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V.A. Berezhna, A.M. Gromova, T.V. Mamontova, N.O. Udovytska, I.I. Starchenko, L.E. Vesnina
Ukrainian Medical Stomatological Academy, Poltava

MORPHOMETRIC ANALYSIS OF PLACENTAL AND M1/M2 MACROPHAGES POLARIZATION IN THE DETECTION OF FETAL GROWTH RESTRICTION

e-mail: berejnayapoltava@gmail.com

The purpose of the present study was to analyzed the morphometric characteristics and CD68 and CD163 expression of macrophages in the placentas of women, whose pregnancies were complicated by fetal growth restriction. The study revealed low indices of the mass and area of the placentas, but high indices of the fetoplacental weight ratio. The level of CD68+ macrophages in fibrously altered terminal villi of the placenta was significantly higher in both groups women whose pregnancies were complicated by fetal growth restriction. There was significant increased CD163+ macrophages in fibrously altered terminal villi. Thus, in the placentas of women whose pregnancy was complicated by fetal growth restriction and resulted preterm birth, there were disruptions of the vascular and stromal component of the chorionic villus with the changes to macrophage polarization from M1 to M2 types in fibrously altered terminal villi of the placenta.

Key words: fetal growth restriction, preterm and term birth, placental morphometry, M1 and M2 macrophages

В.А. Бережна, А.М. Громова, Т.В. Мамонтова, Н.О. Удовицька, І.І. Старченко, Л.Е. Весніна

МОРФОМЕТРИЧНИЙ АНАЛІЗ ПЛАЦЕНТИ ТА ПОЛЯРИЗАЦІЇ М1/М2 МАКРОФАГІВ ПРИ ВИЗНАЧЕННІ ЗАТРИМКИ РОСТУ ПЛОДА

Метою цього дослідження було проаналізувати морфометричні характеристики та експресію CD68 та CD163 макрофагів у плацентах жінок, вагітність яких ускладнилася затримкою росту плода. Дослідження виявило низькі показники маси та площі плаценти, але високі показники плацентарно-плодного коефіцієнту. Рівень CD68 + макрофагів у фіброзно змінених термінальних ворсинах плаценти був значно вищим в обох групах жінок, вагітність яких ускладнювалась затримкою росту плода. Спостерігалося значне збільшення CD163 + макрофагів у фіброзно змінених термінальних ворсинах. Таким чином, у плаценті жінок, вагітність яких ускладнилася затримкою росту плода та призвела до передчасних пологів, відбулися порушення судинного та стромального компонента ворсин хоріона із зміщенням поляризації макрофагів від типів М1 до М2 у фіброзно змінених термінальних ворсинах плаценти.

Ключові слова: затримка росту плода, передчасні та своєчасні пологи, плацентарна морфометрія, макрофаги М1 та М2.

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Fetal growth restriction (FGR) continues to be a significant perinatal problem. A growth restricted fetus is one with an estimated fetal weight of less than the tenth percentile for that gestational age [14]. The prevalence of growth restricted fetuses is known to be about 8%. FGR is a complex multi-factorial condition and manifested as a result of several fetal and maternal disorders [1]. FGR is challenging because of the difficulties in reaching a definitive diagnosis of the cause and planning management. FGR is associated not only with a marked increased risk in perinatal mortality and morbidity but also with long-term outcome risks. Therefore, obstetricians aim to identify fetuses with early FGR so delivery can be planned according to gestational age and severity of the condition.

Despite decades of intense research, the exact cause of FGR remains unknown. It is generally accepted that one of the causes of intrauterine FGR can be pathology of the placenta: deficient trophoblast invasion, generalized endothelial dysfunction, and anomalies in placental development [4]. Immune cells are critically involved in placental development and functioning, and inadequate regulation of the maternal immune system is associated with placental pathology and pregnancy complications.

Few attempts have been made for comparative studies of FGR versus normal placental morphology. Mitra S.C. et al. (2000) reported that pregnancies with FGR are associated with smaller placentas, increase in the thickness of villi vessel wall, and decrease in lumen circumference. Thickening of the villous trophoblastic basal membrane, incidence of villous infarction and the incidence of villitis were more common in the FGR group. There were, however, no significant differences in perivillous fibrin deposition, stromal fibrosis and cytotrophoblast proliferation between the groups [15]. As pointed out recently the FGR placenta is smaller than normal placentae throughout gestation and displays maldevelopment of both the placental villi and the fetal vasculature within these villi, trophoblasts exhibit abnormal function and development in FGR placentae [11]. Thus, the placenta undergoes aberrant changes at the macroscopic to cellular level in FGR, which can limit exchange capacity and downstream fetal growth.

Macrophages play a crucial role in the initiation and development of placenta during pregnancy. Studies of human placental macrophages populations in FGR are limited. Recent studies show that decreased levels of CD68+ cells in the placental villus in association with both advancing gestational age as well as FGR [6]. These cells possibly take part in the control of villous development by remodeling of the villous core, controlling villous angiogenesis, or controlling trophoblast turnover. Conversely, another group documented elevated numbers of decidual macrophages with a lower M2/total macrophage ratio in FGR suggest a role for a macrophage surplus in its pathogenesis and could specifically indicate involvement of inflammatory macrophages [2, 5]. Overall, therefore, the precise composition of macrophages in pregnancy complicated by FGR remains controversial and unclear.

The purpose of the study was to analyse the morphometric characteristics, CD68, and CD163 expression of macrophages in the placentas of women, whose pregnancies were complicated by FGR and resulted term births after 37 weeks of gestation and preterm births up to 37 weeks of gestation.

Materials and methods. The present study was carried out in the City Clinical Maternity Hospital of Poltava. For this study, 10 cases of normal fetuses from women with physiological pregnancies and births (control group).Were selected 16 cases of growth restricted fetuses: 1-st group – 7 cases from women, whose pregnancies were complicated by FGR and whose pregnancies resulted in term births after 37 weeks of gestation. 2-nd group – 9 cases from women, whose pregnancies were complicated by FGR and whose pregnancies resulted in preterm births within 37 weeks of gestation. The study was approved by the Commission on Bioethics of Ukrainian Medical Stomatological Academy. Written consent was obtained from all study participants.

Placentae of those deliveries were collected for the study. Was examined and performed anthropometry of the placenta. Next the fetoplacental weight ratio was calculated by using following formula: F/P=Weight of fetus in grams/weight of the placenta in grams.

Next, the placenta was immersed in 10 % formalin. After fixation the placenta was examined in to and then cut into sections 4 μm thick longitudinally for microscopic evaluation. These slides were stained with haematoxylin and eosin and were mounted under cover slips and finally examined under microscope. On microscopy the following points were noted such as placental infarcts, calcifications, leucocyte infiltration, villus changes, and haemorrhages. Stereometric calculation was applied using a square multipurpose stereometric grid with dot counting (mg, x400). The sections were examined under microscope followed by photographing (x200, x400; Olympus BX-41, Olympus, Germany).

The expression of CD68+ and CD163+ macrophages was investigated in all samples by using immunohistochemical streptavidin peroxidase method. Paraffin sections, 4 μm thick, were deparaffinized and dehydrated, antigens were recovered in citrate buffer in the microwave oven, and endogenous peroxidase was blocked. Further, the sections were incubated at 4°C overnight with murine monoclonal antibodies anti-CD68 (1 : 25, clone PG-M1, REF PD M065-S, Diagnostic BioSystems, USA) and anti-CD163 (1 : 100, clone 10D6, REF Mob460-01, Diagnostic BioSystems, USA). Afterwards, the sections were treated in two steps with the Mouse/Rabbit PolyVue™ HRP/DAB Detection System (Diagnostic BioSystems, USA), with visualization by chromogen; the nuclei were counterstained with Mayer's haemalaun. We used Antibody Diluent buffer as a negative control instead of primary antibodies, and lymph node tissues were used as a positive control. Quantitative indicators were obtained by counting immunopositive $CD68^+$ and $CD163^+$ cells over the entire field of view with a large magnification lens ×40 (high power field, HPF) of placenta. We took into account all obtained quantitative individual data from all fields of view with calculating the mean value. The sections were examined under microscope followed by photographing (x200, x400; Axio Lab.A1, Zeiss, Germany)

Analyses were performed using Prism 7.0 (GraphPad, CA, USA). P-values <0.05 were considered to indicate statistical significance. Normally distributed data were reported using the means with standard deviations, categorical variables were reported using counts and proportions. Comparisons between groups were performed using parametric T-test and nonparametric methods: χ^2 Fischer exact test, Spearman´s correlation test.

Results of the study and their discussion. Analysis of the mass and area of the placentas (table 1) revealed significantly lower rates in women of the 2nd group compared to those of women in the control group (p=0.001; p=0.006; respectively). F/P ratio, reflecting the circulatory-metabolic balance of the fetoplacental system, was significantly higher in women of the 2nd group than in women of the control group (p=0.01).

Table 1

Placental parameters of women with physiological pregnancies (n=10), women of group 1 (n=7) and group 2 (n=9)

Parameter	Women with physiological pregnancies, n=10	Women of group 1, n=7	Women of group 2, n=9	p
Placental weight, g	484.2±25.44 (392.0-600.0)	421.1±50.95* (290.0–590.0)	347.8±22.9 (238.0-478.0)	p_1=0.24 p_2=0.001 p_3=0.17
Placental area, sm^3	788.5±58.4 (480-1140)	631.3±134.0* (324-1194.4)	537.4±52.8 (336-765)	p_1=0.24 p_2=0.006 p_3=0.49
Fetoplacental weight ratio	0.145±0.0007 (0.111-0.174)	0.153±0.02* (0.098–0.280)	0.224±0.03 (0.138–0.409)	p_1=0.75 p_2=0.01 p_3=0.08

The morphometric evaluation of placenta (table 2) in the study of both groups with FGR revealed signs of transformation of chorionic villi and the vascular component of the placenta, characteristic of placental insufficiency. Significant decrease in the diameter and area of terminal villi (p=0.0001; p=0.0001, respectively) and an increase in the volume density of syncytiotrophoblast (p=0.009; p=0.006, respectively) were shown in the 1-st and 2-nd groups in comparison with the control group. It demonstrated that significant increase in the average diameter and area of hemocapillaries (p=0.006; p=0.016, respectively) and a significant decrease in the thickness of syncytiotrophoblast and connective tissue (p=0.0001; p=0.009, respectively) were revealed in the 1-st group in comparison with the control group. Whereas, the indicators of the thickness and volume density of syncytiotrophoblast significantly increased (p=0.0001; p=0.006, respectively) in the 2-nd group in comparison to the control group. A significant difference between 2-nd and 1-st groups was noted in pronounced signs of capillary hypovascularization due to an increase in the stroma of the villi. Determsned a significant decrease in the average diameter, area and volume density of hemocapillaries (p=0.005; p= 0.03, p=0.01, respectively). Formation of syncytiocapillary membranes (p=0.0001) against the background of an increase in the thickness of syncytiotrophoblast (p=0.001) and the volume density of connective tissue (p=0.03).

Table 2

Morphometric indices of the placentas of women with physiological pregnancies (n=10), women of group 1 (n=7) and group 2 (n=9)

Parameter	Women with hysiological pregnancies, n=10	Women of group 1, n=7	Women of group 2, n=9	p
Mean diameter of terminal villi, μm	41.42±0.7	33.2±0.54	34.14±0.67	p_1=0.0001 p_2=0.0001 p_3=0.3
Mean area of terminal villi, $μm^2$	1452.7±42.0	930.3±25.4	1024.4±44.56	p_1=0.0001 p_2=0.0001 p_3=0.11
Mean diameter of hemocapillaries, μm	11.03±0.56	13.74±0.61	11.7±0.28	p_1=0.006 p_2=0.323 p_3=0.005
Mean area of hemocapillaries, $μm^2$	102.7±21.63	162.9±14.09	116.6±23.98	p_1=0.016 p_2=0.274 p_3=0.03
Mean number of hemocapillaries	3.93±0.05	3.17±0.28	3.16±0.06	p_1=0.006 p_2=0.0001 p_3=0.97
Thickness of syncytiotrophoblast terminal villi, μm	3.62±0.03	3.11±0.03	4.12±0.02	p_1=0.0001 p_2=0.0001 p_3=0.0001
% Formation of syncytiocapillary membranes from all capillaries of the terminal villi of the placenta	24.47±0.24	32.34±0.49	16.2±0.23	p_1=0.0001 p_2=0.0001 p_3=0.0001
Volume density of hemocapillaries	24.12±0.74	27.52±1.88	19.13±1.65	p_1=0.07 p_2=0.01 p_3=0.004
Volume density of syncytiotrophoblast	33.27±1.0	38.25±1.42	38.92±1.58	p_1=0.009 p_2=0.006 p_3=0.76
Volume density of connective tissue	42.59±1.55	34.21±1.83	41.92±2.53	p_1=0.003 p_2=0.82 p_3=0.03

It was found a correlation between the weight of the newborn and the area of the placenta in women in the control group (r=0.839, p=0.002); a correlation between the weight of the newborn and the average number of hemocapillaries in the villous chorion of the placenta in the 1-st group (r=0.912, p=0.04). At the same time, in the 1-st group the weight of the newborn was associated with the placenta area (r=0.71, p=0.03), F/P ratio (r=-0.728, p=0.03), average the area of terminal villi (r=0.712, p=0.03), the average area of hemocapillaries in the terminal villi (r=0.811, p=0.008).

The immunohistochemical expression of CD68 in the placental macrophages was cytoplasmic and expression of CD163 in the placental macrophages was membranous in the fibrously altered terminal villi (fig. 1).

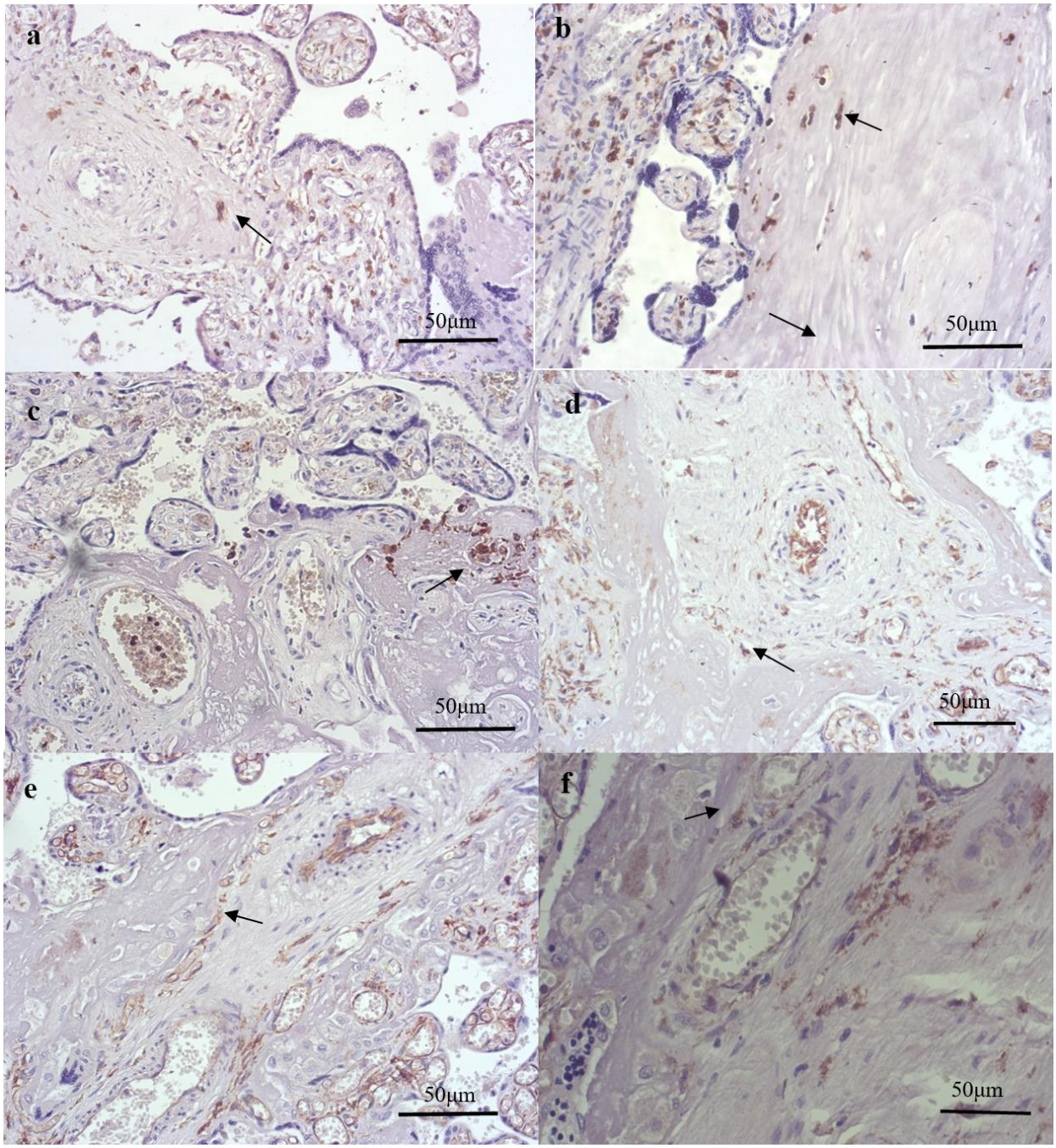

Fig. 1. (a) Expression of CD68 in women with physiological pregnancies; (b) Expression of CD68 in the placentas of women 1-st group; (c) Expression of CD68 in the placentas of women 2-nd group; (d) Expression of CD163 in the placentas of women with physiological pregnancies; (e) Expression of CD163 in the placentas of women 1-st group; (f) Expression of CD163 in the placentas of women 2-nd group; staining with hematoxylin, magn. x200.

The level of CD68+ macrophages in fibrously altered terminal villi of the placenta in the control group was 7.36±0.89%, and the level of CD163+ macrophages was 12.35±0.87% (p=0.0008). The level of CD68+ macrophages in fibrously altered terminal villi of the placenta in the 1-st group was 14.0±1.44%, and the level of CD163+ macrophages was 15.93±2.34% (p=0.49). The level of CD68+ macrophages in

fibrously altered terminal villi of the placenta in the 2-nd group was 10.85±1.22%, and the level of CD163+ macrophages was 16.29±1.02% (p=0.003).

The level of CD68+ macrophages in fibrously altered terminal villi of the placenta was significantly higher in the 1-st and 2-nd groups than in women of the control group (p=0.0008, p=0.03, respectively). However, there was not significant difference between 1-st and 2-nd groups. When compared the level of CD163+ macrophages in fibrously altered terminal villi in the control group, only in the 2-nd group showed significant increased expression of marker (p=0.009).

Next, we analyzed the relationship of the morphometric indices with the CD68+ or CD163 expression in macrophages. It was found in the control group significant negative correlation between the level of CD68 macrophage in fibrously altered stroma of terminal villi and volume density of syncytiotrophoblast (r=-0.782, p=0.008) and volume density stroma of connective tissue in terminal villi (r=0.823, p=0.003); in the 2-nd group there was a significant positive correlation between the level of CD68 macrophages in fibrously altered areas of terminal villi and the average diameter of hemocapillaries in the terminal villi (r=0.67, p=0.048).

In the current study it was shown that in FGR in women of the 2-nd group, there were lower indicators of placenta mass, area, but higher indices of fetoplacental weight ratio, in contrast to women of the control or 1-st group. Our findings are consistent with studies that investigated the relationship between postpartum placental morphometry and infant birth weight. It was shown that low birth weight is comparable to low parameters of weight, volume and area of the placenta in FGR [3]. It was suggested that morphological changes in the placenta in FGR may be caused by placental perfusion due to a decrease in the vascular bed and hypovascularization of terminal villi [7]. However, the data exist concerning the study of morphometric parameters in women whose pregnancy was complicated with FGR and resulted in timely or premature delivery with scarce remaining.

Our studies indicate that in FGR there are characteristic signs of placental insufficiency. Vascularization, development and growth of villi are particularly important morphological indicators of the state of the placenta. The distinctive morphofunctional parameters of the placentas in women of the 2-nd group include more pronounced signs of hypovascularization and hypoplasia of chorionic villi, which was manifested by increased formation of fibrosis, increase volume density of syncytiotrophoblast of terminal villi and decreased average area of hemocapillaries in contrast to the placentas of the 1-st group. The obtained data are consistent with the findings of other authors [9], which showed that in FGR, there is a decrease in the number, length, volume and area of capillaries in terminal villi. In another study [10], conducted in the population with preeclampsia and FGR, it was shown that the characteristic morphological changes of the placenta in FGR that resulted preterm labor is a combination of lesions of the placental bed and stroma of villi, namely excessive fibrosis and hypovascularization. Consequently, the revealed changes in the placenta may indicate a single mechanism and systemic damage to the chorionic villi not only at the tissue, but also at the cellular level, which causes dysregulation of adaptive homeostatic reactions.

Macrophages (Hofbauer cells) have been associated with numerous complications of pregnancy such as chorioamnionitis, pre-eclampsia, miscarriage, and preterm birth. The potential role of macrophages in the pathophysiology of complications of pregnancy such as FGR is completely unknown. During pregnancy development, regulated tissue remodeling following cellular senescence and activation fibrotic process contributes to embryonic growth and patterning. Removal of senescent cells by macrophages is critical, thus When this mechanism does not work, senescent cells continue to secrete inflammatory cytokines, and the retention of the inflammatory status disrupts the tissue remodeling process, which leads to tissue dysfunction.

In this study, we investigated the potential of tissue macrophages to repolarize in the placenta and undergo a phenotypical switch in fibrously altered terminal villi of the placenta. We observed changes to macrophage polarization with higher level CD163 (M2 type) in contrast to CD68 (M1 type) macrophages in fibrously altered terminal villi of the placenta at preterm birth pregnancy complicated with FGR. It was concluded that macrophages predominately are M2 polarized in the placenta from women which pregnancy complicated with FGR. In pre-eclamptic pregnancies, one study investigated CD163, CD68, CD206, and DC-SIGN in histology and on mRNA level. A dramatic decrease in all markers was observed [12]. However, no M1 markers were investigated, so one can only speculate if there is shift from M2 to M1 polarized macrophages or an absolute decrease in Hofbauer cells numbers in pre-eclampsia. Throughout different stages in pregnancy, predominance of either M1 or M2 macrophages has been observed and the balance between those subsets is crucial for successful pregnancy outcome. Importantly, alterations in

Hofbauer cells activity have been associated with second trimester pregnancy loss [8] and glucocorticoids, which are used as medication against pre-term labor, alter Hofbauer cells function via induction of CD163 [13]. Therefore, a stable, homeostatic, and tolerance-inducing M2 phenotype that only adapts minimally to pro-inflammatory conditions might actually be desired in pregnancy.

Conclusions

In the placenta of women whose pregnancy was complicated by FGR and resulted preterm birth, there were disruptions of the vascular and stromal component of the chorionic villus with the changes to macrophage polarization from M1 to M2 types in fibrously altered terminal villi of the placenta.

Therefore, having knowledge of morpological and immunological factors may help in early diagnosis, prompt intervention and better management, which can ultimately lead to good obstetric care during fetal growth restriction.

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