ORIGINAL ARTICLE

Low 2-methoxyestradiol levels at the first trimester of pregnancy are associated with the development of pre-eclampsia

Alejandra Pérez-Sepúlveda[1], María José Torres[1], Francisco J. Valenzuela[1], Raimundo Larraín[1], Horacio Figueroa-Diesel[1,2], José Galaz[1], Jyh Kae Nien[1,2], Ramón Serra[1], Luis Michea[3] and Sebastián E. Illanes[1,2]*

[1]Fetal Medicine Unit, Department of Obstetrics and Gynecology, Universidad de los Andes, San Carlos de Apoquindo, Santiago, Chile
[2]Clinical Perinatal Unit, Clínica Dávila, Santiago, Chile
[3]Biomedical Sciences Institute, Universidad de Chile, Santiago, Chile
*Correspondence to: Sebastián E. Illanes. E-mail: sillanes@uandes.cl

ABSTRACT

Objective To determine whether maternal plasma levels of 2-methoxyestradiol (2-ME) are decreased early in pregnancies that subsequently develop pre-eclampsia (PE) and whether this difference could be attributed to the presence of Val158Met catechol-*O*-methyltransferase (*COMT*) polymorphism in the placenta.

Methods Clinical characteristics and plasma samples were collected at 11 to 14 weeks prospectively in a cohort of patients. From them, 13 PE and 72 control pregnant women were chosen. Plasma soluble fms-like tyrosine kinase1 and placental growth factor levels were measured by electrochemiluminescence and 2-ME was measured by high-performance liquid chromatography with mass spectrometry/mass spectrometry detection. At delivery, placental tissue was collected and the Val158Met *COMT* polymorphism was determined by restriction fragment length polymorphism–PCR.

Results At 11 to 14 weeks, patients who would develop PE have significantly lower plasma levels of 2-ME than controls [1.9 ± 2 standard error of the mean (SEM) vs 61.7 ± 27 pg/mL, $P < 0.05$]. The Val158Met polymorphism was more frequent in controls than in PE patients and the placental presence of *COMT* polymorphism was associated with a decreased risk of developing PE [PE: 23.1% vs control: 66.6%; $\chi^2 = 10.9$, $p = 0.0041$].

Conclusions Lower plasma concentrations of 2-ME during early pregnancy in patients who subsequently develop PE were found. Presence of placental Val158Met *COMT* polymorphism is associated with a decreased risk to develop PE, suggesting a protective role against PE. © 2012 John Wiley & Sons, Ltd.

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INTRODUCTION

Pre-exclampsia (PE) is one of the leading causes of maternal and fetal morbidity and mortality, affecting ≈5% of all pregnancies. The etiopathogenesis of this disease is still not completely understood; however, it is clear that PE is associated with a poor trophoblastic invasion early in pregnancy, placental hypoxia (therefore elevated HIF-1a levels) and angiogenic imbalance with increased expression of soluble fms-like tyrosine kinase 1 (sFlt-1) [at 12 weeks: control: 973 ± 490 pg/mL vs PE: 1048 ± 657 pg/mL][1] and decreased levels of placental growth factor (PlGF) [at 12 weeks: control: 63 ± 145 pg/mL vs PE: 23 ± 24 pg/mL].[1] All these alterations produce endothelial dysfunction that contributes to the clinical manifestations of the disease.

Recent evidence suggests that 2-methoxyestradiol (2-ME) might be necessary for cytotrophoblast invasion of the maternal decidua, and therefore could be involved in the etiopathogenesis of PE.[2] 2-ME is synthesized by the *O*-methylation of the catechol ring of a metabolite of 17-β-estradiol by catechol-*O*-methyltransferase (*COMT*).[3,4] It is involved in the catabolism of HIF-1α and in the regulation of the appropriate cytotrophoblast invasion process.[2] Normally, the levels of 2-ME in human plasma are in the picomolar range and during normal pregnancy 2-ME levels increase more than 1000-fold (11th–16th week: 0.7 ng/mL; 37th–40th week: 3.8 ng/mL).[5] Low levels of 2-ME (0.8 ng/mL) have been reported in a cohort of women with clinical PE (at late second trimester and third trimester) with respect to a control group matched by gestational age (PE: 0.8 ng/mL, $n = 8$; controls: 1.5 ng/mL, $n = 13$).[6] One possible explanation for this observation is the presence of a G-to-A transition in codon 158 of the *COMT* gene that results in a valine to methionine substitution at position 108/158 in soluble-bound and membrane-bound COMT proteins, respectively.[7,8] This single-nucleotide

polymorphism (SNP) affects protein abundance and enzyme activity.[9,10] Recently, it has been described that the deletion of the *COMT* gene in mice is associated with the development of a PE-like phenotype, characterized by high blood pressure (BP), proteinuria and preterm delivery. Administration of 2-ME to *COMT*-deficient mice reverts the PE-like phenotype without toxicity, supporting the role of *COMT* activity and 2-ME as pathogenic factors in PE.[6]

No studies have been conducted to determine the association between the presence of the G-to-A transition in codon 158 in the *COMT* gene, decreased levels of 2-ME and the development of PE. Our hypothesis was that women who would develop PE have lower plasma levels of 2-ME at the first trimester of pregnancy compared with control women. We also sought to determine whether the development of PE and/or low levels of 2-ME were associated with the presence of the Val158/108Met *COMT* polymorphism in placental tissue.

METHODS

Study design

A prospective case–control study was conducted in the Obstetrics and Fetal Medicine Unit from Hospital Parroquial de San Bernardo, Santiago, Chile. In total, 13 pre-eclamptic women (6 mild PE and 7 severe PE) were recruited from an ongoing prenatal cohort. The controls, who do not differ in racial origin from PE patients, were randomly selected from the same cohort, to generate a sample of 72 healthy control subjects without pregnancy complications or chronic medical problems. They did not take multivitamins at enrolment and they were not prescribed during pregnancy. Both groups consisted of women with singleton gestation. Maternal anthropometric data and blood samples were collected at 11 to 14 weeks' gestation. PE was defined as hypertension (PA higher or equal to 140/90 mmHg on two occasions separated by 6 h or PA higher or equal to 160/110 mmHg) that occurred after 20 weeks of gestation, in women with previously normal blood pressure, accompanied by proteinuria (300 mg/24 h). Severe PE was diagnosed when at least one of the following symptoms was present: severe proteinuria (>3 g/24 h), elevated blood pressure (≥160 mm Hg systolic BP and ≥100 mm Hg diastolic BP) or low platelet counts (<100 000/mm ^{3}).

Written informed consent was obtained from the women who agreed to participate in the study, which was approved by Hospital Parroquial de San Bernardo and the Universidad de los Andes Ethics Committee.

Blood and tissue sample collection

Individual maternal blood samples were drawn at 11 to 14 weeks of gestation into two types of BD Vacutainer® tubes: K2 EDTA and serum. The samples were then transferred to 1.5 mL polypropylene tubes and centrifuged at 4 °C with a relative centrifugal force of 1600*g* for 10 min. The supernatants were transferred to clean 1.5 mL polypropylene tubes and centrifuged again at 4 °C, at 8700*g* for 10 min. Aliquots were stored at −80 °C until use.

Placental tissues were obtained within 15 min of delivery from all of the subjects. Placental samples (≈1 cm ^{3}) were taken from a standardized location, a placental cotyledon between cord insertion and placental border midway, avoiding tissue from areas showing placental infarcts. The tissue was washed in ice-cold physiologic solution (NaCl 0.9%) to remove maternal blood contamination. Samples were processed and stored at −80 °C until use.

sFlt-1 and placental growth factor measurement

A blind assay was performed to measure total serum sFlt-1 andbiologically active PlGF levels in plasma samples by electrochemiluminescence immunoassay (Elecsys, Roche, Mannheim, Germany, Cat. No. 05109523 and 05144671, respectively) on a Cobas e 411 analyzer (Roche, Mannheim, Germany), following the manufacturer's recommendations.

2-Methoxyestradiol measurement

Measurement of EDTA-tube plasma 2-ME was performed in a subgroup of patients (13 control women and all the women that developed PE, 6 with mild PE and 7 with severe PE) by Pharmaceutical Product Development, Inc. (PPD, Richmond, VA, USA). To ensure a lack of bias, this measurement was designed as a blind assay. Briefly, a 100 μL plasma sample aliquot was fortified with 50 μL of internal standard working solution. A 10% methanol in water solution and hydrolysis buffer was added and the sample was then vortexed. Extraction solvent was added and the sample was vortex-mixed and centrifuged. The aqueous layer was frozen and the organic layer was transferred to a clean tube. The organic-soluble extract was evaporated and the remaining residue was reconstituted with 200 μL of 20% mobile phase B in A. A 50-μL volume of the final extract was injected and analyzed via high-performance liquid chromatography (HPLC) with mass spectrometry/mass spectrometry (MS/MS) detection. The specificity for 2-ME determination of this method is 100%, without cross reactivity for other similar species. The quantification limit of the validated assay is 1 ng/mL.

Val158/108Met *COMT* polymorphism genotyping

To determine the presence of placental Val158/108Met (rs4680; http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=4680) COMT SNP, tissue DNA was isolated from samples by using MasterPure DNA purification kit (Cat# MC85200, Epicentre Biotechnologies, Madison, USA), following the manufacturer's recommendations. After extraction, DNA samples were stored at −20 °C. The identification of the Val158/108Met *COMT* SNP was performed using PCR–restriction fragment length polymorphism (RFLP) technique. Briefly, to amplify the fragment of interest, the following primers were used: sense 5′-CATCACCATCGAGATCAAC-3′ and antisense 5′-CAGTGAACGTGGTGTGAAC-3′. A 182 bp product was obtained with initial denaturation at 95 °C for 2 min and the following PCR amplification protocol: denaturation at 95 °C for 20 s; annealing at 57 °C for 20 s, and extension at 72 °C for 30 s (35 cycles). A final extension was performed at 72 °C for 5 min. PCR products were digested by restriction enzyme Hsp92II (New England Biolab, MA, USA). When the SNP was absent, four restriction fragments were generated: 114pb, 67pb, 54pb, 32pb. The presence of the SNP

created an additional restriction site, resulting in five fragments of 96pb, 67pb, 54pb, 32pb and 18pb observed in the separation by polyacrylamide gel electrophoresis.

COMT single-nucleotide polymorphism association analysis

The *COMT* SNP association analysis with PE included the three possible genotypes (Val/Val; Val/Met; Met/Met) and was carried out using a Pearson's Chi-square statistic test. The relationship between the *COMT* SNP and PE was also tested separately for the subgroups of pre-eclamptic women against non-pre-eclamptic control women, using a dichotomous method (positive-SNP vs negative-SNP). This was carried out to analyze whether carrying the *COMT* SNP was associated with the development of the disease, and a Chi-square test was used for this purpose. A threshold of $\alpha = 0.05$ was set for statistical significance of all computed analyses. Hardy–Weinberg proportions concordance was tested using a Chi-square goodness of fit statistic.

Statistical analysis

Statistical analysis was performed using a Mann–Whitney test to establish differences in parameters between controls and cases. Values are expressed as mean ± SEM. Significant differences were considered when $p < 0.05$.

RESULTS

Clinical characteristic of the population

The clinical characteristics of the total group of patients enrolled in this study are shown in Table 1. At 11 to 14 weeks of gestation, women who developed PE later in gestation showed a significantly higher systolic and diastolic arterial pressure ($p = 0.007$ and $p = 0.001$, respectively) than controls. No significant differences were observed in maternal age, maternal weight or body mass index at this time of pregnancy.

Table 1 **Clinical characteristics of controls and pre-eclampsia patients, at 11 to 14 weeks of gestation**

	Control patients ($n = 72$)	Pre-eclampsia patients ($n = 13$)	*p*-value
Maternal age (years)	25.46 ± 0.74	30.0 ± 2.39	0.1560
At 11 to 14 weeks			
Systolic pressure (mmHg)	106.88 ± 1.28	118.46 ± 3.90	0.0069*
Diastolic pressure (mmHg)	64.76 ± 1.02	73.69 ± 2.16	0.001**
Maternal weight (kg)	66.07 ± 1.60	71.42 ± 3.54	0.1298
Body mass index (kg/m^2)	26.72 ± 0.61	28.16 ± 1.08	0.1872

Values are given as mean ± SEM. Statistical significance was assessed using Mann–Whitney test.
*$p < 0.05$.
**$p < 0.005$.

Also, no significant difference was observed in *s*Flt-1 and PlGF plasma levels (Table 2).

Low 2-methoxyestradiol levels in women who will develop pre-eclampsia

Plasma concentrations of 2-ME were measured at 11 to 14 weeks of gestation in a subgroup of patients. We used HPLC/MS to measure plasma levels of 2-ME.[6] As shown in Figure 1, women who developed PE had significantly lower plasma levels of 2-ME than women with a normal pregnancy [1.9 ± 2 (SEM) vs 61.7 ± 27 pg/mL, $p < 0.05$].

Placental frequency of *COMT* polymorphism

To determine the placental presence of Val158Met polymorphism in *COMT* gene and its relation with PE development, we determined the genotype of placental tissue from control and pre-eclamptic women. Table 3 shows the frequencies of the Val158Met *COMT* polymorphism. The presence of the SNP was more frequent in controls than in PE women [Control: 66.7% vs PE: 23.1%; $\chi^2 = 10.9$, $p = 0.0041$]. The odds ratio (OR) calculated based on these results gives a

Table 2 **PlGF and sFLT-1 levels of controls and preeclampsia patients at 11 to 14 weeks of gestation**

	Control patients ($n = 15$)	Pre-eclampsia patients ($n = 13$)	*p*-value
PlGF	55.48 ± 6.50	43.2 ± 4.02	0.2894
sFLT-1	1522.64 ± 130.10	1595.85 ± 283.63	0.9633

Values are given as mean ± SEM. Statistical significance was assessed using Mann–Whitney test.

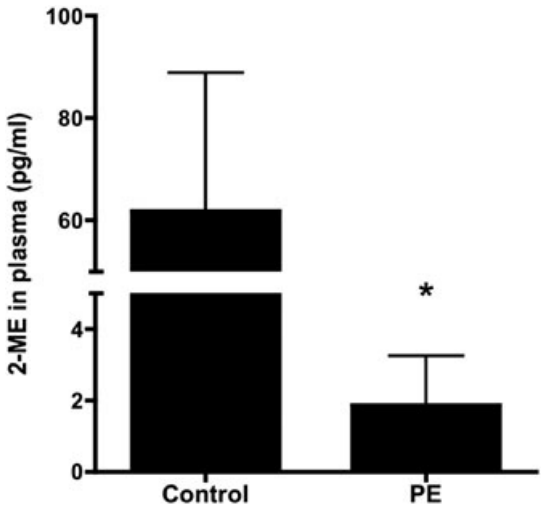

Figure 1 **Low levels of 2-ME in plasma samples of control women and women who will developed PE. Samples were taken at 11 to 14 weeks of gestation. The measurement of 2-ME was assessed by HPLC associated with MS, under optimal conditions. Values are means ± SEM, from 13 control women and 13 women who will be diagnosed with PE at 22 to 24 weeks of gestation. * $P < 0.05$ versus control by unpaired *t*-test**

Table 3 **Frequencies of genotypes of the *COMT* gene in controls and pre-eclamptic patients**

Genotypes	Control patients ($n=72$)	Pre-eclampsia patients ($n=13$)	χ^2(df)	p-value	OR	[95% Conf. interval]
COMT						
Wild type	24 (33.33%)	10 (76.9%)	10.97 (2)	0.0041*	0.15	[0.038–0.597]
Heterocygote/homocygote	48 (66.66%)	3 (23.1%)				

Wild type (*COMT*/AA), heterozygote (*COMT*/AG) and homozygote (*COMT*/GG). Data show the number of control and pre-eclamptic patients with or without the polymorphism. Statistical significance was assessed using Chi-square test.
*$p<0.005$.

probability of 13% for the development of disease [OR = 0.15; 95% confidence interval (CI) = 0.038 to 0.6].

DISCUSSION

In this study we report lower levels of 2-ME in plasma samples obtained at the first trimester from women that developed PE later in pregnancy, as compared with controls. At 11 to 14 weeks of gestation, our cohort showed significantly higher systolic and diastolic blood pressure in women who developed PE compared with controls ($p<0.005$). Despite that our angiogenic marker measurements did not differ statistically between control and women who would develop PE, due probably to our sample size, our results followed the known trend of these markers.[11] In the same way, the body mass index of patients who developed PE was higher compared with control women, without reaching statistical difference. These last features have been described previously as predictors of PE or related to the development of this disease.[12–14]

2-Methoxyestradiol is a naturally occurring metabolite of estradiol. It is generated by hydroxylation at the 2-position of 17-β-estradiol by Cytochrome P450 enzymes, and subsequently *O*-methylation of the catechol ring by *COMT*.[15] Human plasma levels of this metabolite are, in normal conditions, in the picomolar range. Berg *et al.* published, in 1983, the measurements of 2-ME levels during normal pregnancy, assessed by radioimmunoassay.[5] With this technique they showed that during pregnancy the plasma levels of 2-ME increase more than 1000-fold, proportional to the gestational age (11th–16th week: 0.77 ng/mL; 37th–40th week: 3.76 ng/mL).[4] Kanasaki *et al.* in 2008 reported that plasma levels of 2-ME are decreased significantly in pre-eclamptic women at 22 to 29 weeks, compared with women with normal pregnancies at the same gestational age. They assessed the 2-ME levels by HPLC/MS, one of the most sensitive and specific approaches to measure this molecule. Our results using this technique are consistent with previous observations,[6] and provide new evidence showing low concentration of 2-ME at 11 to 14 weeks of gestation in women who would develop PE.

One possible pathway that explains low 2-ME levels during pregnancy is the presence of the *COMT* Val158Met polymorphism.[3] Considering that the placental *COMT* could be responsible for the majority of 2-ME production during pregnancy,[2,6,16,17] we evaluated the placental genotype, to establish an association between the presence of the *COMT* polymorphism in the placenta and the development of PE. Contrary to our expectations, the presence of the Val158Met polymorphism is more frequent in control women placentas than in placental tissue of PE women, and the presence of this SNP in placental *COMT* gene is significantly associated with a reduced risk to develop PE. This observation suggests a protective role against PE of this polymorphism in the placenta.

It has been recently reported by Hill *et al.* that maternal 'low-activity' *COMT* genotype is associated with a significantly reduced risk for PE.[18] TIn this study was conducted in a Chilean population of similar ethnic characteristics as ours and four SNPs were analyzed, including the *COMT* SNP analyzed in this paper. The other three SNPs are indirectly involved in the enzymatic activity change of *COMT*. With this approach, the authors have found that the maternal low activity haplotype of *COMT* is associated with a significantly lower risk for PE. Despite our results being obtained from the analysis of one *COMT* SNP, they suggest that the presence of the Val158Met *COMT* polymorphism in placenta could be a protective factor against PE. This protective effect could be achieved by inducing compensation mechanisms in the mother to respond to pregnancy requirements of 2-ME. Thus, PE could appear also when placental 2-ME production is insufficient and the maternal side cannot compensate for this failure. Although more research is needed to clarify these results, it is possible that both maternal and placental systems responsible for 2-ME production would be complementary and redundant to ensure sufficient production of this metabolite in response to the pregnancy requirement.

Also, low levels of 2-ME could be caused by an alteration in the methionine-homocysteine metabolism (MHM) because of abnormalities in the re-methylation pathway. MHM plays a critical role in determining the availability of methionine that is essential for placental and fetal development.[19] Defects in the re-methylation pathway reduce the availability of methionine, needed for the production of S-adenosylmethionine, which is the main donor of the methyl groups inside cells and essential for the activity of *COMT*.[20] Alterations in MHM are caused mainly by SNPs of the genes that code for the enzymes participating in this metabolic pathway, such as the methionine synthase, the methionine synthase reductase and methylenetetrahydrofolate reductase,[27] which can lead to increased hyperhomocysteinemia, causing endothelial cell dysfunction and oxidative stress, which are key factors in the etiopathogenesis of PE. Moreover, it

is known that hyperhomocysteinemia and diminished levels of S-adenosylmethionine are associated with PE.[21–26]

CONCLUSION

This study shows that during the first trimester of pregnancy, women who would develop PE have lower plasma levels of 2-ME compared with controls. Placental presence of the Val158Met *COMT* polymorphism is associated with a decreased risk to develop PE, suggesting a protective role of this polymorphism against PE. More investigations are needed to elucidate the role of 2-ME in the development of PE and the mechanism involved.

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WHAT'S ALREADY KNOWN ABOUT THIS TOPIC?

- 2-Methoxyestradiol could be an important factor in the development of PE. *COMT* knock out mice have a PE-like phenotype, that is reverted by the administration of 2-ME.

WHAT DOES THIS STUDY ADD?

- This study shows that low plasma levels of 2-methoxyestradiol (2-ME) at the first trimester of pregnancy, are associated with the development of pre-eclampsia (PE). The presence of *COMT* polymorphism, a key enzyme in the metabolism of 2-ME, is associated with a decreased risk to develop PE, suggesting a protective role against PE.

CONTRIBUTION TO AUTHORSHIP

AP-S: designed the main aspects of the study, conceived and designed experiments. She also performed experiments and analyzed data. She participated in the writing of the manuscript. SEI: contributed to elaborate the initial idea about the study and to design experiments. He also participated in data analysis and the writing of the paper. LM and HF-D: contributed to the analysis of data and also participated in the writing of the paper. JKN and RS: participated in the analysis of the data and results. MJT, FJV, and RL: participated in the performance of experiments and data analysis. JG: helped perform experiments.

DETAILS OF ETHICS APPROVAL

This study was approved by the Universidad de los Andes Ethics Committee on August 23, 2010 (Reference Number EC0810)

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