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Syncytin-2: a potential early marker of preeclampsia

LOKOSSOU A.G.G.[1 2*], DAVITO L.[1], BADAROU A.[1], ACCROMBESSI M.[3], AZONNAKPO J.[2], KOUNOUDJI G[2], LOZES E.J.[2], HOUNKPATIN B.[1], PERRIN R.X.[1], BARBEAU B.[4*]

[1] Centre Hospitalier et Universitaire de la Mère et de l'Enfant de la Lagune (CHU-MEL), Cotonou, Bénin
[2] École Polytechnique d'Abomey Calavi (EPAC), Abomey-Calavi, Bénin
[3] Centre d'Étude et de Recherche sur le Paludisme Associé à la Grossesse et l'Enfance (CERPAGE), Cotonou, Bénin
[4] Université du Québec à Montréal (UQAM), Département des sciences biologiques and Centre de recherche BioMed, Montréal, Canada.

[*] Correspondance : gatien.lokossou@epac.uac.bj et barbeau.benoit@uqam.ca

Abstract

Syncytin-2 is an envelope protein that plays a key role in placental formation through the fusion of villous cytotrophoblasts (CTV), which leads to the formation of the syncytiotrophoblast (ST). The syncytiotrophoblast plays a key role in the function of the placenta. Defects in syncytiotrophoblast formation are associated with marked reduction in the expression of syncytin-2 and with placental pathologies such as preeclampsia (PE). Pre-eclampsia is responsible for a large number of maternal and fetal mortality and morbidity. However, there is no biomarker allowing an early diagnosis of PE. The aim of this study was to compare the abundance of syncytin-2 in serum derived-exosomes of pregnant women with pre-eclampsia *vs* normal pregnant women. Thus, 44 pregnant women in a pre-eclampsia cohort at the Centre Hospitalier Universitaire-Mère et Enfant de la Lagune (CHU-MEL) in Cotonou were selected. Women were recruited at the beginning of their pregnancy for the first 20 weeks of gestation and included 22 normal pregnant women and 22 pregnant women with PE. Serum derived-exosomes were purified and their content was analyzed. Lower abundance in syncytin-2 was measured in serum derived-exosomes of pregnant women with PE compared to those of normal pregnant women. This difference was observed between 9.6 and 13 weeks of gestation ($p<0.05$). This study confirms that syncytin-2 is a promising marker for early diagnosis of pre-eclampsia.

Keywords: Preeclampsia, early diagnosis, exosomes, syncytine-2

Résumé

La syncytine-2 est une protéine d'enveloppe qui joue un rôle clé dans la formation du placenta grâce à la fusion des cytotrophoblastes villeux (CTV), qui conduit à la formation du syncytiotrophoblaste (ST). Le syncytiotrophoblaste joue un rôle clé dans la fonction du placenta. Les défauts de formation des syncytiotrophoblastes sont associés à une réduction marquée de l'expression de la syncytine-2 et à des pathologies placentaires telles que la pré-éclampsie (PE). La pré-éclampsie est responsable d'un grand nombre de mortalité et de morbidité maternelles et fœtales. Cependant, il n'existe pas de biomarqueur permettant un diagnostic précoce de la PE. Le but de l'étude était de comparer l'abondance de la syncytine-2 dans les exosomes dérivés du sérum des femmes enceintes avec la pré-éclampsie par rapport aux femmes enceintes normales. Pour ce faire, 44 femmes enceintes ont été sélectionnées dans une cohorte de pré-éclampsie au Centre Hospitalier Universitaire-Mère et Enfant de la Lagune (CHU-MEL) à Cotonou. Les femmes ont été recrutées au début de leur grossesse pendant les 20 premières semaines de gestation et comprenaient 22 femmes enceintes normales et 22 femmes enceintes qui ont fait la PE. Au cours de la grossesse, les exosomes dérivés du sérum ont été purifiés et leur contenu a été analysé. La plus faible abondance de syncytine-2 a été mesurée dans les exosomes dérivés du sérum des femmes enceintes atteintes de PE comparativement à celles des femmes enceintes normales. Cette différence a été observée entre 9,6 et 13 semaines de gestation ($p<0,05$). Cette étude confirme que la syncytine-2 est un marqueur prometteur pour le diagnostic précoce de la pré-éclampsie.

Mots clés : Pré-éclampsie, diagnostic précoce, exosomes, syncytine-2

1. Introduction

Preeclampsia (PE) is a pathology linked to both vascular and immunological dysfunction, leading to placental disorder and chronic immune activation (Luppi and DeLoia, 2006; Luppi *et al.* 2006; Prins *et al.* 2009; Hsu and Nanan, 2014). This disorder is associated with changes in the levels of cytokines, chemokines, blood coagulation factors, and apoptotic markers (McKelvey *et al.,* 2018). In certain cases, PE leads to multi-organ failure in the mother and is one of the major causes of maternal and perinatal morbidity and mortality. PE affects 2 to 15% of pregnancies and occurs more frequently in low and middle-income countries (LMIC) (Steegers *et al.,* 2010; Maher *et al.,* 2017; McLaren *et al.,* 2017). It has been established that each year around 10 million pregnant women develop PE worldwide with an estimated rate of mortality of 100,000 pregnant women and 500,000 newborns related to PE. Ninety nine percent of these deaths

occur in LMIC (Duley, 2009; von Dadelszen and Magee, 2014). PE is preceded by placental dysfunction in early pregnancy resulting later in maternal clinical signs after 20 weeks of pregnancy (WP). Symptoms associated to PE result from systemic inflammation, oxidative stress, and endothelial dysfunction (Chaiworapongsa *et al.,* 2014) and can even be detected as late as 4-12 weeks postpartum (Stone and Franzblau 1995). In the absence of adequate care, PE can lead to a fatal outcome for the mother in addition to increased risk of sequalae associated with uterine growth retardation to the infant and iatrogenic premature childbirth (Sibai *et al.,* 2005; McLaren *et al.,* 2017). Management of PE is limited to treatment of symptoms and in severe cases, require early childbirth to prevent further deterioration of the mother's and fetus' health. In sub-Saharan Africa, severe forms of PE and eclampsia are diagnosed in more than 15% of pregnancy (von Dadelszen and Magee 2014). Effective management of PE requires the identification of early markers, detectable before 16 weeks of pregnancy as well as modulation of specific immune cells to restore the normal function of the immune system.

Syncytin-2 (S2) is a protein encoded by a human endogenous retrovirus (HERV) gene (Dupressoir and Heidmann, 2011). It is believed to play a determinant role in placenta formation (Toufaily *et al.,* 2015; Lokossou *et al.,* 2016). In addition to its role in maternal-fetal exchange, the placenta produces microvesicles called exosomes that seem to be endowed with an immunosuppressive action (Kshirsagar *et al.,* 2012; Redman *et al.,* 2012; Lokossou *et al.,* 2014; Vargas *et al.,* 2014; Mitchell *et al.,* 2015). Interestingly, we have recently demonstrated that syncytin-2 is present on the surface of the exosomes (Vargas *et al.,* 2014). These published results further show lower abundance of syncytin-2 in serum-derived exosomes of PE women (Vargas *et al.,* 2014). We thereby hypothesized that exosome-associated syncytin-2 could be used as an early marker of PE. Our overall goal is to provide an early preeclampsia diagnostic kit that will result in a closer medical follow-up of women susceptiebl to PE development.

2. Materials and methods

2.1. Patients and serum samples

One hundred women who were confirmed to be pregnant at the HOMEL facilities, accepted to be part of the current study after having signed the informed consent form. A sample of 7 ml of peripheral blood were monthly harvested in dry tubes from gestational week 1 to 20 at the HOMEL (Cotonou, Bénin). Women were then monitored during pregnancy for PE symptoms. based on recommendation from the International Society for the Study of Hypertension in Pregnancy, the Canadian Hypertension Society, and the Society of Obstetricians and Gynaecologists of Canada and Benin. Patients with PE (n=22) without adverse outcomes or sustained hypertension or diastolic blood pressure exceeding 90 mmHg and with proteinuria ≥ 0.3 g/d (in 24-hour harvest) for more than 4 hours were defined as moderate PE (MPE). A control group consisting of 22 normal pregnant women was included in this study (**table I**). Sera was first isolated from blood samples and stored at -20°C. Exosomes were next purified, using the ExoQuick™ kit (EXOQ20A-1, System Bioscience, Mountain View, CA, USA) (Vargas *et al.,* 2014). Briefly, sera were thawed on ice and centrifuged at 3000g for 15 minutes to remove cells and cell debris. The supernatant was then transferred to a sterile eppendorf tube. Serum-derived exosomes were isolated by adding 250 µl of serum to 63 µl of exosome precipitation solution. Tubes were inverted three times and the mixture was then incubated for 30 minutes at 4°C. After centrifugation at 1500 g for 30 minutes at 4°C, the supernatant and excess of Exoquick were gently removed. After 2 consecutive washes in 0.1 M sodium phosphate, the pellet was resuspended by vortexing of the samples in 100 µl binding buffer solution. Exosome preparations were next incubated at 37°C for 20 minutes, and after centrifugation at 1500 g for 5 minutes, supernatants (containing exosomal proteins) were transferred to a tube previously cooled and kept in ice. Protein content was then quantified using Pierce™ BCA Protein Assay Kit (2322; Waltham, Ma, USA), bicinchoninic acid (BCA) test**.**

2.2. Direct ELISA

The CD63 ExoELISA kit (EXOEL-CD63A-1; System Biosciences) was used to normalize the amount of exosomes from sera, according to manufacturer's recommendations and further optimized for syncytin-2 quantification. Briefly, 96 well plates were incubated overnight at 37°C with 50 µg of exosomal proteins. Plates were washed three times with PBS and incubated with anti-CD63 (1:100; System Biosciences) or our purified anti-syncytin-2 antibody (4 µg/ml in blocking solution; (Vargas *et al.,* 2009)). Bound anti-CD63 or anti-syncytin-2 antibodies were then detected with an horseradish peroxidase (HRP)-

conjugated anti-rabbit antibody (1:5000; System Biosciences) added for 1 h at room temperature. After three washes, HRP activity was assessed by the addition of the TMB (TetraMethylBenzidine) substrate. After addition of the stopping solution (H_2SO_4 1 M), absorbance (450 nm) of each sample was measured on a microplate reader (F 200 pro Tecan). Syncytin-2/CD63 ratios were finally determined from the measured absorbances.

2.3. Statistical analyses

Data were analysed by a paired Student's t test. Statistical analyses were performed with Stata (College Station, TX, USA). Significance was determined at $p < 0.05$. The median of the ratios of syncytin-2/CD63 were calculated and error bars were calculated using the 25th and 75th percentile values.

Table I. Clinical characteristics of healthy pregnant women and patients with preeclampsia

Group	Observed	Mean of Syn-2/CD63	SD	[95% Conf. Interval]	P value
Control	57	23.20	22.84	17.14-29.27	0.03
Preeclampsia	55	14.58	19.68	9.26-19.90	

Age and gestational age data are expressed as means ±SD.

Table II: Syncytin-2 incorporation in serum-derived exosomes of healthy pregnant women *vs* patients with preeclampsia

Group	Observed	Mean of Syn-2/CD63	SD	[95% Conf. Interval]	P value
Control	57	23.20	22.84	[17.14-29.27]	0.03
Preeclampsia	55	14.58	19.68	[9.26 -19.90]	

3. Results

Serum-derived exosomes from patients with PE have reduced syncytin-2 levels. The mean age in the PE group was 27,70 ± 6,46 years in comparison to 30,76 ± 4,28 years in the normal pregnant women group. Serum was harvested at different times during pregnancy with a mean of 11,81 ± 3,74 gestational weeks (WG) in normal pregnant group vs. 71 ± 6,46 WG for the PE group (table I). After comparing the mean of the syncytin-2/CD63 ratio in all serum-derived exosomes before gestational week 20, we observed higher levels of syncytin-2 in samples from normal pregnant women compared to those from PE women ($p<0.05$) (table II).

Pregnant women were further analysed according to their gestational age. As depicted in Figure 1, increased abundance of syncytin-2 was observed in exosomes from normal pregnant women vs. PE women between 0 and 13 WG. But this difference was not significantly between 0 and 9,5 WG (Table 3), while the difference was significantly accentuated in samples between 9.6 and 13 WG ($p<0.05$) (table III). After 13 WG, a threshold of syncytin-2 incorporation in serum-derived exosomes was reached in both pregnant group, followed by a decrease until 16 WG. After this decrease in syncytin-2 levels, levels increased again between 16 and 20 WG only in normal pregnant women serum-derived exosomes (figure 1 & table III). No statistically significant differences were noted for measured syncytin-2 levels at other gestational age (table III).

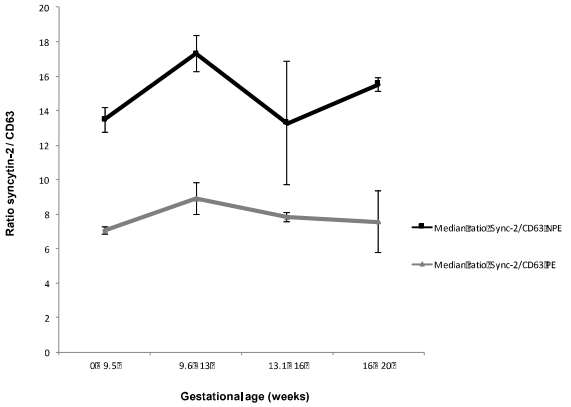

Figure 1: **Syncytin-2 incorporation into serum-derived exosomes of normal pregnant women and PE women according to gestational age.**

4. Discussion

Several studies have shown that preeclampsia is the result of a defect in placentation (Chaiworapongsa *et al.* 2014). This defect is followed by an exaggerated systemic inflammatory response (Matthiesen *et al.,* 2005;

Mangeney *et al.,* 2007; Rusterholz *et al.,* 2007; Messerli *et al.,* 2010; Schlecht-Louf *et al.,* 2010; Steegers *et al.,* 2010; Al-ofi *et al.,* 2012; Lee *et al.,* 2012; Redman *et al.,* 2012; Raghupathy, 2013; Liu *et al.,* 2015, 2016; Molvarec *et al.,* 2015; Siwetz *et al.,* 2015; Al-Ofi and Anumba, 2017). Strong evidence support the essential role played by the human endogenous retrovirus protein syncytin-2 in the formation of the placenta and the maintenance of the immunosuppressive environment needed for proper fetal development (Lokossou *et al.,* 2014; Toufaily *et al.,* 2015) (Mangeney *et al.,* 2007; Schlecht-Louf *et al.,* 2010).

Table II: Syncytin-2 incorporation in serum-derived exosomes of healthy pregnant women *vs* patients with preeclampsia

Group	Observed	Mean of Syn-2/CD63	SD	[95% Conf. Interval]	P value
Control	57	23.20	22.84	[17.14-29.27]	0.03
Preeclampsia	55	14.58	19.68	[9.26 -19.90]	

Table III: Syncytin-2 incorporation in serum-derived exosomes between gestational weeks (WG) interval

Serum-derived exosomes	Group	Observed	Mean of Syn-2/CD63	SD	[95% Conf. Interval]	P value
Between 0 and 9,5 WG	Control	17	19.00	17.36	[10.08-27.93]	0.11
	Preeclampsia	13	9.97	10.48	[3.64-16.30]	
Between 9,6 and 13 WG	Control	20	27.38	25.10	[15.64-39.13]	0.04
	Preeclampsia	14	12.38	9.94	[6.64 -18.13]	
Between 13,1 and 16 WG	Control	7	14.15	10.13	[4.78-23.53]	0.94
	Preeclampsia	14	14.72	19.37	[3.54-25.91]	
Between 16 and 20 WG	Control	13	27.14	29.45	[9.34-44.93]	0.59
	Preeclampsia	14	20.93	31.54	[3.00-38.85]	

In the current study, using a cohort consisting of 44 pregnant women, we demonstrate that the incorporation of syncytin-2 into the serum-derived exosomes of normal pregnant women is more abundant compared to those of PE patients between 9.6 and 13 weeks of gestation (Vargas *et al.,* 2014). As clinical signs of PE only appear at 20 weeks of gestation, diagnosis of PE before this period of gestation should be beneficial for medical follow-up of suspected pregnant women (Steinborn *et al.,* 2007; Vargas *et al.,* 2014). Moreover, between 9.6 and 16 weeks after reaching a threshold, the level of syncytin-2 incorporation were found to decrease in exosomes of normal pregnant women and PE patients. From 16 weeks of pregnancy, this decrease in syncytin-2 levels continued slightly in PE exosomes whereas in normal pregnant women, we observed an increase. Such data correlate with reduced syncytin-2 levels in placentas or women suffering from PE and could be a direct cause of defective placenta formation of significant fusion activity of cytotrophoblasts in normal pregnant women (Vargas *et al.,* 2011). Our results are further in line with our previous study showing a lower incorporation of syncytin-2 on the surface of serum-derived exosomes of women with PE during the second and third trimester (Vargas *et al.,* 2014), and support that this exosome-associated protein could be a promising early PE diagnostic marker.

5. Conclusion

In this study, we show that, syncytin-2 incorporation into serum-derived exosomes from PE women is reduced when compared to normal pregnant women before 13 WG, thus confirming that this protein is a promising marker for early PE diagnosis. Further multicenter studies will be needed to validate the use of syncytin-2 as such a marker.

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Study approval

This study was approved by the Ethic Committee from the Institute of Applied Biomedical Science of Cotonou, Benin. Peripheral blood samples were obtained in accordance with the established guidelines of the ethic committees of the Institute of Applied Biomedical Science (Cotonou, Benin). All participating pregnant women had signed an informed consent form agreeing to blood donation.

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