Urinary bisphenol A in children, mothers and fathers from Slovenia: Overall results and determinants of exposure

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ABBREVIATIONS

BPA, Bisphenol A; DCM, dichloromethane; EtAc, ethyl acetate; GM, geometric mean; EDC, endocrine disrupting chemical; LOD, limit of detection; LOQ, limit of quantification; MTBSTFA, *N-tert*-Butyldimethylsilyl-*N*-methyltrifluoroacetamide; PVC, polyvinyl chloride plastics; UGT, uridine diphosphate-glucuronosyl transferase

ABSTRACT

In the present study, urinary bisphenol A (BPA) levels were reported for the first time in the Slovenian general population and were evaluated with regard to dietary and non-dietary exposure sources, and compared according to age, gender and area of residence. First morning urine was collected from children (6-11 years), their mothers (30-52 years) and fathers (30-53 years), living in urban and rural areas of Slovenia. Besides basic questionnaire data on general population characteristics, socio-economic status and dietary habits, BPA-specific data was also collected, including consumption of food and beverages from plastic and canned containers, presence of white dental fillings, the use of specific consumer products and hormonal treatments. Urine samples were analysed for both free and conjugated BPA using GC-MS/MS. The urinary levels of total BPA in children, mothers and fathers were low, with geometric means of 1.51, 0.79, and 0.20 μg/g creatinine, respectively. The levels were comparable with the levels reported for other European countries and were all below the current health-based guidance values. In line with large-scale surveys, the data revealed age-dependant BPA urinary levels, with the highest levels in the youngest age group. In mothers, urinary levels of BPA were determined by hormonal interactions more than dietary sources, while a positive association between urinary BPA and diet was apparent in children (canned food/drink and food from plastic material) and fathers (canned food/drink). The study clearly shows that physiological and behavioural differences account for differences in levels of urinary BPA among study groups, a finding that sets the priorities for future research.

Key words: bisphenol A; urine; human biomonitoring; internal exposure; exposure sources

Role of funding source

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The study was reviewed and approved by the Republic of Slovenia National Medical Ethics Committee (NMEC) with numbers of accordance 64/06/11. Informed written consent was obtained from all the participants. Participation was voluntary and a participant could withdraw from the study at any time.

1 INTRODUCTION

Bisphenol A (BPA) is a non-persistent industrial chemical used in the manufacture of polymers such as polycarbonate and epoxy resins, as an antioxidant and inhibitor of end polymerization in polyvinyl chloride plastics (PVC), and as a precursor for the synthesis of the flame retardant tetrabromobisphenol-A (Geens et al., 2012). Polycarbonate is widely used in articles such as baby bottles, tableware, microwave ovenware, storage containers, returnable water and milk bottles, and refillable water containers, while epoxy-phenolic resins are used as an internal protective lining for food and beverage cans and as a coating on metal lids for glass jars and bottles (EFSA, 2006). BPA is also used in a variety of other, non-food applications, among them in printing inks, carbonless and thermal paper, flame retardants, resin-based composites and sealants used in dentistry (EFSA, 2006). BPA is defined as an endocrine disrupting chemical (EDC) that interacts with and elicits responses through endogenous receptors (reviewed by Mørck, 2012). The normal functioning of the endogenous hormonal system is essential for the development and growth of the foetus and the reproductive organs, therefore it has been proposed that exposure to BPA may disrupt foetus and young child development as a consequence of the interaction with oestrogen, androgen and thyroid receptors (Mørck, 2012). Many *in vitro* and *in vivo* (animal) studies have demonstrated that adverse health effects due to BPA exposure can occur also at environmentally relevant levels (Rochester, 2013). In addition, along with laboratory studies, there is growing literature correlating environmental BPA exposure to adverse effects in humans, providing increasing evidence that BPA is associated with adverse health outcomes (as reviewed by Rochester, 2013). These include: reduced human reproduction (e.g. infertility, reduced sperm quality, changes in endogenous sex hormone concentrations, polycystic ovary syndrome), metabolic disease, cardiovascular disease, obesity, altered thyroid hormone concentrations, altered epigenetic markers and gene expression; with the most firm evidence relating to behavioural and other developmental effects in children.

The migration of BPA from the food container to the food itself means that dietary ingestion is a major source of exposure in all age groups of the general population (Pirard and Charlier, 2014). Among non-dietary sources, skin contact with thermal paper used in receipts might be a significant source of exposure for cashiers (Geens et al., 2012; Ndaw et al., 2016) and composite dental fillings for the general population, while indoor air and dust are also potential exposure sources for the non-occupational population (Geens et al., 2012).

When BPA is administered orally, it is rapidly absorbed and efficiently conjugated with glucuronic acid into the conjugate BPA-glucuronide in the liver by first-pass metabolism (Edginton and Ritter, 2009) and rapid elimination occurs within 24 h (a single peak) after BPA

administration (Mørck, 2012). BPA conjugation results in detoxification of the parent compound, since only the free plasma (unbound) BPA is able to interact with molecular receptors. The rapid and complete excretion of orally administered BPA, means urine samples are a suitable matrix for BPA biomonitoring since it reflects recent exposure i.e. within the last few hours (Dekant and Völkel, 2008; Lakind and Naiman, 2008; Vandenberg et al., 2007).

Considering the importance of BPA exposure, several biomonitoring programs have included BPA. European-wide and country-specific levels of urinary BPA were obtained through a harmonized protocol for participant recruitment, sampling and quality controlled biomarker analysis in the frame of the twin projects COPHES and DEMOCOPHES (Becker et al., 2014); within which BPA levels were measured in 6-11 years old children and their mothers in six EU member states: Belgium, Denmark, Luxembourg, Slovenia, Spain, and Sweden (Covaci et al., 2015). Overall, the geometric means (GMs) of all countries (95% CI) adjusted for urinary creatinine, age and gender were 2.04 (1.87–2.24) μg/L and 1.88 (1.71–2.07) μg/L for children (n=653) and mothers (n=639), respectively, which is below the current HBM values of 0.1 mg/L (children) and 0.2 mg/L (adults), updated by the Human Biomonitoring Commission of the German Environment Agency in 2015 (UBA, 2017). Only few of the measured BPA values were higher than the current HBM values.

The aim of this study was to compare the levels of urinary BPA among three different study groups within the Slovenian non-occupationally exposed population and compare the data with other European studies. The study population included children (6-11 years), their mothers (30-52 years) and fathers (30-53 years) living in two areas with distinct population densities (urban and rural). Besides setting the background levels for the three study groups of different ages and genders (girls vs. boys, women vs. men) living in urban and rural areas, the aim was also to identify the main sources of exposure to BPA. In order to do this, a BPA-specific questionnaire was developed, including consumption of food and drinks from plastic and canned containers, generally, and during a 24 h period prior to urine collection. The questionnaire was also used to collect information about other sources of BPA exposure (dental fillings, mouthwash, sunscreen, disinfectant use and occupational exposure) and about confounding factors such as contraception and hormonal pills intake, pregnancy and breastfeeding.

2 MATERIAL AND METHODS

2.1 Study population and sampling

Three study groups were addressed: children (age group 6-11 years), accompanying mothers representing women of childbearing age (age 30-52 years) and accompanying fathers or current male partners (age 30-53 years). Families were recruited from two sampling locations chosen according to the degree of urbanization (city vs. rural), while excluding contaminated sites. The capital of Slovenia (Ljubljana) was chosen as the urban location; and an independent rural area in the south-eastern region of Slovenia free of industrial activities as the rural location (an area located around the two small towns of Šmarje pri Jelšah and Bizeljsko). The target population was recruited from primary schools taking into account inclusion/exclusion criteria as described by Becker et al. (2014). In total, 155 children, 155 mothers and 71 fathers were recruited between December 2011 and February 2012. The study was approved by the Republic of Slovenia National Medical Ethics Committee (NMEC) with numbers of accordance 64/06/11. Informed written consent was obtained from all the participants. Participation was voluntary and a participant could withdraw from the study at any time.

Urine samples were collected from all participating families following the COPHES protocol (Becker et al., 2014). Sample collection took place in the selected schools. Urine collection vessels were provided to the families prior to their interview at the school; first morning urine was collected by all participants and stored in a cool box provided before the interview. Urine samples were aliquoted into 10 ml-tubes (each containing 5 mL of urine) already in the field by one of the field workers from the clinical laboratory. Urine samples were transported in refrigerated in cool boxes and stored in freezers (at -20 ºC) at the Jožef Stefan Institute.

2.2 Questionnaire data

During the visit at the school, trained field workers interviewed participants. The field workers collected data on (a) residential environment and type of residence, (b) nutrition, (c) smoking habits, (d) exposure-relevant behaviour, (e) occupation, (f) socio-demography, and (g) special data on potential determinants of BPA exposure. These included namely consumption of food from plastic containers or cans, consumption of food re-heated in plastic, use and age of plastic sport bottles, use of products potentially containing BPA – sunscreen, mouthwash and disinfectants, dental fillings, and consumption of contraceptive and hormonal pills, pregnancy and breastfeeding.

2.3 Analysis of BPA in urine samples

2.3.1 Analytical method

Urine samples were homogenized by vortexing for 15 s. From each sample, a 1 mL aliquot was deconjugated (determination of total BPA), while a second 1 mL aliquot was put aside (determination of free BPA). Deconjugation was performed by adding sodium acetate buffer (pH 5.2) and β-glucuronidase at a final concentration of 100 U/mL. Samples were then incubated at 37 ºC on a rotary shaker (140 rpm) for 18 h. After the addition of the internal standards (final concentration 10 ng/mL), samples were extracted by solid phase extraction (SPE) on Oasis HLB cartridges (30 mg / 1 cc, Waters Corp.). First, each cartridge was conditioned with 1 mL dichloromethane (DCM), 1 mL ethyl acetate (EtAc), 1 mL MeOH and equilibration with 1mL Milli-Q water. One mL samples diluted in 2 mL of Milli-Q water were then loaded on the equilibrated SPE cartridges, and washed with 1 mL 20 % v/v MeOH. Finally, the analytes were eluted with 3 × 0.5 mL MeOH:EtAc (1:1 v/v). The extracts were dried under a gentle stream of nitrogen, reconstituted in 0.5 mL of EtAc and derivatized with 20 µL of *N*-*tert*-Butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA) at 80 ºC for 30 min. Clean-up was performed using silica columns (500 mg, Isolute Si, Biotage), preconditioned with 2.5 mL DCM and 2 x 2 mL EtAc, while the analyte derivatives were eluted with 4 mL of DCM. The samples were dried under a gentle stream of nitrogen and resuspended in 0.1 mL EtAc. Analysis was performed on a 450-GC gas chromatograph coupled to 240-MS ion trap mass spectrometer (Agilent, former Varian Inc.). The GC injector temperature was 250 °C and operated in the splitless mode for 4 min, then switched to 1/30 split mode. The oven was held at 100 °C for 2 min, then ramped at 20 °C / min to 220 °C and held for 1 min, ramped at 1 °C / min to 222 °C, and finally ramped at 20 °C / min and held for 5 min. Mass spectrometric detection was performed in the MS/MS mode after external electron impact ionization.

2.3.2 Quality control

BPA eluted at 15.6 min and its identity was confirmed based on the ratio between the two MS/MS transitions, m/z 442>327 and m/z 442>233. Limits of detection (LOD) and quantification (LOQ) were 0.04 µg/L and 0.11 µg/L, respectively. Recovery was ≥ 80 %, irrespective of the concentration in the relevant calibration range (up to 50 µg/L). Method accuracy was 95 %, which was defined as the agreement between the correct value ascertained in the COPHES/DEMOCOPHES Inter-laboratory exercise and the determined value. Since no certified reference material was available, a spiked urine, synthetic urine and Milli-Q water samples, and an Inter-laboratory exercise sample were used for Quality Control, which was performed once per week for the whole procedure and for each operator involved in the sample preparation.

The laboratory also participated in an Inter-laboratory Comparison Investigation and in two External Quality Assessment exercises, conducted within the COPHES/DEMOCOPHES, which were designed to monitor both total and free BPA and check for possible blank issues involving free BPA. Based on the analysis of the test samples and the evaluation of the Quality Assurance Unit, the testing laboratory was found to be competent to perform BPA analysis. More details on the participating laboratories and the specific procedures for quality assurance are presented in (Schindler et al., 2013). The Results and Discussion sections in this paper refer to the levels of total BPA.

2.4 Statistical analysis

Values below the LOQ were treated as LOQ/2. Values measured in urine samples with a creatinine content below 300 mg/L or above 3000 mg/L were excluded from the dataset because of suspected abnormal urinary excretion. All variables were categorized. Descriptive statistics were generated from the questionnaire data variables and from the total BPA levels in urine. Log-normally distributed data was normalized using natural logarithm. Comparison of exposure levels between the selected population groups (mothers, children, and fathers) was performed using analysis of variance (ANOVA). Single linear regression was used to test correlation between total BPA concentrations and selected questionnaire data variables, and between total BPA concentrations among the study groups. Multiple linear regression models were used to evaluate the association between total BPA levels and all covariates (questionnaire data variables) that were correlated with the biomarker with a probability value (p-value) of <0.25. Predefined confounders (age, gender of the child) were fixed in the models. Probability values <0.05 were considered statistically significant and p-values <0.1 marginally significant. Statistical analyses were performed using STATA 12 (USA).

3 RESULTS

3.1 General characteristics of the study population

The demographic characteristics of the study population are presented in Table 1. The age of the children was evenly distributed between 6 and 11 years, while the ages of the fathers and mothers were between 30 and 53 years. Based on the BMI of mothers and fathers, and creatinine levels of all participants, the study population was normal; all participants had delivered their first morning void and most had had their last void more than 8 hours prior to sampling. Among mothers and fathers, there was a prevalence of tertiary education. Seventeen percent and 26 % of mothers and fathers were current smokers, respectively. The study population was equally distributed between urban and rural areas.

Table 1. Demographic characteristics of the study population.

	Category	Mothers	Children	Fathers
N		140	145	69
Age (years)	Median	39	9	41
	Min-Max	30-52	6-11	30-53
Age distribution	6-8 years	-	59 (40.7%)	-
	9-11 years	-	86 (59.3%)	-
	≤35 years	36 (25.7%)	-	6 (8.6%)
	35-40 years	50 (35.7%)	-	24 (34.3%)
	>40 years	54 (38.6%)	-	40 (57.1%)
Body-mass index (kg/m^2)	Median	23.0	-	26.0
	Min-Max	18.6-37.6	-	19.4-35.3
Urinary creatinine (mg/L)	Median	1355	1200	1670
	P25-P75	980-1880	910-1660	1430-2080
	Min-Max	204-3080	350-2990	380-3050
Urine sampling period*	≤ 5 h	8 (6.2%)	16 (11.3%)	0 (0%)
	5-8 h	38 (29.5%)	38 (26.7%)	24 (43.6%)
	> 8 h	83 (64.3%)	88 (62.0%)	31 (56.4%)
Smoking status	Smoker	24 (17%)	-	18 (26%)
	Non-smoker	116 (83%)	-	50 (74%)
Area of residence	Urban	70 (50.0%)	73 (50.3%)	37 (52.9%)
	Rural	70 (50.0%)	72 (49.7%)	33 (47.1%)
Highest education level	Primary or lower secondary education	18 (12.9%)	-	12 (17.4%)
	Higher sec. or post-sec. non-tertiary education	55 (39.3%)	-	24 (34.8%)
	Tertiary education	67 (47.9%)	-	33 (47.8%)

*Period of time between the last urination and the sample collection.

Bisphenol A-specific questionnaire data is presented in Table 2. Among the study population, there were a similar proportion of participants who consumed food stored in plastic containers 24 h prior to sampling and those who had not. The majority of the participants had not consumed canned food or drink prior to sampling. In general, the study groups consumed canned food or food from plastic containers equally frequently. The majority of the participants re-heated food in plastic containers only occasionally or never. A sports bottle was used by most of the study participants less than once per week, and about 2/3 of the participants did not know the age of the bottle. Most of the participants did not re-use single-use plastic bottles. The presence of white dental fillings was highest in mothers, followed by fathers, and evenly distributed among children. Use of sunscreen and mouthwash in the last 4 weeks before sampling was less frequent, more frequent was the use of disinfectants, particularly by mothers. Use of contraceptive pills was declared by 12 % of the participating mothers. Most parents were not occupationally exposed to BPA.

Table 2. Description of the BPA-specific questionnaire data of the study population.

	Category	Mothers	Children	Fathers
N		140	145	69
Consumption of food from PVC containers	Yes	70 (50.0%)	61 (43.3%)	36 (54.5%)

within 24 h before sampling	No	70 (50.0%)	80 (56.7%)	30 (45.5%)
Consumption of canned food/drinks within 24 h before sampling	Yes	23 (16.4%)	16 (11.3%)	18 (27.7%)
	No	117 (83.6%)	126 (88.7%)	47 (72.3%)
Frequency of food consumption from PVC containers (last 4 weeks)	Once per week or more	68 (48.6%)	70 (48.3%)	35 (53.0%)
	Less than once per week	72 (51.4%)	75 (51.7%)	31 (47.0%)
Frequency of canned food consumption (last 4 weeks)	Once per week or more	50 (36.0%)	48 (33.3%)	41 (62.1%)
	Less than once per week	89 (64.0%)	96 (66.7%)	25 (37.9%)
Consumption of food re-heated in PVC containers within 24 h before sampling	Yes	3 (2.2%)	2 (1.4%)	2 (3.0%)
	No	136 (97.8%)	142 (98.6%)	65 (97%)
Frequency of re-heating food in PVC containers	At least once per month	18 (12.9%)	19 (13.1%)	9 (13.4%)
	Occasionally or never	122 (87.1%)	126 (86.9%)	58 (86.6%)
Consumption of food stored in plastic bags within 24h prior to sampling	Yes	37 (26.4%)	23 (15.9%)	15 (21.7%)
	No	103 (73.6%)	122 (84.1%)	54 (78.3%)
Frequency of sports bottle use	Once per week or more	20 (14.3%)	37 (25.5%)	14 (20.9%)
	Less than once per week	120 (85.7%)	108 (74.5%)	53 (79.1%)
Re-filling single-use plastic bottle	Yes	49 (35.0%)	54 (36.2%)	24 (36.4%)
	No	91 (65.0%)	91 (62.8%)	42 (63.6%)
White dental fillings	Yes	112 (80.0%)	63 (43.8%)	43 (65.2%)
	No	28 (20.0%)	81 (56.2%)	23 (34.8%)
Number of white dental fillings	Median	4	0	2
	P25-P75	1-7	0-2	0-4
	Min-Max	0-20	0-9	0-20
Use of sunscreen (last 4 weeks)	Yes	4 (2.9%)	4 (2.8%)	0 (0%)
	No	136 (97.1%)	141 (97.2%)	67 (100 %)
Use of mouthwash (last 4 weeks)	Yes	27 (19.3%)	20 (13.8%)	9 (13.4%)
	No	113 (80.7%)	125 (86.2%)	58 (86.6%)
Use of disinfectants (last 4 weeks)	Yes	51 (36.4%)	14 (9.7%)	15 (22.4%)
	No	89 (63.6%)	131 (90.3%)	52 (77.6%)
Use of contraceptive pills	Yes	17 (12.1%)	-	-
	No	123 (87.9%)	-	-
Occupational exposure	Yes	3 (2.2%)	-	1 (1.5%)
	No	136 (97.8%)	-	66 (98.5%)

3.2 Bisphenol A levels in urine of the Slovenian population

Bisphenol A was detected in 94%, 88% and 81% of the urine samples from children, mothers and fathers, respectively. The descriptive statistics for total BPA levels in the study groups is given in Table 3. Creatinine levels differed significantly between the study groups, and for this reason the results were creatinine-normalized. Among the study groups, fathers had the lowest levels of total BPA (GM of 0.2 µg/g creatinine), followed by mothers (GM of 0.8 µg/g creatinine) and children (GM of 1.5 µg/g creatinine) ($p<0.001$) (Fig 1). The levels of BPA did not exceed the current HBM values established by the German Human Biomonitoring Commission (0.1 mg/L for children and 0.2 mg/L for adults) (UBA, 2017). Although somewhat higher levels were observed in boys than in girls (Fig 1), the difference was not significant, neither in the levels expressed per volume ($p=0.085$) nor per creatinine ($p=0.243$). Among children, there was a significant trend of higher levels in younger children ($r=-0.22$, $p=0.009$). In mothers and fathers, this trend was not significant ($p=0.418$ and 0.433, respectively). A positive trend with BMI was observed in mothers ($r=0.25$, $p=0.007$), while a negative trend was observed in fathers ($r=-0.23$, $p=0.061$).

Urinary BPA levels differed between urban and rural areas among all the study groups. In mothers, the levels were higher in rural than in urban area (GM_{urban}=0.61 µg/g creatinine; GM_{rural}=1.03 µg/g creatinine, p=0.067), while in children (GM_{urban}=1.99 µg/g creatinine; GM_{rural}=1.14 µg/g creatinine, p=0.062). and fathers (GM_{urban}=0.29 µg/g creatinine; GM_{rural}=0.13 µg/g creatinine, p=0.005) the opposite was observed.

Table 3. Descriptive statistics for total BPA levels in mothers, their children and fathers, expressed as µg/L or µg/g creatinine. LOQ (limit of quantification)=0.11 µg/L, GM=geometric mean. 95% CI=95 % confidence interval.

Population	N	>LOQ (%)	min-max	GM	95% CI	P10	P25	P50	P75	P90
				Total BPA (µg/L)						
Mothers	140	88	<LOQ-69.8	**1.02**	0.77-1.34	<LOQ	0.35	1.14	3.47	6.81
Children	145	94	<LOQ-31.4	**1.81**	1.42-2.31	0.25	0.67	2.39	4.57	12.8
-Girls	76	93	<LOQ-15.8	**1.54**	1.13-2.08	0.28	0.53	1.94	3.71	13.8
-Boys	69	96	<LOQ-31.4	**2.17**	1.46-3.21	0.24	0.89	3.27	5.72	16.6
Fathers	69	81	<LOQ-40.9	**0.32**	0.24-0.44	<LOQ	0.22	0.32	0.48	1.11
Population	**N**	**>LOQ (%)**	**min-max**	**GM**	**95% CI**	**P10**	**P25**	**P50**	**P75**	**P90**
				Total BPA (µg/g creatinine)						
Mothers	140	88	<LOQ-25.8	**0.79**	0.61-1.03	<LOQ	0.32	1.08	2.24	4.07
Children	145	94	<LOQ-36.8	**1.51**	1.18-1.92	0.20	0.58	1.86	3.84	9.29
-Girls	76	93	<LOQ-36.8	**1.37**	1.01-1.87	0.31	0.54	1.41	3.37	7.07
-Boys	69	96	<LOQ-36.8	**1.67**	1.13-2.46	0.15	0.66	2.45	4.74	11.5
Fathers	69	81	<LOQ-36.2	**0.20**	0.15-0.27	<LOQ	0.11	0.19	0.34	0.97

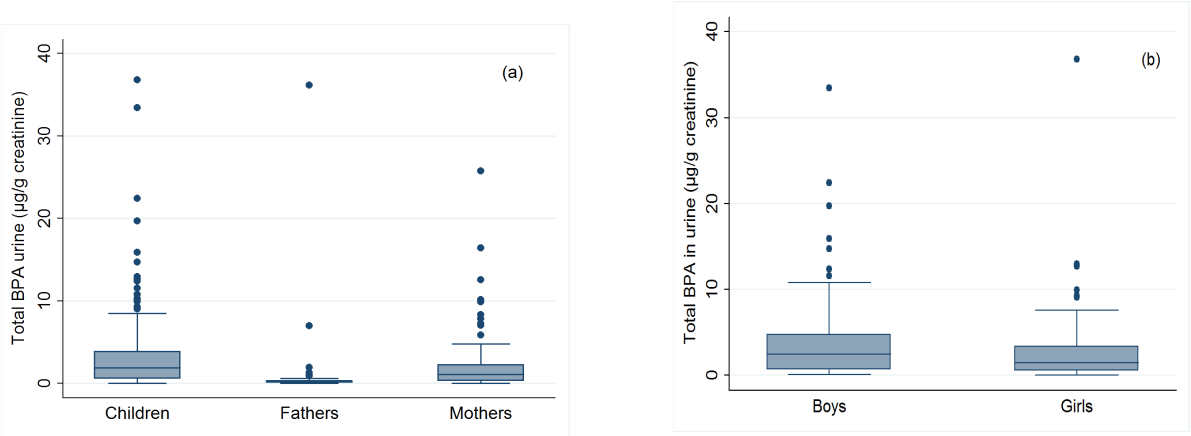

Fig 1. Total BPA levels in urine of the study groups from Slovenia: (a) children, their mothers and fathers, (b) boys and girls.

3.3 Determinants of exposure

Bivariate analysis of urinary BPA levels and questionnaire data revealed certain associations between creatinine-normalized urinary levels and consumption of food from plastic

containers and food or drinks from cans. However, there were marked differences in the observed associations depending on gender or the study group. In mothers and children, consuming food from plastic containers (24 h prior to urine collection) only contributed marginally to higher BPA levels (p=0.093, and 0.083, respectively), but not significantly in fathers (p=0.660). There was no association observed between BPA levels and consumption of food (frozen or dried) stored in plastic bags (p>0.1). A marginal association between food re-heated in plastic containers and consumed 24 h prior to sampling and higher urinary BPA levels was observed in children (p=0.091), while there was no significant association in mothers (p=0.522) and fathers (p=0.525). A positive association between canned food and drink and BPA levels was observed among all groups, in mothers with an overall frequency of consumption (p=0.070), while in children and fathers with current consumption, i.e. 24 h prior to sampling (p=0.080 and 0.003, respectively). Drinking from a plastic sports bottle was not significantly associated with urinary BPA levels, and neither was re-filling a single-use bottle (p>0.1).

Among the consumer products included in the questionnaire (sunscreen, mouthwash and disinfectants), the level of urinary BPA was associated positively with frequency of mouthwash and disinfectant agent use in fathers (p=0.052 and p=0.075, respectively), but not in children or mothers. The presence of white dental fillings was associated negatively with levels of BPA in children's urine (p=0.038), while their number marginally significantly (r=-0.14, p=0.097). In mothers and fathers, BPA levels in urine were not found to associate neither with the presence of dental fillings (p=0.879 and 0.471, respectively) nor the number of fillings (r=-0.09, p=0.277 and r=0.17, 0.177, respectively).

After adjustment for possible confounders and co-variates (Table 4), levels of maternal urinary BPA were associated significantly with the area of residence (higher in rural) and marginally significantly with BMI (positive association), consumption of contraception pills (higher in mothers taking the pills) and smoking (higher in non-smoking mothers). In children, the observed bivariate associations remained significant in the model; younger children and those living in the urban area and those having consumed food/drinks from plastic or canned containers 24 h before sampling had higher urinary BPA levels. The model confirmed an inverse association with the presence of white dental fillings, but no gender-related difference was observed. Despite the lower sample size for fathers, their model had the highest R^2 value. Adjustment revealed higher urinary BPA levels in urban area (marginal significance) and confirmed a positive association with consumption of canned food/drinks within 24 h before sampling. Education level was not associated with urinary levels in any of the study groups.

Table 4. Multiple linear regression. Determinants of BPA exposure. *Coeff* - estimate of change in urinary BPA level. CI – 95% confidence interval. P-value – significance value.

Total BPA in urine (µg/g creatinine)		***Coeff.* (95% CI)**	**p-value**
Mothers *(model R^2=0.09, p=0.015, n=138)*			
Age	≤35 (n=36)	1.00	
	>35 and ≤ 40 (n=48)	0.85 (0.43-1.70)	0.649
	>40 (n=54)	1.30 (0.63-2.69)	0.478
BMI	Change of 10 %	1.006 (1.000-1.0012)	0.052
Area	***Urban (n=69)***	***1.00***	
	Rural (n=69)	***1.85 (1.01-3.37)***	***0.046***
Consumption of food from PVC containers within 24h before sampling	No (n=70)	1.00	
	Yes (n=68)	1.42 (0.84-2.39)	0.186
Frequency of canned food/drink consumption	Less than once/week (n=49)	1.00	
	Once/week or more (n=89)	1.48 (0.85-2.57)	0.160
Presence of white dental fillings	No (n=28)	1.00	
	Yes (n=110)	1.40 (0.68-2.86)	0.358
Contraceptive pills	No (n=121)	1.00	
	Yes (n=17)	2.07 (0.91-4.70)	0.081
Smoking	No (n=115)	1.00	
	Yes (n=23)	0.55 (0.27-1.11)	0.097
Use of disinfectant agents	Occasionally (n=50)	1.00	
	At least once/week (n=88)	1.47 (0.82-2.63)	0.198
Children *(model R^2=0.13, p<0.001, n=136)*			
Gender	Boys (n=66)	1.00	
	Girls (n=70)	0.84 (0.53-1.34)	0.464
Age	***6-8 years (n=53)***	***1.00***	
	9-11 years (n=83)	***0.59 (0.37-0.94)***	***0.027***
Area	***Urban (n=68)***	***1.00***	
	Rural (n=68)	***0.56 (0.35-0.89)***	***0.015***
Consumption of food from plastic containers within 24h before sampling	***No (n=77)***	***1.00***	
	Yes (n=59)	***1.67 (1.04-2.67)***	***0.034***
Consumption of canned food/drink within 24h before sampling	***No (n=120)***	***1.00***	
	Yes (n=16)	***2.21 (1.08-4.53)***	***0.030***
Consumption of food re-heated in plastic containers within 24h before sampling	No (n=134)	1.00	
	Yes (n=2)	3.16 (0.45-22.1)	0.245
Presence of white dental fillings	***No (n=77)***	***1.00***	
	Yes (n=59)	***0.56 (0.34-0.91)***	***0.019***
Fathers *(model R^2=0.22, p=0.004, n=64)*			
Age	≤35 (n=6)	1.00	
	>35 and ≤ 40 (n=21)	1.50 (0.52-4.34)	0.444
	>40 (n=37)	0.80 (0.28-2.28)	0.675
Area	Urban (n=34)	1.00	
	Rural (n=30)	0.54 (0.28-1.02)	0.056
Consumption of food packed in a plastic bag within 24 h before sampling	No (n=50)	1.00	
	Yes (n=14)	1.85 (0.91-3.76))	0.089
Consumption of canned food/drink within 24 h before sampling	***No (n=46)***	***1.00***	
	Yes (n=18)	***2.54 (1.33-4.85)***	***0.006***
Presence of white dental fillings	*No (n=23)*	1.00	
	Yes (n=41)	1.01 (0.53-1.93)	0.977
Use of disinfectant agents	Occasionally (n=15)	1.00	
	At least once/week (n=49)	1.49 (0.74-3.00)	0.255

3.4 Correlation between family members

Levels of BPA correlated positively between mothers and children (R^2=0.06, p=0.005) (Fig 2), while no significant correlation was observed between mothers and fathers (R^2=0.004, p=0.627) and between children and fathers (R^2=0.0001, p=0.951).

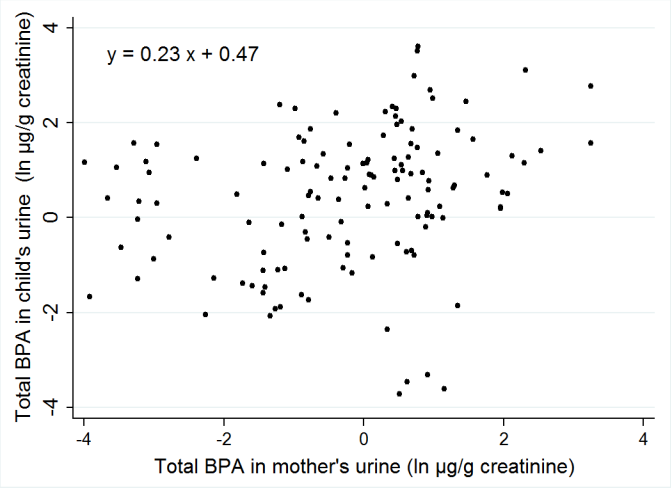

Fig 2. Correlation between total BPA levels in urine of mothers and children (r=0.25, p=0.005).

4 DISCUSSION

The present paper presents the first data on BPA exposure in the Slovenian general population according to age, gender and residence, and identifies potential sources of BPA exposure among the selected study groups. Levels were consistent with current low BPA exposure within Europe, i.e. 1.67 µg/g creatinine in 6-11 year olds in Sweden (Larsson et al., 2014), 3.55 µg/L in 3-5 year olds and ~2.5 µg/L in 6-14 year olds in Germany (Becker et al., 2009), 6.0 µg/g creatinine in 6-11 year olds in Denmark (Frederiksen et al., 2013). In adults from Belgium, a GM of 2.54 µg/g creatinine (<LOD-29.4 µg/g creatinine) was observed, with neither gender- nor age-related differences (Pirard et al., 2012). Outside of Europe, similar exposure levels were reported in the U.S. population, GMs being 2.36 µg/g creatinine in 6-11 years old children, 1.70 µg/g creatinine in 12-19 years old adolescents, and 1.90 µg/g creatinine in adults (CDC, 2015); and for the Canadian population, GMs were 2.3 µg/g creatinine in 3-5 year olds, 1.5 and 1.0 µg/g creatinine in 6-11 and 12-19 year olds, respectively, and 1.0 µg/g creatinine in 20-39 years old adults (Health Canada, 2015). Recently, BPA exposure was established for young Chinese adults (18-22 years), the GM for total urinary BPA was 0.79 µg/g creatinine (range 0.07-30.1 µg/g creatinine), with no significant difference between males and females, as well as between urban and rural populations (Gao et al., 2016). For the Korean population, the mean exposure was 1.87 µg/g creatinine (95% CI: 1.74-2.01) in males and 2.16 µg/g creatinine (95% CI: 2.04-2.28) in females, with no statistical difference (Park et al., 2016).

In comparison to the countries included in the DEMOCOPHES (2013) study, levels in the children and the mothers from Slovenia were similar to those from Belgium (children 2.4 and mothers 2.6 µg/L), Denmark (1.9 and 2.0 µg/L), Luxembourg (1.8 and 1.6 µg/L), Spain (1.8 and 2.0 µg/L) and Sweden (1.5 and 1.3 µg/L) (Covaci et al., 2015). In general, the DEMOCOPHES study identified low inter-individual variability, narrow distribution within each country and across all countries, and similar levels in children and mothers. However, significant differences were observed between the countries, children in Slovenia having significantly higher urinary BPA levels than children from other countries and mothers from Slovenia the lowest. Moreover, in Slovenia, the largest difference between BPA levels in children and mothers was observed in comparison to other countries (Covaci et al., 2015).

In addition to mother-child pairs, BPA exposure in the Slovenian study was assessed also in the fathers. BPA levels differed significantly among the study groups, being the highest in children and the lowest in fathers. In all three population groups, the levels of BPA in urine depended on the area of residence: levels in mothers were higher in the rural area, while in fathers and children levels were higher in the urban area. The differences remained even after adjusting for potential confounders and co-variates. Age-related difference was the most prominent in children, i.e. younger children (6-8 years) had markedly higher BPA levels in urine than older age groups (9-11 years). Age-dependent BPA variability has been clearly demonstrated in the large-scale (n>2000) cross-sectional national surveys: the German GerES IV (Becker et al., 2009), U.S. NHANES (CDC, 2015), Canadian CHMS (Health Canada, 2015) and the Korean (Park et al., 2016) surveys, but not in a small scale (n=131) Belgian population with an age span from 0 to 60 years (n=131) (Pirard et al., 2012) . However, there is consistent evidence from multiple national HBM programs to show higher body burdens of BPA in children (Choi et al., 2017). Covaci et al. (2015) reviewed worldwide data by age groups and observed that urinary levels of BPA tended to be higher in the youngest age category. Similarly in the DEMOCOPHES study, weighted GMs tended to be higher in children than in mothers across the EU. Both, the EU-wide (Covaci et al., 2015) and the present study (Table 4) confirmed that there are no significant differences between boys and girls. Similarly, no notable gender differences were reported for children and adults of the U.S., Canadian, Chinese and Korean HBM surveys.

In mothers, none of the potential dietary and non-dietary sources included in the questionnaire showed any firm association with urinary BPA levels, after adjustment for age, place of residence and BMI. Taking contraceptive pills does seem to have a role in BPA exposure (Table 4). Together with BPA's oestrogenic activity, changes in sex hormones, particularly androgens, have been linked with BPA levels in human tissues (Galloway et al., 2010; Takeuchi et al., 2004). Knowing that androgens can downregulate the activity of

uridine diphosphate-glucuronosyl transferase (UGT), the enzyme that catalyses the metabolism of BPA in the intestine and liver, yielding the major urinary metabolite BPA-glucuronide (Teeguarden et al., 2005), and that contraceptive pills contain small amounts of oestrogens, reverse causation may be hypothesised. The glucuronidation of BPA might be suppressed under a hyperandrogenic environment (Galloway et al., 2010; Takeuchi et al., 2004). A higher rate of glucuronidation might be expected with oestrogen intake through contraception resulting in a higher amount of total BPA detected in urine. Furthermore, there was a marginal significance observed with levels of urinary BPA and a mother's BMI (Table 4). This was found to be the case in many studies, including large-scale surveys (Andra and Makris, 2015; Carwile and Michels, 2011; Galloway et al., 2010; Rochester, 2013; Song et al., 2014; Takeuchi et al., 2004), but the role of BPA in obesity or in weight gain is questionable. It is possible that the increased levels of BPA in these individuals is due to increased body fat rather than BPA inducing increased BMI (Rochester, 2013). Indeed, an *in vitro* study of human adipose tissue found no association between BMI and levels of BPA in adipose tissue (Fernandez et al., 2007). The statistical model (Table 4) revealed marginally significant lower urinary BPA levels in smoking mothers, which was not the case in the bivariate analysis. This finding is consistent with the NHANES data (Lakind and Naiman, 2011), where an inverse association with cigarette smoking was revealed in the adult population (n=2548). The levels were comparable to this study. In the Chinese population (n=952) with similar exposure to the U.S. and Slovenian populations, the authors examined personal factors in relation to non-occupational BPA exposure and found a positive association between urinary BPA and smoking (He et al., 2009). The rationale for the link between internal BPA exposure and smoking is unclear, and could be linked with some other trait associated with smoking habit (Lakind and Naiman, 2011) .

Unlike in mothers, BPA levels adjusted for age, gender and place of residence in children were significantly associated with recent consumption of canned food or drink and food stored in plastic containers (Table 4), demonstrating a higher influence of dietary intake in children as opposed to mothers. The inverse association between BPA in a child's urine and the presence of white dental fillings, confirmed by the statistical model, is confusing, since BPA can be present as an impurity in the polymers used in fillings, which can then leach out as a result of hydrolysis through esterases present in saliva (Geens et al., 2012). Elevated BPA associated with presence of dental fillings has been shown by different *in vitro*, *in vivo* (Geens et al., 2012) and population-based studies in children (Chung et al., 2012). On the basis of *in vivo* studies, the highest exposures were measured immediately after sealant placement (Fleisch et al., 2010), and there is evidence that the released BPA depends on the producer of the dental resins/sealants (Fleisch et al., 2010; Joskow et al., 2006). Our observation is not in line with the literature data, however, it could be explained by co-

linearity with some other variable not included in the study questionnaire and is yet to be examined.

BPA levels in fathers, like in children, when adjusted for age and place of residence showed a noticeable association with dietary BPA - consumption of canned food or drinks, and a marginally significant association with frozen convenience food stored in plastic bags (Table 4). Migration of BPA from the resin coatings can occur during the sterilization processes and from polycarbonate plastics (particularly baby bottles) after manufacturing and hydrolysis/aminolysis of the polymer. In addition, migration increases at higher temperatures and with longer testing periods (reviewed by Geens et al., 2012). Unlike cans and baby bottles, other food packaging material has not been tested to the same extent, however there is evidence that BPA migrates into food/drinks also from glass, plastic, paper and laminated paperboard/polyethylene carton (Geens et al., 2010). The study by Geens et al. (2010) suggested that sources other than canned foods and beverages contribute to BPA exposure in humans, which is in agreement with BPA exposure observed in the study population, particularly among children (Table 4). In line with this, Von Goetz et al. (2010) clearly showed that exposure in the youngest age groups (<6 years) was predominantly from PC feeding bottles, while in older consumer groups it was from canned food.

In this study, a positive association between mothers and their children (Fig 2) demonstrated significance of shared behaviour in a household. The same was observed in the overall DEMOCOPES cohort (Covaci et al., 2015) and also by Pirard et al. (2012) who compared samples of people living in the same home collected on the same day. This is an indication of dietary intake as a primary route of BPA exposure, as opposed to intake from personal care products. Lack of association between fathers and mothers/children might be due to different dietary patterns and/or different physiology, however, the lower number of participating fathers in comparison to mother-child pairs did not allow a direct comparison of the observed within-family associations.

Strengths and limitations of the study

The main strength of the present study is in the evaluation of exposure and sources of exposure among three study groups covering gender and age. The basic study population established within DEMOCOPHES was enlarged by including fathers/male partners. This allowed a comparison of the levels of BPA, not only among different study groups, but also to construct multiple regression models identifying potential sources of exposure separately for each group.

An important amendment to the DEMOCOPHES protocol was the collection of detailed data on the recent (24 h before sampling) consumption of food stored in different types of food

contact material; data on contraceptive/hormonal pills, which may influence BPA metabolism within the body (Takeuchi et al., 2004; Teeguarden et al., 2005), and the use of different consumer products. The intake of contraceptive pills is usually not accounted for in HBM studies.

One important limitation of this study is the collection of spot urine samples. However, the study conducted by Heffernan et al. (2014) suggests that single spot samples obtained from young children can provide a reliable characterization of absolute and relative exposure over a short study period. Another limitation is the limited sample size, which is not wholly representative of the Slovenian population; nevertheless, it covers a broad range of demographic characteristics, age classes, body-mass index, residency, parental educational status and nutrition habits.

5 CONCLUSIONS

Urinary BPA levels were recorded for the first time in children, their mothers and fathers living in Slovenia, and revealed that exposure in the general population is within the worldwide exposure. Statistical differences among the three study groups were observed, with the highest levels found in children and the lowest levels in fathers. An age-dependant trend that is consistent with the literature was observed in children, but not adults. Evaluation of the results with regard to potential sources of exposure revealed physiological and behavioural differences between adults and children and between males and females, but not between boys and girls. In mothers, urinary levels of BPA were determined by hormonal interaction more than dietary sources, while a positive association between urinary BPA and diet was apparent in children (canned food/drink and food from plastic material) and fathers (canned food/drink). Determining potential sources of exposure will help to prioritize future research, which should ideally involve measuring BPA in plasma, genotyping of UGT and collecting relevant parameters outlined in the present study.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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