4p15.2-q11(23369297-52586923)x2~3 dn	29.0 Mb	TOP	Yes
3-36175	US: hygroma colli	CVS native	arr Xp11.23p11.22(49370299-52687306)x0 mat	3.3 Mb	TOP	na
4-36248	US: diaphragm hernia, pulmonary hypoplasia, urogenital anomalies, singular umbilical artery	AF cultivated	arr 16p13.11p12.3(15509406-18172740)x1 pat	2.6 Mb	Spontaneous birth at 38 6/7 weeks of gestation and neonatal death after 12 h	na
5-36519	AMA	CVS native	arr 4q34.1q35.2(176530315-190708858)x1 dn	14.2 Mb	TOP	Yes
6-36806	AMA	CVS native	arr 7q35q36.1(147864512-151418870)x1 dn	3.5 Mb	nk	na
7-36809	NT > 3 mm	CVS native	arr 15q26.3(100987918-101612249)x1 dn	624 kb	TOP	Yes
8-37259	US: IUGR	AF cultivated	arr Xp22.31(6458939-8135644)x0	1.7 Mb	Familial X-linked ichthyosis	na
9-37466	US	AF cultivated	arr 1q21.1(146043713-147897962)x3 dn	1.8 Mb	TOP	na
10-37458	NT = 3.5 mm	CVS native	arr 22q11.21(18916842-21465659)x3 dn	2.5 Mb	Spontaneous birth, several facial, dysmorphisms,	na
11-37641	NT (hygroma colli)	CVS native	arr 1p36.23p36.13 (9101154-18339522)x1 dn arr 14q11.2q12(22252419-26263013)x1 dn arr 14q11.2(20511672-21089393)x1 dn	9.2 Mb 4.0Mb 578kb	TOP	na
12-37683	AMA	CVS native	arr 15q13.3(31073735-32446830)x1 dn	1.4 Mb	TOP	Yes
13-38045	US	AF cultivated	arr 3q26.33(179685964-182441561)x1	2.6 Mb	TOP	na
14-38134	FH	CVS native	arr 15q24.3q25.2(777443690-82020162)x1 pat	4.58 Mb	TOP due to NTD diagnosed later in pregnancy, mild phenotype in father	na
15-38543	US: hydrops fetalis	AF cultivated	arr 17q11.2q25.3(30120803-80933290)x2~3 dn arr Xp22.33p11.3(1502618-45412465)x1~2 dn	50.8 Mb 43.9 Mb	ni, hydrops resolved	na

US, ultrasound; VSD, ventricular septal defect; AF, amniotic fluid; nk, not known; TOP, terminations of pregnancies; na, not available; PA, parental anxiety; CVS, chorionic villi samples; IUGR, intrauterine growth retardation; AMA, advanced maternal age; NT, nuchal translucency; FH, family history; NTD, neural tube defect; ni, no pregnancy interruption.

In 0.2% of cytogenetically normal samples, we found an aberration representing a carriership status for an X-linked recessive intellectual disability (MIM #300495) in a female fetus. Because the aberration was inherited from a healthy mother, this finding was important for future pregnancies in the carrier family. Such findings have not been reported in previous studies, which may be due to the higher resolution of our array platform or differences in reporting policies.

We identified a pathologic finding in 3.2% of cytogenetically normal samples. For those cases, with an indication conferring a high pretest probability (US or NT) and in those cases referred for AMA, our detection rates were 6.9% and 1.6%, respectively. Thus, our findings using high-resolution CMA are in line with those of previous reports using low-resolution array if only cases with normal conventional karyotyping were considered and VISL are excluded (Table 6).

Table 6 **Overview over the largest data sets published using microarray testing**

Study	Author	Number of cases	Microarray type	Results overall[a]	High pretest probability (US, NT)	Low pretest probability (AMA)	PA	MSS	FH
1	Fiorentino *et al.*[10]	3000 (incl. 1037 first cohort)	BAC array Resolution 1 Mb across the genome, 100 kb in selected regions	0.8% (24/2929)	8% (6/75)	0.6% (6/1090)	0.7% (11/1658)	0% (0/26)	0% (0/25)
2	Lee *et al.*[9]	3171	BAC-oligo array 100 kb to 1 Mb	1.1% (34/3083)	10.4% (19/182)	0.5% (10/1891)	0.5% (5/966)	0% (0/26)	—
3	Srebniak *et al.*[13]	207	SNP array Resolution: 150/200 kb	n.a.	8.0% (16/199)	—	—	—	—
4	Armengol *et al.*[11]	906	Targeted-BAC microarray	1.6% (14/864)	3.8% (6/157)	1.1% (3/265)	1.7% (1/60)	0.4% (1/224)	2.1% (3/140)
5	Wapner *et al.*[12]	4406	Targeted array, resolution 1 Mb	2.5% (96/3822)	6% (45/755)[b]	1.7% (34/1966)	—	n.k.	—
6	Shaffer *et al.*[14]	5003	Targeted array	5.5% (140/2533)	n.k.	0.3% (1/346)	n.k.	n.k.	n.k.
Studies 1–6 combined		16 693		2.3% (308/13 231)	6.7% (92/1368)	1.0% (54/5558)	0.6% (17/2684)	0.4% (1/276)	1.8% (3/165)
Our study	Oneda *et al.*	463	CNV-SNP array 20–100 kb across the genome	3.2% (15/463)	6.9% (10/144)	1.6% (3/187)	10% (1/10)	0% (0/86)	2.7% (1/37)
				$p<0.2$	$p<0.9$	$p<0.6$	$p<0.06$	$p<1$	$p<0.7$

US, ultrasound; NT, nuchal translucency; AMA, advanced maternal age; PA, parental anxiety; MSS, maternal serum screening; FH, family history; CNV, copy number variant.

[a]In order to compare the reported detection rates with our study, we excluded cases, which could have been detected by conventional karyotyping, and categorized the indications according to the definitions we describe in Section on Materials and Methods. Values might therefore differ from those of the original publications, and moreover, the numbers reported in 'results overall' might differ from the sum of subcategories in cases, when the authors reported 'other indication'. n.k. is not known, because of differences in category definition (for example, the authors state 'pathologic Down syndrome screening' but do not clarify whether this is MSS or NT or both). n.a., not applicable: all the cases in this study were pre-selected and only of high pretest probability.

[b]Only cases with structural ultrasound anomalies or NT > 3.5 mm, as defined by the authors.

There is an ongoing debate regarding how to categorize VISL. We believe that such variants should be distinguished from the VOUS category because of their well-known pathologic potential, differently from the VOUS group, which should contain only *de novo* CNVs with significant gene content, for which phenotypic consequences are difficult to predict because of lack of scientific information.[15] We therefore propose the introduction of a third category termed VISL, in which we classified our three cases with the microduplications 22q11.23 and 16p13.11. For these CNVs, there is clear literature evidence showing their association with a spectrum of neurodevelopmental disorders.[16,20,21] Moreover, a very recent publication shows that control carriers of the 16p13.11 duplication, who were classified as phenotypically normal at first glance, performed at a level that is between that of patients and the population control in various cognitive tests (performance IQ, verbal IQ, and spatial working memory).[22] Genetic counseling in these VISL cases was not problematic because the aberrations were all inherited and the parents did not regret having performed CMA testing. Given the better breakpoint definition and therefore better genotype–phenotype correlation from high-resolution CMA data, we consider the latter as the first choice for prenatal CMA testing.

Of note, neither our data nor data published previously show an advantage of CMA testing in cases with indication MSS. This might be due to the fact that all the pathologic cases with abnormal MSS are already detected by conventional karyotyping showing mainly the common trisomies.

So far, prenatal high-resolution CMA testing was considered potentially harmful because of its risk to detect VOUS, which may induce unnecessary PA and TOP. Applying the policy neither to follow nor to report CNVs associated with late onset disorders or CNVs affecting single genes with unknown significance in normal US cases, our results show a very low rate of VOUS (0.4). Of note, in one of the two VOUS cases, further genetic testing revealed a monogenic diagnosis: like in all cases with NT and normal chromosomal studies, we performed Noonan syndrome mutational analysis, which turned out to be positive. The parents decided to terminate the pregnancy because of this diagnosis, and hence, the VOUS did not provoke any counseling problems. We believe that the low VOUS frequency we observed is due to our comparison of the samples with a very large cohort of healthy controls ($n = 1270$). This is crucial in CMA testing, particularly when moving to higher-resolution platforms. Moreover, the online databases of genomic variations on normal individuals and on pathologic aberrations in defined patients are steadily growing and help considerably in minimizing the risk to detect a VOUS. We speculate that ultimately, as CMA testing continues to be used, VOUS will almost disappear and already today should not speak against prenatal CMA testing. However, when rare variants are detected and parental samples are asked for, we consider expert genetic counseling important to keep PA low. Notably, pregnancies were terminated in all cases with clearly pathologic *de novo* events but not in the VOUS cases. In cases with inherited pathologic aberrations, TOP was only performed in one case with severe neural tube defect and in the X-linked recessive conditions associated with intellectual disability. TOP was not performed in the male fetus with the CNV causing X-linked *ichthyosis*, a benign skin disorder. Interestingly, we observed two cases with three *de novo* CNVs each. In one of these cases, the mother was under methotrexate treatment for rheumatoid arthritis, which might possibly explain the finding, although the literature does not support a strong mutagenic potential for this drug used at low doses.[23] The pregnancy was then terminated, and the patient stopped treatment. A few months later, she became pregnant again, and a CMA analysis on CVS was normal.

A further interesting finding was that we observed two fetuses (one male fetus and one female fetus) with a duplication of 343 and 843 kb, respectively, encompassing the gene NLGN4X. Although mutations and deletions of NLGN4X causing ID, Asperger syndrome, and autism in boys have been reported (MIM #300495), the significance of duplications was not clear. We therefore analyzed segregation in the first case and found the mother and the maternal grandfather to be healthy carriers. A benign NLGN4X duplication was also reported by Hillmann,[4] and therefore, it seems to be quite a common finding.

Taken together, our results show that high-resolution CMA testing in karyotypically normal prenatal samples is feasible with a very low VOUS detection rate, which, after proper genetic counseling, does not cause TOP. From our data, we would therefore suggest offering CMA testing as a complement to conventional cytogenetic testing not only to patients with US/NT but also in cases with indication AMA. A more cost-effective alternative to this strategy would be a rapid test (Quantitative fluorescent polymerase chain reaction (QF-PCR) or FISH) on native material to exclude the most frequent aneuploidies (chromosomes 13, 18, 21, X, and Y), followed by CMA analysis in normal cases. This strategy would obviously miss balanced translocations occurring in 1/500 of the general population,[24] which, however, usually does not permit to draw prognostic conclusions in the prenatal setting.

WHAT'S ALREADY KNOWN ABOUT THIS TOPIC?

- Previous studies showed an advantage of prenatal low-resolution microarray analysis over conventional karyotyping in cases with fetal ultrasound abnormalities.
- A concern was the detection of variants of unknown significance (VOUS).

WHAT DOES THIS STUDY ADD?

- Our study is the first assessing prenatal high-resolution chromosomal microarray (CMA) and showing the feasibility of this type of analysis in the prenatal setting for any clinical indication.
- High-resolution CMA for any clinical indication increases diagnostic yield by at least 17.3% when compared with conventional karyotyping.
- We do not find an increased rate of VOUS.

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