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Original Contribution

Association between maternal micronutrient status, oxidative stress, and common genetic variants in antioxidant enzymes at 15 weeks' gestation in nulliparous women who subsequently develop preeclampsia

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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. Deficiencies of specific micronutrient antioxidant activities associated with copper, selenium, zinc, and manganese have previously been linked to preeclampsia at the time of disease. Our aims were to investigate whether maternal plasma micronutrient concentrations and related antioxidant enzyme activities are altered before preeclampsia onset and to examine the dependence on genetic variations in these antioxidant enzymes. Predisease plasma samples (15 ± 1 weeks' gestation) were obtained from women enrolled in the international Screening for Pregnancy Endpoints (SCOPE) study who subsequently developed preeclampsia ($n=244$) and from age- and BMI-matched normotensive controls ($n=472$). Micronutrient concentrations were measured by inductively coupled plasma mass spectrometry; associated antioxidant enzyme activities, selenoprotein-P, ceruloplasmin concentration and activity, antioxidant capacity, and markers of oxidative stress were measured by colorimetric assays. Sixty-four tag–single-nucleotide polymorphisms (SNPs) within genes encoding the antioxidant enzymes and selenoprotein-P were genotyped using allele-specific competitive PCR. Plasma copper and ceruloplasmin concentrations were modestly but significantly elevated in women who subsequently developed preeclampsia (both $P<0.001$) compared to controls (median (IQR), copper, 1957.4 (1787, 2177.5) vs 1850.0 (1663.5, 2051.5) μg/L; ceruloplasmin, 2.5 (1.4, 3.2) vs 2.2 (1.2, 3.0) μg/ml). There were no differences in other micronutrients or enzymes between groups. No relationship was observed between genotype for SNPs and antioxidant enzyme activity. This analysis of a prospective cohort study reports maternal micronutrient concentrations in combination with associated antioxidant enzymes and SNPs in their encoding genes in women at 15 weeks' gestation that subsequently developed preeclampsia. The modest elevation in copper may contribute to oxidative stress, later in pregnancy, in those women that go on to develop preeclampsia. The lack of evidence to support the hypothesis that functional SNPs influence antioxidant enzyme activity in pregnant women argues against a role for these genes in the etiology of preeclampsia.

Abbreviations: BMI, body mass index; Cu/Zn SOD, copper/zinc superoxide dismutase; dBP, diastolic blood pressure; FRAP, ferric-reducing ability of plasma; GPx, glutathione peroxidase; ICP–MS, inductively coupled plasma mass spectrometry; Mn SOD, manganese superoxide dismutase; ROS, reactive oxygen species; sBP, systolic blood pressure; SCOPE, Screening for Pregnancy Endpoints; SGA, small for gestational age; SNP, single-nucleotide polymorphism; TBARS, thiobarbituric acid-reactive substances

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Introductiuon

Preeclampsia is a hypertensive condition affecting 2–7% of pregnant women and is associated with maternal multiorgan dysfunction. This disease, which manifests as high blood pressure and proteinuria, is probably mediated by endothelial damage and

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may affect multiple systems of the body and contribute to adverse pregnancy outcomes including maternal death, preterm birth, fetal growth restriction, and fetal death [1]. The etiology remains unknown, but placental defects and oxidative stress have been implicated and have been reported to develop at early gestation in affected pregnancies [2,3]. Several dietary micronutrients and in particular selenium, which plays a critical role in antioxidant defense, have been proposed to play a contributory role [4]. Dietary selenium intake in the United Kingdom, as in some other European countries, is below the recommended nutrient intake (60 μg/day for women) [5] and associations between lower blood selenium concentration and preeclampsia at delivery [6,7] have been reported. Selenium-dependent glutathione peroxidases (GPx's), which play a key role in protection against oxidative damage [5,8], have been shown to have reduced activity during pregnancy, with the lowest activity at delivery [7,9,10]. In addition, in women with preeclampsia, total GPx activity seems compromised, because we have reported a reduction in maternal and fetal activity and placental protein expression/activity of GPx at the time of delivery compared with controls [7]. Activity of the selenoproteins is determined by activity/translation of the relevant genes, among which a number of single-nucleotide polymorphisms (SNPs) are described in nonpregnant subjects, including some with reported effects on gene expression, mRNA translation, or protein stability [11–13]. Preeclampsia has a familial component [14–19], but it is not known whether these SNPs could, independently or in association with lower selenium status, be determinants of the putative impairment in antioxidant defense.

An increase in the plasma copper concentration has also been reported in women with established preeclampsia and this may generate oxidative stress by catalyzing the synthesis of reactive oxygen species (ROS) [20,21]. In addition, abnormally high serum ceruloplasmin concentrations in preeclampsia at delivery have been observed (approximately 96% of plasma copper is bound to ceruloplasmin) [22]. Lower plasma concentrations of zinc [21,23] and placental mRNA expression of copper/zinc superoxide dismutase (Cu/Zn SOD), as well as inadequate manganese status, have also been recorded in affected women [24,25]. Cu/Zn SOD, the cytosolic form of the enzyme, and mitochondrial manganese SOD (Mn SOD) provide important antioxidant defense [26], and lower plasma SOD concentrations have also been linked with preeclampsia [27–29]. The genotypes of SNPs in genes encoding Mn and Cu/Zn SOD [30,31] have been assessed in two studies, but with contradictory results [32,33], and lower plasma catalase activity has also been previously noted in preeclamptic women, after clinical diagnosis [21,28,29].

Nutrigenomics aims to reveal relationships between nutrition and the genome, allowing investigation of how genetic factors can influence nutritional requirements. It is likely that interactions between genotype and diet are important in determining the risk of common complex diseases [34,35], such as preeclampsia. Nutrients can interact with the genome and with SNPs to modulate disease risk such as cancer and cardiovascular disease, and SNPs in genes coding for antioxidant enzymes have been linked to cancer development [35–37].

We have therefore undertaken a study in a well-characterized prospective cohort of pregnant women to address (a) the hypothesis that inadequate antioxidant defenses contribute to the generation of oxidative stress in women before the development of preeclampsia and (b) whether this is confounded by suboptimal micronutrient status and (c) whether relationships between micronutrient status and antioxidant enzyme activities in pregnant women may be influenced by genetic variants affecting the function of the antioxidant enzymes.

Materials and methods

Participants

This was a nested preeclampsia case–control study of participants in the SCOPE (Screening for Pregnancy Endpoints; www.scopestudy.net) study, an international multicenter prospective cohort study of nulliparous singleton pregnancies with the aim of developing predictive screening tests for preeclampsia, preterm birth (PTB), and small-for-gestational-age (SGA) infants. Eligible women were recruited to the study between November 2004 and February 2011 at centers in Auckland, Adelaide, Leeds, London, Manchester, and Cork. Women were excluded if they were judged to be at high risk for preeclampsia, PTB, or SGA owing to underlying medical conditions, preexisting hypertension, or more than three previous miscarriages or terminations and those undergoing interventions that may affect pregnancy outcome, as previously described [38,39]. All participants provided informed written consent under ethical approval granted from local ethics committees (New Zealand AKX/02/00/364; Australia REC 1712/5/2008; London, Leeds, and Manchester 06/MRE01/98; and Cork ECM5(10) 05/02/08).

Samples and clinical data

Nonfasting heparinized plasma samples were selected from white women recruited at 15 ± 1 weeks' gestation: 244 women who developed preeclampsia and 472 controls were matched for BMI and age (Table 1 & 2). Women were considered to have a normal pregnancy outcome if they remained normotensive (<140 mm Hg systolic blood pressure (sBP) and/or <90 mm Hg diastolic blood pressure (dBP) before labor), showed no proteinuria, delivered a live baby after 37 weeks' gestation who was not SGA, and had no other signs of pregnancy-related complications. Definition of preeclampsia was blood pressure measurements, at least 4 h apart, after 20 weeks' gestation and before the onset of labor, in which sBP was ≥ 140 mm Hg and/or dBP was ≥ 90 mm Hg (Korotkoff V) plus proteinuria (≥ 300 mg per 24 h or spot urine protein:creatinine ratio ≥ 30 mg mmol^{-1} creatinine or urine dipstick protein $\geq ++$). For additional analysis, cases were subdivided into severe preeclampsia (sBP >160 or dBP >110 mm Hg), mild preeclampsia (sBP 140–160 and dBP 90–110 mm Hg), and also early- (delivery <34 weeks) and late-onset (delivery ≥ 34 weeks) groups. Relevant clinical data were extracted from the SCOPE database; biochemical analyses were conducted on heparinized plasma; DNA for genotyping was extracted from buffy coat samples.

Laboratory investigations

All biochemical analyses were carried out in triplicate, unless otherwise stated, with standard curves calculated using four-parameter logistic curve analysis using GraphPad Prism 5 (San Diego, CA, USA).

Micronutrient concentrations

Plasma concentrations of copper, zinc, selenium, and manganese were assayed by inductively coupled plasma mass spectrometry (ICP–MS) at *m/z* 65, 66, 78, and 55, respectively, as previously described [40]. Samples and standards (SPEX Certiprep, Inc.) were prepared identically in a diluent containing 0.1% Triton X-100 nonionic surfactant (+antifoam-B, Sigma), 2% methanol, and 1% HNO_3 (trace analysis grade), including the internal ICP–MS standards iridium (5 μg/L), rhodium (10 μg/L), gallium (25 μg/L), and scandium (50 μg/L). For all analytes, the ICP–MS was run in collision-reaction cell mode with pure H_2 as the cell gas to maximize sensitivity for ^{78}Se determination. Aspiration was

through a single sample line via a Burgener–Miramist PEEK nebulizer. Calibrations for all micronutrients were in the range 0–50 μg/L. Quality of analysis was ensured by the use of appropriate reference materials (Seronorm and UTAK; Nycomed Pharma AS). Trace-element-free techniques were used during collection and analysis, following guidelines from the International Zinc Nutrition Consultative Group. Both intra- and interassay coefficients of variation were $<5\%$.

Selenoprotein P

Plasma selenoprotein P concentrations were measured essentially as described previously [41]. Briefly, an immunoluminometric sandwich assay (Selenotest, ICI GmbH, Berlin, Germany) using two monoclonal mouse anti-human selenoprotein P antibodies was used. The analytical detection limit was 0.008 mg/L and the assay was linear on dilution. The inter- and intra-assay coefficients of variation were 7.5 and 2%, respectively.

Ceruloplasmin (activity)

Ceruloplasmin activity was assessed in plasma diluted 1:50, using a commercially available kit following the manufacturer's protocol (DetectX Ceruloplasmin Colorimetric Activity Kit K035-H1, Arbor Assays, Ann Arbor, MI, USA). The inter- and intra-assay coefficients of variation were 22.2 and 3.9%, respectively.

Ceruloplasmin (concentration)

Ceruloplasmin protein concentrations were measured using a competitive enzyme-linked immunoabsorbent assay (ELISA) as per the manufacturer's instructions (AssayMax Human Ceruloplasmin ELISA Kit EC4001–1, Assaypro LLC, St. Charles, MO, USA). Plasma samples were diluted 1:100 and analyzed in duplicate. The standards and plasma samples were added to plates precoated with a polyclonal antibody specific for human ceruloplasmin. The ceruloplasmin present in the samples competed with biotinylated ceruloplasmin sandwiched by the immobilized polyclonal antibody and streptavidin–peroxidase conjugate. The addition of tetramethylbenzidine, a peroxidase enzyme substrate, instigated a color development that was stopped with hydrochloric acid after 10 min. The optical density was subsequently measured at 450 nm; inter- and intra-assay coefficients of variation were 8.4 and 1.3%, respectively.

Glutathione peroxidase activity

GPx activity was measured using a commercially available kit, following the published instructions (Cayman Chemicals, Ann Arbor, MI, USA). Plasma samples were diluted 1:5 and analyzed in duplicate. Change in optical density (at 340 nm) was determined over a 6-min period and the baseline subtracted to calculate the rate of enzyme activity. The inter- and intra-assay coefficients of variation were 18.6 and 9.4%, respectively.

Genotyping

DNA was extracted from buffy coat samples by LGC Genomics (formerly KBioscience, Hertfordshire, UK), using KlearGene chemistry (DNA-binding and elution method followed by ethanol precipitation). Fourteen genes encoding antioxidant enzymes were selected for genotyping (Table 3). TagSNPs were identified using Tagger software applied to data from the HapMapII-CEU population of northern and western European ancestry, using a minor allele frequency of >0.1 and $r^2>80\%$. Nonsynonymous SNPs with a reported minor allele frequency of ≥ 0.03 were also included in the genotyping strategy. Genotyping was performed by LGC Genomics, with the use of their proprietary fluorescence-based competitive allele-specific PCR genotyping assay, KASP. As quality assurance measures, 15% of samples were assayed in duplicate; DNA-free negative controls were included in each 96-sample plate; samples that failed more than 10% of genotyping assays were excluded from the genotyping study. Further quality control measures included checks for deviations from Hardy–Weinberg equilibrium using a χ^2 goodness-of-fit statistic and comparison of interplate allele frequencies.

Thiobarbituric acid-reactive substances (TBARS)

The measurement of TBARS is a well-established method for monitoring lipid peroxidation utilizing the reaction between malondialdehyde (MDA), a product of lipid peroxidation, and thiobarbituric acid (TBA) at high temperatures of 90–100 °C. The MDA–TBA product formed is measured colorimetrically at 530–540 nm. Plasma samples were assayed using a commercial kit (Cayman Chemicals 10009055) according to a modified version of the manufacturer's protocol. After the reaction at high temperature, the vials were centrifuged for 15–20 min, longer than the recommended time and at room temperature rather than 4 °C: this minimized cloudiness in the samples, which interfered with the optical density assessments. The inter- and intra-assay coefficients of variation were 11.4 and 2.1%, respectively.

Ferric-reducing ability of plasma (FRAP)

The ferric-reducing ability of plasma was measured using a commercially available kit developed on the Benzie & Strain assay [42], according to the manufacturer's protocol (DetectX FRAP colorimetric detection assay K043-H1, Arbor Assays). At low pH a ferric–tripyridyltriazine complex is reduced to the ferrous (Fe^{II}) form provided a reductant (antioxidant) is present. In the FRAP assay, excess Fe^{III} is used so that the rate-limiting factor of the production of the Fe^{II} form of the complex is the reducing ability of the plasma sample. The workable assay range was 31.25–1000 μM and the plasma samples were assayed at a dilution of 1:2. In our laboratory, the inter- and intra-assay coefficients of variation were 9.3 and 4.3%, respectively.

Superoxide dismutase

Plasma SOD activity was measured using a commercially available kit, following the manufacturer's protocol for plasma samples (DetectX SOD Colorimetric Activity Kit K028-H1, Arbor Assays). Samples were assayed at a dilution of 1:5. The inter- and intra-assay coefficients of variation were 22.6 and 1.7%, respectively.

Catalase

Plasma catalase activity was measured using a commercially available kit, following the manufacturer's protocol (DetectX Catalase Colorimetric Activity Kit K033-H1, Arbor Assays). Samples were diluted 1:20 and analyzed in triplicate. The inter- and intra-assay coefficients of variation were 15.7 and 3.4%, respectively.

Power calculations

Micronutrient status

The number of samples used in this study provided 94% power to detect a difference of 0.4 standard deviations between cases and controls with an $\alpha=0.0005$.

SNP analysis

Statistical power for assessment of the relationships between SNPs and relevant enzyme activity was estimated using Quanto beta version 0.5.5. Using our previous pregnancy data [7] on GPx activity (mean 50 ± 10 nmol/min/ml) and selenium (mean 58.4 ± 14.9 μg/L) in an additive model for minor allele frequencies between 0.1 and 0.5 the study had 82% power to detect a difference in allelic expression that accounts for 4% or more of

Table 1
Characteristics at booking, at enrollment, and after onset of preeclampsia.

Parameter	Normotensive controls ($n=472$)	Preeclampsia ($n=244$)
Maternal age (years)	29 [23, 32]	28 [23, 32]
Booking body mass index (kg/m^2)	25.4 [22.9, 29.4]	26.3 [23.2, 31.1]
Highest first-trimester systolic BP (mm Hg)	107 [100, 112]	111 [104.8, 120]
Highest first-trimester diastolic BP (mm Hg)	63 [60, 69]	69 [63.5, 74]
Current smoker	58 (12)	24 (10)
Quit smoking	81 (17)	34 (14)
Never smoked	333 (71)	186 (76)
Gestational age at enrollment, weeks	15 [15, 16]	15 [14, 16]
After clinical diagnosis		
Highest systolic BP	120 [115, 130]	150 [145, 162]*
Highest diastolic BP	74 [70, 80]	98 [92, 104]*
Dipstick proteinuria		
Not done	258 (55)	142 (58)
Negative	214 (45)	–
Present 1+ or greater	–	102 (42)
Protein creatinine ratio (mg/mmol)	–	94 [46, 214] ($n=84$)
Early-onset preeclampsia	–	38 (15.6)
Severe preeclampsia	–	116 (47.5)

Data are presented as median [IQR] or number (percentage). Definitions: severe preeclampsia, sBP >160 or dBP >110 mm Hg; mild preeclampsia, sBP 140–160 and dBP 90–110 mm Hg; early onset, delivery <34 weeks; and late onset, delivery ≥ 34 weeks. Mann–Whitney *U* test was used for between-group comparisons.

* $P<0.0001$ for BP (blood pressure).

Table 2
Characteristics at delivery for women in each group.

Parameter	Normotensive controls ($n=472$)	Preeclampsia ($n=244$)
Gestation at delivery, weeks	40 [39,41]	38 [37,40]
Preterm delivery $<37/40$ weeks	0	34 (15.2)
Birth weight, g	3600 [3328,3866]	3190 [2570,3565]*
SGA (<10th customized birth weight centile)	0	61 (25)

Data are presented as the median [IQR] or number (percentage). SGA, small for gestational age. Mann–Whitney *U* test was used for between-group comparisons.

* $P<0.0001$.

the total variation in the 472 controls, equivalent to a change of 0.3 to 0.5 nmol/min/ml per minor allele for GPx activity, 4 to 7 μg/L for selenium. The Bonferroni correction for testing of multiple SNPs was applied to the power calculations, using $\alpha=0.0005$; this is a conservative adjustment in the light of the lack of independence between nearby SNPs, owing to linkage disequilibrium; moreover only 69 SNPs were in fact considered.

Statistical analyses

All statistical analysis was carried out in Stata version 11.2 (StataCorp, College Station, TX, USA) and the diagt package [43]. Data were checked for normality of distribution using appropriate distributional plots, and Box-Cox regression was used to determine optimal transformations where needed [44,45]. Summary data are presented as median (quartiles) or *n* (%) as appropriate. Between-group comparisons were made using Mann–Whitney *U* tests. Spearman's rank correlation test was used to test associations. SNP genotypes were coded 0, 1, or 2 dependent on the number of minor alleles, and genetic association with relevant enzyme activities was tested by linear regression analysis under an additive model in control and case samples separately (Table 4).

A formal Bonferroni correction to the *P* values for multiple testing was not used for interpretation of the results of micronutrient analyses, as the number of tests required for adjustment is a matter of opinion. Instead, the level of multiple testing, the coherent pattern of results, and the level of significance were borne in mind when considering how to interpret related results. Confidence intervals (95%) are given where appropriate [46,47].

Results

Subjects

The characteristics of 244 women who developed preeclampsia and 472 controls recruited at 15 ± 1 weeks' gestation are shown in Table 1; infant outcomes are shown in Table 2; the two groups were matched for age and BMI.

Genetic analysis

Fourteen genes were selected for genotyping, focusing on antioxidant enzymes and metalloproteins. Individuals were genotyped for 69 SNPs. Assay design was unsuccessful for 4 SNPs (rs8136921 in *TXN2*, rs1050450 in *GPX1*, rs4958872 in *GPX3*, rs4713167 in *GPX6*), and genotypes of rs480496 in the catalase gene deviated significantly from Hardy–Weinberg equilibrium; these 5 SNPs were excluded from further investigation. The genotyping success rate was over 97.5% for all the remaining 64 SNPs; allele frequencies in cases and controls are shown in Table 3. Ten subjects in whom genotyping was unsuccessful in 5 or more SNPs were excluded from further analysis of all genetic data.

Table 3
SNPs used to genotype.

Gene	SNP	Major/minor allele	Type	MAF controls	MAF cases	*P* value[a]
SOD1	rs10432782	T/G	Intron	0.13	0.11	0.51
SOD1	rs1041740	C/T	Intron	0.29	0.30	0.93
SOD1	rs2234694	A/C	Intron	0.04	0.05	0.80
SOD2	rs2855116	A/C	3′UTR	0.48	0.47	0.94
SOD2	rs5746136	C/T	3′UTR	0.31	0.30	0.79
SOD2	rs7855	A/G	3′UTR	0.04	0.05	0.86
SOD2	rs4880	A/G	Missense	0.49	0.49	0.92
SOD3	rs2536512	G/A	Missense	0.36	0.35	0.87
SOD3	rs1799895	C/G	Missense	0.01	0.01	0.18
CAT	rs2284365	T/C	Intron	0.21	0.19	0.07
CAT	rs533425	G/A	Intron	0.39	0.40	0.84
CAT	rs12270780	G/A	Intron	0.28	0.28	0.72
CAT	rs11032700	A/C	Intron	0.33	0.35	0.83
CAT	rs480496	G/A	Intron	Excluded—not in HWE		
CAT	rs10488736	C/T	Intron	0.31	0.32	0.67
CAT	rs11032703	C/T	Intron	0.13	0.09	0.08
CAT	rs1001179	C/T	5′FR	0.21	0.22	0.81
CAT	rs566979	A/C	Intron	0.37	0.40	0.59
CAT	rs480575	A/G	Intron	0.28	0.28	0.21
TXN	rs4135187	A/T	Intron	0.09	0.11	0.32
TXN	rs4135179	T/C	Intron	0.23	0.19	0.21
TXN	rs4135203	C/G	Intron	0.21	0.22	0.29
TXN	rs4135225	A/G	Intron	0.33	0.34	0.86
TXN	rs4135221	G/A	NCE	0.12	0.11	0.70
TXN	rs1410051	T/C	Intron	0.22	0.21	0.34
TXN	rs4135192	A/G	Intron	0.31	0.30	0.95
TXN	rs4135208	A/G	Intron	0.32	0.33	0.65
TXN	rs4135168	T/C	Intron	0.24	0.25	0.19
TXN	rs4135165	G/A	Intron	0.08	0.08	0.79
TXN	rs4135215	T/C	Intron	0.15	0.16	0.91
TXN	rs4135220	T/G	NCE	0.47	0.48	0.24
TXN	rs2418076	C/T	Intron	0.26	0.26	0.84
TXN	rs4135212	C/T	Intron	0.14	0.14	0.13
TXN	rs4135218	T/C	NCE	0.45	0.44	0.64
TXN2	rs8136921	T/C	Intron	Failed assay design		
TXN2	rs9622400	T/C	Intron	0.09	0.10	0.95
TXN2	rs2267337	C/T	Intron	0.18	0.20	0.68
TXN2	rs6519008	A/C	Intron	0.09	0.09	0.49
TXN2	rs8139906	G/C	Intron	0.17	0.17	0.46
TXN2	rs5756202	A/C	Intron	0.15	0.14	0.59
SEPP1	rs7579	C/T	3′UTR	0.27	0.30	0.48
SEPP1	rs3877899	C/T	Missense	0.24	0.22	0.54
SEPP1	rs230819	C/A	NCE	0.46	0.45	0.91
SEPP1	rs13168440	T/C	Intron	0.16	0.14	0.53
GPX1	rs1050450	G/A	Missense	Failed assay design		
GPX1	rs1800668	G/A	5′UTR	0.30	0.32	0.46
GPX1	rs8179169	C/G	Missense	Nonpolymorphic		
GPX2	rs2412065	C/G	Intron	0.19	0.21	0.60
GPX2	rs2737844	G/A	Intron	0.28	0.31	0.53
GPX2	rs17880492	G/A	Missense	Nonpolymorphic		
GPX3	rs4958434	G/A	Intron	0.17	0.16	0.60
GPX3	rs8177431	A/G	Intron	0.37	0.29	0.007
GPX3	rs4958872	T/C	Intron	Failed assay design		
GPX3	rs3763011	G/A	Intron	0.21	0.13	0.001
GPX3	rs2070593	G/A	3′UTR	0.19	0.13	0.01
GPX4	rs713041	C/T	Missense	0.44	0.42	0.70
GPX5	rs2273106	A/G	Intron	0.09	0.08	0.85
GPX5	rs440481	A/C	Intron	0.17	0.15	0.39
GPX5	rs445870	A/G	Intron	0.30	0.29	0.88
GPX5	rs58554303	T/C	Missense	0.02	0.01	0.16
GPX6	rs4713166	C/T	Intron	0.09	0.08	0.85
GPX6	rs2859358	T/A	Intron	0.18	0.16	0.58
GPX6	rs4713167	T/C	Intron	Failed assay design		
GPX6	rs35394555	C/G	Missense	< 0.01	< 0.01	0.77
GPX6	rs35062161	A/T	Missense	0.08	0.06	0.24
GPX6	rs406113	A/C	Missense	0.31	0.31	0.99
GPX7	rs946154	C/G	Intron	0.33	0.35	0.84
GPX7	rs3753753	G/C	Intron	0.28	0.28	0.97
GPX7	rs1047635	A/C	3′UTR	0.47	0.49	0.60

TagSNPs were genotyped in 14 genes encoding antioxidants and selenoproteins. MAF, minor allele frequency; HWE, Hardy–Weinberg equilibrium; 5′FR, 5′ flanking region; NCE, noncoding exon.

[a] *P* values are shown for case–control comparisons of genotype frequencies using χ^2 analysis.

The genetic component of this study was designed to investigate the effect of genetic variations on the activity of antioxidant enzymes. There was no association between SNP genotypes in the relevant genes and the activity of the antioxidant enzymes SOD, GPx, or catalase; selenoprotein P concentration; or TBARS or FRAP in cases or controls ($P > 0.05$ for all analyses; Table 4).

This study was not designed for case–control analysis of genetic association, for which considerably larger sample sizes are required to achieve adequate statistical power. Three SNPs within *GPX3*, which display partial linkage disequilibrium with one another ($D' = 1$; $r^2 = 0.3$ to 0.9), showed nominal association with preeclampsia ($P = 0.01$ to 0.001), but this was not significant after correction for multiple testing.

Micronutrients

The maternal copper concentration was significantly ($P < 0.0001$) higher in the women who later developed preeclampsia, compared to controls (Table 5). This difference was maintained when copper concentrations were analyzed in the individual participating countries, and overall only a small variation between countries and no significant differences were observed. All other micronutrients did not differ between groups ($P > 0.05$; Table 5). No additional differences were observed in micronutrient concentrations between either mild or severe/early or late onset preeclampsia ($P > 0.05$ for all).

Biochemical measurements

Ceruloplasmin concentrations were significantly increased in the women who subsequently developed preeclampsia compared with controls ($P = 0.02$; Table 5), whereas ceruloplasmin activity did not differ between groups. We measured TBARS as an indirect marker of oxidative stress via lipid peroxidation and FRAP to measure antioxidant capacity. There were no differences between groups in any of the associated antioxidant enzyme activities, FRAP, or TBARS ($P > 0.05$ for all; Table 5). No additional differences were observed between either mild or severe/early or late onset preeclampsia ($P > 0.05$ for all).

Significant positive correlations were observed between copper and both ceruloplasmin activity ($r = 0.24$, $P < 0.0001$) and ceruloplasmin concentration ($r = 0.16$, $P < 0.0001$). Copper and ceruloplasmin concentrations were positively associated with TBARS concentrations ($r = 0.15$, $P < 0.0001$; $r = 0.39$, $P < 0.0001$, respectively). In addition, significant associations were also found between plasma selenium status and both GPx activity ($r = 0.12$, $P = 0.005$) and selenoprotein P concentration ($r = 0.53$, $P < 0.0001$).

Table 4
Genetic variants tested for association with antioxidant markers.

Gene	Marker	Modifier
SOD1–3	SOD activity	Copper, zinc, manganese
GPX1–7	GPx activity	Selenium
CAT	Catalase activity	
SEPP1	Selenoprotein P concentration	Selenium; BMI
All genes	TBARS	
All genes	FRAP	

Genetic associations were tested separately in case and control groups by regression analysis of measures of antioxidant activity against genotype, with and without adjustment for modifying factors; $P > 0.05$ for all analyses. CAT, catalase; SOD, superoxide dismutase; GPx, glutathione peroxidase; TBARS, thiobarbituric acid-reactive substances; FRAP, ferric-reducing ability of plasma; BMI, body mass index.

Discussion

To our knowledge, this study is the first to report maternal plasma micronutrient concentrations in combination with associated antioxidant enzymes and markers of both antioxidant capacity and oxidative stress in women at 15 weeks' gestation who subsequently developed preeclampsia. Although many of the measured micronutrients, and associated enzyme activities, particularly selenium and GPx, have been shown to be reduced in women with established preeclampsia at time of delivery [5,7], we found no evidence of this in predisease samples. This observation suggests that the reduced selenium concentration and GPx activity and increased oxidative stress at delivery may be a consequence of the oxidative stress associated with preeclampsia, rather than a cause. Therefore this finding does not support the suggestion that supplementation with these micronutrients could help prevent the development of preeclampsia, which has its origins early in pregnancy.

The genetic component of this study was designed to assess the influence of SNPs on markers of plasma antioxidant activity at 15 ± 1 weeks' gestation, using a comprehensive tag-genotyping strategy. In contrast to some previous reports (discussed below) this study, which was powered to detect a genetic effect accounting for 4% of variation in target antioxidant markers in controls, did not identify any associations with genotype.

In a nonpregnant population, a study of two of the four SNPs in SEPP1, which were also included in the present study (rs7579 in

Table 5
Maternal plasma micronutrient concentrations, antioxidant activities, and associated concentrations in women who had a healthy pregnancy and those who developed preeclampsia.

Micronutrient/biochemical measurement	Normotensive controls ($n = 472$) (median [IQR])	Preeclampsia ($n = 244$) (median [IQR])
Selenium concentration (μg/L)	79.6 [73.1, 86.8]	79.0 [71.8, 87.4]
Copper concentration (μg/L)	1850.0 [1663.5, 2051.5]	1957.4 [1787, 2177.5]**
Zinc concentration (μg/L)	575.7 [515.6, 641.7]	579.6 [521.1, 638.6]
Manganese concentration (μg/L)	1.4 [0.7, 1.7]	1.4 [1.0, 1.7]
Ceruloplasmin activity (U/ml)	158.6 [133.5, 184.5]	162.3 [132.4, 187.1]
Ceruloplasmin concentration (μg/ml)	2.2 [1.2, 3.0]	2.5 [1.4, 3.2]*
Selenoprotein P (μg/ml)	3.4 [3.0, 3.8]	3.4 [3.0, 3.7]
FRAP activity (U/ml)	741.2 [651.6, 848.1]	715.5 [625.6, 822.0]
GPx activity (nmol/min/ml)	45.7 [26.8, 57.2]	43.3 [25.5, 58.8]
Superoxide dismutase activity (U/ml)	0.5 [0.3, 0.6]	0.5 [0.4, 0.6]
Catalase activity (U/ml)	25.4 [19.1, 33.7]	24.8 [19.0, 33.8]
TBARS (μM)	6.7 [5.6, 9.2]	6.5 [5.4, 8.3]

Data are presented as median [IQR]. GPx, glutathione peroxidase; TBARS, thiobarbituric acid-reactive substances; FRAP, ferric-reducing ability of plasma. Mann–Whitney *U* test was used for between-group comparisons.

* $P < 0.05$.

** $P < 0.001$.

the 3′UTR and the missense SNP rs3877899), reported a significant interaction between genotype, gender, and BMI, affecting plasma selenium and selenoprotein P concentrations and the activity of the selenoenzymes GPx1, GPx3, GPx4, and thioredoxin [48]. The interactive effects of *SEPP1* genotype and BMI were most evident after selenium supplementation, suggesting that the effects of genotype may be modified by nutritional status. A further study of four tagSNPs in *SEPP1* showed that baseline plasma selenium and genotype were not associated [49], although there is evidence that the relative abundance of the two isoforms of selenoprotein P at baseline are influenced by genotype [50]. In the present study, plasma selenium, selenoprotein P, and GPx activity were significantly correlated, as in the nonpregnant state, but *SEPP1* genotype was not associated with any of these markers, either alone or after adjustment for BMI (data not shown). Plasma selenium concentrations are low in normal pregnancy, suggesting that the operation of regulatory factors, including nutritional status and plasma dilution, may mask the effect of *SEPP* genotype in pregnancy.

For completeness, genotyping covering all SNPs in the *GPX1–7* genes was undertaken as part of this study, although GPx5 and GPx7 are not selenoproteins. GPx activity in plasma, which is predominantly due to GPx3 expression, was not associated with genotype for SNPs in any of the GPX genes tested. After their finding of an association between two intronic SNPs in *GPX3* and gastric cancer, Wang et al. conducted reporter gene studies that supported functional effects of these SNPs on transcriptional expression [51]. SNP rs4958872, which is a perfect proxy for one of these intronic SNPs, failed assay design in the present study, but it is partially captured by rs4958434 and rs8177431 ($D'=1$; $r^2=0.75$), which showed no evidence of association with plasma GPx activity. Associations between SNP genotypes in *GPX1* and *GPX4* and GPx activity measured in erythrocytes or lymphocytes have been reported in nonpregnant subjects [11–13]. We did not have access to suitable material for investigation of intracellular GPx activity, which may have limited our ability to detect SNP-dependent variability.

This study did not demonstrate any association between tagSNPs in the three SOD genes encoding soluble Cu/Zn SOD (*SOD1*), Mn SOD (*SOD2*), or extracellular Cu/Zn SOD (*SOD3*) and preeclampsia. Polymorphisms within the SOD genes have shown statistical association in case–control studies with disorders featuring oxidative damage, including cancers and diabetes [52], but there have been relatively few studies of the functional basis for these associations. An Ala → Thr missense polymorphism in *SOD3* (rs2536512) was associated with preeclampsia complicated by fetal growth restriction in a relatively small study (159 cases and 114 controls) [33]. This finding was not replicated in the present study, nor was there any evidence for an association between rs2536512 and plasma SOD activity. Decreased expression of Cu/Zn SOD in trophoblast cells harvested from preeclamptic placentae has been demonstrated [53]; studies of fetal SOD genotypes would therefore be of interest.

Reporter gene assays, supported by measurements of catalase activity in genotyped individuals, have demonstrated that catalase expression is influenced by a polymorphism in the 5′ flanking region of the gene (rs1001179), which affects transcription factor binding [54]. The association of the minor allele at this SNP with lower red cell catalase activity has been confirmed, and an interaction with fruit and vegetable consumption has been documented [55]. In the present study, the lack of association of plasma catalase with genotype at this or any other tagSNP may be due to the overriding influence of the oxidative stress associated with normal pregnancy or the moderating effects of diet.

The raised copper and ceruloplasmin concentrations in women who subsequently develop preeclampsia are in accord with reports of increased copper concentrations in women with preeclampsia at the time of disease [20,21], suggesting a potential etiological role. Copper is a component of several metalloenzymes and an essential cofactor of antioxidant enzymes, but can also act as an oxidant [56]. Fattah et al. reported increased copper concentrations in maternal blood but not placenta or cord blood in preeclampsia before delivery, suggesting that the placenta is not the source of the copper; rather it may be released from other tissues such as the liver [22].

Ceruloplasmin is known to be an acute-phase reactant, being stimulated by inflammatory cytokines leading to increased serum concentrations rising during infection, inflammation, and tissue trauma [57]. During pregnancy, plasma copper concentrations increase owing to induction of ceruloplasmin by estrogen, returning to normal nonpregnant values after delivery [58–60]. In this cohort, the estrogen concentrations were not available. Because copper is a redox-active transition metal and can participate in single-electron reactions and catalyze free radical formation, the modest elevation in plasma copper observed in the women who later developed preeclampsia could contribute to oxidative stress in association with the onset of disease later on in pregnancy [20]. Conversely, the raised copper concentration may also be a protective response to prevent excessive ROS production and oxidative stress, via its antioxidant capacity, through the induction of copper-dependent antioxidant enzymes. Our data are in accordance with previous reports of raised serum ceruloplasmin and copper in women with established preeclampsia, which, unlike this study, also showed a correlation with elevated serum TBARS and prompted the suggestion that synthesis occurred in response to lipid peroxidation [20,57,61]. The discordance between the increased ceruloplasmin concentrations and activity in the women who later developed preeclampsia could reflect a disproportionate increase in copper binding capacity, rather than its secondary role as a ferroxidase enzyme, as Vitoratos et al. have reported increased ceruloplasmin concentrations in serum of preeclamptic women, but no differences in ferroxidase activities [57]. This may indicate biological dysfunction of the ceruloplasmin molecule. However, further studies are required to substantiate this hypothesis.

A limitation of the study was its restriction to women of white European ancestry, to avoid the confounding effects of population admixture on interpretation of the results of SNP genotyping. Future studies are required to confirm the increased copper and ceruloplasmin concentrations in other ethnic groups.

In conclusion, this study reports maternal micronutrient concentrations in combination with associated antioxidant enzymes and SNPs in their encoding genes in women at 15 weeks' gestation that subsequently developed preeclampsia. The modest elevation in copper may contribute to oxidative stress, later in pregnancy, in those women that go on to develop preeclampsia. The lack of evidence to support the hypothesis that functional SNPs influence antioxidant enzyme activity in pregnant women argues against a role for these genes in the etiology of preeclampsia.

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