

Article

Exposure to Phthalates and Alternative Plasticizers Is Associated with Methylation Changes of *ESR1* and *PGR* in Uterine Leiomyoma: The ELENA Study

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Abstract: Uterine leiomyomas are estrogen-dependent benign tumors with unknown etiologies. Phthalates are endocrine-disrupting chemicals and ubiquitous in the environment; thus, it has been suggested that they play a role in the development of uterine leiomyoma. We aimed to investigate whether the pathogenesis of uterine leiomyoma is related to methylation changes in promoter regions of estrogen receptor α (*ESR1*) and progesterone receptor (*PGR*) genes in response to phthalates and alternative plasticizers exposure. Urinary concentrations of 20 phthalate metabolites and seven metabolites of di-2-ethylhexyl terephthalate (DEHTP) and di (isononyl) cyclohexane-1,2-dicarboxylate (DINCH) were measured by UHPLC-MS/MS in thirty leiomyoma patients, who provided both paired leiomyoma and myometrium tissues. Methylation levels of *ESR1* and *PGR* were analyzed by pyrosequencing assay. A total of 12 phthalate metabolites and 5 alternative metabolites (3 DEHTP and 2 DINCH) were detected >70% among study participants. The methylation of *ESR1* and *PGR* were significantly lower in leiomyoma tissues compared to those in myometrium (18.10 ± 4.41 vs. 28.72 ± 4.95 ; 2.32 ± 0.81 vs. 3.27 ± 0.56 , respectively). *ESR1* methylation in leiomyoma was negatively associated with mono-2-carboxymethyl-hexyl phthalate (2cx-MMHP) and mono-3-carboxyl-propyl phthalate (MCP) after adjusting for confounding factors. However, 1-mono-2-ethyl-5-oxohexyl-benzene-1,4-dicarboxylate (5OXO-MEHTP), one of the alternatives, showed positive association with *ESR1* methylation in leiomyoma. *PGR* methylation in leiomyoma was significantly associated with mono butyl phthalate (MnBP), but negatively associated with cyclohexane-1,2-dicarboxylate-mono-7-hydroxy-4-methyl-heptyl ester (cx-MINCH). Our results suggest that phthalates exposure may contribute to leiomyoma pathogenesis via *ESR1* and *PGR* methylation changes.

Keywords: leiomyoma; phthalates; alternative plasticizers; methylation; *ESR1*; *PGR*

1. Introduction

Phthalates are high-volume-produced chemicals that have been used in many settings, including industrial, commercial, personal, and household applications. As a result, phthalates have become ubiquitous in the environment, attributable to their widespread

and frequent use in daily life [1]. Phthalates are known to be estrogenic and androgenic endocrine disruptors; thus, adverse effects of phthalates on the human reproductive system have been reported [2–7]. Considering the negative health effects of phthalates to animals and humans, there is a need to develop and/or use alternative plasticizers with low toxicity and less severe consequences for human health [8,9]. Subsequently, alternative plasticizers have been recently and rapidly used; however, there is very limited information regarding their toxicity and health effects [10]. One murine study showed that prenatal exposure to di-iso-nonyl-cyclohexane-1,2-dicarboxylate (DINCH), one of the alternative plasticizers, was associated with premature aging of testes and impaired liver metabolic capacity in offspring [11]. Therefore, some animal studies may raise the concern that adverse human health effects will emerge with increasing use and exposure to these materials.

Uterine leiomyomas are estrogen-dependent benign tumors with unknown etiologies; thus, it has been suggested that endocrine-disrupting chemicals (EDC), including phthalates, play a role in the development of uterine leiomyoma. Our group previously reported that exposures to phthalate metabolites, including mono-2-ethylhexyl phthalate (MEHP), mono-2-ethyl-5-hydrohexyl phthalate (MEHHP), mono-2-carboxymethylhexyl phthalate (2cx-MMHP), Σ MEHP-3 (combining MEHP, MEHHP, and mono-2-ethyl-5-oxohexyl phthalate (MEOHP)), and Σ MEHP-5 (Σ 3-DEHP plus mono-2-ethyl-5-carboxypentyl phthalate (5cx-MEPP), and 2cx-MMHP) were associated with the prevalence of leiomyoma in Korean women [12]. Several studies also examined the effects of phthalates on the development of uterine leiomyoma; however, the results are controversial and the underlying mechanisms remain unclear. [12–17].

It is becoming increasingly evident that environmental influences can result in physiological changes through epigenetics, which is the study of heritable changes in gene expression that occur without directly altering the DNA sequence. Epigenetic alterations are associated with the development and progression of numerous pathological states and diseases [18]. Thus, there is a great deal of potential for the use of epigenetics as biomarkers to better understand the early-stage biological response and molecular mechanisms by which environmental exposures to phthalates and alternative plasticizers lead to uterine leiomyoma. Several studies have reported that estrogen receptor α gene (*ESR1*) expression in leiomyoma tissues is regulated by DNA methylation [19,20]. It was also reported through a study on genome-wide DNA methylation analysis that a methylation change of *ESR1* contributed to an aberrant response to estrogen, and this was subsequently associated with development of uterine leiomyoma [21].

Overexpression of *ESR1* and aromatase was shown in myoma tissues compared to normal myometrium tissues for development of uterine leiomyomas. Estrogen is known to support both progesterone receptor (PR) induction and the facilitation of PR ligands' action on target cells. Thus, estradiol's primary role in uterine leiomyoma was suggested to be maintenance of PR levels, with progesterone promoting uterine leiomyoma growth and progression [22]. In the present study, we aimed to investigate whether the pathogenesis of uterine leiomyoma is related to methylation changes in promoter regions of *ESR1* and PR gene (*PGR*) in response to phthalates and alternative plasticizers exposure.

In the present study, we analyzed urinary concentrations of twenty phthalate metabolites as well as four di-2-ethylhexyl terephthalate (DEHTP) and three DINCH metabolites as alternative plasticizers, which have rapidly increased in use [23,24]. We also measured methylation levels of *ESR1* and *PGR* in leiomyoma tissues from 30 cases, and these methylation levels were compared to those in myometrium tissues—adjacent normal tissues—as internal controls from the same patients.

2. Materials and Methods

2.1. Clinical Subjects

This study was performed utilizing data and bio-samples of patients from the study of Environmental exposure for Leiomyoma, ENdometriosis, and Adeomyosis of reproductive women (ELENA). We recruited patients who had undergone laparoscopic surgery and

exploratory laparotomy between March 2017 and February 2019 at the department of Obstetrics & Gynecology in Inje University Ilsan Paik Hospital. Written informed consents were obtained from each patient using consent forms. The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board of Inje University Ilsan Paik Hospital (Goyang-si, Korea, Code: 2017-06-024, Date of approval: 17 August 2017).

All study participants for the ELENA study were initially examined by gynecologic ultrasonography to detect uterine leiomyoma and any other gynecologic problems including intramural, submucosal, and subserosal fibroids before surgery. Final uterine leiomyoma diagnoses were confirmed by direct visualization during operation and subsequent pathological analysis. Medical and pain/disease histories of all study subjects were assessed and recorded in a standard format. Patients with a history of occupational exposure to reproductive toxicants, hormone therapy, any malignancy, or other reproductive treatment within the last 3 months were excluded from this study. The patients for the ELENA study consisted of 97 patients, including those with leiomyoma ($n = 45$), leiomyoma with endometriosis ($n = 29$), leiomyoma with adenomyosis ($n = 10$), and leiomyoma with endometriosis and adenomyosis ($n = 13$). Thus, among a total of 97 patients, we randomly selected 30 patients, from whom samples of both leiomyoma and adjacent normal myometrium tissues were obtained.

2.2. Data and Biospecimens (Urine and Tissues) Collection

The demographic data of patients were obtained from an interviewed questionnaire before operation. The questions included age, body mass index (BMI), waist circumference, age of menarche, duration of menstrual cycle, history of dysmenorrhea and operation, gravidity, parity, cigarette smoking, alcohol consumption, exposure to secondhand smoke, and exercise.

Once the presence of fibroids and need for surgery were confirmed in each patient, all subjects were requested to undergo blood tests in preparation for their surgery. A 20–30 mL urine sample was collected in a phthalate-free polypropylene container at the time of blood tests as previously described [25,26]. Samples were immediately centrifuged and stored at -80°C until further analysis and after measurement of creatinine levels. Paired leiomyoma and myometrium tissues of the study participants who underwent hysterectomy and myomectomy were dissected immediately after the removal of the uterus or leiomyoma, immersed in liquid nitrogen, and stored at -80°C until further analysis.

2.3. Analysis of Urinary Phthalate and Alternative Plasticizer Metabolites

Twenty urinary phthalates and seven alternative plasticizers (four DEHP and three DINCH) were analyzed in the urine samples as previously described [25,26]. Standards of phthalate metabolites, including: MEHP, mono-butyl phthalate (MnBP), mono-isobutyl phthalate (MiBP), mono-benzyl phthalate (MBzP), mono-cyclo-hexyl phthalate (MCHP), MEHHP, MEOHP, 5cx-MEPP, 2cx-MMHP, mono-n-octyl-phthalate (MnOP), mono-isonomy phthalate (MiNP), mono-3-carboxyl-propyl phthalate (MCP), mono-isodecyl phthalate (MiDP), mono-ethyl phthalate (MEP), mono-methyl phthalate (MMP), mono-n-pentyl phthalate (MnPP), mono-isopropyl phthalate (MiPrP), mono-hexyl phthalate (MHxP), mono-hydrocyl-isononyl phthalate (OH-MiNP), and mono-carboxy-isodecyl phthalate (cx-MiDP); and alternative plasticizer metabolites, including: mono-2-ethylhexyl terephthalate (MEHTP), 1-mono-2-ethyl-5-carboxypentyl-benzene-1,4-dicarboxylate (5cx-MEHTP), 1-mono-2-ethyl-5-hydroxylhexyl-benzene-1,4-dicarboxylate (5OH-MEHTP), 1-mono-2-ethyl-5-oxohexyl-benzene-1,4-dicarboxylate (5OXO-MEHTP), cyclohexane-1,2-dicarboxylate-mono-4-methyloctyl ester (MINCH), cyclohexane-1,2-dicarboxylate-mono-7-hydroxy-4-methyl-octyl ester (OH-MINCH), and cyclohexane-1,2-dicarboxylate-mono-7-hydroxy-4-methyl-heptyl ester (cx-MINCH); as well as their respective $^{13}\text{C}_4$ or $^{13}\text{C}_2$ -labeled internal standards, were purchased from Cambridge Isotope Laboratory (Cambridge Isotope Laboratory, Cambridge, MA, USA) and Toronto Research Chemical (North York, ON, Canada).

After enzymatic hydrolysis, samples were pretreated using the Strata X SPE Cartridge (30 mg/cc, Phenomenex, Torrance, CA, USA), following a method by Servaes et al. [27] with a minor modification. Ultrahigh performance liquid chromatography mass spectrometry (UHPLC-MS/MS, UHPLC, Nexera X2, Shimadzu Corporation, Kyoto, Japan and API 4500, AB SCIEX, ON, Canada) was used for measurement.

The limits of detection (LOD) ranged from 0.01 ng/mL (2cx-MMHP) to 0.8 ng/mL (MiDP) as shown in Table S1. For each instrumental run, reagent blanks and QC samples were included for quality control. The method accuracies were between 80% and 120%, and precisions were lower than 10% relative standard deviation (RSD) for high and low concentrations of spiked samples ($n = 7$).

2.4. DNA Methylation Analysis

Genomic DNA was isolated from the frozen leiomyoma and myometrium tissues according to the manufacturer's protocol with the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA). The DNA concentration and purity ratio were measured using an ND1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Subsequently, 1 µg of the genomic DNA was sodium bisulfite-converted using the EZ DNA MethylationTM Kit (Zymo Research, Irvine, CA, USA) for Pyrosequencing assay.

Pyrosequencing assay was used for selected gene-specific methylation analysis. Gene specific primers were designed for *ESR1* and *PGR* using PyroMark Assay Design 2.0 software (Qiagen) as previously described [28]. Table S2 shows the detail of the primers and PCR conditions used in this study. Gene regions were located using NCBI and GenBank.

PCR amplification of the bisulfite converted DNA was conducted and PCR reactions were performed using Pyromark PCR Kit (Qiagen) with cycling parameters consisting of denaturation at 95 °C for 5 min, and 45 cycles of 95 °C for 30 s, 51–53 °C for 30 s, 72 °C for 30 s, and a final extension of 72 °C for 5 min, and with the annealing temperature varying slightly for each gene-specific primer set. Pyrosequencing was conducted using a PyroMark Q96 MD instrument (Qiagen), with subsequent quantification of methylation levels determined by the PyroMark-CpG software (Qiagen). All samples were duplicated; each pyrosequencing run included a non-template control, and repeatability of the assay was confirmed using human methylated and non-methylated DNA sets (Zymo Research).

2.5. Statistical Analysis

We summarized the distribution of urinary phthalate metabolites using median and interquartile range. For statistical analysis, t-test, ANOVA, and Spearman correlation were performed using software from the R Foundation for Statistical Computing (version 4.0.4) and SPSS statistical software (version 26.0, Chicago, IL, USA). The urinary concentrations of four phthalate metabolites (i.e., MiPrP, MHxP, OH-MiNP, and cx-MiDP) and alternative plasticizers were not fully analyzed from all study participants (only $n = 21$ were available for those), attributable to insufficient urine samples. Thus, correlation and regression analyses were performed with <30 samples for the listed metabolites above. Phthalate metabolites that were detected at concentrations of more than 70% were selected for further analyses. All of the phthalate metabolites data were assessed by the Kolmogorov–Smirnov test to evaluate whether they were normally distributed. Since the distribution of phthalate metabolite concentrations was most likely right-skewed, all the phthalate metabolite measurements were logarithm-transformed to approximate normal distribution. The total concentration of phthalate and alternative plasticizer metabolites was creatinine standardized using the following formula to address urinary dilution as stated in previous studies: Total [phthalate metabolites] = $100 \times \text{phthalate (ng/mL)} / \text{creatinine (mg/dL)}$ [29,30].

Multiple regression analyses were performed to identify the association of methylation changes (by leiomyoma and myometrium tissues type) with each urinary phthalate and alternative plasticizer metabolite level. A separate regression model was conducted for each log-transformed urinary phthalate and alternative plasticizer metabolite due to a possible correlation with each other. The regression model was adjusted for age, dysmenorrhea,

menstrual phase, smoking status, alcohol drinking, secondhand smoking, and BMI. The p values less than 0.05 were considered statistically significant.

3. Results and Discussion

3.1. Clinical Characteristics of Patients

The demographic and clinical characteristics of patients with uterine leiomyoma are shown in Table 1. The mean age ($\pm$ SD) and BMI were 44.67 (4.99) years old and 24.25 (4.43), respectively. The size of leiomyomas ranged from 12 mm to 108 mm and the mean number of leiomyoma was 3.93 (3.16). Sixteen leiomyoma patients were accompanied by endometriosis ($n = 3$), adenomyosis ($n = 9$), or endometriosis and adenomyosis ($n = 4$).

Table 1. Demographic and clinical characteristics of the study population.

Variable	Number (%) ($n = 30$)	Mean $\pm$ SD (Range)
Age (years)		44.67 $\pm$ 4.99 (29–54)
BMI (kg/m ²)		24.25 $\pm$ 4.43 (17.7–35.9)
Waist circumference (cm)		73.95 $\pm$ 6.54 (61–86.4)
Diameter of the largest leiomyoma (mm)		6.79 $\pm$ 2.42 (12–108)
Number of leiomyoma		3.93 $\pm$ 3.16 (1–12)
Operation status		
Myomectomy	12 (40)	
Hysterectomy	18 (60)	
Disease status		
Leiomyoma only	14 (46.7)	
Leiomyoma + Endometriosis (E)	3 (10)	
Leiomyoma + Adenomyosis (A)	9 (30)	
Leiomyoma + E + A	4 (13.3)	
Parity		
Primipara	13 (43.7)	
Multipara	17 (56.7)	
Menstrual phase		
Proliferative	14 (46.7)	
Secretory phase	13 (43.3)	
Atrophy	3 (10)	
Menstrual cycle		
Regular	22 (73.3)	
Irregular	8 (26.7)	
Dysmenorrhea		
No	13 (43.3)	
Yes	17 (56.7)	
Smoking status		
No	21 (75)	
Yes	7 (25)	
Alcohol drinking status		
No	1 (3.6)	
Yes	27 (96.4)	

If $n < 30$, missing values are present.

3.2. Urinary Concentrations of Phthalate and Alternative Plasticizer Metabolites

Twenty phthalate metabolites and seven alternative plasticizer metabolites (i.e., 4 DEHTP and 3 DINCH) are shown in Table 2. A total of 12 phthalate metabolites (di-2-ethylhexyl phthalate (DEHP) (i.e., MEHP, MEOHP, MEHHP, 2cx-MMHP, and 5cx-MEPP); di-n-octyl phthalate (DnOP), and others (i.e., MBzP, MiBP, MEP, MnBP, MMP, MCP, and OH-MiNP)) were detected >73%, while MiNP, MnOP, MCHP, MiDP, MnPP, MiPrP, MHxP, and cx-MiDP were rarely detected. Urinary concentrations of 4 DEHTP and 3 DINCH

metabolites were measured, while their monoesters such as MEHTP and MINCH were rarely detected. Three DEHTP metabolites (i.e., 5cx-MEHTP, 5OH-MEHTP, and 5OXO-MEHTP) and two DINCH metabolites (i.e., OH-MINCH and cx-MINCH) were detected >75%, showing that alternative plasticizers are widespread and frequently exposed.

Table 2. Urinary concentrations of phthalate and alternative plasticizer metabolites.

Phthalates and Alternatives	Measured >LOD <i>n</i> (%)	GM $\pm$ GSD ($\mu\text{g/g}$ Creatinine)	Median (IQR) ($\mu\text{g/g}$ Creatinine)
MEHP	27/30 (90)	14.91 $\pm$ 13.19	14.39 (8.31–22.34)
MEOHP	30/30 (100)	18.53 $\pm$ 14.92	17.41 (11.45–28.22)
MEHHP	30/30 (100)	32.28 $\pm$ 23.40	31.07 (18.22–54.36)
2cx-MMHP	30/30 (100)	20.84 $\pm$ 15.87	19.29 (13.86–26.90)
5cx-MEPP	30/30 (100)	35.47 $\pm$ 33.24	28.48 (22.15–55.80)
MBzP	29/30 (96.7)	10.15 $\pm$ 12.52	8.73 (4.25–25.63)
MiBP	30/30 (100)	19.87 $\pm$ 26.80	41.20 (7.73–47.63)
MEP	27/30 (90)	11.23 $\pm$ 13.99	11.41 (7.06–29.87)
MnBP	29/30 (96.7)	32.54 $\pm$ 27.28	37.76 (17.20–55.22)
MMP	30/30 (100)	9.73 $\pm$ 11.61	6.79 (3.96–30.11)
MCP	22/30 (73.3)	2.91 $\pm$ 1.92	2.77 (1.94–3.96)
MiNP	6/30 (20)	1.16 $\pm$ 1.36	1.17 (0.70–2.06)
OH-MiNP	21/21 (100)	4.35 $\pm$ 2.89	4.24 (3.21–6.00)
MnOP	0/30 (0)	NA	NA
MCHP	0/30 (0)	NA	NA
MiDP	0/30 (0)	NA	NA
cx-MiDP	6/21 (28.6)	1.42 $\pm$ 1.20	1.56 (1.38–1.79)
MnPP	1/30 (3.3)	0.77	0.77
MiPrP	2/21 (9.5)	0.23 $\pm$ 0.55	0.23 (0.20–0.27)
MHxP	4/21 (19)	0.48 $\pm$ 2.50	0.30 (0.19–2.20)
MEHTP	4/21 (19)	1.15 $\pm$ 0.31	1.20 (1.03–1.29)
5cx-MEHTP	21/21 (100)	28.78 $\pm$ 16.31	26.43 (22.77–44.47)
5OH-MEHTP	20/21 (95.2)	4.07 $\pm$ 2.24	3.69 (2.71–6.54)
5OXO-MEHTP	19/20 (95)	2.35 $\pm$ 1.62	2.12 (1.52–4.36)
MINCH	1/21 (4.8)	1.45	1.45
OH-MINCH	21/21 (100)	0.66 $\pm$ 0.94	0.58 (0.41–0.84)
cx-MINCH	16/21 (76.2)	0.78 $\pm$ 1.21	0.82 (0.43–1.05)

All phthalate and alternative plasticizer metabolites were creatinine (mg/dL) standardized. LOD = limit of detection; GM = geometric mean; GSD = geometric standard deviation; IQR = interquartile range; NA = not available.

In phthalates, the creatinine-adjusted levels of 5cx-MEPP (35.47 $\pm$ 33.24 $\mu\text{g/g}$ creatinine) were the highest, followed by MnBP, MEHHP, 2cx-MMHP, MiBP, MEOHP, MEHP, MEP, MBzP, MMP, OH-MiNP, and MCP. Among alternative plasticizers, creatinine-adjusted levels of 5cx-MEHTP (28.78 $\pm$ 16.31 $\mu\text{g/g}$ creatinine) were the highest, followed by 5OH-MEHTP, 5OXO-MEHTP, cx-MINCH and OH-MINCH. Both 5cx-MEPP and 5cx-MEHTP are carboxylated monoester metabolites and major oxidized metabolites excreted in urine.

Metabolites of DEHP (i.e., MEOHP, MEHHP, 2cx-MMHP), DEHTP (i.e., 5cx-MEHTP, 5OH-MEHTP, 5OXO-MEHTP), and DINCH (i.e., OH-MINCH, and cx-MINCH) were significantly correlated within each group as shown in Figure S1. In particular, the most widely and frequently used phthalates, including di-ethyl phthalate (DEP), DEHP, and di-n-butyl phthalate (DnBP), showed significant correlations among their metabolites (Spearman's rho = 0.55 for MEP vs. MEHP, 0.39 for MEP vs. MCP, rho = 0.53 for MiBP vs. MnBP, rho = 0.83 for MnBP and MCP). This could be attributable to various phthalates that are mixed and used in products. Therefore, factor analyses were further performed to consider the possible mixing effects; however, there were no significant associations, as demonstrated by the single phthalate–leiomyoma association (data not shown).

There has recently been a shift from the use of traditional phthalates to alternative plasticizers due to environmental and health concerns [31]. One of the well-known al-

ternative plasticizers is DEHTP, which is a structural isomer and a direct substitute for high-molecular-weight plasticizers of DEHP. Like DEHP, DEHTP is rapidly metabolized to its monoester and then further modified by a side-chain hydroxylation and oxidation reaction (5OH-MEHTP, 5oxo-MEHTP, 5cx-MEPTP, and 2cx-MMHTP) [32]. Another alternative plasticizer, DINCH, is a non-aromatic compound similar in viscosity and flexibility to DEHP [33]. OH-MINCH is the predominant metabolite, followed by cx-MINCH and cyclohexane-1 [34]. DINCH has chemical properties comparable to those of DEHP; however, it has been reported to have very limited toxic effects in animal studies. Therefore, DINCH has been approved for the production of food packaging plastic material [35], and as a result it has almost completely replaced DEHP in plastic production in Europe [23].

Although there is increased use of alternatives and political focus on the phasing-out of phthalates, little information is available regarding the metabolic pathways as well as distribution of exposure to alternative plasticizers. From one birth cohort of Puerto Rico, DEHTP metabolites were detected in at least 82% of samples, representing widespread prenatal exposure [36]. DINCH metabolites were also detected in 18% to 35% of samples, but detection rates of DINCH metabolites also increased over time. Our results of alternative plasticizers had a similar pattern; however, the exposed levels of phthalate and alternative plasticizer metabolites should be considered as those in the specialized leiomyoma patients' group.

3.3. Methylation of *ESR1* and *PGR* in Leiomyoma and MYOMETRIUM Tissues

Methylation levels of *ESR1* and *PGR* in both leiomyoma tissues and myometrium tissues are shown in Figure 1. The methylation levels of *ESR1* (18.10 ± 4.41 vs. 28.72 ± 4.95) and *PGR* (2.32 ± 0.81 vs. 3.27 ± 0.56) were significantly lower in leiomyoma tissue compared to those in myometrium tissue.

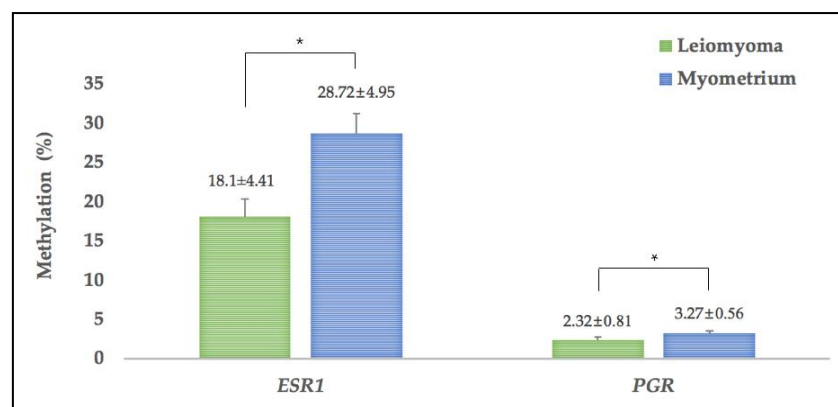


Figure 1. Methylation levels of *ESR1* and *PGR* in leiomyoma and myometrium tissues. Data indicate mean $\pm$ standard deviation, and * $p < 0.05$.

The methylation of *ESR1* and *PGR* showed no significant differences by status of parity, menstrual phases, menstrual pattern, smoking, indirect smoking, education, and family incomes. However, there was significant difference in the level of *PGR* methylation between patients with and without dysmenorrhea (2.07 ± 0.71 vs. 2.51 ± 0.85) (Table S3).

Epigenetic changes are known to play an important role in the pathogenesis of uterine leiomyoma. DNA methylation profiles in leiomyoma tissue are clearly distinguished from those in myometrium tissue, while quite similar DNA methylation profiles are observed in myometrium tissues in women with and without leiomyomas [21]. Furthermore, significant methylation changes in genome-wide DNA and X chromosomes, as well as promoter regions of specific genes, have been observed in leiomyoma tissues [37–41]. Asada et al. [20] reported that highly expressed *ESR1*, attributable to *ESR1* hypomethylation, is associated with pathogenesis of uterine leiomyoma. Our results—significant hypomethylation of

ESR1 and *PGR* in leiomyoma tissues compared to adjacent myometrium tissues—are in concord with their finding.

PR's role in pathogenesis of leiomyoma is crucial, but methylation of *PGR* have not been extensively reported. Recently, one paper showed that PR integrates the effects of mutated *mediator complex subunit 12 (MED12)* and altered DNA methylation to stimulate *receptor activator of nuclear factor kappa-B ligand (RANKL)* expression and stem cell proliferation in uterine leiomyoma [41]. Progesterone action is essential for uterine leiomyoma growth and development via interaction with PR. Thus, our results could explain pathogenesis of uterine leiomyoma partially, but further investigations should be performed to validate this.

3.4. Methylation Changes and Exposure to Phthalates and Alternative Plasticizers

Our group previously reported that the urinary concentrations of MEHP, MEHHP, 2cx-MMHP, Σ MEHP-3, and Σ MEHP-5 were associated with risk of uterine leiomyoma, suggesting that phthalates could have the potent effects on pathogenesis of uterine leiomyoma [12]. Further associations between exposure to phthalates and alternative plasticizers and DNA methylation changes in two different tissues were analyzed as shown in Table 3. *ESR1* methylation in leiomyoma was negatively associated with 2cx-MMHP ($\beta = -9.389$, 95% CI: $-15.425, -3.353$) and MCPP ($\beta = -10.670$, 95% CI: $-20.571, 0.769$) after adjusting for confounding factors. However, 5OXO-MEHTP, one of the alternatives, showed positive association with *ESR1* methylation in leiomyoma ($\beta = 17.928$, 95% CI: $6.420, 29.437$). *PGR* methylation in leiomyoma was significantly associated with MnBP ($\beta = 1.128$, 95% CI: $0.055, 2.201$), but negatively associated with cx-MINCH, another alternative metabolite ($\beta = -1.220$, 95% CI: $-2.210, -0.230$). In addition, *PGR* methylation in myometrium was also positively associated with MEP ($\beta = 0.637$, 95% CI: $0.093, 1.181$) and MCPP ($\beta = 1.259$, 95% CI: $0.250, 2.268$).

Table 3. Association of phthalate and alternative plasticizer metabolites with *ESR1* and *PGR* methylation in leiomyoma and myometrium tissues.

Phthalates and Alternatives ^a	ESR1		PGR	
	Leiomyoma ^b	Myometrium	Leiomyoma	Myometrium
MEHP	−4.267 (−9.432, 0.898)	2.773 (−3.1171, 8.663)	−0.330 (−1.381, 0.722)	0.566 (−0.080, 1.213)
MEOHP	−4.797 (−11.150, 1.574)	2.932 (−3.678, 9.541)	0.583 (−0.612, 1.777)	0.003 (−0.805, 0.812)
MEHHP	−4.797 (−12.060, 2.466)	2.010 (−5.541, 9.561)	0.726 (−0.612, 2.063)	−0.644 (−0.975, 0.846)
2cx-MMHP	−9.389 (−15.425, −3.353) *	2.086 (−5.374, 9.545)	−0.066 (−1.432, 1.301)	−0.222 (−1.117, 0.672)
5cx-MEPP	−3.772 (−9.280, 1.735)	2.231 (−3.461, 7.923)	0.569 (−0.446, 1.585)	0.068 (−0.624, 0.761)
MBzP	−1.588 (−6.032, 2.856)	−0.419 (−4.858, 4.019)	0.047 (−0.748, 0.842)	−0.315 (−0.832, 0.201)
MiBP	1.482 (−2.487, 5.451)	0.308 (−3.718, 4.334)	0.630 (−0.036, 1.296)	−0.254 (−0.720, 0.213)
MEP	0.015 (−5.046, 5.076)	0.541 (−4.900, 5.982)	0.557 (−0.353, 1.467)	0.637 (0.093, 1.181) *
MnBP	2.199 (−4.481, 8.879)	0.917 (−5.785, 7.619)	1.128 (0.055, 2.201) *	−0.253 (−1.056, 0.550)
MMP	−2.107 (−6.888, 2.674)	0.568 (−4.306, 5.443)	−0.569 (−1.412, 0.275)	0.421 (−0.127, 0.969)
MCPP	−10.670 (−20.571, 0.769) *	8.301 (−1.153, 17.753)	−0.836 (−2.979, 1.308)	1.259 (0.250, 2.268) *
OH-MiNP	−1.453 (−6.580, 3.675)	2.794 (−1.775, 7.364)	−0.114 (−1.016, 0.789)	−0.131 (−0.683, 0.421)
5cx-MEHTP	7.7836 (−5.812, 21.380)	1.635 (−12.068, 15.339)	1.498 (−0.823, 3.819)	−0.830 (−2.288, 0.627)
5OH-MEHTP	4.457 (−9.944, 18.857)	−5.732 (−18.016, 6.551)	1.426 (−0.972, 3.824)	−1.304 (−2.641, 0.032)
5OXO-MEHTP	17.928 (6.420, 29.437) *	−11.974 (−25.960, 2.011)	1.702 (1−1.645, 5.048)	−1.424 (−3.304, 0.457)
OH-MINCH	−1.649 (−8.029, 4.731)	1.776 (−3.531, 7.083)	−0.868 (−1.809, 0.073)	0.339 (−0.328, 1.006)
cx-MINCH	1.606 (−8.18, 11.393)	−1.932 (−10.033, 6.169)	−1.220 (−2.210, −0.230) *	0.128 (−1.033, 1.290)

^a All metabolites of phthalates and alternative plasticizers were log-transformed and creatinine (mg/dL) standardized ^b Data represent beta coefficient (95% CI). Model adjusted for age, dysmenorrhea, menstrual phase, alcohol drinking, smoking, secondhand smoking, and BMI. * $p < 0.05$.

The epigenetic effect of phthalates was reported in several experimental and animal studies. One murine study showed that maternal exposure to DEHP caused testicular dysfunction via DNA hypermethylation, leading to increased expression of *DNA methyltransferases (Dnmts)* and downregulated expression of *insulin-like hormone-3 (InsI3)* [42]. Exposure to phthalates mixture induced abnormal expression of miR-141-3p and miR-184 in rats, and their downregulated target genes were associated with prostate development and oncogenesis [43]. Another study found that MCF7 cells treated with dibutyl phthalate (DBP) or butyl benzyl phthalate (BBP) led to demethylation of promoter regions of

ESR1 [44]. In addition, phthalates are known to adversely affect estrogen signaling by interacting with ER α and ER β [45]. Growth of uterine leiomyoma depends on ovarian steroids, estrogen and progesterone [46,47]. However, there are very limited studies to determine the association between phthalates and expression of *ESR1* and *PGR* in relation to uterine leiomyoma.

Our results showed that two phthalate metabolites (i.e., 2cx-MMHP and MCPP) showed significant association with hypomethylation of *ESR1* in leiomyoma tissue, while MnBP was associated with hypermethylation of *PGR*, suggesting that phthalate metabolites might contribute to pathogenesis of uterine leiomyoma via increased expression of *ESR1*. A recent study by Zota et al. [48] showed that mono-hydroxybutyl phthalate (MEBP) and MEHHP are significantly associated with overexpression of miR-10a-5p and miR-577 in leiomyoma patients. The consequent target genes of phthalates-associated miRNAs are linked to multiple fibroid-related processes including angiogenesis, apoptosis, and proliferation of connective tissues [48]. This study supports our findings suggesting that epigenetic alteration is associated with phthalates exposure and uterine leiomyoma development. Furthermore, EDC, including phthalates, are known to induce oxidative stress and its associated burden such as DNA methylation changes (i.e., hypermethylation and hypomethylation). EDC-induced reactive oxygen species (ROS) interact with androgen and ERs, leading to sex-hormone pathologies [49]. Thus, phthalates-generated ROS induce methylation changes in *ESR1*; this, in turn, may cause estrogen pathologies and uterine fibroids.

There is little data related to phthalates and *PGR* expression; a few mechanistic, pre-clinical, and clinical studies reported that phthalates interfere with normal function of PR. DEHP treatment inhibited progesterone secretion from human luteal cells [50] and decreased secretion of progesterone in mice [51]. One study on the structural binding mechanism of DEHP and its metabolites with PR found that phthalates induced dysfunction of progesterone signaling and adverse effects due to their high binding affinity [52]. Our results also showed that phthalates seemed to inhibit *PGR* expression in leiomyoma; however, additional studies are needed to confirm this.

In this study, we also analyzed alternative plasticizer metabolites in leiomyoma patients and examined the association between exposed levels of alternative plasticizers and methylation of *ESR1* and *PGR* in leiomyoma. Interestingly, alternative metabolite 5OXO-MEHTP had an opposing association with *ESR1* methylation compared to phthalate metabolites, while cx-MINCH showed another opposing association with *PGR* methylation in leiomyoma. DINCH and DEHTP have been used as the substitute for DEHP, but their metabolites' effects on methylation changes in *ESR1* and *PGR* are opposed to those of DEHP metabolites. These differences in biological activity at the molecular level may be associated with different structures and physical properties with different chemodynamics. Furthermore, DINCH showed significant impacts on steroidogenesis, testicular development, and function in some studies [53,54], and an in vitro study by Engel et al. showed that DINCH metabolites had a weak estrogenic property [55]; these results support our results. However, further findings, including in vivo and in vitro should be done to confirm the correct epigenetic mechanism.

There are some limitations in our study; the relatively small sample size with large variation in disease status may restrict the statistical power of the results. We also obtained only one spot urine sample from each of the cases, which would be only moderately predictive for long-term exposure, and exposure misclassification cannot be fully ruled out. Some of the study specimens accompany endometriosis and adenomyosis, which may be associated with phthalates exposure. Therefore, further study for only leiomyoma cases is needed. Despite these limitations, this study, to our knowledge, is the first to report methylation changes of *ESR1* and *PGR* in related to uterine leiomyoma and exposure to phthalates and alternative plasticizers.

4. Conclusions

Our results suggest that exposure to some phthalates and alternative plasticizers is associated with *ESR1* and *PGR* methylation changes, which may contribute to leiomyoma pathogenesis. However, further studies with larger sample size and additional genes are needed for strengthening these results.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/app11094234/s1>, Figure S1: Correlations between metabolites of target phthalates and alternatives from leiomyoma patients Table S1: Levels of limit of detection for phthalate and alternative plasticizer metabolites, Table S2: Primer sequences and PCR conditions for gene-specific methylation analysis, Table S3: Methylation of *ESR1* and *PGR* in leiomyoma and myometrium tissues by characteristics.

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