Ultrasound Obstet Gynecol 2019; **53**: 615–622
Published online 12 April 2019 in Wiley Online Library (wileyonlinelibrary.com). **DOI:** 10.1002/uog.20103

Premature placental aging in term small-for-gestational-age and growth-restricted fetuses

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KEYWORDS: aging; apoptosis; fetal growth restriction; placenta; senescence; small-for-gestational age

ABSTRACT

Objective *To perform a comprehensive assessment of the placental aging process in small term fetuses classified as being small-for-gestational age (SGA) or having fetal growth restriction (FGR) through analysis of senescence and apoptosis markers.*

Methods *This was a prospective nested case–control study of singleton pregnancies delivered at term, including 21 control pregnancies with normally grown fetuses and 36 with a small fetus classified as SGA (birth weight between the 3rd and 9th percentiles and normal fetoplacental Doppler;* n = *18) or FGR (birth weight < 3rd percentile and/or abnormal cerebroplacental ratio and/or uterine artery Doppler;* n = *18). Telomerase activity, telomere length (quantified by comparing the amount of amplification product for the telomere sequence (T) to that of a single copy of the gene 36B4 (S)) and RNA expression of senescence (Sirtuins 1, 3 and 6) and apoptosis (p53, p21, BAX and Caspases 3 and 9) markers (analyzed using the* $2^{-\Delta\Delta Ct}$ *method) were determined in placental samples collected at birth and compared between the three groups.*

Results *Compared to pregnancies with a normally grown fetus, both SGA and FGR pregnancies presented signs of accelerated placental aging, including lower telomerase activity (mean ± SD, 12.8 ± 6.6% in controls* vs *7.98 ± 4.2% in SGA* vs *7.79 ± 4.6% in FGR;* P = *0.008), shorter telomeres (mean ± SD T/S ratio, 1.20 ± 0.6 in controls* vs *1.08 ± 0.9 in SGA* vs *0.66 ± 0.5 in FGR;* P = *0.047) and reduced Sirtuin-1 RNA expression (mean ± SD* $2^{-\Delta\Delta Ct}$*, 1.55 ± 0.8 in controls* vs *0.91 ± 0.8 in SGA* vs *0.63 ± 0.5 in FGR;* P = *0.001) together with increased p53 RNA expression (median (interquartile range)* $2^{-\Delta\Delta Ct}$*, 1.07 (0.3–3.3) in controls* vs *5.39 (0.6–15) in SGA* vs *3.75 (0.9–7.8) in FGR;* P = *0.040). FGR cases presented signs of apoptosis, with increased Caspase-3 RNA levels (median (interquartile range)* $2^{-\Delta\Delta Ct}$*, 0.94 (0.7–1.7) in controls* vs *3.98 (0.9–31) in FGR;* P = *0.031) and Caspase-9 RNA levels (median (interquartile range)* $2^{-\Delta\Delta Ct}$*, 1.21 (0.6–4.0) in controls* vs *3.87 (1.5–9.0) in FGR;* P = *0.037) compared with controls. In addition, Sirtuin-1 RNA expression, telomerase activity, telomere length and Caspase-3 activity showed significant linear trends across groups as severity of the condition increased.*

Conclusions *Accelerated placental aging was observed in both clinical forms of late-onset fetal smallness (SGA and FGR), supporting a common pathophysiology and challenging the concept of SGA fetuses being constitutionally small. Copyright © 2018 ISUOG. Published by John Wiley & Sons Ltd.*

INTRODUCTION

Over the last 20 years, clinical evidence has shown consistently that there are at least two main prenatal forms of fetal smallness[1,2]: fetal growth restriction (FGR), in which there are changes in fetoplacental Doppler[3,4] and a higher risk for *in-utero* deterioration/stillbirth[5–9], and small-for-gestational-age (SGA) fetuses, usually referred to as constitutionally small since they fail to show evident changes in fetoplacental Doppler and have near normal perinatal outcome[10]. However, recent studies

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Accepted: 10 August 2018

have shown that both FGR and SGA are associated with suboptimal neurodevelopment[11,12] and increased cardiovascular risk[13–15], suggesting that SGA may in fact be a milder form of FGR rather than a group of normal smaller fetuses.

Placental dysfunction is the most commonly accepted etiology of fetal smallness[16,17]. However, in approximately 25% of pregnancies complicated by FGR, the placenta lacks any morphological abnormality on routine macroscopic and histological examination[18,19]. Hence, new approaches more suited to detect subtle placental changes have been proposed[20]. During pregnancy, the placenta normally suffers some degree of aging, promoting cell death and consequently presenting decreased activity related to normal post-term changes[21]. The most studied aging-associated phenomenon is related to modifications in telomerase homeostasis[22]. However, placental aging encompasses not only telomere homeostasis but also different patterns of cell-cycle arrest, such as apoptosis and cell senescence, both involving signaling of transformation-related protein 53 (p53) and Sirtuin 1.

Altered placental aging has been described in several obstetric complications[23] including stillbirth[24,25], spontaneous preterm labor[26–28], poorly controlled maternal diabetes[29], pre-eclampsia and FGR[30,31]. Previous studies in placentas of pregnancies complicated by FGR showed markers of placental aging, including shorter telomeres, telomerase activity suppression[32–34] and increased apoptosis mediated by the p53 pathway[35–37]. However, it is unclear whether SGA pregnancies with apparently normal placental function on ultrasound examination have significant placental aging or if this phenomenon is restricted solely to fetuses with FGR.

The objective of this study was to perform a comprehensive assessment of the placental aging process in FGR and SGA pregnancies delivered at term, including fetoplacental Doppler ultrasound, conventional placental morphometric and histological evaluation, and in-depth analysis of placental aging markers.

METHODS

Study population

This was a prospective nested case–control study of singleton pregnancies delivering at term, conducted at the Department of Maternal-Fetal Medicine at BCNatal Barcelona from April to October 2016. The study population comprised 57 singleton pregnancies delivered after 37 weeks of gestation, classified into 21 normally grown and 36 small fetuses, defined by estimated fetal weight and birth weight $< 10^{th}$ percentile according to local standards[38]. Small fetuses were further subdivided into SGA (birth weight between the 3^{rd} and 9^{th} percentiles and normal fetoplacental Doppler; $n = 18$) and FGR (birth weight $< 3^{rd}$ percentile and/or cerebroplacental ratio $< 5^{th}$ percentile and/or mean uterine artery pulsatility index (PI) $> 95^{th}$ percentile; $n = 18$). SGA and FGR cases were followed up every 1 or 2 weeks according to our clinical protocol. Doppler findings did not change later in pregnancy in any of the cases. Controls were selected randomly from uncomplicated low-risk pregnancies with a confirmed birth weight $> 10^{th}$ percentile. In all pregnancies, gestational age was calculated based on crown–rump length measurement on first-trimester ultrasound examination[39] and weight percentiles were calculated using local reference curves[38]. Pregnancies with congenital malformation, chromosomal abnormality or fetal infection were excluded.

The study protocol included maternal baseline and perinatal characteristics, comprehensive fetoplacental ultrasonographic assessment and placental sampling for subsequent conventional histopathological and in-depth aging analysis. The study protocol was approved by the local ethics committee and all patients provided written informed consent.

Fetoplacental ultrasound examination

Fetoplacental Doppler was performed at diagnosis of fetal smallness by experienced physicians and included assessment of: umbilical artery PI, calculated from three or more consecutive waveforms obtained from a free-floating portion of the umbilical cord at insonation angles of $< 30°$; fetal middle cerebral artery PI at the proximal portion of the vessel; cerebroplacental ratio, calculated as the ratio of middle cerebral artery PI to umbilical artery PI[40]; and mean uterine artery PI, calculated as the average PI of the right and left uterine arteries[41].

Placental sample collection

Placental samples were collected immediately after delivery. Tissue pieces of selected villous parenchyma ($1–2\,cm^3$) were sampled at four different sites in four lobules (free of visible infarction, calcification, hematoma or damage) and immediately snap frozen in liquid nitrogen and stored at $-80°C$ for subsequent nucleic acid extraction. The rest of the placenta was fixed in 10% buffered formalin and the following samples of each specimen were taken for routine processing and staining with hematoxylin and eosin: one transverse section of cord; one rolled strip of membranes; three blocks of villous parenchyma; and any macroscopic lesions.

Placental morphometric and histopathological assessment

Placental examinations were supervised by a single senior pathologist (A.N.) who was blinded to neonatal outcome. Placental diameter and thickness were recorded. Trimmed placenta was weighed (after removal of the membranes, cord and any blood clots) and weight percentiles were assigned based on gestational age-specific placental weight charts[42]. Fetoplacental weight ratio was calculated as birth weight/fresh placental weight. Placental lesions were categorized histologically according to the 2015 Redline Classification (Appendix S1)[43]. Briefly, placental findings were subdivided into three groups: maternal and

fetal vascular processes, immuno-inflammatory processes and other processes.

Placental aging markers

In order to assess placental aging markers, protein, DNA and RNA were extracted using standard protocols (Appendix S1). The following measurements were performed in duplicate, per sample and in two different areas of the placental tissue.

Telomerase activity was determined in placental homogenates using the commercially available kit TeloTAGGG Telomerase PCR ELISA (Roche Diagnostics GmbH, Mannheim, Germany). The assay is based on the principle of the Telomeric Repeat Amplification Protocol (TRAP) described by Kim *et al.*[44]. Values were expressed as a percentage of the absorbance obtained in a positive control (cell extract from immortalized telomerase-expressing human kidney 293 cells).

Relative telomere length was determined using quantitative real-time PCR (qPCR) according to the methodology described by Cawthon[45]. Telomere length was quantified by comparing the amount of amplification product for the telomere sequence (T) to that of a single copy of the gene 36B4 (S), and calculating the T/S ratio.

Gene expression analysis was carried out for apoptosis markers (Caspase 3 (CASP3), Caspase 9 (CASP9) and BCL2-associated X protein (BAX)) and senescence markers (p53, cyclin-dependent kinase inhibitor protein 21 (p21) and Sirtuins 1, 3 and 6 (SIRT1, SIRT3 and SIRT6)) using qPCR (Appendix S1). Cycle threshold (Ct) values obtained for each gene were referenced to the average of 18S and β-actin gene Ct (ΔCt) and converted to the linear form using the term $2^{-\Delta Ct}$. The quantity of cDNA transcript copies in each experimental group (SGA and FGR) was established relative to a reference sample (control) and expressed as $2^{-\Delta\Delta Ct}$.

Statistical analysis

Statistical analyses were performed using STATA 14 (StataCorp., College Station, TX, USA). Normal distributions were assessed using the Kolmogorov–Smirnov test. Data are presented as mean ± SD, median (interquartile range) or *n* (%). Chi-square test or Fisher's exact test and analysis of variance (ANOVA) or Kruskal–Wallis test (non-parametric) were used to compare categorical and continuous variables, respectively, between groups. Student's *t*-test or Mann–Whitney *U*-test were used to compare two groups. In addition, linear polynomial orthogonal contrast or Jonckheere–Terpstra tests were used to test for linear association between the groups as case severity increased (i.e. controls to SGA to FGR), when appropriate. Linear correlations were measured using Pearson or Spearman's correlation coefficient. Following standard methodology, data were adjusted for smoking by linear regression analysis. Two-sided *P*-values < 0.05 were considered to indicate statistical significance.

RESULTS

Study population

Baseline characteristics, fetoplacental Doppler and perinatal outcome are shown in Table 1. Maternal baseline characteristics were similar between groups with the

Table 1 Maternal characteristics, fetoplacental Doppler and perinatal outcome of term pregnancies with a normally grown fetus (controls) and of those with a small fetus, classified as small-for-gestational age (SGA) or growth restricted (FGR)

Parameter	*Controls* (n = *21*)	*SGA* (n = *18*)	*FGR* (n = *18*)
Maternal characteristic			
Age (years)	31.8 ± 6.5	30.2 ± 6.0	31.9 ± 4.7
Caucasian ethnicity	71.4	77.8	83.3
Smoker	4.8	38.9*	33.3*
Nulliparous	42.9	55.6	72.2
Fetoplacental Doppler			
GA at scan (weeks)	32.6 (29.0–36.0)	32.0 (29.0–35.0)	33.3 (31.0–35.0)
Mean UtA-PI *Z*-score	−0.50 ± 1.2	−0.05 ± 1.2	0.67 ± 1.5*
UA-PI *Z*-score	−0.16 (−0.6 to 0.3)	−0.04 (−0.5 to 0.3)	0.68 (−0.2 to 1.0)*†
MCA-PI *Z*-score	0.14 ± 1.3	0.22 ± 0.9	−0.22 ± 1.0
CPR *Z*-score	−0.20 (−0.9 to 0.7)	−0.20 (−0.9 to 0.3)	−1.28 (−1.6 to −0.1)*†
Perinatal outcome			
GA at delivery (weeks)	39.3 (38.4–39.5)	39.5 (38.5–40.2)	37.3 (37.2–38.2)*†
Male gender	42.9	50.0	61.1
Birth weight (g)	3258 ± 291	2728 ± 234*	2311 ± 247*†
Birth-weight percentile	42 (33–76)	7 (4–9)*	1 (0–2)*†
Cesarean section	42.9	33.3	44.4
Emergency Cesarean section	0	5.6	5.6
Umbilical cord artery pH	7.20 ± 0.1	7.23 ± 0.1	7.20 ± 0.1
5-min Apgar score < 7	0	0	0

Data are given as mean ± SD, % or median (interquartile range). *$P < 0.05$ when compared with controls. †$P < 0.05$ when compared with SGA. CPR, cerebroplacental ratio; GA, gestational age; MCA, middle cerebral artery; PI, pulsatility index; UA, umbilical artery; UtA, uterine artery.

exception of a higher prevalence of smokers in SGA and FGR pregnancies compared with controls. Prevalence of gestational diabetes, pre-eclampsia and use of *in-vitro* fertilization was also similar between groups. As expected, FGR cases showed significantly worse fetoplacental Doppler parameters when compared with SGA cases and controls, and a linear trend towards worsening results with increasing severity of the condition was observed. SGA and FGR newborns had significantly lower birth weight and birth-weight percentile compared with controls. As defined by the inclusion criteria, all cases and controls were delivered at term, but FGR cases had a lower gestational age at delivery (37 weeks *vs* 39 weeks in the other groups) as expected based on our clinical management protocol recommending induction of labor at 37 weeks of gestation in such cases.

Table 2 Placental morphometric and histopathological findings in term pregnancies with a normally grown fetus (controls) and in those with a small fetus classified as being small-for-gestational age (SGA) or growth restricted (FGR)

Finding	*Controls* (n = *21*)	*SGA* (n = *18*)	*FGR* (n = *18*)
Placental morphometry			
Weight (g)	486 ± 130	414 ± 82*	326 ± 67*†
Weight < 10th percentile	9.5	27.8	72.2*†
Weight < 3rd percentile	4.8	11.1	44.4*†
Length (cm)	18.5 ± 2.4	16.6 ± 1.8*	15.4 ± 1.7*†
Breadth (cm)	16.5 ± 1.8	14.9 ± 1.9*	14.1 ± 1.4*
Thickness (cm)	1.83 ± 0.6	1.97 ± 0.7	1.91 ± 0.6
Fetoplacental ratio	7.13 ± 1.9	6.79 ± 1.2	7.13 ± 1.3
Placental histopathological lesions			
Maternal vascular	38.1	44.4	66.7
Fetal vascular	38.1	33.3	38.9
Infectious	14.3	16.7	0
Immune	9.5	11.1	22.2
Other	19.0	11.1	22.2

Data are given as mean ± SD or %. *$P < 0.05$ when compared with controls. †$P < 0.05$ when compared with SGA.

Placental morphometric and histopathological findings

Table 2 details placental morphometric and histological findings in the study populations. As expected, placental weight, length and breadth were lower in small fetuses compared with controls, with a significant linear trend across groups as severity of the condition increased. Placental thickness and fetoplacental ratio were similar between groups. The prevalence of histopathological lesions was similar between the study groups, with a non-significant trend towards increased rates of maternal malperfusion and immune lesions in pregnancies complicated by FGR when compared to controls and those complicated by SGA.

Placental aging

Placental senescence and apoptosis findings are shown in Table 3 and Figure 1. When compared with normally grown fetuses, both SGA and FGR cases presented signs of boosted placental senescence with reduced SIRT1 RNA expression, lower telomerase activity, shorter telomeres and increased p53 RNA expression. In addition, significant linear trends towards lower SIRT1 RNA expression, telomerase activity and telomerase length, and higher Caspase-9 activity, were observed with increasing severity of the condition. Compared with controls, FGR cases also presented increased signs of apoptosis as reflected by increased CASP3 and CASP9 RNA expression. Similar RNA expression levels of p21, BAX, SIRT3 and SIRT6 were observed between the study groups. A significant positive correlation was observed between birth weight and telomerase activity ($R = 0.37$; $P = 0.007$), telomere length ($R = 0.28$; $P = 0.03$) and

Table 3 Markers of placental aging and apoptosis in term pregnancies with a normally grown fetus (controls) and in those with a small fetus classified as small-for-gestational age (SGA) or growth restricted (FGR)

Marker	*Controls* (n = *21*)	*SGA* (n = *18*)	*FGR* (n = *18*)	P*
Telomerase activity (%)	12.80 ± 6.6	7.98 ± 4.2†	7.79 ± 4.6†	0.008
Telomere length (T/S)	1.20 ± 0.6	1.08 ± 0.9	0.66 ± 0.5	0.017
Gene expression level				
Sirtuin 1 ($2^{-\Delta\Delta Ct}$)	1.55 ± 0.8	0.91 ± 0.8†	0.63 ± 0.5†	< 0.001
Sirtuin 3 ($2^{-\Delta\Delta Ct}$)	1.55 (1.1–2.8)	2.48 (0.6–3.8)	2.18 (0.9–7.1)	0.302
Sirtuin 6 ($2^{-\Delta\Delta Ct}$)	2.00 (0.6–3.7)	3.14 (1.4–6.6)	1.49 (0.5–4.2)	0.899
p53 ($2^{-\Delta\Delta Ct}$)	1.07 (0.3–3.3)	5.39 (0.6–15.0)†	3.75 (0.9–7.8)†	0.075
p21 ($2^{-\Delta\Delta Ct}$)	1.30 (0.8–4.3)	1.09 (0.5–5.6)	1.22 (0.5–4.2)	0.715
BAX ($2^{-\Delta\Delta Ct}$)	1.12 (0.7–1.4)	1.41 (0.5–3.0)	0.72 (0.4–1.1)	0.199
Caspase 3 ($2^{-\Delta\Delta Ct}$)	0.94 (0.7–1.7)	1.78 (0.8–8.0)	3.98 (0.9–31.0)†	0.007
Caspase 9 ($2^{-\Delta\Delta Ct}$)	1.21 (0.6–4.0)	2.95 (0.7–7.1)	3.87 (1.5–9.0)†	0.063

Data are given as mean ± SD or median (interquartile range). *Linear tendency *P*-value calculated using linear polynomial orthogonal contrast or Jonckheere–Terpstra test. †$P < 0.05$ when compared with controls. $2^{-\Delta\Delta Ct}$, level of gene expression normalized by cycle threshold of β-actin and 18S, in reference to control levels; BAX, BCL2-associated X protein; p21, cyclin-dependent kinase inhibitor protein 21; p53, transformation-related protein 53; T/S, ratio of amount of amplification product for telomere sequence (T) to that of single copy of gene 36B4 (S).

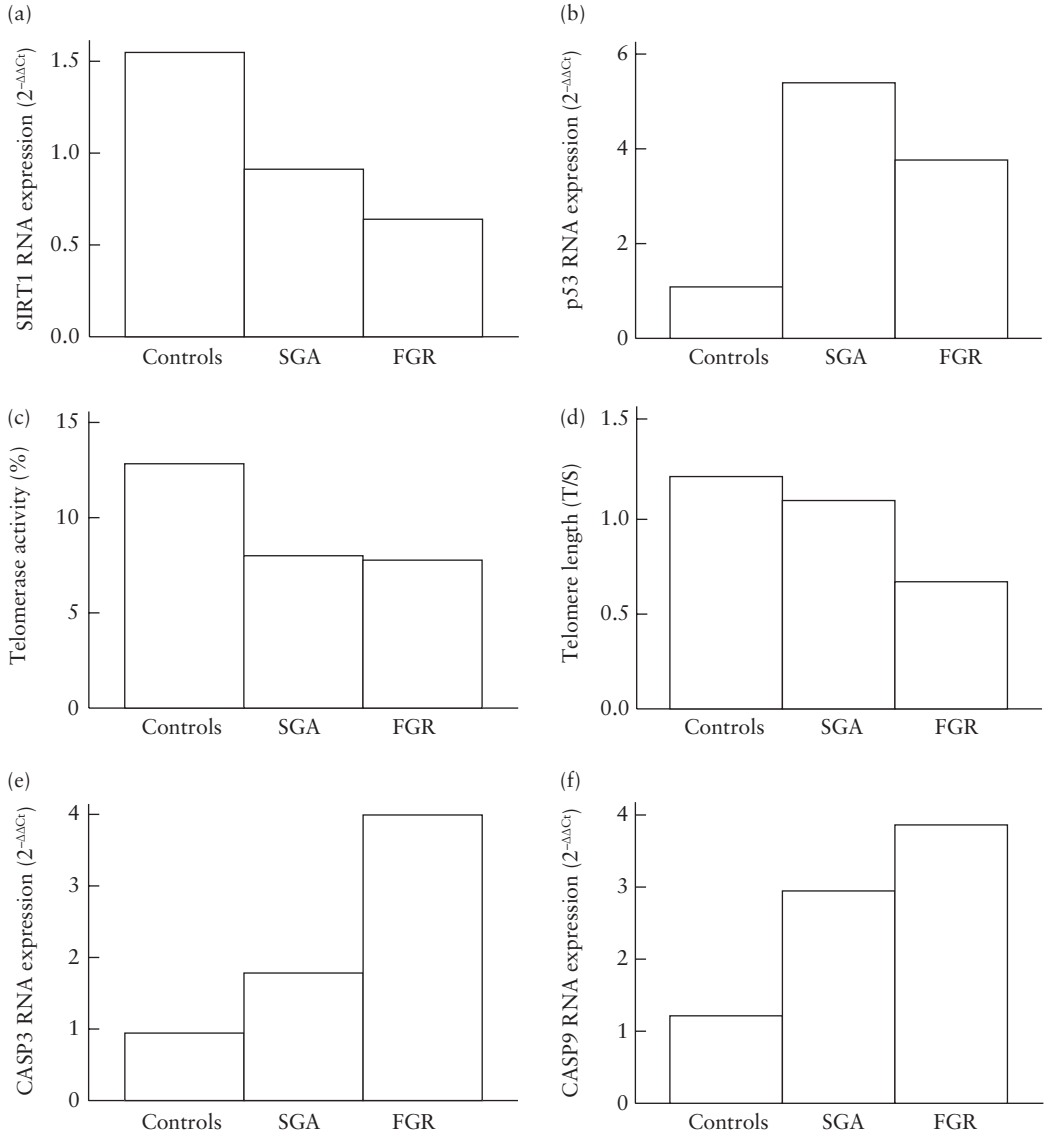

Figure 1 Markers of placental senescence and apoptosis in term pregnancies with a normally grown fetus (controls) ($n = 21$) and in those with a small fetus classified as small-for-gestational age (SGA; $n = 18$) or growth restricted (FGR; $n = 18$): (a) Sirtuin-1 (SIRT1) RNA expression; (b) transformation-related protein 53 (p53) RNA expression; (c) telomerase activity; (d) telomere length; (e) Caspase-3 (CASP3) RNA expression; (f) Caspase-9 (CASP9) RNA expression. Data are given as mean (a,c,d) or median (b,e,f). $2^{-\Delta\Delta Ct}$, level of gene expression normalized by cycle threshold of β-actin and 18S, in reference to control levels; T/S, ratio of amount of amplification product for telomere sequence (T) to that of single-copy of gene 36B4 (S).

SIRT1 RNA expression level ($R = 0.49$; $P = 0.002$). When adjusted for maternal smoking status, placental aging results remained similar, with the exception of telomerase activity, which did not show any statistically significant difference when comparing controls with SGA pregnancies (adjusted $P = 0.1$), but maintained a significant linear trend across groups.

DISCUSSION

The present study provides an in-depth analysis of the placental aging process in fetal smallness, demonstrating accelerated placental aging in both phenotypes, FGR and SGA, suggesting that they are both true forms of fetal restriction.

Our results suggesting premature placental aging in small fetuses are consistent with those of previous studies showing reduced telomerase activity, shorter telomeres[23,30,32,46] and increased expression of cell senescence markers (p21, p16 and EF-1α) in the placenta of pregnancies with a small fetus[34]. Telomeres are nucleoprotein structures located at the termini of chromosomes that become progressively shorter in each mitotic cycle or due to environmental factors. Once telomeres reach a critically short length, cell senescence and apoptosis are triggered. Telomere length is regulated by telomerase, an enzyme that adds DNA repeats onto the ends of chromosomes, maintaining cell integrity and stability[47,48]. This process seems to be disrupted in the placenta of SGA and FGR pregnancies, with reduced telomerase activity and subsequent telomere shortening that could activate

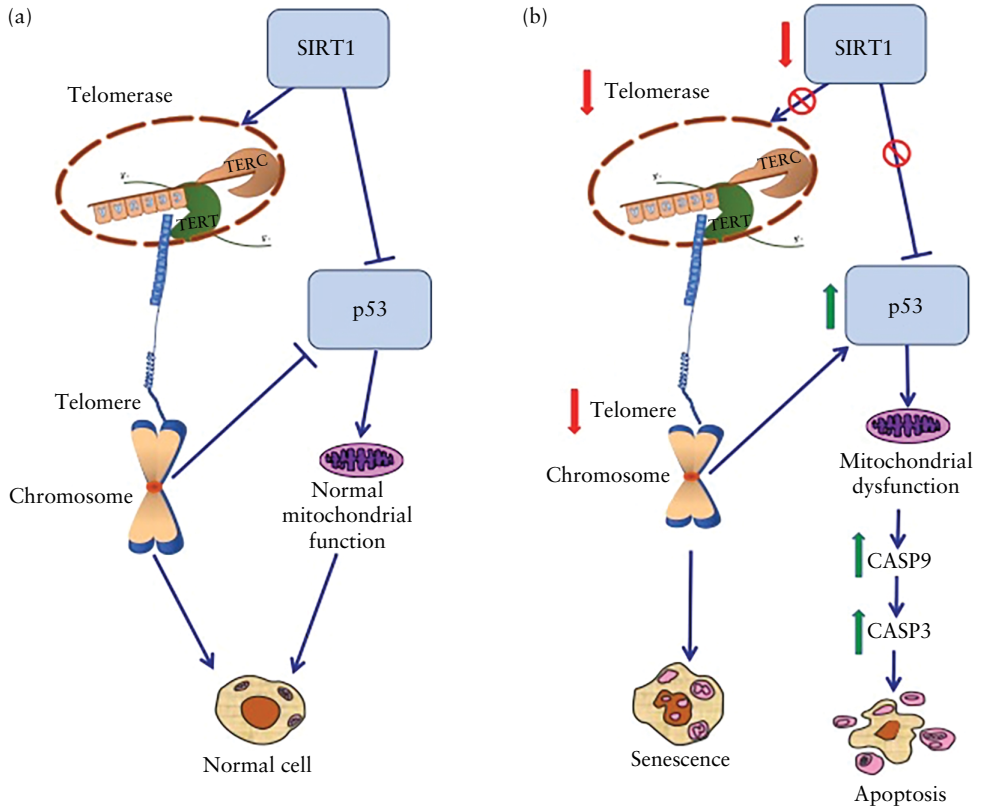

Figure 2 Molecular changes underlying placental aging process. In normal placental aging (a) Sirtuin 1 (SIRT1) activates telomerase, thereby maintaining telomere length and inhibiting expression of transformation-related protein 53 (p53), hence contributing to cell stability. Conversely, accelerated placental aging (b) is characterized by reduced levels of SIRT1, which diminishes telomerase activity, leading to telomere shortening, DNA damage and p53 activation. Subsequently, p53 activation induces mitochondrial dysfunction and increases expression of proapoptotic proteases such as Caspase 9 (CASP9) and Caspase 3 (CASP3). TERC, telomerase RNA component; TERT, telomerase reverse transcriptase.

cell senescence and p53 (Figure 2). While most previous studies focused only on telomere homeostasis, we provide further evidence of SIRT1 and p53 dysregulation in the placenta of pregnancies with a small fetus. SIRT1 is a stress-activated enzyme involved in various nuclear events such as DNA transcription, replication and repair, and acts as an antiaging agent that modulates telomerase activity and direct inactivation of p53. In addition, continued telomere shortening and associated DNA damage also promote the activation of p53 to boost DNA repair[49]. Therefore, the observed placental SIRT1 downregulation and p53 overexpression in pregnancies with a small fetus is consistent with the telomere results. Overall, sustained telomere shortening, DNA damage and p53 activation may lead to compromised mitochondrial function, cell senescence and apoptosis (as reflected by increased Caspase activity in FGR)[50]. In summary, significant changes in placental SIRT1, telomeres and p53 observed in small fetuses are consistent with accelerated activation of the entire placental aging process, potentially contributing to an insufficient placenta unable to meet the demands of the growing fetus[51].

The present study further demonstrates signs of accelerated placental aging both in FGR and SGA pregnancies delivering at term. As anticipated, FGR cases showed ultrasonographic signs of placental dysfunction and activation of the entire process of placental cell senescence as well as apoptosis. Although FGR cases showed the most prominent changes, we could demonstrate significant differences in placental SIRT1, p53 and telomerase activity in SGA cases with apparently normal placental Doppler and histology. Indeed, the increased, compared with controls, RNA expression of p53 is more prominent in SGA than in FGR pregnancies, which might be explained by p53 being involved not only in the aging process, but also in processes such as cell development/differentiation and tissue homeostasis[52]. We also observed a significant linear trend across groups, as severity of the condition increased, in RNA expression of senescence and some apoptosis markers, such as SIRT1 and CASP3, which correlate well with birth weight. According to these results, SGA fetuses presented a certain degree of premature placental aging, challenging the concept of SGA fetuses being constitutionally small and suggesting they have some degree of growth restriction. These findings are in line with the suboptimal cardiovascular and neurodevelopmental outcomes reported previously in SGA cases[53,54]. Previously, our group reported that fetal cardiovascular programming occurs in SGA fetuses, even if brain Doppler and neonatal outcomes were similar to those in controls[14]. We also demonstrated

fetal brain reorganization and microstructural changes and suboptimal postnatal neurodevelopment in SGA cases[55,56]. Thus, this evidence supports the hypothesis that SGA fetuses suffer some degree of placental insufficiency, which is not severe enough to show Doppler alterations or macroscopic placental lesions but is adequate enough to produce a certain degree of growth restriction[1]. We hypothesize that different degrees and timing of placental disease could lead to different clinical phenotypes of fetal smallness.

A strength of the present study is the comprehensive assessment of placental aging together with fetoplacental Doppler and histological placental examination in the same population. While both groups of small fetuses showed significant reductions in placental size and weight, a similar rate of placental histological lesions was observed among cases and controls. These data provide further evidence on the low sensitivity and specificity of conventional histology for identifying subtle placental dysfunction.

We acknowledge several limitations of our study. The relatively low sample size might have limited the ability to detect statistical significance for some placental markers. We decided to include only term pregnancies in order to avoid the effect of prematurity, although this limits the ability to extrapolate our results to early FGR. Despite FGR fetuses being delivered slightly earlier than controls (37 *vs* 39 weeks), they exhibited an even higher degree of placental aging. Our study provides information about one type of cell death, apoptosis, without analyzing autophagy, which is another type of cell arrest involving fusion of acidic lysosomes with the autophagosomes. Future studies are warranted to assess further placental aging and cell death in larger cohorts of small fetuses, including early-onset cases.

In conclusion, this study provides a comprehensive picture of the placental aging process in fetal smallness. Our findings demonstrate accelerated placental aging in both clinical forms of fetal smallness at term, SGA and FGR, challenging the concept of SGA fetuses being constitutionally small and supporting that this subset of small fetuses also presents a certain degree of placental dysfunction. Placental aging could be considered a potential mechanism of placental insufficiency and growth restriction, contributing to a better understanding of the different phenotypes of fetal smallness. Future studies are needed to evaluate its role as a potential biomarker or therapeutic target for placental disease.

ACKNOWLEDGMENTS

We are indebted to the IDIBAPS Biobank and Biobanc de l'Hospital Infantil Sant Joan de Déu per a la Investigació, integrated in the Spanish National Biobank Network of ISCIII, for sample and data procurement. This project was funded partly by the Instituto de Salud Carlos III (PI14/00226, INT16/00168 and PI15/00130) integrados en el Plan Nacional de I + D + I, the ISCIII-Subdirección General de Evaluación, the Fondo Europeo de Desarrollo Regional (FEDER) Otra manera de hacer Europa and the Erasmus+ Programme of the European Union (Framework Agreement number: 2013-0040). Additionally, this project received funding from: la Caixa Foundation; Cerebra Foundation for the Brain Injured Child (Carmarthen, Wales, UK) and AGAUR 2014 SGR, Grant No 928. J.M. was supported by a predoctoral governmental Bolivar Gana con Ciencia grant from Colombia. C.P. was supported by a Rio Hortega Grant from Instituto de Salud Carlos III (CM16/00142) and by a grant from Fundació Dexeus Mujer.

REFERENCES

1. Figueras F, Gratacós E. Update on the diagnosis and classification of fetal growth restriction and proposal of a stage-based management protocol. *Fetal Diagn Ther* 2014; **36**: 86–98.
2. Figueras F, Gratacós E. An integrated approach to fetal growth restriction. *Best Pract Res Clin Obstet Gynaecol* 2017; **38**: 48–58.
3. Oros D, Figueras F, Cruz-Martinez R, Meler E, Munmany M, Gratacós E. Longitudinal changes in uterine, umbilical and fetal cerebral Doppler indices in late-onset small-for-gestational-age fetuses. *Ultrasound Obstet Gynecol* 2011; **37**: 191–195.
4. Romero R, Hernandez-Andrade E. Doppler of the middle cerebral artery for the assessment of fetal well-being. *Am J Obstet Gynecol* 2015; **213**: 1.
5. Flenady V, Wojcieszek AM, Middleton P, Ellwood D, Erwich JJ, Coory M, Khong TY, Silver RM, Smith GC, Boyle FM, Lawn JE, Blencowe H, Leisher SH, Gross MM, Horey D, Farrales L, Bloomfield F, McCowan L, Brown SJ, Joseph KS, Zeitlin J, Reinebrant HE, Ravaldi C, Vannacci A, Cassidy J, Cassidy P, Farquhar C, Wallace E, Siassakos D, Heazell AE, Storey C, Sadler L, Petersen S, Frøen JF, Goldenberg RL. Stillbirths: Recall to action in high income countries. *Lancet* 2016; **387**: 691–702.
6. Moraitis AA, Wood AM, Fleming M, Smith GCS. Birth weight percentile and the risk of term perinatal death. *Obstet Gynecol* 2014; **124**: 274–283.
7. Gardosi J, Madurasinghe V, Williams M, Malik A, Francis A. Maternal and fetal risk factors for stillbirth: population-based study. *BMJ* 2013; **346**: f108.
8. Mendez-Figueroa H, Truong VTT, Pedroza C, Khan AM, Chauhan SP. Small-for gestational-age infants among uncomplicated pregnancies at term: a secondary analysis of Maternal-Fetal Medicine Units Network studies. *Am J Obstet Gynecol* 2016; **215**: 628.e1–7.
9. Figueras F, Gratacós E. Stage-based approach to the management of fetal growth restriction. *Prenat Diagn* 2014; **34**: 655–659.
10. O'Dwyer V, Burke G, Unterscheider J, Daly S, Geary MP, Kennelly MM, McAuliffe FM, O'Donoghue K, Hunter A, Morrison JJ, Dicker P, Tully EC, Malone FD. Defining the residual risk of adverse perinatal outcome in growth-restricted fetuses with normal umbilical artery blood flow. *Am J Obstet Gynecol* 2014; **211**: 420.e1–5.
11. Egaña-Ugrinovic G, Sanz-Cortes M, Figueras F, Bargalló N, Gratacós E. Differences in cortical development assessed by fetal MRI in late-onset intrauterine growth restriction. *Am J Obstet Gynecol* 2013; **209**: e1–8.
12. Eixarch E, Meler E, Iraola A, Illa M, Crispi F, Hernandez-Andrade E, Gratacós E, Figueras F. Neurodevelopmental outcome in 2-year-old infants who were small-for-gestational-age term fetuses with cerebral blood flow redistribution. *Ultrasound Obstet Gynecol* 2008; **32**: 894–899.
13. Crispi F, Bijnens B, Figueras F, Bartrons J, Eixarch E, Le Noble F, Ahmed A, Gratacós E. Fetal growth restriction results in remodeled and less efficient hearts in children. *Circulation* 2010; **121**: 2427–2436.
14. Crispi F, Figueras F, Cruz-Lemini M, Bartrons J, Bijnens B, Gratacós E. Cardiovascular programming in children born small-for-gestational-age and relationship with prenatal signs of severity. *Am J Obstet Gynecol* 2012; **207**: e1–9.
15. Tang X, Hernandez-Andrade E, Ahn H, Garcia M, Saker H, Korzeniewski SJ, Tarca AL, Yeo L, Hassan SS, Romero R. Intermediate diastolic velocity as a parameter of cardiac dysfunction in growth-restricted fetuses. *Fetal Diagn Ther* 2016; **39**: 28–39.
16. Lackman F, Capewell V, Gagnon R, Richardson B. Fetal umbilical cord oxygen values and birth to placental weight ratio in relation to size at birth. *Am J Obstet Gynecol* 2001; **185**: 674–682.
17. Brosens I, Pijnenborg R, Vercruysse L, Romero R. The "Great Obstetrical Syndromes" are associated with disorders of deep placentation. *Am J Obstet Gynecol* 2011; **204**: 193–201.
18. Fox H, Sebire NJ. The placenta in abnormalities and disorders of the fetus. In *Pathology of the Placenta: Major Problems in Pathology* 7 (3rd edn), Fox H, Sebire NJ (eds). Saunders Elsevier: Philadelphia, PA, 2007.
19. Redline RW. Placental pathology: a systematic approach with clinical correlations. *Placenta* 2008; **29**: S86–91.
20. Sultana Z, Maiti K, Aitken J, Morris J, Dedman L, Smith R. Oxidative stress, placental ageing-related pathologies and adverse pregnancy outcomes. *Am J Reprod Immunol* 2017; **77**. DOI: 10.1111/aji.12653.
21. Velarde MC, Menon R. Positive and negative effects of cellular senescence during female reproductive aging and pregnancy. *J Endocrinol* 2016; **230**: R59–76.
22. Xu Z, Duc KD, Holcman D, Teixeira MT. The length of the shortest telomere as the major determinant of the onset of replicative senescence. *Genetics* 2013; **194**: 847–857.

23. Sultana Z, Maiti K, Dedman L, Smith R. Is there a role for placental senescence in the genesis of obstetric complications and fetal growth restriction? *Am J Obstet Gynecol* 2018; **218**: S762–S773.
24. Maiti K, Sultana Z, Aitken RJ, Morris J, Park F, Andrew B, Riley SC, Smith R. Evidence that fetal death is associated with placental aging. *Am J Obstet Gynecol* 2017; **217**: 441.e1–14.
25. Ferrari F, Facchinetti F, Saade G, Menon R. Placental telomere shortening in stillbirth: a sign of premature senescence? *J Matern Fetal Neonatal Med* 2016; **29**: 1283–1288.
26. Polettini J, Dutta EH, Behnia F, Saade GR, Torloni MR, Menon R. Aging of intrauterine tissues in spontaneous preterm birth and preterm premature rupture of the membranes: a systematic review of the literature. *Placenta* 2015; **36**: 969–973.
27. Behnia F, Taylor BD, Woodson M, Kacerovsky M, Hawkins H, Fortunato SJ, Saade GR, Menon R. Chorioamniotic membrane senescence: a signal for parturition? *Am J Obstet Gynecol* 2015; **213**: 359.e1–16.
28. Gomez-Lopez N, Romero R, Plazyo O, Schwenkel G, Garcia-Flores V, Unkel R, Xu Y, Leng Y, Hassan SS, Panaitescu B, Cha J, Dey SK. Preterm labor in the absence of acute histologic chorioamnionitis is characterized by cellular senescence of the chorioamniotic membranes. *Am J Obstet Gynecol* 2017; **217**: 592.e1–17.
29. Biron-Shental T, Sukenik-Halevy R, Naboani H, Liberman M, Kats R, Amiel A. Telomeres are shorter in placentas from pregnancies with uncontrolled diabetes. *Placenta* 2015; **36**: 199–203.
30. Biron-Shental T, Sukenik-Halevy R, Sharon Y, Goldberg-Bittman L, Kidron D, Fejgin MD, Amiel A. Short telomeres may play a role in placental dysfunction in pre-eclampsia and intrauterine growth restriction. *Am J Obstet Gynecol* 2010; **202**: 381.e1–7.
31. Sukenik-Halevy R, Amiel A, Kidron D,Liberman M, Ganor-Paz Y, Biron-Shental T. Telomere homeostasis in trophoblasts and in cord blood cells from pregnancies complicated with preeclampsia. *Am J Obstet Gynecol* 2016; **214**: 283.e1–7.
32. Biron-Shental T, Sadeh-Mestechkin D, Amiel A. Telomere homeostasis in FGR placentas - a review. *Placenta* 2016; **39**: 21–23.
33. Toutain J, Prochazkova-Carlotti M, Cappellen D, Jarne A, Chevret E, Ferrer J, Idrissi Y, Pelluard F, Carles D, Maugey-Laulon B, Lacombe D, Horovitz J, Merlio JP, Saura R. Reduced placental telomere length during pregnancies complicated by intrauterine growth restriction. *PLoS One* 2013; **8**: e54013.
34. Davy P, Nagata M, Bullard P, Fogelson NS, Allsopp R. Fetal growth restriction is associated with accelerated telomere shortening and increased expression of cell senescence markers in the Placenta. *Placenta* 2009; **30**: 539–542.
35. Levy R, Smith SD, Yusuf K, Huettner PC, Kraus FT, Sadovsky Y, Nelson DM. Trophoblast apoptosis from pregnancies complicated by fetal growth restriction is associated with enhanced p53 expression. *Am J Obstet Gynecol* 2002; **186**: 1056–1061.
36. Erel CT, Dane B, Calay Z, Kaleli S, Aydinli K. Apoptosis in the placenta of pregnancies complicated with IUGR. *Int J Gynaecol Obstet* 2001; **73**: 229–235.
37. Heazell AE, Sharp AN, Baker PN, Crocker IP. Intra-uterine growth restriction is associated with increased apoptosis and altered expression of proteins in the p53 pathway in villous trophoblast. *Apoptosis* 2011; **16**: 135–144.
38. Figueras F, Meler E, Iraola A, Eixarch E, Coll O, Figueras J, Francis A, Gratacós E, Gardosi J. Customized birth weight standards for a Spanish population. *Eur J Obstet Gynecol Reprod Biol* 2008; **136**: 20–24.
39. Robinson HP, Fleming JEE. A critical evaluation of sonar crown–rump length measurements. *Br J Obstet Gynaecol* 1975; **82**: 702–710.
40. Baschat AA, Gembruch U. The cerebroplacental Doppler ratio revisited. *Ultrasound Obstet Gynecol* 2003; **21**: 124–127.
41. Gómez O, Figueras F, Fernández S, Bennasar M, Martínez JM, Puerto B, Gratacós E. Reference ranges for uterine artery mean pulsatility index at 11–41 weeks of gestation. *Ultrasound Obstet Gynecol* 2008; **32**: 128–132.
42. Almog B, Shehata F, Aljabri S, Levin I, Shalom-Paz E, Shrim A. placenta weight percentile curves for singleton and twins deliveries. *Placenta* 2011; **32**: 58–62.
43. Redline RW. Classification of placental lesions. *Am J Obstet Gynecol* 2015; **213**: S21–28.
44. Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, Coviello GM, Wright WE, Weinrich SL, Shay JW. Specific association of human telomerase activity with immortal cells and cancer. *Science* 1994; **266**: 2011–2015.
45. Cawthon RM. Telomere measurement by quantitative PCR. *Nucleic Acids Res* 2002; **30**: e47.
46. Biron-Shental T, Sukenik-Halevy R, Sharon Y, Laish I, Fejgin MD, Amiel A. Telomere shortening in intra uterine growth restriction placentas. *Early Hum Dev* 2014; **90**: 465–469.
47. Egan, E.D. and K. Collins. Biogenesis of telomerase ribonucleoproteins. *RNA* 2012; **18**: 1747–1759.
48. Collins, K. Physiological assembly and activity of human telomerase complexes. *Mech Aging Dev* 2008; **129**: 91–98.
49. Lee, J.M. and A. Bernstein. p53 mutations increase resistance to ionizing radiation. *Proc Natl Acad Sci USA* 1993; **90**: 5742–5746.
50. Sahin, E. and R.A. DePinho. Axis of aging: telomeres, p53 and mitochondria. *Nat Rev Mol Cell Biol* 2012; **13**: 397–404.
51. Smith R, Maiti K, Aitken R. Unexplained antepartum stillbirth: a consequence of placental aging? *Placenta* 2013; **34**: 310–313.
52. Harris SL, Levine AJ. The p53 pathway: positive and negative feedback loops. *Oncogene* 2005; **24**: 2899–2908.
53. Figueras F, Oros D, Cruz-Martinez R, Padilla N, Hernandez-Andrade E, Botet F, Costas-Moragas C, Gratacós E. Neurobehavior in term, small-for-gestational-age infants with normal placental function. *Pediatrics* 2009; **124**: e934–941.
54. Fick TA, Backes CH. Cardiovascular remodeling in the small-for-gestational-age infant - implications and future directions. *Circ J* 2016; **80**: 2096–2097.
55. Sanz-Cortes M, Figueras F, Bargallo N, Padilla N, Amat-Roldan I, Gratacós E. Abnormal brain microstructure and metabolism in small-for-gestational-age term fetuses with normal umbilical artery Doppler. *Ultrasound Obstet Gynecol* 2010; **36**: 159–165.
56. Sanz-Cortés M, Figueras F, Bonet-Carne E, Padilla N, Tenorio V, Bargalló N, Amat-Roldan I, Gratacós E. Fetal brain MRI texture analysis identifies different microstructural patterns in adequate and small-for-gestational-age fetuses at term. *Fetal Diagn Ther* 2013; **33**: 122–129.

SUPPORTING INFORMATION ON THE INTERNET

The following supporting information may be found in the online version of this article:

Appendix S1 Classification of placental findings and experimental protocols

This article has been selected for Journal Club.

A slide presentation prepared by Dr Yael Raz,
one of UOG's Editors for Trainees, is available online.

Spanish translation by Dr Rubén D. Fernández Jr.
Chinese translation by Dr Ying Ma and Prof. Qingqing Wu, ISUOG China Task Force.

Ultrasound Obstet Gynecol 2019; **53**: 615–622
Published online 12 April 2019 in Wiley Online Library (wileyonlinelibrary.com). **DOI:** 10.1002/uog.20103

Envejecimiento prematuro de la placenta en fetos pequeños para la edad gestacional y con restricción del crecimiento

RESUMEN

Objetivo Realizar una evaluación integral del proceso de envejecimiento de la placenta en fetos a término clasificados como pequeños para la edad gestacional (PEG) o con restricción del crecimiento fetal (RCF) mediante el análisis de los marcadores de senescencia y apoptosis.

Métodos Este fue un estudio prospectivo de casos y controles anidados de embarazos únicos a término, que incluyó 21 embarazos de control con fetos de crecimiento normal y 36 con un feto clasificado como PEG (peso al nacer entre los percentiles 3º y 9º y Doppler fetoplacentario normal; n=18) o con RCF (peso al nacer menor del percentil 3º y/o relación cerebroplacentaria anómala y/o Doppler de la arteria uterina; n=18). La actividad de la telomerasa, la longitud de los telómeros (cuantificada comparando la cantidad de producto de amplificación para la secuencia de telómeros (T) con la de una sola copia del gen 36B4 (S)) y la expresión del ARN de la senescencia (Sirtuinas 1, 3 y 6) y los marcadores de apoptosis (p53, p21, BAX y Caspasas 3 y 9) (analizados usando el método $2^{-\Delta\Delta Ct}$) se determinaron en muestras de placenta obtenidas en el momento del nacimiento y se compararon entre los tres grupos.

Resultados En comparación con los embarazos con un feto de crecimiento normal, tanto los embarazos PEG y con RCF presentaron signos de envejecimiento placentario acelerado, como una menor actividad de la telomerasa (media ± SD, 12,8 ± 6,6% en los controles frente a 7,98 ± 4,2% en PEG frente a 7,79 ± 4,6% en RCF; P=0,008), telómeros más cortos (media ± SD razón T/S, 1,20 ± 0,6 en los controles frente a 1,08 ± 0,9 en PEG frente a 0,66 ± 0,5 en RCF; P=0,047) y expresión reducida de la Sirtuina 1 en el ARN (media ± SD $2^{-\Delta\Delta Ct}$, 1,55 ± 0,8 en los controles frente a 0,91 ± 0,8 en PEG frente a 0,63 ± 0,5 en RCF; P=0,001), junto con una mayor expresión del p53 en el ARN (mediana (rango intercuartil) $2^{-\Delta\Delta Ct}$, 1,07 (0,3-3,3) en los controles frente a 5,39 (0,6–15) en PEG frente a 3,75 (0,9–7,8) en RCF; P=0,040). Los casos de RCF presentaron signos de apoptosis, con un aumento de los niveles en ARN de la Caspasa 3 (mediana (rango intercuartil) $2^{-\Delta\Delta Ct}$, 0,94 (0,7–1,7) en los controles frente a 3,98 (0,9–31) en RCF; P=0,031) y Caspasa 9 (mediana (rango intercuartil) $2^{-\Delta\Delta Ct}$, 1,21 (0,6-4,0) en los controles frente a 3,87 (1,5–9,0) en RCF; P=0,037) en comparación con los controles. Además, la expresión de la Sirtuina 1 en el ARN, la actividad de la telomerasa, la longitud de los telómeros y la actividad de la Caspasa 3 mostraron tendencias lineales significativas entre los grupos en función del aumento de la severidad de la anomalía.

Conclusiones Se observó un envejecimiento acelerado de la placenta en ambas formas clínicas de tamaño pequeño del feto de inicio tardío (PEG y RCF), lo que apoya una fisiopatología común y pone en tela de juicio el concepto de que los fetos PEG son en pequeños por su propia condición.

足月小于胎龄儿和生长受限胎儿胎盘过早老化

摘要

目的：通过衰老和细胞凋亡标志物分析，对足月小于胎龄儿（SGA）或生长受限（FGR）胎儿的胎盘老化过程进行综合评估。

方法：这是一项对足月分娩的单胎妊娠进行的前瞻性巢式病例对照研究，研究纳入 21 例胎儿正常发育的对照组妊娠和 36 例小胎儿：SGA（出生体重介于 3%和 9%之间，胎儿胎盘多普勒正常；n=18）或 FGR（出生体重<3%和/或脑胎盘比值异常和/或子宫动脉多普勒异常；n=18)。出生时采集胎盘标本，分别检测端粒酶活性、端粒长度（通过将端粒序列（T）的扩增产物数量与基因 36B4（S）的单一拷贝扩增产物数量进行比较来量化）及衰老的 RNA 表达（Sirtuins 1、3、6）和细胞凋亡（p53、p21、BAX、Caspases 3、9）标志物（利用 $2^{-\Delta\Delta Ct}$ 法分析），并在三组中进行比较。

结果：与正常发育的胎儿相比，SGA 和 FGR 妊娠均表现出胎盘老化加速的迹象，包括降低的端粒酶活性（均数 ± 标准差，对照组 12.8 ± 6.6% vs SGA 组 7.98 ± 4.2% vs FGR 组 7.79 ± 4.6%；P=0.008）、缩短的端粒（均数 ± 标准差 T/S 比值，对照组 1.20 ± 0.6 vs SGA 组 1.08 ± 0.9 vs FGR 组 0.66 ± 0.5； P=0.047）及 Sirtuin-1 RNA 表达减弱（均数 ± 标准差 $2^{-\Delta\Delta Ct}$；对照组 1.55 ± 0.8 vs SGA 组 0.91 ± 0.8 vs FGR 组 0.63 ± 0.5；P=0.001）和 P53 RNA 表达增强（中位数（四分位数间距）$2^{-\Delta\Delta Ct}$，对照组 1.07（0.3 – 3.3） vs SGA 组 5.39（0.6 – 15）vs FGR 组 3.75（0.9 – 7.8）； P=0.040）。与对照相相比，FGR 病例出现细胞凋亡迹象，并伴随 caspase-3 RNA 水平升高（中位数（四分位数间距）$2^{-\Delta\Delta Ct}$，对照组 0.94（0.7 – 1.7）vs FGR 组 3.98（0.9 – 31）；P=0.031）及 caspase-9 RNA 水平升高（中位数（四分位数间距）2 – ΔΔCt，对照组 1.21（0.6 – 4.0）vs FGR 组 3.87（1.5 – 9.0）；P=0.037）。此外，随着病情的加重，Sirtuin-1 RNA 的表达、端粒酶活性、端粒长度和 Caspase-3 活性在各组间呈明显的线性趋势。

结论：两种临床形式的迟发性胎小症（SGA 和 FGR）均出现胎盘加速老化的现象，支持共同的病理生理机制，并对 SGA 胎儿天生小的概念提出质疑。