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Mistry, Hiten D. and Wilson, Vicky and Ramsay, Margaret M. and Symonds, Michael E. and Broughton Pipkin, Fiona (2008) Reduced selenium concentrations and glutathione peroxidase activity in preeclamptic pregnancies. *Hypertension*, 52 . pp. 881-888. ISSN 0194-911X

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**REDUCED SELENIUM CONCENTRATIONS AND GLUTATHIONE PEROXIDASE
ACTIVITY IN PREECLAMPTIC PREGNANCIES**

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Short title: Selenium, glutathione peroxidase & preeclampsia.

Word Count: 5,715

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Abstract

Preeclampsia is a pregnancy-specific condition affecting 2-7% of women and a leading cause of perinatal and maternal morbidity and mortality. Preeclampsia may also predispose the fetus to increased risks of adult cardiovascular disease. Selenium, acting through the selenoprotein glutathione peroxidases, has critical roles in regulating antioxidant status. Recent reports implicate poor maternal selenium status as a nutritional factor predisposing the mother to preeclampsia but the fetus and placenta have not been studied in tandem. Measurement of selenium concentrations, expression and activity levels of glutathione peroxidase and markers of oxidative stress were performed on maternal and umbilical venous blood samples or the placenta from 27 normal pregnant, 25 preeclamptic and 22 healthy age-matched non-pregnant women. The results of this study revealed highly significant reductions in serum selenium concentrations and plasma glutathione peroxidase activity in pregnancy *per se* compared to non-pregnant controls. Moreover, these levels were further decreased in the preeclamptic mothers and babies compared to normal pregnancies. Umbilical venous selenium was particularly low (42.1 ± 11.8 and 29.0 ± 9.9 $\mu\text{g/L}$; mean \pm s.d.; $P < 0.05$). Both mother and baby had significantly increased levels of markers for oxidative stress in the preeclamptic group. The placental glutathione peroxidase activity and immunohistochemical staining were also reduced in the preeclampsia placentae. Oxidative stress associated with preeclampsia may be a consequence of reduced antioxidant defence pathways specifically involving glutathione peroxidases, perhaps linked to reduced selenium availability. Reduced glutathione peroxidases could be associated with increased generation of toxic lipid peroxides contributing to the endothelial dysfunction and hypertension of preeclampsia.

Key Words: pregnancy, human; preeclampsia; hypertension; oxidative stress; selenium; glutathione peroxidase; placenta.

Introduction

Preeclampsia is estimated to occur in 2-7% of all pregnancies and is a leading cause of maternal and perinatal mortality and morbidity in the Western world¹; together with other hypertensive disorders of pregnancy it is responsible for approximately 60,000 deaths each year². Moreover, the effects of the disease are not restricted to pregnancy as it also predisposes both the mother and baby to adult cardiovascular disease³. Preeclampsia is now commonly regarded as being a state of oxidative stress, thought to arise from a biochemical imbalance which occurs from excessive generation of free radical formation and/or inadequate antioxidant capacity (see: ⁴). It is thought that excessive production of reactive oxygen species (ROS), resulting in oxidative stress secondary to reduced placental perfusion plays a critical role as a possible mediator of endothelial cell dysfunction⁵, hypertension and thus clinical manifestations of preeclampsia⁶.

The trace element selenium is an essential component of the antioxidant selenoproteins, including glutathione peroxidases (GPx). These remove the products of attack by ROS (hydroperoxides and oxidized lipoproteins) which can break down to further reactive free radicals and cytotoxic agents⁷, and so limit adverse effects on the endothelium⁸. Various forms of GPx are found in vertebrates: the cellular and cytosolic GPx (GPx1), the cytosolic gastrointestinal GPx (GPx2), the extracellular plasma GPx (GPx3) and the phospholipid hydroperoxide GPx (GPx4)⁹. Two studies have shown decreases in maternal serum or toenail selenium concentrations in preeclamptic patients compared to normal pregnant controls^{10, 11} and other studies have reported significant reductions in maternal plasma¹² and placental^{13, 14} GPx activities in preeclamptic patients. We are not aware of any studies linking selenium and GPx in the mother, placenta and baby. We hypothesized that fetal selenium and GPx concentrations would also be reduced in preeclamptic pregnancy, which could contribute to

present morbidity and future cardiovascular risk. We have therefore conducted a cross-sectional study to explore these factors in tandem comparing patients and their babies from normal and preeclamptic pregnancies and, when appropriate, non-pregnant controls.

Methods

Power calculations: We calculated that a sample of 25 normal pregnant and 25 preeclamptic women would give us a 95% power of detecting a 30% difference in serum selenium concentration.

Subjects: The study population consisted of three groups of Caucasian women: 27 normal, 25 preeclamptic and 22 healthy age-matched women. The investigations were approved by the Hospital Ethics Committee of the Queen's Medical Centre, Nottingham and written, informed consent was obtained from each participant. Cases were defined on admission with a clinical diagnosis of preeclampsia, defined as a systolic blood pressure of ≥ 140 mm Hg and diastolic pressure (Korokoff V) of ≥ 90 mm Hg on 2 occasions after 20 weeks gestation in a previously normotensive women and proteinuria > 300 mg/L, 500 mg/day or 2+ on dipstick analysis of midstream urine (MSU) if 24-hour collection result was not available. Medical and obstetric histories, including delivery data, were obtained for each woman. The birthweight centile for each baby was computed, correcting for gestation age, sex, maternal parity and body mass index (BMI)¹⁵.

Sample collection: Venous blood samples were collected in chilled tubes containing ethylenediaminetetraacetic acid (EDTA; GPx assays), heparin (thiobarbituric acid reactive substances (TBARS) assay) and plain tubes for serum (selenium assays) from both maternal and, where possible, umbilical cord before delivery and immediately after respectively.

Plasma and serum were stored at -80°C until biochemical analysis were performed. In addition placental tissue samples were collected from three standardized locations between cord insertion and placental border; one set were snap frozen and stored at -80°C. Another set were formalin fixed and wax-embedded for immunohistochemical analysis.

Tissue preparation: Placental tissue fragments (100 mg) were thawed and homogenized in 3 volumes of RIPA buffer (1 x PBS (pH 7.4), 0.1 % SDS, 1 % Igepal CA-630 (Sigma-Aldrich, Poole, UK) and 0.5% Sodium Deoxycholate) using an Ultra Turrax homogenizer. Samples were centrifuged (3000 g, 10 mins, 4°C) and supernatants were stored at -80°C. Protein concentrations were determined using the Lowry method¹⁶.

Biochemical measurements: The plasma concentration of TBARS (a global measure of lipid peroxidation) was measured by the method of Urchiyama and Milhara¹⁷. Samples were assayed in duplicate; the within- and between-assay coefficients of variation were 4% and 5% respectively

Serum selenium concentrations were determined by a Varian SpectrAA graphite furnace atomic absorption spectrophotometer¹⁸. Standard reference samples (Seronorm and serum control; purchased from Nycomed Pharma AS, Norway and UTAK trace element control, normal level) were used. The intra- and inter-assay coefficients of variances were < 5% for both.

mRNA expression measurements: Total RNA was extracted from a known amount of placental tissue (between 50 – 100 mg) using Tri-Reagent (Sigma-Aldrich, Poole, UK). Following RNA extraction, 1 µg of each samples was reverse transcribed in a 20 µl reaction

buffer (250 mM Tris.HCl, 40 mM KCl, 5 mM dithioerythritol pH 8.5), 2 mM dNTPs, 1 x hexanucleotide mix, 10 units RNase inhibitor, 10 units Reverse transcriptase (RT) (Roche Diagnostics, Lewes, UK) in a Touchgene Gradient thermocycler (Techne, Barloworld Scientific Ltd, Stone, UK). The conditions used to generate first strand cDNA were 72°C (5 min), 4°C (2 min), 25°C (5 min), 25°C (10 min), 42°C (60 min), and 72°C (10 min). RNA concentration and quality were verified by gel electrophoresis and spectrophotometrically; all samples had an A_{260}/A_{280} ratio greater than 1.96 and were stored at -20°C.

Standards for GPx 1, 2, 3, 4, and for the housekeeping gene 18s ribosomal RNA were made from cDNA obtained from a randomly selected control placental sample using semi-quantitative polymerase chain reaction (PCR). The method used oligonucleotide primers to GPx 1, 2, 3, 4, and 18s genes generating specific intron-spanning products (Table 1). The PCR program comprised an initial denaturation stage (95°C, 15 min), amplification (stage I, 94°C (30 s); stage II, annealing temperature (60°C, 30 s); stage III, 72°C (1 min), and final extension (72°C, 7 min; 8°C 'hold'). The PCR mixture (final volume 20 µl) contained 7 µl nuclease-free water (Ambion), 10 µl thermo-start PCR master mix (ABgene, Epsom, UK), 1 µl forward primer, 1 µl reverse primer, and 1 µl RT (cDNA) product. The annealing temperature (60°C) and cycle number of all primers were optimized and used in their linear range. The resultant PCR product was extracted after agarose gel electrophoresis (QIAquick gel extraction kit, Qiagen®, cat no. 28704), sequenced, and results cross-referenced against the Genbank website to determine specificity of the target gene. Extracted PCR products were resuspended in nuclease-free water and a 10-fold serial dilution performed. Standards were stored at -20°C until use in quantitative PCR.

Quantitative PCR was used to examine the expression of GPx 1, 2, 3, 4, and 18s. PCR reactions, set up in duplicate, were carried out in 20 µl volumes consisting of 1 × SYBR® PCR mastermix (with ROX passive reference dye) (Qiagen Ltd, Crawley, UK), 500 nM forward primer, 500 nM reverse primer, and 7 µl nuclease-free water. Real time PCR was performed in a Techne Quantica™ 14 real-time thermocycler (Techne, Barloworld Scientific Ltd) on all samples at 95°C (15 min) followed by 45 cycles of 95°C (15 s), annealing temperature at 60°C (25 s), 72°C (25 s). Three negative control reactions were carried out with each set of samples analyzed: (1) no RNA template but RT and polymerase provided; (2) RNA and polymerase provided but no RT; and (3) RNA and RT provided but no polymerase.

GPx activity assay: GPx activity was determined by a modified method of Paglia and Valentine¹⁹ in both plasma and placental extracts. Activity was measured spectrophotometrically in duplicate by coupling the oxidation of glutathione and nicotinamide adenine dinucleotide phosphate (NADPH) using glutathione reductase. Briefly, 900 µl of assay mix containing 0.1 M potassium phosphate, pH 7.0; 2 mM EDTA; 0.5 U/ml glutathione reductase; 10 mM glutathione and 0.3 mM reduced NADPH was placed into a WPA Biotech spectrophotometer set at 340 nm. Diluted tissue extracts or plasma (50 µl of 1/10 dilution) were added to the cuvette along with 50 µl of 20 mM tert-butyl hydroperoxide, a suitable substrate for GPx. The decrease in A₃₄₀ was determined over a 3-min period and rate calculations were performed. GPx activity was standardised against protein concentrations and expressed as mmol/min/mg protein or mmol/min/ml for plasma samples; the inter- and intra-assay variations were < 5%.

Histological analysis: Serial sections of 5µm were cut in the same orientation from paraffin-embedded tissue blocks (Sledge Microtome, Anglia Scientific, Norwich, UK) and mounted

onto Superfrost plus glass microscope slides (Menzel-Glaser, Braunschweig, Germany). Before use, sections were dewaxed by immersion in xylene followed by rehydration in descending concentrations of alcohol (3 min each).

Immunohistochemistry: Immunohistochemical analysis was performed using the Dako Envision™ visualization system (Dako, Ely, UK). All GPx antibodies were purchased from Autogen Bioclear; Table 2 provides further details on antibody dilutions and positive controls. Antibodies to cytokeratin (CK-7) and CD-68 (Santa Cruz Biotechnology, Calne, UK) were used to confirm positive GPx staining in cytotrophoblast and Hofbauer cells respectively (Table 2). A negative control was performed for each test section by omitting incubation in the primary antibody. Sections were dehydrated in ascending concentrations of alcohol and xylene before coverslips were mounted (DPX mountant, BDH).

Quantification was performed at x400 magnification (Leica DM RB microscope) using a previously described method that allows counting of positively stained cells/areas and requires no corrections based on other estimated quantities²⁰; Openlab software (Improvision Openlab 4.0.2) was used for quantification. All analysis was performed blinded as to group by the same assessor using a reference slide to check for consistency.

Statistical analysis: All tests were performed using SPSS for Windows version 14.0.

Summary data are presented as means \pm SDs or median (interquartile range) as appropriate for their distribution. Between group comparisons were made using 1-way ANOVA (with *post hoc* Gabriel's test if significant)/Kruskal-Wallis tests or 2-tailed students *t* test/Mann-Whitney *U*-tests depending on the distribution. Correlations between the parameters were tested with either Pearson's or Spearman's Ranks correlation tests. Discriminant function

analysis (DFA)²¹ using the method of Wilks was used to determine which of the markers studied allowed best discrimination between normal pregnancy and preeclampsia. The null hypothesis was rejected where $P < 0.05$.

Results

Subjects: Table 3 describes the demographic, obstetric and pregnancy data of the 74 women who participated in the study. All pregnancy groups conceived spontaneously and carried singleton pregnancies. The normal pregnancy group gave birth without developing hypertension or proteinuria, to infants weighing > 2500 g, delivered 37 weeks or later. The systolic and diastolic blood pressure levels were, by definition, significantly raised in preeclampsia compared to normal pregnancy ($P < 0.0001$). Overall, the preeclamptic women all had moderate to severe disease and also had lower gestational ages at delivery than the control group ($P < 0.0001$) (Table 3). No preeclamptic woman had HELLP. All neonates from both pregnancy groups survived.

Biochemical and molecular measurements: The values of plasma TBARS, serum selenium concentrations and plasma GPx activities are given in Table 4. Maternal plasma TBARS levels were significantly higher in preeclampsia compared to both normal pregnancy and non-pregnant controls ($P < 0.001$); no significant differences were observed between non-pregnant and normal pregnancy samples. Umbilical venous TBARS concentrations were also significantly higher in preeclamptic than normal pregnancy ($P = 0.04$).

There was a highly-significant trend for decreasing plasma selenium concentrations from non-pregnant, to normal pregnant and pre-eclamptic women (Table 4; Kendall's τ ; $P < 0.001$).

Selenium concentrations were also significantly reduced in umbilical venous samples in

preeclampsia by comparison with samples from babies of normotensive mothers ($P < 0.0001$). There was also a highly-significant trend for decreasing plasma GPx activity from non-pregnant, to normal pregnant and pre-eclamptic women (Table 4; Kendall's τ ; $P < 0.001$). Umbilical venous plasma GPx activities were also significantly lower in preeclampsia than in normal pregnancy samples ($P < 0.005$). There was a highly-significant overall correlation between maternal serum selenium concentrations with both maternal plasma and placental GPx activity (Figure 1a & b; $r = 0.49$; $P < 0.001$ and $r = 0.38$; $P = 0.005$ respectively), but no significant association within individual groups, or between umbilical venous serum selenium and GPx activity.

No significant differences were observed in placental mRNA expression levels of GPx1, 3 and 4 ($P > 0.1$) (Table 5). As expected, no biologically significant placental GPx2 mRNA expression was observed. Significantly lower activity of placental GPx activity was seen in preeclampsia compared to normal pregnancy placentae ($P < 0.0001$) (Table 5). No significant differences in placental GPx activities were seen between sampling sites ($P > 0.1$), thus mean values are represented. A significant negative correlation was observed between placental GPx activity and maternal TBARS concentrations in preeclampsia only (Figure 2).

Due to the large spread of the corrected birthweight centiles in the preeclampsia group (Table 3), we examined maternal GPx activity in mothers who gave birth to infants above and below the 50th centiles. Univariate analysis of variance indicated that both pregnancy group and centile above/below 50th centile significantly react with maternal GPx activities ($P < 0.001$, $P = 0.021$ respectively) (Figure 3).

Immunohistochemistry: Immunohistochemistry of GPx 1, 3 and 4 indicated positive expression in cytotrophoblast and Hofbauer cells (Figure 4). Quantification revealed that the expression was significantly reduced in preeclampsia for all three GPx antibodies (GPx1 and GPx3: $P < 0.05$; GPx4: $P < 0.001$) (Figure 4). Location of positively stained areas was confirmed by staining with CK-7 and CD-68 antibodies (Figure 5). No significant differences in expression were observed from the different placental sampling sites for any of the antibodies tested.

Discriminant function analysis: Stepwise discriminant function analysis was applied to the whole sample using maternal serum selenium concentrations and plasma GPx activity produced a significant Wilks' lambda test (assessed using the F test)²¹ (Table 6) ($P < 0.0001$). DFA using these measurements correctly predicted 82% of the subjects, with correct classification of 84.6% of normal pregnancies and 78.3% of preeclamptic patients (Table 6).

Discussion

At present the source of the lipid peroxides in preeclampsia is unknown, but it has been suggested that poorly perfused placental tissue may evoke a free radical cascade and increase in generalised lipid peroxidation²². By entering the maternal circulation, these lipid peroxides could affect the maternal endothelial cellular membranes by the increased production of ROS, thus contributing to the maternal vascular dysfunction¹⁰. The significantly raised concentration of TBARS, a surrogate measure of lipid peroxidation, in the maternal circulation confirms previous reports^{10, 12}. However, previous studies investigating oxidative stress have focussed on maternal parameters and only a single study appears to have been carried out in the fetus²³. Our study also investigated umbilical venous concentrations of TBARS, which were significantly increased after preeclamptic pregnancy compared with

normal pregnancy, indicative of increased oxidative stress. Defective placentation in preeclampsia could prevent the active placental transport of these lipid peroxides away from the fetus thus increasing the levels of TBARS in the umbilical venous samples. This prenatal exposure to raised lipid peroxidation might be one of the factors predisposing the baby of the preeclamptic mother to cardiovascular disease in later life^{24, 25}.

Worryingly, the selenium concentrations in the non-pregnant women were lower than the recommended levels, as observed in other studies (eg²⁶), suggesting that the selenium concentrations may not actually be high enough for optimum GPx activities even in the non-pregnant population of the United Kingdom. The selenium concentrations are further reduced in normal pregnancies (Table 4), illustrating a possible increased requirement for selenium during pregnancy. However, this could also be due to inadequate absorption from the gastrointestinal tract, or inadequate renal reabsorption in the face of the increased glomerular filtration rate of pregnancy. Moreover this study also observed a further significant reduction in maternal selenium concentrations in the preeclamptic samples compared to both the normal pregnant and non-pregnant controls; this is in line with other studies^{10, 11} which measured serum selenium in samples with widely varying gestational ages and in toenail selenium concentrations respectively. The lower selenium concentrations seen in preeclampsia might adversely affect the functional activities of the selenoproteins, GPx, thus compromising the protection against oxidative stress. Also, a recent report linked increased selenium intake over two years with significantly decreased excretion of the major thromboxane metabolite²⁷. An early imbalance between thromboxane and prostacyclin synthesis has been implicated in the pathogenesis of preeclampsia over the last 20 years (eg^{28, 29}).

The source of selenium to the fetus is via placental transport across a concentration gradient from the maternal circulation³⁰. In the present study, selenium concentrations were significantly lower in the neonates of both groups, as compared to matched maternal serum; selenium is transported by simple diffusion across the placenta³¹. It is evident (Table 4) that the reduced selenium in the maternal circulation in preeclampsia is having a profound effect on the fetal selenium concentrations and thus could also compromise the mechanisms to protect the fetus from oxidative stress.

The lower maternal and umbilical venous GPx activities observed in the preeclamptic samples indicates that insufficient antioxidant defence may be a contributing factor to the pathophysiological mechanisms associated with oxidative stress and preeclampsia. However, it must be remembered that measured GPx activities should only be viewed in combination with other biomarkers, as its activity in plasma, unless organ specific, can be misleading. Nevertheless, our results provide information regarding a component of the global antioxidant defence levels in both the mother and baby. The reduced plasma activities in the babies born to preeclamptic pregnancies in this study parallels the observation of reduced erythrocyte GPx¹² and suggests that, as with the maternal samples, these babies may potentially have reduced antioxidant status.

Small size at birth has been postulated to increase the risks of cardiovascular disease in later life²⁴. Maternal plasma GPx activity clearly differed by pregnancy outcome, in terms both of the presence or absence of preeclampsia, and whether the birthweight centile was above or below 50% (Figure 3). The higher plasma GPx in smaller babies was unexpected, but might reflect an adaptive response in these intrauterine growth restricted babies to try and protect these fetuses from oxidative stress. To our knowledge, this is a novel observation.

This is the first study to show the presence of three of the main forms of GPx in the human placenta. Wang & Walsh, who did not differentiate the different GPxs, used Northern analysis and reported a significant decrease in expression in preeclampsia³². Whereas our real-time PCR analysis and found no significant differences in any of the GPxs. The immunohistochemical staining of all three GPx antibodies confirmed that as with the activities, the expression levels were significantly lower in the preeclamptic compared to normal placentae (Figure 4). GPx in the cytotrophoblast cells is ideally located for the transportation of these GPxs into both the maternal and fetal circulations; these cells are exposed to relatively high oxygen concentrations and thus the GPxs may also protect the cells from oxidative stress³³. Due to the role of Hofbauer cells in vasculogenesis and angiogenesis, the GPxs may play essential roles in antioxidant protection throughout placental development and may indeed be the first line of antioxidant defence as these Hofbauer cells are found from week 4 of pregnancy until term³⁴.

Total placental GPx activities were also significantly lower in the preeclamptic placenta compared to normal pregnancy placentae, confirming previous studies^{13, 14}. This result therefore supports the hypothesis that in the placental compartment, at least, there is decreased GPx expression and activity contributing to the oxidative stress and enhanced biological oxidation in these tissues during preeclampsia. The fact that no differences were found in placental GPx mRNA expression levels between pregnancy groups but the GPx activity was reduced in the preeclamptic placentae indicates a possible post-translational modification/mutation reducing the GPx antioxidant activity; this has been observed outside pregnancy³⁵. Moreover, the negative association seen between mean placental GPx activities

and maternal TBARS concentrations (Figure 2) in preeclampsia provides a further indication that the reduced placental antioxidant defence in the placenta could have functional effects.

The GPxs are selenoproteins, but direct associations between the selenium concentrations and GPx activities have not previously been investigated with respect to preeclampsia. The significant positive relationships seen in pregnancy *per se* (Figure 1a and b) suggest that at lower maternal selenium concentrations, there is a reduction in GPx; however as the selenium concentrations increases, the placental GPx activity begins to rise rapidly. This is in line with the reports that the maternal Se concentrations must be clearly deficient to have a detrimental affect on GPx activities³⁶ and this appears to be the case in some of the women suffering from preeclampsia in this study. A possible hypothesis could be that the selenium concentrations may be below a threshold value for optimum GPx synthesis; this has been observed in studies of selenium deficiency in mice³⁷.

Discriminant functional analysis using only maternal biochemical measurements (serum selenium concentrations and plasma GPx activities) classified 82% of the subjects into the correct pregnancy outcomes. Caution must be exercised; this study was performed at the time of delivery, when the disease was established. Longitudinal studies are needed to determine whether similar differences antedate the onset of clinically-detectable disease, that is, whether they are cause or effect. If they do occur early, they would provide a novel addition to the analytes currently suggested as predictive.

Perspectives

These data are from this cross-sectional group of women with well characterised moderate-to-severe preeclampsia. The study illustrates how trace quantities of selenium can have very

significant physiological effects. There is currently considerable interest in the role of dietary selenium supplementation as prophylaxis for various forms of cancer, such as prostate cancer³⁸, another condition of aberrant vascular development. If selenium deficiency is confirmed in women suffering from preeclampsia, and continues to be linked with GPx inadequacy, consideration could be given to a randomised controlled trial of selenium supplementation in pregnancy. Furthermore, as assay methods become more readily available, measurements of serum selenium and GPx activity might contribute to the early identification of women at high risk of developing the disease. Further longitudinal studies are required to elucidate a 'cause or effect' relationship for these factors.

Acknowledgements

We thank all the women who participated in the study and the midwives and doctors whose support made this study possible. Thanks also to Mr John Corrie and Mr Darren Hepworth from the School of Environmental Sciences, Nottingham for their help with the selenium measurements. We are grateful to Ms Chrissi Dunster from King's College London for running the TBARS assays and for the help and advice regarding immunohistochemical quantification from Dr. Paula Williams, University of Nottingham.

Sources of Funding

H.Mistry was supported by a studentship from Biotechnology and Biological Sciences Research Council (BBS/S/P/2003/10412A) as well the Nottingham Hospital Special Trustees charity (reference number: RAP 0003; Fund No: N7050).

Disclosures

None

References

1. Sibai B, Dekker G, Kupferminc M. Pre-eclampsia. *Lancet* 2005;365:785-99.
2. Broughton Pipkin F. Risk factors for preeclampsia. *N Engl J Med* 2001;344:925-6.
3. Wilson BJ, Watson MS, Prescott GJ, Sunderland S, Campbell DM, Hannaford P, Smith WC. Hypertensive diseases of pregnancy and risk of hypertension and stroke in later life: results from cohort study. *BMJ* 2003;326:845.
4. Poston L. The Role of Oxidative Stress. In: Critchley H, MacLean A, Poston L, Walker J, eds. *Pre-eclampsia*. London: RCOG Press, 2004.
5. Brosnan MJ. One Step Beyond: Glutathione Peroxidase and Endothelial Dysfunction. *Hypertension* 2008.
6. Hubel CA. Oxidative stress in the pathogenesis of preeclampsia. *Proc Soc Exp Biol Med* 1999;222:222-35.
7. Rayman MP. The importance of selenium to human health. *Lancet* 2000;356:233-41.
8. Oster O, Prellwitz W. Selenium and cardiovascular disease. *Biol Trace Elem Res* 1990;24:91-103.
9. Brigelius-Flohe R. Tissue-specific functions of individual glutathione peroxidases. *Free Radic Biol Med* 1999;27:951-65.
10. Atamer Y, Kocyigit Y, Yokus B, Atamer A, Erden AC. Lipid peroxidation, antioxidant defense, status of trace metals and leptin levels in preeclampsia. *Eur J Obstet Gynecol Reprod Biol* 2005;119:60-6.
11. Rayman MP, Bode P, Redman CW. Low selenium status is associated with the occurrence of the pregnancy disease preeclampsia in women from the United Kingdom. *Am J Obstet Gynecol* 2003;189:1343-9.
12. Bulgan Kilicdag E, Ay G, Celik A, Ustundag B, Ozercan I, Simsek M. Oxidant-antioxidant system changes relative to placental-umbilical pathology in patients with preeclampsia. *Hypertens Pregnancy* 2005;24:147-57.
13. Vanderlelie J, Venardos K, Clifton VL, Gude NM, Clarke FM, Perkins AV. Increased biological oxidation and reduced anti-oxidant enzyme activity in pre-eclamptic placentae. *Placenta* 2005;26:53-8.
14. Walsh SW, Wang Y. Deficient glutathione peroxidase activity in preeclampsia is associated with increased placental production of thromboxane and lipid peroxides. *Am J Obstet Gynecol* 1993;169:1456-61.
15. Gardosi J, Francis A. Customised Centile Calculator: Gestational network - GROW - centile v 5.1, 2006; www.gestation.net.
16. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;193:265-75.
17. Uchiyama M, Mihara M. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. *Anal Biochem* 1978;86:271-8.
18. Sanders J. Selenium in Serum measured by Zeeman GFAAS. *Varian AA Instruments at Work* 1995;AA-122.
19. Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J Lab Clin Med* 1967;70:158-69.
20. Gundersen HJ. Notes on the estimation of the numerical density of arbitrary profiles: the edge effect. *Journal of Microscopy* 1977;111:219-223.
21. Abu Alhaja ES, Richardson A. Growth prediction in Class III patients using cluster and discriminant function analysis. *Eur J Orthod* 2003;25:599-608.
22. Dekker GA, Sibai BM. Etiology and pathogenesis of preeclampsia: current concepts. *Am J Obstet Gynecol* 1998;179:1359-75.

23. Tastekin A, Ors R, Demircan B, Saricam Z, Ingec M, Akcay F. Oxidative stress in infants born to preeclamptic mothers. *Pediatr Int* 2005;47:658-62.
24. Barker D. Mothers, Babies and Disease in Later Life: BMJ Publishing Group, London, 1994.
25. Lamireau D, Nuyt AM, Hou X, Bernier S, Beauchamp M, Gobeil F, Jr., Lahaie I, Varma DR, Chemtob S. Altered vascular function in fetal programming of hypertension. *Stroke* 2002;33:2992-8.
26. Rayman MP. The argument for increasing selenium intake. *Proc Nutr Soc* 2002;61:203-15.
27. Arnaud J, Bost M, Vitoux D, Labarere J, Galan P, Faure H, Hercberg S, Bordet JC, Roussel AM, Chappuis P. Effect of low dose antioxidant vitamin and trace element supplementation on the urinary concentrations of thromboxane and prostacyclin metabolites. *J Am Coll Nutr* 2007;26:405-11.
28. Fitzgerald DJ, Entman SS, Mulloy K, FitzGerald GA. Decreased prostacyclin biosynthesis preceding the clinical manifestation of pregnancy-induced hypertension. *Circulation* 1987;75:956-63.
29. Zhao S, Gu Y, Lewis DF, Wang Y. Predominant basal directional release of thromboxane, but not prostacyclin, by placental trophoblasts from normal and preeclamptic pregnancies. *Placenta* 2008;29:81-8.
30. Nandakumaran M, Dashti HM, Al-Zaid NS. Maternal-fetal transport kinetics of copper, selenium, magnesium and iron in perfused human placental lobule: in vitro study. *Mol Cell Biochem* 2002;231:9-14.
31. Nandakumaran M, Dashti HM, Al-Saleh E, Al-Zaid NS. Transport kinetics of zinc, copper, selenium, and iron in perfused human placental lobule in vitro. *Mol Cell Biochem* 2003;252:91-6.
32. Wang Y, Walsh SW. Antioxidant activities and mRNA expression of superoxide dismutase, catalase, and glutathione peroxidase in normal and preeclamptic placentas. *J Soc Gynecol Investig* 1996;3:179-84.
33. Burton GJ, Jauniaux E. Placental oxidative stress: from miscarriage to preeclampsia. *J Soc Gynecol Investig* 2004;11:342-52.
34. Benirschke K, Kaufmann P, Baergen R. Pathology of the human placenta. New York: Springer, 2006.
35. Voetsch B, Jin RC, Bierl C, Benke KS, Kenet G, Simioni P, Ottaviano F, Damasceno BP, Annichino-Bizacchi JM, Handy DE, Loscalzo J. Promoter polymorphisms in the plasma glutathione peroxidase (GPx-3) gene: a novel risk factor for arterial ischemic stroke among young adults and children. *Stroke* 2007;38:41-9.
36. Rotruck JT, Pope AL, Ganther HE, Swanson AB, Hafeman DG, Hoekstra WG. Selenium: biochemical role as a component of glutathione peroxidase. *Science* 1973;179:588-90.
37. Weitzel F, Ursini F, Wendel A. Phospholipid hydroperoxide glutathione peroxidase in various mouse organs during selenium deficiency and repletion. *Biochim Biophys Acta* 1990;1036:88-94.
38. Duffield-Lillico AJ, Reid ME, Turnbull BW, Combs GF, Jr., Slate EH, Fischbach LA, Marshall JR, Clark LC. Baseline characteristics and the effect of selenium supplementation on cancer incidence in a randomized clinical trial: a summary report of the Nutritional Prevention of Cancer Trial. *Cancer Epidemiol Biomarkers Prev* 2002;11:630-9.

Table Legends

Table 1. Primers, sequences, product sizes and BLAST sequence numbers for the different glutathione peroxidases measured in this study.

Table 2. Details of antibodies used.

Table 3. Demographic and pregnancy data of subject groups. Data are presented as mean±SD or median [IQR] as appropriate, except for proteinuria: (median (min, max) and parity and Caesarean sections (number (percentage))). † $P < 0.05$ between non-pregnant and both pregnancy groups; ‡ $P < 0.05$ between normal and preeclamptic pregnancies.

Table 4. Maternal and umbilical venous TBARS, selenium concentrations and GPx activities. Data are shown as mean±SD or median [IQR] as appropriate. ‡ $P < 0.0001$ between non-pregnant and both pregnancy groups; * $P < 0.005$, ** $P < 0.0001$ between normal and preeclamptic pregnancies.

Table 5. Placental GPx mRNA expression and activities. Data are shown as (median (IQR)) as normalized ratios x1000. ‡ $P < 0.001$ between normal and preeclamptic pregnancies.

Table 6. Constant and discriminant function coefficients for the maternal predictors

Figure Legends

Figure 1. There were positive associations between maternal serum selenium concentrations with a) maternal plasma ($r = 0.49$; $R^2 = 0.24$; $P < 0.001$) and b) placental ($r = 0.38$; $R^2 = 0.14$; $P = 0.005$) GPx activities in pregnancy *per se*.

Figure 2. Maternal TBARS concentrations and placental GPx activities were inversely correlated in the preeclamptic pregnant women ($r = -0.54$; $R^2 = 0.27$; $P = 0.008$); no significant association was observed in the normal pregnancy samples.

Figure 3. Maternal plasma GPx activity in normal and preeclamptic pregnancies split by baby birthweight centiles above and below the 50th centile.

Figure 4. Immunohistochemical staining of GPx 1 (A1-A4), 3 (B1-B4) and 4 (C1-C4) placental sections from normal (A1, B1 & C1) and preeclamptic (A2, B2 & C2) pregnancies. (A3-A4; B3-B4; C3-C4 are positive and negative staining for each GPx). Positive staining is shown in brown; black arrows indicate cytotrophoblast cells and white arrows show Hofbauer cells. x400 magnification. * $P < 0.05$ between normal and preeclamptic placentae.

Figure 5. Representative placental sections of positive staining of CK-7 (A1) and CD68 (B1). Positive staining is shown in brown; black arrows indicate cytotrophoblast cells and white arrows show Hofbauer cells; negative controls are shown in A2 (cytotrophoblast cells) and B2 (Hofbauer cells). x400 magnification.

Table 1. Primers, sequences, product sizes and BLAST sequence numbers.

Primer	Sequences (F=forward; R= reverse)	BLAST Sequence No.
	<i>F</i> : CAACCAGTTTGGGCATCA	
GPx1	<i>R</i> : CCGTTCACCTCGCACTTC	NM_000581
	<i>F</i> : TGTGGCTTCGCTCTGAGG	
GPx2	<i>R</i> : GTTCTCCTGATGTCCAAA	NM_002083
	<i>F</i> : GATGGGAGGAGTACATCC	
GPx3	<i>R</i> : ACCGAATGGTGCAAGCTC	NM_002084
	<i>F</i> : GGCTACAACGTCAAATTCG	
GPx4	<i>R</i> : GCAGGTCCTTCTCTATCA	NM_002085

Table 2. Antibody details for immunohistochemistry

Antigen	Clone	Titre ($\mu\text{g/ml}$)	Positive control	Supplier
				Autogen
GPx1 (polyclonal)	-	1:1000 dilution	Thymus	Bioclear, UK
				Autogen
GPx3 (monoclonal)	23B1	1:100 dilution	Prostate	Bioclear, UK
				Autogen
GPx4 (polyclonal)	-	1:250 dilution	Tongue	Bioclear, UK
	OV-TL			Santa Cruz
Cytokeratin-7 (monoclonal)	12/30	0.5	-	Biotechnology
				Santa Cruz
CD-68 (monoclonal)	PG-M1	1:50 dilution	-	Biotechnology

Table 3. Demographic, pregnancy, clinical and biochemical data of subject groups

Parameter*	Normal		
	Non-pregnant	pregnant	Preeclampsia
Maternal age (years)	30±10.2	29±6.8	32±5.8
Primipara (No. (%))	-	16(59.3)	17(68)
Booking body mass index (Kg/m ²)	22.7±4.8 [†]	26.6±5.8	27.3±5.6
Max. systolic blood pressure outside labour (mmHg)	-	116±4.3	159±8.8 [‡]
Max. diastolic blood pressure outside labour (mmHg)	-	76±2.8	98±4.9 [‡]
Proteinuria (g/L)	-	-	1.0(0.3, 11.5)
Gestation age at delivery (weeks)	-	40±1.1	36.4±3.8 [‡]
Caesarean section (No. (%))	-	4(15)	11(44) [‡]
Birthweight (Kg)	-	3.55[3.25, 3.86]	2.92[1.92,3.51] [‡]
Birthweight centile	-	45[23, 67]	35[2, 87]
Placental/birthweight ratio	-	1.9±0.3	2.3 ± 1.1 [‡]

Table 4. Maternal and umbilical venous TBARS, selenium concentrations and GPx activities

Biochemical measurement	Non-pregnant	Normal pregnant	Preeclampsia
Maternal TBARS $\mu\text{mol/L}$			
(median[IQR])	0.40[0.2, 0.7]	0.45[0.2, 0.8]	1.2[0.6, 1.6] [‡]
Umbilical venous TBARS $\mu\text{mol/L}$			
(median[IQR])	-	0.6[0.2, 0.8]	0.8[0.5, 1.0] [‡]
Maternal selenium $\mu\text{g/L}$ (mean \pm SD)	68.8 \pm 2.5 [†]	58.4 \pm 14.9	39.7 \pm 13.8 [‡]
Umbilical venous selenium $\mu\text{g/L}$			
(mean \pm SD)	-	42.1 \pm 11.8	29.0 \pm 9.9 [‡]
Maternal plasma GPx activity			
nmol/min/ml (median[IQR])	0.82[0.7, 0.9] [‡]	0.53[0.4, 0.6]	0.32[0.1, 0.4]**
Umbilical venous GPx activity			
nmol/min/ml (median[IQR])	-	0.51[0.4, 0.6]	0.39[0.2, 0.5]*

Table 5. Placental GPx mRNA expression and activities

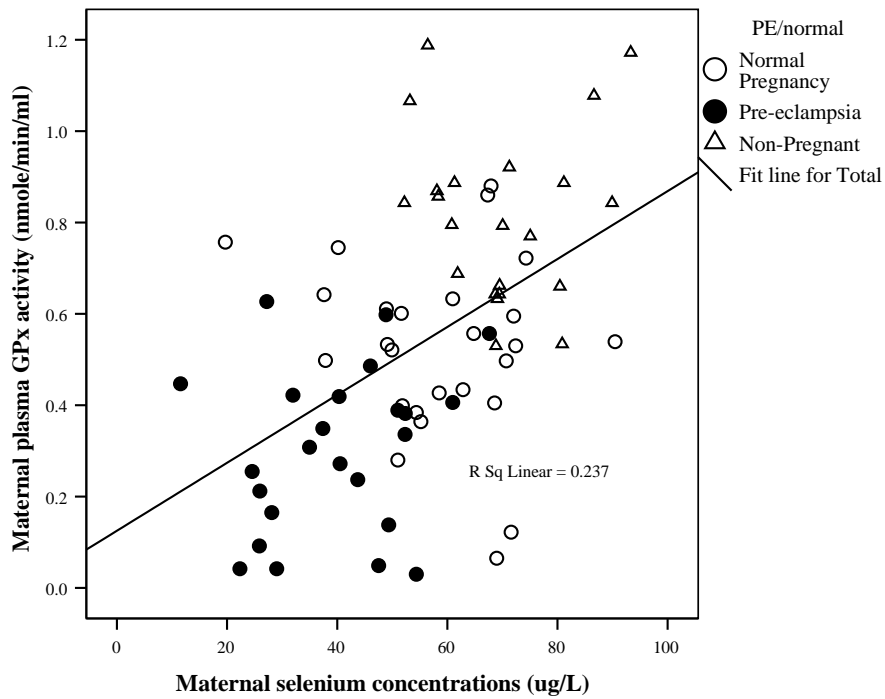
Placental GPx measurements	Normal pregnant	Preeclampsia
GPx1 normalized ratio mRNA expression		
(median([QR]))	5[1, 13]	4[2, 13]
GPx3 normalized ratio mRNA expression		
(median[IQR])	75[23, 116]	47[18, 91]
GPx4 normalized ratio mRNA expression		
(median[IQR])	5[2, 28]	12[3, 42]
GPx activity nmol/min/mg (median[IQR])	0.33[0.2, 0.6]	0.16[0.1, 0.2] [‡]

Table 6. DFA data for maternal biochemical results

Maternal predictive variable	Coefficients
Constant	-4.06
Serum Se concentrations ($\mu\text{g/L}$)	0.05
Plasma GPx activity (nmole/min/L)	3.47

Figure 1.

a)



b)

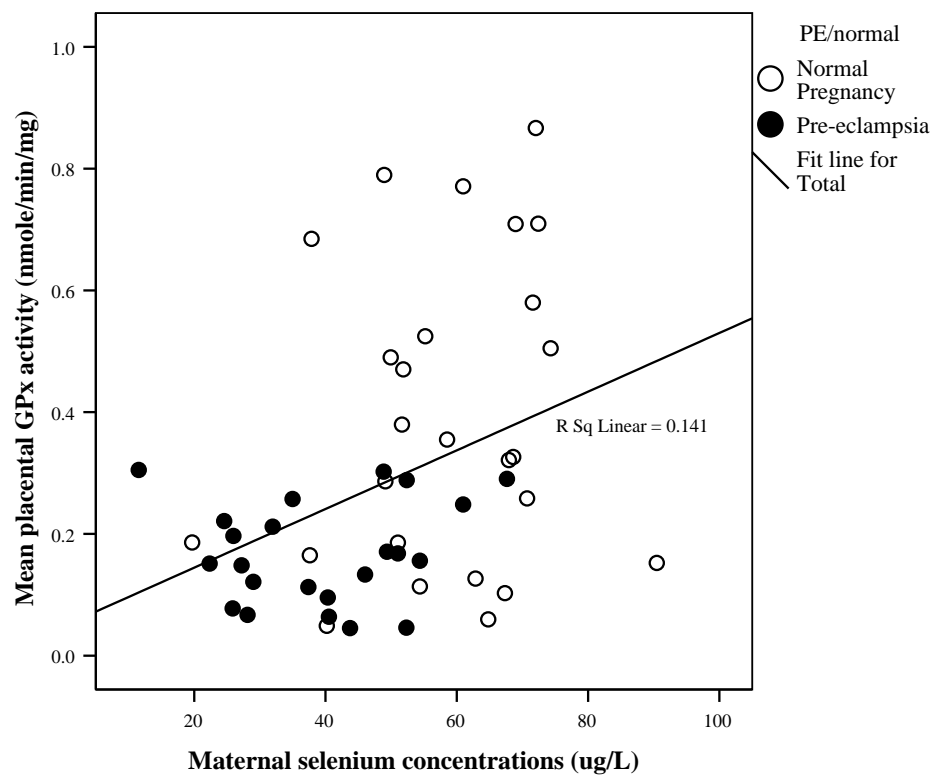


Figure 2.

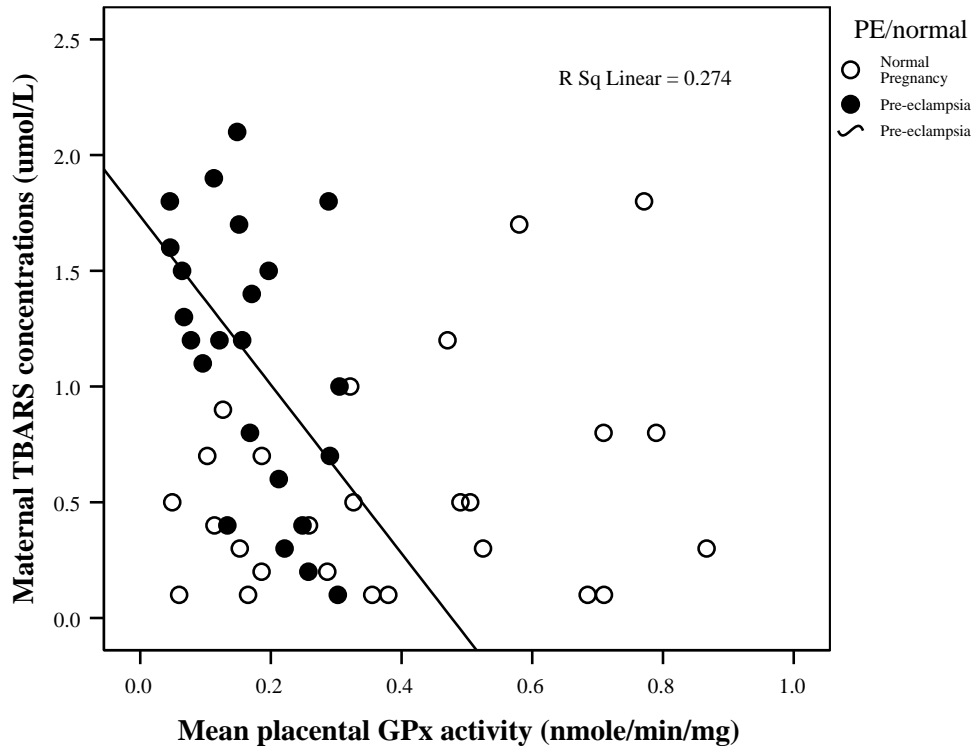


Figure 3

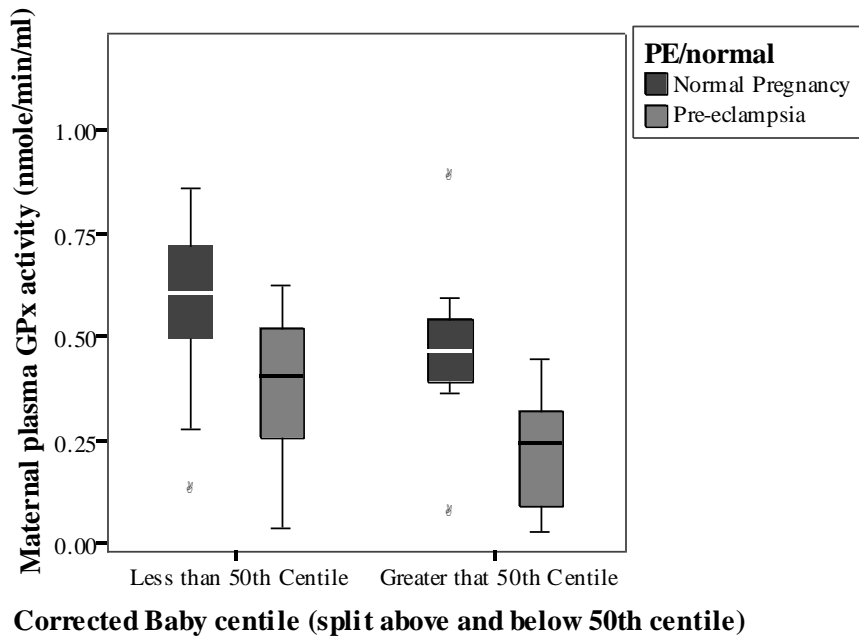


Figure 4

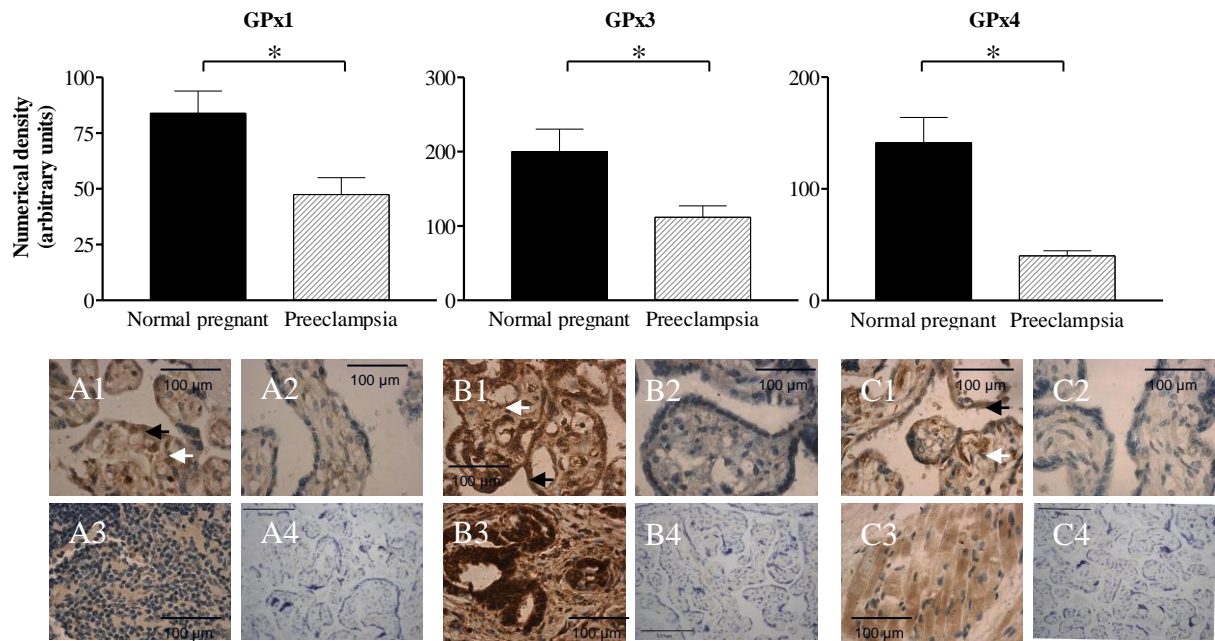


Figure 5.

