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From Cell Biology to Tissue Engineering

Preterm birth and/or low birth weight are associated with periodontal disease and the increased placental immunohisto-chemical expression of inflammatory markers

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Summary. The objective of this study was to determine whether gynecological and periodontal clinical parameters and the immunohistochemical expression in placental chorionic villi of the markers cyclooxygenase-2 (COX-2), interleukin (IL)-1β, vascular endothelial growth factor receptor 1 (VEGFR1), podoplanin, and Heat Shock Protein (HSP70) are associated with preterm birth (PB) and/or low birth weight (LBW) neonates.

Material and methods: An observational case-control study was performed in 130 puerperal women: mothers of PB/LBW neonates (cases, n=65) and mothers of full-term normal-weight neonates (controls, n=65). Data were gathered from all participants on socio-demographic, gynecological, and periodontal variables and on placental immunohistochemical COX-2, IL-1β, VEGFR1, podoplanin, and HSP70 expression.

Results: Among the 42 women with mild/moderate periodontitis or gingivitis, the studied periodontal variables were significantly worse and the placental COX-2 (p=0.043), HSP70 (p=0.001), IL-1β (p=0.001), VEGFR1 (p=0.032), and podoplanin (p=0.058) expressions were significantly higher in the cases than in the controls. In comparison to the mothers without periodontitis, only COX-2 (p=0.026) and VEGFR1 (p=0.005) expressions were significantly increased in those with the disease. Increased COX-2 values were detected in the women with a history of genitourinary infection (p=0.036), premature rupture of membrane (p=0.012), or drug treatment (p=0.050).

Conclusions: The etiology of preterm birth and/or low birth weight is multifactorial and involves consumption habits, social-health factors, and infectious episodes. These adverse pregnancy outcomes were associated with periodontitis and the increased placental expression of IL-1β, COX-2, VEGFR1, and HSP70.

Key words: Cyclooxygenase 2, Immunohistochemistry, Placenta, Chronic periodontitis, Gingivitis

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Introduction

Pregnancy and parturition involve a complex interaction between mother and fetus that is not fully understood. Adverse pregnancy outcomes, such as preterm birth (PB) and low birth weight (LBW), are the main cause of late perinatal and neonatal morbidity and mortality (McCormick, 1985). Almost two decades ago, it was proposed that PB and LBW are associated with maternal periodontal status and oral microbial burden (Offenbacher et al., 1996, 1998). The pathogenesis of PB is considered multi-factorial, involving multiple mechanisms such as infection, uteroplacental ischemia,

hemorrhage, stress, and other immunologically-mediated processes (Romero et al., 2006). The periodontitis-associated dental biofilm is thought to serve as an important source of oral bacteria and related virulence factors disseminated via the blood stream to the fetoplacental unit (Arce et al., 2009), where an elevation in inflammatory cytokines and mediators is produced directly by the bacteria and their pathogenic products and indirectly through an increase in these levels at gingival level in response to periodontal pathogens (Bobetsis et al., 2006).

A recent systematic review reported a consistent association between periodontitis and PB and/or LBW, but data must be taken with reservation (Chambrone et al., 2011). However, contradictory results were reported by six meta-analyses on the association between maternal periodontal disease and the risk of PB and/or LBW (Khader and Ta'ani, 2005; Vergnes and Sixou, 2007; Polyzos et al., 2009, 2010; Fogacci et al., 2001; Corbella et al., 2012). Some authors proposed that periodontitis may not be causally related to negative pregnancy outcomes and that both may result from the same hyperinflammatory and/or environmental influences on the mothers (Ferguson et al., 2007) (Klebanoff and Searle, 2006). Mixed results have been published by periodontal intervention studies designed to explore the possible causal relationship between maternal periodontal infection/inflammation and the risk of PB and/or LBW delivery. Discrepant results have also been published by randomized trials evaluating the effect of periodontal treatment on the risk of preterm birth (Boutin et al., 2013). A multicenter clinical trial that enrolled 823 patients found no significant treatment effect of periodontal disease on live PB/LBW rates or fetal growth but a borderline significant effect on fetal loss through spontaneous abortion and still birth (Michalowicz et al., 2006).

The mechanisms of human preterm labor appear inextricably linked to cytokine biosynthesis by gestational tissues (Mitchell et al., 2012). Several causal factors have been related to these pregnancy disorders. Expressions of cytokines and inflammatory mediators, growth factors and proteins related to vascularization, and oxidative stress have been studied in maternal blood (Sata et al., 2009; Sert et al., 2011) and oral cavity (Konopka et al., 2004). However, there have been no reports on placental levels of these molecules.

Inflammatory cytokines have been associated with preterm birth, low birth weight, and even intrauterine growth restriction (Konopka et al., 2004). Interleukin (IL)-1β, a pro-inflammatory cytokine, has been implicated in various chronic diseases, including periodontitis. IL-1β is able to activate chemotactic cytokines, adhesion molecules, and osteoclastic bone resorption (Kornman et al., 1997; Kornman and Duff, 2001; Moreira et al., 2005) and may influence the onset of premature labor (Genc and Ford, 2010). Inflammatory mediators such as cyclooxygenase-2 (COX-2) are also involved in the induction of parturition, and COX-2 expression is known to increase in fetal membranes at the onset of labor (Slater et al., 1999).

The vascular endothelial growth factor (VEGF) family comprises angiogenic factors essential for the development of the placenta. It regulates angiogenesis, the degradation of the extracellular matrix, and vascular endothelium by stimulating VEGFR-1 and VEGFR-2 endothelial cell surface receptors (Suthin et al., 2003).

Podoplanin is also related to angiogenesis. Immunohistochemical studies have demonstrated the presence of this sialoglycoprotein in placental stroma cells during pregnancy, and a significant reduction in its expression was recently reported in cases with adverse outcomes (Wang et al., 2011). However, the role of this protein and its function in the placenta remains unknown.

Spontaneous mature birth and PB begin and proceed in a similar manner. This is confirmed by the similar involvement in both processes of corticotropin-releasing hormone, urocortin, amniotic fluid extracellular stress protein HSP70, prostaglandins, proinflammatory cytokines or glucocorticosteroids (Pawelec et al., 2013). There is an increase in oxidative stress during labor. Heat shock proteins (HSPs), especially HSP90 and HSP70, are activated to help cells recover from this type of stress. HSP70, a cellular defense protein, is essential for the normal function of cells under stress and acts as an antiapoptotic protein to protect the fetus from this stress during development of the placenta (Padmini et al., 2012).

The aim of this study was to determine the association of PB and/or LWB with gynecological and periodontal clinical variables and with the expression in placental chorionic villi of the markers IL-1β, COX-2, VEGFR1, podoplanin, and HSP70.

Material and methods

An observational case/control study was conducted in pregnant women hospitalized in Our Institution, who were consecutively recruited between January 2013 and December 2013. Inclusion criteria for cases were age ≥ 18 years with PB (<37 weeks of gestation) and/or LBW (<2.5 Kg) newborn. The control group included women over 18 years old with full-term (≥35 weeks of gestation) and normal-weight (>2.5 Kg) newborn. Exclusion criteria for both cases and controls were: age <18 years, presence of diabetes or systemic disease not related to the pregnancy, a history of multiple pregnancies, and interruption of the pregnancy for medical reasons. After application of the study eligibility criteria, the final study sample included 130 puerperal women. The study was approved by the hospital ethics committee. Written informed consent was obtained from all study participants.

Data on the mothers were gathered from their clinical records, while information on the gestational age and newborn weight values was obtained from the Neonatology Department records. The periodontal health

status of each woman was established by dental examination.

Socio-demographic and gynecological variables

A single researcher (E.P.) interviewed the mothers and looked for clinical records to gather socio-demographic data on: age; ethnicity; marital status at the delivery (married, single, separated, widow), employment (yes/no); socioeconomic level, according to the modified classification of J. Goldthorpe cited by Regidor (2001), years of schooling; and consumption during the pregnancy of tobacco (cigarettes/day), alcohol (grams/day), and coffee (cups/day). Gynecological data were collected on the number of previous pregnancies, family history of PB/LBW, maternal weight before pregnancy, weight gain during pregnancy, genitourinary infections during pregnancy, and premature rupture of membrane.

Periodontal variables

Variables were measured at a single time-point during the 24 h after delivery (48-72 h after a cesarean delivery). The periodontal examination was performed in the hospital room, following World Health Organization recommendations for oral examinations and using a calibrated periodontal probe (University of North Carolina No. 15 Probe, Hu-Friedy, Chicago, IL). Periodontal status was assessed by determining: the gingival bleeding index proposed by Ainamo and Bay (1975); gingival recession for all teeth present, except for third molars; and the probing depth (PD) and clinical attachment (CA) loss at four sites per tooth (mesial, distal, vestibular, and palatine/lingual). Periodontitis was diagnosed when four or more teeth showed one or more sites with PD ≥4 mm and CA loss ≥3 mm, based on the definition by López et al. (2002) Patients who were not diagnosed with periodontitis but showed bleeding on probing were diagnosed with gingivitis.

Histopathological and immunohistochemical studies

Placentas were assessed by a single pathologist (F.O.), and samples were collected consistently from the same paracentral placental region. Samples of approximately 2x2 cm were obtained from all participants, immediately fixed in 10% buffered formalin for 48 h, and then embedded in paraffin. Tissue arrays were prepared from punch biopsies of the paraffin blocks using a disposable 4 mm punch (Kai Europe GmbH, Solingen, Germany), and 4-μm sections were dewaxed, hydrated, and stained using the hematoxylin-eosin technique.

Tissue arrays of paraffin-embedded sections were dewaxed, hydrated, and heat-treated in 1 mM EDTA pH 8 for antigenic unmasking in an antigen retrieval PT module (Thermo Fisher Scientific Inc., Waltham, MA) at 95°C for 20 min. Sections were incubated for 10 min at room temperature with prediluted monoclonal antibodies against COX-2 (clone SP21) and podoplanin (clone: D2-40), and polyclonal antibodies against HSP70, VEGFR1, and IL-1β. All antibodies were purchased from Master Diagnóstica, Granada, Spain. An appropriate isotype for each antibody was used as negative control.

The immunohistochemical study was conducted in an automatic immunostainer (Autostainer 480, LabVision Fremont, CA) using the micropolymer-peroxidase-based method (Ultravision Quanto, Master Diagnóstica, Granada, Spain), followed by development with diaminobenzidine. A quantitative study of the different immunostainings was done automatically using ten black and white images per case of five independent antibodies without hematoxylin staining. The algorithms were implemented using specific analysis software ImageJ v.1.46 (rsbweb.nih.gov/ij). Results were expressed in terms of area (mm^2) and grey levels (integrated optic density [IOD]).

Statistical analysis

A statistical software package (SPSS 20.0 IBM Inc., Chicago, IL) was used for the statistical analysis. The normality of the distribution of variables was examined with the one-dimensional Kolmogorov-Smirnov test. The bivariate tests used are reported in the table footnotes. Patients were stratified in four groups (PB and full-term pregnant with or without LBW) for application of the Kruskal-Wallis test. The general linear model was applied with the group as dependent variables and the immunohistochemical placental expression of markers as independent variables. A p-value of 0.05 was accepted as statistical significance threshold.

Results

This study included 130 puerperal women, 65 mothers with full-term normal-weight newborns (controls) and 65 with PB/LBW (cases). In the control group, the mean age was 29.4±4.4 yrs, mean gestation period 39.6±1.1 weeks, and mean newborn weight 3.34±0.38 Kg. In the cases group, the mean age was 29.9±7.1 yrs, mean gestation 35.2±2.1 weeks, and mean newborn weight 2.29±0.4 Kg; in this group, 10 children had normal weight (≥2.5 Kg) but <37 weeks of gestation, 10 children weighed <2.5 Kg but were full-term deliveries, while 45 children weighed <2.5 Kg and were preterm deliveries.

Table 1 compares socio-demographic and gynecological data between cases and controls. Significantly higher frequencies of a family history of premature labor, genitourinary infections, hypertension during pregnancy, and coffee consumption were found in cases than in controls. Table 2 compares periodontal data between groups, showing significantly worse values for all variables in the cases group; 88 women had no periodontal disease. Among the 42 women with mild/moderate periodontitis or gingivitis, the studied

periodontal variables were significantly worse in cases than in controls. Chronic periodontitis was diagnosed in 11(16.41%) of the cases and 5 (7.81%) of the controls (p<0.01 chi-square test with Yates correction).

Biopsies of placental cotyledons were obtained from all cases (n=65) and controls (n=65). In all samples, the morphological study evidenced: third-trimester chorionic villi with appropriate vascular development; no inflammatory phenomena or histopathological signs of chronic villitis, chorioamnionitis, or placental chorangiosis; a normal number of intravillous Hofbauer cells; and no villous trophoblast abnormalities. COX-2 immunoreactivity was detected in amniotic epithelial cells, chorion cells, and decidual cells (data not shown),

Table 1. Socio-demographic and gynecological data of the pregnant women (n=130).

Variable	Controls (n=65)	Cases (PB/LBW) (n=65)	P-value
Ethnicity (% Caucasian)	83.1	84.6	0.793‡
Marital status (% married)	69.2	52.3	<0.001‡
Employed (% yes)	53.8	55.4	0.337‡
Social level.(range, I-VII), median	VI	VII	0.219§
Schooling (yrs), mean±sd	11.5±4.8	10.2±4.1	0.106†
Nº previous pregnancies, mean±sd	1.36±1.10	1.45±1.69	0.725†
Family history of premature labor, %*	9.2	18.5	<0.001‡
Genitourinary infection, %	21.5	35.4	<0.001‡
Premature rupture of membrane, %	6.2	55.4	<0.001‡
Coffee (No-1 cup week -1 cup daily)	57-4-4	43-22-0	0.016§
Tobacco (Never/ex-smoker.- ≤10/day - >10/day)	49-3-4-9	32-15-5-13	0.793‡
Alcohol, % (None-≤ 5 g/day- >5 g/day	64-1-0	64-0-1	0.328§
Arterial hypertension during pregnancy,%	1.5	4.6	<0.001‡

*Data not gathered in 1 case and 2 controls; †Student's t test; ‡chi-square test with Yates correction; §Mann-Whitney U-test.

Table 2. Description and comparison of periodontal status of puerperal women (n=130).

Variable	Controls (n=65)	Cases (PB/LBW) (n=65)	p-values
Gingival bleeding index*(%)	18.2±24.7	24.6±30.6	0.188‡
Gingival recession* (mm)	0.04±0.09	0.10±0.23	0.097‡
Depth of sulcus* (mm)	1.59±0.34	1.76±0.62	0.050‡
Clinical attachment loss* (mm)	1.64±0.38	1.86±0.81	0.049‡
Patients with periodontitis (%)	7.81	16.41	<0.01†
Nº sites measured*	109±6	108±7	0.378‡

*Values are expressed as mean ± standard deviation; †chi-square test with Yates correction; ‡Student's t test.

Table 3. Comparison of placental immunohistochemical expression of different molecules between groups (n=126).‡

Variable	Control	Cases (PB/LBW)	p-values†
Podoplanin#	1.05±0.92	1.56±1.09	0.058
IL-1β (mm^2)	0.03±0.04	0.03±0.03	0.207
IL-1β*	4.49±7.30	6.48±6.10	0.001
COX-2 (mm^2)	0.13±0.02	0.14±0.02	0.043
COX-2*	39.48±25.75	47.38±31.42	0.124
HSP70 (mm^2)	0.03±0.03	0.03±0.03	0.219
HSP70*	4.53±7.61	6.69±6.91	0.001
VEGFR1 (mm^2)	0.05±0.01	0.05±0.01	0.706
VEGFR1*	5.36±2.55	6.18±3.77	0.383

Values are expressed as mean±standard deviation; #Semi-quantitative scale (0-3 points); *Optic density values (gray levels). †Mann-Whitney U-test. ‡Four samples were non-assessable.

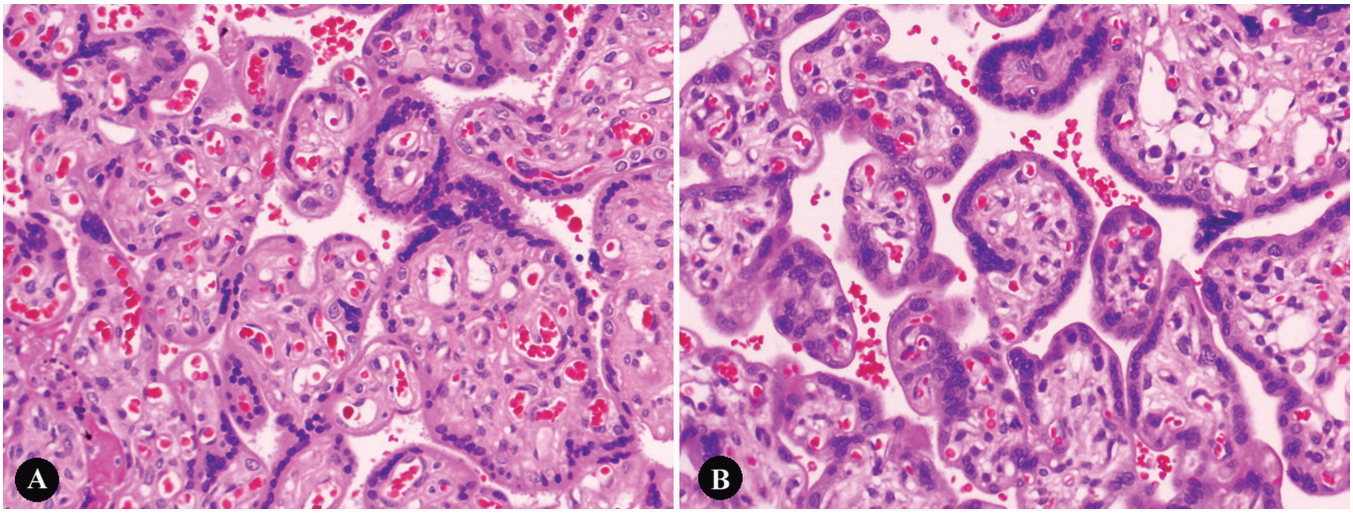

Fig. 1. Morphological features of third-trimester chorionic villi with appropriate vascular development, with no histopathological signs of chronic villitis or chorioamnionitis. **A.** Control group. **B.** Case group (Hematoxylin-eosin). x 20

and strong COX-2 expression was observed in the syncytiotrophoblast, which was increased in the PTB/LBW cases. IL-1β, VEGFR1, and HSP70 expression was observed in trophoblastic cells and was more evident on the apical surface of the syncytiotrophoblast layer than in stromal or muscle cells of the blood vessels or Hofbauer cells; podoplanin was only expressed in villous stroma. Table 3 compares the quantitative immunohistochemical expression of COX-2, IL-1β, VEGFR1, podoplanin, and HSP70 in chorionic villi between cases and controls. Significantly increased placental expressions of COX-2 (p=0.043), HSP70 (p=0.001), IL-1β (p=0.001), VEGFR1 (p=0.032), and podoplanin (p=0.058, Kruskal-Wallis test) were detected in the cases versus controls when patients were stratified in four groups. The linear regression was statistically significant (R=0.264, p=0.034) when the model included the areas of immunostaining for COX-2 (p=0.042), HSP70 (p=0.042), and IL-1β (p=0.041).

A higher placental expression of COX-2 (p=0.026) and VEGFR1 (p=0.005, Mann-Whitney U-test) was found in the women with periodontitis than in those without this disease. Increased COX-2 values were detected in the women with a history of genitourinary infection during pregnancy (p=0.036), premature membrane rupture (p=0.012), or drug treatment (p=0.050, Mann-Whitney U-test).

Discussion

In this study of 130 puerperal women, PB and LBW were associated with fewer schooling years, a heavier smoking habit, and a higher frequency of genitourinary infections and hypertension during pregnancy. Various authors have reported the influence of these factors on pregnancy outcomes. Kitsantas and Christopher (2013) found a relationship between tobacco and PB/LBW in their study of more than a million mothers. In addition, infections have emerged as confounding variables in the few published analyses of their role in the pathogenesis of adverse pregnancy outcomes (Nigro et al., 2011). A finding of particular interest in the present investigation was the association found between PB/LBW and the elevation of placental inflammatory markers.

The incidence of periodontitis was low in this series of puerperal women, but it was higher in the mothers with PB or LBW than in the controls. All periodontal disease indicators examined were higher in the cases, who evidenced more sites with bleeding on probing, greater gingival recession, and worse PD and CAL values. As noted by Manau et al. (2008) the significance of the association between periodontal disease and pregnancy outcomes can be influenced by the definition of periodontal disease employed. We adopted the most demanding definition of periodontitis, proposed by Lopez et al. (2002) which requires the presence of four or more teeth showing one or more sites with PD of ≥4 mm and CAL of ≥3 mm (at the same site). The application of less strict criteria, as well as differences in ethnic background and age among the study populations, may explain the approximately three-fold higher prevalence of periodontitis reported by Hasegawa-Nakamura et al. (2001) (39.13%) and Tateishi et al. (2012) (37.5%) in comparison to our finding of 12.21%.

Placental morphology findings were similar between the groups and compatible with disease-free third-trimester chorionic villi. However, the cases and controls differed in the immunohistochemical expression of pro-inflammatory, vasculogenic, and oxidative stress markers in the placenta.

The increased expression of IL-1β and COX-2 in placental trophoblasts from the cases suggests that a subclinical protoinflammatory state at the maternal-fetal interface may have contributed, among other factors, to

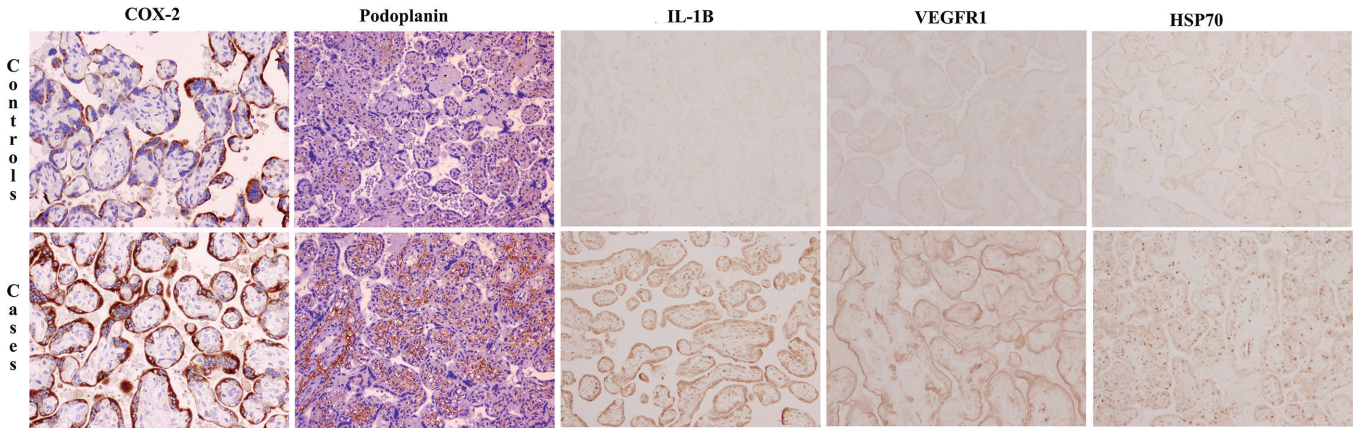

Fig. 2. Representative immunohistochemical expression of different markers in placentas. The cases showed increased expression versus the controls. Note that COX-2 and podoplanin are counterstained with hematoxylin, while the microphotographs for IL-1β, VEGFR1, and HSP70 are without counterstaining because they were taken for image analysis study. (Micropolymer peroxidase-based method). x 10

triggering preterm delivery. This supports the proposition that the regulation of COX-2 in placental membranes may mediate an increase in prostaglandin synthesis, which promotes parturition. It therefore appears reasonable to conclude that the elevated COX-2 and IL-1β levels produced by a pro-inflammatory state in the mother may have a negative effect on the pregnancy outcome. Similar findings were reported in a previous study by our group (Mesa et al., 2013), although the higher expression of COX-2 was not significant, possibly because it was assessed in a semi-quantitative manner, unlike in the present quantitative study, in which statistical significance was reached.

Recent studies have linked the VEGF family and its receptors to adverse pregnancy outcomes. Some authors have implicated decreased placental levels of this family of angiogenic growth factors in the pathogenesis of preterm birth (Andraweera et al., 2012), and an increase in these levels has been reported to indicate a response to hypoxia or ischemia (Kumazaki et al., 2002). Serum levels of VEGF and VEGFR-1 and 2 may also be associated with periodontal disease through an increase in vascular permeability and angiogenesis (Sert et al., 2011; Horton and Boggess, 2012).

The placental expression of podoplanin has been related to fetal angiogenesis during placenta development, and a reduced podoplanin level was recently detected in preeclamptic placentas (Pawelec et al., 2013). In the present study, the expression of podoplanin in placental stromal cells was higher in the PB/LBW cases than in the controls, and this higher expression might have contributed to the adverse pregnancy outcomes. However, the role of this protein in the placenta has yet to be elucidated.

Extracellular HSP70 is known to be involved in activation of the innate and adaptive immune response, and increased serum HSP70 levels appear to reflect systemic inflammation and oxidative stress (Molvarec et al., 2009). High placental HSP levels were reported to indicate hypoxic or ischemic changes and the struggle of cells to survive (Padmini et al., 2012). This enhanced expression probably represents a protective response to cellular stress. Intra-amniotic infection, histologic chorioamnionitis, and delivery have been associated with elevated amniotic fluid HSP70 concentrations (Chaiworapongsa et al., 2008). HSP70 plays a role in the host defense mechanism by activating the innate arm of the immune response in women with intrauterine infection, and extracellular HSP70 may be involved in the mechanisms of preterm and term parturition (Chaiworapongsa et al., 2008). In our study, increased HSP70 expression in trophoblastic cells was related to preterm parturition.

In conclusion, the etiology of preterm birth and/or low birth weight is multifactorial and involves consumption habits, social-health factors, and infectious episodes. These adverse pregnancy outcomes are associated with periodontitis and the increased placental expression of IL-1β, COX-2, VEGFR1, and HSP70.

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