Morphometric image analysis of placental C-type lectin domain family 2, member D (CLEC2D) immuno-expression in HIV associated pre-eclampsia

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A B S T R A C T

Objective: C-type lectin domain family 2, member D (CLEC2D) is implicated in the immune response. Pre-eclampsia and HIV infection have opposing immune responses. In view of the high prevalence of HIV infection and pre-eclampsia in South Africa, this study assessed the placental immuno-expression of CLEC2D in HIV associated pre-eclampsia.
Method: Placental tissue was obtained from 60 pregnancies which were categorized according to pregnancy type (pre-eclamptic or normotensive) and HIV status (positive or negative). Immunohistochemistry and morphometric image analysis were used to evaluate placental CLEC2D immuno-expression.
Results: CLEC2D expression was significantly decreased in the conducting villi of pre-eclamptic vs normotensive placentae ($p = 0.0418$) but was increased in the exchange villi, albeit non-significant ($p = 0.4948$). HIV positive status intensified placental CLEC2D immuno-expression in conducting ($p = 0.0312$) and exchange ($p = 0.0025$) villi. CLEC2D expression was significantly different in exchange vs conducting villi ($p < 0.0001$) and across study groups ($p = 0.0003$). Normotensive; HIV negative placentae (control) had a non-significant difference in CLEC2D expression across villi types, however significant difference was noted within the remaining groups: normotensive; HIV positive ($p < 0.05$), pre-eclamptic; HIV positive ($p < 0.01$ and pre-eclamptic; HIV negative ($p < 0.001$).
Conclusion: The contrasting expression of CLEC2D in HIV infection and pre-eclampsia is demonstrative of the immunosuppressive and pro-inflammatory roles of the respective pathologies. However, this implication may be confounded by highly active anti-retroviral treatment (HAART).

Introduction

The top 3 causes of maternal mortality in South Africa are non-pregnancy related infections (HIV and TB), hypertension in pregnancy and obstetric hemorrhage [1]. Moreover, one-fifth of women in their reproductive ages are HIV infected [2].

Pre-eclampsia (PE) is a pregnancy-specific hypertensive disorder, defined as a BP $\geq$ 140 mmHg systolic and/or $\geq$90 mmHg diastolic [3]. It is a multifactorial condition, accompanied by one or more of the following: proteinuria, thrombocytopenia, hemolysis, acute kidney injury, neurological features, liver dysfunction, or fetal growth restriction [3]. Pre-eclampsia may be classified as early onset (EOPE) or late onset (LOPE) PE based on gestational age, the former associated with intrauterine growth restriction [4,5]. Pre-eclampsia is a two-stage disorder, the first 1st stage involves inadequate placentation and the non-physiological conversion of spiral arteries [6]. This creates a hypoxic micro-environment, leading to elevated oxidative stress and anti-angiogenic factors [7]. The 2nd stage involves the clinical manifestation of this systemic disease [6].

During normal pregnancy, due to the presence of the placenta there is a shift from Th1 to Th2 immune response, however, in PE

this shift does not occur [8,9]. In contrast, during HIV infection, there is a shift from Th1 to Th2 immune response [10]. Hence, it is likely that co-morbidity of these two conditions may stabilize the immune response [11].

C-type lectins (CLECs) contain at least one characteristic C-type lectin-like domain and function physiologically in development, angiogenesis and inflammation because of their ability to identify exogenous and endogenous ligands [12]. CLEC2D transcripts include lectin like transcript (LLT1) and osteoclast inhibiting lectin (OCIL) [13,14].

In PE, there is an increased activation of circulating natural killer (NK) cells, this may be liable for the induction of lysis of trophoblast cells, which lack HLA-G [15]. Nevertheless, NK cells produce angiogenic factors which are imbalanced in PE [16]. HIV infection induces CLEC2D expression on lymphocytes, resulting in the inhibition of NK cell mediated cytotoxic response and the initiation of IFN-γ release by CD8 + T lymphocytes [17,18]. Moreover, CLEC2D is associated with the protection of B lymphocytes following viral infection [19,20]. In addition, HIV-CLEC2 interaction causes platelet activation which mediates the platelet capture of HIV virions [20,21]. The binding of HIV to platelets leads to the internal replication and multiplication of HIV virions [20,22].

Whilst the role of CLECs in immunity is well established, there is a paucity of data regarding the role of CLEC2D in homeostasis and angiogenesis [23]. Furthermore, the role of this protein in the exaggerated immune response of PE remains obscure. Moreover, to our knowledge, there is a lack of data on placental CLEC2D expression in HIV associated PE. Therefore, this study aims to morphometrically analyze CLEC2D immuno-expression in placental conducting and exchange villi, based on pregnancy type (normotensive *vs* PE), HIV status (HIV- *vs* HIV+) and across all study groups.

Materials and methods

This prospective study was performed during the period January–December 2018.

Ethical considerations

This study received institutional ethics approval (BCA 338/17), hospital managers consent, and informed consent was obtained from all participants.

Study population

The study population (n = 60) consisted of pre-eclamptic (n = 30; PE) and normotensive (n = 30; N) women attending the obstetric unit of a large regional hospital in Durban, KwaZulu-Natal. Study groups were stratified into HIV negative (n = 15; PE- and N-) and HIV positive (n = 15; PE + and N+).

Inclusion criteria

Pre-eclampsia was defined as a sustained systolic blood pressure ≥140 mmHg and a diastolic blood pressure ≥90 mmHg at least 4 h apart, and proteinuria after 20 weeks' gestation in a previously normotensive patient [24]. HIV status was determined by a rapid test.

Exclusion criteria

Participants with chronic hypertension, diabetes mellitus, gestational diabetes, heart failure, epilepsy chronic renal disease, unknown HIV status, intrauterine death, abruptio placentae, systemic lupus erythematous, antiphospholipid antibody syndrome, chorioamnionitis, thyroid disease, sickle cell anemia, and participants treated with aspirin, non-steroidal anti-inflammatory drugs and asthma medication were excluded.

Methods

This study utilizes wax embedded placental samples that were retrospectively collected. The original samples were fixed in 10% buffered formaldehyde and embedded into paraffin wax blocks as per standard laboratory procedure [25]. Sections of placental tissue were cut (3 μm) using a rotary microtome and mounted onto coated slides (X-tra Adhesive, Leica Microsystems, Germany). Samples were de-paraffinized and rehydrated.

Immunostaining

Immunostaining was performed using the EnVision FLEX mini kit, high pH, (DAKO, Denmark) as per the manufacturer's instructions. Antigen retrieval was achieved by preheating (5 min) and incubating in a target retrieval solution (Envision FLEX DAKO, Denmark) for 20 min at 95 °C. Slides were rinsed and washed in wash buffer (5 min). Post encircling sections with a de-limiting pen (pap pen; DAKO, Denmark), endogenous peroxidase was blocked (5 min). Thereafter, sections were incubated in the primary CLEC2D antibody for 2 h (Abcam, ab197341;1:200; diluent-DAKO REAL diluent) at room temperature. Post washing, the sections were incubated with horse-radish peroxidase (20 min.). Detection of immunoreactivity was performed with diamino-benzidine (DAKO, Denmark). All sections were counterstained with Mayer's hematoxylin, dehydrated and mounted. Negative controls were performed by replacing the primary antibody with non-immune sera of the same IgG isotype as the primary antibody. The primary antibody was replaced with PBS in buffer controls. Thyroid cancer served as the positive control.

Morphometric analysis

The Axioscope A1 microscope (Carl Zeiss, Germany) was used to view the prepared placental tissue sample and a random selection of 4 fields of view per slide was analyzed at an objective magnification of ×20. AxioVision Image Analysis Software Version 4.8.3 (Carl Zeiss, Germany) was used to acquire, capture, process and analyze the images. By using the auto-measure mode of the software, CLEC2D immuno-expression was evaluated as a percentage of immunostaining per frame area. Frame size was expressed as field of frame in μm². The entire frame was selected for exchange villi. The amount of CLEC2D immunostaining (green) within the placental exchange villi (red) was established by a two-phase threshold, thus areas of immunostaining were segmented and defined as a field area percentage for each villi type.

Data analysis techniques

An excel database was created to compare CLEC2D expression across all groups, between pregnancy types and by HIV status.

Statistical analysis

Sample size was determined using Cohens' formula. Results were tested for normality prior to analysis. Two-way ANOVA was performed to examine the effect of HIV status (HIV- *vs* HIV+) and pregnancy type (normotensive *vs* pre-eclamptic) and across all groups. Bonferroni *post hoc* test was used to further assess the data between subcategories.

A probability level of $p < 0.05$ was considered statistically significant. All statistical analyses were conducted using GraphPad Prism™ version 5.01 (San Diego, CA, USA).

Results

Clinical characteristics

Maternal age ($p = 0.0046$), parity ($p = 0.0055$) and placental weight ($p = 0.0259$) were significantly different across groups. As expected, the diastolic ($p = <0.0001$) and systolic ($p = <0.0001$) blood pressure differed significantly (Table 1). There was one twin pregnancy in the HIV positive PE group.

CLEC2D placental immunolocalization

Conducting (stem) and exchange (intermediate and terminal) were immunostained with CLEC2D across all study groups. CLEC2D immuno-reactivity was observed in syncytiotrophoblast and cytotrophoblast cell populations across villi types [conducting (Fig. 1a–d) and exchange (Fig. 2a–d)]. The mesenchymal core of the villi was non-reactive. The positive control displayed CLEC2D immunostaining while buffer and method controls produced no staining.

Morphometric image analysis of placental CLEC2D

Conducting villi

The frame area of conducting villi within each group was: PE+: 267036 μm^2 $\pm$ 17574 μm^2; PE-: 266350 μm^2 $\pm$ 18185 μm^2; N+: 239141 μm^2 $\pm$ 25923 μm^2; N-: 253306 μm^2 $\pm$ 14722 μm^2.

The field area percentage of CLEC2D immunoreactivity within each group was PE+: 7.399 $\pm$ 0.6334%; PE-: 6.001 $\pm$ 0.5664%; N+: 9.713 $\pm$ 1.364%; N-: 7.289 $\pm$ 0.6374%. CLEC2D immuno-expression within conducting villi differed based on HIV status (irrespective of pregnancy type) [F(1.56) = 4.89, $p = 0.0312$] (Fig. 3b). Similarly, CLEC2D immuno-expression within conducting villi was significantly different between normotensive and PE, irrespective of HIV status [F(1.56) = 4.34, $p = 0.0418$] (Fig. 3a). However, the Bonferroni multiple comparison test showed no interaction across all groups [PE + vs PE-; PE + vs N+; PE- vs N-; N + vs N-; F(1.56) = 0.35, $p = 0.5552$] (Fig. 5).

Exchange villi

The frame area of was kept consistent throughout (598907.2739 μm^2), however the area of exchange villi within each group was PE+: 229770 μm^2 $\pm$ 13392 μm^2, PE-: 287870 μm^2 $\pm$ 13692 μm^2, N+: 253084 μm^2 $\pm$ 12650 μm^2, N-: 256649 μm^2 $\pm$ 13198 μm^2.

The field area percentage of CLEC2D immunoreactivity within each group was PE+: 11.40 $\pm$ 0.7062%, PE-: 10.99 $\pm$ 0.9482%, N+: 12.96 $\pm$ 0.7358%, N-: 8.336 $\pm$ 0.7628%. CLEC2D immuno-expression within exchange villi was significantly different based on HIV status (irrespective of pregnancy type) [F(1.56) = 10.05, $p = 0.0025$] (Fig. 4b) and interaction across all groups [F(1.56) = 7.03, $p = 0.0104$]. However, there was no significant difference in the CLEC2D immuno-expression within exchange villi between PE and normotensive women, irrespective of HIV status [F(1.56) = 0.47, $p = 0.4948$] (Fig. 4a).

CLEC2D immuno-expression between villi types

A two-way ANOVA was used to assess the interactions based on villi type. CLED2D immuno-expression was significant between exchange and conducting villi irrespective of study group [F (1.112) = 32, $p < 0.0001$]; the study groups had a very significant effect on CLEC immuno-expression [F(3.112) = 6.78, $p = 0.0003$]. The Bonferroni *post hoc* test was used to determine significance between each group (based on percentage CLEC2D immuno-expression of conducting vs exchange villi), [N-: $p > 0.05$, N+: $p < 0.05$, PE-: $p < 0.001$, PE+: $p < 0.01$] (Fig. 5).

Discussion

This novel study demonstrates a significant decrease of CLEC2D expression in the conducting villi of pre-eclamptic vs. normotensive placentas, irrespective of HIV status. Whilst there is no existing literature on placental CLEC2D expression in PE, Apps et al., (2011) reported a high expression of CLEC2D in villous and extra-villous trophoblasts in normotensive pregnancy [26]. The CLEC2D down-regulation observed in our study may be attributed to the immune exacerbation that occurs in PE [27–29].

In normal pregnancy, inflammatory modulators such as CLEC2D maintain a balance between maternal-fetal immune responses, by regulating NK cell activity [30]. Whilst NK cells are important for placentation, an exaggerated presence of NK cells may lead to complications during pregnancy [29]. In PE, an exacerbated inflammatory response is a result of elevated NK cells [31]. Since CLEC2D binds to the NK cell receptor- NKR-P1, it is no longer available to mediate its function [30,32].

Suzuki-Inoue, (2014) discussed CLEC2-podoplanin interactions in promoting platelet activation and the impediment of migration and growth of lymphatic endothelial cells [33]. Furthermore, platelet activation by CLEC2-podoplanin interactions assist in remodeling of the spiral arteries during normal

Table 1
Patient demographics in normotensive HIV negative; normotensive HIV positive; pre-eclamptic HIV negative and pre-eclamptic HIV positive pregnant women.

n	Normotensive pregnant women		Pre-eclamptic women		*p* value
	HIV negative (N−) 15	HIV positive (N+) 15	HIV negative (PE−) 15	HIV positive (PE+) 15	
Maternal age (years)	18 (18–20)	23 (20–27)	20 (18–24)	23 (20–26)	p = 0.0046[**]
Gestational age (weeks)	38 (37–39)	40 (39–40)	38 (35–40)	38 (37–39)	p = 0.0569
Parity	1(1)	1(1)	1(1)	1 (1–2)	p = 0.0055[**]
Systolic blood pressure (mmHg)	120 (116–125)	117 (112–121)	152 (146–162)	144 (136–169)	p = <0.0001[***]
Diastolic blood pressure (mmHg)	70 (63–74)	68 (63–72)	101 (92–108)	95 (92–100)	p = <0.0001[***]
Maternal weight (kg)	80 (62–85.9)	80 (68.5–89.1)	71 (64–82.95)	71 (64.1–89.7)	p = 0.6304
Maternal BMI (kg/m²)	32.07 (28.08–35.09)	29.38 (26.1–33.18)	29.26 (27.31–33.18)	29.01 (26.84–36.39)	p = 0.8118
Placental weight (g)	450 (400–500)	550 (490–600)	510 (450–560)	500 (480–510)	p = 0.0259[*]
Baby weight (kg)	3.1 (2.77–3.4)	3.5 (3–3.65)	3.2 (2.65–3.5)	3 (2.8–3.6)	p = 0.3427

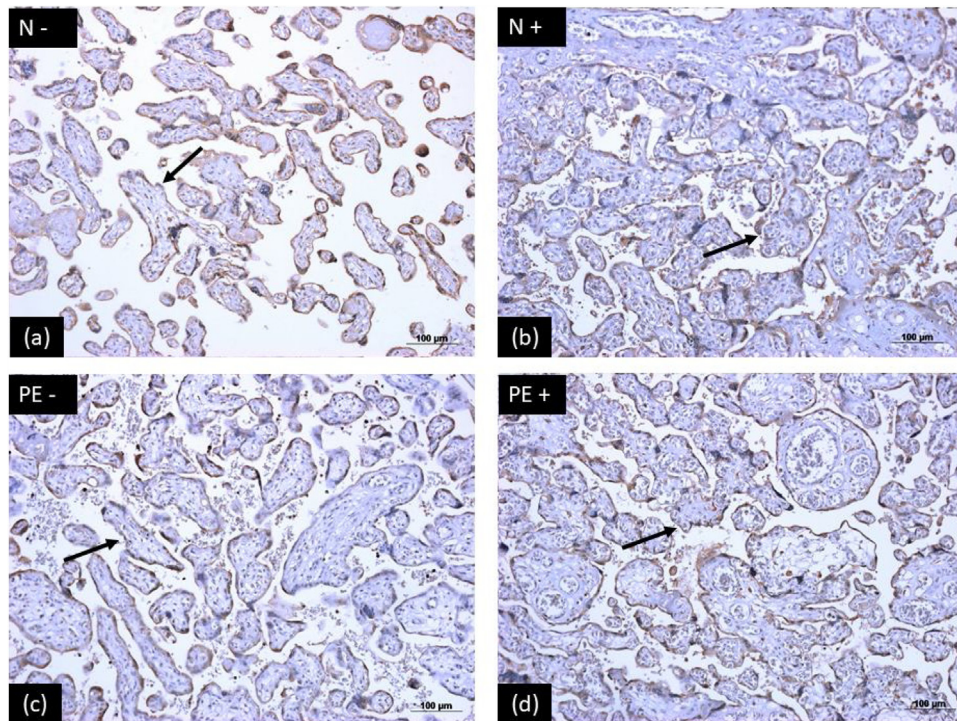Summary statistics are presented as median and interquartile range, n = 60.
[*] p < 0.05.
[**] p < 0.01.
[***] p < 0.001.
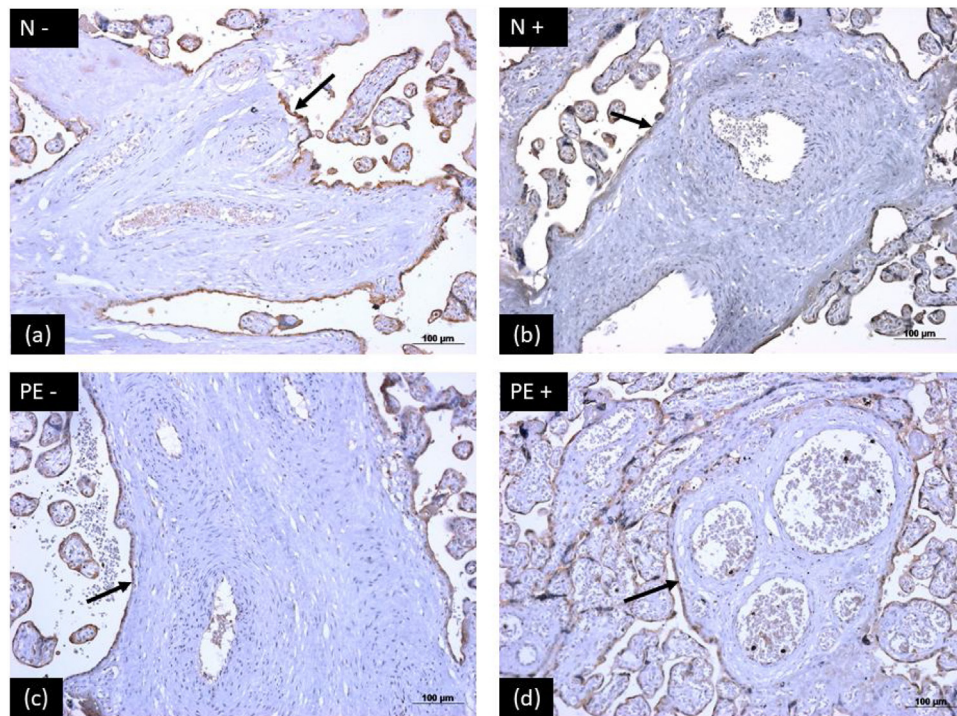
Fig. 1. CLEC2D immunoreactivity in conducting villi. (a) HIV negative, normotensive healthy controls (N−); (b) HIV positive, normotensive (N+); (c) HIV negative, pre-eclamptic (PE−) and (d) HIV positive, pre-eclamptic (PE+).
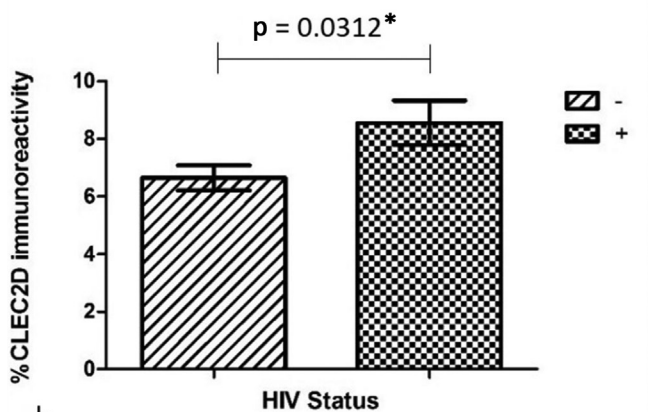

Fig. 2. CLEC2D immunoreactivity in exchange villi. (a) HIV negative, normotensive healthy controls (N−); (b) HIV positive, normotensive (N+); (c) HIV negative, pre-eclamptic (PE−) and (d) HIV positive, pre-eclamptic (PE+).

placentation [33,34]. Additionally, CLECs facilitate immune suppression, metastasis and tumor invasion by assisting the interactions of cancer cells with platelets, endothelial cells and leukocytes [35]. CLEC2 and cancerous-podoplanin expressing cells may mediate platelet aggregation, thus playing a protective role against leukocytes and shear stress whilst promoting angiogenesis, metastasis and tumor development [36]. In normal pregnancy, the villous stroma of the placenta contains a strong expression of podoplanin [37]. Platelets may be exposed to higher levels of placental podoplanin during angiogenesis, and suppression of CLEC2D may decrease platelet activation [38]. Hence, the diminished presence of CLEC2D in our findings, may also contribute to thrombocytopenia and the anti-angiogenic properties associated with PE.
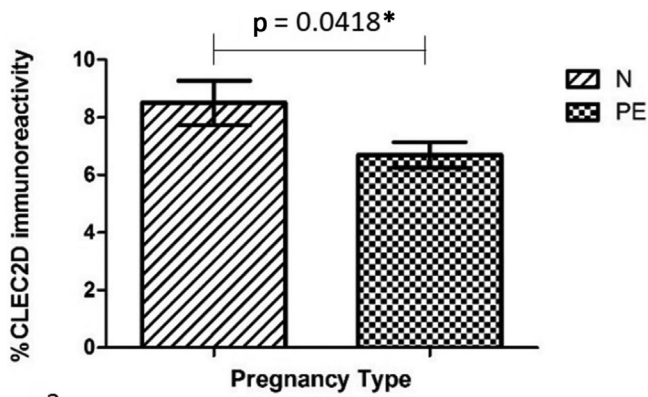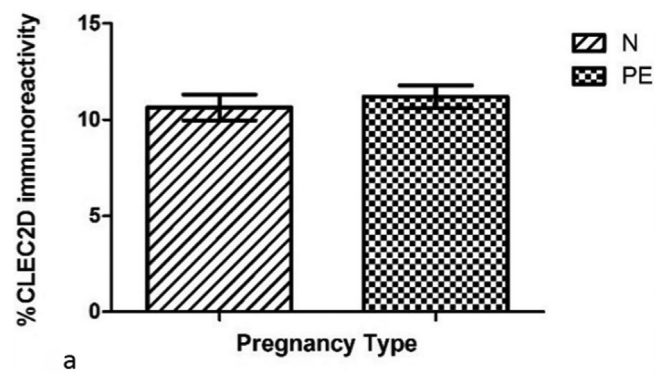
Fig. 3. CLEC2D immunoreactivity in conducting villi based on (a) pregnancy type and (b) HIV status.

Fig. 4. CLEC2D immunoreactivity in exchange villi based on (a) pregnancy type and (b) HIV status.
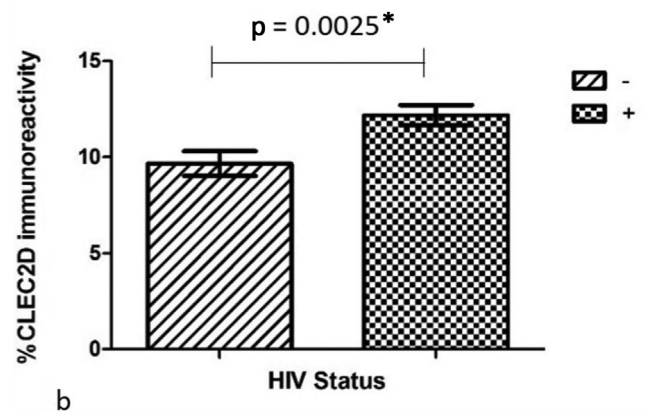
We further report an up-regulatory, albeit non-significant trend of placental CLEC2D expression in exchange villi of PE *vs* normotensive pregnancies, regardless of HIV. Lin et al. indicated that villous tree geometry affects the oxygen exchange capabilities, which influences angiogenesis [39]. An increased villi density was observed in PE as a compensatory response to the hypoxic micro-environment [40]. Additionally, Sankar et al., (2013) found that PE has an area reductive effect of conducting villi, but no effect on exchange villi was observed [41]. Notably, a reduced area in conducting villi would result in a decreased CLEC2D expression.

Although the exaggerated micro-environment of PE suppresses the anti-inflammatory effector function of CLEC2D, HIV progression is enhanced by CLEC2D. This is the first demonstration of a significant up-regulation of CLEC2D in both conducting and exchange villi based on HIV status. Viral glycoproteins are recognized by cells expressing CLECs and promotes an anti-viral response. Alternatively, DC-SIGN (CLEC2 family member) recognizes HIV leading to infection of dendritic cells and subsequent trans-infection in CD4 + T cells [42]. C type lectin polymorphisms correlate with viral disease susceptibility and progression [43]. Activated endothelial cell CLECs induce a pro-inflammatory response that contributes to pathology in conditions of viral infection such influenza and Ebola virus [44,45].

CLEC2D is responsible for detecting alterations in the expression of MHC class 1 molecules and thus mediates NK cell activity accordingly [46]. HIV compensates for this by its mimicry of platelet integrins ($\alpha IIb\beta 3$) and by the expression of CLEC2 on viral proteins [22]. This result is representative of the immunosuppressive role of HIV and the inhibitory mechanisms of CLEC2D, as well as the protective effect CLEC2D serves for HIV infected lymphocytes.

Our study further demonstrates a significant increase in CLEC2D immune-expression in exchange *vs* conducting villi, irrespective of study group. Additionally, a significant difference was noted between groups, irrespective of villi type. Furthermore, we demonstrated a significant difference in CLEC2D immuno-expression in villi types, between groups. The control group (normotensive; HIV negative group) yielded a non-significant result suggesting that CLEC2D remains unchanged under non-pathological conditions. Moreover, the highest significance existed between the PE- group ($p < 0.001$), suggesting that this was an exaggerated inflammatory response, followed by the PE+ group ($p < 0.01$), and the N+ ($p < 0.05$).

The combined effect of the exaggerated immune response in PE and the immunosuppressive influence of HIV, the maternal-fetal interface, requires a finessing of the immunological balance between exhibiting the maternal defence to pathogens whilst encouraging tolerance to the allogenic fetus [47]. Thus, the combined actions of CLEC2D play protective and antagonistic roles in HIV associated PE. Additionally, Highly Active Anti-retroviral Therapy (HAART) is the standard of care for HIV infected individuals. Phoswa et al. suggests that although HAART suppresses HIV by restoring NK cell abilities, this activation may lead to dysregulated trophoblast invasion during pregnancy and potentially lead to PE [48].

Limitations of this study include the heterogeneity of the study population; early and late onset PE. The duration and time of initiation of HAART therapy was absent. It is possible that HAART may have confounded immuno-expression of CLEC2D.
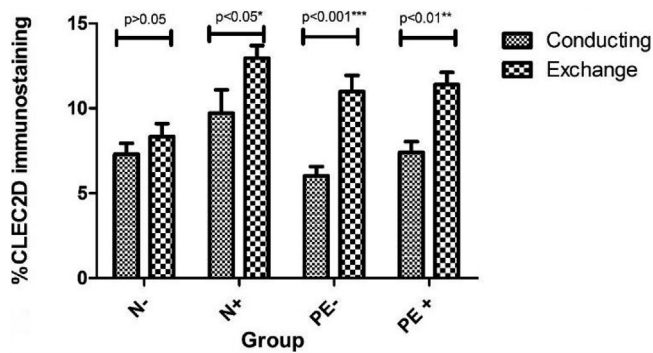
Fig. 5. CLEC2D immunoreactivity based on villi type across all groups.

In conclusion, this novel study highlights a down-regulation of CLEC2D immuno-staining in the conducting villi of PE which may be attributable to the CLEC2D- NKR-P1 binding that occurs in the high inflammatory milieu of PE. Moreover, this decline may contribute to thrombocytopenia and the anti-angiogenic state associated with PE. We also demonstrate an enhanced trend of placental CLEC2D expression in exchange villi of PE occurring as a compensatory response of the villous tree geometry in the hypoxic micro-environment. Moreover, we show significant difference in CLEC2D expression based on villi type. Additionally, we have established a significant increase of CLEC2D in botconducting and exchange villi based on HIV status validating the anti-viral response of CLEC2D. It is plausible that antagonistic action of CLEC2D are dependent on the mediation of PE, HIV infection and PE co-morbid with HIV infection, and more importantly may be confounded by HAART. Future recommendations include investigating polymorphisms and SNP analysis of CLEC2D on a larger sample size and considering gestational age of PE.

Conflict of interest

The authors have no conflicts of interest to declare.

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