# Impact of exposure to environmental tobacco smoke, arsenic and phthalates on locally advanced cervical cancer treatment – preliminary results

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**Background.** Cancer research is a national and international priority, with the efficiency and effectiveness of current anti-tumor therapies being one of the major challenges with which physicians are faced. **Objective.** To assess the impact of exposure to environmental tobacco smoke (ETS), arsenic, and phthalates on cervical cancer treatment. **Methods.** We enrolled 43 patients with locally advanced cervical carcinoma who underwent chemotherapy and radiotherapy. We determined cotinine and five phthalate metabolites in urine samples collected prior to cancer treatment, by gas chromatography coupled to mass spectrometry, and urinary total arsenic by atomic absorption spectrometry with hydride generation. We used linear regression to evaluate the effects of cotinine, arsenic, and phthalates on the change in tumor size after treatment, adjusted for confounding variables. **Results.** We detected no significant associations between urinary cotinine, arsenic, or phthalate monoesters on change in tumor size after treatment, adjusted for urine creatinine, age, baseline tumor size, and cotinine (for arsenic and phthalates). However, higher %mono-ethylhexyl phthalate (%MEHP), a putative indicator of phthalate diester metabolism, was associated with a larger change in tumor size (β=0.015, 95%CI=0.003-0.03, P=0.019). **Conclusion.** We found no statistically significant association between the urinary levels of arsenic, cotinine, and phthalates metabolites and the response to cervical cancer treatment as measured by the change in tumor size. Still, our results suggested that phthalates metabolism may be associated with response to treatment for locally advanced cervical cancer. However, these observations are preliminary and will require confirmation in a larger, more definitive investigation.

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[1] Health Department, Environmental Health Center, Cluj-Napoca, Romania

[2] Imogen Research Institute Cluj-Napoca, Romania

[3] Departments of Environmental Health Sciences and Epidemiology and Biostatistics, University at Albany, State University of New York, Rensselaer, NY, USA

[4] Physico-chemical and Biotoxicological Analysis Laboratory, Environmental Health Center, Cluj-Napoca, Romania

[5] Faculty of Environmental Science and Engineering, Babes-Bolyai University, Cluj-Napoca, Romania

[6] Radiotherapy II, Department, Institute of Oncology "Prof. Dr. Ion Chiricuta", Cluj-Napoca, Romania

[7] Functional Genomics, Proteomics and Experimental Pathology Laboratory, Institute of Oncology "Prof. Dr. Ion Chiricuta", Cluj-Napoca, Romania

[8] University of Medicine and Pharmacy "Iuliu Hatieganu", Cluj-Napoca

Corresponding Author:

Iulia A. Neamtiu[1,2]

58 Busuiocului Street, Cluj-Napoca, postal code 400240, Romania

Email address: iulianeamtu@ehc.ro

## Abstract

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**Methods.** We enrolled 43 patients with locally advanced cervical carcinoma who underwent chemotherapy and radiotherapy. We determined cotinine and five phthalate metabolites in urine samples collected prior to cancer treatment, by gas chromatography coupled to mass spectrometry, and urinary total arsenic by atomic absorption spectrometry with hydride generation. We used linear regression to evaluate the effects of cotinine, arsenic, and phthalates on the change in tumor size after treatment, adjusted for confounding variables.

**Results.** We detected no significant associations between urinary cotinine, arsenic, or phthalate monoesters on change in tumor size after treatment, adjusted for urine creatinine, age, baseline tumor size, and cotinine (for arsenic and phthalates). However, higher %mono-ethylhexyl phthalate (%MEHP), a putative indicator of phthalate diester metabolism, was associated with a larger change in tumor size ($\beta$=0.015, 95%CI=0.003-0.03, P=0.019)

**Conclusion.** We found no statistically significant association between the urinary levels of arsenic, cotinine, and phthalates metabolites and the response to cervical cancer treatment as measured by the change in tumor size. Still, our results suggested that phthalates metabolism may be associated with response to treatment for locally advanced cervical cancer. However,

these observations are preliminary and will require confirmation in a larger, more definitive investigation.

**Keywords:** Environmental tobacco smoke, arsenic, phthalates, exposure, locally advanced cervical cancer, treatment response.

## 1. Introduction

Cervical cancer is among the leading causes of cancer-related morbidity in women worldwide; in 2012 there were approximately 4,343 new cases in Romania alone, accounting for 1,909 deaths (Ferlay et al., 2013). Response to treatment consisting of radiotherapy combined with cisplatin-based chemotherapy in late stages, in most cases of locally advanced cervical cancers, is good, however, 5-year free survival rates are unsatisfactory (Eifel et al., 2009).

Human papilloma virus (HPV) infection (particularly types 16 and 18) is present in more than 90% of cervical cancer cases (Bosch et al., 1995), however, not all HPV infections result in cervical cancer (Burd, 2003). In addition to HPV infection, evidence from epidemiologic studies also supports an association between active cigarette smoking and cervical neoplasia, suggesting that exposure to environmental tobacco smoke (ETS) may increase the risk for cervical cancer (Haverkos et al., 2003; Trimble et al., 2005). Even in non-smokers, lifetime exposure to ETS may contribute to the development of pre-cancerous cervical intraepithelial neoplasia (Wu et al., 2003).

Exposure to arsenic via drinking water has also raised concern worldwide, as it has been associated with the development of lung, bladder, and skin cancer among others (Smith et al., 1992). Arsenic induces higher levels of reactive oxygen species (Flora, 2011), an excess of which peroxidzies lipids and oxidizes proteins and nucleic acids, leading to abnormal function and DNA lesions (Ercal, Gurer-Orhan & Aykin-Burns, 2001).

A growing body of evidence indicates that arsenic may impair the immune system function and so play a role in carcinogenesis (Acharya et al., 2010). Exposure to arsenic suppressed T and B

lymphocyte maturation, induced apoptosis of macrophages and lymphocytes, and impeded T cell specific cytokine expression (Vega et al., 1999; Cheng et al., 2004). Immune system suppression promotes cancer development (Acharya et al., 2010). Oncogenesis triggered by arsenic exposure also leads to immune suppression, further facilitating tumor growth and generating a vicious circle of positive feedback between cancer and immune function (Acharya et al., 2010), that may also impact the effectiveness of treatment.

Phthalates are widely used in many consumer goods, facilitating frequent human exposures (Kamrin, 2009). Sources of exposure include personal care products that come into contact with the skin, home improvement products and building materials from which phthalates may be released into the air, and contamination during the production of food and beverages (Kamrin, 2009). Importantly, phthalates appear to play a role in inflammation, which at a chronic level precedes tumorigenesis (Anand et al., 2008). Phthalates-mediated increases in chronic inflammation have been demonstrated in the prostate, uterus, ovary, and breast, all common locations for neoplastic proliferation (Singh & Li, 2012).

While environmental factors are likely to contribute as component causes in the etiology of cancer (Wu et al., 2016), there appears to be little if any data available to assess effects of environmental pollutants on cancer treatment. As part of a larger investigation into the factors that modify response to treatment among women with invasive cervical cancer, we conducted an interim, exploratory analysis to assess the impact of exposures to ETS, arsenic and phthalates. To the best of our knowledge, no prior investigations have assessed the impact of these widely

distributed environmental pollutants on the effectiveness of treatment for locally advanced cervical cancer.

## 2. Materials and Methods

*Study population*

We recruited 43 women receiving treatment for locally advanced cervical cancer at the Oncology Institute "Prof Dr Ion Chiricuta" (Cluj-Napoca, Romania), between 2013 and 2014. Patients were eligible for participation if: 1) diagnosed with histologically-confirmed squamous cell carcinoma of the cervix at clinical stages IIB, IIIA, or IIIB according to the MD Anderson Cancer Center modification of the International Federation of Gynecology and Obstetrics (FIGO) diagnostic criteria (Benedet et al., 2000); 2) aged 20-79 years at the time of diagnosis; 3) diagnosed with Zubrod score ≤ 2 (an overall well-being index scored as 0-5, for which 0 = asymptomatic and 5 = death) (Oken et al., 1982); 4) blood hemoglobin ≥ 9 g/dL, leucocytes ≥ 3000/$mm^3$, and platelets ≥ 100 000/$mm^3$; 5) urine creatinine < 1.2 mg/L and urine nitrogen < 80 mg/L; and 6) normal transaminases. We excluded patients with: 1) a history of a prior malignancy, including previous cervical cancer; 2) interrupted treatment (women who stopped therapy for any reason); or 3) cardiovascular, kidney, or liver function that was deemed too poor to initiate treatment. Of the eligible patients contacted, 97.7% enrolled in our study. All participants provided written informed consent prior to study participation and the research protocol was approved by the Ethics Committee in Research and Development and Quality Assurance for Clinical Studies at the Oncology Institute "Prof. Dr. Ion Chiricuta" (approval stated in the Ethics Committee Evaluation Report no. 6490/2013).

All study participants underwent three cycles of chemotherapy with Taxol and Carboplatin (AUC5) followed by radiotherapy, according to the usual clinical cervical cancer treatment protocol, as previously described in detail (Balacescu et al., 2014). For some patients, additional surgical treatment included hysterectomy and the removal of positive for malignant cells pelvic lymph nodes. We assessed treatment response as the difference in tumor size measured before and after therapy, using a computerized tomography scan. Treatment outcome data were available for 37 (86%) enrolled participants.

*Urine samples collection and analysis*

We analyzed one urine sample, collected prior to cancer treatment, for each study participant. Study nurses collected urine specimens at the time of the cancer diagnosis, into 50 mL polyethylene containers previously decontaminated with nitric acid and then rinsed with water. Within 15 minutes of urine collection, samples were frozen at –20°C and then transferred to the Environmental Health Center (Cluj-Napoca, Romania), where they were stored until analysis for cotinine, arsenic, and five phthalate monoesters. Urinary creatinine was measured according to a previously described procedure, (Neamtiu, Dumitrascu & Roba, 2014), for which the intra-assay coefficient of variation (CV) was 6.5%.

*Urinary cotinine* – The analytic method for determination of urinary cotinine was previously described in detail (Neamtiu, Dumitrascu & Roba, 2014). In brief, cotinine was extracted with dichloromethane, dissolved in toluene and analyzed using a QP 2010 Plus NCI gas chromatograph (Shimadzu, Japan) coupled to a mass spectrometer (GC-MS) operated in the

selective ion monitoring mode. The method limit of detection (LOD) was 10 µg/L and the intra-assay CV was 3.47%.

*Urinary arsenic* – The total arsenic concentration in urine samples was analyzed using a Zeenit 700P atomic absorption spectrometer with hydride generation system (Analytik Jena, Germany). To determine arsenic, 5 mL of urine was mixed with 5 mL $HNO_3$ and 2 mL $H_2O_2$ and mineralized using a Mars 6 microwave digestor (CEM Corporation, USA). The mineralized sample was then diluted to 25 mL with ultrapure water. The mineralized sample reacts with sodium borohydride in an acid environment and forms volatile metal hydrides, which were atomized in a quartz cell heated at 960°C. After plotting the calibration curve (arsenic specific wavelength, λ=193.7nm), the processed samples were atomized and their absorbencies were measured. The method LOD was 0.5 µg/L and the intra-assay CV was 2.36%.

*Urinary phthalates* – We determined urinary phthalate monoester metabolites of dibutyl phthalate (DBP), including mono-butyl phthalate (MBP), benzyl-butyl phthalate (BzBP), including mono-benzyl phthalate (MBzP), and di-ethylhexyl phthalate (DEHP), including mono-(2-ethyl hexyl) phthalate (MEHP), mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), and mono-(2-ethyl-5-oxo-hexyl) phthalate (MEOHP), based on a recently published method (Kim et al., 2014). Briefly, after enzymatic (β-glucuronidase) hydrolysis of urine samples, phthalate metabolites were extracted with a solvent mixture (hexane, acetone) by sonication. The organic phase was dried and evaporated to dryness. A derivatization agent, N,O-bis(trimethylsilyl) trifluoroacetamide with trimethylchlorosilane 1% (BSTFA with TMCS 1%), was added, and the samples were kept in a thermoreactor (Techne, UK) at 65°C for 1 hour. For the analysis, we used

a GC-MS QP 2010 Plus NCI (Shimadzu Corporation) in the single ion monitoring mode. The method LOD was 2.5 µg/L and intra-assay CVs were 9.64% (MBP), 9.01% (MBzP), 5.43% (MEHP), 8.7% (MEOHP), and 4.78% (MEHHP).

*Data analysis*

We characterized distributions for exposures and covariates, and imputed urinary cotinine, arsenic, and phthalates values below the method LODs as LOD/√2 prior to analysis (Hornung & Reed, 1990). We also calculated %MEHP as [MEHP/(MEHP + MEOHP + MEHHP) x 100] on a molar basis, to assess the impact of phthalates metabolism (Hauser, 2008). To evaluate the unadjusted effects of environmental exposures on response to cervical cancer therapy, defined as the difference in tumor size before and after treatment, we used a series of individual regression models, including only urine creatinine as a covariate in addition to either cotinine, arsenic, or each phthalate as the sole predictor. We also constructed a series of comprehensive multiple linear regression models to evaluate the impact of confounder adjusted environmental exposures on cervical cancer treatment response, including age, baseline tumor size, and urine cotinine (arsenic and phthalates models) as covariates.

Given our use of spot collections for biomarkers of exposure, we adjusted for diurnal variation in urine volume by including creatinine as a covariate in the regression models (Kim et al., 2011). However, in a second set of regression models, we also used a more 'traditional' creatinine correction, in which urinary cotinine, arsenic, and phthalates were divided by urine creatinine and the 'normalized' variables entered into the regression models. We examined the distribution of residuals from all regression models to verify the tenability of the normality assumption and to

identify outlying and influential observations for further examination. Stata v.12 (StataCorp LP, College Station, TX USA) was used for the statistical analysis, and statistical significance was defined as $p < 0.05$ for a two-tailed test.

## 3. Results

As described in Table 1, 37 participants were 52 years of age on average at the time of the cervical cancer diagnosis (range 26-76 years). Approximately 59% (n = 22) of patients responded well to the cancer treatment, presenting an 80-100% reduction of the initial tumor size, although the tumor size was reduced by less than 80%, in 41% of women. Most participants (59%) were non-smokers (including 13 women who reported ETS exposure), 6 were former smokers (16%), and 9 (24%) women self-identified as smokers.

Table 2 shows the distributions for measured urinary cotinine, arsenic, and phthalates levels on a creatinine basis. Values are reported on a wet-weight basis in the Supplementary Material (Table S1). Urinary cotinine values ranged from < LOD-395.4 μg/g creatinine with a geometric mean of 13.9 μg/g creatinine. Total arsenic values ranged from < LOD-115.4 μg/g creatinine with a geometric mean of 13.1 μg/g creatinine. Using ANOVA, cotinine was higher in smokers compared to non-smokers ($p=0.04$) and to former smokers ($p=0.08$). On a creatinine basis, geometric mean values for 35 women with sufficient urine volume available for phthalates determination were 8.8 μg/g MBP, 5 μg/g MBzP, 15.8 μg/g MEHP, 3.5 μg/g MEOHP, and 8.7 μg/g MEHHP. The highest maximum values were measured for MBP (295.5 μg/g) and MBzP (182.9 μg/g), whereas maximum values for MEHP (91.9 μg/g), MEOHP (34.1 μg/g), and MEHHP (88.6 μg/g). The geometric mean %MEHP was 68.2, ranging from a minimum value of

10.7% to a maximum value of 97.2%.

Table 3 describes the multiple linear regression analysis of cervical cancer treatment response, measured as the change in tumor size, upon environmental exposures, adjusted for confounding variables. All effect estimates were of small magnitude and 95% confidence intervals (95%CI) included the null hypothesis; we detected no statistically significant confounder-adjusted associations between urinary cotinine, total arsenic, or phthalate metabolites and the change in tumor size. In contrast, the results for the unadjusted regression analysis of total arsenic were statistically significant (β=0.01, 95% CI 0.0003, 0.02; P=0.045), although other results were similarly null. The results were also similar when we used a traditional creatinine correction procedure in lieu of adjustment for urinary creatinine as a covariate in regression models (Supplementary Material Table S2). However, as described by Figure 1 we detected a statistically significant positive association between %MEHP and response to cervical cancer therapy, in the multivariable regression model adjusted for confounders (β=0.015; 95% CI 0.003, 0.03; P=0.019). The unadjusted association was not statistically significant for %MEHP (β=0.004; 95% CI -0.01, 0.02; P=0.634).

## 4. Discussion

We evaluated the impact of exposure to ETS, arsenic, and phthalates on locally advanced cervical cancer treatment using urine biomarkers from 37 women aged 26-76 years. More than a half of the study participants responded well to cancer treatment, presenting a reduction of the initial tumor size between 80% and 100%. We found no meaningful effects on the change in tumor size for urine cotinine or total arsenic, or for five monoester metabolites of three widely

distributed phthalate diesters. Yet, our analysis of %MEHP suggested an enhanced response to cervical cancer treatment, indicating that less efficient conversion of the primary hydrolytic DEHP phthalate monoester MEHP, to its secondary-oxidative metabolites MEOHP and MEHHP, may confer benefit (Hauser, 2008). It is tempting to speculate the existence of a common metabolic pathway impacting hydrolysis and oxidation of DEHP and response to the cervical cancer treatment protocol. However, given the limited sample size available for our study, and the absence of more comprehensive indicators to characterize cervical cancer therapy response, these observations should be considered preliminary and will require confirmation in a more definitive investigation.

Several human biomonitoring studies from nearby European areas reported urinary levels of cotinine, arsenic, and phthalates measured in general population samples. Geometric mean urinary cotinine was modestly lower in 120 Romanian mothers 25 to 45 years of age (9.10 µg/g creatinine) (Lupsa et al., 2015) than for women in our study. However, the geometric mean total urine arsenic value for 4,730 Germans aged 18 to 69 (3.1 µg/g) was substantially lower than for women in our study (Becker et al., 2003). In our study, we determined urinary metabolites of DBP, BzBP, and DEHP as those phthalates most prevalent in Central and Eastern European populations (Černá et al., 2015). The highest urinary phthalates values in our study population were measured for MBP, MBzP, MEHP and MEHHP, while MEHP had the highest geometric mean. The geometric mean concentrations of MBzP and MEHP were higher in our study than were reported for 117 women from the Czech Republic (4.35 and 3.13 µg/g creatinine, respectively), 125 women from Slovakia (3.81 and 3.22 µg/g creatinine, respectively), and 115 women from Hungary (3.82 and 3.70 µg/g creatinine, respectively) (Černá et al., 2015). In

255 contrast, levels of MEOHP and MEHHP were lower in our study, than reported for Czech (11.68
256 and 18.45 µg/g creatinine, respectively), Slovakian (11.54 and 18.20 µg/g creatinine,
257 respectively), and Hungarian (10.37 and 15.54 µg/g creatinine, respectively) women (Černá et
258 al., 2015). Overall, the distribution of exposures among our study population was unique, yet we
259 identified similarities with values reported for several nearby, non-clinical European populations.
260
261 Our study had several important limitations, and so, our results should be interpreted only as
262 hypothesis generating. The small number of study participants may have limited our ability to
263 detect modest associations, and it precluded an analysis of multiple exposures included in a
264 single regression model. We hope to expand the sample size in the future to generate more
265 precise effect estimates and to more comprehensively analyze exposure to the mixture of tobacco
266 smoke, arsenic, and phthalates. While our study was prospective in nature, we measured
267 exposure at only a single time point prior to initiation of cancer treatment; the short in vivo ½-
268 lives and the episodic nature of phthalates exposure may have misclassified some patients
269 (Fromme et al., 2007). Our use of a total arsenic variable, including comparatively innocuous
270 organic species with toxic inorganic species, may have further misclassified exposure for some
271 women (Marchiset-Ferlay, Savanovitch & Sauvant-Rochat, 2012). Yet, misclassification is
272 unlikely to have differed by study outcome and so any bias will have led to underestimated
273 effects. Finally, we did not incorporate recent data suggesting an important role for gene
274 expression on cancer treatment response in our study population (Balacescu et al., 2014). Still,
275 we do anticipate a link to exposure and so bias was unlikely. A larger future investigation should
276 incorporate longitudinal collection of urine specimens during cancer treatment to reduce
277 exposure misclassification as well as gene expression information to assess the impact on

278 treatment response.

279

280 To the best of our knowledge, this preliminary report describes the first investigation on the
281 impact of widely distributed environmental pollutants on the effectiveness of locally advanced
282 cervical cancer therapy. These results should help to reassure clinicians that even levels of
283 cotinine, total arsenic, MBzP, and MEHP higher than reported from other European study
284 populations are unlikely to interfere with the effectiveness of radio and chemotherapy for
285 invasive cervical cancer. Still, %MEHP may prove important. To better understand the impact of
286 these results, particularly in women diagnosed with advanced stage cervical cancer, a larger
287 sample size is needed for detecting potentially modest effects, and to comprehensively assess the
288 exposure. Current cancer treatments are both expensive and induce serious side effects, and so
289 characterizing the potential impact of widely distributed environmental pollutants is critical to
290 establish new indicators for predicting the effectiveness of cervical cancer treatments.

291

292 **Conclusions**

293 Exposure to tobacco smoke, arsenic, and phthalates did not appear to impact cervical cancer
294 treatment at the levels of exposure experienced by our study population. Phthalates metabolism
295 may be associated with locally advanced cervical cancer treatment response, although the
296 clinical relevance is unclear. A more comprehensive investigation with a larger sample size will
297 be necessary for a more definitive result.

298

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## Table 1(on next page)

Demographic and clinical characteristics of women receiving cervical cancer treatment and participating in the study (n=37)

1

| Characteristics | Mean (n) | SD (%) | Min. | 50th %tile | Max. |
|---|---|---|---|---|---|
| Age (years) | 52.2 | 11.1 | 26 | 54 | 76 |
| Body mass index (kg/m$^2$) | 27.1 | 5.4 | 17 | 26.8 | 41.2 |
| Smoker (yes) | (9) | (24.3) | - | - | - |
| Tumor Stage | | | | | |
| IIB | (13) | (35.2) | - | - | - |
| IIIA | (14) | (37.8) | - | - | - |
| IIIB | (10) | (27) | - | - | - |
| Change in tumor size (cm) | 3.3 | 1.3 | 1 | 3 | 6 |

2

## Table 2 (on next page)

Urinary cotinine, arsenic, and phthalates metabolites measured in n=37 women undergoing cervical cancer treatment and participating in the study (µg/g creatinine)

LOD = method limit of detection; MBP = mono butyl phthalate; MBzP = mono benzyl phthalate; MEHP = mono (2-ethylhexyl) phthalate; MEOHP = mono (2-ethyl-5-oxohexyl) phthalate; MEHHP = mono (2-ethyl-5-hydroxyhexyl) phthalate; %MEHP = 100 x (MEHP/(MEHP + MEOHP + MEHHP) on a molar basis

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| Analyte | Min. | 25th %tile | 50th %tile | 75th %tile | Max. | Geometric mean |
|---|---|---|---|---|---|---|
| Cotinine | < LOD | < LOD | 9.3 | 35.3 | 395.4 | 13.9 |
| Arsenic | < LOD | 7.4 | 13.2 | 20.8 | 115.4 | 13.1 |
| MBP | < LOD | < LOD | 9.8 | 22 | 295.5 | 8.8 |
| MBzP | < LOD | 1.8 | 5.4 | 10.7 | 182.9 | 5 |
| MEHP | 1.5 | 10.6 | 15.5 | 28.3 | 91.9 | 15. 8 |
| MEOHP | < LOD | < LOD | 2.8 | 8.2 | 34.1 | 3.5 |
| MEHHP | < LOD | 4.6 | 8.8 | 13.8 | 88.6 | 8.7 |
| %MEHP | 10.7 | 63.6 | 85.4 | 91.6 | 97.2 | 68.2 |

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## Table 3 (on next page)

Associations for urinary cotinine, arsenic, and phthalates (µg/l) with cervical cancer therapy response, adjusted for covariates using multiple linear regression models [a]

[a] Adjusted for baseline tumor size (cm), age (years), urinary creatinine (mg/l), and urinary cotinine for arsenic and phthalates (µg/l); [b] %MEHP = 100 x (MEHP/(MEHP + MEOHP + MEHHP)) on a molar basis.

MBP = mono butyl phthalate; MBzP = mono benzyl phthalate; MEHP = mono (2-ethylhexyl) phthalate; MEOHP = mono (2-ethyl-5-oxohexyl) phthalate; MEHHP = mono (2-ethyl-5-hydroxyhexyl) phthalate.

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| Predictors | n | β | 95% CI | | P-value |
|---|---|---|---|---|---|
| Cotinine | 37 | -0.001 | -0.005 | 0.003 | 0.590 |
| Arsenic | 37 | 0.005 | -0.002 | 0.013 | 0.173 |
| MBP | 35 | 0.0003 | -0.001 | 0.002 | 0.752 |
| MBzP | 35 | -0.001 | -0.013 | 0.010 | 0.831 |
| MEHP | 35 | 0.0007 | -0.006 | 0.007 | 0.817 |
| MEOHP | 35 | -0.016 | -0.044 | 0.012 | 0.256 |
| MEHHP | 35 | 0.0006 | -0.006 | 0.007 | 0.855 |
| **%MEHP [b]** | **35** | **0.015** | **0.003** | **0.03** | **0.019** |

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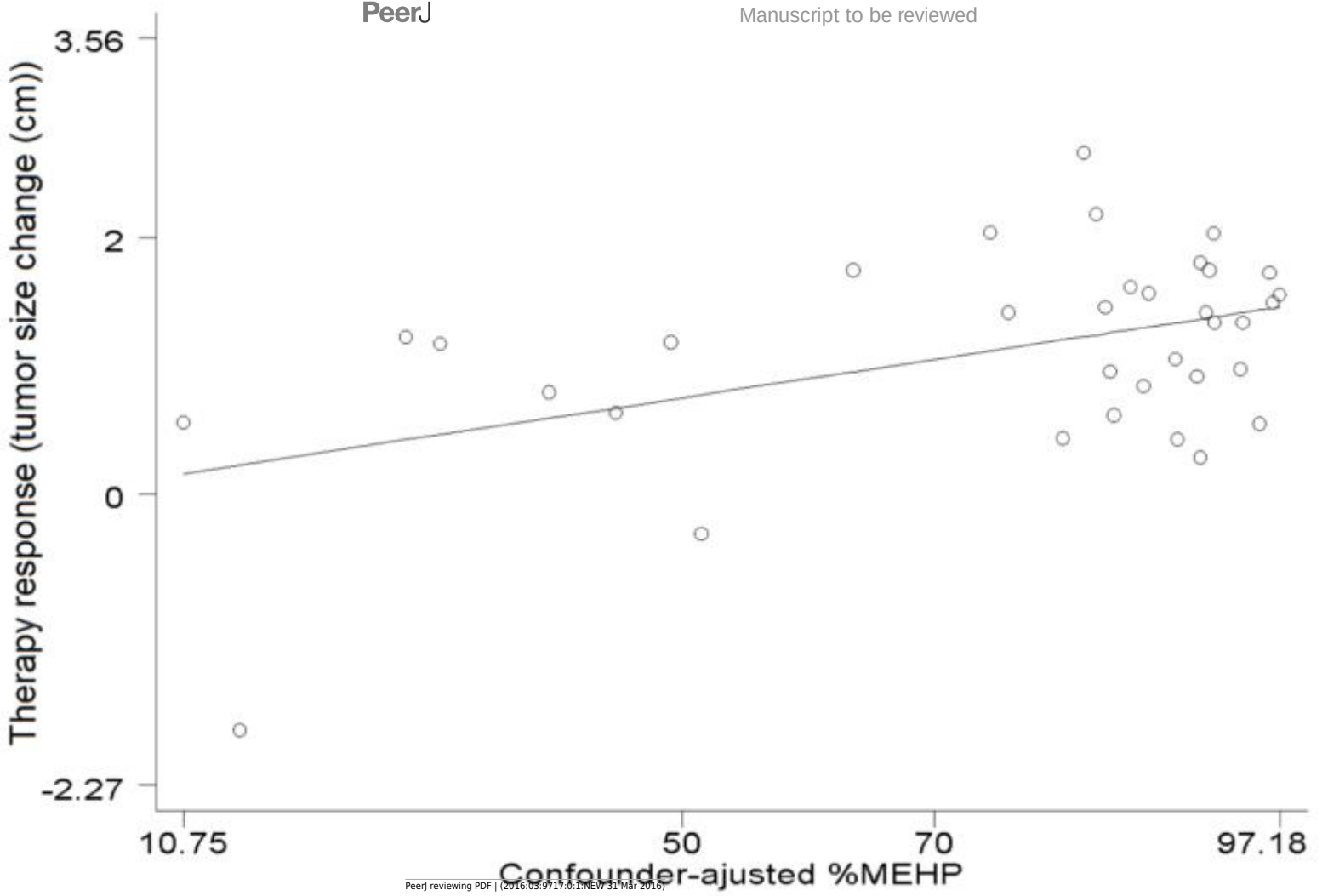
## Figure 1(on next page)

Association between %MEHP and response to cervical cancer treatment[a]

[a] Adjusted for initial size of the tumor, urine cotinine, creatinine, and age %MEHP = 100 x (MEHP/(MEHP + MEOHP + MEHHP)) on a molar basis


3.56
2
0
-2.27
Therapy response (tumor size change (cm))
10.75
50
70
97.18
Confounder-ajusted %MEHP