

## Possible aggravating impact of gene polymorphism in women with endometriosis

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**Background & objectives:** Endometriosis is one of the most commonly encountered benign problems in gynaecology. It is frequently associated with chronic pelvic pain, dysmenorrhoea, menorrhagia and dyspareunia, which lead to infertility. To determine the possible association between polychlorinated biphenyls (PCBs) and GSTM1 null (\*0/\*0) mutation and their possible impact in the pathogenesis of endometriosis.

**Methods:** Ninety seven women with endometriosis mean age ( $28.5 \pm 6.5$  yr) diagnosed by laparoscopy and 102 women without endometriosis ( $28.4 \pm 4.8$  yr) were included. Heparinised blood samples were collected from all for DNA isolation and estimation of PCBs. GSTM1 genotyping was done by PCR and PCBs were estimated by gas chromatography.

**Results:** Women with endometriosis showed significantly higher concentrations of PCBs compared with control group. Twenty six (26.8%) women with endometriosis and 15 (14.7%) of the controls had the GSTM1 null (\*0/\*0) genotype [odds ratio (OR) = 2.12, 95% confidence interval (CI) = 1.045-4.314], which showed significant association ( $P=0.03$ ) with endometriosis. The association between the concentrations of PCBs, GSTM1 null genotype and different severity of endometriosis was significant ( $P<0.05$ ) for all four compounds and GSTM1 (PCB1:  $r = +0.5388$ ,  $P<0.0001$ ; PCB5:  $r = +0.6753$ ,  $P<0.0001$ ; PCB29:  $r = +0.6471$ ,  $P<0.0001$ ; and PCB98:  $r = +0.4357$ ,  $P<0.0001$ ; GSTM1:  $r = +0.9439$ ,  $P=0.05$ ).

**Interpretation & conclusions:** The study results suggested that women having higher concentration of PCBs and GSTM1 null (\*0/\*0) polymorphism might have an increased susceptibility of endometriosis. The findings need to be confirmed in a larger sample.

**Key words** Endometriosis - GSTM1 - PCBs - polymorphism

Endometriosis is a debilitating gynaecological disorder characterized by the presence of endometrial-like tissue outside the uterus, most commonly the pelvic peritoneum, ovaries, and rectovaginal septum<sup>1</sup>. It is frequently associated with chronic pelvic pain, dysmenorrhoea, menorrhagia and dyspareunia, leading

to infertility<sup>2,3</sup>. It affects approximately 15 per cent of women of childbearing age<sup>4,5</sup>. Despite extensive research on endometriosis, our knowledge of its aetiology and pathogenesis is quite limited<sup>6,7</sup>. The widely accepted retrograde menstruation theory<sup>8</sup> also fails to explain the development of endometriosis in the majority of cases.

Environmental pollutants that have previously shown to be linked to endometriosis are polyhalogenated aromatic hydrocarbons (PHAH), a class of widespread environmental contaminants that includes polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polychlorinated biphenyls (PCBs) and phthalate esters (PEs)<sup>9,10</sup>. Persistent exposure to these chemicals results in a variety of toxic effects in experimental animals, including immunologic, neurochemical, neurotoxic, carcinogenic, and endocrine changes<sup>11</sup>. With the availability of cheaper and quicker genotyping technologies, there is a rapidly increasing interest in identifying genes and genetic polymorphisms that predispose women to increased risk of developing endometriosis<sup>12,13</sup>. Further, earlier studies have indicated a possible involvement of genetic component in endometriosis susceptibility<sup>14</sup>. We hypothesize that the compromised detoxification, determined genetically, might be a risk factor for the development of endometriosis. Enzymes belonging to the glutathione S-transferases (GSTs) and Cytochrome P450 (CYP) families are involved in the two-stage detoxification process of number of pro-carcinogens. The genes for these are part of the aryl hydrocarbon (Ah) gene family and are under the Ah receptor control<sup>15</sup>.

The GSTs are a family of enzymes that are believed to have a significant role in cellular protection against toxic foreign chemicals and oxidative stress<sup>16</sup>. These enzymes not only catalyze the conjugation of glutathione (GSH) to variety of electrophilic compounds<sup>17</sup>, but also detoxify organic peroxides, acting as non-selenium-dependent GSH peroxidases<sup>18</sup>.

In humans, six classes of GST enzymes,  $\alpha$ ,  $\mu$ ,  $\omega$ ,  $\pi$ ,  $\theta$  and  $\zeta$  have been identified, with each class being encoded by a separate gene or gene subfamily<sup>19</sup>. The *GSTM1* gene located on chromosome 1p13.3<sup>20</sup>, codes for cytosolic GST  $\mu$  class enzyme, and has a deletion polymorphism that, when homozygote (*GSTM1* null), results in the complete absence of functional gene product. An elevated frequency of inactive variant of the *GSTM1* gene has been reported in endometriosis patients from France, Russia and Ukraine<sup>21-23</sup>. But other studies<sup>24,25</sup> failed to prove the association in the US and UK populations. The frequency of the Glutathione S-transferase M1 null genotype varies from population to population and was reported to be about 53 per cent in Caucasians and Asians<sup>26,27</sup>.

To evaluate the association between PCBs and *GSTM1* null mutation and their impact on the risk of

endometriosis, the present study was undertaken to assert whether women with endometriosis having higher concentrations of PCBs were more prone to carry *GSTM1* null mutation compared to those free from the disease.

### Material & Methods

In this prospective case-control study women undergoing infertility treatment at the three collaborating centres, viz., Meternal Health and Research Trust and Owaisi Hospital and Research Centre, Hyderabad, were included. The period of study from 2005 to 2007, all consecutive women in these two centres were included.

The ethical committee of Owaisi Hospital and Research Centre approved the research protocol. Informed written consent was obtained from all the participants. Proforma to obtain information on the general, obstetric and gynaecological details including family history, marital status, age at menarche, length of menstrual cycle, associated symptoms, duration and amount of blood loss, duration of infertility, and socio demographic details like social status, occupation, lifestyle, age, body mass index (BMI) and limited information on diet was used. Pelvic ultrasound scan was done prior to surgery to know the uterine and ovarian status followed by laparoscopy to confirm the diagnosis.

**Study group:** The study group consisted of 97 unrelated women of Indian origin, who were diagnosed to have endometriosis by laparoscopy. The severity of the disease was staged according to the revised American Society for Reproductive Medicine (rASRM) classification of endometriosis<sup>28</sup>. Endometriosis was staged as minimal (rASRM stage I) in 37 women, mild (rASRM stage II) in 33, moderate (rASRM stage III) in 16 and severe (rASRM stage IV) in 11. All these women were infertile [primary infertility in 74 (76.2 % of women) and secondary infertility in 23 (23.7% of women)], with mean age of 28.5 yr (SD 6.5), mean age at menarche of 12.6 (SD 1.3), mean duration of infertility of 5.5 yr (SD 4.0), and with the following clinical symptoms: dyspareunia 33 (34%) and dysmenorrhoea [mild 29 (29.8%), moderate 4 (4.1%) and severe 4 (4.1%)]. The remaining 27 (27.8%) of women were asymptomatic.

**Control group:** One hundred and two women who attended the same hospital for other gynaecological pathology (e.g., fibroids, tubal defects, polycystic

ovaries, idiopathic infertility and pelvic inflammatory disease) and tubal sterilization but were laparoscopically confirmed to be without endometriosis were included in the control group. One hundred two (67.6%) women were infertile [primary infertility in 59 (57.8%) of women, secondary infertility in 10 (9.8%) of women and 33 (32.3%) of women were proven fertile], with mean age of 28.4 yr (SD 4.8), mean age at menarche of 12.5 (SD 1.1) and mean duration of infertility of 3.9 yr (SD 4.2). The following symptoms were also present in the women in control group: dyspareunia 17 (16.6%) and dysmenorrhoea [mild 26 (25.4%), moderate 5 (4.9%) and severe 1 (0.9%)]. The remaining 53 (51.9%) women were symptom free.

**Methodology:** Heparinised blood samples (6-8 ml) were collected from all the 97 cases and 102 controls (total=199) for DNA isolation and estimation of PCBs. Plasma was isolated from 86 of 97 endometriosis and 91 of 102 control cases because of insufficient blood samples for GC analysis.

**GSTM1 genotyping:** Genomic DNA was extracted from peripheral<sup>29</sup>, the pellet was washed with 300 µl of 70 per cent ethanol, air dried and DNA was stored at -20°C in hydrated form in Tris- EDTA buffer (Tris HCl- 10 mM, EDTA- 1 mM) until analysis was undertaken.

GSTM1 genotyping for deletion status for each participant was carried out by polymerase chain reaction (PCR) using a MJ Research Minicycler (Waltham, USA) to amplify the specific *GSTM1* gene exon 7 by using the following primers: forward: 5'-GAA CTC CCT GAA AAG CTA AAG C-3'<sup>1</sup> and reverse: 5'- GTT GGG CTC AAA TAT ACGG TGG-3'<sup>1</sup> (MWG-Biotech AG Ltd, Bangalore), which produced a 219 bp product based on the published sequence<sup>30</sup>. Positive and negative controls were included in the study and GSTM1 null genotypes were confirmed by amplification of a 340 bp fragment in exon 7 of the *CYP1A1* gene as an internal positive control.

PCR was performed in a final volume of 25 µl, consisting of DNA 0.5 µl, 0.5 µl dNTP Mix (10 mM; MBI-Fermentas), 2.5 µl MgCl<sub>2</sub> (25 mM), 0.5 µl Taq polymerase (3U/µl) (Bangalore Genei Pvt Ltd), 2.5 µl of 10 x reaction buffer (15 mM), 0.5 µl of each forward and reverse primers and sterile water (17.5 µl). Amplification was performed with an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55.2°C for 30 sec and elongation at 72°C for 30 sec, and final elongation of 72°C for 10 min. The amplified PCR

products were electrophoresed on 2 per cent agarose gel, with ethidium bromide to visualize the DNA bands using UV transilluminator. Gel documentation and analysis were carried out using Chemi Imager (Alpha Innotech Corporation, USA).

**Estimation of PCBs by gas chromatography with flame ionization detector (GC FID):** Plasma was separated from the heparinized blood samples within in 24 h after collection by centrifugation (2,500 x g for 15 min). The plasma (4-5 ml) was pooled and kept frozen at -20°C until PCBs were analyzed. All solvents used were of analytical grade purity (HPLC grade; Qualigens, Ltd., Mumbai, India). Eight standard PCB congeners mix (PCB Mix 525, Supelco, Bellefonte, PA) were selected at a concentration 500 µg/ml in hexane of each PCB. Gas chromatography for the extraction of PCBs was divided into five phases. Extraction of PCBs was performed by the method Burse *et al*<sup>31</sup>, with modifications by<sup>32</sup>. The concentrated organic phases in phase four were pooled and dried under nitrogen gas. The sample was resuspended in hexane and then injected for gas chromatography. Gas chromatography analysis was carried according to the method given by Reddy *et al*<sup>33</sup> done at Hetero Research Foundation on the GC-2010 series gas chromatograph (Shimadzu, Kyoto, Japan).

**Statistical analysis:** Statistical analysis was performed using Medcalc 7.6 version software (MedCalc Software, Mariakerke, Belgium). Independent two-sample t test was performed for age, BMI, age at menarche and the concentration of PCBs between the endometriosis and control group. The GSTM1 genotype frequencies amongst the cases and control group were compared using the two-tailed Fisher exact test. Crude odds ratio (OR), along with 95 per cent confidence intervals (CI), were estimated to indicate the risk of GSTM1 null polymorphism for developing endometriosis. Two-tailed fishers exact tests were used to determine the association between concentration of PCBs, GSTM1 null genotype and the severity of endometriosis.  $P < 0.05$  was considered significant.

## Results

No significant difference in age, BMI age at menarche and duration of infertility was observed between the two groups (Table I). Significant increase in the concentrations of co-planar (PCB-1, 5, 29) and non-coplanar (PCB-98) PCBs was observed in plasma of women as the severity of endometriosis

increases (Table II). The frequencies of GSTM1 null (\*0/\*0) genotypes were 26.8 per cent in the cases, and 14.7 per cent in the controls. Significant association was found between the endometriosis and GSTM1 null mutation with an odds ratio of 2.12 (95% CI = 1.045-4.314,  $P < 0.05$ ) (Table III). Correlation analysis

**Table I.** Comparison of socio-demographic and reproductive history among cases and controls

Characteristics	Endometriosis group (n=97)	Control group (n=102)
Age (yr)	28.5 ± 6.5	28.4 ± 4.8
Body mass index (kg/m <sup>2</sup> )	23.7 ± 2.0	23.6 ± 1.7
Age at menarche (yr)	12.6 ± 1.3	12.5 ± 1.1
Duration of infertility (yr)	5.5 ± 4.0	3.9 ± 4.2
Primary infertility [n (%)]	74 (76.2)	59 (57.8)
Secondary infertility [n (%)]	23 (23.7)	10 (9.8)
Proven fertility [n (%)]	NR	33 (32.3)
Dyspareunia [n (%)]	33 (34)	17 (16.6)
Dysmenorrhoea		
Mild [n (%)]	29 (29.8)	26 (25.4)
Moderate [n (%)]	4 (4.1)	5 (4.9)
Severe [n (%)]	4 (4.1)	1 (0.9)
No dyspareunia & dysmenorrhoea [n (%)]	27 (27.8)	53 (51.9)

Data are presented as mean ± SD  
NR, not reported

of serum concentrations of PCBs and GSTM1 null (\*0/\*0) polymorphism with the different severity of endometriosis was computed to determine their strength of association. The correlations were strong and statistically significant for all four compounds and GSTM1 null genotype (PCB1:  $r = +0.5388$ ,  $P < 0.0001$ ; PCB5:  $r = +0.6753$ ,  $P < 0.0001$ ; PCB29:  $r = +0.6471$ ,  $P < 0.0001$ ; and PCB98:  $r = +0.4357$ ,  $P < 0.0001$ ; GSTM1: CI= 1.045-4.314,  $P = 0.03$ ).

## Discussion

In confirmation with our earlier report<sup>33</sup> there was a significant association between PCBs and PEs with endometriosis. In the present study we showed an association between PCBs and GSTM1 null genotype and their possible impact in developing endometriosis in women. Our results supported the previously published findings reporting an association between the GSTM1 null mutation and endometriosis<sup>23,34</sup>. However, no significant association in the frequency of the GSTM1 null mutation and endometriosis have been shown by various other groups<sup>24,35,36</sup> and the OXEGENE collaborative group<sup>25</sup>. In the Japanese and Korean population also association of endometriosis with the GSTM1 mutations was not found<sup>30,37</sup>.

**Table II.** PCBs concentrations in different stages of endometriosis and control group

Congener	Endometriosis (n=86)				Control group (n=91)	(r) correlation coefficient	P value
	Stage I (µg/ml)	Stage II (µg/ml)	Stage III (µg/ml)	Stage IV (µg/ml)			
PCB-1(co-planar)*	0.23 ± 0.26	0.42 ± 0.28	0.60 ± 0.27	0.84 ± 0.56	0.05 ± 0.14	+0.5388	<0.0001
PCB-5 (co-planar)*	0.10 ± 0.12	0.24 ± 0.21	0.62 ± 0.39	0.75 ± 0.43	0.01 ± 0.06	+0.6753	<0.0001
PCB-29 (co-planar)*	0.13 ± 0.15	0.29 ± 0.31	0.50 ± 0.34	0.99 ± 0.54	0.02 ± 0.08	+0.6471	<0.0001
PCB-98 ((Non-coplanar)*)	0.03 ± 0.10	0.11 ± 0.18	0.34 ± 0.32	0.26 ± 0.31	0.00 ± 0.03	+0.4357	<0.0001

Data are represented as mean ± SD.  $P < 0.05$

**Table III.** Frequencies of GSTM1 null gene polymorphism in women with and without endometriosis

Gene	Endometriosis					Controls (n=102)	OR	95% CI
	Stage 1 (n=37)	Stage II (n=33)	Stage III (n=16)	Stage IV (n=11)	Total (n=97)			
Null (%)	4 (10.8)	6 (18.1)	8 (50)	8 (72.7)	26 (26.8)	15 (14.7)	2.12	1.045-4.314 ( $P = 0.03$ )*
Non-null (%)	33 (89.1)	27 (81.8)	8 (50)	3 (27.2)	71 (73.1)	87 (85.2)		

\* $P < 0.05$ . Two-tailed Fishers exact test

We observed lower GSTM1 null (\*0/\*0) deletion frequency in our study population compared to others<sup>23,24,30,34,36</sup>. These discrepancies may be due to differences in selection of cases and/or controls, inadequate sample sizes, disease classification differences and demographic location.

There might be an association between GSTM1 null (\*0/\*0) mutation and development of endometriosis in women having higher concentrations of PCBs. We observed a significant increase in co-planar PCBs such as PCB-1, 5, 29 and non-co-planar PCB-98 in the plasma of women as the severity of endometriosis increased.

Our study protocol was well designed and the sample size was more compared to previous studies<sup>37</sup>. One of the strengths of our study was the estimation of plasma concentrations of PCBs in cases and controls, in conjugation with GSTM1 null genotyping and their possible impact in the development of endometriosis. Our sample size is lower end of what is required for studies of complex diseases, but to date our knowledge this is the first study attempted to resolve the conundrum of conjugation of both PCBs and GSTM1 gene deletion frequency in the pathogenesis of endometriosis.

No significant differences in mean plasma concentrations of the 14 PCB congeners and 11 chlorinated pesticides was observed among women with or without endometriosis as confirmed by laparoscopy. Gerhard & Runnebaum<sup>38</sup> reported elevated concentrations of three PCB congeners in women with endometriosis in contrast to women free from the disease. It was not possible to compare our results with those of other investigators due to varying methodologies.

In conclusion, our study results suggested that women having higher concentration of PCBs and GSTM1 null (\*0/\*0) polymorphism might have an increased susceptibility of endometriosis.

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