Clinical and Morphological Study of Single and Twin
Pregnancies Placenta

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ABSTRACT: Placental morphology is very important in both single and multiple pregnancies. It can dictate certain aspects such as: fibrin depositions, calcifications, infarctions, type of vascularization, which can be directly related to placental weight and implicitly to foetal weight, both in single and twin pregnancy. Our study highlighted the macroscopic morphological aspects and through the classical and immunohistochemical colours the microscopic placental morphological aspects, both in single and in dichorionic diamniotic twin pregnancy and showed that the placenta of the foetuses from the twin pregnancy has a higher vascular density compared to the single pregnancy, and the areas of placental fusion are poor in blood vessels, but rich in fibrin depositions, calcifications and placental infarctions. We also pointed out that maternal weight can increase with age, foetal weight can be directly proportional to maternal weight, as well as placental weight is directly proportional to foetal weight and implicitly to maternal weight, but in terms of vascularization, we observed that there is an inversely proportional connection between placental, foetal weight and vascular density.

KEYWORDS: Placenta, vascularization, fibrin depositions, calcifications.

Introduction

Placenta is a vile organ, which has vascularization since the 21st day of gestation. This structure undergoes various architectural changes until the end of the pregnancy. It is an essential organ for the supply of nutrients, for the transport of respiratory gases between mother and foetus, but also for the elimination of metabolism products [1].

Depending on the type of pregnancy, there are several types of amnionicity and chorionicity. In pregnancy with a single foetus, the foetal attachments are unique. In the multiple polyizygous pregnancy, the placenta, the chorion and the amniotic membranes are separated, but during pregnancy, the two placentas can also fuse and form vascular anastomoses, with various implications on the foetuses. In monozygotic pregnancy, amnionicity and chorionicity differ depending on the period during which the division took place, being monozygotic dichorionic diamniotic, monozygotic monochorionic diamniotic, or monozygotic monochorionic monoamniotic [2].

Aims

The aim of our study was to analyse the morphology of placental structures and compare the density of placental vascularization from single pregnancy, dichorionic diamniotic twin pregnancy for both foetuses (Foetus 1: the highest weight, foetus 2: the lowest weight) and their placental fusion areas.

Materials and Methods

Our study was performed on a number of 60 patients, 30 patients who gave birth to a single live foetus and 30 patients who gave birth to twins (dichorionic diamniotic pregnancies). The patients were admitted and investigated in the 2nd Clinic of Obstetrics-Gynaecology of the County Clinical Emergency Hospital in Craiova, during the period 2017-2020. The study was approved by the Ethical Committee of U.M.F. of Craiova, and the patients gave their written
informed consent regarding the publication of their anonymized data. A clinical-statistical study was performed using the Microsoft Excel 2010 program, based on: mother's age, weight, foetus weight and sex.

After birth (eutocious or segment-transverse C section) the placentas were collected, analyzed macroscopically and subsequently were collected placental fragments that were sent to the Centre for Studies of Microscopic Morphology and Immunology (University of Medicine and Pharmacy of Craiova) for histological and histopathological analysis. The tissues were fixed in 10% buffered neutral formalin, at room temperature for no more than 1 week. After fixation, the tissue was routinely processed for paraffin embedding, and sectioned as 5μm-thick sections using a HM350 rotary microtome, equipped with a system to transfer water bath sections (Thermo Scientific). Sections were collected on poly-L-lysine slides and utilised for Haematoxylin-Eosin (HE), Trichrome Masson (TM) and Periodic Acid Schiff (PAS) stains, in order to evaluate overall tissue architecture, collagen fibers and neutral mucus, but also for immunohistochemistry.

For the immunohistochemical study, we dewaxed the slides in xylene, delimited the tissue with a hydrophobic marker (Agilent, Santa Clara, CA, USA), and rehydrated the tissue to distilled water. Antigen retrieval was performed by boiling the slides in a microwave oven (650W, 7 cycles x 3 minutes) in citrate solution (pH=6) or Ethylenediaminetetraacetic acid (EDTA) solution (pH=9). Next, the endogenous peroxidase was inactivated by incubation for 30 minutes in a 1% solution of hydrogen peroxide, then the non-specific antigenic-binding sites were blocked with a 3% skim milk solution for another 30 minutes. Starting with this point, all washings were done in 1x Phosphate-buffered saline (PBS) solution (0.1M). Subsequently, the histological sections were covered with the primary antibody solutions (Table 1) and incubated at 4°C for 18 hours. The next day, the slides were washed in 1xPBS in order to remove the excess antibody solutions, and corresponding species-specific secondary antibodies (mouse/rabbit IgG antibody, VC002-025, R&D Systems, VisUCyte HRP Polymer) were applied for 1 hour, then after thorough washing, the signal was developed with 3,3'-Diaminobenzidine (DAB) (Agilent). Finally, the nuclei were counterstained with Haematoxylin, the slides were dehydrated, clarified in xylene and mounted with a xylene-based medium.

Slides were imaged on a Nikon 55i microscope (Nikon Imaging Inc, Tokyo, Japan), equipped with a 5-megapixel cooled charge coupled device camera (Nikon DS-5MC) and the Image ProPlus AMS software (Media Cybernetics, Bethesda, MD, USA).

Data were analyzed using SPSS 10.0 (SPSS Inc., Chicago, IL, USA), and graphs plotted in Microsoft Excel 2016. Ages, weights, and microscopic measured features were presented as average ± standard deviation of the means (SD). Two-groups comparisons were performed utilizing a Student's t-tests, while multi-group comparisons were analyzed using a one-way analysis of variance (ANOVA) test. P<0.05 was considered statistically significant in all testing.

Table 1. Antibody properties.

| Antibody | Clone    | Antigen retrieval | Dilution | Labeling      | Manufacturer |
|----------|----------|-------------------|----------|---------------|--------------|
| Anti-CD34| QBEnd 10 | Citrate, pH 6     | 1:50     | Blood vessels | Dako         |

CD: Cluster of differentiation; CK: Cytokeratin

Results

Each pregnant patient, at term (over 37 weeks of pregnancy), introduced to the study was subjected to clinical and imaging investigation and had no pregnancy-related conditions.

The clinical observation sheet was accompanied by the written consent of each patient for the use of personal information, photographing of tissue samples during and after eutocious birth or by segmental-transverse C-section.

The age of the patients ranged between 18-40 years old for single-born mothers and between 17-41 years for mothers with twin pregnancies. Mother’s average age wasn’t significantly lower for the mothers with one newborn (27.13±6.50) compared to the mothers with two newborns (29.66±6.27), t(60)=−1.534, p=0.065 (Figure 1).
Mother’s average age wasn’t significantly lower for the mothers with one newborn compared to the mothers with two newborns, t(60)=1.534, p=0.065.

Also, the patients were weighed, their weight varied as follows: for single-pregnancy mothers between 51-93 kilograms (Kg), and for twin pregnancy mothers it varied between 57-103kg. Mother’s average weight was significantly lower for the mothers with the first newborn (70.96±11.82) compared to the mothers with the second newborn (86.16±11.63), t(60)=-5.017, p<0.001 (Figure 2).

Regarding the sex of the new-borns introduced in the study, we noticed that the single foetuses were 24 males and 6 females, and for the dichorionic diamniotic twin pregnancies, we noted the foetuses with 1 and 2, foetus 1 having the highest weight, foetus 2-the lowest weight, and we noticed that for foetus 1 there was an equal number (15+15) between the two sexes, and for foetus 2 there were 20 females and 10 males. The distribution of males/females was significantly different for the 1 new born, 2 newborn fetus 1 and 2 newborns-fetus 2 groups, χ²(2, N=90)=13.529, p=0.0011 (Figure 3).
The weight of single female foetuses from single pregnancy ranged from 2510-3470 grams (g), with a mean weight equal to 2961.66 g (±344.46), the weight of single males from single pregnancy ranged from 2330 to 3830g, with a mean weight equal to 3186.25 g (±420.90); for the female foetus 1 newborn from twin pregnancy, the weight ranged from 2440 to 3400g, with a mean weight equal to 2770.66 g (±280.36), for the male foetus 1 newborn from twin pregnancy, the weight ranged between 2370-3250g, with a mean weight equal to 2724.66 g (±266.10); for female foetus 2 newborn from twin pregnancy, the weight ranged between 1820-3230g, with a mean weight equal to 2440.25 g (±350.08), and for male foetus 2 newborn from twin pregnancy, the weight ranged from 2100 to 2860g, with a mean weight equal to 2575g (±253.25). There was a globally very significant difference between the weight of the newborns for these groups, F(5, 89)=11.854, p<0.001 (Figure 4).

The placental weight of single female foetuses ranged from 430-575g, with a mean placental weight equal to 491.66 g (±57.32), of single male foetuses from single pregnancy ranged between 410-650g, with a mean placental weight equal to 532.91g (±76.41); Placental weight of female foetus 1 newborn from twin pregnancy ranged from 405-530g, with a mean placental weight equal to 454g (±44.76), placental weight of male foetus 1 newborn from twin pregnancy ranged from 415-530g, with a mean placental weight equal to 445.33g (±43.44); the placental weight of male foetus 2 newborn from twin pregnancy ranged from 300-510g, with a mean placental weight equal to 402.75g (±48.40), and the placental weight of male foetus 2 newborn from twin pregnancy ranged from 380 to 480g, with a mean placental weight equal to 432g (±38.38). There was a globally very significant difference between the placental weight of the newborns for these groups, F(5, 89)=13.43, p<0.001 (Figure 5).
By macroscopic analysis of the placenta from single pregnancy and twin pregnancy, we observed its maternal and foetal sides, amniotic membranes, fibrin depositions, placental vascularization but also the placental fusion area of the placenta from the dichorionic diamniotic twin pregnancy (Figures 6-9).

Figure 6. Placental maternal side of the single pregnancy. The fibrin depositions are observed in the red boxes, and in the area highlighted with the green arrow, placental calcification is observed.

Figure 7. Placental foetal side from single pregnancy. The amniotic membrane (red arrow), umbilical cord (white arrow) and placental vascularization (green arrow) are observed.

Figure 8. Placental maternal side from the dichorionic diamniotic twin pregnancy. Both umbilical cords (blue arrows), amniotic membranes (red arrow) and placental fusion area (white arrow) can be observed.

Figure 9. Placental foetal side from the dichorionic diamniotic twin pregnancy. Both centrally fused placentas, umbilical cords (blue arrows) and distinct amniotic membranes (red arrows) can be observed.

Histopathology revealed mature placental tissues with preponderance of terminal and mature intermediate villi, fibrin depositions, blood vessels and placental calcifications (Figures 10-13).
Figure 10. Placental microscopic aspects of single pregnancy. A. Normal mature placental villi, with normal-looking intravillous capillaries, small areas of calcifications (purple areas) at the villi tissue periphery. Hematoxylin-Eozine staining,×200; B. Normal placental mature intermediate villi showing basal vascular membranes stained with PAS-Hematoxylin and small fibrinoid deposition areas (pink-purple),×200; C. Placental terminal villi with fibrinoid and intravillous fibrin depositions (stained in red). Masson’s classic trichrome staining,×200; D. Immunohistochemical staining with anti-CD34 antibody. We observe the brown endothelial labeling of small intravillous vessels,×200.

Figure 11. Placental microscopic aspects of twin pregnancy-Foetus 1: highest weight. A. Normal placental mature intermediate and terminal villi, with normal-looking intravillous capillaries, small areas of calcifications (purple areas) at the villi tissue periphery. Infarcted placental mature intermediate villi with perivillous fibrinoid deposition is also observed. Hematoxylin-Eozine staining,×200; B. Mature intermediate placental villi at which the basal vascular membranes stained with PAS- Hematoxylin and fibrinoid deposition areas (pink-purple),×200; C. Stem and terminal villi with fibrinoid and intravillous fibrin depositions (stained in red). Masson's classic trichrome staining,×200; D. Immunohistochemical staining with anti-CD34 antibody. We observe the brown endothelial marking of small intravillous vessels,×200.
Figure 12. Placental microscopic aspects of twin pregnancy—Foetus 2: lowest weight. A. Normal placental mature intermediate villi, with normal-looking intravillous capillaries, small areas of calcifications (purple areas) at the villi tissue periphery and fibrinoid deposition areas (stained in pink). An infarcted placental area with terminal villi with perivillous fibrinoid deposition is also observed. Hematoxylin-Eosine staining, ×200; B. Placental terminal villi at which the basal vascular membranes stained with PAS-Hematoxylin and intra and perivillous fibrinoid deposition areas (stained in purple-pink), ×200; C. Mature intermediate placental villi with intra and perivillous fibrinoid deposition area (stained in red) and areas of villous calcification (purple). Masson’s classic trichrome staining, ×200; D. Immunohistochemical staining with anti-CD34 antibody. We observe the brown endothelial marking of small intravillous vessels, ×200.

Figure 13. Placental microscopic aspects of twin pregnancy—placental fusion area. A. Normal placental villi, with normal-looking, but numerically reduced, intravillous capillaries, small areas of calcification (purple areas) on the periphery of villi tissue and fibrinoid deposition areas (stained in pink). Hematoxylin-Eosine staining, ×200; B. Terminal placental villi at which the basal vascular membranes stained with PAS-Hematoxylin and intra and perivillous fibrinoid deposition areas (pink-purple), ×200; C. Mature intermediate and terminal placental villi with fibrinoid and intravillous fibrin depositions (stained in red) and areas of villous calcification (purple). Masson’s classic trichrome staining, ×200; D. Immunohistochemical staining with anti-CD34 antibody. We observe the brown endothelial marking of the small, intravillous, reduced numerically vessels, ×200.
Through the classic HE stainings, we observed placental villi, collagen fibres, syncytiotrophoblast, calcification areas and light fibrinoid areas. Through the classic PAS-Hematoxylin staining, we highlighted the basal membranes of the blood vessels intravascularization and extra-vascularization and the glycosaminoglycans from the fibrin depositions (pink-purple areas). Through the classic TM staining we labelled placental villi tissues, collagen fibres (stained in blue), syncytiotrophoblast, calcification areas and fibrinoid areas. Through the immunohistochemical reaction with the anti-CD34 antibody, we labelled the vascular endothelium of the intravillous and intervillous blood vessels (brown labelling) and observed that in the twin pregnancy their number was higher, compared to the single pregnancy, and especially with the placental fusion areas of the twin pregnancies, where vascular density was very low.

We captured 4 images with the objective×200 from the same tissue section of each case (30 cases single pregnancy placenta; 30 cases multiple pregnancy placenta foetus A/B+placental fusion area), immunohistochemically labelled section, then we counted the intravillous and intervillous blood vessels from the placenta in the single and twin pregnancies, but also from the placental fusion areas from the dichorionic diamniotic twin pregnancies and we compared them. We noticed that the highest vascular density was present in the case of the placenta from the twin pregnancy-foetus 1: the one with the highest weight, with a mean vascular density equal to 133.70 vessels/×200 (±10.04), followed by the placental vascularity density of foetus 2 from twin pregnancy, with a mean of 80.85 vessels/×200; the placental vascular density of the single pregnancies averaged 71.21 vessels/×200), and the lowest vascular density was present in the placental fusion areas of the dichorionic diamniotic twin pregnancies, equal to 24.10 vessels/×200 (±3.6). There was a globally very significant difference between the number of blood vessels/×200 of the newborns placenta for these groups, F(3, 119)=1117.18, p<0.001, (Figure 14).

We also compared the mother's weight according to age and observed that was a globally very significant difference between age and weight of the mothers introduced in this study (Figure 15), (Table 2).
We compared the weight of the foetus with the placental weight and observed that was a globally very significant difference between foetal and placental weight (Figure 16), (Table 3).

Table 3. Comparison between the mean foetal weight and the mean placental weight according to the type of pregnancy.

|                  | Newborn 1-F | Newborn 1-M | Newborn twins 1-F | Newborn twins 1-M | Newborn twins 2-F | Newborn twins 2-M |
|------------------|-------------|-------------|-------------------|-------------------|-------------------|-------------------|
| t Stat           | 15.564      | 30.385      | 31.601            | 32.74             | 25.782            | 26.456            |
| P(T<=t) one-tail | p<0.005     | p<0.005     | p<0.005           | p<0.005           | p<0.005           | p<0.05            |

We compared the vascular placental density of the foetus with the placental weight and observed that was a globally very significant difference between vascular placental density and placental weight (Figure 17), (Table 4).

Table 4. Comparison between mean placental vascular density and foetal weight.

|                  | Newborn 1-F | Newborn 1-M | Newborn twins 1-F | Newborn twins 1-M | Newborn twins 2-F | Newborn twins 2-M |
|------------------|-------------|-------------|-------------------|-------------------|-------------------|-------------------|
| t Stat           | 15.564      | 30.385      | 31.601            | 32.74             | 25.782            | 26.456            |
| P(T<=t) one-tail | p<0.005     | p<0.005     | p<0.005           | p<0.005           | p<0.005           | p<0.05            |

Figure 16. Comparison between the mean foetal weight and the mean placental weight according to the type of pregnancy: single/twin and the sex of the new-born. We noticed that the foetal weight values increase directly proportional to the placental weight values. F: female, M: male.

Figure 17. Comparison between mean placental vascular density and foetal weight. We noticed that the foetal weight values increase directly proportional to the placental weight values, so in the case of twin pregnancies the placental weight may decrease directly proportional to the foetal weight, but there is an inverse relation with the vascular density, resulting in the placenta from the twin pregnancy increasing the number of small blood vessels. F: female, M: male.
Table 4. Comparison between mean placental vascular density and foetal weight.

|                | Newborn twins 1-F | Newborn twins 1-M | Newborn twins 2-F | Newborn twins 2-M |
|----------------|-------------------|-------------------|-------------------|-------------------|
| t Stat         | 43.811            | 40.629            | 55.002            | 56.661            |
| P(T<=t) one-tail| p<0.005           | p<0.005           | p<0.005           | p<0.005           |

Discussions

In the placenta there are two circulatory systems: maternal open (intervillous) and foetal closed (intravillous). The blood from the two types of systems circulates closely, being separated by the villous epithelium, the villous stroma and the villous capillary endothelium. Thus the metabolic exchange between the two systems is achieved. The circulatory blood of the foetus is provided by a vein and two umbilical arteries. The umbilical arteries enter the chorionic plate, perform a safety shunt, branch into the chorale branches, which in turn send in the villous trunks two central cotyledonary arteries, of 1st, 2nd, or 3rd order [3] and reach a terminal villi, where they spread to the surface of the villi in a capillary network [4]. CO₂ and foetal catabolites are transported through the venous system of the intervillous spaces. The venous system is composed of large calibre vessels that reach the level of the intervillous space and the marginal sinus, located at the junction of the chorion with the basal plate [4].

Placental vascularization is an essential element for normal growth and development of the foetus, whether it is single pregnancy or multiple pregnancy. This topic is a very dynamic area in modern obstetric research [5].

After spontaneous or surgical birth, we examined the placenta from a macroscopic and microscopic point of view. From a macroscopic point of view, we observed both in the single pregnancy and in the dichorionic diamniotic twin pregnancy, the existence of the areas of calcifications, infarctions, the presence of fibrinoid depositions, but also of vascularization. In the twin pregnancy we identified the areas of placental fusion, with the same changes specific to the third trimester of pregnancy.

Generally, the placenta has a round, oval or discoidal shape, with an average diameter of 20-25 cm, with an average weight of 500g, being an organ with a hemochorionic structure, at the level of which, the vascular branches ensure the permanent exchange of nutrients, gases and elimination products, between mother and foetus [6]. In our study we observed that the mean placental weight varied according to the type of pregnancy, single or twin as follows: the heaviest placenta was the one with the single pregnancy with male foetus, followed by the placenta from the single pregnancy with female foetus, placenta from multiple pregnancy of the heaviest female foetus, placenta from twin pregnancy of the heaviest male foetus, placenta of small male foetus from twin pregnancy, and placenta of the small female foetus from twin pregnancy.

After the macroscopic morphological examination, we analysed the placenta microscopically and applied quantitative methods (vascular density measurements). The technological advances of the last decades have opened the doors to advanced studies in the field of microscopy [7].

We observed that the patients’ age and weight, as well as the foetuses’ weight and the placental weight were in a directly proportional relation, and the vascular density varied according to the type of pregnancy, single or multiple. Most small blood vessels, marked with the anti-CD34 antibody, were identified in the case of heaviest foetuses from twin pregnancies, followed by low weight foetuses from twin pregnancy, single pregnancy foetuses and then by placental fusion areas from dichorionic diamniotic twin pregnancy.

The villous vascularization is created by the appearance of neoformation capillaries. The first precursor of the capillary endothelium is located in the villous stroma and is called hemangioblast. This endothelium was highlighted in this study using the immunohistochemical marker QBend10 monoclonal antibody that highlights the presence of CD34 [8, 9].

Through the classic HE, TM and PAS-Hematoxylin stains, similar to other studies on the heart or placenta [10-17], by highlighting the areas of collagen in pink (HE), blue (TM), or fibrin depositions where the glycosaminoglycans are stained in pink (PAS-Hematoxylin), we observed that infarctions, fibrinoid depositions and calcifications are more intense in the placenta of twin pregnancy, especially in the placenta of low weight foetus, but also in the fusion areas of the two placentas.
Placental infarction can be identified both macroscopically by the presence of parenchymal focal lesions and microscopically by highlighting the areas of villous necrosis [18,19]. Most placental infarctions occur in the last trimester of pregnancy, with a discouraging or even chaotic picture of foetal development [18]. Large infarcted areas have been associated with pre-eclampsia and/or intrauterine growth restriction [18-21].

Several studies have been conducted on placental infarctions and it has been established that they may be frequent and common, but if they are large or multiple, they may have adverse effects on foetal growth. The pathogenesis of placental infarctions is similar to that of infarctions produced in other organs, by rapid loss of arterial flow, by thrombi migration or by the presence of spiral arteries in the placental bed [22,23].

Placental infarctions and intravillous thrombosis are histopathological changes that can usually be microscopically differentiated. However, intravillous thrombosis belong to a wide spectrum of focal or diffuse intervillous fibrin depositions, microscopically identified in most placentas, and when they are large, the less they can be differentiated. Fox [24, 25] made a clear description of these lesions, separating them from other "white infarcts". He was referring to "plates" under the name of "massive intervillous fibrin depositions", a terminology that is no longer appropriate, as this name may also refer to severe diffuse fibrin depositions [26]. However and Rushton [27] have argued that intravillous fibrin plates can be easily confused with infarctions and require morphopathological confirmation [27]. Ruston [28] used at one time the terminology: "diffuse depositions of perivillous fibrin", for the lesions initially described by Fox [24,25], believing that the "plates" also highlight their macroscopic nature. Maternal and foetal associations with placental infarction have been reported in the past, but the small number of studies on these changes and the presence of intravillous thrombosis or intervillous fibrin depositions have concluded that they are of low clinical importance. However, in our study, we could observe that by increasing the infarction / fibrin depositions/calcifications areas, vascular density decreased and implicitly the placental weight decreased, but also foetal weight, thus arguing that these morphopathological changes may have clinical implications on the foetus from single pregnancy, but also from twin pregnancy.

Conclusions
Through this study we have observed that the morphopathological changes (placental infarction, calcifications, fibrin depositions) identified by the classic histopathological examination can influence placental vascularization, foetal growth and development.

The presence of these changes leads to a decrease in the number of capillary vessels from the level of placental villi, and by decreasing the vascularity, the placental weight decreases and implicitly the foetal weight.

Also, the foetal weight varies depending on the type of pregnancy: single or twin, but also on the foetal gender.

Conflict of interests
None to declare.

Authors’ contribution
Nicoleta-Loredana Voicu and Roxana Elena Bohițea equally contributed to this article.

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