INVASIVE DIAGNOSTIC PROCEDURES IN MULTIPLE PREGNANCIES
INVAZIVNI DIJAGNOSTI^KI POSTUPCI U VI[EPODNOJ TRUDNO\]I

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SUMMARY. Over the past few years, the rising rate of multiple pregnancies, attributed to both increasing reliance on infertility treatment modalities and delayed childbearing, has expanded the need for prenatal invasive genetic testing. In multiples, first-trimester chorionic villus sampling and second-trimester amniocentesis are relatively safe and efficient alternative procedures, whereas fetal blood sampling is reserved for cases where an indefinite result of fetal karyotyping needs elucidation. The choice of invasive technique should be based on gestational age at referral date, procedure related risks and technical demands, but experience of the center performing the modality should be emphasized in decision making. Technological advances in modern high resolution ultrasound equipment along with increasing operator experience available today result in more accurate and efficacious invasive prenatal diagnosis in twin or higher-order pregnancies, minimizing potential post-procedural fetal loss rate.

INTRODUCTION

The prevalence of multiple pregnancy varies worldwide from 6.7 per 1000 deliveries in Japan to 40 per 1000 deliveries in Nigeria. The respective prevalence in Europe and North America is estimated to be 11 per 1000 deliveries. The incidence of multiple births has increased dramatically over the past three decades since in vitro fertilization (IVF) was first introduced in modern obstetrics and gynaecology. Tremendous advances achieved in assisted reproductive techniques (ART) including IVF and non-ART procedures such as ovulation induction, rendered infertility treatment increasingly popular among infertile and subfertile couples. It has been postulated that women undergoing ovulation induction have an approximately 6% chance of conceiving multiples.1 Furthermore, implementation of ART is accompanied by a 35% chance or more of accomplishing twin or higher-order pregnancy.2 Hence, increasing reliance on assisted conception modalities noted nowadays, has been considered the major causative factor of the rising rate of multiple pregnancies. In a lesser degree, delayed childbearing in progressively advanced maternal age currently adopted by many prospective mothers accounts for the rising rate of spontaneously conceived multiples.

There is no doubt that infertility treatment is associated with an increase in the rate of monozygotic (MZ) twins to greater than 10-fold, the latter being at high risk of functional and structural abnormalities, affecting 10–15% of these twins.3–5 On the other hand, the incidence of chromosomal abnormalities is strongly related to maternal age. Available data confirms that twin pregnancies per se are at increased risk for fetal chromosomal abnormalities than those with singletons.6,7 Therefore, the increasing incidence of multiple pregnancies illustrates a concomitant increase in the need for invasive genetic testing in these pregnancies.

Zygocity and chorionicity

It is widely accepted that the number of fetuses itself does increase possible maternal and fetal risks and
Thereby the potential of an adverse pregnancy outcome. However, the cornerstone in prenatal diagnosis, surveillance and management of a multiple gestation is chorionicity as well as zygocity determination. Chorionicity refers to the placentation whereas zygocity implies the genetic profile of the pregnancy and therefore determines the degree of risk and whether or not the fetuses may be concordant or discordant for chromosomal abnormalities.

It is estimated that more than 30% of twins are identical or MZ and nearly 70% are fraternal or dizygotic (DZ). **Monozigotic twins** originate from the division of a single fertilized ovum with an incidence rate of about 3.5/1000 pregnancies. \(^8\) The rate of spontaneous MZ twin pregnancies is constant contrary to the increased frequency reported among pregnancies derived from infertility treatment modalities. MZ twins may be dichorionic diamniotic (DC-DA), monochorionic diamniotic (MC-DA), monochorionic monoamniotic (MC-MA), and even conjoined, determined by the period of embryonic development when zygotic splitting takes place. In about 20–30% of cases, splitting occurs within three days of fertilization resulting in separate fetuses with independent placental circulations, therefore being DC-DA, even if placentas may seem to be in continuity or fused. In the majority of cases (about 70%) splitting occurs within the first week but later than the third day, it results in a single monochorionic plate and two distinct amniotic sacs, hence MC-DA twins originate. Delayed zygotic splitting leads to MC-MA twins, accounting for 1% of MZ twins, though later than 13\(^{th}\) day is extremely rare, resulting in the formation of the abnormal conjoined (Siamese) twins.

**Dizygotic twins** arise from the fertilization of two distinct ova, thus may be of the same or different sex. Each twin has its own placenta and amniotic sac (DC-DA). Very infrequent cases of MC-DZ twins originating from the fusion of two separate blastocysts have been recently reported in association with ART, \(^9\) staggering the categorical general rule by far having been accepted in obstetrics that MC twins are exclusively MZ. \(^10\) The incidence rate of DZ twins varies significantly, influenced by race (higher in blacks, lower in Asians), heredity, maternal age (peak between 35–40 years of age), history of previous DZ twin pregnancy, nutrition habitus and anthropometric features (height and weight) of the woman.

ART including in vitro fertilization and non ART procedures such as ovulation induction and subsequent intrauterine insemination using human pituitary gonadotrophic hormones increase the incidence of multiple pregnancy, both MZ and DZ, while clomiphene citrate increases the occurrence rate of DZ pregnancies to about 5–10%. \(^3,3.11\)

It should be emphasized the general aspect that the incidence of multiple pregnancies is correlated with increasing maternal age stands for DZ twins. It is well established that the frequency of MZ twinning remains relatively constant, independent of the age of the woman.

**The risk of structural anomalies in multiple gestations**

The incidence of structural anomalies in twins is higher compared to singletons. However, the frequency of malformations in DZ twins is thought to be similar to that of singletons (2–3%) contrary to that observed to MZ, which has been reported to be 2–3 times higher. \(^12\)

The exact underlying mechanism of the increased prevalence of structural defects in MZ twins remains obscure, although the teratogenic nature of the twinning process itself and vascular events occurring during intrauterine development may account for part of them. Of interest, concordance (both fetuses similarly affected) for a structural anomaly, even in MZ twins, is rare (less than 20%). \(^13\)

Neural tube defects, anencephaly, holoprosencephaly, sirenomelia complex, cloacal extrophy and abnormalities that fit into the expanded VATER/VACTERL associations are more common in MZ twins. However, the risk of fetal abnormalities in twins may be biased, because multiple pregnancies are intensively scanned, increasing the chances of detecting underlying anomalies. Moreover, twinning is much more common in women of advanced age, in whom prenatal screening is more likely to yield the diagnosis of fetal defects as far as aging is associated with increased risk for fetal abnormalities.

**The risk of chromosomal abnormalities in multiple gestations**

Inasmuch as zygocity represents the genetic make-up of the developing entity, accurate determination of this parameter is considered a prerequisite in multiple pregnancy prenatal screening for aneuploidies. In the clinical setting, zygocity is usually inferred from the ultrasound diagnosis of chorionicity, \(^7\) the latter best achieved in the first trimester. In DA pregnancies with fused placentas, measurements of the thickness of the dividing membrane using a cut-off value of 2 mm can differentiate MC from DC twinning, though a high inter and intra-observer variability has been reported. Sonographic detection of the »lambda« or »twins peak« sign is reported as a more reliable indicator of DC placentation with an accuracy of 100% at 10–14 weeks’ gestation. \(^14\) Delayed in the second trimester sonographic evaluation is associated with a 10–12% chorionicity misinterpretation rate, \(^15,16\) while after 20 weeks’ gestation the determination may turn out impossible. Therefore, in the absence of the »lambda« or »twins peak« sign in a DA twin pregnancy, single placentation and monogygocity is concluded, when the rare cases of MC-DZ gestations following ART reported are not taken into consideration. However, when a single amniotic sac is detected, monochorionicity is indisputable. Given that the great proportion (80–90%) of DC twins are DZ, \(^17,18\) chorionicity may roughly correspond to zygocity. \(^19\)

MZ twins are almost always of the same sex and genetically identical and therefore the risk for chromo-
Prenatal screening for fetal anomalies in multiples

Unambiguously, it has been a common practice to extrapolate data derived from singletons to multiples. However, implementation of ultrasound as well as maternal serum analyze screening for fetal abnormalities in twins or higher-order pregnancies, seems to be more complex. Cautious interpretation of screening results is considered mandatory in order to minimize possible erroneous high false positive rate and subsequent high rate of undue invasive procedures.

Given that chorionicity has been definitely determined, in DC multiple pregnancies, first-trimester ultrasound scan offer an invaluable aid in fetal risk assessment for chromosomal abnormalities. In particular, fetal nuchal translucency (NT) screening has yielded comparable results regarding detection rates as well as false positive rates with singleton pregnancies.29

In MC twins, a cautious evaluation of increased NT thickness should be reserved in respect to the possible early twin-to-twin transfusion syndrome (TTTS) origin of this finding. A rational approach has been proposed to be the application of the average value of NT measurement of both fetuses in risk assessment in an effort to reduce misinterpretation.30

The inability to determine the degree to which each fetus contributes to the overall maternal biochemistry level may reflect a significant shortcoming in first trimester biomarkers’ sensitivity and specificity in multiple gestations. Nevertheless, the altered biochemical markers of the aneuploid fetoplacental unit are partly masked by the normal biochemical profile of the euploid co-twin.

Since the application of maternal biochemistry in risk assessment for aneuploides in twin or higher-order multiple gestations remains arguable, ultrasound scan has been proven of great value for early determination of chorionicity and subsequent standardized NT measurement as well as genetic sonogram targeting to illuminate possible sonographic markers of fetal aneuploidy.

Invasive procedures for prenatal diagnosis

It is uniformly accepted that invasive procedures for fetal karyotyping in twins or higher-order multiples are more challenging than in singletons. First-trimester chorionic villus sampling (CVS) and second-trimester amniocentesis are alternative techniques requiring experienced hands to ensure sampling both fetuses and minimize procedure related risks. Fetal blood sampling for genetic studies is rarely used today.

Amniocentesis

Genetic amniocentesis performed later than 15 weeks of gestation has been proven a safe and accurate procedure for sampling all fetuses of a multiple gestation. Currently, there is little information regarding the risk of amniocentesis between 13 and 15 weeks though this invasive procedure has been associated with increased risk for fetal loss, amniotic fluid leakage and fetal talipes equinovarous and therefore is not recommended.19,31,32

Amniocentesis in twins can be reached through a single or double uterine entry. Three methods of tapping multiple sacs have been described so far. One of them uses the single whereas the remaining two use the double uterine entry approach. Each technique can be performed either freehand or with a needle guide.

The first one, initially described by Elias et al in 1980, involves two or more needle insertions, one for each sac, also called the double needling technique or technique of double amniocentesis.33 In a twin or higher-order multiple pregnancy, two or more 22 gauge 3.5 inch spinal needles are separately and sequentially inserted transabdominally under ultrasound visualization into each sac and about 20 ml of amniotic fluid is readily aspirated and sent for cytogenetic evaluation or fetal karyotyping. A problem not infrequently faced with this
The needle entry is made into the proximal sac near the insertion of the dividing membrane and 20 ml of amniotic fluid are retrieved. After the stylet is replaced, the needle is advanced through the second sac under direct ultrasound guidance. In order to avoid contamination the first few milliliters of amniotic fluid are discarded and aspiration of 20 ml from the second sac integrates the procedure. Many advantages linked to this technique have been reported: requiring only one needle insertion and being swifter and shorter reduces woman’s discomfort as well as the risk of post-procedural complications. Moreover, advancing the needle through the septum between the two sacs under ultrasound guidance provides positive proof of tapping both of them, diminishing the need for dye insertion. However, potential disadvantages render this approach less popular. Possible contamination of the second sample with amniotic fluid and fetal cells from the first one, may lead to an incorrect diagnosis of mosaicism in the second fetus. This complication can be avoided by strictly adhering to the technique, by replacing the stylet prior to intertwin membrane penetration and by discarding the first few milliliters from the second sac. Besides, the possibility of converting DA to pseudo-MA twin pregnancy with the correspondence risks for cord entanglement and the formation of the amniotic band syndrome cannot be precluded. In addition, technical difficulty in penetrating a »tenting« dividing membrane has been reported.

Two years later, in 1992, the double simultaneous visualization technique or double simultaneous amniocentesis, was introduced by Bahado-Singh et al. This technique involves two needles inserted separately into the amniotic sacs under ultrasound visualization like in the technique of double amniocentesis. The difference is that after aspiration of the amniotic fluid from the first sac, the needle is left in place indicating the sampled cavity and the second insertion is made into the other sac. The main advantage seems to be the documentation of correct sampling from each sac. However, it is not widely used mainly because it is more time consuming and thereby the experience with this approach is limited.

Prenatal diagnostic invasive procedures and thus amniocentesis must be preceded by a detailed ultrasound evaluation of the multiple pregnancy involving chorionicity and amnionicity determination and documentation of the location of the placenta(s). Moreover, relative position, size, anatomy and gender (if possible) consisting distinguishing features of each fetus should be specified, and »labeling« of the multiples using text and diagram should be performed to ensure correct sampling from each of them. Recently, the role of amniotic fluid alpha-fetoprotein (AFAFP) values was evaluated in confirmation of both sacs in a DC pregnancy being sampled.

Concern regarding potential post-amniocentesis increase in fetal loss rate in multiples has led to a plethora of studies evaluating this parameter. Early reports suggested a higher fetal loss rate in twin pregnancies than in those with singletons. However, these studies did not take into account the possibility that the increased fetal wastage might be attributed to the twin pregnancy itself rather than the invasive procedure. Later on, it was reported that the maternal history of twins per se carries a pregnancy loss rate up to 24 weeks of about 6.3% and severe prematurity (24–28 weeks) rate of about 8%. Most series of single pregnancy outcome following second-trimester amniocentesis report loss rates before 20 weeks’ gestation of between 1% and 2.5% and a much higher loss rate before 28 weeks. In a multicenter European study, the pregnancy loss rate was estimated to be 2.3% and 3.7% before 20 and 28 weeks’ gestation respectively. In a case control study, a similar fetal loss rate was reported between sampled twins and unsampled matched twin controls (3.5% vs 3.2%).

In conclusion, amniocentesis in twin pregnancies is thought to be a relative safe and accurate diagnostic procedure providing that sampling involves both sacs regardless the zygocity and chorionicity.

Chorionic villus sampling

CVS, also called placental biopsy, is a safe alternative invasive procedure to amniocentesis for prenatal diagnosis in multiples. The major advantage of CVS is early diagnosis, obtained in the first trimester of pregnancy. In particular, genetic results are feasible either within hours by direct preparations of the cytotrophoblast layer, or within 3–7 days by tissue culture of chorionic villus mesenchymal core. Early diagnosis provides earlier reassurance of fetal well being and thereby eliminates both maternal anxiety and uncertainty. On the other hand, the diagnosis of one or both abnormal twins allows subsequent selective reduction of the affected fetus or surgical termination of pregnancy rather than
medical induction of labour as early as in the first tri-

mester where complication rates are lower. Moreover,
fetal reduction performed earlier in pregnancy may be
associated with a higher survival rate of the unaffected
twin. In terms of privacy and maternal psychology, the
earlier an abnormal pregnancy is terminated the lesser
the chance of being widely recognized.

CVS is best performed between 11 and 13 weeks’ ges-
tation. Data derived from singleton pregnancies illustrate
an association of CVS performed earlier in pregnancy
and fetal transverse limb abnormalities, micrognathia and
microglosia. In general, first-trimester CVS in multiple
gestations is technically more demanding than second-
trimester amniocentesis. Transabdominal as well as
transcervical approach have been used. Some suggest
that the highest success rates are achieved when the cli-

nician is comfortable using either technique. Transcervical
CVS is performed either by an aspiration catheter or by a
biopsy forceps. Technically, it may be more difficult to
perform and the “learning curve” appears to involve
many patients. Transabdominal approach uses an aspira-
tion needle and is technically more similar to second-tri-
mester amniocentesis and thus more familiar to the
majority of obstetricians. Both techniques can be performed
either freehand or with a needle guide.

Continuous ultrasound visualization of the tip of the
needle, catheter or biopsy forceps is essential to ensure
sampling both chorions. If in doubt, a follow-up pro-
dure should be performed either by an immediate repeat
CVS or by second-trimester amniocentesis. A serious
drawback of CVS is potential contamination of one
sample by villi belonging to the other chorion or less fre-
quent by maternal cells. At that case, a confusing or even
misleading diagnosis is unfortunately possible. Al-
though early studies suggested a contamination rate as
high as 4%, more recent studies report a much lower
rate, almost nullified. Still, Weisz and Rodeck sug-
gest that it would be prudent to counsel patients that
about 2–3% of twin pregnancies having CVS will need
re-sampling because of uncertainty of results.

In rare cases, the combination of transcervical and
transabdominal approach along with the increasing cli-
nician experience available today can eliminate such an
unfortunate possibility. Furthermore, obtaining samples
adjacent to the cord insertion site far away from the di-

viding membrane is reasonably recommended.

Genetic counseling must include the possibility of a
discordant abnormal result necessitating cautious inter-
pretation. Therefore, detailed documentation and »la-

beling« of the fetuses and the chorions is equally as im-
portant with CVS as it is with amniocentesis. Although
the position of sacs will remain unchanged during the
2–3 weeks-time following sampling, it is standard prac-
tice to re-confirm the original diagnosis in both fetal and
chorionic tissues before selective reduction of the af-

ected twin.

The estimated risk of CVS-associated fetal loss in sin-

gletons varies widely (1.3–4.3%). Two or more sam-

plings during one procedure have been linked to in-
creased risk of post-procedural miscarriage, implying that the risk may be higher in twin sampling. Overall an estimated risk of 2–4% in twin pregnancies has been reported. However, available data demonstrate significant variations. In one study, the risk of CVS-ass-

ociated fetal loss before 28 weeks’ gestation did not seem to differ between twin and singleton pregnancies (4.9 vs 4%). When only chromosomal normal preg-

nancies are considered, the overall loss rate found in a

study of 202 twin pregnancies that underwent CVS be-

came 3.7%, a figure that is considerably less than that of

amniocentesis. In another study, the pregnancy loss
rate before 20 weeks following CVS was found 3.3%
comparable to 2.8% in a control group of twin pregnan-
cies undergone amniocentesis. Hence, it may be claimed
that in experienced centers, CVS is as safe as amnion-
centesis for sampling twins.

The choice of invasive technique for fetal karyo-
typing should depend on the procedure related risks, on
accuracy of obtaining a result from both fetuses, on
technical demands and on clinicians’ experience. Gesta-
tional age at referral date may be crucial in decision
making. Eventually, is there a clear benefit of perform-
ing CVS than amniocentesis or vice versa that would
render one procedure by far superior than the other? The
answer is absolutely no. Amniocentesis is technically
easier and widely adopted, whereas CVS’s results are
available about one month earlier, thus therapeutic as
well as selective terminations are safer. However, it
should be emphasized that if the prenatal center is not
skilled and experienced in CVS, amniocentesis should
be preferred. A rational approach may be as follows:
the choice of invasive technique should be based on individ-
ual risk calculated from the combination of maternal age
and fetal NT thickness measured in the first trimester.
When the risk for a chromosomal defect, in at least one
of the fetuses, is greater than 1 in 50, it may be prefera-
table to perform CVS. For pregnancies with a lower risk,
amniocentesis after 15 weeks may be more appropriate.

Fetal blood-sampling

Fetal cordocentesis for prenatal genetic testing has
been previously used to validate abnormal findings in
amniocentesis or CVS. It has also been used in case that
a rapid chromosomal diagnosis (rapid karyotyping) was
pending, since the results are offered in 2–3 days-time.
Nowadays, novel molecular techniques allow accurate
rapid karyotype determination thereby limiting fetal
blood sampling’s application.

Likewise in singletons, cordocentesis in multiples is
technically challenging requiring skilled operators with
extensive experience in other invasive ultrasound-guid-
ded needle procedures, such as amniocentesis and CVS.
Umbilical cord is usually punctured proximal to its in-
sertion into the placenta. A needle guide or freehand

technique may be used.

In a study conducted in 2003, involving 84 twin preg-
nancies, mainly screened for hemoglobinopathies, the
overall procedure-related fetal loss (up to 2 weeks post-
procedurally) was 8.2%, about fourfold higher than the
consequence risk in singletons. However, this tech-
nique can be used as an alternative to amniocentesis af-
after 20 weeks’ gestation to confirm an abnormal karyo-
type in a DC pregnancy, when selective feticide is con-
sidered a few weeks after the initial procedure.21

Conclusions
In conclusion, the rising rate of multiple pregnancies
mainly attributed to the widely use of infertility treatment
modalities has increased the need for invasive genetic
studies in these pregnancies. Diagnosis of fetal aneu-
ploidies and genetic defects can be achieved by first-
trimester CVS or by second-trimester amniocente-
sis, whereas it is postulated that they are equally safe in
experienced hands. The choice of invasive procedure in
multiple pregnancies depends on several factors, but the
experience of the center performing the modality should
be emphasized in decision making. The indications of fe-
tal blood sampling are currently limited and progres-
sevly surrogated by novel molecular techniques imple-
mented in CVS or amniocentesis’ specimen. High reso-
lution ultrasound equipment available today, along with
increasing operator experience gained throughout the
years, results in more accurate and efficacious invasive
prenatal diagnosis in twin or higher-order pregnancies,
minimizing potential post-procedural fetal loss rate.

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