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Research article

Thrombospondin-2 in HIV-Associated Preeclampsia

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ABSTRACT

To determine the serum concentration of TSP-2 in HIV-associated preeclampsia, archived serum samples from normotensive (n=36) and preeclamptic (n=36) pregnant groups, which were further subdivided by HIV status, were used to measure TSP-2 levels, using MilliPlex immunoassays. A statistical difference was noted for gestational age, systolic blood pressure, diastolic blood pressure and baby weight ($p < 0.0001$). Based on pregnancy type (normotensive vs preeclamptic), no significant difference in TSP-2 levels was observed regardless of HIV status (26.61 ng/ml; 95% CI: 17.52-34.67 vs 25.35ng/ml; 95% CI: 18.32-37.49). Based on HIV status, a significant increase of TSP-2 levels ($p = 0.04$) was observed in HIV-positive (29.66 ng/ml; 95% CI: 21.99-38.01) vs HIV-negative (24.34 ng/ml; 95% CI: 16.24-31.48) women. Based on pregnancy type and HIV status, TSP-2 levels were statistically significant between the P+ve and N-ve; and P+ve and P-ve groups ($p = 0.01$) respectively. The significant TSP-2 elevation noted in preeclamptic versus normotensive pregnancies, may account for the defective trophoblast cell invasion in preeclampsia. Based on HIV status, TSP-2 levels was significantly upregulated, which may be attributed to the action of HIV tat protein. TSP-2 may be a potential biomarker for the early detection of preeclampsia development.

Keywords: *endothelial damage; HIV; preeclampsia; TSP-2, pregnancy*

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INTRODUCTION

South Africa has a HIV prevalence rate of 13.7%, of which a quarter comprises women in their reproductive age (Statistics South Africa, 2021). The competing COVID-19 pandemic has increased the maternal mortality among South African pregnant women, with hypertensive women being more susceptible to death (Basu *et al*., 2021). Preeclampsia (PE) accounts for majority of deaths emanating from hypertension in pregnancy (Department of health, 2017). It usually develops after 20 weeks of pregnancy and is diagnosed by the onset of high blood pressure (Brown, 2018). Preeclampsia is associated with intrauterine growth restriction, immune activation, and multi-organ endothelial dysfunction (Phipps *et al*., 2019). Likewise, HIV infection also confers endothelial injury emanating from an imbalance in oxidative stress, thereby exacerbating endothelial injury. This systemic endothelial activation and dysfunction in PE results in endothelial secretion of thrombospondin-2 (TSP-2) (Stenczer *et al*., 2011, Jiang *et al*., 2019).

The thrombospondin (TSP) family are a group of calcium binding glycoproteins that contribute to cell-to-cell and cell-to-matrix interaction, thereby controlling extracellular matrix (ECM) assembly and cell phenotype (Adams *et al*., 1995, Adams and Lawler, 2004). These glycoproteins include TSP-1, -2, -3, -4 and -5 isoforms (Lawler, 2000), each with distinct roles emanating from the regulation of its genetic transcription (Adams *et al*., 1995). Thrombospondins 1 and 2 have similar functions arising from their ability to interact with cytokines, cell receptors, growth factors and proteases hence they regulate apoptosis, angiogenesis, cell proliferation, cell invasion, platelet aggregation, inflammation, connective tissue assembly and wound healing (Adams and Lawler, 2004, Bornstein *et al*., 2000). Despite TSP-2 being an angiogenic inhibitor, it is highly expressed in developing blood vessels (Volpert *et al*., 1995). This raised expression maybe related to its TGF-β independent activity localised on its properdin type 1 domain (Volpert *et al*., 1995). It is well established that an angiogenic imbalance presides in PE in favour of an elevation of anti-angiogenic factors (Nikuei *et al*., 2015). Similarly, angiogenesis is also dysregulated in HIV infection (Paydas *et al*., 2009). In view of this dysregulation, this study aimed to compare the circulating levels of TSP-2 in HIV-associated preeclampsia versus normotensive pregnancies.

MATERIALS AND METHODS

Ethical approval: Institutional ethical approval together with gatekeeper permission (BCA338/17) was obtained from the Biomedical Research Ethics Committee, University of KwaZulu-Natal. The procedures followed in this study were

in accordance with the Declaration of Helsinki (October 2008 revision). Informed consent for use of specimens was obtained from all participants.

Study population: This study was a retrospective cross-sectional study. Archived serum samples collected from women who attended a large regional hospital in Durban, South Africa was used to determine the serum level of TSP-2. As determined by institutional biostatistician, normotensive pregnant (n=36) and preeclamptic (n=36) participants were further stratified by HIV status into normotensive HIV-negative (N-ve: n=18), normotensive HIV-positive (N+ve: n=22), preeclamptic HIV-negative (P-ve: n=18) and preeclamptic HIV-positive (P+ve: n=18) pregnant women. Preeclampsia was defined as new onset high blood pressure (systolic blood pressure ≥140mmHg or diastolic blood pressure ≥90mmHg) together with one or more of the following conditions: proteinuria (urinary protein ≥300mg per 24 hours), maternal organ dysfunction or uteroplacental dysfunction, developing at or after 20 weeks of gestation (Brown, 2018). The exclusion criteria for the P+ve group was polycystic ovarian syndrome, eclampsia, chronic hypertension, intrauterine death, abruption placentae, pre-gestational or gestational diabetes, chronic diabetes, systemic lupus erythematous, chronic renal disease, sickle cell disease, thyroid disease, antiphospholipid antibody syndrome, cardiac disease, pre-existing seizure disorders, active asthma that required medication during the gestation period and unknown HIV status as well as patients that were not booked into the hospital.

Determination of circulating TSP-2 levels: The concentration of TSP-2 was quantified using the MilliPlex Human Angiogenesis Magnetic Bead Panel 2 kit according to the manufacturer's instructions (MILLIPLEX® MAP Human Angiogenesis Panel 2, catalogue no: HANG2MAG-12K). Assay buffer (200µl) was added to a 96-well plate. Thereafter, 25µl of standards, controls, assay buffer, serum matrix solution, serum samples and antibody-immobilized beads were added to the appropriate wells. The plate was then incubated with agitation at 2-8℃ overnight. After incubation, the plate was washed 3 times with 200µl wash buffer; detection antibodies were dispensed into each well, followed by incubation with agitation at room temperature for 1 hour. The reporter conjugate Streptavidin-Phycoerythrin was added to each well and incubated with agitation at room temperature for 30 minutes. Lastly, the plate was washed with wash buffer 3 times and sheath fluid was added to each well. The plate was read using the Bio-Plex® MAGPIX™ Multiplex Reader (Bio-Rad Laboratories Inc., USA). Data was analysed using the Bio-Plex Manager™ analysis software version 4.1.

Statistical analysis: All data was analysed using STATA (version 12, STATACORP) and Graph Pad Prism version 8 (California, USA), and are presented as median and interquartile range (IQR). Data was tested for normality using the D' Agostino test and histograms. A Mann-Whitney U test was used to determine significance based on pregnancy type (normotensive vs preeclamptic) and HIV status (negative vs positive). The Kruskal Wallis test and the Dunn's post hoc test were used to determine if there was a significant difference between groups. The Spearman's correlation coefficient was used to assess the relationship between clinical and demographic factors; and between TSP-2 and clinical factors between groups. Missing data were excluded. A p value ≤ 0.05 was considered statistically significant at a 95% confidence level.

RESULTS

Clinical characteristics: The clinical profile of all study participants is shown in Table 1. As expected, the systolic (SBP) and diastolic (DBP) blood pressures were significantly higher in PE compared to normotensive pregnancies ($p<0.0001$).

Table 1:
Clinical and biochemical characteristics of all study groups [N=72; Median (25th–75th percentile]

	Normotensive (n=36)		Pre-eclamptic (n=36)		
	HIV negative (n=18)	**HIV positive (n=18)**	**HIV negative (n=18)**	**HIV positive (n=18)**	*p-value*
Maternal age (years)	24.50 (21.00-29.00)	24.50 (22.00-29.00)	27.50 (21.00-36.00)	27.00 (22.00-38.00)	*p*=0.58
Parity	1.00(0.00-2.00)	1.00(0.00-2.00)	1.00(1.00-2.00)	1.00(0.00-2.00)	*p*=0.48
Gravidity	2.00(1.00-2.00)	2.00(1.00-3.00)	2.00(2.00-3.00)	2.00(1.00-3.00)	*p*=0.46
Gestational age (wks)	38.00 (37.00-40.00)*	37.50 (37.00-38.00)*	30.00 (28.00-33.00)*	30.50 (28.00-32.00)*	*p*=0.0001*
Systolic BP (mmHg)	105.50 (103.00-113.00)*	108.50 (101.00-115.00)*	154.00 (149.00-165.00)*	157.50 (147.00-168.00)*	*p*=0.0001*
Diastolic BP (mmHg)	68.50 (63.00-74.00)	71.50 (65.00-75.00)	102.00 (96.00-109.00)*	99.50 (97.00-106.00)*	*p*=0.0001*
Maternal weight (kg)	70.10 (55.00-76.00)*	70.35 (61.00-79.00)	87.00 (65.00-95.00)*	74.00 (67.00-81.00)*	*p*=0.04*
BMI (kg/m^2)	29.85 (24.50-31.20)	28.40 (25.00-31.20)	32.75 (26.70-36.60)	30.90 (29.05-37.80)	*p*=0.19
Baby weight (kg)	3.34 (3.00-3.87)*	3.30 (3.04-3.73)*	1.88 (1.54-2.91)*	2.35 (1.36-2.67)*	*p*=0.0001*
TSP-2 (ng/ml)	24.80 (16.30-34.67)*	28.99 (18.82-34.76)	21.97 (16.19-30.70)*	31.98 (23.89-44.21)*	*p*=0.01*

**p<0.05 considered statistically significant*

Lower gestational age at delivery was noted in PE compared to normal pregnancies ($p<0.0001$). A statistically significant difference was noted for maternal weight between the P+ve vs N-ve; and between P-ve vs N-ve ($p=0.04$). Likewise, baby weight significantly differed between the P+ve vs N-ve; P+ve vs N+ve; and between P-ve vs N+ve ($p=0.0001$), being lower in the preeclamptic versus the normotensive groups.

Serum TSP levels: Based on pregnancy type (normotensive vs preeclamptic), no significant difference was observed in TSP-2 concentrations, regardless of HIV status (26.61 ng/ml; 95% CI: 17.52- 34.67 vs 25.35ng/ml; 95% CI: 18.32-37.49; $p = 0.70$). In contrast, based only on HIV status (ie. negative vs positive) and irrespective of pregnancy type, we observed a significant increase in TSP-2 levels ($p = 0.04$) in the HIV-positive group (29.66 ng/ml; 95% CI: 21.99-38.01) compared to the HIV-negative group (24.34 ng/ml; 95% CI: 16.24-31.48). Also, based on pregnancy type and HIV status, circulating TSP-2 levels differed significantly between the P+ve and N-ve; and P+ve and P-ve groups respectively ($p=0.01$; Table 1).

Spearman's correlation between circulating TSP-2 levels and clinical parameters: A bivariate Spearman's correlation analysis was conducted to determine the relationship between clinical and demographic factors; and between circulating TSP-2 levels and clinical parameters in the HIV-positive normotensive and preeclamptic groups. A statistically significant positive association was observed between parity and maternal age for the N-ve ($r=0.70$; $p=0.04$), the P-ve ($r=0.67$; $p=0.01$) and P+ve ($r=0.79$; $p=0.003$) groups respectively. A similar association was observed between parity and gravidity across these groups. For BMI, a positive correlation was noted with maternal weight in the N-ve ($r=0.82$, $p=0.006$); N+ve ($r=0.82$, $p=0.006$); P-ve ($r=0.94$, $p=0.000$) and P+ve ($r=0.94$, $p=0.000$) groups respectively.
In the N-ve groups, a strong negative correlation was demonstrated between TSP-2 and DBP ($r=-0.78$; $p=0.01$). Similarly, in the P-ve pregnancies, a strong negative correlation was demonstrated between TSP-2 levels and BMI ($r=-0.79$; $p=0.002$) and maternal weight ($r=-0.75$; $p=0.005$) respectively

DISCUSSION

Our main findings demonstrate a significant elevation of serum TSP-2 concentration in PE versus normal pregnancies, irrespective of HIV status. These findings are corroborated by Stenczer and co-workers, who also demonstrated elevated TSP-2 levels in PE compared to normotensive pregnancies (Stenczer *et al*., 2011). Thrombospondin-2 aids in communication between cells and the extracellular matrix, effecting proliferation and invasion and in cellular events such as apoptosis and angiogenesis (Jiang *et al*., 2019, Streit *et al*., 1999, Daniel *et al*., 2007). Elevated TSP-2 may account for the defective trophoblast invasion in PE.

An earlier report confirms that TSP-2 expression in cardiomyocyte progenitor cells (hCMPCs) is stimulated by hypoxia, influencing matrix metalloproteinase modulators, MMP-2 and MMP-9 and consequently degrades the ECM (Van Oorschot *et al*., 2011). More recently, an elevated placental hypoxia inducible factor-1α (HIF-1α) expression was reported in PE compared to normotensive pregnancies (Verma *et al*., 2018). This implicates the placenta as the source of TSP-2 where hypoxia modulates its expression (Stenczer *et al*., 2011). Moreover, TSP-2 knockout mice are associated with increased proliferation and invasion of hCMPCs (Van Oorschot *et al*., 2011). Off note, SM22α-cre HIF-1α KO mice also show increased TSP-2 mRNA levels under hypoxic conditions (Maclauchlan *et al*., 2018). This data supports our finding of elevated TSP-2 in the hypoxic state of PE indicating that TSP-2 may participate in controlling trophoblast migration and invasion in PE development.

Preeclampsia is associated with an angiogenic dysregulation (Steegers *et al*., 2010, Venkatesha *et al*., 2006), as validated by an upregulation of soluble fms-like tyrosine kinase-1 and soluble endoglin and a concurrent decline in placental growth factor and vascular endothelial growth factor (VEGF) (Govender *et al*., 2015, Ngene *et al*., 2019). The elevated serum TSP-2 levels observed in PE may emanate from the extensive systemic endothelial damage and its potent antagonistic effect on VEGF function (Lawler and Lawler, 2012). TSP-2 antagonizes NO signaling through its interaction with endothelial surface receptors, CD36 and CD47 (Simantov *et al*., 2005, Lawler and Lawler, 2012). The CD36/TSP structural homology repeat (TSR) link is involved in the angiogenic switch that converts a pro-angiogenic phenotype to an anti-angiogenic response via the non-receptor tyrosine kinase fyn, p38 MAP kinase, and caspase pathways (Simantov *et al*., 2005, Jiménez *et al*., 2000). Since TSP-2 regulates the bioavailability of proteases, its action in ECM-cell communication may be counteracted by the TGF-β/SMAD axis on endothelial cells (Bornstein *et al*., 2000, Pellerin *et al*., 1994).

Integrins have a vital role in regulating cell behaviour, proliferation and migration (Hynes, 2002), especially the α4β1 integrins, which are receptors for TSP-2 (Li *et al*., 2002). Therefore, the dysregulation of TSP-2 noted in our study may be attributed to an aberrant integrin expression that influences trophoblast cell invasion (Merviel *et al*., 2001). Also, microRNAs play an essential role in regulating migration and invasion of trophoblast cells (Yang *et al*., 2019). An overexpression of miR-221-3p was recently shown to stimulate the growth, migration and invasion of trophoblasts, in contrast to that observed during knockout of miR-221-3p (Yang *et al*., 2019). The latter study also noted an elevation in TSP-2 mRNA expression in PE; that negatively correlated with miR-221-3p, corroborating our findings, and highlighting the possible role of TSP-2 in trophoblast cell migration. TSP-2 is an established anti-angiogenic, pro-apoptotic and immunomodulatory protein (Stenczer *et al*., 2011). The elevated TSP-2 levels observed in our study may thus also correlate with the elevated trophoblast apoptosis that characterises PE (Naicker *et al*., 2013).

Furthermore, we report a significant difference between PE and normotensive pregnancies based on HIV status, with TSP-2 levels increasing in HIV-positive women. Like PE, HIV infection is also associated with endothelial injury

(Naidoo *et al*., 2021). The HIV-1 transactivator protein (tat) is a potent angiogenic factor due to its similar arginine and lysine-rich sequence to VEGF (Zhou *et al*., 2013). TSP binds with high affinity to Tat protein in the ECM via glutathione-S-transferase (GST)-tat protein but not to GST (Rusnati *et al*., 2000). Thus, TSP would regulate the bioavailability of extracellular tat protein. The antiviral property of TSP-2 observed in our study is corroborated by other studies (Crombie, 2000, Rusnati *et al*., 2000); implicating the binding of the tat protein in the modulation of TSP function (Rusnati *et al*., 2000). It is also plausible that the binding of the tat protein to TSP-2 may promote an angiogenic imbalance in HIV infection.

Based on HIV status, we report an upregulation of TSP-2 regardless of pregnancy type. The upregulation of TSP-2 observed in the HIV-positive group in our study may be attributed to the binding affinity of TSP-2 to gp120 of HIV-1 via CD36 (Crombie, 2000). More specifically, the conserved regions flanking the V3 loop of gp120 provide the antiviral property for direct HIV-1 inhibitory action of TSP. Notably, the HIV-positive group in our study received dual antiretroviral therapy (HAART + Nevirapine). A previous study suggests that HAART may influence the HIV-1 matrix protein p17 to induce TSP secretion (Caccuri *et al*., 2014).

The effect of TSP-2 in the hypoxic, inflammatory microenvironment of PE combined with the anti-angiogenic effect of TSP-2 reduces its bioavailability for VEGF binding. We believe that increased TSP-2 levels observed in the pregnancy group complicated by HIV infection, may be related to the routine use of the triple ART regimen; of which HAART reconstitutes immune response (Maharaj *et al*., 2017).

After tissue damage, thrombospondins regulate remodelling and inflammation (Nakao and Morita, 2019), as demonstrated by an elevated TSP-2 expression during tissue remodelling, which is associated with inflammation (Bornstein *et al*., 2004). Additionally, in autoimmune disease such as rheumatoid arthritis, TSP-2, a constituent of the synovial microenvironment, regulates tissue inflammation (Park *et al*., 2004). Thus, it is plausible that the elevated TSP-2 observed in PE reflects a similar hyper inflammatory environment.

In conclusion, and to the best of our knowledge, this is the first study to report the serum concentrations of TSP-2 in HIV-associated preeclamptic versus normotensive pregnancies. Limitations of this study include small sample size. Additionally, antiretroviral therapy is shown to reconstitute the immune response which could be considered as a limitation as all HIV-positive women in this study received dual antiretroviral therapy. This study demonstrates for the first time a significant upregulation of TSP-2 in HIV-associated PE compared to normotensive pregnancies. This elevation may emanate from the anti-angiogenic, pro-apoptotic and immunomodulatory role of TSP-2. Notably, we also show an upregulation of TSP-2 in the HIV-positive group; attributed to the binding of TSP-2 to gp120 of HIV-1. Thrombospondin-2 may have a predictor test value in the early diagnosis of PE due to its role in the remodelling of vasculature, angiogenesis regulation, apoptosis, and inflammation. Finally, further large-scale studies are required to confirm its biomarker value in PE development.

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