Monochorionicity: Unveiling the Pandora Box

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ABSTRACT

This article reviews the phenomenon of monozygosity by conducting an exhaustive literature review of this special type of twin pregnancies. An epidemiological update is presented showing the substantial contribution to perinatal morbidity and mortality as well as the increasing incidence associated with assisted reproductive techniques. Molecular mechanisms of zygosity and chorionicity are explored and the processes that lead to differences in monozygotic twin pairs are identified. A diagnostic sequence is proposed considering the modifications relative to singleton pregnancies as well as the pathology unique to this pregnancy type.

Keywords: Chorionicity, Discordance, Genotype, Malformations, Mosaicism, Perinatal outcome, Phenotype, Placentation, Screening, Twins, Zygosity.

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INTRODUCTION

Multiple pregnancies represent about 1.2% of all pregnancies. About 30% of these pregnancies are iatrogenic. An “epidemic” of multiple pregnancies is being observed in the world, as a consequence of both the increase in the reproductive age of pregnant women¹ and the widespread use of ovulation induction and assisted reproduction techniques (ART).^{2–4}

The contribution of ART toward the increase in the rate of twins is well known. Namely in the USA, an increase of 30% in the prevalence of twins was observed since the beginning of the 90s and 65% since the 80s. Assisted reproduction techniques, naturally associated with multiple ovulation and polyzygotic twins, show a twin rate 20 times higher than that of spontaneous conceptions, and a dizygotic twin rate even higher than the MZ twin rate (10:1). However, the rate of MZ twinning as a consequence of ART seems to be increased,^{5–8} mainly associated with the transfer of blastocysts (when compared with the transfer of embryos on the third day), and seems not to be affected by the manipulation of the *zona pellucida*. In a study that included 15,644 cycles and the transfer of a single embryo in 7,832 cases of IVF, the monozygosity rate was 2.3%, a figure 6 times higher than the 0.4% described in the literature.⁷ In contrast, the frequency of MZ twins recorded after ovulation induction (6.4%) was 14 times higher than the rate of spontaneous twins, and more than twice the rate of MZ twins after IVF.

The growing concern with multiple pregnancies is mainly related to the higher mortality and greater incidence of adverse perinatal outcomes when compared with singleton pregnancies.^{9–12} There is a duplication of risk for structural defects^{13–15} and a higher risk of chromosomal anomalies.¹⁶ Though multiple pregnancies represent 1.2% of the population, they heavily contribute toward perinatal mortality representing 12.6% of deaths. In the case of monochorionic twin pregnancies, the known perinatal morbidity and mortality are even more dramatic¹⁷ and this should encourage more differentiated and specialized surveillance.¹²

MONOZYGOSITY PHENOMENON

The human female was programmed by evolution to ovulate only once in every menstrual cycle, leading to mono-fetal pregnancies and to the nurturing of only one infant at a time. Therefore, in about 99% of spontaneous pregnancies, a unique fetus derives from a

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single zygote. As a consequence of a reproductive disorder, which occurs in 0.8% of spontaneous conceptions, more than an oocyte is produced and fertilized in each cycle, resulting in polyzygotic multiple pregnancies. In another 0.4% of spontaneous pregnancies, as a result of a reproductive anomaly, a single ovum normally destined to produce a single embryo splits to form monozygotic multiples.

The MZ twinning is a form of “vegetative” reproduction whereby more than one individual results from a single zygote. MZ twins, at birth, weigh somewhat less than twice the birth weight of singletons of corresponding gestational age. Hence, a single fertilized egg is capable of producing much more somatic and placental mass than a singleton pregnancy, though the mechanisms of overgrowth in MZ twinning are not fully understood. We can consider the existence of extra mitotic cycles and/or reduced apoptosis, but it is a tribute to the plasticity of early embryogenesis that anatomically and well-grown normal MZ twins, triplets, or higher-order multiples, can be produced from a single fertilized egg.

The prevalence of MZ twinning is fairly constant worldwide, strongly suggesting that MZ twinning derives from an intrinsic “anomalous” property of human zygotes. Based on this stability, Weinberg’s law was established and allows the accurate prediction of zygosity based on the frequency assessment of twin pairs of different sexes. In spontaneous conceptions, the rate of MZ twins represents about a third of all twin pregnancies, i.e., about 4:1,000

births and about two-thirds of MZ twin pregnancies will develop monochorionic placentation, i.e., 2–3:1,000 births. In iatrogenic pregnancies, the ratio is altered and MZ twin pregnancies are more prevalent (1:15–20 instead of 1:3). In certain cases, a familial tendency to MZ twinning was disclosed, probably due to molecular mechanisms that cause post-zygotic cells to disaggregate more easily than usual, forming two or more internal masses.

It is awkward to consider MZ twins “genetically identical”, based on an equal number of multipotential “founder” cells exposed to the same intrauterine milieu. These assumptions seem untrue, and they are more inaccurate in some fetuses than in others.^{18,19} The complexity and variety of the initial development of MZ twins lend a degree of sophistication and fascination toward the understanding of MZ twinning, introducing biases in twin studies. A lot of them do not take into consideration the possibility of discordance at birth that can exist caused by the different environmental, genetic, post-zygotic, and epigenetic factors of prenatal occurrence.

Why does the division of a single zygote occur? Though scientific evidence does not exist, different hypotheses have been proposed but all are at present merely speculative. Presently, four theories are available to explain the mechanism of zygotic splitting: the so-called repulsion hypothesis²⁰—cells in the developing zygote express genetic differences that translate into repulsive forces that lead to the splitting of the zygote; the existence of co-dominant axes¹⁴—the continuous presence of a co-dominant axis will cause the zygote to split; depressed calcium levels in the early embryo,²¹ and the blastomere herniation hypothesis—the integrity of the *zona pellucida* is breached during embryonic development, thereby losing its sequestering and protective role and permitting herniation of pluripotent cells through a gap in the *zona pellucida*.^{22,23} All these theories are, however, not convincingly clear in explaining the origin of monozygotic triplets and quadruplets, and do not elucidate the higher rate of MZ twins related to ART techniques (with or without manipulation of the *zona pellucida*). This particular difficulty is due to the non-existence of animal models of monozygosity, in which, except for the human race and the Armadillo (*Dasypus novemcinctus*), in no other mammals is this process expected to occur. More recently, the hypothesis that human fertilized oocytes more splitting-prone can undergo one or two successive binary fissions, or various combinations offered by subsequent secondary fissions, just as the case for the 9-banded armadillo, and give rise to various combinations of monozygotic pregnancies has been proposed (zona pellucida assisted binary fission theory).²⁴ This latter theory would be able to explain monozygotic triplets and quadruplets, as well as “mirror-image” characteristics²⁵ and some midline asymmetries in MZ twins.²⁶

The true frequency of zygotic division is not exactly known but inferred by the number of fetuses that exceed the number of transferred embryos. Clearly, this estimation is not reliable. If we consider that after the transfer of two embryos, one is lost and the other originates an MZ pregnancy with dichorionic placentation, no excess of fetuses would be found. Moreover, it is not clinically possible to differentiate between dichorionic dizygotic twins of same-sex from MZ twins with dichorionic placentation. Finally, Weinberg’s law is based on proportions observed in spontaneous conceptions that are not necessarily reproducible in ART pregnancies.

In a hypothetical model, we can calculate the risk of iatrogenic twins per 1,000 births: natural conceptions comprise 1.2% of twins including 0.4% MZs, whereas ART conceptions comprise 25% of

twins, of which 2.5% are MZs. In a department with 10% deliveries after ART, 6 MZ twins are predictable for every 1,000 deliveries as compared with 4 MZs after spontaneous conception. An increase of 1% in iatrogenic conceptions will translate into an increase of monozygosity by 6.25% compared to a 50% increase when 10% of the conceptions are iatrogenic. It can be concluded that the number of MZs increases as a linear function of iatrogenic pregnancies and the overall increase in iatrogenic multiples will significantly increase the total number of MZs in any given population.²³

Still addressing the issue of iatrogenic pregnancies, it was noticed that the early spontaneous embryonic/fetal loss was slightly superior in the pregnancies resulting from ART than in spontaneous pregnancies. Nevertheless, whenever the end result of ART was a twin pregnancy, this early loss rate appeared importantly reduced. Thus, multiple pregnancies may be seen as a marker of reproductive advantage.^{27,28} A sensible explanation for this advantage of twins over singletons would be that the higher levels of placental hormones produced by a larger placental mass in twin gestations would improve the implantation capacity of the egg. Several studies support this evidence,^{27–33} showing loss rates of 24% for singleton pregnancies vs 3–11% for twin pregnancies. The early fetal loss rate associated with ART is 2–5 times higher in singleton pregnancies.

TIME MATTERS IN MONOZYGOSITY: SHARING LEVELS AND A MODEL OF PLACENTATION

The timing of MZ twinning events can be inferred from the type of placentation, X chromosome inactivation in female MZ twins, asymmetric language function in the cerebral hemispheres, and asymmetric dermatoglyphics. Thus, the type of membranes and placentation is assumed to represent the time of splitting, according to Corner’s hypothesis, which was never proven, since no evidence of such a split exists in observations from *in vitro* fertilization.

Dizygotic twins, fraternal or non-identical, result from the fertilization of two ova by different spermatozooids, possessing therefore different genetic content. The type of placenta resulting from this process of fertilization, in which the trophoblast formation precedes the egg implantation, has two chorions and two amniotic sacs, defining a dichorionic diamniotic pregnancy.

Monozygotic twins comprise one-third of all twin pregnancies. These identical or uni-ovular twins result from the early division of a single egg (originating from the fertilization of an oocyte and a single spermatozoid) in two cell masses more or less identical, which contain the same genotype (exception: heterokaryotypic monozygotes). This cleavage will occur between the fertilization and the gastrulation period. Individual variations are the result of post-zygotic mutations and arbitrary division of mitochondrial DNA. The placentation inherent to these twins is more complex, depending on the very moment in which the separation occurs.

Dichorionic diamniotic (DC-DA) MZ twins (18–36%) have separate membranes and placentas and result from abnormal splitting at the 2 cell stage to morula (day 0–3). Consequently, each twin receives trophoblastic and somatic stem cells into their cell masses. Therefore, twinning must have happened before the differentiation and physical separation of somatic and trophoblastic cells into the outer and inner cell masses, respectively, in the cavitated blastocyst at about 3 days post-conception. Dichorionic diamniotic placentas are derived from the splitting of about eight blastomeres.

Splitting at any time thereafter results in a single MC placenta that continues as such even in the face of subsequent MZ twinning in the inner cell mass. Monochorionic diamniotic (MC-DA) twins (~80%) separate at the inner cell mass, after the chorion has formed (day 4–7). Consequently, two fetuses will develop with the same genome, a single placenta and two amniotic cavities, as the amniotic cavity will only be formed after the 8th day after conception. The sharing of a single placenta is in itself an anomaly, creating unequal vascular territories for each twin and a more common eccentric location of the umbilical cords.^{34,35} Eventually, 5–15% will develop a twin-to-twin transfusion syndrome (TTTS), and a late discrepancy in fetal growth can be a frequent finding.

If the division occurs between the 9th and 12th day, at the late blastocyst stage, a single placenta and a unique amniotic cavity will be formed, i.e., all placental structures will be shared, resulting in a monochorionic monoamniotic (MC-MA) twin pregnancy (1%).

The highest level of sharing occurs very rarely when the division happens circa the 13th day, resulting in the incomplete division of the embryonic disc, originating conjoined twins (estimated prevalence of 1 in 100,000). After that time, the undivided egg will maintain the expected course to produce a singleton pregnancy.

If MZ twinning events would take place at a constant rate during the first 12 days post-conception, proportions of DC, MC-DA, and MC-MA twins would vary from those observed. The under-representation at the birth of MC-MA and conjoined twins is explained by the higher intrauterine lethality. The relative excess of DC-MZ twins may be the result of their having fewer placental complications. Considering the level of sharing, it is inversely proportional to the incidence of twins that result from this sharing [the incidence of MZ twins is about 1:250, the incidence of monochorionic twins is 2/3 of the MZ pregnancies (1:350–400 births). The incidence of monochorionic monoamniotic twins is 1:2,500 liveborns, and the one from conjoined twins is <1:40,000 liveborns]. On the other hand, perinatal mortality and morbidity are related directly to the level of sharing, i.e., the greater the level of sharing the greater the risk of pregnancy adverse outcomes. Whereas MZ with dichorionic placentation and dizygotic twins present pregnancies with similar risks, those with monochorionic placentation have an important increase in worse outcomes, mainly if the shared circulation is unbalanced or if we are dealing with monoamniotic or conjoined twins.

HOW IDENTICAL ARE MZ TWINS?— PHENOMENA OF GENETIC AND EPIGENETIC DISCORDANCE

Unexpectedly MZ twins may be of a different chromosomal composition (heterokaryotypic twins).³⁶ Heterokaryotypic twins may be explained by two mechanisms: when the mitotic error occurs before the twinning event, the mosaic will be present in both fetuses with different distribution of the two cell lines between the twins; when the mitotic error occurs after the twinning event, the mosaic will be present in only one twin.³⁷ All possible combinations of karyotypes observed in twins can be attributed to the unequal allocation of the abnormal cells to each twin: abnormal/normal, mosaic/mosaic, abnormal/mosaic, and normal/mosaic.³⁸

Adding to the complexity of the situation, we must consider the timing of the event (responsible for the presence of the mitotic error in somatic regions of one twin) and the placental status of the chromosomes.³⁹

MZ twins with chromosomal anomalies and discordant phenotypes have been reported, including discordant sex phenotype with mosaicism 46XY/45X, Turner's syndrome in female twins with mosaicism 46XX/45X,^{37,40,41} trisomy 21,⁴⁰ and trisomy 13.⁴² There are a few case reports of MZ twins with discordant phenotype and rare partial chromosomal anomalies including 22q11 deletion, 7q syndrome,⁴³ monosomy 21,⁴⁴ and partial trisomy 1.⁴³ Trisomy 11p has already been reported³⁸ either associated with a non-specific clinical pattern or with the Beckwith–Wiedemann syndrome (BWS) when the additional region causes paternal disomy.³⁸ MZ pairs discordant for 45X emerging from 47XXY, 47XXX, 46XY, and 46XX zygotes have been reported.³⁹

Therefore, various post-zygotic events determine a discordant phenotype for MZ twins, which are not so identical as would be expected.⁴⁵

Concordant or Reciprocal X Chromosome-inactivation

X-inactivation (or lyonization) refers to the inactivation of one of the X chromosomes in females to achieve dosage compensation of X-linked genes with males.

Several studies link random inactivation of the X chromosome of maternal or paternal inheritance to subtypes and timing of MZ twins and indicate that allocation of cells to the twins is not always equal. Despite the female excess in MC twinning, it is not clear that X-inactivation is a major stimulus to MZ twinning *per se*. The discordance in the inactivated X may justify the phenotypic discordance for aspects linked to chromosome X, such as Duchenne muscular dystrophy. This inactivation precedes the MC placentation and confirms that the production of a monoamniotic sac is a late event in the monochorionic placentation.

An excess of females over males has been observed in surviving MZ twins, mainly with monochorionic placentation, suggesting a relationship with the time of twinning. Discordance for several X-linked recessive conditions has been reported in MZF twins, suggesting and often demonstrating non-random X-inactivation.²²

Finally, the highly similar patterns of X chromosome inactivation among monochorionic twins may indicate that X chromosome inactivation occurs before the twinning event in this subgroup of MZ twins.

Chromosomal Mosaicism

The presence of two or more cell lines derived from the same zygote having different chromosomal constitutions (secondary to post-zygotic events) is well recognized in aneuploid singletons. Depending on the relative timing of the twinning and chromosomal events, two or more cell lines may be distributed largely or exclusively in somatic cells of one twin or the other. Blood mosaicism might also be present in normal twins, as a result of interfetal anastomoses, and therefore, karyotyping in MZ twins that are discordant for some fetal abnormality should be performed in amniocytes rather than in fetal blood.³⁷

This mosaicism is particularly problematic for those fetuses initially 46,XY with loss of a Y chromosome, which will result in a male fetus and a mosaic 46,XY/45,XO reared as a female. This female fetus may suffer masculinization due to the fetal passage of testosterone produced by the 46,XY co-twin through the vascular anastomoses.

Whether derived from a 46,XX or 46,XY zygote, the MZ twin with a predominant or total 45,XO constitution is likely to develop fetal jugulolymphatic obstruction, endangering the life of the co-twin.

Imprinted Genes

Genomic imprinting is a phenomenon in which one of two alleles, from the maternal or paternal origin, is inactivated by DNA methylation.⁴⁶

Imprinting is initiated during gametogenesis and transmitted to embryos via mature male and female gametes. Appropriate imprinting of the two gametes is critical and implicated in prenatal growth, development and differentiation, behavior, and human disease.⁴⁷

Discordance between MZ twins has been reported for several diseases where genomic imprinting is suspected or implicated, such as BWS. Beckwith–Wiedemann syndrome has a higher prevalence than expected between MZF twins and they are mostly discordant.^{22,46} Although MZ twin concordance for BWS has been described, most MZM twins are discordant for BWS.⁴⁸ May well be that unequal splitting of the inner cell mass results in differential methylation between the two cell masses. These parent-of-origin effects, together with the finding of paternal uniparental disomy of chromosome 11p15 in 20% of BWS cases, suggest that abnormal genomic imprinting might play an important role in the etiology of BWS. Discordance for hyperinsulinemic hypoglycemia⁴⁹ and Russell–Silver syndrome⁵⁰ has also been reported and although these are likely to be heterogeneous conditions, imprinted genes may play nonetheless an important role.²²

Discordance in MZ twin pairs is well recognized for diseases thought to result from abnormal imprinting of genes. Beckwith–Wiedemann syndrome is caused by abnormal imprinting of one or more of a cluster of genes in the p15 region chromosome 11. There is an excess of females among MZ twin pairs discordant for the syndrome. It is not clear how the abnormal imprinting relates to the MZ twinning process, through unequal splitting, post-zygotic chromosomal rearrangements, or abnormal imprinting at the crucial moment of twinning.

Trinucleotide Repeat Sequence

Several progressive neurological disorders are characterized by phenotypic expression only when a threshold of trinucleotide repeat sequence number is exceeded, such as fragile X syndrome. Discordance has been found in some MZ twin pairs. A more severely affected twin had a complete mutation, whereas his less severely affected sibling has “mosaic” for pre-mutation and full mutation. Therefore, there may be some difficulty in offering predictive tests for these kinds of diseases to MZ twins.

Epigenetics

Epigenetic phenomena are characterized by “modifications in gene expressions that are controlled by heritable but potentially reversible changes in DNA methylation and/or chromatin structure”.^{51,52} Epigenetic modifications consist of methylation of cytosines, as well as modifications of histones by methylation, acetylation, phosphorylation, and ubiquitination.⁵³

DNA methylation may occur more frequently in MZ twins and may influence susceptibility to bipolar disorder and schizophrenia. MZ twins discordant for schizophrenia have been more frequently reported. Different DNA methylation patterns have been implicated in the discordance for schizophrenia in relation to specific schizophrenia candidate genes. Other studies also focused on the role of epigenetics in twin discordance, implying different methylation patterns in age-related diseases, risk-taking behavior, and caudal duplication anomaly.^{53–55}

More recently, Bianchi⁵⁶ as well as Liao et al.,⁵⁷ Leonard and Martin,⁵⁸ Caroto et al.,⁵⁹ and Judah et al.⁶⁰ pointed out in different meta-analyses to alternative methods for evaluating *in vivo* genetic differences in MZ twins, such as cell-free fetal DNA (cfDNA) in maternal blood, mRNA in amniotic fluid and RNA single-nucleotide polymorphism (SNP) allelic ratio analysis. Therefore, prenatal gene expression investigation *in vivo* may open a new field for prenatal twin research.

Phenotypic Discordance for a Single Gene Mutation

It would be expected that MZ twins affected by an autosomal dominant disorder such as type I neurofibromatosis might show different somatic patterns of distribution of discrete lesions because of somatic mosaicism. However, major somatic differences may be attributed to a post-zygotic discordance for the causative point mutation.

Phenotypic Discordance with Same Genetic Predisposition

MZ twins are usually discordant for the extent and severity of congenital heart diseases when they have microdeletion 22q11.1, probably due to a second epigenetic effect on the phenotype.

Discordance for Major Malformation

The prevalence of structural defects in a dizygotic twin pregnancy is the same as in a singleton pregnancy, whereas in monozygotic twins it will be 2–3 times higher.^{14,15} The concordant defects (both fetuses are affected) are rare, occurring in 10% of DC twin pregnancies and 20% of MC twin pregnancies.⁶¹

The discordance in DZ twins is due to a genetic predisposition. In the MZ pregnancy, this discordance is more prevalent and may be explained by:

- Variable gene expression (post-zygotic mutation, asymmetrical X inactivation).
- Asymmetric splitting of the cellular mass.
- Splitting after laterality gradients have been established (midline defects and cardiac).
- Hemodynamic factors, in monochorionic gestations with TTTS (putatively responsible for disruptions).

MZ are usually discordant for malformations such as cardiac and urinary tract defects, omphalocele, and neural tube defects, but on the contrary, are usually concordant for malformations such as cloacal dysgenesis and omphalocele-exstrophy-imperforate anus-spinal defects syndrome.

Pseudo-concordance of malformations may occur in MZ twins. Though they are frequently discordant for thyroid dysgenesis, both may only be mildly ill. Due to interfetal connections, the affected fetus may be protected from hypothyroidism by the transplacental transfusion of thyroxine from the thyroid of the unaffected co-twin and present at birth almost normal thyroxine levels.

Another example of pseudo-concordance of phenotype exists for monoamniotic twins with urinary tract malformations, in which the anuric fetus is protected from pulmonary hypoplasia and deformities by the urine output from the other fetus.

Disruptions in MZ twins including limb reduction defects, hemifacial microsomia, and amyoplasia may be the result of shared placental circulation leading to secondary disruptions and sometimes to single intrauterine fetal death.

Finally, clubfeet, dislocated hips, and cranial synostosis are deformations associated with spatial constraint and intrauterine

crowding, which are related to limited space *in utero* for the bearing of two fetuses.

“Mirroring” in Monozygotic Twins

In about 25% of MZ twins, the development of normal lateralization may be impaired by the twinning process itself in such a way that for some of the asymmetrical features, the resulting twins would not be duplicated, but mirror-images.⁶²

The concept of mirror-image MZ twins is based on inverse laterality. This may suggest that the event that originated the twins began after the cells of the embryonic plate were beginning to lateralize but before the formation of the primitive streak.

Later MZ twinning could occur when the molecular determinants of left/right asymmetry are beginning to be expressed with lateral discordance. Opposite-handedness is more prevalent among both DZ and MZ twin pairs.

“Fine-Tuning”: Detailed Peripheral Patterning

MZ twins are not concordant for dermatoglyphics, this being one of the examples by which MZ twins are not “identical”. The extent of discordance in dermatoglyphic patterns varies with chorionicity and MC twins show more within-pair variability than DC-MZ twins. There is also evidence of an effect from placental crowding: MZ twins with DC placentation show greater variability than those with separate placentas. This effect was also noted in like-sexed DC twin pairs.

Unequal Placental Territoriality

The unequal allocation of stem cells/blastomeres to the twins can determine the existence of unequal placental masses or the differential sharing of placental territory in MZ twins. As a consequence, discrepant fetal growth and different phenotype can occur in MZ twins.⁶³

LIMITS OF ZYGOSITY TESTING: POST-NATAL IMPORTANCE

The estimation of MZ twins is frequently based on the number of monochorionic twins observed in obstetric ultrasound. Though all monochorionic placentas correspond to MZ twins and all twin pairs of different sex are DZ, the majority of like-sexed twins with dichorionic placentation are blind to their zygosity. The recognition of zygosity in dichorionic MZ twins is based on physical similarities but even the most experienced practitioner may misclassify zygosity in about 6% of cases.^{20,64} In like-sex dichorionic twins, we are blind to zygosity in about 44% of the cases. Despite observations of DZ monochorionic twins, the so-called “gold standard” defining monozygosity is that all monochorionic twins are MZ. Biochemical characteristics such as blood type, enzyme polymorphisms, and HLA types have also been used to classify zygosity. However, the “gold standard” for the determination of zygosity should be based on several genetic markers (including “DNA fingerprinting”) in buccal swabs or blood sampling, applied to like-sexed dichorionic twin pregnancies. Though this determination would have more scientific than clinically justified, in particular cases it can be life-saving as in solid organ transplantation. This may be, however, a somewhat misleading approach as all MZ twins share vascular placental connections and might have exchanged DNA (chimerism).

In reality, the final diagnosis of zygosity cannot be accomplished in about 43% of cases without DNA evaluation.⁶⁵ In daily practice, the estimation of MZ twins is based roughly on chorionicity and fetal

sex, disregarding about 1/3 of MZ twins. Therefore, whatever the method is used, it will underestimate the real incidence of MZ twins.

Considering the data from the East Flanders Prospective Twin Survey, in which zygosity was studied in all like-sexed dichorionic twins, the frequency of MZ twins after ART was 4.5% (10× superior to the 0.45% MZ rate found in twins after spontaneous conception). The frequency of MZ twins after IVF was 2.6% (6× the rate of spontaneous conceptions).⁶⁵

CHORIONICITY: A (RE)DEFINITION OF PERINATAL PROGNOSIS

Clearly, it is chorionicity rather than zygosity that determines several aspects of antenatal management and perinatal outcome. Thus, the routine assignment of chorionicity and the earliest possible diagnosis of monochorionic twinning are highly desirable, though seldom achieved in practice.

Zygosity refers to the type of conception whereas chorionicity reflects the type of placentation. The type of placentation depends on the time of splitting of the fertilized ova. One study suggests the possibility of determining zygosity, using ultrasound, at the beginning of the first trimester by counting the number of yolk sacs;⁶⁶ however, a unique placental mass and the same fetal sex suggest but do not prove monozygosity. It is not possible to determine zygosity in about 45% of cases because dizygotic twins of the same sex (about half of the dizygotic twins) and monozygotic dichorionic twins (a 1/3 of all monozygotic twins) cannot be differentiated unless molecular tests are used.

Caution should be taken when using the number of chorionic sacs and the number of yolk sacs alone to determine the number of embryos. Shortly after the sixth postmenstrual weeks, embryonic heartbeats are visible and one can confidently count the number of embryos by the number of beating hearts. By that time, to determine the number of amnions in a monochorionic twin pregnancy, in which two embryos are seen within the chorionic sac, it is wise to wait until the 8th postmenstrual week to ascertain amnionity. The amniotic membrane is so thin that it may remain inconspicuous until 8–9 weeks, leading to the incorrect consideration of a monochorionic-monoamniotic gestation.

After that gestational age, it becomes clear that no amniotic membrane is present between the embryos and only one yolk sac is visualized. When two embryos or fetuses assume a parallel, head-to-head position, conjoined twins should be suspected. A jerk with the probe should be inflicted to induce movement between the two fetuses, away from each other or close together when conjoined twins are in question. From then on it is important to look for sonographic signs of cord entanglement, potentially depicted as early as 12 weeks, with the help of color Doppler.

At 10–14 weeks, the gold standard “window” for chorionicity definition, the chorion frondosum is sufficiently thick to be identified between the two layers of amnion. We can see with ultrasound a wedge-shaped structure in a dichorionic twin pregnancy (not obligatorily a dizygotic twin pregnancy), yielding the fully diagnostic “twin peak” or “lambda” or “delta” sign^{67–69} (Fig. 1). However, the number of “twin-peak” signs is no secure indication of how many chorionic sacs exist within a given pregnancy. If two fused layers of amnion, without any chorion interposed, are found in the scan creating a T-shaped “take-off” (T sign), showing a very thin membrane with strictly two layers, a monochorionic twin pregnancy can be diagnosed with a 100% certainty^{69,70} (Fig. 1).

Fig. 1: Diagnosis of chorionicity by ultrasound: lambda sign (dichorionic placentation) and T-sign (monochorionic placentation) at 10–14 weeks of gestation. Lambda sign: dichorionic placentation; T sign: monochorionic placentation

After 16 weeks physiologically, the chorion frondosum regresses and chorionicity becomes a more confounding issue (in the second trimester, a false characterization of chorionicity can occur in about 10% of cases).^{68,71} The delta sign disappears^{72,73} and, if sexes are alike, monochorionicity can be wrongly inferred. It is the conjugation of several sonographic criteria that approaches the correct diagnosis of chorionicity, the ultimate determinant of perinatal prognosis. Therefore, an adequate first-trimester scan of multifetal pregnancies makes the subsequent second- and third-trimester evaluation more meaningful, simpler, and faster.

If one finds two separate placentas of a different location, or two fetuses of different sex or a thick interfetal membrane >2 mm with more than two layers, dichorionicity is strongly suggested. Note that to identify the interfetal membranes, a right-angled orientation of the probe in relation to the membranes should be obtained to take advantage of the axial over the lateral resolution. To count the number of layers one must “zoom in”. In contrast, a membrane placed parallel to the ultrasonic beam will appear thinner and poorly imaged, rendering it impossible to be sure about the number of layers of the intertwin membrane.

The confirmation of monochorionic placentation by the detailed examination of the placenta is the most reliable proof of monozygosity. Not only is it possible to identify the pathological aspects that interfere with placental function but also the relationship between the chorion and fetal membranes (chorionicity), and the pattern and the type of anastomoses of the chorionic vessels. This exam includes the careful macroscopic examination of the placenta with consideration of aspects such as the fusion of the chorion and amnion, thickness and translucency of the septum, and the vascular pattern of the fetal surface. The histological evaluation includes cord fragments, membranes and placental parenchyma, and fragments of the transitional zone. To study the vascular anastomoses, the placenta should not be fixed and the amnion should be removed.

When chorionicity is established, stratification of risk is possible, anticipating the complications of each type of placenta. Complications of MC twin pregnancies are by far more common, such as preterm delivery, fetal malformations, TTTS, intrauterine death of one fetus, and cerebral palsy. Recently, an update on the management of twin pregnancies was published, discussing the peculiarities of clinical orientation in MC twins.⁷⁴

PRENATAL SCREENING AND DIAGNOSIS: PATHOLOGY UNIQUE TO TWINS

Twins present unique issues in prenatal diagnosis. The performance of screening tests designed for singleton pregnancies is altered. Having correctly established the chorionicity, specific aspects of prenatal screening and diagnosis can be adequately programmed. Chromosomal abnormalities in twin pregnancies give rise to serious clinical, ethical, and moral problems that need to be addressed.⁷⁵

- Effective methods of screening, such as maternal serum biochemistry, are not applicable and have lower detection rates.
- In the presence of a “screen-positive” result, there is no feature to suggest which fetus may be affected.
- Non-invasive prenatal test (NIPT) is prone to quality issues in case of multiple gestations: the minimum total amount of cell-free fetal DNA must be higher to reach a comparable sensitivity and vanishing twins may cause results that do not represent the genetics of the living sibling.^{58–60,76}
- Invasive testing techniques are more demanding in twins and it may be difficult to ensure that fetal tissue is obtained from each fetus.
- Increased risk of a miscarriage of an invasive test in twins.
- Which invasive test to offer.
- The paucity of data in abnormally affected pregnancies when the fetuses are either concordant or discordant for an abnormality.
- The difficulties of clinical management of fetal reduction and the potential increased risk to the unaffected co-twin.

The overall probability that multiple gestations contain an aneuploid fetus is directly related to its zygosity. In dizygotic pregnancies, each fetus has an independent risk of aneuploidy, thus, the maternal age-related risk for chromosomal abnormalities for each twin may be the same as in singleton pregnancies, but the chance that at least one fetus will be affected by a chromosomal defect is twice as high as in singleton pregnancies⁷⁷ (Fig. 2). This means that for dizygotic twin pregnancies, the pregnancy-specific risk is calculated by adding the individual risk estimates for each fetus. Furthermore, since the rate of dizygotic twinning increases with maternal age, the proportion of twin pregnancies with chromosomal defects is higher than in singleton pregnancies. The 10% of dichorionic twin pregnancies that are monozygotic will incorrectly have their risks calculated by addition rather than

Fig. 2: Screening for trisomy 21 in dichorionic twins (summing method): the lambda sign is evident at 10–14 weeks. We can observe discrepant nuchal translucencies (NT = 1.1 and 5.7 mm). Ductus venosus Doppler blood flow is abnormal in the fetus with increased NT that eventually revealed to be a fetus affected by trisomy 21

the averaging method. However, the ultimate effect on screening performance and clinical meaning will be a negligible one.

In monozygotic twins, the risk of an affected fetus is similar to the maternal age risk of a singleton pregnancy and, in the vast majority of cases, the risk for one fetus is the same as the risk for the other.^{75,78} There is no reason to attribute different risks to the two fetuses because, presumably, both will be affected or both will be unaffected. The most reliable and reproducible method of screening is nuchal translucency (NT) and it is, therefore, appropriate to take the average of the two NT measurements, so that a single risk estimate can be calculated (averaging method).

This ignores the small possibility of heterokaryotypic monozygotic twins resulting from a mitotic non-disjunction after the zygote splits. There are occasional reports of monozygotic twins discordant for abnormalities of autosomes or sex chromosomes, most commonly with one fetus presenting a Turner syndrome and the other either a normal male or female phenotype, but usually with a mosaic karyotype or a Klinefelter syndrome.

The relative proportion of spontaneous dizygotic to monozygotic twins is about 2:1 and, therefore, the prevalence of chromosomal abnormalities affecting at least one fetus in a twin pregnancy would be expected to be about 1.6 times that of singletons.

If zygosity is unknown, the risk of at least one aneuploid fetus can be approximated as five-thirds that of the singleton risk. This is based on the assumption that a third of all twin pairs are monozygotic.¹⁶ Counseling based on chorionicity, clinically more feasible than zygosity, means that in monochorionic twins both fetuses can be affected equally. If the pregnancy is dichorionic, then the parents should be counseled that the risk of discordance for a chromosomal abnormality is about twice that in singleton pregnancies, whereas the risk that both fetuses are affected is a much rarer event, corresponding to the singleton risk squared. However, with higher risk conditions, such as autosomal recessive disorders, this could be as high as 1 in 16. In a dizygotic twin pregnancy, the risk that one fetus would be affected would be 1 in 50 (1 in 100 plus 1 in 100), whereas the risk that both fetuses would be affected is 1 in 10,000 (1 in 100 $\times$ 1 in 100). This is, however, an oversimplification since, unlike all monochorionic pregnancies that are always monozygotic, only about 90% of dichorionic pregnancies are dizygotic.

While calculating the risk of higher-order multiples, estimates can be made by multiplying the singleton risk by the number of fetuses.⁷⁹ This method assumes unique chorionicity for each fetus, though monozygosity can occur more frequently than usually thought at higher rates in ART multiple gestations.^{5,6}

The possibility of deriving a risk for trisomy 21 from NT assessment in the first trimester of pregnancy shifted the consideration of a pregnancy-specific risk to a fetus-specific risk.⁸⁰ This assumption was based on the observation that the distribution of NT measurements in twin fetuses with trisomy 21 was similar to that in singletons.^{9,81,82} The higher rate of false positives for NT among MC twins (8.4%) should be ascribed to the possibility of early hemodynamic imbalance (the risk of developing TTTS if NT is increased is augmented 3–5 fold).^{81,83,84}

Though assessing nasal bones in multiple pregnancies can be more demanding due to the more difficult acquisition of adequate fetal face planes, whenever they are assessed they can be combined with NT and biochemical screening for calculating first-trimester risks. With the addition of nasal bone evaluation, sensitivity for trisomy 21 screening increased from 79 to 89%, for the same false-positive rate of 5%.^{85,86}

In contrast, though biochemical screening in twins was the first alternative to age-derived risk it can still be a source of confusion and clearly has a lower detection rate for fetal aneuploidies (50%) and higher rates of false positives.^{87,88} This kind of screening only allows the calculation of pregnancy and not a fetus-specific risk. In twin pregnancies, the levels of maternal serum markers are, on average, expected to be about twice as high in unaffected twin pregnancies as in unaffected singleton pregnancies,⁸⁹ i.e., proportional to the number of fetoplacental units. However, as biochemical screening in twins is still investigational and far less powerful than in singletons, it should not be recommended in general practice without extensive counseling.^{90,91}

MONOCHORIONIC PREGNANCY AS A HIGH-RISK PREGNANCY: TTTS AS A PARADIGM TO TREAT

Over the last decade, perinatal mortality in singleton pregnancies has fallen due to advances in fetal medicine and improvement in perinatal care. A similar reduction has not been observed in multiple pregnancies in which perinatal loss remains six times higher than in singleton pregnancies. Even more striking is the perinatal mortality of monochorionic twins (260 per 1,000) which remains three- to five-fold higher than in dichorionic pregnancies (90 per 1,000): the rate of perinatal loss before 24 weeks in monochorionic compared with dichorionic pregnancies is 12.2 vs 1.8%.⁹

The MC twin placenta is designed and built for a singleton fetus; hence, attempts to cater to the needs of twin fetuses can often be suboptimal. Twin fetal circulations are seldom separate and several intertwin vascular communications of various kinds may be present and quite often there is an unequal sharing of placental parenchyma. An example of a complication almost unique to monozygotic twinning is TTTS. By way of intertwin vascular connections, blood is transfused from the donor, who becomes growth-restricted and develops high output cardiac insufficiency and oligohydramnios (depleted-donor twin), to the recipient, who develops circulatory overload with congestive heart failure and polyhydramnios (volume and an overfilled recipient twin). Therefore, TTTS reflects primarily a pathological form of circulatory imbalance that develops chronically between hemodynamically connected monochorionic twin fetuses.

Twin-to-twin transfusion syndrome affects about 5–15% of monochorionic twin pregnancies (1:400 pregnancies) and thus occurs in 1 in 1,600 deliveries. This syndrome accounts for 17% of perinatal mortality, nearly 12% of neonatal deaths, and 8.4% of

infant deaths in twins. This is 3–10 times higher than that attributed to singletons.

This syndrome was always recognized as a devastating complication of “identical” twins but it took roughly 400 years to understand the way it works. Though clinically identifiable, this condition is still far from being effectively anticipated and treated. Twin-to-twin transfusion syndrome presents unique characteristics and greatest therapeutic challenges in perinatal medicine:

- It affects **two** babies, not one.
- It affects **structurally normal** babies.
- Its basis resides in the **placenta**, not in the babies.
- It is associated with important **perinatal morbidity and mortality**.
- It is amenable to **curative** therapy.

Vascular anastomoses are found invariably in almost all monochorionic placentas. Thus, intertwin transfusion is a normal event in monochorionic twin pregnancies. When intertwin transfusion in MC twins is balanced, clinical manifestations of TTTS are not expected to occur.

More than a century ago, Schatz⁹² suggested that TTTS is due to discordant hemodynamics secondary to transfusional imbalance. Bajoria and coworkers⁹³ related TTTS with unbalanced intertwin transfusion mediated by one or more arteriovenous (AV) anastomoses in association with absent bi-directional superficial anastomoses: those affected by TTTS had fewer arterioarterial (AA) anastomoses present in 24 vs 84% of monochorionic twins without TTTS.⁹⁴ Seventy-eight percent of monochorionic pregnancies in this series with one or more AV anastomoses and no AA anastomoses developed TTTS.⁹⁴ When an AA anastomosis is found, the risk of developing TTTS is reduced 9-fold.

Therefore, due to the particular vascular anatomy of the placenta, some MC twins are unable to compensate for the unidirectional flow in a “causative” AV anastomosis. MC twins have a continuous spectrum of severity in the imbalance between their fetoplacental circulations, depending on an angioarchitectural basis, hemodynamic and hormonal factors. The progressive nature of TTTS *in utero* is thought to be due to one twin (the donor) slowly pumping blood to the other (the recipient) through these anastomoses.

The net result of transfusion between twins depends on:

- **Vascular anastomoses:** a combination of the type of connections (number, type, and diameter) and direction of connections. In some cases, the normal transfusion from the donor’s arterial to the recipient’s venous circulation is not adequately compensated by oppositely directed flow by other deep or superficial anastomoses.⁹³
- **Placental sharing:** unequal placental sharing, both by the discrepant size of the placental territory or by velamentous insertion (VCI) of the umbilical cord, may further impair growth in TTTS fetuses.⁹⁵
- **Asymmetry** in the progressive reduction of an initially large number of bi-directional AV connections formed during the embryonic unification of placental and fetal vessels.
- **The unbalanced renin-angiotensin system (RAS):** upregulation of RAS (donor) and downregulation of RAS (recipient) with the transfer of angiotensin II may cause or contribute to the development of TTTS.⁹⁶
- **Incomplete remodeling** and defective trophoblastic invasion of maternal spiral arteries.

The pathophysiology of TTTS is poorly understood, and, although transfusion has been confirmed *in vivo*, the pathophysiology of TTTS includes more than shunting of blood from donor to recipient. A vicious cycle of hypervolemia-polyuria-hyperosmolality is established, leading in about one-third of the cases to the development of acute polyhydramnios/oligohydramnios sequence in the second trimester of pregnancy.

DIAGNOSIS OF TTTS

In the past, diagnosis of the syndrome was made only after delivery of the affected twin pair and careful examination of the placenta. The standard neonatal criteria comprised:

- A difference in cord hemoglobin concentrations of 5 g/dL or more (false-positives should be considered whenever there is untimely umbilical cord clamping of either donor or recipient, or reversed intrapartum shunts).
- A difference in birthweights of 20% or more (this criterion is not necessarily present in the acute form of feto-fetal transfusion syndrome that occurs in labor, and hydroptic fetuses may obscure the real intertwin size disparity).

Danskin and Neilson,⁹⁷ revisiting the neonatal criteria for diagnosis of TTTS, found that an intertwin hemoglobin disparity of 5 g/dL or more and birthweight differences of >20% was found both in monochorionic and dichorionic twins at similar rates. Wenstrom et al.⁹⁸ concordantly found that weight and hemoglobin level discordance were relatively common among monochorionic twins. Therefore, a definitive diagnosis of TTTS solely based on neonatal criteria seemed insufficient.

Considering the many pitfalls of neonatal findings in TTTS and the more consistent sonographic antenatal criteria,^{99–101} the emphasis of screening and diagnosis of TTTS is being pushed backward in pregnancy. In the early 80s, the contribution of antenatal ultrasound for redefining the diagnostic criteria of TTTS was recognized by Wittmann and Brennan. Wittmann et al. proposed as discriminating findings in TTTS the discrepancy in the sizes of twins and the polyhydramnios surrounding the larger twin. Brennan and colleagues added to the former criteria, the disparity in the size of the vessels in the umbilical cords, same-sex, single placenta showing different echogenicity of the cotyledons supplying the two cords, and evidence of hydrops in either twin or congestive heart failure in the recipient. More recently, more useful sonographic criteria are adopted:

Discordance in Amniotic Fluid Volume (Oligohydramnios Sequence)

In 1988, Chescheir and Seeds¹⁰² disclosed a powerful clue based on the fact that six out of seven twin pregnancies with monochorionic placentas and TTTS had concurrent polyhydramnios and oligohydramnios. This is not surprising when we understand TTTS as a manifestation of a hemodynamic imbalance.¹⁰³ Fetal renal perfusion is asymmetric: the congestive heart failure in the recipient will overperfuse the kidneys with consequent polyuria and excess of amniotic fluid; hypovolemia in the donor causes inadequate perfusion of the kidneys with a decrease in urinary output and oligohydramnios.

More uniform criteria for the oligohydramnios sequence have been proposed for a quantitative definition: deepest vertical pool in the donor sac <2 and >8 cm in the recipient's sac. Not infrequently anhydramnios in the donor sac results in it becoming "stuck",

shrouded by the intertwin membrane, while the recipient's sac becomes severely polyhydramniotic.

One should bear in mind that sonographic pitfalls may exist in the presence of discordant anomalies in twins that imply differences in amniotic fluid volume, such as one twin with esophageal atresia and consequent polyhydramnios, or with renal agenesis, with consequent oligohydramnios/anhydramnios.

Other related confirmatory features include a small or non-visible bladder due to hypovolemia and renal hypoperfusion in the donor, along with a distended urinary bladder with resulting excessive micturition in the recipient.

Discordance in Fetal Size

Discordant growth is a common complication of twin pregnancies. The need for stricter sonographic criteria to define growth has changed gold standards over time. Abdominal circumference rather than head measurements of twins was proposed as the most reproducible and meaningful one. Besides, considering that fetal weight estimations based on singleton growth charts may be inadequate for twins, the abdominal circumference criterion should be definitely used for the sonographic diagnosis of divergent twin growth. A cut-off value of 20 mm for the difference in abdominal circumference between twins indicated a growth discordance of >20%.

Abnormal Doppler Findings

Alterations in cardiac hemodynamics are indirectly put on evidence by alterations in venous blood flow waveforms. The receptor presents with pulsatility in the umbilical vein and absent or reverse flow in the ductus venosus¹⁰⁴ as signs of congestive heart failure due to hypervolemia and increased preload from placental vascular anastomotic transfusion.

Fetal Echocardiography

Both donor and recipient twins have dynamic changes in volume/pressure loading during cardiovascular development constituting a hostile intrauterine environment. Considering the hemodynamic imbalance between the circulations of the twins involving some excess of blood flowing from the donor to the recipient fetus, cardiac involvement is logically expected. Echocardiography is a well-established tool for antenatal assessment of structural and functional heart disease, turning it possible in TTTS to assess cardiovascular adaptation to intertwin transfusion, early recognition of deterioration, and evaluation of antenatal management.

Zosmer et al.¹⁰⁵ showed that some surviving twins of TTTS had a persistent right ventricular hypertrophic cardiomyopathy and proposed that cardiac dysfunction could be induced *in utero* by sustained strain upon the heart by TTTS, predominantly affecting the right ventricle. The right ventricle is stiffer and more afterload-sensitive than the left ventricle, mostly due to the redistribution of blood in the cerebral arteries which decreases the left ventricular afterload. Additionally, recipients remain at increased risk of pulmonary artery stenosis and maintain a slightly reduced early diastolic ventricular filling as compared to donors (diastolic dysfunction).¹⁰⁶ In contrast, the significant reduction of blood flow velocity in the umbilical artery recorded in the "donor" is consistent with hypovolemia and increased placental resistance, increasing cardiac afterload and decreasing umbilical venous return. This is in good agreement with some studies, which show that the donor twin has a trend toward a lower Tei-index than in the normal population. Finally, there have been speculations about an

increased incidence of aortic coarctation in donors due to a lower venous return from the placenta and hence a decreased loading of the left ventricular outflow tract.¹⁰⁶

In the study from Fesslova et al.,¹⁰⁷ all recipient fetuses showed cardiac hypertrophy and dilatation, well-known compensatory mechanisms of blood volume overload, and high cardiac output (Frank-Starling mechanism).

After birth about half of the recipients showed biventricular hypertrophy, with prevalent left ventricular hypertrophic cardiomyopathy.^{105,107,108} A smaller subgroup developed right ventricular tract obstruction (functional pulmonary stenosis) and pulmonary hypertension in the neonatal period, which may be aggravated by systolic right ventricular dysfunction. Recently, diastolic abnormalities were described in the right ventricle, with abnormal filling patterns, prolonged isovolumic relaxation time, and abnormal flow patterns in the inferior vena cava and ductus venosus. In addition to hemodynamic remodeling, it is well recognized the role of increased endothelin-1 in the recipient, mainly in the hydropic recipient, as a mitogenic factor to a smooth muscle cell in the systemic and pulmonary vasculature and for ventricular myocyte proliferation.

More recently, abnormalities of vascular distensibility were described in survivors of TTTS in infancy.¹⁰⁸ The donor fetus shows evidence of chronic hypovolemia resulting in activation of the RAS. This upregulation initially attempts to correct volume depletion, and transfusion of increased concentrations of angiotensin II will probably cause increased vascular stiffness in the surviving donor in childhood.

Signs of Hydrops in the Recipient Twin

In an advanced stage of TTTS, the recipient twin affected by congestive heart failure may present signs of serosa effusions, such as ascites, pleural effusion, and subcutaneous edema.

Other Ultrasonographic Findings

- Identification of cord insertion: VCI of the cord is a frequent finding.
- Funipuncture: theoretically it may allow the antenatal assessment of intertwin hemoglobin difference, the degree of fetal anemia in the donor twin, and the twins' zygosity through blood group studies. However, the possible benefit of this procedure seems to be very poor on clinical grounds and the risks importantly outweigh the informative gain.
- The difference in the color of the placentas: due to blood transfusion from one twin to the other, the placenta of the donor twin tends to be whitish ("pale"), and the placenta of the recipient, of a denser color (excess of blood).

TREATMENT OF TTTS

Fetoscopic laser coagulation of intertwin vascular anastomoses on the monochorionic placenta is the preferred treatment for TTTS. Severe postoperative complications can occur when intertwin vascular anastomoses remain patent including twin-anemia polycythemia sequence or recurrent TTTS. To minimize the occurrence of residual anastomoses, a modified laser surgery technique, the Solomon technique, was developed in which the entire vascular equator is coagulated. This technique was associated with a significant reduction in short-term complications (twin-anemia polycythemia sequence and recurrence of TTTS) when

compared with the standard laser surgery technique. No differences in survival or neurodevelopmental impairment between both techniques were found.¹⁰⁹

PREDICTION OF TTTS

While accounting for only 1.2% of the population, twins are responsible for 12.6% of perinatal mortality. In the particular case of monochorionic twinning, the fetal loss rate is even more relevant and there is an increased risk of adverse perinatal outcomes. Therefore, targeted surveillance of monochorionic twins at earlier stages of gestation could anticipate and provide timely management of the pregnancies at risk of one of the most devastating type-specific complications: TTTS.

Nuchal Translucency

Data gathered from the literature show that increased NT thickness at 10–14 weeks of gestation was found twice as much as in monochorionic than in singleton pregnancies, and the likelihood ratio of developing TTTS in those twins with increased NT was 3.5.^{9,110} Considering that monochorionic pregnancies do not show a higher prevalence of chromosomal abnormalities, the higher prevalence of increased NT in those twins could be ascribed to cardiac dysfunction. With advancing gestation, this transient heart failure eventually resolves with increased diuresis and ventricular compliance. More recently, it was observed that whenever the discrepancy of NT values was above 20%, the detection rate for early fetal death was 63%, and for severe TTTS of 52%.¹¹¹ In a recent study, Lewi et al.¹¹² showed that significant predictors in the first trimester were the difference in crown-rump length (odds ratio = 11) and discordant amniotic fluid (OR = 10). Later in pregnancy, at 16 weeks, significant predictors were the difference in abdominal circumference (OR = 29), discordant amniotic fluid (OR = 7), and discordant cord insertions (OR = 3). Risk assessment in the first trimester and at 16 weeks detected 29 and 48% of cases with a complicated fetal outcome, respectively, with a false-positive rate of 3 and 6%, respectively. Combined first-trimester and 16-week assessment identified 58% of fetal complications, with a false-positive rate of 8%.¹¹²

Ductus Venosus Flowmetry

Can the characteristic circulatory imbalance of TTTS, fully expressed later in pregnancy, disclose indirect signs of cardiac dysfunction in earlier stages of gestation? In recent studies of vascular hemodynamics in fetuses with increased NT at 10–14 weeks, the abnormal flow in DV more frequently recorded in fetuses with chromosomopathies, with or without cardiac defects, was related to heart strain.^{113–116} These findings are in good agreement with the overt hemodynamic alterations found in TTTS later in pregnancy. Therefore, strong evidence suggests that increased NT along with the abnormal flow in the DV, even in the presence of a normal karyotype, may be early signs of cardiac impairment or defect.

In a study from our Unit,⁷⁷ in which NT and Doppler blood flow waveforms in the DV were recorded in both twins between 11 weeks and 14 weeks of gestation. Twin-to-twin transfusion syndrome was recorded in those fetuses which combined increased NT and abnormal flow in the DV. Whenever NT was discrepant but with the normal flow in the DV, no cases of TTTS were found^{77,117} (Fig. 3).

More recently, in a more inclusive study, we showed that discrepant values for NT over 0.6 mm had a sensitivity of 45.5%

Fig. 3: Screening for trisomy 21 in monochorionic twins (averaging method). We can observe discrepant nuchal translucencies (NT = 1.25 and 5.02 mm). Ductus venosus Doppler blood flow is abnormal in the fetus with increased NT. This pair of twins was chromosomally normal and developed a twin-to-twin transfusion syndrome at 16 weeks

and a specificity of 86.9%. The presence of at least one abnormal blood flow waveform in the DV translated into a relative risk for developing TTTS of 11.86 (3.05–57.45) with a sensitivity of 72.7% and a specificity of 91.7%. The combination of abnormal DV blood flow with discrepant NT >0.6 mm, yielded a relative risk for the development of TTTS 21 times higher (IC 95% 5.47–98.33).⁸⁴

In those uncomplicated MC twin pregnancies, in which abnormal DV flow was found in at least one of the fetuses, a higher discordance in birth weight was recorded in the third trimester of pregnancy when compared to those with the normal flow in both fetuses.¹¹⁸

Therefore, both increased NT and abnormal flow in the ductus venosus in monochorionic twins may translate early manifestations of hemodynamic imbalance between donor and recipient. In these pregnancies, in addition to NT measurement at 11–14 weeks, the Doppler assessment of DV blood flow increases relevantly the performance of screening for those at risk of developing TTTS.

More recently, Stagnati et al.¹¹⁹ published a systematic review and a meta-analysis to evaluate the performance of each ultrasonographic early marker (intertwin NT discrepancy, NT > 95th percentile, intertwin CRL discrepancy > 10% or abnormal DV flow) in the prediction of TTTS in the first trimester of pregnancy. An increased risk of TTTS was associated with: intertwin NT discrepancy [positive likelihood ratio (LR+), 1.92 (95% CI, 1.25–2.96); negative likelihood ratio (LR–), 0.65 (95% CI, 0.50–0.84)]; NT > 95th percentile [LR+, 2.63 (95% CI, 1.51–4.58); LR–, 0.85 (95% CI, 0.75–0.96)]; CRL discrepancy > 10% [LR+, 1.80 (95% CI, 1.05–3.07); LR–, 0.92 (95% CI, 0.81–1.05)]; abnormal DV flow [LR+, 4.77 (95% CI, 1.33–17.04); LR–, 0.49 (95% CI, 0.17–1.41)]. The highest sensitivities were observed for intertwin NT discrepancy [52.8% (95% CI, 43.8–61.7%)] and abnormal DV flow [50.0% (95% CI, 33.4–66.6%)].¹¹⁹

Arterioarterial Anastomoses

The search of arterioarterial (AA) anastomoses in the placental plate of MC placentas by color Doppler has until now mainly provided a negative value: only 5% of monochorionic twins will develop TTTS if AA anastomoses are present; if absent, 58% will develop TTTS. In the studies of Taylor and coworkers, the sensitivity and positive predictive value for absent AA anastomoses in predicting TTTS was 74 and 61%, respectively.¹²⁰ The major limitation to the use of absent AA anastomoses in predicting TTTS is the difficulty in being sure that an AA anastomosis is really absent or simply not yet seen, as it frequently happens before 18 weeks.

Intertwin Membrane Folding

At 15–17 weeks of gestation, the disparity in amniotic fluid volume between the two amniotic sacs seems to cause membrane folding: if present, 28% of cases developed severe TTTS and 72% developed mild TTTS. If membrane folding was absent, no cases of TTTS were recorded.

DISCORDANCE OF FETAL GROWTH: WHAT IS ADAPTATION, PROMOTION, AND GROWTH RESTRICTION IN MULTIPLES?

The restriction of intrauterine fetal growth is more frequently found in multiple pregnancies: about 52% of twins and 92% of triplets present low birth weight (<2,500 g) compared to 6% of singletons, whereas 10% of twins and 32% of triplets are born with very low birth weight (<1,000 g) when compared to 1% of singletons. This has not been scrutinized in depth because of three limitations: when we define fetal growth curves of fetuses with comparable

growth potentials should be adopted. At present, it is believed that multiples do not have the same growth potential as singletons and there are serious doubts concerning the appropriateness of singleton standards for multiples. Second, the growth pattern of late pregnancy is practically unknown because preterm birth (by singleton standards) is the rule than the exception in multiple gestations (before 37 weeks, about 50% of twins, and 91% of triplets were already born in comparison with 9% of singletons). Finally, there are few longitudinal studies and most of our knowledge is derived from birth weight by gestational age relationships (growth curves).

Grande et al. demonstrated that early discordant twin pregnancies were at significantly higher risk of chromosomal (OR 11.42; 95% CI, 2.78–46.94) and structural anomalies (OR 5.91; 95% CI, 2.25–15.54), spontaneous fetal loss (OR 4.23; 95% CI, 1.79–10.01), birthweight discordance (OR 2.8; 95% CI, 1.48–5.65), and small-for-gestational-age (OR 3.48; 95% CI, 1.78–6.79). Crown-rump length (CRL) discordance ($\geq 10\%$) presented with higher rates of structural anomalies, stillbirth, birthweight discordance, and small newborns. Fetal anomalies and growth restriction increased in severe CRL discordance ($\geq 16\%$).¹²¹ More recently, Curado et al.¹²² and Litwinska et al.¹²³ demonstrated that a large discordance in CRL is associated with a high risk for fetal loss and ominous perinatal outcomes.

If we monitor fetal growth in multiple pregnancies by singleton standards, $>50\%$ of triplets are considered small for gestational age (SGA) at 35 weeks, and $>50\%$ of twins are considered SGA at 38 weeks. The average birth weight at 39 weeks is 3,357 g, at 35 weeks 2,389 g and at 32 weeks, 1,735 g, for singletons, twins, and triplets, respectively. Though many authors classify twin infants as fetal growth restriction (FGR) by using a gender- and gestational age-specific birth weight below the 10th centile,^{124,125} the truth is that a biological phenomenon with a frequency of over 50% reluctantly will be considered a pathological event. The re-evaluation of the median birthweight centile of twins and triplets compared to the 10th centile for singletons showed that the average multiple is not “SGA” and weighs more than the 10th birth weight percentile for singletons until 35 weeks in triplets and 38 weeks in twins.¹²⁶

The common growth curves show that deviations from singleton standards occur after 28 weeks.¹²⁶ The uterine milieu, comprising uteroplacental, maternal, and fetal components, limits physiologically the growth potential of the individual fetus in multiple pregnancies. This concept implies that most multiples delivered after 28 weeks are growth restricted compared to singletons, as a result of an adaptive process. The total twin and total triplet birthweights exceed that of the 90th birthweight centile for singletons as early as 25 weeks of gestation (data derived from 3.6 million singletons from the Matria database). The uterine potential adaptation to multiple pregnancies is also appreciated by realizing that the average singleton birth weight at 40 weeks is reached as early as 32 weeks in twins and as early as 29 weeks in triplets.

Consequently, the individual multiple is relatively growth-restricted compared to singletons, whereas the entire pregnancy is growth promoted.¹²⁷ This physiological restriction (reduction in fetal size) is a way to promote a more advanced gestation as long as possible, to overcome the tremendous increase in volume, the uterine overdistention, and the higher frequency of preterm

delivery in multiples.¹²⁸ The variables that most strongly influence uterine adaptation are parity (less in nulliparas), maternal age, maternal height, and weight gain.

Discordant growth is another potential way to reduce the uterine volume to promote an advanced gestational age at birth. The problem is to distinguish between natural variation and pathological growth restriction. Differences as large as 15% may be normal, whereas 15–25% discordance may denote adaptation, and differences of $>25\%$ reveal the inability to maintain growth.²³ This latter group presents the best correlation with adverse outcomes, namely neonatal mortality and morbidity and intensive care admission.³

The risk of occurring a FGR in multiple pregnancies is 10 times greater than in a singleton pregnancy,¹²⁸ being even greater in a monochorionic (34%) compared to a dichorionic pregnancy (23%).⁹ More recently, our group put in evidence that in uncomplicated MC twin pregnancies, abnormal DV flow in at least one of the fetuses seems to be associated with a higher discordance in birth weight than in those with the normal flow in both fetuses.¹¹⁸ In fact, in pregnancies with abnormal DV flow in at least one of the fetuses the median discordance in birth weight was higher than in those with normal DV flow in both twins (13.2 vs 7.8%, $p = 0.006$).

Hence, the meaning of fetal growth discordance in multiples depends on chorionicity. The abdominal circumference is the most reliable sonographic criterion for the establishment of growth discrepancy. A difference of 20 mm translates into a birth weight discrepancy of 20% or more. A dichorionic twin pregnancy (dizygotic in 90%) may be ascribed to a different genetic constitution of the twins or an unequal placental function. In a monochorionic pregnancy, as the genetic content is the same, the growth discordance may be due to an unequal division of the cellular mass (unequal sharing of the placenta), VCI of the cord, vasa previa, or to the unbalanced transfusion of blood through the vascular anastomoses. Our group demonstrated that VCI is not associated with the development of TTTS but increases the risk of adverse outcomes. Both VCI and TTTS independently increased the prevalence of intrauterine fetal death and lower gestational age at birth similarly, showing that VCI is an important indicator of adverse perinatal outcomes in monochorionic twins.^{34,35}

By using data from the Matched Multiple Birth Data Set from the National Center for Health Statistics, it was noticed that 10,683 pairs showed a discordance $>25\%$ (8.2% of the entire population of twins). This population was subdivided into three groups according to the birth weight of the smaller twin (<10 th centile, 10–50th centile, or >50 th centile). These subgroups correspond to severely discordant twin pairs who are growth restricted, growth adapted, or growth promoted, respectively. The frequencies of each subgroup were unaltered through the third trimester: 6,668 (62.4%), 3,514 (32.9%), and 501 (4.7%) severely discordant sets.³ Neonatal mortality was significantly higher (29.1%) when the smaller twin weighed less than the 10th centile for gestational age compared with other subgroups (11.2 and 11%). The data prove that even among severely discordant pairs, 40% are appropriately grown twins, of which 6% are growth promoted.

Importantly this growth restriction among twins, mainly the monochorionic subgroup has well-known ominous consequences in fetal neurodevelopment. What is new is that this issue has a negative impact on all three areas of development: cognition, language, and motor skills.¹²⁹

MULTIPLES AND CEREBRAL PALSY: EFFECT OF PREMATURITY OR MORE?

In 1897, Sigmund Freud suggested that multiple pregnancies were the most important cause of cerebral palsy more relevant than perinatal asphyxia or preterm birth. A century later, this postulate remains valid.^{130–134} we know that the risk of one of the fetuses being affected by cerebral palsy is 1.5% in twins, 8% in triplets, and 43% in quadruplets.¹³⁵ In reality, there is an exponential relationship between the number of fetuses in pregnancy and the rate of cerebral palsy. There is a higher prevalence of brain damage in monochorionic and like-sex twins than in dichorionic pregnancies, namely when the death of the co-twin occurs: the incidence of the lesions of the white matter after the death of the co-twin in a monochorionic pregnancy is 25% in contrast with 3% in the survivor of a dichorionic twin pregnancy.¹³⁶ Prematurity and low birth weight are the most relevant risk factors for long-term neurological morbidity. Factors such as zygosity, intrauterine growth restriction, fetal weight discordance, and type of birth are less powerfully correlated with the risk of cerebral palsy in multiple pregnancies.¹³⁷

In a population-based retrospective cohort study comparing neurological problems in Swedish children born after IVF with matched controls, the former were 70% more likely to need rehabilitation. The risk of cerebral palsy was 4-fold increased in children born after IVF. The data confirm a model that suggested a significantly lower estimated cerebral palsy rate (2.7/1,000 neonates) after spontaneous pregnancies as compared with transfer of three embryos (OR = 6.3), two embryos (OR = 3.3), and transfer of three embryos in which all triplets have been reduced to twins (OR = 3.8). Similar estimations suggested that iatrogenic multiples contribute 8% to the annual number of cerebral palsy cases in the USA.

In the specific case of TTTS, Lopriore et al.¹³⁸ presented important data concerning the psychomotor development of survivors from TTTS evaluated until school entry. From a total of 29 children affected *in utero* by TTTS and evaluated during 8 years, 41% presented cerebral anomalies disclosed by ultrasound and about 21% had cerebral palsy.¹³⁸ This figure rose to 50% in the cases of TTTS complicated by the intrauterine fetal demise of the co-twin, whereas rates of 14% of cerebral palsy were found in the cases in which both twins survived.

To anticipate the neurologic risk in cases of TTTS complicated with the death of the co-twin, Senat et al.¹³⁹ proposed the sequential evaluation of the fetal middle cerebral artery peak systolic velocity to predict fetal anemia within 24 hours of the death of one monochorionic twin and to monitor hemoglobin concentration in the surviving fetus at risk for acute anemia.¹³⁹ This method was found to be a reliable non-invasive diagnostic tool and may be helpful in counseling and planning invasive testing.

CONCLUSION

In clinical terms, the assignment of chorionicity is more relevant than zygosity determination and will dramatically influence the perinatal outcome. About 30% of MC twin pregnancies are complicated by TTTS, isolated discordant growth, twin anemia-polycythemia sequence, congenital defects, or intrauterine demise. About 15% will be eligible for invasive fetal therapy. Combining several sonographic markers, ultrasound examination in the first and early second trimester can differentiate monochorionic twins at high risk for an adverse outcome from those likely to be uneventful, useful information for patient counseling and planning of care.

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