Prenatal genetic testing: a pragmatic approach

Boris Tutschek, Debbie Krakow, Olga Bürger

Abstract

Genetic testing in fetal medicine can be used to diagnose suspected disease in symptomatic patients or to identify asymptomatic individuals in the context of screening. The difference between a genetic screening test, which does not give a definite result, and a diagnostic test (verification) is often not well understood. In fetal medicine, screening tests perform a risk assessment for congenital disorders usually in asymptomatic individuals without abnormal family or personal history, i.e. in the so-called low-risk patient.

Any **screening test** must fulfil several criteria to justify its general use. The difference between a screening test (assessing risk in apparently normal pregnancies) and a diagnostic test (verifying the presence of absence of a disease) is often misunderstood. Medical and non-medical reasons influence implementation of a particular screening test. Medical and non-medical reasons influence the use of screening tests. Screening tests require (occasionally extensive) counselling to avoid unnecessary costs or unwarranted or harmful interventions in pregnancy (WHO 2020).

Diagnostic tests are used to detect or exclude a specific condition such as a genetic cause for the fetal anomalies seen on ultrasound.

Over the last decade, genetic testing has expanded rapidly along with the knowledge of genetic causes of diseases. More and more conditions can be diagnosed prenatally. This chapter aims to provide a practical and clinical approach to genetic tests available in fetal medicine today.

Screening tests and diagnostic tests

Screening tests

Screening is testing of asymptomatic individuals to assess the risk for a particular disease (Tutschek et al. 2002, WHO 2020). The condition that is screened for must neither be extremely rare nor very common: Screening for very rare diseases in a general population cannot be justified since it may have high costs to be performed in the entire population with an effect (detection) in only few individuals and many false alarms. Screening for very common conditions is not applicable either.

With few exceptions, screening tests do not diagnose disease directly, but identify a higher-risk group that then may go on to diagnostic testing. Screening tests can reduce morbidity or mortality by early detection and treatment, for example mammography screening for breast cancer, measuring blood prostate specific antigen for prostate cancer detection and measuring blood cholesterol levels to identify individuals at higher risk for coronary heart disease.
The main criteria for screening tests are that (1) the patient understands the aim of the test and gives consent to the test performed, (2) that the condition screened for is medically relevant, recognizable and treatable; (3) screening does not create excessive cost or harm; and (4) early detection of the condition is advantageous in terms of treatment. Epidemiologically, screening tests are followed by secondary preventive measures (as opposed to primary prevention, i.e., preventing the development of a condition). Table 1 lists general principles for screening tests proposed by the World Health Organization.

1. The disorder should be a significant health problem.
2. There must be recognized treatment for detected cases.
3. There must be facilities that diagnose and treat.
4. There must be a recognizable latent or early symptomatic stage.
5. There must be an appropriate test.
6. The test must be accepted by the population.
7. There should be sufficient research on the natural history of the disease, including the progression from the latent to the clinically symptomatic stage.
8. There should be agreement on who should be treated as a patient.
9. Costs should be reasonable.
10. Discovery of cases should be a continuous process and not a one-time exercise.

Tab. 1 Principles of screening tests (adapted from Wilson and Jungner 1968)

Screening tests in fetal medicine have evolved greatly with new techniques becoming available. Historically, pregnant women over the age of 35 were classified “at high risk” for offspring with chromosomal abnormalities (commonly trisomy 21) and were offered invasive testing and diagnosis. However, at that time, only about 30% of pregnancies with trisomy 21 were identified using this approach, since 70% of babies with trisomy 21 were born to those younger than 35 years. In addition, many women over 35 underwent an unnecessary invasive procedure that carries a risk of miscarriage. Today, screening for fetal Down syndrome has a detection rate of up to over 99%, and between 5% and less than 1% of pregnant women will

receive an abnormal screening test result. “Combined first trimester screening” combines the measurement of the nuchal translucency and crown-rump length (typically between 45 and 84 mm, corresponding to 11 to 14 postmenstrual weeks), maternal age and the maternal serum parameters free beta-hCG and PAPP-A to calculate a risk for the three most common autosomal trisomies. Initially a side effect, it is now recognized that an increased NT and/or pathological biochemical markers can also identify fetuses at risk for structural fetal anomalies including heart defects, other aneuploidies and Mendelian disorders including the so-called RASopathies (Sinajon et al. 2020, Mastromoro 2022). In the presence of an increased NT direct chromosomal testing (aneuploidies, gene mutations) should be discussed.
In clinical practice, counselling patients about screening tests is demanding. The patient needs to be informed about the condition for which screening is offered; what the likely outcomes are; and that screening tests usually do not give a definite diagnosis but categorizes individuals usually as “low risk” or “high risk”. After a “high risk” result, the patient is usually offered a diagnostic test (in case of aneuploidy risk chorionic villus sampling, CVS, or amniocentesis, AC) to confirm or rule out the at-risk condition.
Caregivers and patients must also be aware of the emotional and social aspects of a “high-risk” screening result and of a definite, abnormal diagnosis from invasive testing before screening is performed.
The aim pf prenatal screening is to detect conditions on the fetus and provide information to the expecting parents to enable them to make informed choices (WHO 2020). The WHO has adopted strict theoretical criteria for screening tests proposed by Wilson and Jungner (1986), but in reality not all screening tests in clinical use fulfil these criteria.

Diagnostic tests

Diagnostic tests for genetic conditions in fetal medicine are usually performed on fetal material obtained by invasive procedures (typically chorionic villus sampling and amniocentesis). These procedures carry a small risk of miscarriage, which in experienced hands is less than 0,5%.
Counselling also before an invasive procedure is essential. The overall aim of offering tests (screening or diagnostic) must be the usefulness of a test result to the pregnant women, respecting and enabling autonomy. The clinical challenge in selecting the correct test is to identify its utility to the patient and to obtain the informed consent before executing any test.
Regardless of maternal age, all women should be given information about screening and follow-up diagnostic testing. In the United States it is recommended that all women regardless of maternal age can also be offered diagnostic testing.

Parameters to quantify and compare screening tests

It is important to understand test performance parameters in general and genetic tests in particular (Tutschek et al. 2002, WHO 2020). There are several characteristics or terms used to explain test properties.

Detection rate (DR) and false positive rate (FPR)

The detection rate (DR) refers to the subgroup within the studied population that has an abnormal screening test for a condition **and** that is also affected by the condition. The DR is expressed as a proportion of the truly affected, not of the entire population studied. Ideally, a screening test should detect all affected individuals (100% detection rate). Screening tests have a DR of between 60 and 99%. The higher the DR, the better the test.

By far not all individuals with an abnormal screening ("screen positives") are also truly affected by the condition ("true positives"; see fig. 1). Indeed, typically, the majority of those labeled as "high risk" (screen positive) by screening are, in fact, not affected; they are "false positives".

If screening detects 85 out of 100 of the affected individuals for fixed other screening parameters, the detection rate is 85%.

Screening is never fully specific, i.e. screening will always "identify" individuals as "possibly affected" or at "high risk" who are, indeed, unaffected. These are called "false positives". Their proportion, expressed as fraction of the entire screened population, is the false positive rate (FPR). The proportion of individuals labeled by screening as "positive" or "abnormal" comprises true positives and false positives. Typically, in screening, there are more false positives than the true positives, which explains the unwarranted anxiety often caused by screening. The lower the FPR, the better the test.

The FPR can also be understood as the proportion of the population that will have to be offered definitive (diagnostic) testing. Because (1) the condition for which screening is performed is usually uncommon and (2) screening is not fully specific, the FPR is roughly equal to the rate of diagnostic testing that will have to be offered. For example, combined screening for trisomy 21 by maternal age, nuchal translucency and serum biochemistry has a FPR of 5% and a DR of 85%, of short "85% DR for 5% FPR": The entire population will be offered screening, which sort all individuals by their risks. The top 5% in terms of calculated risk will have to undergo diagnostic testing to detect 85% of cases of Down syndrome. Of these 5% screen positives, most well still be unaffected. Combined screening formally has a low "positive predictive value" (see below).

For trisomy 21 screening only, done by “non-invasive prenatal testing” (NIPT, see below), the DR is more than 99% for a FPR of only 0,3% (DR 99% for FPR=0,3%). Hence, NIPT technically is the better test for Down syndrome screening. Is NIPT, therefore, “better” than combined screening? This depends on other factors beyond mere DR and FPR: NT-based screening requires an anatomical assessment with attention to small details, which provides very useful additional, anatomical information beyond a mere risk assessment for trisomy 21, e.g. the option to detect structural anomalies early in pregnancy.

Another important statistical term to describe abnormal screening test results is the positive predictive value (PPV). The PPV indicates, how often an abnormal screening test result correctly predicts the presence of the condition. NIPT for example has a very high PPV for trisomy 21, mirroring its low FPR for Down syndrome: If NIPT is positive for trisomy 21, unfortunately, fetal Down syndrome is very likely, but not proven. Proof can only be achieved with a diagnostic test. If combined screening is positive for trisomy 21, it is still far more likely that the fetus will not have Down syndrome (low PPV), but further testing is required. Tests with a low PPV may be very useful, but require specific prior counseling, addressing also their PPV.

Only diagnostic testing can diagnose or exclude all affected individuals, but it usually has higher cost and/or a risk due to the test itself, such as miscarriage after invasive testing.

Table 1 lists typical screening and diagnostic tests used in fetal medicine.

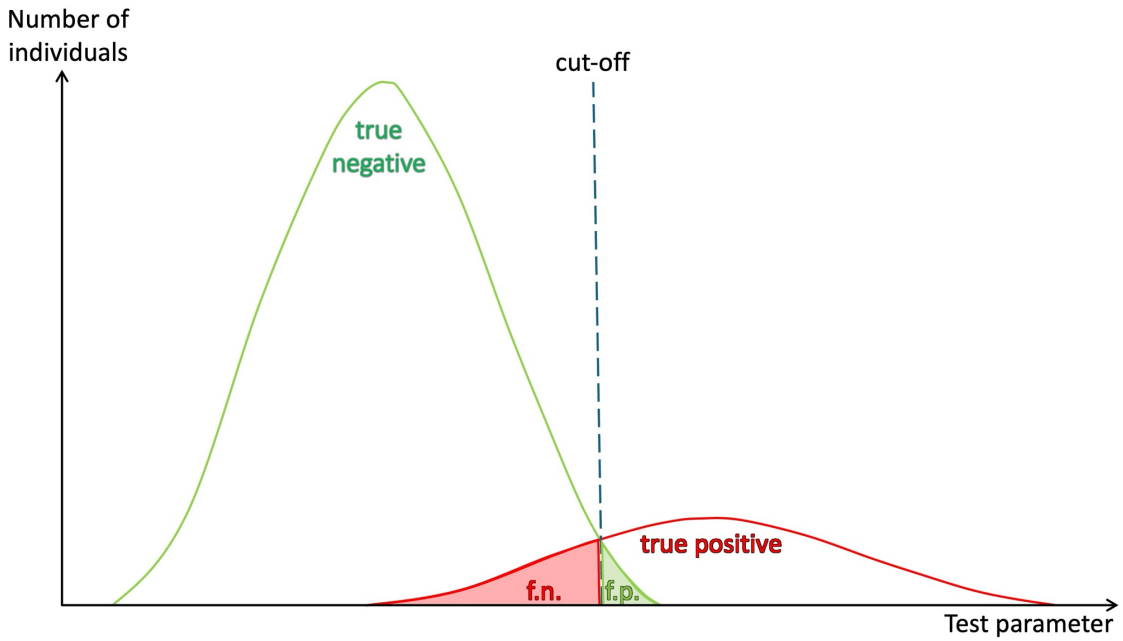


Fig. 1 Screening test to detect as many affected individuals as possible (true positive, few false negatives) while avoiding “identification” of actually unaffected individuals (false positives), using a cut-off for a test parameter which increases the risk of being affected. f.n.: false negative; f.p.: false positives.

Type	*Aim*
Screening tests	
Parental carrier screening for recessive and X-linked disorders	Identification of pregnancies at risk for inherited Mendelian disorders
Viability scan in early pregnancy (CRL < 45 mm) *	confirmation of viability, detection of multiple gestation *
first trimester ultrasound at time of nuchal translucency screening (CRL 45 – 84 mm)	combined risk assessment for trisomy 13, 18, 21; "early anomaly scan" *
Second trimester biochemical marker screening	risk assessment for trisomy 13, 18, 21
Non-Invasive Prenatal Testing (NIPT)	screening for common trisomies or genomewide screening for aneuploidies and copy number changes
Second trimester maternal blood screening using alpha-fetoprotein (msAFP)	Detection of cases at risk for spina bifida
Anatomy/anomaly scan (often as second trimester screening) *	Assessment of fetal anatomy and growth*, detection of placental abnormalities
Diagnostic tests	
Chorionic Villus Sampling (CVS)/placental biopsy	Source for cytogenetic and molecular tests
Amniocentesis (AC)	Source for cytogenetic and molecular tests and to detect fetal infection
Other invasive fetal sampling (e.g. chordocentesis, pleural effusions tap, bladder tap)	Source for cytogenetic and molecular diagnostics, biochemical tests; may occasionally be therapeutic

Tab. 1 Typical tests used for fetal screening or diagnosis. * may also be (fully or partially) diagnostic

Genetic techniques in fetal medicine

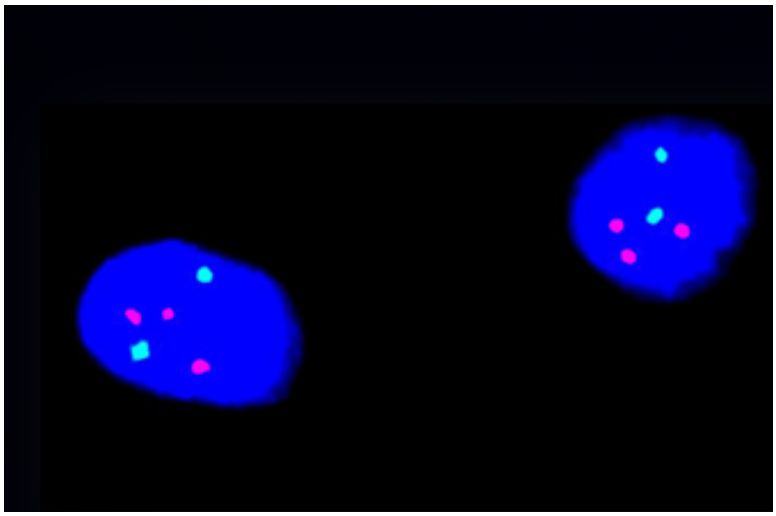
Chromosome analysis with FISH-technique (fluorescence-in-situ-hybridization)

FISH (Fluorescence-in-situ-hybridization) technique uses fluorescent probes that bind to chromosomal DNA in both metaphase and interphase chromosomes (fig. 2; Klinger et al 1992). This allows rapid information in non-dividing cells, for example uncultured amniotic fluid cells. The most used FISH probes target specific regions on chromosomes 13, 18, 21 and the sex chromosomes, allowing for rapid detection of aneuploidy involving these chromosomes[2].

FISH can be used both on uncultured and cultured cells. In fetal medicine, most FISH analyses are performed on uncultured chorionic villi or amniocytes and give results after 24 –

48 hours. FISH analysis can only distinguish numerical anomalies for the chromosomes that the probes are specific for. Studies for structural chromosomal imbalances require either chromosomal microarray analysis or conventional karyotyping (light microscopy from metaphase preparations).

A.



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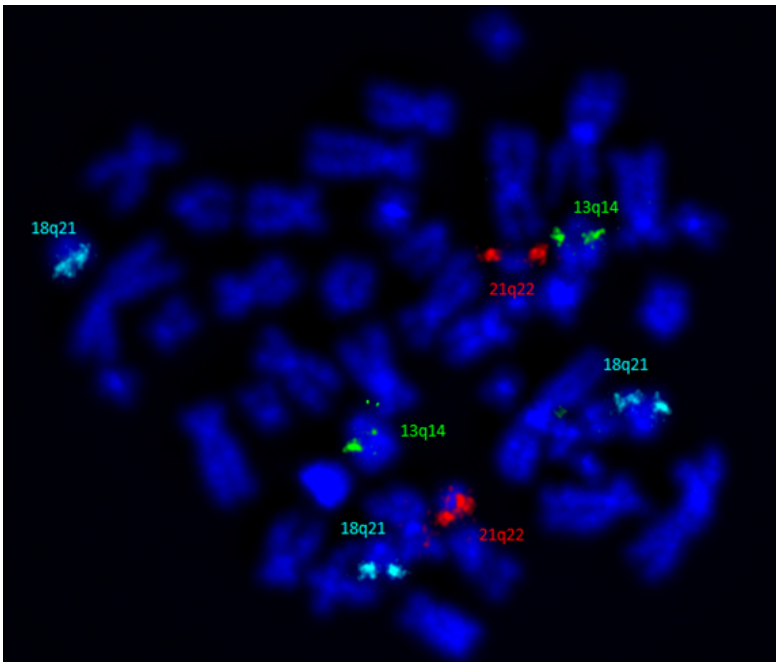

Fig. 2 Microscopic examples of locus-specific fluorescence in-situ hybridisation (FISH) for common trisomies. A. Two interphase nuclei (DAPI stained in royal blue) with three red signals for a chromosome 21 specific probe and two green signals for a chromosome 13 specific probe indicating trisomy 21. B. Metaphase chromosome spread of one nucleus after direct preparation of chorionic villi (all chromosomes stained in royal blue by DAPI) with specific probes for chromosome 13 (region q14, green; disomy 13), 18 (region q21, light blue; trisomy 18) and 21 (region q22, red; disomy 21) Note double dots per chromosome due to mitotic chromatid replication. *Images courtesy of University of Zurich, Institute of Medical Genetics.*

Chromosome analysis using quantitative fluorescent polymerase chain reaction QF-PCR)

QF-PCR uses specific amplification of chromosome-specific DNA sequences (short tandem repeat marker, STR), using fluorescence, in a multiplex reaction. The amplified DNA segments can be identified by their fluorescence intensity, representing the amount of product, and fragment size, appearing as "peaks" in the automatic analysis. A diploid (normal) fetus will show either two peaks with an approximate peak ratio of 1:1 if it is heterozygous for the investigated STR, or one (larger) peak, if it is homozygous (non-informative) for each chromosome analyzed. Trisomies are visualized either as three peaks or as two peaks with a 2:1 peak area ratio[3] for the markers of the respective chromosome. QF-PCR can provide results within 24-48 hours (Nicolini et al. 2004). Like FISH, QF-PCR cannot detect structural chromosomal imbalances beyond the specificity of the primers used. It should be followed by chromosomal microarray analysis or conventional karyotyping. QF-PCR with several markers for a particular chromosome studied increases its specificity. FISH has a higher sensitivity to detect and quantify mosaicism, because it allows assessment of many individual cells. This is particularly useful in suspected sex chromosome anomalies.

Chromosomal microarray (CMA)

CMA or "array" refers to molecular karyotyping, using tiny arrays of specific chromosome sequences to which patient chromosome fragments are bound ("hybridized"; Oneda et al. 2017). Clinically, two microarray techniques are used: In "array comparative genomic hybridization" (aCGH) DNA from both the patient and a normal control sample are labelled with different fluorochromes and hybridized to complementary DNA probes on a chip. The fluorescent intensities at every specific site are measured, and the fluorescence intensities of differentially labeled patient and control sample fragments bound to every site are compared. Abnormal ratios between the patient and control sample show aneuploidies and copy number variants (CNV, i.e. deletions or duplications; see fig. 3). The second microarray technique uses single nucleotide polymorphisms (SNP) to assess the number of alleles in a sample. SNP-based microarrays have the advantage of being able to detect triploidy, low-level mosaicism and regions of homozygosity suggestive of either parental consanguinity or uniparental disomy[4].

CMA resolution differs according to the number of DNA probes used on the array platform but can detect CNVs up to a few kilobases. Balanced rearranged chromosomes (balanced translocations) cannot be detected by CMA.

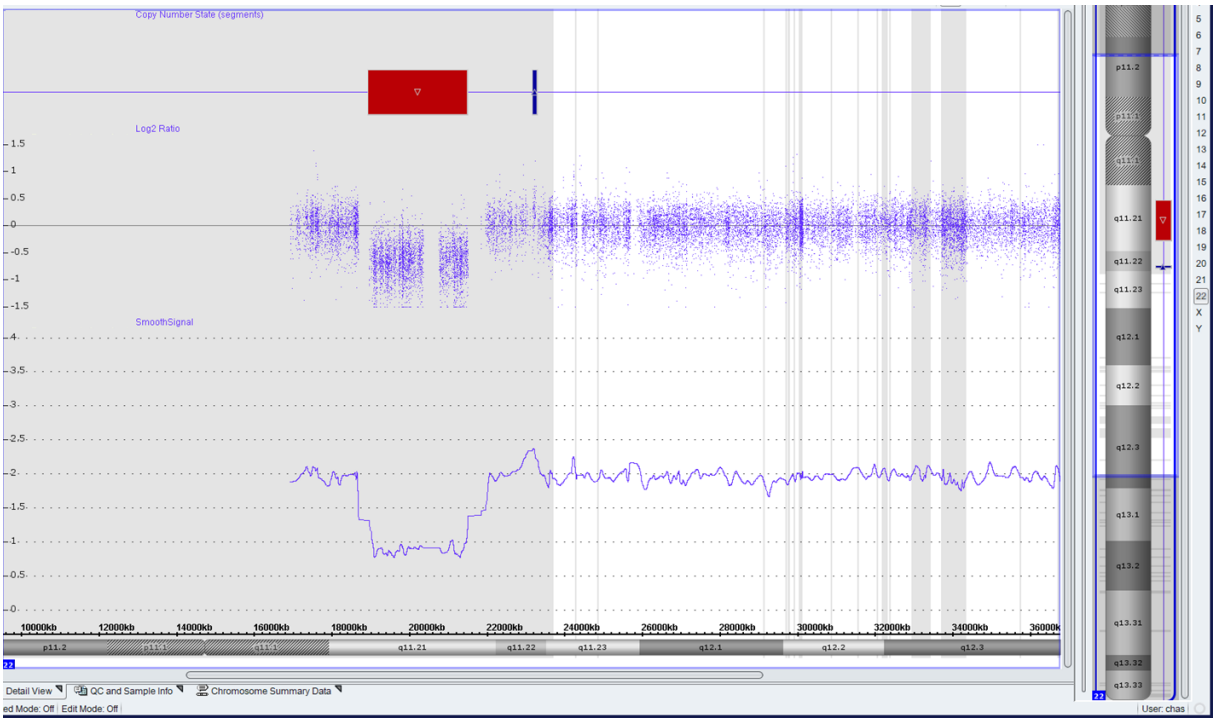

Fig. 3 Chromosomal microarray analysis to detect copy number changes of chromosomal material. In this example, the relative fluorescent intensities of patient DNA bound to many loci on chromosome 22 (displayed on the Y axes on the left) are examined. The equivalent of the conventional G-banding locations is used as the X axis along with the chromosomal length in kilobases. There is a typical microdeletion 22q11.2, associated among others with the DiGeorge syndrome phenotype. *Image courtesy of University of Zurich, Institute of Medical Genetics.*

Conventional karyotyping

Conventional karyotyping (microscopic analysis of metaphase chromosomes) can detect numerical aneuploidies (including trisomies and monosomies), relatively large chromosomal aberrations (>5-10Mb; partial aneuploidy) and sufficiently large translocations. The sample needs to be cultured to accumulate cells undergoing mitosis. The cells are arrested in metaphase by blocking mitotic spindle formation and stained to show characteristic banding patterns when analyzed light-microscopically (fig. 4). As dividing cells are required, and results can usually only be achieved after successful cell culture, typically only after 7-10 days.

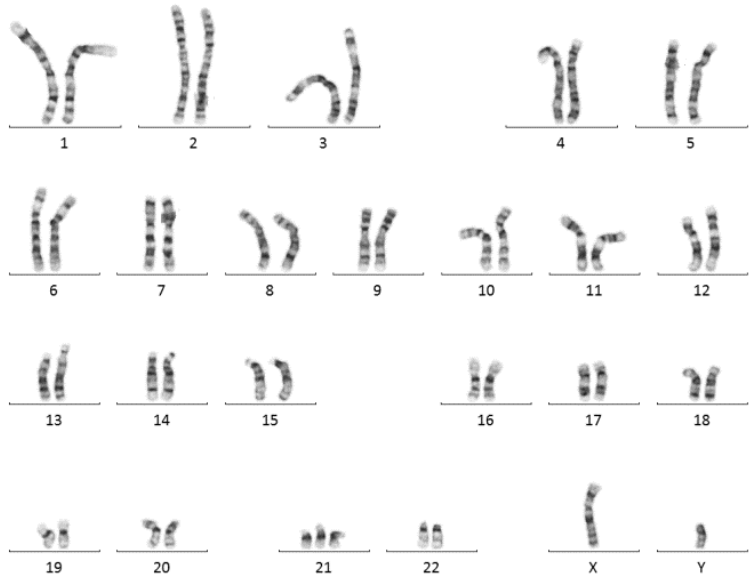

Fig. 4 Conventional metaphase karyotype after G-banding, light microscopy. The chromosomes are arranged by size and banding pattern, and there is a free trisomy 21 in a male patient (47,XY,+21). *Image courtesy of University of Zurich, Institute of Medical Genetics.*

Sequencing

In conventional or "Sanger" sequencing, the nucleotide sequence of DNA is analyzed using primers for specific DNA regions. Sanger sequencing is used to detect point mutations and short sequence alterations and is an effective way to analyze single, specific DNA sites, to test for familial variants and to validate next generation sequencing (NGS) results. Sanger sequencing is not effective for larger DNA regions.

Multiplex ligation-dependent analysis (MLPA)

Multiplex ligation-dependent analysis (MLPA) is used to detect copy number variants (CNV, deletions and duplications) on the level of exons. Exons are the regions that code for amino acids during processing of the RNA transcript, whereas introns are the sequences found between exons. MLPA is often used in combination with sequencing to examine genes with both point mutations or CNV.

Next generation sequencing (exome or genome sequencing)

Next generation sequencing (NGS) is used to detect DNA variants (point mutations and short sequence alterations), but automatically and on a much larger scale than Sanger sequencing.

Through massive parallel sequencing, many genes can be sequenced at the same time; some specific regions or mutational mechanisms, however, will still require targeted testing. In addition, these sequencing data can be used for copy-number analysis. In whole exome sequencing (WES), only the protein-coding regions of genes (exons) are analyzed (1-2 % of the genome). In whole-genome-sequencing (WGS), the entire genome (exon and introns) is sequenced. Due to limitations in interpreting non-coding variants, analysis of WGS data is usually also focused on the coding regions (exons) but has a much higher sensitivity and specificity for copy-number changes than WES data. Currently WES analysis can be achieved at a much lower cost and with less input material than WGS. In some cases, WGS may be required to give an additional diagnostic yield over WES (Grether et al. 2023).
Genome sequencing is required in cases with markedly increased nuchal translucency or (other) structural anomalies that have a normal microarray result (Mastromoro et al. 2022).

NIPT (non-invasive prenatal testing)

Non-invasive prenatal testing (NIPT) from peripheral maternal blood has been used in aneuploidy screening and fetal RhD genotyping since 2012. It requires a maternal blood sample and analyses cell-free fetal DNA (cfDNA; Bianchi et al. 2004). The majority of fetal cfDNA circulating in maternal plasma is derived from the syncytiotrophoblast layer of the placenta[5]. Fetal cfDNA can be detected in the maternal plasma before seven weeks of gestation and increases with gestational age. The ratio of fetal cfDNA compared to maternal cfDNA is called the fetal fraction and varies among pregnant women. cfDNA can be analyzed through different techniques using next generation sequencing, a targeted approach with SNP or microarray analysis.
Standard NIPT targets the common aneuploidies (trisomy 13, 18, 21) and the sex chromosomes only. “Genome-wide” (GW) NIPT expands analysis to all chromosomes (1-22 and the sex chromosomes) and can detect CNVs (deletions and duplications) up to a size of 2 – 3 Mb depending on the technique used, the chromosome segment in question and the fetal fraction (Oneda et al. 2020). An example for a genetic condition detectable with a GW NIPT is the 2.5-3 Mb deletion on 22q11.2 causing DiGeorge syndrome.
Since the cfDNA analyzed in NIPT (regardless of the technique used) is mainly derived from the syncytiotrophoblast layer of the placenta, NIPT may give false positive results in cases with confined placental mosaicism (Reilly et al. 2023) and represents more a screening than a diagnostic test. A diagnostic test (CVS or AC) must be offered to confirm or rule out the

condition suspected on NIPT in the fetus proper. Multiple reports of false positive and false negative NIPT in euploid fetuses exist to confirm this recommendation.

Test	*Objective*	*Comment*
QF-PCR or FISH	aneuploidy testing for specific chromosomes	limited to a number of aneuploidies that can be tested at once
Conventional karyotyping (microscopic analysis of metaphase chromosome)	Diagnosis of aneuploidy or major structural chromosomal anomalies	Requires cell culture, resolution for the detection of deletion/duplication is commonly ~10-20 Mb
Chromosomal microarray	Detailed analysis of chromosomal copy number for detection of aneuploidy including deletions and duplications	Detection of deletions/duplications at the submicroscopic level (< 5-10 Mb); SNP arrays: low-level aneuploidy mosaics, parental consanguinity or uniparental disomy
Targeted gene analysis	Exclusion or confirmation of specific genetic anomalies suspected on prenatal ultrasound or known from family history	DNA sequence analysis is definitive if the familial variant is known and maternal contamination has been excluded
Whole exome sequencing (WES) or whole genome sequencing (WGS)	Targeting of most clinically known genes or of gene panels relevant for the fetal anomalies	“one step” approach to genetic disease etiology (e.g. incase of abnormal ultrasound findings) for Mendelian disorders; variants of unknown significance can confound interpretation
Polygenic risk score or genome wide association (GWAS)	Identification of multiple genomic regions associated with structural birth defects (e.g. cleft lip, hypospadias)	At present a research tool to assess common traits with normal distribution or novel susceptibility genes for complex disorders

Tab. 2 Genetic tests possible from on pure fetal material obtained using an invasive procedure

Clinical scenarios

The historical distinction between “low risk” and “high risk” pregnancies is no longer applicable. Rather, individual risk spectrums for a of number condition can be recognized; then appropriate testing should be offered. Some condition such as the autosomal trisomies increase with maternal age, but others may affect predominantly younger pregnant women (e.g. gastroschisis).

Anamnestic and family risks

Familial risk factors can include a family member with a chromosomal abnormality or a single gene disorder or an unclarified anomaly that could be chromosomal or genetic in origin. Affected family members may constitute a surprising risk factor, for example in case of familial translocations that may be passed on through healthy family members (carriers), who have a balanced form, but convey a risk to their offspring for an unbalanced form of up to 50%.

Depending on the type of genetic condition, the recurrence risk in a family with an affected child in a subsequent pregnancy for a child with the same genetic condition ranges from 1–2% in suspected or diagnosed “de novo” aneuploidies or mutations (this empirical risk is attributed to potential germ line mosaicism) to 25 % in recessive diseases to 50% in dominant diseases or familial translocations.

"Marker(s)" or fetal anomalies detected by ultrasound

Ultrasound findings can indicate certain genetic risks. Traditionally, structural fetal anomalies are considered “hard markers”, often carrying chromosomal or genetic risks in the double-digit percent range. In contrast, “soft markers” are often variants of the norm, present predominantly in healthy individuals. Soft markers usually only mildly increase the genetic risk.

A special “soft” marker is the increased nuchal translucency because of its strong association with certain anomalies if markedly increased. For example, a markedly increased nuchal translucency with cystic hygroma at 12 weeks raises the suspicion of Turner syndrome. First-line genetic tests for Turner syndrome should include chromosomal analysis (FISH or QF-PCR and/or microscopic karyotyping).

Increased nuchal translucency and an atrioventricular defect (AVSD) at 12 weeks for example might indicate trisomy 21. First-line genetic testing should also include chromosomal analysis (FISH or QF-PCR and/or microscopic karyotyping; Morlando et al. 2017).

Another example for “hard markers” detectable by ultrasound, i.e. fetal malformations, are fetal long bones much shorter than expected for the gestational age, short ribs and, possibly, curved femora and an unusual head shape. These findings point towards thanatophoric dysplasia type (TD), one of the most common lethal fetal skeletal dysplasias. First-line diagnostic testing should target the known causative gene (*FGFR3*).
In fetuses with major ultrasound anomalies or markedly increased nuchal translucency without a clear suspicion for a certain condition, high-resolution chromosomal microarray analysis and, if found normal, a search for gene mutations by high-throughput sequencing should be offered (Mastromoro 2022).

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Recommended reading

WHO. Screening programmes: a short guide. Increase effectiveness, maximize benefits, minimize harm. World Health Organization 2020, ISBN 978 92 890 5478 2 (https://iris.who.int/bitstream/handle/10665/330829/9789289054782-eng.pdf?sequence=1)

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