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Increased Maternal and Fetal Cholesterol Efflux capacity and Placental CYP27A1 Expression in Pre-eclampsia

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Abbreviations: AGA: appropriate-for-gestational-age; AIBP: apoA1 binding protein; ApoA1: apolipoprotein A1; ApoE: apolipoprotein E; BMI: body mass index; Cav-1: caveolin-1; CYP27A1: sterol 27-hydroxylase; CVD: cardiovascular disease; FBS: fetal bovine serum; FGR: fetal growth restriction; HDL: high-density-lipoprotein; HELLP: haemolysis, elevated liver enzymes and low platelet count syndrome; OHC: hydroxycholesterol; LDL: low-density-lipoprotein; RCT: reverse cholesterol transport; SGA: small-for-gestational-age; TC: total cholesterol; TG: triglyceride; VEGFR1: vascular endothelial growth factor receptor 1

Abstract

Pre-eclampsia is a pregnancy-specific condition, leading to increased cardiovascular risk in later life. A decrease in cholesterol efflux capacity is linked to cardiovascular disease.

We hypothesised that, in pre-eclampsia there would be a disruption of maternal/fetal plasma to efflux cholesterol as well as differences in the concentrations of both placental sterol 27-hydroxylase (CYP27A1) and apoA1-binding-protein (AIBP).

Total, HDL- and ABCA1-mediated cholesterol efflux were performed with maternal and fetal plasma from women with pre-eclampsia and normotensive controls (both n=17). ApoA1/apoE were quantified by chemiluminescence; 27-hydroxycholesterol (27-OHC) by GC-MS. Immunohistochemistry was used to determine placental expression/localisation of CYP27A1, AIBP, apoA1, apoE and SRB1.

Maternal and fetal total and HDL-mediated cholesterol efflux capacities were increased in pre-eclampsia (by 10-20%), but ABCA1-mediated efflux was decreased (by 20-35%; $P<0.05$). Maternal and fetal apoE concentrations were higher in pre-eclampsia. Fetal plasma 27-OHC levels were decreased in pre-eclamptic samples ($P<0.05$). Placental protein expression of both CYP27A1 and AIBP were localised around fetal vessels and significantly increased in pre-eclampsia ($P=0.04$). Placental 27-OHC concentrations were also raised in pre-eclampsia ($P<0.05$).

Increased HDL-mediated cholesterol efflux capacity and placental CYP27A1/27-OHC could be a rescue mechanism in pre-eclampsia, to remove cholesterol from cells to limit lipid peroxidation and increase placental angiogenesis.

Keywords: lipid/efflux; 27-hydroxycholesterol; apolipoproteins; cholesterol; pregnancy; hypertension.

Introduction

Pre-eclampsia is one of the three leading causes of maternal morbidity and mortality worldwide, complicating around 2–8% of pregnancies (1). This disorder increases the chance of adverse perinatal outcomes such as fetal growth restriction (FGR) and preterm delivery (2). Both, the women who develop pre-eclampsia and their babies, are at increased risk of hypertension in later life. Moreover, women with a history of pre-eclampsia have an 8-fold greater risk of death from cardiovascular disease (CVD) (3). The American Heart Association has included pre-eclampsia as a risk factor in guidelines for preliminary risk evaluation for CVD in women (4). The cause of pre-eclampsia remains unknown; different lines of evidence indicate that abnormal lipid metabolism is involved in the pathogenesis; the acute atherosclerosis seen in uteroplacental beds in pre-eclamptic women is one of these (5).

Cholesterol is an essential component required for both placental and fetal development as a precursor of metabolic mediators such as oxysterols (6). The sterol 27-hydroxylase (CYP27A1), a ubiquitously expressed mitochondrial enzyme belonging to the cytochrome P450 family, catalyses the hydroxylation of cholesterol at C-27 to form 27-hydroxycholesterol (27-OHC) and cholestenoic acid (7). CYP27A1 is also involved in cholesterol efflux, the first and rate-limiting step of reverse cholesterol transport (RCT). RCT is defined as the transport of cholesterol from peripheral organs/macrophages to an acceptor in plasma, followed by its transfer to the liver in order to be eliminated/recycled. Overexpression of CYP27A1, as seen in Chinese hamster ovary cells, expressing polyoma LT antigen (CHOP) cells and macrophages (8, 9) results in enhanced cholesterol efflux. Indeed, the appearance of 27-OHC may produce conformational changes in the plasma membrane and facilitate the release of cholesterol and 27-OHC to an acceptor in plasma. 27-OHC is also a ligand of the liver X-activated receptor, which regulates a number of genes involved in cholesterol homeostasis, in particular ABCA1 (8). Little is known about CYP27A1 expression in the placenta, and we are not aware of any study reporting placental 27-OHC concentrations.

Apolipoproteins (apo) are proteins that interact with lipids to form soluble lipid-complexes, allowing the transport of lipids via the circulation. ApoE is a major constituent of very low-density lipoproteins and is an essential ligand

for the uptake and clearance of atherogenic lipoproteins, playing important roles in atherosclerosis by modifying inflammatory responses and facilitating cholesterol efflux from foam cells (10, 11). ApoA1 is a major apolipoprotein of high-density-lipoprotein cholesterol (HDL-C) and is significantly involved in the regulation of lipid transport and metabolism of HDL-particles (12). ApoAI binding protein (AIBP) is secreted from tissue and physically binds with apoAI (13). AIBP accelerates cholesterol efflux from endothelial cells to HDL-C thereby regulating angiogenesis via alterations in the composition of the lipid rafts, possibly due to reduced caveolin-1 (CAV-1) and as well as interfering with VEGFR2 signalling (14, 15). Alteration in angiogenic factors have been repeatedly observed in pre-eclamptic placentae (16) with decreased VEGF-A and VEGFR1 in the cytotrophoblast. Increased plasma apoE polymorphisms have been reported in women with pre-eclampsia (17, 18), however findings relating to apoA1 measurements in pre-eclampsia are conflicting (19, 20). To-date, it is not known whether AIBP is expressed in the placenta or altered in pre-eclampsia.

In our previous study with the same patients used in this study, no significant differences could be demonstrated between groups with regards to the maternal and umbilical venous (fetal) serum low-density-lipoprotein (LDL-C), HDL-C, total cholesterol (TC) and triglycerides (TG). However, fetal serum concentrations were significantly lower compared to maternal concentrations, in both groups (21).

In a recent study of 2924 non-pregnant individuals, where baseline assessments were compared to measurements at a mean follow-up of 9.4 years, the addition of cholesterol efflux to traditional risk factors was associated with improved discrimination/reclassification indexes of cardiovascular risk (22). The authors suggested that cholesterol efflux capacity may be a more important factor than simply HDL-C concentrations alone when assessing the risk of a cardiovascular event occurrence. Increased cholesterol efflux capacity was associated with a reduced cardiovascular risk (22).

We hypothesised that maternal and fetal cholesterol efflux as well as placental CYP27A1 expression would be increased in pre-eclampsia. This could be an attempt to remove cholesterol from cells and thus prevent further

lipid peroxidation and subsequent oxidative stress, whilst simultaneously having an athero-protective role, during pregnancy.

Material and Methods

Subjects and selection criteria

The study consisted of two groups of white European women (17 normotensive, 17 with pre-eclampsia; Table 1), where matched maternal, fetal and placental samples were available. Detailed demographics and outcome data have previously been published (23). Back power calculations using our cholesterol efflux capacity data revealed an 85.5% power with α of 0.5 to detect the differences observed. The study was approved by the Hospital Ethics Committee of the Nottingham University Hospitals; written, informed consent was obtained from each participant. Pre-eclampsia was defined as systolic blood pressure of $\geq 140/90$ mmHg on 2 occasions, and proteinuria ≥ 300 mg/L, 500 mg/day or $\geq 2+$ on a dipstick analysis of midstream urine after 20 weeks (24). The birthweight centile for each baby was computed, correcting for gestation age, sex, maternal parity and body mass index (BMI) (25). Small-for-gestational-age (SGA) was defined as a centile below the 10th, and appropriate-for-gestational-age (AGA) when the individualised birthweight ratio was between the 10th and 90th percentile (26). All women who took part in this study were labouring and either delivered vaginally or by emergency Caesarean section; no differences were observed in any measurements between Caesarean section and vaginal deliveries.

Sample collection and measurements

Maternal venous blood samples were taken prior to delivery. Where possible, fetal blood samples were collected from the umbilical cord vein, immediately after delivery of the placenta as previously described (23). All blood samples were collected in EDTA (plasma) or plain tubes (serum).

Full depth tissue biopsies were collected within ten minutes of the placenta being delivered as previously described (23) sampling half way between the cord insertion and periphery of the placenta, avoiding infarcts.

The lipoproteins (LDL-C, HDL-C, TC, TG) were previously measured in maternal and fetal serum from the same sample collection (21).

Cholesterol Efflux

Cholesterol efflux was determined in RAW264.7 cells (ATCC) as previously described (8), using maternal or fetal plasma as acceptors. Briefly, following labelling for 48h with 0.2 $\mu\text{Ci/mL}$ [^3H]-cholesterol (Anawa, Switzerland), cells were washed with PBS (pH 7.4), equilibrated for 18h in OptiMem and incubated for 2h in the presence or absence of 5% maternal or fetal plasma. The HDL-mediated cholesterol efflux was performed as described above, using 5% apoB-depleted plasma as the acceptor. For preparation of apoB-depleted plasma, 40 μl polyethylene glycol (20% in 200 mmol/l glycine buffer) was added to 100 μl plasma, incubated at room temperature for 20 minutes, and the supernatant was recovered after centrifugation (10,000 rpm, 20 minutes, 4°C) as described by Holzer *et al* (27). To measure the ABCA1-mediated efflux, the difference in efflux between control and 8-bromoadenosine 3',5'-cyclic monophosphate sodium salt-cAMP (8Br-cAMP) -stimulated cells was assessed using 5% plasma as the acceptor. For this, the incubation with OptiMem was replaced with DMEM containing 0.2% fatty acid free bovine serum albumin and 0.3 mM 8Br-cAMP.

For all 3 types of assays, radioactivity was measured in both supernatant and cells. Cholesterol efflux was calculated as percentage of labelled cholesterol released to the medium divided by the amount of total labelled cholesterol in the medium and cells in each well. To control for inter-assay plate variability, standard pooled plasma was prepared from several healthy (non-pregnant) volunteers and run on each plate as a reference; all assay plates were processed together in a single run. Both intra- and inter-assay coefficients of variation were < 5%.

Quantification of apoA1 and apoE

ApoA1 and apoE concentrations were determined in plasma using ELISA kits following the manufacturer's protocol (apoA1: 3710-1HP-2; apoE: 3712-1HP-2; Mabtech, Nacka Strand, Sweden). The inter- and intra-assay coefficients of variations were: apoA1 2.5% and 2.2% and apoE 2.5% and 2.8% respectively.

Cholesterol concentrations

The lipoproteins (LDL, HDL, TC, TG) were measured in serum as previously described (21) using MicroSlide technology on the Vitros Fusion 5.1 Chemistry System (New York, NY). All samples were analysed in triplicate, with the inter-assay variation being less than 10% and the intra-assay variation less than 5%.

27-OHC concentrations

27-OHC was quantified by gas chromatography-mass spectrometry in plasma (28) and placenta. For plasma, 200 μ l was used. The volume was adjusted to 500 μ l with saline, and 100 μ l butyl hydroxytoluol in 1% pyridine was added to avoid auto-oxidation. 5 α -cholestan-3 β ,6 α -diol (100 ng) was used as an internal standard. Saponification was performed with 1.5 ml of ethanolic NaOH 1M for 2h at 50°C, neutralized with 1 ml of phosphate buffer 0.1M pH 7.0 and pH adjusted to 7 with phosphoric acid. Samples were centrifuged for 5 min at 1000 g, and supernatants were harvested for the following clean-up procedure performed by solid-phase extraction: Sep Pak C18 cartridges were preconditioned with n-heptane/2-propanol (1:1, vol/vol), methanol and water before application of the supernatants. The cartridges were washed with methanol/water (3:1, vol/vol) and dried with nitrogen. 27-OHC was eluted by n-heptane/2-propanol (1:1) in a tube containing 100 μ l butyl hydroxytoluol in 1% pyridine and 100 ng of stigmasterol as a standard for chromatography and derivatisation. After evaporation of the solvent under nitrogen, samples were derivatised with 100 μ l pyridine and 100 μ l N,O-bis-trimethylsilyl-trifluoroacetamide for 1h at 60°C (29, 30). Samples were diluted with 200 μ l of cyclohexane and analysed by GC/MS by selected ion monitoring. A standard curve containing a mixture of known amounts of each 27-OHC was performed.

For 27-OHC quantification in placenta, 100 mg powdered tissue was homogenized in 1 ml Potassium phosphate buffer pH 7.2 (containing 300 mM sucrose, 0.5 mM dithiothreitol, 10 mM EDTA) and 100 μ l butyl hydroxytoluol in 1% pyridine, in Lysing Matrix tubes (MPI, Lucerna Chem AG, Switzerland). Cells debris was removed by centrifugation at 1500g for 1 min. The supernatant was collected, 100 ng internal standard 5 α -cholestan-3 β ,6 α -diol was added, and lipids were extracted with 19 volumes chloroform/methanol (2:1, vol/vol) for 30 min.

Samples were centrifuged at 2000g for 5 min and the lipid-containing chloroform layer was collected and transferred into a fresh tube containing 100 µl butyl hydroxytoluol in 1% pyridine. Solid phase extraction, desorption and derivatisation was performed as described for plasma.

CYP27A1 activity was determined in plasma by calculating the ratios of 27-OHC/(27-OHC + cholesterol). Cholesterol values were previously measured and reported in these samples (21).

RNA extraction, cDNA synthesis and quantitative real time polymerase chain reaction (qPCR)

Total RNA was extracted from ~100 mg placental tissue using QIAzol lysis reagent (Qiagen, UK) as previously described (31). RNA (1 µg) was reverse transcribed using the QuantiTect Reverse Transcription kit (Qiagen, UK) in a Primus96 thermocycler (Peqlab Ltd, UK).

Real-time PCR was carried out using SYBR Green chemistry (2x QuantiFast SYBR Green, Qiagen, UK) on a AB7500 Fast (Life Technologies, UK) using the primers in Supplemental Table S1. Abundance data for the genes of interest were expressed following normalisation using GeNORM (<http://medgen.ugent.be/~jvdesomp/genorm/>), with stably expressed housekeeping genes (GAPDH, β -2-microglobulin and β -actin), expressed as normalised copy number.

Immunohistochemical staining

Placental protein expression was analysed by immunohistochemistry as previously described (31), using CYP27A1 rabbit- (14739-1-AP; dilution 1:20; Proteintech, USA); AIBP goat- (sc-161321; dilution 1:50; Santa Cruz biotechnology, USA); SRB1 rabbit- (SAB3500048; dilution 1:600; Sigma-Aldrich, UK); ApoA1 rabbit- (A8599; dilution 1:300; Sigma-Aldrich, UK) polyclonal antibodies and ApoE mouse- (HPA001352; dilution 1:250; Sigma-Aldrich, UK) monoclonal antibody. All of the slides were assessed by the same observer (YTM), blinded to pregnancy outcome. Quantification was performed as described previously (31), using the Positive Pixel Algorithm of Aperio ImageScope software; a visual check was also performed.

Statistical analysis

All tests were performed using SPSS version 22. Summary data are presented as means \pm standard deviation (SD) or median and interquartile range (IQR) as appropriate. The Kruskal-Wallis test, followed by Mann-Whitney *U*-test was used for multiple group analysis. The Student's *t* test or Mann-Whitney *U*-tests were used depending on the distribution of the data, after testing using the Kolmogorov-Smirnov test. The null hypothesis was rejected where $P < 0.05$.

Results

Subjects

Demographic and obstetric data of the 34 participants are shown in Table 1. Within the pre-eclamptic group, 6 women delivered SGA infants and 11 delivered AGA infants (Table 2); no woman in the normotensive control group delivered an SGA infant.

Cholesterol Efflux

Cholesterol efflux capacity of both maternal and fetal plasma was increased in pre-eclampsia (by 10.2% and 9.3% respectively; $P < 0.05$ for both; Fig. 1A); cholesterol efflux capacity was lower in fetal compared to maternal plasma in both study groups (31% and 24% respectively; $P < 0.001$ for both; Fig. 1A). In addition, the same differences were observed when HDL-mediated cholesterol efflux capacity was performed with apoB-depleted plasma as an acceptor (increased in pre-eclamptics by maternal: 19.3%; fetal: 12.7%; Fig. 1B). Conversely, both maternal and fetal ABCA1-mediated efflux was significantly reduced in the pre-eclampsia samples (by 35% and 20% respectively; $P < 0.05$; Fig. 1 C). Data of cholesterol efflux capacity of stimulated and non-stimulated cells can be seen in Supplemental Figure S1.

Despite no differences being observed in the maternal cholesterol efflux capacity between groups (Figure 2A), the fetal cholesterol efflux capacity was lower in SGA compared to AGA infants only within the pre-eclamptic group (median [IQR]; SGA: 2.3 [2.1, 2.3]; AGA: 2.6 [2.4, 3.2] % cholesterol efflux; $P < 0.05$; Figure 2B).

ApoA1 and apoE concentrations

Maternal apoA1 concentrations were higher than fetal concentrations in both groups ($P < 0.05$; Table 3); no differences were observed for either maternal or fetal apoA1 between normotensive control and pre-eclamptic women ($P > 0.05$ for all; Table 3).

Maternal plasma apoE was higher than fetal apoE only in pre-eclampsia ($P<0.0001$; Table 3). ApoE concentrations were higher in maternal and fetal plasma in the pre-eclampsia group ($P<0.05$; Table 3). In the pre-eclamptic group, maternal apoE was not changed for SGA infants (Figure 2C), but fetal apoE was lower in SGA compared to AGA infants (median [IQR]; SGA: 0.42 [0.37, 0.47]; AGA: 0.56 [0.48, 0.64] g/L; $P<0.05$; Figure 2D).

Lipoprotein and 27-OHC concentrations

As previously reported (21), circulating total cholesterol, triglycerides and lipoproteins were not significantly different between groups, although lower levels were observed in both fetal normotensive control and pre-eclamptic group compared to the maternal samples (Table3).

We analysed 27-OHC concentration as a marker of CYP27A1 activity: this was reduced in fetal compared to maternal plasma, irrespective of diagnostic pregnancy group (Fig. 3A and 3B). Maternal 27-OHC was slightly, but not significantly, higher in pre-eclampsia (Fig. 3A). Fetal 27-OHC however, was significantly reduced in the pre-eclamptic group ($P<0.05$; Figure 3B). CYP27A1 activity (assessed by the ratio of 27-OHC/(27-OHC+ cholesterol)) was unchanged between normotensive women and those with pre-eclampsia in both maternal (mean \pm SD; 0.97 ± 0.01 vs. 0.97 ± 0.03 ng/ml) and fetal (0.97 ± 0.02 vs. 0.96 ± 0.02 ng/ml) plasma ($P>0.05$ for both).

Placental 27-OHC concentrations were significantly increased in tissues obtained from pre-eclamptic mothers (mean \pm SD; normotensive controls: 15.1 ± 1.8 ; pre-eclampsia 17.2 ± 3.4 ng/ml; $P<0.05$; Fig. 3C).

Placental CYP27A1, AIBP, ApoA1, ApoE & SCB1 expression

Expression of both *CYP27A1* and *AIBP* mRNA was detected in placental tissue but there were no significant differences between groups for both (median [IQR] normalised copy numbers; normotensive controls vs. pre-

eclampsia) *CYP27A1* (1201 [293, 2667] vs. 1013 [677, 6657]) or *AIBP* (284 [154, 526] vs. 310 [220, 616]); $P>0.05$ for both.

CYP27A1 protein was localised by immunohistochemistry around fetal vessels and in Hofbauer cells (Figure 4). *CYP27A1* protein expression was increased in pre-eclampsia (mean \pm SD; pre-eclampsia: 0.06 ± 0.07 ; normotensive controls: 0.02 ± 0.02 ; $P=0.04$; Figure 4). Within the pre-eclamptic group, *CYP27A1* expression was higher in pregnancies delivering SGA compared to AGA infants (mean \pm SD; SGA: 0.11 ± 0.01 ; AGA: 0.04 ± 0.04 ; $P=0.02$). No differences were observed between early- and late-onset pre-eclampsia and normotensive controls (ANOVA: $P>0.05$).

AIBP protein was localised to fetal vessels and syncytiotrophoblasts (Figure 5). *AIBP* protein expression was also increased in pre-eclampsia compared to the control group (mean \pm SD; pre-eclampsia: 0.78 ± 0.10 ; normotensive controls: 0.66 ± 0.14 ; $P=0.004$; Figure 5). No differences were found between pre-eclamptic women delivering an AGA or SGA infant ($P>0.05$).

Protein expression of ApoA1, ApoE & SCB1 was also confirmed and localised to syncytiotrophoblasts; there were no differences between groups for any of these proteins ($P>0.05$; data not shown).

Discussion

It is currently recognised that women who develop pre-eclampsia have an increased risk of developing atherosclerosis later on in life (32). Low birth weight is one of the classic risk factors for developing cardiovascular disease in adult life: children born to women who experience pre-eclampsia in their pregnancy are susceptible to these risk factors in such a way as to develop increased BMI and hypertension at an earlier stage in adulthood than may otherwise occur purely due to aging (33).

Therefore in this study, we compared normotensive women to those with pre-eclampsia, analysing the ability of maternal and fetal plasma to remove cholesterol from the cell. We further supplemented our previous lipid profile analyses by quantifying 27-OHC in plasma and CYP27A1 activity, and by assessing the efflux capacity of plasma under various conditions. Additionally, we were able to assess the concentration of 27-OHC in matched placental tissue. This is a novel and detailed study of placental CYP27A1, 27-OHC and AIBP proteins, strengthened by the determination of cholesterol efflux capacity in matched maternal and fetal plasma samples.

It is well established that in addition to hypertension, altered cholesterol metabolism is also linked to cardiovascular events. In non-pregnant individuals, a low level of HDL-C is a major independent risk factor for atherosclerotic CVD (34). However, randomised clinical trials aimed at improving HDL-C concentrations have not shown any significant protective effect in cardiovascular outcomes (35), strongly suggesting the importance of alternative mechanisms or combinations of diagnostic biomarkers. It has recently been advocated that a more important factor may actually be the ability of HDL-C to act as an acceptor for the cholesterol discharged from macrophages. Cholesterol efflux is the first and rate limiting step of RCT (22) and animal studies support a direct and causal link between macrophage-specific cholesterol efflux capacity and prevention of atherosclerosis (36). Furthermore, studies have revealed inverse relationships between cholesterol efflux capacity and prevalent coronary artery disease, which are independent of HDL-C levels (37). The role of CYP27A1 in cholesterol efflux has been shown (8), and the deleterious effect of its mutation in patients with cerebrotendinous xanthomatosis underlines the importance of this enzyme in RCT and atherosclerosis prevention.

The increased maternal and fetal cholesterol efflux capacity in the pre-eclamptic group suggests a potential adaptation to extrude cholesterol out of cells in pre-eclampsia. It is conceivable that a reduction in the oxidation of LDL-C would result in attenuation of lipid peroxidation (Figure 6). We have previously reported that these fetal samples had lower circulating HDL-C concentrations (21), but when corrected for HDL concentrations, the differences in efflux capacity remained as did the HDL-mediated efflux. Thus, we might hypothesise that the increased cholesterol efflux capacity in both the women with pre-eclampsia and their fetuses could be a protective mechanism to prevent excessive lipid peroxidation in these babies. In light of the fact that overall cholesterol efflux capacity is increased in pre-eclampsia, the specific reduction in ABCA1-mediated cholesterol efflux in pre-eclampsia suggests that other components of this efflux system may be increased in order to compensate. Since apoE complexed to HDL-C has also been reported to promote cholesterol efflux (38), and as apoE is increased in our pre-eclampsia group, both maternal and fetal, the apoE-mediated efflux might counteract the reduction in ABCA-1 mediated efflux. The increased apoE levels may also have a positive contribution towards offsetting the oxidative stress and inflammatory states reported in pre-eclampsia (39), through its reported antioxidant and anti-inflammatory role (40).

The localisation of CYP27A1 protein, around fetal vessels and within Hofbauer cells in the placenta, also points towards a functional role in cholesterol efflux process facilitating the removal of the cholesterol from the tissue via the vascular circulation. The increased placental CYP27A1 expression and production of 27-OHC in pre-eclampsia may reflect placental dysfunction, contributing to increased lipid peroxidation within the tissue. Furthermore, placental ABCA1 is known to be down-regulated in pre-eclampsia (41) thus increased CYP27A1 activity could, at least in part, contribute to a protective mechanism by providing sufficient ligand to activate the LXR-ABCA1 pathway.

In addition, the higher placental CYP27A1 in pre-eclamptic women delivering SGA infants further supports the notion of inappropriate fetal cholesterol uptake in these cases. Higher fetal and placental oxidised LDL-C concentrations have been reported from women delivering FGR infants (42), suggesting a disturbed cholesterol

supply to these affected fetuses. Our current study further strengthens this hypothesis reporting even further reduction of fetal cholesterol efflux and apoE concentration in SGA babies from women experiencing pre-eclampsia in their pregnancy.

Fang *et al.*, have recently reported that effective cholesterol efflux is critical for proper angiogenesis (14). They observed that both AIBP and HDL-C mediated cholesterol efflux alters the composition of the lipid rafts, due to reduced CAV-1, as well as interfering with vascular endothelial growth factor receptor 1 (VEGFR2; KDR) dimerization and signalling, thus inhibiting VEGF-induced angiogenesis (14, 15). The authors concluded that AIBP positively regulates cholesterol efflux from endothelial cells and that effective cholesterol efflux is critical for proper angiogenesis (14). Our current result demonstrating increased placental AIBP together with our previously observed reduction in placental CAV-1 expression in pre-eclampsia (43) and the reported reduction of VEGFR2 (44), all considered together intimates at an inappropriate lipid homeostasis. This may inhibit/disrupt placental angiogenesis, contributing to the endothelial dysfunction that is characteristic of pre-eclampsia. The raised expression in early-onset pre-eclampsia further supports this theory as placental dysfunction is only seen in these pregnancies but not in late-onset pre-eclampsia. Further studies are required to tease out the balance of positive *vs.* detrimental effects of increased cholesterol efflux in pre-eclampsia.

Despite there being no significant differences between groups in the mRNA expression of neither *CYP27A1* nor *AIBP*, both proteins showed an increased expression in placentae from pre-eclamptic but not normotensive women (Figures 3 & 4). This suggests possible existence of miRNAs, some post-translational modifications of these proteins or changes in protein half-life in pre-eclampsia contributing to the lipid peroxidation. This is further substantiated by the additional increases in protein expression of *CYP27A1* and 27-OHC in placentae from pre-eclamptic mothers.

Melchiorre *et al.*, showed that the majority (56%) of early-onset pre-eclampsia had stage B asymptomatic heart failure 1 year postpartum (compared to just 14% late-onset and 8% of controls). Even more concerning was the

observation that 40% of these women with previous early-onset pre-eclampsia, developed hypertension within 2-years of pregnancy (45). Another prospective study, with a long-term follow-up of 37 years, reported the mutually adjusted hazard ratio of subsequent cardiovascular death in pre-eclamptic women was 2.14, with a 95% confidence interval (CI) of 1.29-3.57. For the women who had early-onset pre-eclampsia, the hazard ratio increased to 9.54 (95% CI: 4.5-20.3), further emphasising the importance of severity of the syndrome in terms of cardiovascular disease risks (46). Although in the present investigation, sample numbers were small, our data demonstrate increased cholesterol efflux capacity only in late-onset pre-eclampsia. This indicates that mothers with the more severe form of pre-eclampsia (i.e. early-onset), as well as their babies, may not have the capacity to protect against lipid peroxidation or have not been exposed to metabolic challenge as those with late-onset disease. Additionally, poor placentation, characteristic of early-onset pre-eclampsia, has been previously associated with increased lipid peroxidation, further compromising lipid metabolism in both the mother and the fetus. It is possible that this phenomenon may also continue postpartum, but further work is required to test this hypothesis.

Figure 6 summarises the potential mechanisms relating CYP27A1, 27-OHC and cholesterol efflux capacity in pre-eclampsia. The raised placental AIBP may contribute to the impaired angiogenesis and extravillous trophoblast (EVT) invasion, possibly leading to increased oxidative stress and endothelial dysfunction. In contrast, the increased placental CYP27A1, which parallels the increased 27-OHC placenta concentration may lead to the observed increased cholesterol efflux, thus trying to dampen the lipid peroxidation and associated oxidative stress/endothelial dysfunction, providing a compensatory response.

A limitation of this study was the relatively low participant numbers, which prevents sub-group analysis between the more severe, early-onset and more moderate late-onset, forms of pre-eclampsia (diagnosis before or after 34 weeks' gestation). Future work includes postpartum follow-up of these women and their offspring, both in the short- and long-term. Exploring alterations in their cholesterol efflux capacity in combination with other known risk factors may provide a novel and more accurate biomarker for prediction of cardiovascular disease.

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Table 1 Clinical and obstetric data of subject groups*

Parameter	NC	PE
	n = 17	n = 17
Age (yrs)	28.2 ± 7.2	31 ± 6.5
Body mass index at first visit (<14 weeks' gestation; Kg/m ²)	26.4 ± 5.5	24.8 ± 3.5
Smoking status		
Non-smoker	9 (53)	11 (65)
Smoker	8 (47)	6 (35)
Parity		
Nulliparous	11 (65)	10 (59)
Multiparous	6 (35)	7 (41)
Max. systolic blood pressure (mmHg)	114 ± 3.8	155 ± 3.8 [†]
Max. diastolic blood pressure (mmHg)	75 ± 2.1	97 ± 5.0 [†]
Proteinuria (g/L) Median [min, max]	-	1.0 [0.3, 9.4]
Gestational age at delivery (Wks)	40 ± 1.0	37.7 ± 1.8
Mean birthweight (g)	3439 ± 498	2993 ± 735 [†]
Corrected birthweight centile	27.9 [17.5, 66.8]	35.1 [8.6, 76.3]
Caesarean section	4 (24)	13 (76)

*Data represented as mean ± SD or median [IQR] as appropriate, except for smoking status, parity and Caesarean sections and early-onset PE, which are shown as number (percentage). NC: normotensive control; PE: pre-eclampsia; BMI: body mass index. [†] $P < 0.05$ between NC and women with PE.

Table 2 Clinical and obstetrics data of the pre-eclamptic women delivery AGA and SGA infants*

Parameter	PE-AGA	PE-SGA
	n = 11	n = 6
Age (yrs)	30.6 ± 7.1	31.5 ± 3.9
Gestational age at delivery (weeks)	38.8 ± 1.2	36.5 ± 2.4
Mean birthweight (g)	3505 ± 658	2175 ± 611 [†]
Corrected birthweight centile	64.5 [33.5, 86.2]	4.1 [0.5, 8.5] [†]

*Data represented as mean ± SD or median [IQR] as appropriate. PE: pre-eclampsia; AGA: adequate-for-gestational age; SGA: small-for-gestational-age; [†] $P < 0.05$ between PE-AGA and PE-SGA

Table 3 Maternal and fetal lipoprotein, apoA1 and apoE concentration*

Parameter	NC	PE
	n = 17	n = 17
<i>Lipoprotein concentrations (mmol/L)[#]</i>		
Maternal total cholesterol	5.6 ± 2.2	6.4 ± 1.1
Fetal total cholesterol	2.6 ± 1.5 [‡]	1.7 ± 0.3 [‡]
Maternal triglycerides	2.2 ± 0.7	2.6 ± 0.6
Fetal triglycerides	1.1 ± 0.9 [‡]	0.7 ± 0.9 [‡]
Maternal HDL	1.5 ± 0.3	1.4 ± 0.9
Fetal HDL	0.9 ± 0.4 [‡]	0.6 ± 0.1 [‡]
Maternal LDL	3.5 ± 1.9	3.8 ± 0.1
Fetal LDL	1.2 ± 0.8 [‡]	0.8 ± 0.3 [‡]
<i>ApoA1 & apoE concentrations (g/L)</i>		
Maternal apoA1	19.3 ± 9.3	23.6 ± 7.4
Fetal apoA1	2.4 ± 0.9 [‡]	2.8 ± 1.4 [‡]
Maternal apoE	0.6 ± 0.4	1.0 ± 0.5 [†]
Fetal apoE	0.4 ± 0.1	0.5 ± 0.1 ^{†‡}

*Data represented as mean ± SD. NC: normotensive control; PE: pre-eclampsia; OHC: hydroxycholesterol;

[†]*P*<0.05 between normotensive controls and women with pre-eclampsia; [#]lipoprotein concentrations previously measured (21), [‡]*P*<0.05 between maternal and fetal samples.

Figure 1: Cholesterol efflux with maternal and fetal plasma. A) Cholesterol efflux capacity (%); B) HDL-mediated (PEG treated) cholesterol efflux (%) and C) ABCA1-mediated cholesterol efflux from normotensive controls (NC) and pre-eclamptic (PE) plasma. Data are represented as median [IQR]; * $P < 0.05$; *** $P < 0.0001$.

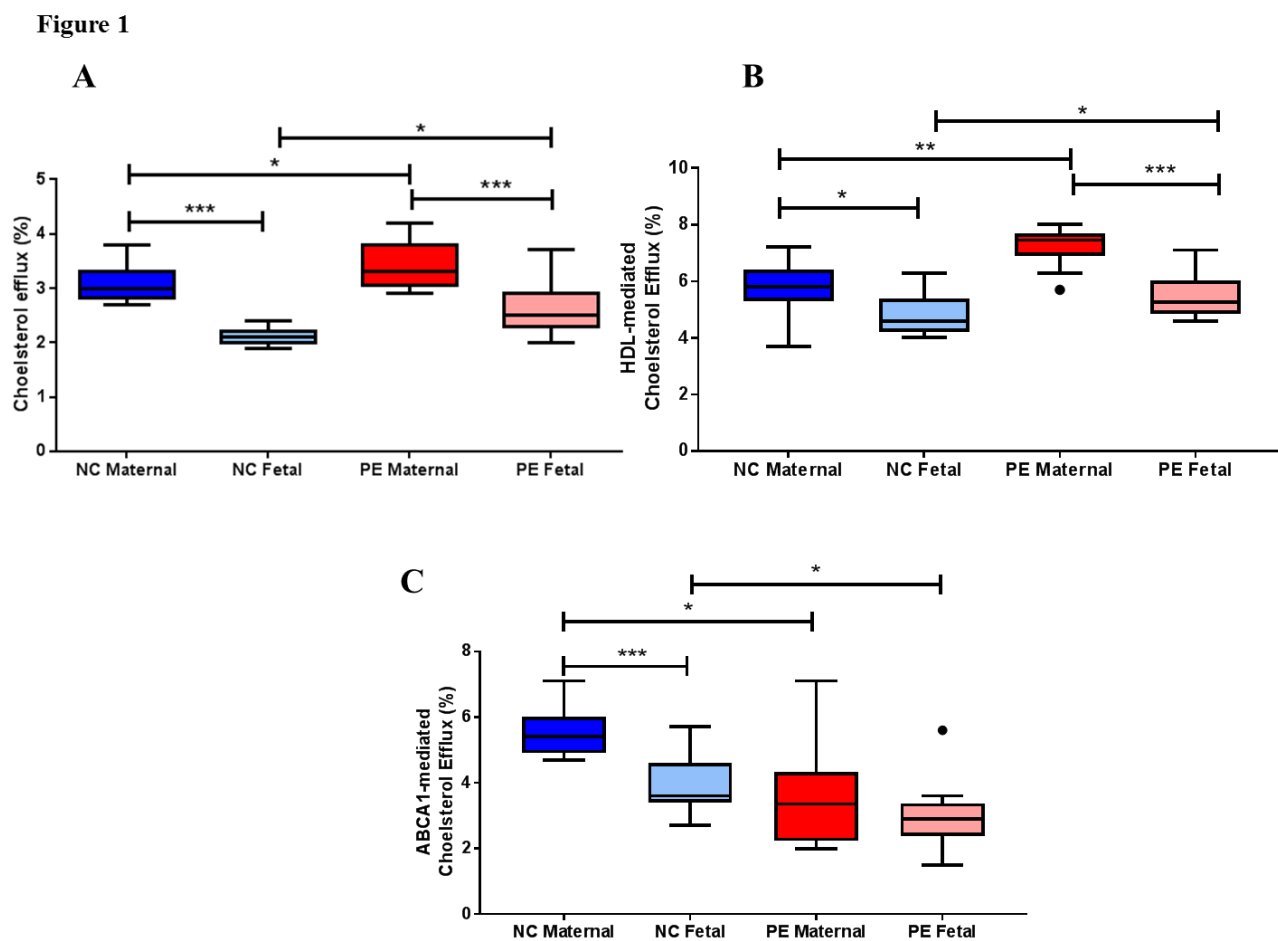


Figure 2: Cholesterol efflux capacity and apoE in PE with AGA or SGA. Maternal (A) and fetal (B) cholesterol efflux was quantified in pre-eclamptic pregnancies from adequate-for-gestational-age (AGA; n = 11) and small-for-gestational-age (SGA; n = 6). ApoE was quantified in maternal (C) and fetal (D) plasma from pre-eclamptic pregnancies with and without SGA. Data are represented as median [IQR]; * $P < 0.05$.

Figure 2

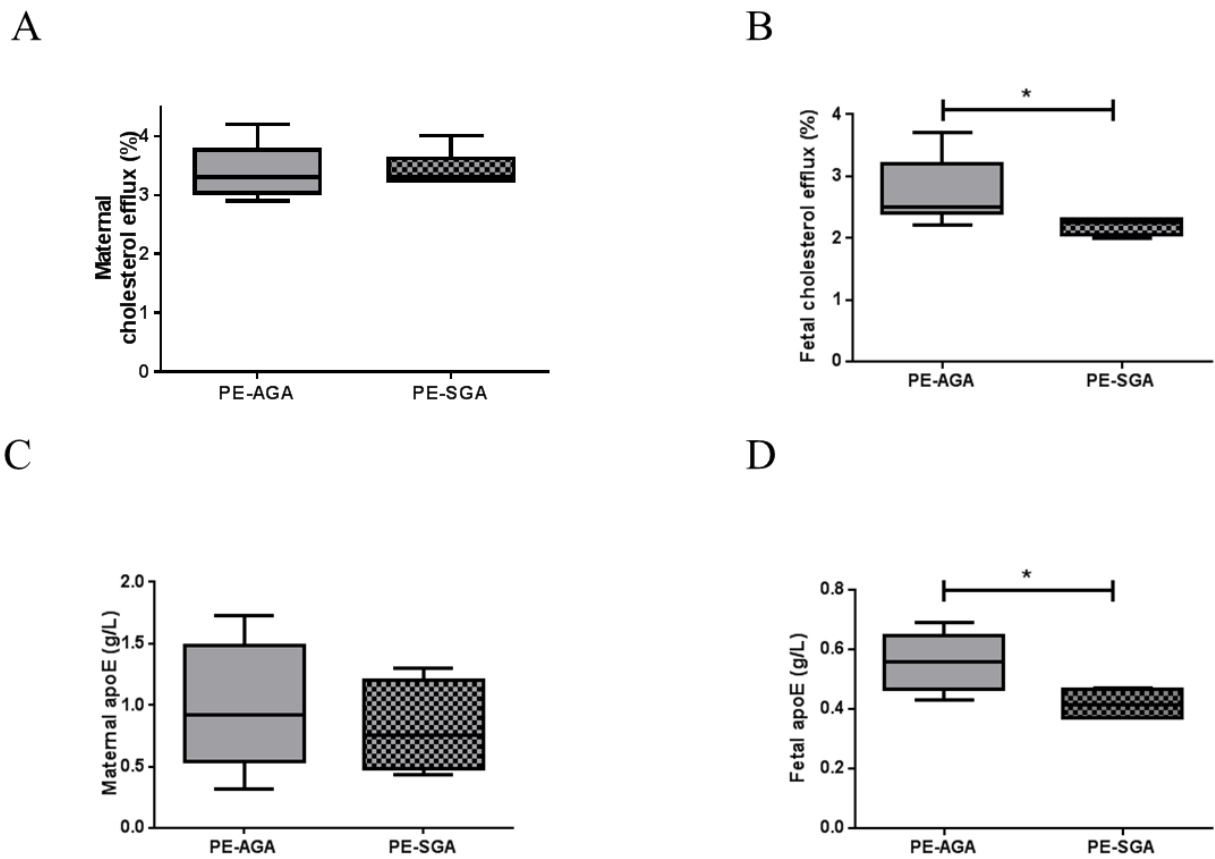


Figure 3: Quantification of 27-OHC in maternal plasma (A); fetal plasma (B) and placental tissue C). 27-hydroxycholesterol (27-OHC) concentrations were quantified by GC-MS in plasma from normotensive controls (NC) and pre-eclampsics (PE). In the graphs data are represented as mean \pm SD; * P <0.05.

Figure 3

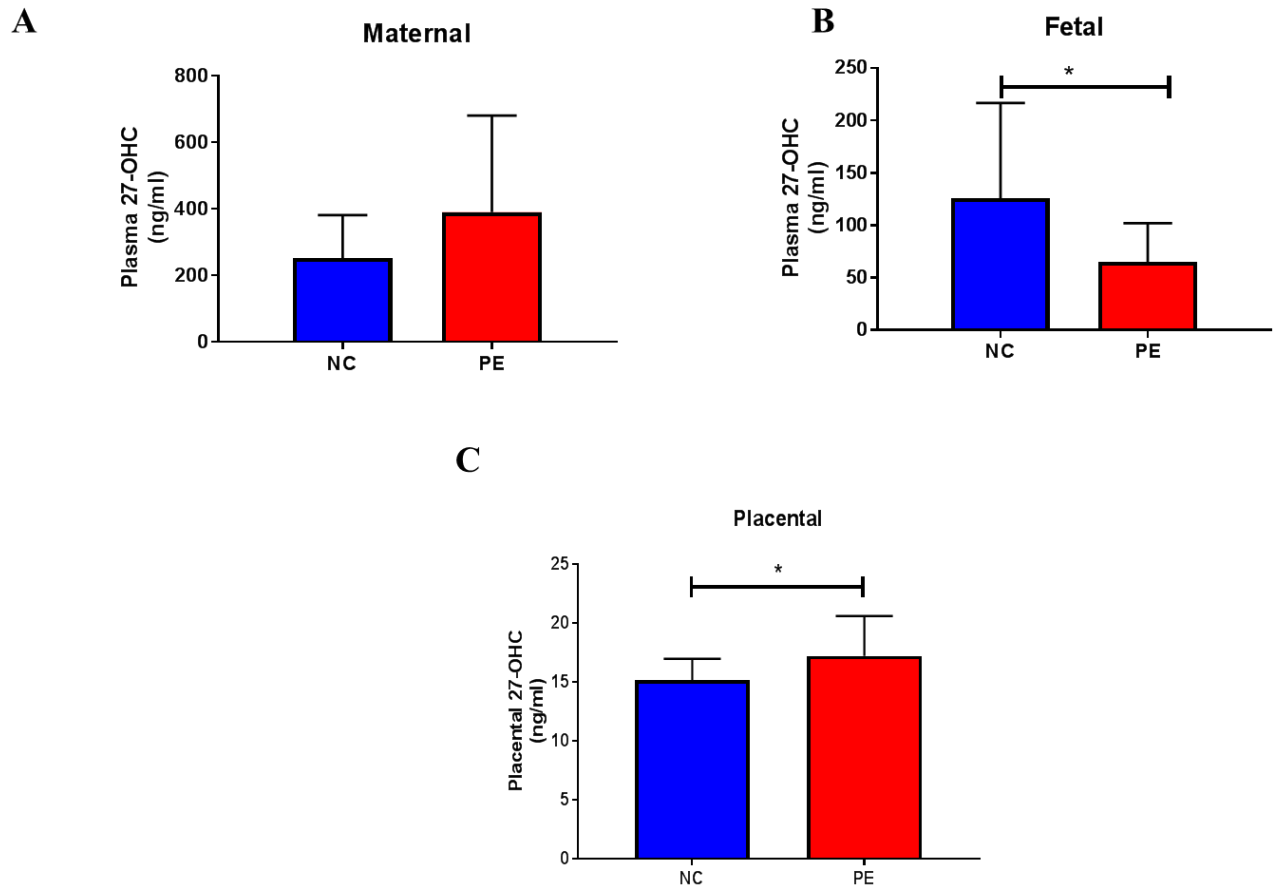


Figure 4: Localisation and quantification of placental CYP27A1 immunostaining in placentae from A) normotensive controls (NC; n = 17) and B) pre-eclampsia (PE; n = 17) and C) negative control. In photomicrographs, positive cells appear in brown; magnification x400. Protein expression was localised to Hofbauer cells (black arrows) and fetal vessels (red arrows). In the graph (D), data are represented as mean \pm SD; * P <0.05.

Figure 4

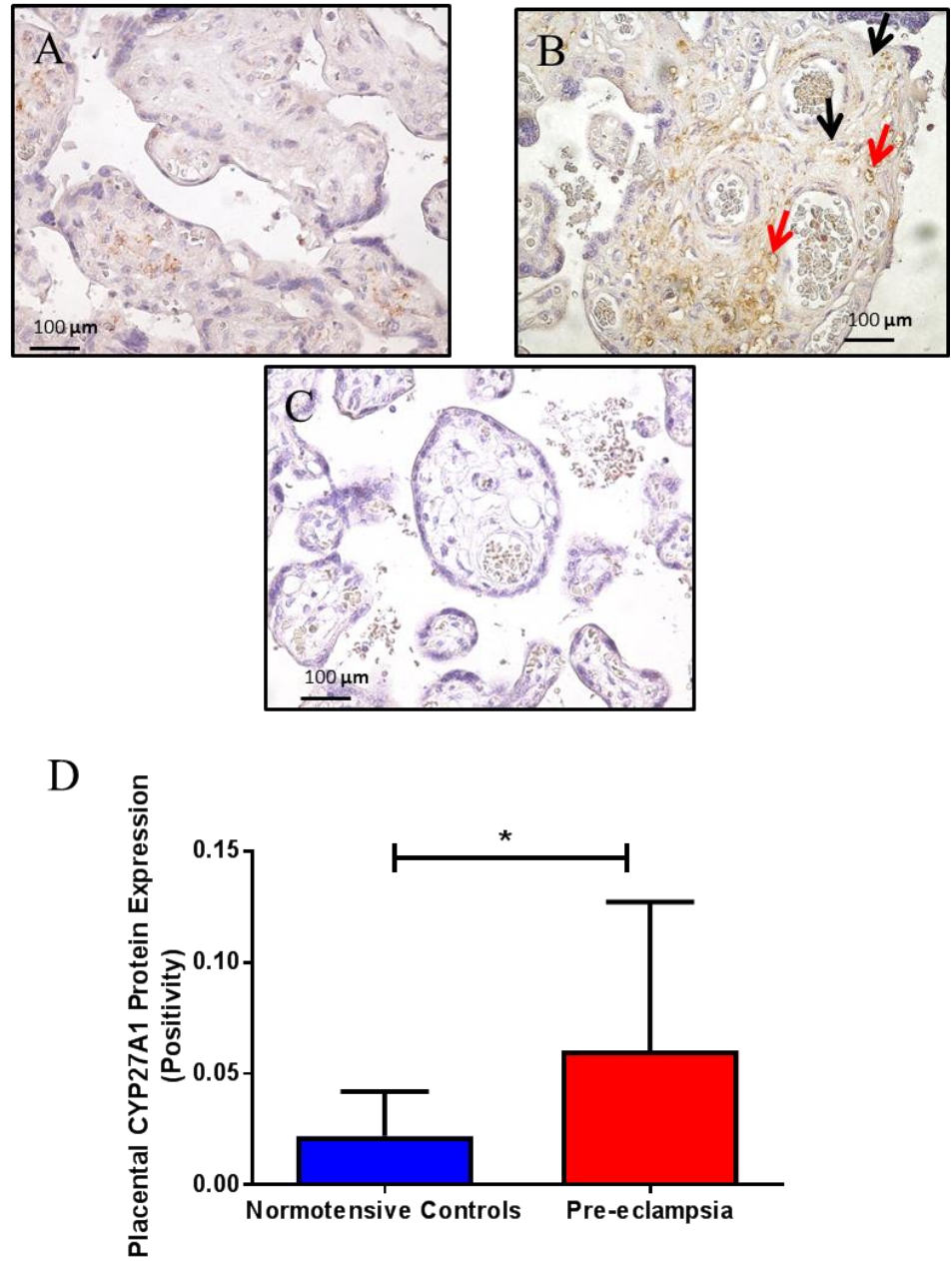
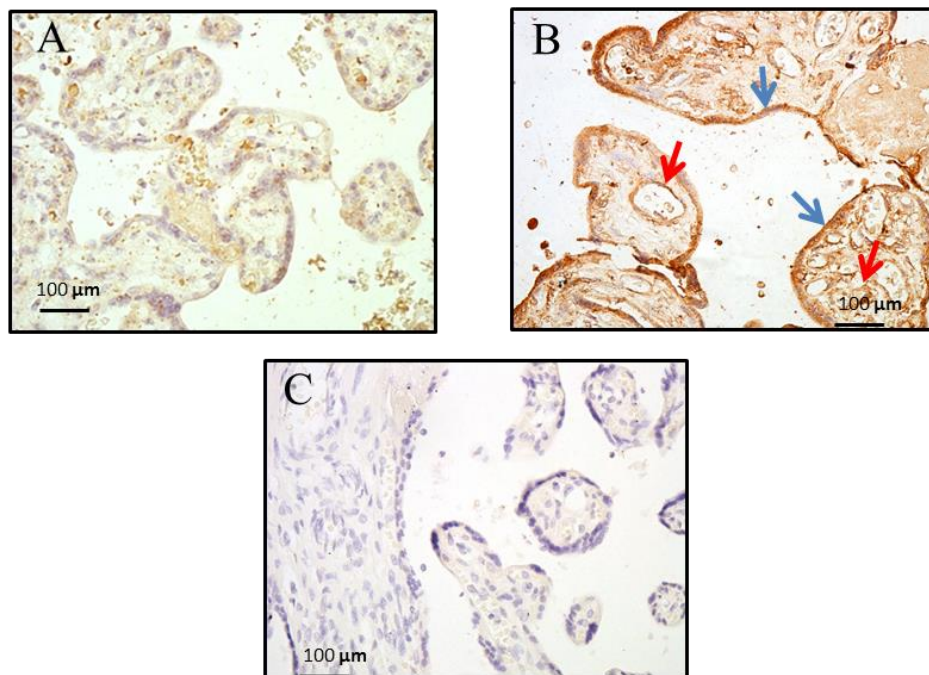


Figure 5: Localisation and quantification of placental AIBP immunostaining in placentae from A) normotensive controls (NC; n = 17) and B) pre-eclampsia (PE; n = 17) and C) negative control. In photomicrographs, positive cells appear in brown; magnification x400. Protein expression was localised to fetal vessels (red arrows) and syncytiotrophoblasts (blue arrows). In the graph (D), data are represented as mean \pm SD; $**P < 0.001$.

Figure 5



D

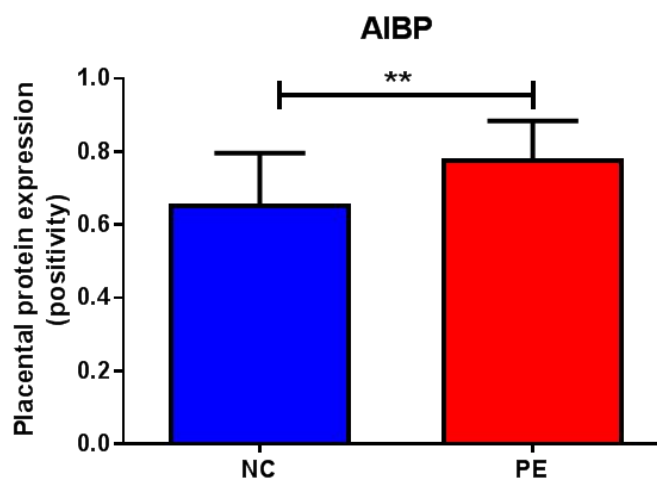


Figure 6: Schematic diagram illustrating the changes observed in pre-eclampsia to suggest a potential link between changes in cholesterol efflux and the alteration in cholesterol metabolism. The raised placental AIBP may contribute to the impaired angiogenesis/extravillous trophoblast (EVT) invasion, possibly leading to increased oxidative stress, and endothelial dysfunction. In contrast, the increased placental CYP27A1, which parallels the increased 27-OHC placenta concentration may lead to the increased cholesterol efflux observed, thus dampening the lipid peroxidation and associated oxidative stress/endothelial dysfunction, providing a compensatory response.

Figure 6

