**Differential expression and distribution of placental glutathione peroxidases 1, 3 and 4 in normal and preeclamptic pregnancy**

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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; it may also predispose the mother and fetus to increased risks of adult cardiovascular disease. The selenoprotein glutathione peroxidases (GPxs) have critical roles in regulating antioxidant status.

**Objectives, study design and main outcome measures:** Immunohistochemical measurements of GPx 1, GPx3 and GPx4 protein expression were performed on samples taken from three standardised sampling sites between the cord insertion and the periphery of the placenta from 12 normotensive, and 12 preeclamptic women to establish if their expression differed between sampling sites. Total GPx activities were also examined from the three sampling sites of these placentae.

**Results:** There were highly significant reductions in overall immunohistochemical staining of all 3 GPxs in the preeclampsia compared to normotensive placentae (GPx1: $P = 0.016$; GPx3: $P = 0.003$; GPx4: $P < 0.001$). Furthermore, graded differences in expression between the standardised placental sampling sites were also found for GPx3 (higher in the inner region, $P = 0.05$) and GPx4 (higher in the periphery, $P = 0.02$) but not GPx1. Placental GPx enzyme activity was also significantly reduced in tissue from preeclamptic women as compared to normotensive women ($P = 0.007$; the difference was more pronounced nearest the cord insertion).

**Conclusions:** We have shown highly significant reductions in expression of all three major classes of GPx in placentae from women with preeclampsia, and distribution gradients in activity, which may relate to the differential oxygenation of regions of the placenta.

**Keywords:** Preeclampsia; hypertension; oxidative stress; glutathione peroxidase; placenta.

**Abbreviations:** Glutathione peroxidases: GPxs; Intrauterine growth restriction: IUGR; intraclass correlation coefficient: ICC.

## 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 world [1]; together with the other hypertensive disorders of pregnancy it is responsible for approximately 60,000 maternal deaths each year [2] and increases perinatal mortality five-fold [3]. Preeclampsia is now commonly regarded as being a state of oxidative stress, thought to arise from a very early biochemical imbalance due to excessive 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 dysfunction [5], hypertension and thus clinical manifestations of preeclampsia [6]. Recent evidence has shown that the deleterious effects of pre-eclampsia in a woman and her baby extend beyond pregnancy into her later life, including increased risk of cardiovascular disease, diabetes and end stage renal disease [7-9].

The glutathione peroxidases (GPx) are antioxidants which provide protection from oxidative damage by reducing lipid hydroperoxides to their corresponding unreactive alcohols and reducing free hydrogen peroxide to water. This limits adverse effects on the endothelium [10] from reactive free radicals and cytotoxic agents [11]. 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) [12]. The regulation of this type of enzyme alters in different situations depending on oxygen tension, cell differentiation and tissue maturation [13] . The trace element selenium is an essential component of the antioxidant selenoproteins, including the GPxs. Our group and others have shown decreases in maternal and fetal serum selenium concentrations in preeclamptic patients compared to normotensive controls and have also reported significant

parallel reductions in maternal and fetal plasma and placental GPx activities in preeclamptic patients [14-17].

Although the placenta is a discrete organ, its complex structure results in heterogeneous function in different areas. The degree of vascularisation differs across the placenta resulting in differences of oxygen availability in these locations [18] with most gaseous exchange occurring at the periphery where blood flow is greatest. There are known regional variations in tissue sensitivity to polypeptides [19]; cytokine protein concentrations can vary up to 3-fold across different sampling sites of the placenta [20] and expression of hypoxia-related genes, such as vascular endothelial growth factor, is lower near the basal plate close to the cord insertion site and higher at the peripheral chorionic plate [21]. Although the distribution of selenium within the placenta has not been examined, other elements such as iron and calcium, have been reported to be differentially distributed across the placental disc [22]. A single study of GPx mitochondrial activity found this to be 41 per cent lower in the periphery compared to the pre-insertion region [23]. In an earlier paper [17] we reported that sampling site did not affect expression of GPx, but we did identify an apparent trend, and only examined 15 placentae (7 preeclampsia and 8 normotensive). We are not aware of any other studies examining GPx placental protein expression from different sampling sites of the placenta.

We hypothesised that GPx1, GPx3 and GPx4 expression, identified by immunohistochemistry, would alter in different areas of the placenta, as well as being reduced in preeclamptic pregnancy. We have therefore conducted a cross-sectional study to explore these factors, comparing placentae from normotensive and preeclamptic pregnancies.

**Methods**

*Subjects:* The study population consisted of White European women who had either a normotensive or preeclamptic pregnancy (Table 1). The investigations were approved by the Nottingham Hospital Ethics Committee 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 or more and diastolic pressure (Korotkoff V) of 90 mm Hg or more on 2 occasions after 20 weeks gestation in a previously normotensive woman 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 [24]. 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) [25].

*Sample collection*: Twelve placentae from preeclamptic women, and 12 controls were studied. Data from 7 preeclampsia and 8 normotensive placentae were reported in [17]. Two full depth placental tissue samples were collected from three standardised locations between cord insertion and placental border (1 cm from the cord insertion, 1 cm from the periphery, and midway between the two), avoiding placental infarcts. The placental samples were taken within 10 minutes of delivery, membranes removed and tissue washed in ice cold 1x PBS to remove maternal blood contamination. One set was snap frozen in liquid nitrogen and stored at -80$^{o}$C for GPx enzyme activity and the other formalin fixed and wax-embedded for immunohistochemical analysis.

*Histological analysis:* Serial sections of 5 μm 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 staining 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, which were optimised by performing a dilution series for each antibody. 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 [17]. The heat induced epitope retrieval was achieved by heating in a citrate buffer (pH 6.0) using a microwave oven for 15 minutes. A negative control was performed for each test section by omitting incubation with the primary antibody. A positive control for each antibody was used to verify specificity: human thymus (GPx1), prostate (GPx3), tongue (GPx4) as recommended by Autogen Bioclear; both positive and negative controls were completed on serial sections. Sections were dehydrated and cleaned in ascending concentrations of alcohol and xylene before coverslips were mounted (DPX mountant, BDH).

Quantification was performed at x400 magnification (Nikon Eclipse II microscope) using a previously described method [26]. This is a grid-based method which allows unbiased counting of positively stained cells/areas and requires no normalisation. NIS Elements F 2.20 software (Laboratory Imaging Ltd) was used for quantification. All analysis was performed blinded as to group by the same assessor (HDM) and independently double counted by a trained second assessor (PJW); a reference slide was used throughout to check for consistency. The intraclass correlation coefficient (ICC) (ratio of between-groups variance of

the total variance) was used to assess agreement between judges [27, 28]. The ICCs for each antibody were $r = 0.996$ ($P < 0.001$) for GPx1; $r = 0998$ ($P < 0.001$) for GPx3 and $r = 0.995$ ($P < 0.001$) for GPx4.

*GPx activity assay:* Placental GPx activity was determined by a modified method of Paglia and Valentine [29]. Placental tissue (100 mg) was extracted using RIPA buffer (1 x PBS (pH 7.4), 0.1 % SDS, 1 % Igepal CA-630 (Sigma-Aldrich, Poole, UK) and 0.5% sodium deoxycholate) and protein concentrations were determined by the Lowry protein assay. Activity was measured spectrophotometrically in triplicate by coupling the oxidation of glutathione and nicotinamide adenine dinucleotide phosphate (NADPH) using glutathione reductase, described in detail previously [17]. GPx activity was standardised against protein concentrations and expressed as mmol/min/mg protein; the inter- and intra-assay variations were < 5%.

*Statistical analysis:* All tests were performed using SPSS for Windows version 16.0. Summary data are presented as means ± SD or median (interquartile range) as appropriate for their distribution. Between-group comparisons were made using repeated measures ANOVA /Kruskal-Wallis tests or 2-tailed student's *t* test/Mann-Whitney *U*-tests depending on the distribution. Kendall's τ tests were used to test for differential staining intensities between placental sampling sites. The null hypothesis was rejected where $P < 0.05$.

## Results

*Subjects:* Table 1 describes the demographic, obstetric and pregnancy data of the 24 women (12 normotensive, 12 preeclampsia) who participated in the study. All women conceived naturally and carried singleton pregnancies. The normotensive 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 had lower gestational ages at delivery than the control group ($P < 0.05$) (Table 1). No preeclamptic woman had HELLP (Hemolysis Elevated Liver enzymes, and Low Platelet count). All neonates from both pregnancy groups survived.

*Immunohistochemistry:* As we have previously shown GPx 1, GPx3 and GPx4 were all positively expressed in both cytotrophoblast and Hofbauer cells [17] and the expression of all three enzymes was significantly reduced in preeclampsia (GPx1: $P = 0.016$; GPx3: $P = 0.003$; GPx4: $P < 0.001$; Figures 1-3). The three samples were analysed in parallel to control for placental inhomogeneity with respect to potential gradient of oxygenation between the cord insertion and the peripheral border.

Comparison of staining intensity across the placental sampling sites demonstrated differential localisation of the enzyme forms for GPx3 and GPx4, but not GPx1 (Figures 1- 3). Visual inspection indicated more intense staining for GPx3 towards the inner part of the placental disc, nearest the cord insertion (Figure 2; $P = 0.05$, Kendall's $\tau$) in placentae from normotensive, but not preeclamptic, women. Five of the infants in the preeclamptic group had birthweight centiles below 10% (intrauterine growth restriction; IUGR) while 7 were above this value. The median (range) GPx3 staining near the cord insertion was 84 (65 – 97) units in

pre-eclamptic placentae associated with IUGR and 120 (70 – 151) units in preeclampsia without IUGR ($P$ = 0.042).

GPx4 positive staining, showed an opposite pattern, being significantly reduced in the inner part of the placental bed, with higher intensity at the outer edge (Fig 3; $P$ = 0.02, Kendall's $\tau$) only in the preeclamptic placentae. When GPx4 was examined in relation to IUGR in the preeclamptic group, the median (range) GPx staining in the outer edge was 75.7 (64.5 – 91.7) units in pre-eclamptic placentae associated with IUGR and 53.3 (22.5 – 80) units in preeclampsia without IUGR ($P$ = 0.028).

Placental GPx enzyme activity was also significantly reduced in tissues from preeclamptic women. The median placental GPx activity was 0.43 (0.19 – 0.56) nmol/min/mg in normotensive placentae, compared with 0.16 (0.10 – 0.28) nmol/min/mg in placentae from preeclamptic women ($P$ = 0.007). This difference was most pronounced nearest the cord insertion (Table 3).

**Discussion**

The main finding of our study is the differential changes in GPx expression and activity between preeclamptic and normotensive placentae. This is one of the first detailed studies to show the distribution of three of the main forms of GPx (GPx1, 3 and 4) in the human placenta. To ensure that the observed differences in GPx activity were not related to gestation age at delivery, we also compared these data with controls only for the preeclamptic women who delivered at ≥37 weeks' gestation. All between-group differences remained statistically-significant.

These three forms have different locations and perform slightly different functions, though all GPxs reduce hydroperoxides via glutathione. GPx3 is the predominant GPx in the plasma, and not only scavenges hydrogen peroxide and metabolises peroxidised organic molecules but also maintains the bioavailability of vascular nitric oxide, thus decreasing systemic oxidative stress. GPxs in the cytotrophoblast are ideally located for release into both the maternal and fetal circulations; these cells are exposed to relatively high oxygen concentrations and thus may protect the cells from oxidative stress [30].

Regulation of specific GPxs has been demonstrated at a number of levels; the effects of hypoxia on up-regulation of GPx3 expression are transcriptional whilst selenium and oxidative stress are believed to regulate GPx3 through both transcriptional and translational mechanisms [31]. Stability of mRNA for selenoproteins is controlled by nonsense-mediated decay [32, 33] which in turn is controlled by selenium supply; the available selenium being prioritised for synthesis of particular selenoproteins [34]. Regulation of GPx3 remains mainly uncharacterised; in inflammatory conditions, oxidative stress upregulates GPx3 at the

transcriptional level [35] yet surprisingly, the response to a more oxidised state in cancer cells is down-regulation [35].

The differential distribution of GPx4 in preeclampsia and in relation to IUGR has not been previously reported. GPx4 has been implicated in the functional modulation of cellular metabolic pathways, or more specifically, silencing eicosanoid formation by suppressing the activity of 15-lipoxygenase [36]. Hence the lower levels of this enzyme in preeclampsia may be contributing to the increased production of 15-hydroxyeicosapentaoic acid (15-HETE) by the placental trophoblast [37]. 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 peroxidation [38]. 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 dysfunction [16]. The defective placentation in preeclampsia could prevent the active placental transport of these lipid peroxides away from the fetus. We have previously reported [17] increased levels of thiobarbituric acid reactive substances (TBARS) in umbilical venous blood from cases with preeclampsia. The increased GPx4 expression in the periphery of the placenta could be due to this area having the highest density of terminal vasculature. This area of the placenta is known to be more sensitive to a variety of vasoactive substances [19], and so this increase in GPx4 may be a compensatory increase. At present, there is little known about the mechanism of GPx4 activation and so we cannot speculate any further.

The functional effect of such gradients cannot be determined at present. At least in the second trimester, both anatomical and Doppler studies have shown lower impedance to flow in the spiral arteries of the peri-insertion area of the placenta [39] where we identified the greatest

GPx3 expression. The reduced GPx3 expression in preeclamptic placentae might thus be of particular significance here and larger studies are required to fully investigate this. Studies of human placental samples *in vitro* suggested that enhanced villous maturation, syncytial knots and fibrin deposits, all features of preeclamptic placentae, were more frequent in samples from the outer edge under hypoxic conditions, and correlated with up-regulation of hypoxia-related transcripts [21]. Hypoxia has been shown to up-regulate GPx3 expression almost 3-fold in a renal cell line [40]; we are not aware of comparable studies of GPx4. Our group has previously shown no differences in placental GPx1, 3 and 4 mRNA expression between pregnancy groups [17]. However, the reduction of GPx activity and protein expression in the preeclamptic placentae indicates a possible post-translational modification/mutation reducing the GPx antioxidant activity; this has been observed outside pregnancy [41].

This study also observed no differences in GPx1 expression between sampling sites; GPx1 is the major cellular/cytosolic form of the enzyme. The expression of GPxs has been shown to be hierarchical, dependent on the availability of selenium, with GPx1 ranked low among the GPxs and other selenoproteins [12]. It has also been suggested that GPx1 may be more important as a buffer or storage form of selenium than as a glutathione peroxide [42, 43]. We speculate that this may be a contributing factor relating to this lack of regulation of GPx1 which requires further investigations.

Due to the limitations of the activity assay in specificity for each GPx isoforms, we plan to measure GPx 1, GPx3 and GPx4 expression in the three placental locations using Western Blot analysis, which would enable further justification of the immunohistochemical data. Also, although the numbers of Caesarean sections were significantly higher in the pre-eclamptic group compared to the normal pregnancies, all were performed in established

labour. We unfortunately did not record the duration of labour. It has been reported that labour increases maternal leukocyte GPx activity in normal pregnancies [44]. Therefore, it could be suggested that placentae from preeclamptic pregnancies may not have the same ability to respond to the ischemic hypoxic stress during labour that the normal pregnant placentae have, thus resulting in the reduction of both placental GPx activity and expression in preeclampsia observed in this study, but this requires further investigation.

There are morphological differences between placentae from women with preeclampsia alone, IUGR alone and preeclampsia with IUGR [45]. The surface area of peripheral villi is relatively decreased in preeclampsia with IUGR and the trophoblast volume is decreased but its thickness increased, which would impair transport of gases and nutrients. We observed a significantly lower placental GPx3 staining in preeclampsia with IUGR pregnancies but greater GPx4 staining. Whether such differences are cause or consequence is at present entirely speculative and requires further investigation.

In conclusion, we have shown distribution gradients in GPx expression and activity, which may relate to the differential oxygenation of regions of the placenta. As noted in the Introduction, there is increasing realisation that standardisation of placental sampling site is important, because both expression of various genes and vascular function differ across the placenta [18-21]. Our demonstration of significant gradients of GPx activity further supports the need for clear description of how placental sampling is standardised.

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**Conflict of Interest**

None

**Contributors**

All authors have contributed to this manuscript and have seen and approved the final version. The authors' responsibilities were as follows: H.D. Mistry completed this study as part of a PhD funded by BBSRC and wrote the majority of this article. P.J. Williams was the second assessor for the quantification of stained tissues and L.O. Kurlak completed some of the immunohistochemical work. F. Broughton Pipkin and M.E. Symonds were the principle investigators; M.M. Ramsay provided the clinical input for this study.

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