Morphological and ultrasound findings in multiple pregnancy placentation

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Abstract
The incidence of multiple pregnancy has significantly increased over the past decades, reaching different statistics to double, triple, or even overcome these numerical orders globally. Zygosity and chorionicity are the key elements in the multiple pregnancy but the placentation issue should be correlated primarily with zygosity, unlike chorionicity that should be correlated with the outcome and complications of multifetal gestation. Multiple pregnancy is by itself a special maternal–fetal condition, and the monochorionic one, moreover, due to specific complications. These aspects make early assessment of chorionicity and amnionicity a priority. Ultrasound is essential in pregnancy but pathological placental examination after delivery is complementary, in order to have a complete overview of potential mechanisms and pathogenesis affecting twin gestation. In this review, we highlight both ultrasound aspects specific to multifetal placentation, complemented by macro and microscopic morphological aspects, which underpin the obstetric imaging.

Keywords: twins, chorionicity, amniotic membranes, specific complications, microscopic analysis.

Introduction
Chorionicity is undoubtedly the essential condition for multiple pregnancy outcome. Any ultrasound (US) sign that raises the suspicion of a multiple pregnancy, must make the diagnosis of chorionicity a priority.

Each embryo is genotypically structured to express itself morphologically, during embryonic development, with a trophoblast and a set of amniotic membranes [1, 2]. The frequency of multiple pregnancy has steadily increased over the last few decades, mainly due to maternal age at the time of conception, and the use of assisted reproductive technology (ART) [3].

Dizygotic (DZ) twin pregnancy, respectively fraternal type or non-identical twin gestation, is phenotypically expressed in the above manner, even if the two placental masses become later, due to limited intrauterine space, clustered or apparently fused, sometimes making chorionicity difficult to diagnose [1].

In monozygotic (MZ) multiple pregnancy, the embryonic disk is split and placentation in this case is, in turn, determined by the moment at which this splitting occurs in relation to the theoretical moment of fecundation [1, 3, 4].

Thus, if splitting occurs within the first three days from the time of fecundation, meaning that splitting occurs before the time of trophoblast differentiation, then each embryo that develops will have its own trophoblast and then its own placenta, resulting the monozygotic–dichorionic–diamniotic (MZ-DC-DA) variety, otherwise the most favorable in outcome [1, 4, 5].

Later on, after the theoretical moment of fecundation, if splitting takes place in the blastocyst between the third and eighth days after fertilization of the ovocyte, when only the trophoblast, not the amniotic cavity, will be differentiated, the two embryos will share in different degrees the same placenta and will each have their own amniotic sac, resulting monozygotic–monochorionic–diamniotic (MZ-MC-DA) type [1, 5].

Further on, if splitting occurs later, between days eighth and thirteenth, when the amniotic cavity is undergoing differentiation, the monozygotic–monochorionic–monoamniotic (MZ-MC-MA) variety arises [1, 4, 5].

Finally, if embryonic splitting takes place after the thirteenth day of fertilization, when the embryonic disk is completely differentiated, the embryonic cleavage will be incomplete, in varying degrees and at different levels, generating conjoined twins [1, 6, 7].

Assessing chorionicity is an essential milestone in managing multiple pregnancy. In terms of the optimal time for US evaluation, several studies specify the interval...
between six and nine gestational weeks, but it is certain that the maximum accuracy of the determination is before 14 weeks and the accuracy of the chorionicity assessment decreases after that time, making it difficult counting the planes of the interfetal membrane [8–14].

Amnionicity is also an important criterion of maternal–fetal outcome in multiple gestation, and its specification should not be accomplished until nine weeks of gestation [8, 10, 15–17].

The placation issue in multiple pregnancy should be correlated primarily with zygosity, unlike chorionicity that should be correlated with the outcome and complications of multifetal gestation [1].

**Chorionicity assessment**

The identification in the first trimester of pregnancy of two or more distinct placental masses or, on the other hand, of two fetuses of different sexes is an almost axiomatic criterion of dichorionicity (DC), or even more of dizygosity (Table 1).

The US expression of monochorionicity (MC) is represented by the T sign. This US expression has as a morphological background the fine, supple and almost perpendicular insertion of the two thin amniotic membranes on the plane of the single chorial disk (Figure 1) [8, 9, 18].

From the morphological point of view, in the case of MZ-MC-DA gestation, the interfetal membrane is the result of joining of the two amniotic membranes, the chorion being common (Table 1; Figure 2) [8, 9, 19–23].

The lambda and twin peak signs respectively, are specific to DC gestation (Figure 3). When membrane insertion appears thick and dense, the US expression is the lambda or twin peak sign, and from a morphological point of view, it is a DC-DA twin pregnancy [1, 8, 9, 24, 25].

Both the lambda and the twin peak signs are the US expression of the morphology of DC, both of which appear as a hyper or iso-echogenic triangular image at the base of the placentation insertion of interfetal membrane on the fetal face of the apparently unique placental mass (Figure 3) [2, 8, 10, 26].

**Table 1 – Ultrasound of chorionicity and amnionicity**

<table>
<thead>
<tr>
<th>US of chorionicity</th>
<th>US of amnionicity</th>
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<tbody>
<tr>
<td>▪ Number of gestational sacs;</td>
<td>▪ Assessment of the interamniotic membrane:</td>
</tr>
<tr>
<td>▪ T sign (MC-DA);</td>
<td>▪ 4 layers or more than 2 layers ⇒ DC-DA;</td>
</tr>
<tr>
<td>▪ Lambda sign (DC-DA);</td>
<td>▪ 2 layers or less ⇒ MC-DA.</td>
</tr>
<tr>
<td>▪ Epsilon sign (higher than two numerical order);</td>
<td>▪ Assessment of the Yolk sacs:</td>
</tr>
<tr>
<td>▪ Two distinct placental masses;</td>
<td>▪ 2 Yolk sacs ⇒ MC-DA;</td>
</tr>
<tr>
<td>▪ Fetal sex;</td>
<td>▪ 1 Yolk sac ⇒ MC-MA.</td>
</tr>
<tr>
<td>▪ Detailed assessment of the interfetal membrane;</td>
<td>▪ Assessment of the umbilical cords (MC-MA):</td>
</tr>
<tr>
<td>▪ Yolk sac;</td>
<td>▪ cords entanglement;</td>
</tr>
<tr>
<td>▪ Evaluation of amniotic cavities.</td>
<td>▪ cords proximity.</td>
</tr>
<tr>
<td>▪ Conjoined twins:</td>
<td>▪ fused embryos/fetuses, concomitance of movements, single/double cardiac activity.</td>
</tr>
</tbody>
</table>

US: Ultrasound; MC: Monochorionic; DC: Dichorionic; MA: Mono-amniotic; DA: Diamniotic.

**Figure 1** – MZ-MC-DA twin pregnancy: (A) US demonstrating the T sign (arrow); (B) Transvaginal (TV) first trimester US demonstrating two fetuses (red arrows) and the presence of two planes in the interfetal membrane (blue arrows); (C) First trimester TVUS using tomographic ultrasound imaging (TUI), demonstrating two fetuses and a two planes interfetal membrane; (D) First trimester TVUS using TUI in a 10 weeks (+ 2 days) twin pregnancy demonstrating two planes interfetal membrane and Yolk sac. MZ-MC-DA: Monozygotic–monochorionic–diamniotic; US: Ultrasound; TVUS: Transvaginal ultrasound.
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Figure 2 – MZ-MC-DA twin pregnancy: (A) Gross placental assessment demonstrating the presence of a single placental mass and flat surface at the level of amniotic membranes insertion (arrows); (B) Detail of the gross placental assessment demonstrating two layers of the interfetal membrane (yellow arrows), unique chorionic membrane (white arrows) and interfetal placental anastomoses, typical of monochorionicity; (C) Umbilical cord fetus A; (D) Umbilical cord fetus B. Note the umbilical cords discordance due to twin-to-twin transfusion syndrome (fetus A – recipient, thick and hydropic umbilical cord, fetus B – donor, thin umbilical cord). MZ-MC-DA: Monozygotic–monochorionic–diamniotic.

Figure 3 – MZ-DC-DA twin pregnancy: (A) US demonstrating the lambda sign (arrow); (B) Transabdominal first trimester US demonstrating intermembrane chorionic tissue interposition, generating the lambda sign (arrows); (C) First trimester transabdominal US and 3D rendering demonstrating two fetuses and an obvious twin peak sign (arrow); (D) 3D US demonstrating the triangular shaped twin peak sign by intermembrane chorionic tissue interposition (arrow). MZ-MC-DA: Monozygotic–monochorionic–diamniotic; US: Ultrasound; 3D: Three-dimensional.
Referring to the morphological perspective, this configuration translates the triangular projection of the villous tissue into the junction of fetal membranes with the apparently unique trophoblastic or placental mass, in fact, from a morphological point of view, being two chorionic disks (Figure 4) [1, 8, 10, 27].

The interfetal membrane in the case of DC placentation is formed by four layers, namely two chorions and two amnions (Figure 4, C and D).

Trizygotic (TZ) triplets occur by fertilizing three oocytes (Figures 5 and 6), while the DZ triplets result from the fertilization of two oocytes and splitting of one of the embryonic disks. MZ triplets occur by fertilizing a single oocyte that is replicated, then another replication occurs on one of the already replicated zygotes [1, 4, 28].

Therefore, the multiple pregnancy with a numerical order higher than two, referring here to the triplet gestation, may be mono, di or trichorionic (TC).

Derom R et al. [29] have put forth a set of aphorisms on the correlation between zygosity and placentation in the twin and multiple pregnancy, precisely to strengthen the rules, without excluding exceptions.

According to these aphorisms, the TZ triplets are trichorionic (Figures 5 and 6), and the DZ triplets are either bichorionic or trichorionic. Also, the MZ triplets may be monochorionic, bichorionic or trichorionic, and dichorionic triplets are either MZ or DZ, while monochorionic triplets are MZ [29].

In the case of triplets, the epsilon or Y sign (Figure 5) is the result of placental insertions and the attachment of the two interfetal membranes (Figure 6). Also, in the case of this type of placentalion, the additional presence of two or three lambda signs, or the concomitant presence of three lambda signs, complements the diagnosis (Figure 5).

In about a half of DC and in quite many TC pregnancies, placentae are fused (Figure 5), and this could be important because the cords may not insert in a central location and thus predispose the fetuses to different intrauterine conditions, or the fusion may not be symmetrical and placental surfaces may comprise different functional territories, these conditions being able to lead to the discordant impairment of fetal growth [29–33].

Postnatal examination of placenta from multiple pregnancies is necessary for a complete pre and postnatal diagnosis of multiple gestation, and this involves the identification of placenta or placental masses, the identification of interfetal membranes and amniotic sacs, the assessment of umbilical cord and insertions, the inspection of the membranes and the inspection and evaluation of the maternal and fetal surfaces of placentae [1, 29, 34–37].

Figure 4 – MZ-DC-DA triplet pregnancy: (A) Gross placental assessment demonstrating two fused placental masses, thick and dense interfetal membrane and abnormal cord insertion; (B) Velamentous cord insertion of one of the twins; (C) Gross assessment of the interfetal membrane demonstrating the morphological background of the lambda sign through the placental insertion of the fetal membrane set and the projection of the villous tissue at their junction; (D) Gross assessment of the interfetal membrane demonstrating four layers. MZ-DC-DA: Monozygotic–dichorionic–diamniotic; a1: Chorion fetus A; a2: Amnios fetus A; b1: Chorion fetus B; b2: Amnios fetus B.
Amnionicity assessment

If the problem of chorionicity is established, amnionicity seems easier to deal with. The presence of the interfetal membrane, either MC or DC type, practically solves the diagnosis of amnionicity.

Probably a substantial differential diagnosis of amnionicity is represented by the presence of the amniotic bands in multiple pregnancy, which besides the specific complications of these abnormal structures could be problematic in determining amnionicity.

Therefore, the interamniotic membrane is the defining marker for the diagnosis of amnionicity, and when the ultrasound examination in the first trimester of pregnancy demonstrates the presence of a twin or multiple pregnancy, but is unable to highlight the interembryonic or interfetal membrane, MA pregnancy must be suspected, especially when there is a single Yolk sac (YS) (Table 1) [38–43].

From the embryological point of view, MA twins are the result of single blastocyst splitting after the appearance of ectodermal and amniogenic cells in the embryonic cell mass at 8–9 days post-fertilization. If this process of splitting and amniogenesis extends to 12th or 13th day, incomplete separation of umbilical cord, respectively single or forked cord may occur [38, 39, 44–47].

Also, Egan & Borgida consider that the appearance of an apparently normal amniotic fluid volume without visualizing an interembryonic or interfetal membrane and two embryos or fetuses inside this amniotic sac is a first element of monoamnionicity (MA) [40].

The classic US pattern of MA is the confirmation of the intrauterine presence of two or more embryos or fetuses,
without a clear interembryonic or interfetal membrane and a single YS, but the presence of two YS do not definitely exclude the existence of MA pregnancy because the number of YS depends on the moment of embryonic disk splitting [38, 48].

Thus, the number of MC twins YS, more precisely if the MA twins have a single YS resulting from the fusion, or two YS, is determined strictly by the time of the germinal disk splitting [12, 15, 38, 39, 46, 49–51].

Maybe more important than diagnosis itself are the complications of MA, represented by the intertwining, entanglement or knotting of the umbilical cords, with increased risk of fetal death in utero of both fetuses [38, 45, 52].

In MA pregnancy, which is by definition MZ and MC, the umbilical cord insertion is usually very close, more precisely within a range of about 6 cm, favoring cords contact, and if their length is significant, increases the risk of complications described above [38–40, 45].

Cord entanglement can be detected by US from 10 gestational weeks, and it has been associated with a high incidence of intrauterine fetal death, therefore regular US follow-up using two-dimensional (2D) or if available, three-dimensional (3D) and color Doppler imaging is recommended [3, 53–55].

Due to MC, in MA twins, the interfetal placental anastomoses are always present but perhaps, paradoxically, twin-to-twin transfusion syndrome (TTTS) has a much lower incidence than in MC-DA twins [45, 64].

**Monochorionic twin-specific complications**

Almost all MC pregnancies have vascular connections or anastomoses between the two umbilical and placental circulations [1, 3, 57–59].

Placental angioarchitecture in MC twins involves two morphological and pathophysiological patterns, namely anastomoses of superficial vessels by arterio-arterial (AA) and veno-venous (VV) connections, and deep vessels that are connected to the superficial vessels of both fetuses, so the input flow is from a fetus and the output flow is to the other, through the arterio-venous (AV) connections, this pattern being valid especially in TTTS [58, 60].

AA anastomoses are generally superficial and bidirectional, while VV connections are superficial, bidirectional and present in only one-fourth of MC placental structures [3, 57–59].

AV anastomoses are present in 95% of MC placenta, are unidirectional, located profoundly in the placental structure, and when they are unbalanced, are liable for the occurrence of the main complications of MC, including TTTS, twin anemia polycythemia sequence (TAPS), and severe growth discordance [3, 57, 59, 61–64].

**Twin-to-twin transfusion syndrome**

From a functional point of view, in MC multiple gestation, functioning of the interfetal transfusion system is a normal process, as long as the circulatory flow is balanced, preventing the occurrence of clinical manifestations [58, 65].

Fisk et al. state that TTTS affects 10–20% of MC-DA twins, resulting in discordant amniotic fluid volume, with hypervolemia and oliguria–oligohydramnios in the donor and hypovolemia and polyuric–polyhydramnios in the recipient [64].

It seems that TTTS is responsible for approximately a half of all perinatal deaths associated with MC multiple gestation [65, 66].

TTTS is a classic complication of MC multifetal gestation that occurs by unbalancing the placental vascular anastomotic flow, resulting in favoring the circulating stream of one of the fetuses, to the detriment of the other [58, 67].

Also, Denbow et al. and Hack et al. consider that there is no unique angio-architectural pattern for TTTS, as large ex vivo studies, show that TTTS occurs considerably in the presence of AV anastomoses without a balancing AA anastomoses [64, 68, 69].

Moreover, several authors show that AV anastomoses are discovered in 90–95% and AA anastomoses in 85–90% of MC-DA placentae, whereas VV anastomoses are uncommon, identified in only 15–22%, when they are associated with poor perinatal survival but not with TTTS [64, 68–70].

AA anastomoses seem to have a compensatory role, and in their absence 43–78% of cases develop TTTS, taking into account that when a single AA anastomosis is present, the TTTS development rate drops to 14% [64, 68, 69, 71].

Essentially, hypervolemia of the recipient and hypovolemia in donor outline polyuria–polyhydramnios and oliguria–oligohydramnios sequences, defining TTTS [58].

These sequences, having the placenta as a central element, underlie the morphological and US semiology of TTTS. This semiology includes volume disorder of amniotic fluid or fetal urinary bladders, Doppler abnormalities, stuck twin, folding intertwin membrane, fetal hydrops and single or double fetal death (Figures 2, 7 and 8) [58, 64, 72].
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Figure 8 – TTTS in a MC-DA twin pregnancy: (A) 3D US demonstrating in the foreground the recipient twin finely rendered because of the polyhydramnios – note the donor twin in the background (arrows) with the interfetal membrane tightly adherent to the fetus and stuck twin appearance; (B) Severe TTTS with double fetal demise – note the recipient twin which appears hydroptic and the donor is smaller. TTTS: Twin-to-twin transfusion syndrome; MC-DA: Monochorionic–diamniotic; 3D: Three-dimensional; US: Ultrasound.

Superficial AA and VV anastomoses are termino-terminal or termino-lateral vascular connections (vascular lumen to vascular lumen), while profound AV anastomoses are connected through a deeply expanded vascular capillary bed to the placental cotyledon [58, 72].

If the AA and VV placental anastomoses are superficial and allow bidirectional blood flow, practically the pathological and morphological substrate of TTTS is at the level of AV deep vascular anastomoses. The AV anastomoses therefore contain an artery vascularized cotyledon from one of the twins, drained by a chorionic vein belonging to the other twin [58, 65, 73].

Thus, the TTTS substrate is an asymmetric resistance to the effective capillary blood flow of the two fetuses with the corresponding chorionic areas, leading to intraplacental intertwin transfusion. Additionally, ex vivo studies have demonstrated that placentae from TTTS pregnancies have a significantly increased number of AV anastomoses as compared to those from MC twin pregnancies without TTTS [61, 65, 73, 74].

Twin anemia polycythemia sequence

TAPS is a rare complication, belonging to MC twins only. TAPS is defined as a chronic form of feto-fetal transfusion, defined by considerable differences in fetal hemoglobin in the absence of amniotic fluid volume disorder, the latter belonging to the TTTS [75].

TAPS is characterized by the existence of anemia, with increased reticulocyte count, in the donor, and polycythemia in the recipient twin, via few and thin vascular anastomoses of placenta [76].

TAPS pathogenesis, unlike TTTS, is based on a unique placental angio-architecture characterized by the presence of only a few and small-scaled AV and also lack of AA anastomoses [64, 70, 75].

TAPS can develop spontaneously or iatrogenically after fetoscopic laser surgery for TTTS [76, 77].

TAPS was first described by Lopriore et al. [78], and then completely characterized by Slaghekke et al. [79]. According to these authors, TAPS is prenatally diagnosed based on Doppler US findings with an elevated middle cerebral arterial peak systolic velocity (MCA-PSV) ≥1.5 multiples of the median (MoM) in the donor twin, suggestive for fetal anemia, and a decreased MCA-PSV ≤1 MoM in the recipient twin, suggestive for fetal polycythemia. Postnatal criteria are stated on the presence of an intratwin hemoglobin difference of ≥8 g/dL and at least one of the following: small anastomoses at the placental surface (<1 mm) and/or reticulocyte count ratio (reticulocyte donor/reticulocyte recipient) ≥1.7 [78, 79].

The absence of amniotic fluid discordance in TAPS may be related to the gradual intratwin blood transfusion, which allows hemodynamic compensatory mechanisms to interfere with and stop the fluid imbalance [70, 79].

The exceedingly increased reticulocyte number in the donor twin, also reveals the chronic condition of TAPS. In addition, the additional criteria are required to differentiate TAPS, which is a chronic pattern of transfusion, from acute peripartum TTTS, which is an intense transfusion model, through large anastomoses, occurring during delivery [80].

From the US point of view, in some cases of TAPS Slaghekke et al. [79] found a striking difference in placental thickness and echodensity. The placental territory of the anemic twin was hydropic and had an elevated echodensity, while the placental sector of the polycythemic twin appeared to be normal.

Thus, TAPS is a form of feto-fetal transfusion completely different from TTTS, which occurs through small, less than 1 mm anastomoses, that may appear in MC pregnancy unexpectedly, or after laser treatment for TTTS [79–81].
Discordant fetal growth

Multiple studies state that in MC twins, intrauterine growth restriction (IUGR) is typically due to an inappropriate placental distribution, with an elevated blood flow transfusion from one twin to the other, through wide diameter AV anastomoses [3, 57, 63, 82, 83].

Actually, selective intrauterine growth restriction (sIUGR) is a routine situation associated with MC twins. It is also increasingly studied as an important issue in MC twins, with potentially serious risks of intrauterine fetal demise (IUDF) or neurological adverse outcome for one or both twins [83–86].

Hubinont et al. and Lewi et al. state that in case of early-onset sIUGR, the placentae are more inaccurate shared, having more and wider AA connections, compared with placentae with late-onset discordant or concordant fetal growth [3, 86].

According to Valsky et al., sIUGR in MC twins is relevant in cases where the estimated fetal weight (EFW) of the small fetus decreases less than the 10th percentile [86].

Regarding the pathophysiology of placental sharing, several authors discuss the hypothesis of a salvage vessel, the risk of major growth discordance is considered to be low [57, 73, 82, 86, 87].

In the same manner, survey in the recent years, stated that sIUGR is correlated with considerable risks for the regularly grown fetus even if both twins are born alive [83].

Table 2 – Classification of different types of sIUGR

<table>
<thead>
<tr>
<th>sIUGR</th>
<th>Characteristics</th>
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<tr>
<td><strong>Type I</strong></td>
<td>• Unequally shared placental mass, average size AA anastomoses [3]; • Doppler pattern – positive diastolic flow in the umbilical artery of the small twin [83]; • Fair number of anastomoses and bidirectional fetal flow interchange [83]; • Generally associated with good outcomes [83]; • Clinical evolution – benign in most instances [83].</td>
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<tr>
<td><strong>Type II</strong></td>
<td>• Persistently absent or reversed end-diastolic flow in the UA [3, 83]; • Unequally shared placenta, absent or small AA anastomoses [3]; • Fetal territory of the IUGR twin – usually extremely small [83]; • The great majority – will show in utero deterioration, but with important differences with respect to DC twins [3, 83].</td>
</tr>
<tr>
<td><strong>Type III</strong></td>
<td>• Major unequal placental sharing [3]; • At least one large AA anastomose [3]; • Intermittently absent/reverse end-diastolic flow in the UA Doppler of the IUGR twin [83]; • The characteristic feature of this Doppler pattern, unique to monochorionic twins – the alternation of phases of positive with phases of absent/reverse diastolic flow, normally but not always in a cyclical fashion [83]; • In most cases – the compensating effect of the large AA allows survival of the IUGR fetus until advanced stages of pregnancy, without showing clear signs of hypoxic deterioration [3, 83]; • Pregnanacies are associated with a significant increase in the risk of unexpected IUFD of the IUGR fetus and of brain injury in the normally grown twin [83].</td>
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Single intrauterine fetal demise (sIUFD)

Generally speaking, IUFD is more common in the multiple pregnancy compared to singletons. In MC twins, sIUFD has far more complex consequences than DCs, secondary to fetal death [94].

Lewi et al. found that sIUFD risk is superior in MC twins (7.5%) compared to DC twins (3%), and MC pregnancies are at special risk due to intertwin placental vascular anastomoses [95, 96]. sIUFD also presents a 20% risk for multicystic encephalomalacia, porencephaly, or preterm birth of survivors in MC pregnancy [94].

According to Hubinont et al., the severity of sIUFR can be assessed according to placental specific features and blood flow in the umbilical arteries [3].

Regarding the potentially injured fetus with normal growth, there may be on the one hand, an elevated prevalence of neurological impairments in the normally grown fetus due to an increased risk of acute feto-fetal transfusion occurrences in utero, and on the other hand, since these pregnancies must be, by default delivered previous to the death of the sIUFD twin, the normal fetus is exposed to serious prematurity with its acknowledged circumstances in terms of neurodevelopmental sequelae [83, 85, 88, 89].

It should also be mentioned that discordant growth between the two fetuses is defined by a birthweight difference of >25% between the twins, being a somewhat different finding from sIUFR. It arises with a comparable incidence of 10–15% in both DC and MC pregnancies [3, 57, 63].

In DC twins as well as in singleton pregnancies, umbilical artery (UA) Doppler is a key point for the diagnosis and management of fetuses with IUGR due to placental insufficiency [83, 90].

Depending on placental characteristics, interfetal anastomoses and UA blood flow, Valsky et al. and Hubinont et al. have established a classification system of different types of sIUFR (Table 2) [3, 83].

Also, many authors assert that UA Doppler in MC twins with sIUFD may present three major waveform models, as characterized by the features of diastolic flow in positive, persistently absent or reverse, and intermittently absent or reverse end-diastolic flow [83, 91–93].
to several mechanisms, most notably hypoxic–ischemic, leading ultimately to intraventricular hemorrhages and periventricular leucomalacia. Another mechanism, not as important but probably associated or concomitant with sIUFD, is the release of thromboplastic material by the deceased fetus, which reaches the survivor’s circulation, functioning as a trigger for generating disseminated intravascular coagulation [94–99].

Several authors also consider the hypovolemic shock that the surviving fetus feels is an important mechanism. According to this theory, the deceased fetus acts by a tap effect on the survivor, which basically bleeds to the deceased fetus, lacking vascular resistance. This rapid transfusion syndrome is accomplished by superficial AA and VV anastomoses [4, 94, 98–99].

In this case, as well, the previous diagnosis of chorionicity is fundamental.

**Monoamniotic twins**

The last frontier of the MZ twin pregnancy that may have a favorable prognosis is the MZ-MC-MA multiple gestation.

Along with all the other risks of monochorionicity, the MA twin pregnancy also has the primary risk of hyperspiraling and torsion of umbilical cord with increased risk of fetal death in utero of both fetuses. Therefore, the major risk of monoamnionicity is represented by the umbilical cord entanglement [4, 94, 99–100].

Obviously, cord entanglement cannot be predicted nor avoided, but it is possible that the length of the umbilical cords, can suggest the possible occurrence of this pathology.

A systematic review of Kuwata et al. suggested that US prenatal diagnosis of cord entanglement did not change neonatal outcome, reporting an overall survival rate as high as 88.6% [54].

Doppler assessment of the umbilical flows in the presence of such complication reveals some characteristic features: diastolic notch, increased systolic/diastolic ratio, absent end-diastolic flow or umbilical vein pulsatility [94, 101].

TTTS is very rare in MA twins. This is due to the unique amniotic cavity that functions in a constant intra-amniotic pressure regime, on the one hand, and on the other hand, the placenta of MA pregnancies have a significantly increased number of superficial and deep anastomoses compared to placenta of DA pregnancies. These two mechanisms have a protective role against the occurrence of TTTS [4, 48, 94, 99, 101].

However, umbilical cord accidents may occur without any prodromal sign, so MA is a subject to hazard by definition.

**Twin reversed arterial perfusion (TRAP) sequence**

TRAP is a rare and severe complication of MC twin pregnancies.

This anomaly occurs because of vascular disturbances occurring early during embryogenesis, in which one of the fetuses, deeply dysmorphic – the acardiac fetus, receives a circulatory support from the other fetus, called the pump fetus, by means of aberrant placental AA anastomoses [102–104]. This condition has been described as being the most severe malformation in humans [105].

Placenta in TRAP is most commonly MC-DA (74%), in which a thin membrane classically separates the gestational sacs, the pump and the acardiac fetuses. MA is present in about 24% of cases [102, 104, 106].

This sequence of early vascular disturbances leads to placental AA and VV intertwin anastomoses that help sustain the abnormal twin [103].

Most commonly, acardiac fetuses are accephalic, with absent superior extremities, because the deoxygenated blood of the umbilical artery preferentially infuses the inferior lower than the upper body [75].

However, the classical diagnostic pattern of TRAP involves a twin pregnancy, most likely a MC-DA, with a normal fetus, at least at first-degree assessment, the pump fetus, and an obviously abnormal, acardiac fetus with multiple hydropic or cystic disturbances, subcutaneous edema and non-classifiable aberrant structures instead of the cephalic extremity, trunk or limbs.

Moreover, regarding the US and maternal–fetal management, estimating the weight of the acardiac fetus is an important aspect in the assessment of pregnancies with the TRAP sequence, as the increase of the acardiac fetus/pump fetus weight ratio, involves an increase of the cardiovascular labor of the pump fetus, which has to bear the mass of the acardiac fetus in addition to its own mass [75, 103, 106].

**Microscopic analysis**

This has been performed after sampling fragments of tissue from the placentall lambda fusion regions. The tissue was fixed in a 10% formalin solution, processed according to the paraffin inclusion technique, and the blocks were cut to a thickness of 4 μm using the HMB350 microtome equipped with a water-based transfer system (STS, microM).

For the classical microscopic study, we used the Hematoxylin–Eosin (HE), Periodic Acid–Schiff (PAS)–Hematoxylin and Masson’s trichrome stainings. For the immunohistochemical (IHC) analysis, histological sections were applied to pre-treated slides with poly-L-Lysine and kept at thermostat for 24 hours, at 37°C.

The IHC technique succeeded the following steps: dewaxing, delimitation of the tissue with Dako hydrophobic marker, dehydration in alcohol with decreasing concentrations: 100%, 96%, 90% and 70%, rehydration in distilled water 3×5 minutes, antigenic exposure in citrate solution pH 7 or ethylenediaminetetraacetic acid (EDTA) pH 9, seven cycles × 3 minutes, washing in distilled water, inhibition of endogenous peroxidase with 3% hydrogen peroxide solution for 30 minutes, washing in distilled water and phosphate-buffered saline (PBS) solution, revealing specific antigenic sites in a 3% dust milk solution for 30 minutes. The primary antibody (Table 3) was then applied for 18 hours, at 4°C.

The next day, the slides were left at room temperature for 30 minutes, washed in PBS, the secondary antibody
Immunoglobulin G 2 alpha; mAb: Monoclonal antibody. Matrix metalloproteinase-13; CD: Cluster of differentiation.

**Table 3 – Immunohistochemical panel of antibodies**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Manufacturer</th>
<th>Clone</th>
<th>Antigenic exposure</th>
<th>Secondary antibody</th>
<th>Dilution</th>
<th>Labeling</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-collagen IV</td>
<td>Dako</td>
<td>CIV22</td>
<td>Citrate</td>
<td>Monoclonal mouse anti-human collagen IV</td>
<td>1:50</td>
<td>Basement membranes</td>
</tr>
<tr>
<td>Anti-NSE</td>
<td>Dako</td>
<td>BBS/NC/V-H14</td>
<td>Citrate</td>
<td>Monoclonal mouse anti-human neuron-specific enolase</td>
<td>1:50</td>
<td>Neuron marker</td>
</tr>
<tr>
<td>Anti-S100</td>
<td>Dako</td>
<td>PC10</td>
<td>Citrate</td>
<td>Polyclonal rabbit S100</td>
<td>1:1000</td>
<td>Neuron marker</td>
</tr>
<tr>
<td>Anti-CK7</td>
<td>Dako</td>
<td>OV-TL 12/30</td>
<td>Citrate</td>
<td>Monoclonal mouse anti-human cytokeratin 7</td>
<td>1:50</td>
<td>Glandular epithelia</td>
</tr>
<tr>
<td>Anti-Ki67</td>
<td>Dako</td>
<td>MIB-1</td>
<td>EDTA</td>
<td>Monoclonal mouse anti-human Ki67</td>
<td>1:50</td>
<td>Cells in division in the G1, S, G2 and M phase</td>
</tr>
<tr>
<td>Anti-PCNA</td>
<td>Dako</td>
<td>PC10</td>
<td>Citrate</td>
<td>Monoclonal mouse anti-proliferating cell nuclear antigen</td>
<td>1:100</td>
<td>Cells in division in the G1, S, G2 and M phase</td>
</tr>
<tr>
<td>Anti-MMP-8</td>
<td>R&amp;D Systems</td>
<td>100608</td>
<td>Citrate</td>
<td>Mouse monoclonal IgG34</td>
<td>1:50</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>Anti-MMP-13</td>
<td>Novus Biologicals</td>
<td>NB110-5919</td>
<td>Citrate</td>
<td>Anti-MMP-13 (VIIIα2) mouse mAb</td>
<td>1:50</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>Anti-CD34</td>
<td>Dako</td>
<td>QBEnd10</td>
<td>Citrate</td>
<td>Monoclonal mouse anti-human CD34 class II</td>
<td>1:50</td>
<td>Endothelial cells of small blood vessels</td>
</tr>
<tr>
<td>Anti-α-SMA</td>
<td>Dako</td>
<td>1A4</td>
<td>Citrate</td>
<td>Monoclonal mouse anti-human smooth muscle actin</td>
<td>1:100</td>
<td>Smooth muscle actin</td>
</tr>
<tr>
<td>Anti-CD68</td>
<td>Dako</td>
<td>KP1</td>
<td>Citrate</td>
<td>Monoclonal mouse anti-human CD68</td>
<td>1:100</td>
<td>Macrophages</td>
</tr>
</tbody>
</table>

NSE: Neuron-specific enolase; CK7: Cytokeratin 7; PCNA: Proliferating cell nuclear antigen; MMP-8: Matrix metalloproteinase-8; MMP-13: Matrix metalloproteinase-13; CD: Cluster of differentiation; α-SMA: Alpha-smooth muscle actin; EDTA: Ethylenediaminetetraacetic acid; IgG34: Immunoglobulin G 2 alpha; mAb: Monoclonal antibody.

From the microscopic point of view, it was observed in classical stainings the fusion region of the placenta, the lambda region, the fused amniotic membranes and various histopathological elements were also present.

After reconstruction of scanned images with a 40× lens, overall images of the lambda area of interest were examined, observing the placenta and amniotic membranes fused, and numerous areas of placental infarction (Figure 9, A and B).

In the classic HE staining, the area of interest shows extensive placental infarction, which has influenced fetal perfusion, perivillous and intervillous fibrinoid necrosis and the presence of syncytial masses and terminal villi of angiomatous type, occurring under hypoxia conditions (Figure 10).

The amniotic membranes of the two fetuses were fused, presenting pavement endothelial epithelium, the chorion accumulating inflammatory infiltration limited to placenta, edema and epithelium with cytotytic lesions. Among the membranes, small sclero-hyalinized placental villi are demonstrated, also necrobioitc, appearing as villous shadows (Figure 11).

Chorionic membranes present amniotic epithelium with cytolysis, edema, and discrete hemat and lymphocytic infiltrate in the underlying chorion (Figure 12).

In the classic Masson’s trichrome staining, placental infarction areas with sclero-hyalinized villi of variable sizes (stained in blue) are observed (Figure 13), and chorionic membranes show cytolysis, edema and the lymphocytic infiltration in the underlying chorion (Figure 14).

Keeling & Khong [107] have described four placental modifications present in the term placenta: the decrease of the villous diameter, the villous capillary system take a broad appearance, similar to the sinusoidal vessels, located at the periphery of the chorionic villi, the development of the syncytial capillary membranes and the fibrinoid degeneration of the villi. All of these have been encountered in the fusion region of the placenta from the twin pregnancy.

Type IV collagen is present throughout pregnancy in specific placental sites and in certain interplacental areas of fetal membranes or in the uterine wall. It is also present in the basal membranes of the epithelium, trophoblast, peritoneum, endometrial surface, glandular epithelium and endothelium. This type of collagen is present at the end of pregnancy in the center of the chorionic villi, forming very fine fibers [108]. By IHC staining with the anti-collagen IV antibody, we noticed that there is a positive reaction in the placental fusion region of a twin pregnancy, in the placental infarction site, and the chorionic edematous membranes did not reacted (Figure 15).

Neuron-specific enolase (NSE) is present in several cell types in placental tissue, representing a protein that has been extensively studied as well, in brain injuries. It has been shown to appear in areas of placental infarction, demonstrating neuroendocrine involvement and nervous system response in affected areas [109, 110].

We obtained a negative reaction in the residual villous trophoblast and a positive cytoplasmic reaction on the extravillous interstitial trophoblast, and the chorionic membranes exhibit negative reactivity to the IHC marker with the anti-NSE antibody (Figure 16).
Figure 9 – The fusion region of the placentae from a DC-DA twin pregnancy. Image obtained by graphic reconstruction: (A) Masson’s trichrome staining, ×40; (B) PAS–Hematoxylin staining, ×40. DC-DA: Dichorionic–diamniotic; PAS: Periodic Acid–Schiff.

Figure 10 – The fusion region of the placentae from a DC-DA twin pregnancy. Placental infarction, perivillous and intervillous fibrinoid necrosis. The presence of syncytial masses and terminal villi of angiomatous type, occurring under hypoxia conditions are observed. HE staining, ×40. DC-DA: Dichorionic–diamniotic.

Figure 11 – The fusion region of the amniotic membranes from a DC-DA twin pregnancy. The pavement endothelial epithelium, inflammatory infiltrated chorion limited to placental structure, small sclero-hyalinized villi, occurring as villous shadows, the presence of edema and epithelium with cytolytic lesions are observed. HE staining, ×40. DC-DA: Dichorionic–diamniotic.

Figure 12 – Chorionic membranes sample with amniotic epithelium demonstrating cytolysis, edema and discreetly hematic and lymphocytic infiltration in the underlying chorion. HE staining, ×100.

Figure 13 – The fusion region of the placentae from a DC-DA twin pregnancy. Placental infarction with sclero-hyalinized villi of variable dimensions. Masson’s trichrome staining, ×40. DC-DA: Dichorionic–diamniotic.

Figure 14 – Chorionic membranes sample with amniotic epithelium demonstrating cytolysis, edema and very discrete hematic and lymphocytic infiltration in the underlying chorion. Masson’s trichrome staining, ×100.
Figure 15 – (A) The fusion region of the placentae from a DC-DA twin pregnancy, placental infarction; (B) Chorionic membranes sample demonstrating edema. Negative reaction to immunohistochemical staining. Anti-collagen IV antibody immunomarking, ×100. DC-DA: Dichorionic–diamniotic.

Figure 16 – (A) The fusion region of the placentae from a DC-DA twin pregnancy – negative reaction to residual villous trophoblast and positive cytoplasmic reaction on extravillous interstitial trophoblast; (B) Chorionic membranes sample with negative reactivity. Anti-NSE antibody immunomarking, ×100. DC-DA: Dichorionic–diamniotic; NSE: Neuron-specific enolase.

The S100 protein family is neurotropic in nervous tissue at low-concentration and neurotoxic in high-concentration. This family is also present in placental tissue but it has not been studied enough. It is reported to occur in tissue lesions with nervous components, syncytio-trophoblast, myofibroblasts, smooth muscle cells in the vascular wall and also in macrophages [109]. Gazzolo et al. [111] have demonstrated the presence of S100 in the umbilical cord blood of the fetuses with IUGR. They determined its low value, when nitric oxide intervened to increase uteroplacental circulation. These authors have suggested that IUGR fetuses may have nerve cell lesions. The IHC study on placental tissue in our interest area, with the anti-S100 antibody, revealed a necrobiotic placenta with increased reactivity on the interstitial extravillous trophoblast and a placental area with necrobiosis and focal site with highly reactive villous and extravillous trophoblast, while chorionic membranes did not react (Figure 17).

Haigh et al. [112] and Blaschitz et al. [113], reported the increased expression of cytokeratin 7 (CK7) in the intermediate filaments of the trophoblastic line. Other authors argue that CK7 is a strong and specific marker of the trophoblast [114]. The IHC labeling with the anti-CK7 antibody was cytoplasmic positive on the villous and extravillous trophoblast as well as in the fibrinoid necrosis area, while the chorionic membranes did not react (Figure 18).

Some studies have demonstrated the positivity of anti-Ki67 and anti-proliferating cell nuclear antigen (PCNA) antibodies in villous cytotrophoblast areas and stromal cells. PCNA, unlike Ki67, is also present in the syncytiotrophoblast. Both markers are positive in the G1, S, G2/M phases of the cell cycle. Immunoreactivity of these two antibodies was present in decidual stromal cells, but PCNA was also located in the glandular epithelium. The occurrence of immunoreactivity to PCNA in syncytiotrophoblast may be the result of an active translation, but is actually unlikely to be, rather it is due to a long half-lives, and therefore, the origin of the fused cytotrophoblast and transformed into the syncytiotrophoblast is observed [115].

In the infarct areas of the interested placental structure, there are also extravillous trophoblast sections where the anti-Ki67 antibody reacted positively, nuclear or focal (Figure 19A), and there was nuclear and cytoplasmic
reactivity on the trophoblastic cell in an infarction area to the IHC labeling with the anti-PCNA antibody (Figure 19B).

Human trophoblastic cells are characterized by a strong invasion capacity of the decidua and the proximal third of the myometrium during normal pregnancy [116]. Initially, these cells attach to the uterine epithelium, degrade the basal membrane and extracellular matrix (ECM) and then migrate to the decidual stroma [117].

The maternal micromedium in the placental bed plays an important role in regulating the migration of trophoblastic cells. At the implantation site, the matrix produced by stromal decidual cells (SDCs) and maternal leukocytes, such as uterine natural killer (uNK), attempt to maintain some degree of control over fetal trophoblast [118, 119]. The invasive process is facilitated by the expression of matrix metalloproteinases (MMPs) activity. They are part of a family of endopeptidases with a zinc-dependent structure, capable of degradation and remodeling of ECM specific components. Most MMPs are produced as proenzymes and secreted in the ECM in the inactive,zymogenous form, subsequently suffering a proteolytic activation process [120]. MMP-8 and MMP-13 collagenases are responsible for the degradation of collagen I and III, which predominates in fetal membranes [121].

Huisman et al. [122] did not detect gelatinolytic activity of MMP-8 and MMP-13 in decidua, which demonstrates that the technique influences the outcome. Other studies have demonstrated the presence of MMP-8 in the early trophoblast cytoplasm, in SDC, as well as in decidual glands, while the expression of MMP-13 is specific for the first trimester fibroblasts [123].

In our interest area, the placental fusion from a DC twin pregnancy, there were outbreaks of fibrinoid necrosis, sclero-hyalinized stem villi that positively responded on the villous and extravillous trophoblast at the immunostaining with the anti-MMP-8 antibody (Figure 20A), whilst chorionic membranes did not respond to this immunostaining (Figure 20B). The IHC labeling with the anti-MMP-13 antibody produced a moderately positive reaction in the extravillous trophoblast and also cytoplasmic positive reaction (Figure 21A).

Cluster of differentiation 68 (CD68) is a highly expressed protein in cells of the monocyte line (phagocytes, osteoclasts) in the blood flow (macrophages), but also in the macrophages from tissues (Kupffer cells, microglia) [124]. The placental infarction or necrobiosis area undergoes a remodeling process, also involving these cells of the inflammatory line.

On our tissue, the anti-CD68 antibody poorly reacted in the necrobiotic area and negative in the placental infarction region, highlighting the reactive macrophages in the sector of interest (Figure 21B).

The CD34 protein is part of the transmembrane sialoglycoprotein family, which shows hematopoietic and vascular expression in associated tissues [125]. It also represents a molecule of adhesion necessary for T-lymphocytes to penetrate into the lymph node. It is expressed in the endothelium of the lymph node, where the L-selectin to which it binds, is at the T-cell level [126, 127]. Anti-CD34 antibody marks the progenitor hematopoietic stem cells, and normally appears in umbilical cord, red marrow, mesenchymal stem cells, endothelial progenitor cells, endothelial cells of blood vessels, mastocytes, also in a subpopulation of interstitial dendritic cells, but not in the lymphatic vessels. The presence of anti-CD34 antibody in non-hematopoietic cells, attests their origin in progenitor stem cells [128].

Using the IHC technique to the sampled lambda tissue, we noticed a lack of reactivity in placental infarction regions (Figure 22A) and a low endothelial reaction in areas adjacent to placental infarction (Figure 22B).

Alpha-smooth muscle actin (α-SMA) is a protein encoded in humans by the ACTA2 gene located on chromosome 10q22-q24 [129]. This protein is one of the six distinct actin isoforms described so far, and is involved in cellular motility, structure and integrity. Alpha actins are an important constituent of the contractile system. It is also used as a marker for myofibroblast formation [130].

In the placental lambda fusion region of DC twin pregnancy, there is a lack of immunoreactivity at the placental infarction site, and adjacent there is an area with blood vessels showing positive reactivity in the middle layer (Figure 23, A and B).

Figure 17 – (A) Placental necrobiosis sample with increased reactivity on the interstitial extravillous trophoblast; (B) Placental sample with necrobiosis area and also focal area with highly reactive villous and extravillous trophoblast and negative reactivity on chorionic membranes. Anti-S100 antibody immunomarking, ×100.
Figure 18 – (A) The fusion region of the placentae from a DC-DA twin pregnancy – positive cytoplasmic reaction on villous and extravillous trophoblast and areas of fibrinoid necrosis; (B) Chorionic membranes sample demonstrating absent reactivity and villous and extravillous trophoblast areas with positive reactivity. Anti-CK7 antibody immunomarking, ×100. DC-DA: Dichorionic–diamniotic; CK7: Cytokeratin 7.

Figure 19 – (A) The fusion region of the placentae from a DC-DA twin pregnancy – focal positive nuclear reaction, on the remaining extravillous trophoblast in the placental infarction area (Anti-Ki67 antibody immunomarking, ×200); (B) The fusion region of the placentae from a DC-DA twin pregnancy – nuclear and cytoplasmic positive reaction on the trophoblastic cell in an infarction zone (Anti-PCNA antibody immunomarking, ×100). DC-DA: Dichorionic–diamniotic; PCNA: Proliferating cell nuclear antigen.

Figure 20 – (A) The fusion region of the placentae from a DC-DA twin pregnancy – there are areas of fibrinoid necrosis, sclero-hyalinized stem villi and positive reaction on the villous and extravillous trophoblast; (B) Chorionic membranes sample demonstrating absent reactivity at this level, positive reactivity on villous and extravillous trophoblast and focally positive on placental stroma. Anti-MMP-8 antibody immunomarking, ×100. DC-DA: Dichorionic–diamniotic; MMP-8: Matrix metalloproteinase-8.
Figure 21 – (A) The fusion region of the placentae from a DC-DA twin pregnancy – moderately positive reaction in the extravillous trophoblast and cytoplasmic positive reaction (Anti-MMP-13 antibody immunomarking, ×200); (B) The fusion region of the placentae from a DC-DA twin pregnancy – slightly positive reaction in the necrobiosis area and negative reaction in the placental infarction area (Anti-CD68 antibody immunomarking, ×100). DC-DA: Dichorionic–diamniotic; MMP-13: Matrix metalloproteinase-8; CD68: Cluster of differentiation 68.

Figure 22 – (A) The fusion region of the placentae from a DC-DA twin pregnancy – negative reaction in the areas of placental infarction; (B) Low endothelial response in the areas adjacent to placental infarction. Anti-CD34 antibody immunomarking, ×200. DC-DA: Dichorionic–diamniotic; CD34: Cluster of differentiation 34.

Figure 23 – The fusion region of the placentae from a DC-DA twin pregnancy: (A) Placental infarction focus and an adjacent area with blood vessels showing positive reactivity in the middle layer; (B) Placental sample with blood vessels showing positive reactivity in the middle layer. Anti-α-SMA antibody immunomarking: (A) ×100; (B) ×200. DC-DA: Dichorionic–diamniotic; α-SMA: Alpha-smooth muscle actin.
Conclusions

Chorionicity is essential for multiple pregnancy outcome. Chorionicity assessment is an essential milestone in managing multiple pregnancy. Because of multiple conditions that can be associated, MC twins at risk until birth, so frequent follow up is important. TTTS is a quantitative disorder, while TAPS is a qualitative one. TTTS is very rare in MA twins, while umbilical cord accidents are the major concerns, which can neither be predicted nor prevented. Discordant fetal growth and IUGR are commonly associated with MC pregnancy. Both in DC, but especially in MC twin pregnancy, fetal growth should be carefully followed up. IUF D is more common in multiple pregnancy, and in MC twins sIUFD has far more complex consequences than DCs. Placental location is also important for the outcome. In TRAP sequence, estimating the weight of the acardiac fetus is a key point in maternal–fetal management. Currently, twins are common and malformations are not rare. Correct dating is also an essential aspect in multifetal gestation.

The placental lambda fusion region undergoes various histopathological changes, such as placental infarction, fibrinoid necrosis, vascularization damage, cell remodeling and cellular proliferation, while fused amniotic membranes exhibit discrete edema and inflammatory or hematic infiltrate.

Conflict of interests

The authors declare that they have no conflict of interests.

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Author contribution

Costin Berceanu and Claudia Meheșințu equally contributed to this article.

References

Morphological and ultrasound findings in multiple pregnancy placentation


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