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

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Running Head: Premature placental aging in fetal growth restriction.

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ABSTRACT

Objective

The aim of this study was to perform a comprehensive assessment of the placental aging process through senescence and apoptotic markers in late-onset small fetuses classified as SGA or FGR.

Study Design

A prospective nested case-control study in singleton pregnancies delivering at term including 21 normally grown fetuses and 36 small fetuses classified into SGA (if birthweight was between the 3rd and 9th centile and normal fetoplacental Doppler; n=18) and FGR (if birthweight <3rd centile and/or abnormal cerebroplacental ratio or uterine artery Doppler; n=18). Telomerase activity, telomere length and RNA expression of senescence (Sirtuin 1,3,6) and apoptotic markers (p53, p21, BAX, Caspase 3 and 9) were analyzed in placental samples collected at birth.

Results

Compared with normally grown fetuses, both SGA and FGR presented signs of accelerated placental aging including lower telomerase activity (controls mean±SD 12.8% ± 6.6 vs SGA 7.98% ± 4.2 vs FGR 7.79% ± 4.6, p=0.008), shorter telomeres (controls 1.20 T/S ± 0.6 vs SGA 1.08 T/S ± 0.9 vs FGR 0.66 T/S ± 0.5, p=0.017), and reduced Sirtuin1 RNA expression

(controls 1.55 $2^{-\Delta\Delta Ct} \pm 0.8$ vs SGA 0.91 $2^{-\Delta\Delta Ct} \pm 0.8$vs FGR 0.63 $2^{-\Delta\Delta Ct} \pm 0.5$, p<0.001) together with increased p53 RNA expression (controls median(IQR) $1.072^{-\Delta\Delta Ct}$ (3.2) vs SGA 5.39 $2^{-\Delta\Delta Ct}$ (15) vs FGR 3.75 $2^{-\Delta\Delta Ct}$ (7.8), p=0.040), with a significant linear tendency across severity stages. In addition, FGR cases presented signs of apoptosis with increased RNA levels of Caspase 3 (controls 0.94 $2^{-\Delta\Delta Ct}$ (1.1) vs FGR 3.98 $2^{-\Delta\Delta Ct}$ (30), p=0.031) and Caspase 9 (controls 1.21 $2^{-\Delta\Delta Ct}$ (4.0) vs FGR 3.87 $2^{-\Delta\Delta Ct}$ (8.7), p=0.037) as compared to controls.

Conclusions

A comprehensive assessment demonstrated accelerated placental aging in both clinical forms of late-onset fetal smallness, supporting a common pathophysiology and challenging the concept of SGA being 'constitutionally small'.

INTRODUCTION

Over the last 20 years, clinical evidences have consistently shown that there are at least two main prenatal forms of small fetuses [1,2]: fetal growth restriction (FGR) with changes in feto-placental Doppler [3,4] and a higher risk for in utero deterioration/stillbirth; [5,6,7,8,9] and small-for-gestational age (SGA) fetuses, usually referred to as "constitutionally" small, since they fail to show evident changes in feto-placental Doppler and have near normal perinatal outcomes. [10] However, recent studies have shown that both FGR and SGA are associated with suboptimal neurodevelopment [11,12] and increased cardiovascular risk, [13,14,15] casting doubt as to whether SGA is not just a group of normal "smaller" fetuses, but a milder form of FGR

Placental dysfunction is the most commonly accepted etiology of fetal smallness. [16,17] However, approximately 25% of FGR placentas lack 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] From early to term 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 not only encompasses telomere homeostasis but also different patterns of cell-cycle arrest such as apoptosis or cell senescence, both involving p53 and SIRT1 signaling.

Altered placental aging process has been described in several obstetric complications [23] including stillbirth, [24,25] spontaneous preterm labor, [26,27,28] poorly controlled maternal diabetes, [29] preeclampsia and FGR. [30,31] Previous studies in placentas from small fetuses showed markers of placental aging, including shorter telomeres and telomerase activity suppression [32,33,34] and increased apoptosis mediated by p53 pathway. [35,36,37] However, it is unclear whether SGA- with

apparent normal placental function on ultrasound- have significant placental aging or if this phenomenon is solely restricted to those fetuses with FGR.

Our objective was to perform a comprehensive assessment of placental aging process in FGR and SGA fetuses. For this purpose, we designed a prospective study in small fetuses delivering at term, which included feto-placental Doppler ultrasound, a conventional placental morphometric and histological evaluation, and in-depth analysis of placental aging markers.

Material and Methods

Study population

We conducted a prospective nested case-control study in singleton pregnancies delivering at term in the Department of Maternal-Fetal Medicine at BCNatal Barcelona from April 2016 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 birthweight below 10th centile according to local standards.[38] Small fetuses were further subdivided into SGA (if birthweight 3-9th centile and normal feto-placental Doppler, n=18) or FGR (if birthweight below 3th centile and/or cerebroplacental ratio below 5th centile and/or uterine artery mean pulsatility index above 95th centile, n=18). SGA and FGR cases were followed up every one or two weeks according to our clinical protocol. None of the cases changed Doppler finings later in pregnancy. Controls were randomly selected from uncomplicated low-risk pregnancies with a confirmed birthweight above 10th centile. In all pregnancies, gestational age was calculated based on crown–rump length measurement on first-trimester ultrasound[39] and weight centiles were calculated using local reference curves.[38] Pregnancies with congenital malformations, chromosomal abnormalities or fetal infection were excluded.

The study protocol included maternal baseline and perinatal characteristics, comprehensive feto-placental 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 patients provided their written informed consent.

Feto-placental ultrasound

Feto-placental Doppler was performed at diagnosis by experienced physicians including: umbilical artery pulsatility index (PI) calculated from three or more consecutive waveforms obtained from a free-floating portion of the umbilical cord at insonation angles of less than 30°; middle cerebral artery PI at the proximal portion of the vessel; cerebroplacental ratio (CPR) calculated as the ratio of middle cerebral artery PI to umbilical artery PI;[40] and uterine arteries mean PI calculated as the average PI of the right and left arteries. [41]

Placental samples collection

Placental samples were collected immediately after delivery. Tissue pieces of selected villous parenchyma (1-2 cm^3) were sampled from 4 different sites of 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 (AN) blinded to neonatal outcome. Diameters and thickness of placentae were collected. Trimmed-placenta was weighted (after removal of the membranes, cord and any blood clots) and weight centiles were assigned based on gestational age-specific placenta weight charts.[42] Fetoplacental weight ratio was calculated as birthweight / fresh-placental weight. Placental lesions were histologically categorized according to the 2015 Redline classification. [43] Briefly, placental findings were

subdivided into three groups: maternal and fetal vascular processes, inmuno-inflammatory processes, and other processes.

Placental aging markers

In order to assess placental aging markers, protein, DNA and RNA were first extracted by standard protocols, as described in the supplementary data. Following measurements were performed in duplicate per sample and in two different areas of the placental tissue:

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

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

- Gene expression analysis of **apoptosis** [caspase 3 (CASP3); caspase 9 (CASP9); BCL2-associated X protein (BAX)] and **senescence markers** [transformation-related protein 53 (TP53); cyclin-dependent kinase inhibitor p21 (p21); and sirtuins 1, 3 and 6 (SIRT1, SIRT3 and SIRT6)] by quantitative real-time PCR (qPCR).

Statistical analysis

Statistical analyses were performed using STATA 14 (Statacorp, College Station, Texas, US). Normal distributions were assessed using the Kolmogorov-Smirnov test. Data are

presented as mean ± standard deviation (SD), median (interquartile range) or number of subjects (%). Chi-square tests or Fisher's exact test and analysis of variance (ANOVA) or Kruskal Wallis (non parametric) test were used to compare categorical and continuous variables among groups, respectively. 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 also used to test the hypothesis of a linear association across severity groups (controls-SGA-FGR) when apropiated. Linear correlations were measured by Pearson or Spearman correlation coefficients. Following standard methodology, data were adjusted for smoking by linear regression analysis. Two-sided *p*-values <0.05 were considered statistically significant.

Results

Study population

Baseline characteristics, feto-placental Doppler and perinatal outcomes are shown in Table 1. Maternal baseline characteristics were similar among groups with the exception of higher prevalence of smokers among SGA and FGR mothers. Prevalence of gestational diabetes, preeclampsia or use of in vitro fertilization was also similar among groups. As expected, FGR cases showed a significantly worse feto-placental Doppler as compared to SGA and controls, with a linear tendency to worse results across severity stages. SGA and FGR newborns had significantly lower birth weights and percentile as compared to controls. As defined by the inclusion criteria, all cases and controls were delivered at term, but FGR cases showed a lower gestational age at delivery (37 weeks in FGR vs 39 weeks in the other groups) as expected due to our clinical management protocol recommending induction of labor at 37 weeks of gestation in FGR cases.

Placental morphometric and histopathological results

Table 2 details placental morphometric and histological findings in the study populations. As expected, placental weight, length, and breath were reduced in small fetuses, with a significant linear tendency across severity stages. Placental thickness and feto-placental ratio were similar among groups. The prevalence of histopathological lesions was similar among the study groups with a non-significant trend to increased maternal malperfusion and immune lesions in FGR as compared to controls and SGA.

Placental aging

Placental senescence and apoptotic results are shown in Table 3 and Figure 1. As compared with normally grown fetuses, both SGA and FGR cases presented signs of boosted placental senescence with reduced Sirtuin1 RNA expression, lower telomerase activity, shorter telomeres and increased p53, with a significant linear tendency across severity stages. FGR cases also presented signs of apoptosis with increased Caspase 3 and 9 RNA expressions as compared to controls. Similar values of p21, Bax, Sirtuin 3 and 6 were observed among the study groups. A significant positive correlation was observed between birthweight and telomerase activity (R 0.37, *P=0.007*), telomere length (R 0.28, *P=0.03*) and Sirtuin 1 (R 0.49, *P=0.002*). When adjusted by maternal smoking habits, placental aging results remained similar with the exception of telomerase activity which lost statistical significance in SGA (adjusted p value =0.1) while maintained a significant linear tendency across stages.

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, and suggesting true forms of fetal restriction.

Our results suggesting premature placental aging in small fetuses are consistent with previous studies showing reduced telomerase activity, shorter telomeres[23],[30],[32, 46] and increased expression of cell senescence markers (p21, p16 and EF-1±) in the placenta of small fetuses.[34] Telomeres are nucleoprotein structures located at the termini of chromosomes that become progressively shorter by each mitotic cycle or environmental factors. Once telomeres reach a critically short length, cell senescence and apoptosis is triggered. Telomere length is regulated by telomerase enzyme that adds DNA repeats onto the ends of chromosomes maintaining cell integrity and stability.[47,48] This process seems to be disrupted in SGA and FGR placentas with reduced telomerase activity and subsequent telomere shortening that could activate cell senescence and p53 (Figure 2). While most previous studies focus only on telomere homeostasis, we provide further evidence on SIRT-1 and p53 dysregulation in placentas from small fetuses. SIRT-1 is a stress-activated enzyme involved in various nuclear events such as DNA transcription, replication and repair, which acts as an anti-aging 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 SIRT-1 down-regulation and p53 over expression in small fetuses 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 caspases activity in FGR).[50] In summary, significant

changes in placental SIRT-1, telomeres, and p53 observed in small fetuses are consistent with an 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 fetuses delivering at term. As anticipated, FGR cases showed ultrasonographic signs of placental dysfunction and activation of the entire process of placental cell senescence and also apoptosis. Although FGR cases showed the most prominent changes, we could demonstrate significant differences in placental SIRT-1, p53, and telomerase activity also in SGA cases with apparently normal placental Doppler and histology. Indeed, p53 results look even more prominent in SGA that might be explained by p53 being involved not only in the aging process but also in others such as cell development/differentiation and tissue homeostasis.[52] We also observed a significant linear tendency across severity stages in senescence and some apoptotic markers as SIRT1 and CASP3 that correlate well with birth weight. According to these results, SGA fetuses presented certain degree of premature placental aging challenging the concept of "constitutionally small" and suggesting some degree of restriction among them. These findings are in line with the suboptimal cardiovascular and neurodevelopmental outcomes previously reported 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 controls.[14] We also demonstrated fetal brain reorganization and microstructural changes and suboptimal postnatal neurodevelopment in SGA cases.[55,56] Thus, these evidences support the hypothesis that SGA fetuses suffer some degree of placental insufficiency, which is not sufficient to show Doppler alterations or macroscopic placental lesions, but enough to produce certain degree of fetal restriction.[1]We hypothesize that different degree and timing of placental disease could lead to different clinical phenotypes of fetal smallness.

One of the strengths of the present study is the comprehensive assessment of placental aging together with feto-placental Doppler and histological placental examination in the same population. While both groups of small fetuses showed significant reductions in placental size and weight, 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 of our study might have limited the statistical significance of some placental markers. We decided to only include term pregnancies in order to avoid the effect of prematurity but limiting the extrapolation of results to early FGR. Despite FGR fetuses delivering slightly earlier than controls (37 vs 39 weeks), they exhibit even a higher degree of placental aging. Our study provides information about one type of cell death, apoptosis, without analyzing autophagy, which is other type of cell arrest involving fusion of acidic lysosomes with the autophagosomes. Future studies are warranted to further assess placental ageing and cell death in larger cohorts of small fetuses also including early-onset cases.

In conclusion, our study provides a comprehensive picture of the placental aging process in fetal smallness. Our study demonstrates accelerated placental aging in both clinical forms of fetal smallness at term, SGA and FGR, challenging the concept of SGA as 'constitutionally small' and supporting that this subset of small fetuses also presents 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.

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Figure Legends

Figure 1. Markers of placental senescence and apoptosis in the study groups.
Data are shown as the mean or median ..SGA = small for gestational age. FGR= fetal growth restricted. SIRT1= sirtuin 1. T/S: ratio of the amount of amplification product (Ct) for the

telomere sequence (T) to that of a single-copy of the gene 36B4 (S). 2-ΔΔCt = levels of gene expression normalized by the Ct of β-actin and 18S and in reference to control levels.

Figure 2. Molecular changes underlying the placental aging process. In normal pregnancy, sirtuin 1 (SIRT1) activates telomerase activity and inhibit p53 expression, and hence, contributes to cell stability by maintenance of telomere length and inhibition of p53. On the contrary, accelerated placental aging is characterized by reduced levels of SIRT1diminishing telomerase activity and leading to telomere shortening, DNA damage and P53 activation. Subsequently, p53 activation induces mitochondrial dysfunction and increases the expression of the pro-apoptotic proteases such as caspase 9 (CASP9) and caspase 3 (CASP3).
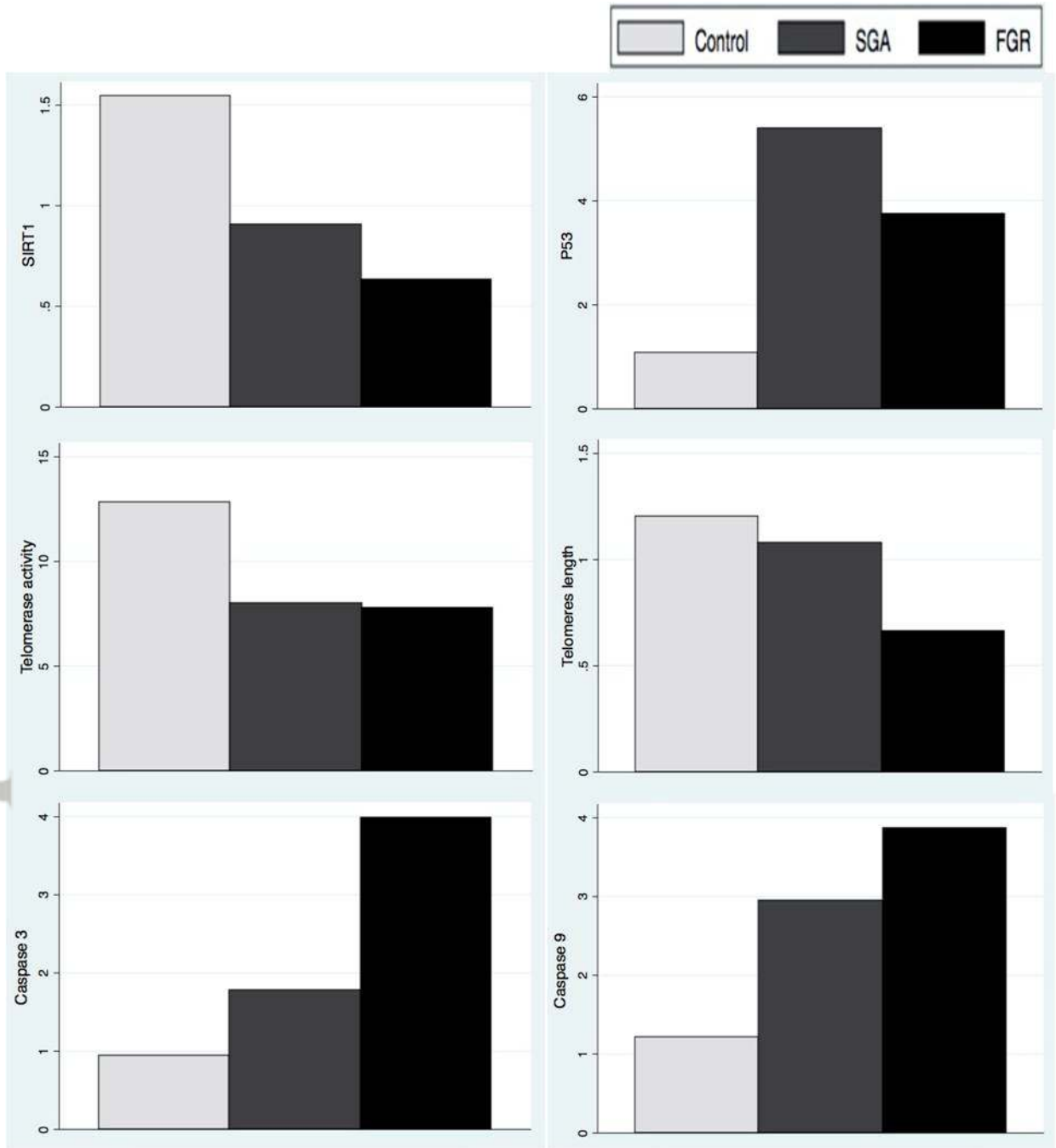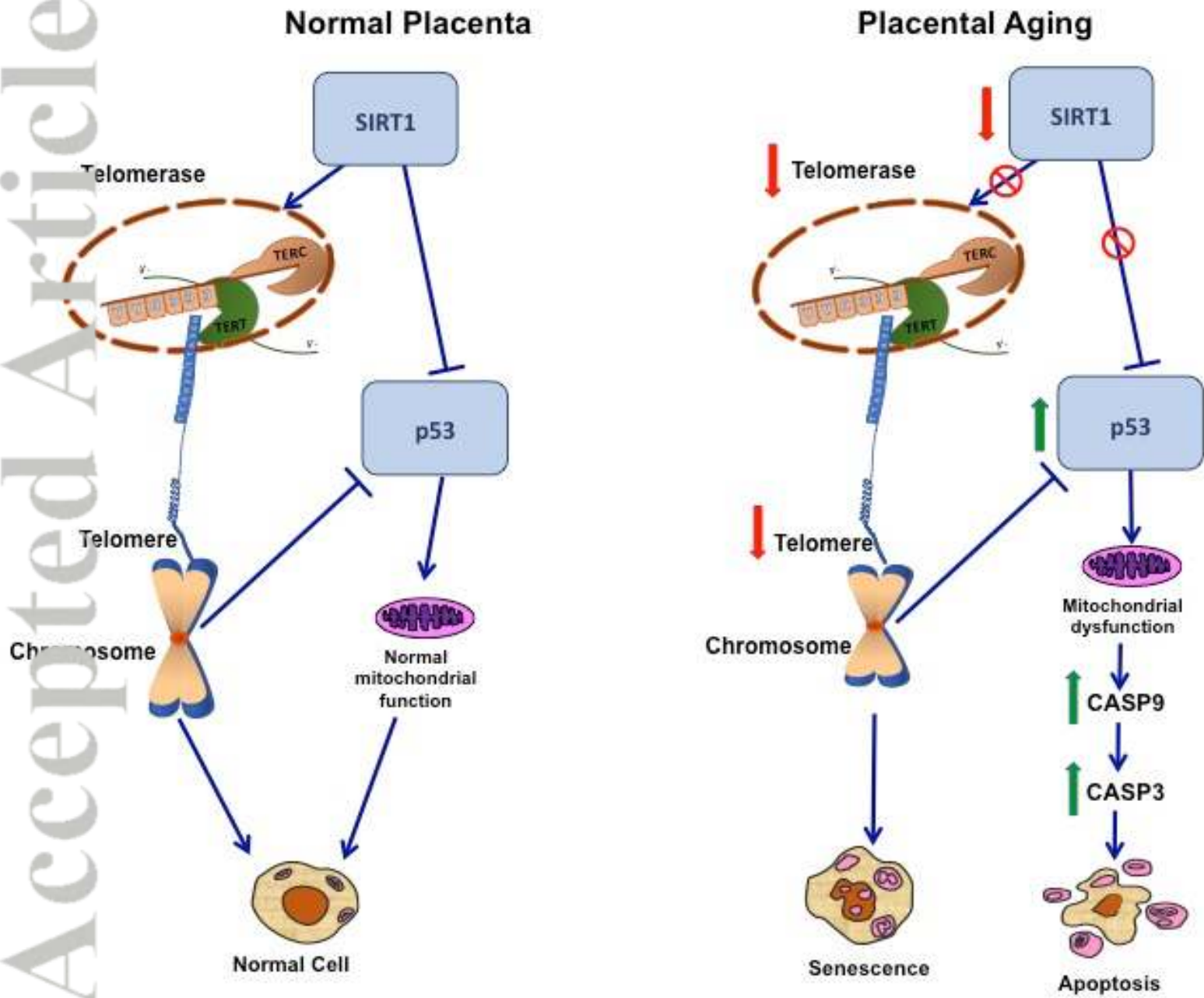
Figure 1.tiff

Figure 2.tiff

Table 1. Baseline and perinatal outcomes of the study populations.

	Control (N=21)	SGA (N=18)	FGR (N=18)
Maternal baseline characteristics			
Age (years)	31.8 ± 6.5	30.2 ± 6.0	31.9 ±4.7
Caucasian (%)	71.4	77.8	83.3
Smoking (%)	4.8	38.9*	33.3*
Nulliparity (%)	42.9	55.6	72.2
Feto-placental Doppler			
Gestational age at scan (weeks)	32.6 (29-36)	32 (29-35)	33.3 (31-35)
Uterine artery mean PI (z-scores)	-0.50 ± 1.2	-0.05 ± 1.2	0.67 ±1.5*
Umbilical artery PI (z-scores)	-0.16 (-0.6-0.3)	-0.04 (-0.5-0.3)	0.68(-0.2-1.0) *¥
Middle cerebral artery PI (z-scores)	0.14 ± 1.3	0.22 ± 0.9	-0.22 ± 1.0
Cerebroplacental ratio (z-scores)	- 0.20 (-0.9-0.7)	-0.20 (-0.9-0.3)	-1.28 (-1.6-(-0.1) *¥
Perinatal outcomes			
Gestational age at delivery (weeks)	39 (38-39)	39 (38-40)	37 (37-38)*¥
Male gender (%)	42.9	50.0	61.1
Birthweight (gr)	3258 ± 291	2728 ± 234*	2311 ± 247*¥
Birthweight percentile	42 (76-33)	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 minutes Apgar score <7 (%)	0	0	0

Data are shown as %, mean±SD or median (IQR).

*P<0.05 as compared to controls . ¥P<0.05 as compared to SGA.

SGA = small for gestational age. FGR = fetal growth restriction.

Table 2. Placental morphometric and histopathological findings in the study populations.

	Control (N=21)	SGA (N=18)	FGR (N=18)
Placental morphometry			
Placental weight (g)	486 ± 130	414 ± 82*	326 ± 67[*][¥]
Placental weight <10th centile (%)	9.5	27.8	72.2*[¥]
Placental weight <3rd centile (%)	4.8	11.1	44.4*[¥]
Placental length (cm)	18.5 ± 2.4	16.6 ± 1.8*	15.4 ± 1.7*[¥]
Placental breadth (cm)	16.5 ± 1.8	14.9 ± 1.9*	14.1 ± 1.4*
Placental thickness (cm)	1.83 ± 0.6	1.97 ± 0.7	1.91 ± 0.6
Feto-placental ratio	7.13 ± 1.9	6.79 ± 1.2	7.13 ± 1.3
Placental histopathologic lesions			
Maternal vascular lesions (%)	38.1	44.4	66.7
Fetal vascular lesions (%)	38.1	33.3	38.9
Infectious lesions (%)	14.3	16.7	0
Immune lesions (%)	9.50	11.1	22.2
Other lesions (%)	19.0	11.1	22.2

Data are shown as %, mean±SD or median (IQR).

*P<0.05 as compared to controls. ¥P<0.05 as compared to SGA.

SGA = small for gestational age. FGR = fetal growth restriction.

Table 3. Markers of placental aging in the study populations

	Control (N=21)	SGA (N=18)	FGR (N=18)	Linear tendency P-value
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
Telomerase activity (%)	12.8 ± 6.6	7.98 ± 4.2*	7.79 ± 4.6*	0.008
Telomeres length (T/S)	1.20 ± 0.6	1.08 ± 0.9	0.66 ± 0.5	0.017
P53 ($2^{-\Delta\Delta Ct}$)	1.07 (0.3-3.3)	5.39 (0.6-15)*	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)*	0.007
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 mean±SD or median (IQR). *P<0.05 as compared to controls. Linear tendency P-value calculated by a linear polynomial orthogonal contrast or Jonckheere–Terpstra test.

SGA = small for gestational age. FGR= fetal growth restricted. T/S = ratio of the amount of amplification product (Ct) for the telomere sequence (T) to that of a single-copy of the gene 36B4 (S). $2^{-\Delta\Delta Ct}$ = levels of gene expression normalized by the Ct of β-actin and 18S and in reference to control levels.