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1 **Determinants of exposure levels of bisphenols in Flemish adolescents**

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18 **Abstract**

19 The broadly used industrial chemical bisphenol A (BPA), applied in numerous consumer products, has
20 been under scrutiny in the past 20 years due to its widespread detection in humans and the
21 environment and potential detrimental effects on human health. Following implemented restrictions
22 and phase-out initiatives, BPA is replaced by alternative bisphenols, which have not received the same
23 amount of research attention. As a part of the fourth cycle of the Flemish Environment and Health
24 Study (FLEHS IV, 2016-2020), we monitored the internal exposure to six bisphenols in urine samples
25 of 423 adolescents (14-15 years old) from Flanders, Belgium. All measured bisphenols were detected
26 in the study population, with BPA and its alternatives bisphenol F (BPF) and bisphenol S (BPS) showing
27 detection frequencies > 50%. The reference values show that exposure to these compounds is
28 extensive. However, the urinary BPA level decreased significantly in Flemish adolescents compared to
29 a previous cycle of the FLEHS (2008-2009). This suggests that the replacement of BPA with its
30 analogues is ongoing. Concentrations of bisphenols measured in the Flemish adolescents were
31 generally in the same order of magnitude compared to recent studies worldwide. Multiple regression
32 models were used to identify determinants of exposure based on information on demographic and
33 lifestyle characteristics of participants, acquired through questionnaires. Some significant
34 determinants could be identified: sex, season, smoking behavior, educational level of the parents,
35 recent consumption of certain foods and use of certain products were found to be significantly
36 associated with levels of bisphenols. Preliminary risk assessment showed that none of the estimated
37 daily intakes (EDIs) of BPA exceeded the tolerable daily intake, even in a high exposure scenario. For
38 alternative bisphenols, no health-based guidance values are available, but in line with the measured
39 urinary levels, their EDIs were lower than that of BPA. This study is, to the best of our knowledge, the
40 first to determine internal exposure levels of other bisphenols than BPA in a European adolescent
41 population.

42

43 **Keywords:** bisphenols, biomonitoring, adolescents, determinants of exposure, estimated daily intake

44

45 **Highlights**

- 46 • BPA and 5 alternatives were measured in a representative Flemish population
- 47 • BPA, BPF and BPS were detected in almost every participant (>80%)
- 48 • Urinary BPA levels decreased significantly from 2008 to 2018
- 49 • Socio-economic status, product use and food were associated with bisphenol levels
- 50 • No participants exceeded the available health-based guidance values for BPA

51 **1. Introduction**

52 Bisphenol A (BPA) is a high production volume industrial chemical, applied in various consumer
53 products, e.g. polycarbonate plastic, epoxy resins used to coat food and beverage cans, thermal paper
54 receipts (Geens et al., 2011; Liao and Kannan, 2011; Geens et al., 2012a; Geens et al., 2012b; Vervliet
55 et al., 2019), dental restoration materials (Vervliet et al., 2018), clothing (Xue et al., 2017; Li and
56 Kannan, 2018) and electronics (Geens et al., 2011). Despite being polymerized in most applications,
57 some amount of free BPA monomer could be present or formed due to degradation. The present free
58 BPA could then leach from these products and humans could thus be exposed, mainly through the
59 dietary intake (Geens et al., 2012a; European Food Safety Authority, 2015). Because of increasing
60 evidence that BPA is harmful to humans due to its endocrine disrupting properties (reproductive,
61 developmental, metabolic toxicity), this extensively used chemical has been phased out of certain
62 applications (e.g. baby bottles, thermal paper) in the past decade worldwide, including in Belgium
63 (Japanese National Institute of Technology and Evaluation, 2003; European Union, 2011; Moniteur
64 Belge, 2012; Kawamura et al., 2014; European Union, 2016). As a consequence, BPA is gradually being
65 replaced by bisphenol analogues, such as bisphenol F (BPF) and bisphenol S (BPS) (Liao et al., 2012c;
66 Bjornsdotter et al., 2017; Vervliet et al., 2019). However, recently some evidence has shown that these
67 BPA-alternatives could have an endocrine disrupting potential similar to that of BPA (Rochester and
68 Bolden, 2015; Gramec Skledar and Peterlin Masic, 2016). Several recent studies have found
69 measurable urinary levels of various alternative bisphenols in different study populations (Liao et al.,
70 2012a; Hoffman et al., 2018; Lehmler et al., 2018; Sakhi et al., 2018). However, data on European
71 human exposure levels to these chemicals are limited, particularly in young people, who might be
72 exposed in different ways and to a different extent than adults (Lehmler et al., 2018; Rocha et al.,
73 2018; Frederiksen et al., 2020). It is suspected that endocrine disrupting chemicals could be more
74 harmful during developmental phases such as puberty, so it is necessary to study exposure in such
75 populations (Vandenberg et al., 2009; Vandenberg et al., 2010; Frye et al., 2012). Urine is the preferred
76 matrix for measuring total internal BPA exposure, as BPA has a short half-life (<7 h) and is excreted
77 quickly. Because it is excreted in the urine in its conjugated form, mainly as its non-toxic glucuronide
78 metabolite, measurements typically include a deconjugation step (Völkel et al., 2002; Teeguarden et
79 al., 2011; Christensen et al., 2012; Thayer et al., 2015). Toxicokinetics of BPA analogues are not yet
80 well characterized, but the available studies on this topic suggest that the total urinary levels are also
81 considered robust biomarkers for internal exposure (Koch et al., 2012; Song et al., 2017; Lehmler et
82 al., 2018; Oh et al., 2018).

83 Since 2002, the Flemish government has established a human biomonitoring network as part of a
84 program on environmental health surveillance. The Flemish human biomonitoring program aims to

85 investigate the complex relationship between environmental contamination and human health by
86 monitoring selected biomarkers of exposure and certain health effects (Schoeters et al., 2012). In
87 previous cycles of the Flemish Environment and Health Study (FLEHS), adolescents were already
88 included. However, only during the second survey (FLEHS II) in 2008-2009, BPA concentrations were
89 monitored (Geens et al., 2014). In the current 4th cycle of FLEHS (2016-2020), a new biomonitoring
90 survey was set up, to repeat measurements and report updated reference values for some chemicals
91 and to report Flemish reference values for other, emerging chemicals for the first time (Steunpunt
92 Milieu en Gezondheid, 2020).

93 The objectives of this study were: 1) to report Flemish reference values of frequently detected
94 emerging bisphenols, 2) to compare the obtained results with international literature and with
95 previously reported levels within FLEHS 3) to evaluate demographic and dietary characteristics as
96 potential determinants of exposure, 4) to compare the observed bisphenol levels with available
97 health-based guidance values from literature for a preliminary risk assessment. This study is, to the
98 best of our knowledge, the first to determine internal exposure levels of other bisphenols than BPA in
99 Flanders and in a European adolescent population.

100

101 **2. Materials and methods**

102 **2.1. Study population**

103 The samples in this study were collected from a group of 423 adolescents who took part in FLEHS IV.
104 The program generates representative reference values for a selected set of chemicals, identifies
105 determinants of exposure, and examines the relation between the exposure measurements and
106 potential effects on human health. Adolescents (14 – 15 years old) were recruited through 20 schools
107 from all five Flemish provinces. The number of schools per province was proportional to the
108 population size of the province and schools in the same province had to be separated at least 20 km
109 from each other. Inclusion criteria were as follows: participants and their parents had to provide
110 written informed consent, participants had to reside in Flanders for at least 5 years and they had to
111 be able to fill in an extensive questionnaire in Dutch.

112 All participants provided a spot urine sample on a school day between September 2017 and June 2018
113 and their body weight (bw) and body height (bh) were measured by trained nurses with calibrated
114 equipment. The urine samples were collected in clean polyethylene (PE) containers and immediately
115 processed during the fieldwork. Samples were divided into aliquots in glass vials and kept at -20 °C
116 until analysis. The adolescents and their parents completed an extensive, self-administered
117 questionnaire at home on health status, food consumption, use of cosmetics, tobacco and alcohol,
118 housing conditions and socio-economic status (e.g. educational level of the parents, household

119 income). Participants filled in an additional short questionnaire including questions on recent
120 exposure (i.e. within the last three days) to smoke, medication and food and on urine collection (e.g.
121 time since last void). The study protocol was approved by the ethical committee of the Antwerp
122 University Hospital (Belgian Registry Number: B300201732753). Collection, storage, transfer, and use
123 of data were carried out in accordance to the European General Data Protection Regulation (GDPR).
124 All data were pseudonymized.

125 In a previous round of the Flemish human biomonitoring program, assessment of BPA exposure was
126 included. In the second cycle of the FLEHS (2008-2009), BPA concentrations were measured in urine
127 of 196 adolescents (Geens et al., 2014). Similar to FLEHS IV, participants were recruited from all 5
128 Flemish provinces in order to examine a representative sample of the population. All adolescents were
129 14-15 years old at the time of sampling. As measurements were carried out at only two points in time,
130 a real temporal trend could not be modelled, but a comparison between the two was made.

131

132 **2.2. Measurement of bisphenols in urine**

133 Bisphenols in urine were measured between July and September 2019. An overview of the used
134 reagents and standards is available in the Supplementary Information (SI)-1. The sample preparation
135 and GC-MS/MS analysis were performed according to the previously validated procedure described
136 elsewhere (Gys et al., 2020a). Briefly, for sample preparation, 1 mL of urine was spiked with isotope-
137 labelled reference standards (4 ng of $^{13}\text{C}_{12}$ -BPA, 2 ng of $^{13}\text{C}_{12}$ -BPF, $^{13}\text{C}_{12}$ -BPS, and $^{13}\text{C}_{12}$ -BPB). Next, 750
138 μL of sodium acetate buffer (1 M, pH 5) and 10 μL of β -glucuronidase/arylsulfatase enzyme solution
139 (30/60 U/mL, respectively) were added. Samples were incubated for 1 h at 37 °C and subsequently
140 sonicated for 15 min. Then, they were extracted using Oasis WAX cartridges (3 mL, 60 mg, Waters,
141 Milford, MA, USA) that were previously washed with 10 mL of methanol and conditioned with 2 mL of
142 water. After loading the samples, the cartridges were washed with 2 mL of water with 5% methanol
143 and dried for 20 min on the vacuum manifold. Elution of bisphenols was carried out using 2 mL of
144 methanol, which was then evaporated to dryness under a gentle stream of nitrogen gas. Analytes
145 were reconstituted in 100 μL of derivatization reagent (10% BSTFA in toluene) and samples were kept
146 at 60 °C during 1 h to complete the trimethylsilyl-derivatization of the target compounds. Final extracts
147 were transferred to glass vials with inserts for GC-MS/MS analysis.

148 Instrumental analysis was performed on an Agilent 7890B gas chromatograph coupled to an Agilent
149 7000D triple quadrupole mass spectrometer (Santa Clara, CA, USA). Chromatographic separation of
150 the derivatized analytes was achieved using an Agilent DB-5MS capillary column (30 m x 250 μm , 0.25
151 μm ; Santa Clara, CA, USA). Target compounds and internal standards were measured using multiple
152 reaction monitoring (Gys et al., 2020a). Limits of quantification (LOQs) were 0.02 ng/mL for BPAF, BPF

153 and BPB, 0.03 ng/mL for BPZ, 0.04 ng/mL for BPS and 0.3 ng/mL for BPA. An overview of the target
154 compounds, their internal standards, linear ranges and LOQs are provided in Table SI-1.

155 Specific gravity (SG) of urine samples was determined by refractometry at Algemeen Medisch
156 Laboratorium (AML, Antwerp, Belgium).

157 FLEHS II and FLEHS IV analyses, both carried out at the Toxicological Centre of the University of
158 Antwerp, employed different analytical methods (Geens et al., 2009; Geens et al., 2014; Gys et al.,
159 2020a). To allow comparison between the two cycles, three blinded duplicate samples from FLEHS II
160 were measured again during analyses for FLEHS IV. As such, their comparability was evaluated. Linear
161 regression was carried out using the results of the two measurements of these three samples. The
162 regression coefficient R^2 was 0.909 and the slope of the curve was 1.119, indicating good accordance
163 between the two measurements.

164

165 **2.3. Quality control and quality assurance**

166 Urine samples were prepared and analyzed in batches consisting of twenty urine samples, two
167 procedural blanks and two quality control (QC) samples. These QC samples were either obtained by
168 participation in international inter-laboratory comparison exercises (see below) or by analysis of a
169 spiked and matching non-spiked pooled urine sample, so that the detected concentration in the non-
170 spiked sample could be subtracted. As BPA is a ubiquitous substance, it is inherently present in the lab
171 environment. Therefore, two procedural blanks (ultrapure water) were included in every batch of 20
172 samples and these blank values were subtracted from concentrations found in samples. All glassware
173 used in the procedure was heated to 400 °C for 2 h and all pipette tips were rinsed twice with methanol
174 beforehand. SPE cartridges were pre-washed with 10 mL of methanol before conditioning and loading
175 samples (Caballero-Casero et al., 2016). Field blanks (from polypropylene containers, used for storing
176 urine samples) were analyzed and did not contain detectable levels of bisphenols. Results of the QC
177 samples and procedural blanks are presented in Table SI-2.

178 External quality control was assured through successful participation in inter-laboratory comparison
179 exercises. This method was thoroughly evaluated in 1) the Human Biomonitoring for Europe External
180 Quality Assurance Scheme (HBM4EU ICI/EQUAS) for BPA, BPF and BPS (four rounds in 2018, 2019 and
181 2020) and 2) the External Quality Assessment Scheme for Organic Substances in urine (OSEQAS) of the
182 *Centre du toxicologie du Québec* for BPA, BPF, BPS and BPZ (four rounds in 2018, 2019 and 2020), and
183 performance was satisfactory. The resulting Z-scores are shown in Table SI-3.

184

185 **2.4. Statistical data analysis**

186 Reference values for analytes with a detection frequency of at least 60% were calculated as geometric
187 means (GM) with 95% confidence intervals. Concentrations below the LOQ were imputed with a
188 random value (between 0 and the LOQ), drawn from the estimation of the lognormal distribution of
189 all values by fitting a truncated lognormal distribution using only values above the LOQ. For analytes
190 detected in less than 60% of the samples, no imputations were applied, and no reference values were
191 calculated. Statistical outliers of urinary bisphenol concentrations were retained as valid data points.
192 For further statistical analysis, concentrations were corrected for urinary dilution with individual
193 specific gravity (SG) values using the following formula (Duty et al., 2005; Pearson et al., 2009; Meeker
194 et al., 2012): $\text{conc}_{\text{SG}} = [\text{conc} * (1.024-1)/(SG-1)]$, where conc_{SG} is the normalized bisphenol
195 concentration, conc is the uncorrected bisphenol concentration, 1.024 is a standardized SG value and
196 SG is the specific gravity level of the individual sample. Corrected concentrations were then
197 transformed by the natural logarithm due to the skewness of the exposure data.

198 For comparison of BPA concentrations between FLEHS II and FLEHS IV, a multiple linear regression
199 model was fitted to test the significance of cohort, corrected for gender, age, smoking and SG.
200 Imputation of values below LOQ was carried out for the FLEHS II values in the same way as for the
201 current FLEHS IV.

202 Associations between questionnaire data (i.e. personal, dietary, socio-economic characteristics) and
203 bisphenol levels were first examined by performing univariate analysis (ANOVA) on the SG-corrected,
204 natural logarithm-transformed concentrations. Variables showing a p-value < 0.2 were subsequently
205 included in a stepwise multiple linear regression model. The SG value was additionally included in the
206 model as a separate, independent variable, to make sure the statistical significance of the relation
207 with other variables in the model was independent of the SG (Barr et al., 2005). During the backward
208 step-by-step building of the multiple regression models the cut-off for the p-value was set at < 0.05
209 and non-significant variables were consecutively excluded until a set of significant variables was
210 retained. Collinearity among independent variables was evaluated beforehand by calculation of the
211 variance inflation factor (VIF), for which the cut-off was set at 0.8. The R-square of the model reflects
212 the percentage of variation in bisphenol levels that could be explained by the remaining independent
213 variables in the final model. Spearman ρ rank correlation was applied to evaluate correlations
214 between analytes. Statistical analyses were carried out using SPSS Statistics software (version 26.0,
215 IBM Corp, Armonk, USA).

216

217 **3. Results and discussion**

218 **3.1. Study population characteristics**

219 The distribution of characteristics of the adolescents who provided a urine sample (n = 423), such as
 220 body mass index (BMI, calculated as bw/bh^2 (kg/m²)), gender, smoking habits and educational level of
 221 the parents and the participant is available in Table 1. In this study, slightly more females (53.4%)
 222 participated compared to males (46.6%), but equal distribution between the sexes is approached. The
 223 adolescents had a mean age of 14.8 ± 0.5 years. Mean BMI of the study population was 21.0 ± 3.7
 224 kg/m² and 72.5% of participants had a normal weight (BMI between 18.5 and 25 kg/m²). The
 225 proportion of adolescents being overweight or obese (BMI > 25 kg/m²) has increased, compared to
 226 previous FLEHS cycles (Geens et al., 2014; Steunpunt Milieu en Gezondheid, 2020). The distribution
 227 over school types of the participants accorded well with that of Flanders in general. The educational
 228 level of the parents was high in comparison with the general Flemish population, which was a typical
 229 finding in previous FLEHS surveys as well, due to better response rates in this group (Morrens et al.,
 230 2012; Geens et al., 2014; Steunpunt Milieu en Gezondheid, 2020). Only 4.3% were active smokers,
 231 which is a decrease compared to previous cycles of FLEHS and in line with the general Flemish numbers
 232 (Rosiers, 2019). Because recruitment was carried out in collaboration with the schools, no samples
 233 were collected during summer (Steunpunt Milieu en Gezondheid, 2020).

234
 235

Table 1 Characteristics of the study population (n = 423).

		N	%
Gender	Male	197	46.6
	Female	226	53.4
Age (years)	Mean, SD	14.8	0.5
BMI class (kg/m ²)	Underweight (≤ 18)	35	8.3
	Normal weight (18 – 25)	307	72.5
	Overweight (> 25)	85	20.1
School type of adolescent	General school	215	50.8
	Technical school	130	30.7
	Vocational school	78	18.4
Educational level parents ^a	Primary	25	5.9
	Secondary	137	32.4
	Tertiary	253	59.8
	Missing	8	1.9
Smoking habits	No active or passive smoking in house	363	85.8
	Non-smoker, passive smoking in house	39	9.2
	Smoker	18	4.3
	Missing	3	0.7
Season of sampling	Winter	136	32.1
	Spring	189	44.7
	Summer	0	0
	Autumn	98	23.2

236 N: number of participants in subgroup; BMI: body mass index. ^aBased on the International Standard Classification
 237 of Education (ISCED); SD: standard deviation

238

239 **3.2. Concentrations of bisphenols in urine**

240 The distribution of bisphenols in the urine of Flemish adolescents is shown in Table 2. All six bisphenols
 241 were detected in the study population, indicating that exposure to this group of rapidly excreted
 242 chemicals is extensive and very common. The most frequently detected compound is BPF (97%),
 243 followed by BPA (86%) and BPS (83%). BPB, BPZ and BPAF were detected in respectively 57%, 37% and
 244 12% of participants. Although BPF was most frequently found, BPA showed the highest concentrations
 245 (median 1.05 ng/mL); while medians for BPF (0.14 ng/mL) and BPS (0.12 ng/mL) were substantially
 246 lower. However, the highest maximal concentrations were 41.5 and 40.0 ng/mL, detected for BPS and
 247 BPF respectively. These values were on the edge or just outside the analytical linear range but were
 248 included as they were very close to the upper limit of the calibration (40.0 ng/mL) and these samples
 249 were reanalyzed for confirmation. After evaluation of potential determinants of exposure (see 3.4),
 250 we found no clear explanation for these high values in the characteristics of the participants. For the
 251 other bisphenols that show lower detection frequencies, the maximum measured concentrations
 252 were low as well. Statistically significant correlations were found between the measured BPA, BPF and
 253 BPS concentrations (Spearman rank, $p < 0.01$). Correlation coefficients ranged from 0.296 to 0.380,
 254 expressing weak, but positive associations. This result indicates the occurrence of co-exposure to
 255 these three chemicals, potentially through common sources or through certain lifestyle habits. The
 256 uncorrected urinary concentrations (in ng/mL) of BPA, BPF and BPS were strongly associated with the
 257 specific gravity of the urine, meaning that the concentration of the contaminant decreased
 258 significantly with increasing dilution of the urine. This was also illustrated by correction of the
 259 aforementioned high maximum concentrations for BPF and BPS for urine dilution using SG (Table 2).

260

261 **Table 2** Reference values of bisphenols urine of Flemish adolescents (n = 423).

Analyte	LOQ	% > LOQ	10 th	25 th	Median	75 th	Maximum	GM	95% CI
ng/mL, uncorrected									
BPAF	0.02	12	<LOQ	<LOQ	<LOQ	<LOQ	0.13	N/A	
BPF	0.02	97	0.04	0.07	0.14	0.29	40.0	0.14	(0.13, 0.16)
BPA	0.30	86	<LOQ	0.55	1.05	1.79	18.1	0.92	(0.82, 1.02)
BPB	0.02	57	<LOQ	<LOQ	0.03	0.05	0.31	N/A	
BPZ	0.03	37	<LOQ	<LOQ	<LOQ	0.04	2.42	N/A	
BPS	0.04	83	<LOQ	0.06	0.12	0.22	41.5	0.11	(0.10, 0.12)
ng/mL, corrected for SG									
BPF			0.04	0.08	0.15	0.30	33.13	0.17	(0.15, 0.19)
BPA			<LOQ	0.69	1.15	1.91	19.41	1.07	(0.98, 1.18)
BPS			<LOQ	0.07	0.14	0.23	35.58	0.13	(0.11, 0.14)

262 LOQ: limit of quantification; GM: geometric mean; CI: confidence interval; N/A: not available.

263 GM was only calculated for compounds showing 60% > LOQ.

264

265 **3.3. Comparison with literature**

266 Urinary bisphenols were measured in several recent international studies. In Table 3, a summary is
267 provided of urinary bisphenol levels reported in other study populations, preferably of similar or
268 overlapping age. Comparisons between the different studies were mainly based on medians for
269 uncorrected concentrations and detection frequencies, keeping in mind that method LOQs and
270 approaches of correcting for urine dilution may differ between studies.

271 In general, the measured concentration of BPA in Flemish adolescents did not differ substantially from
272 levels reported in children and/or adolescents from U.S.A., Canada and Brazil. Although BPA was not
273 the most frequently detected compound in our study population, it was still the predominant
274 bisphenol in terms of measured concentrations. In all studies, BPA was detected with a high frequency,
275 illustrating that it is still used extensively worldwide (Chen et al., 2018; Lehmler et al., 2018; Rocha et
276 al., 2018; Health Canada, 2019). The detection frequencies and levels of BPF and BPS, however, were
277 more variable and depending on the country, the sampling period and the LOQ of the applied
278 analytical method. Median levels of both compounds were considerably higher in U.S.A. (Lehmler et
279 al., 2018). On the other hand, BPF and BPS were only detected in respectively 9 and 23% of urine
280 samples from Brazil and median concentrations were below the method LOQ, which could be
281 (partially) explained by the higher LOQ for BPF (Rocha et al., 2018). Lower levels for BPA and BPS were
282 measured in Chinese children, but the median concentration of BPF was higher than the median for
283 our study population (Chen et al., 2018). A recent study on young Danish men reported slightly higher
284 median concentrations for BPA, BPF and BPS. In their study population, BPA was the most frequently
285 detected bisphenol (92%) (Frederiksen et al., 2020). In comparison to measured bisphenol levels in 7-
286 year-old Japanese school children (Gys et al., 2020a), concentrations in Flemish adolescents are higher
287 but in the same range for BPA, BPF and BPS. Detection frequencies for BPA and BPS were similar, but
288 BPF was less frequently detected in the Japanese children (83% *versus* 97% in FLEHS), although
289 population size was practically equal (396 *versus* 423) and the employed analytical method was the
290 same (Gys et al., 2020a). In a study comprising data for children (5-12 years old) from six European
291 member states (Belgium, Denmark, Luxembourg, Slovenia, Spain and Sweden), the reported median
292 BPA concentration was 1.96 ng/mL (Covaci et al., 2015). This value is slightly higher than our median
293 urinary BPA level, but it is important to keep in mind that the European children's samples were
294 already collected in 2011 and 2012, from six different countries. Comparing data from large (national)
295 biomonitoring surveys should be done with caution, due to intercountry differences in methodology,
296 legislation and behavior (LaKind et al., 2019).

297 BPA is the only bisphenol that was previously included in the Flemish human biomonitoring program.
 298 In the second cycle of the FLEHS (2008-2009), BPA was already measured in urine of 196 adolescents
 299 (Geens et al., 2014). Both GM (95% CI) are corrected for gender, age, smoking and SG. A statistically
 300 significant decrease in the GM BPA concentration in urine was observed ($p < 0.001$) between FLEHS II
 301 (2.56 ng/mL) and the current FLEHS IV (1.07 ng/mL) (displayed in Fig. SI-1). In Belgium, BPA is banned
 302 in baby bottles and food contact materials intended for children < 3 years old (European Union, 2011;
 303 Moniteur Belge, 2012). Since January 2020, BPA can also not be used in thermal paper in a
 304 concentration $\geq 0.02\%$ (European Union, 2016). Because of the age of our study population and the
 305 fact that the samples analyzed in this study were collected during 2017 and 2018, it is unlikely that
 306 these regulations have influenced the measured concentrations directly. However, it is possible that
 307 manufacturers have pro-actively started phasing out BPA in certain consumer applications and are
 308 using alternative bisphenols instead, e.g. in thermal paper (Vervliet et al., 2019). A similar decreasing
 309 trend in urinary BPA was reported for Canadian and American adolescents (12-19 year-olds) and young
 310 Danish men over the past decade as well, while these countries have similar or less strict legislations
 311 in place (Centers for Disease Control and Prevention, 2019; Health Canada, 2019; Frederiksen et al.,
 312 2020). In our study on Japanese children, we also saw a significant decrease in urinary BPA
 313 concentrations between 2012 and 2017 (Gys et al., 2020a).

314

315 **Table 3** Concentrations of urinary bisphenols in Flemish adolescents compared to recent international
 316 literature.

Compound	Country	N (age)	Sampling period	Urinary levels (median, ng/mL)	Reference
BPA	BE	423 (14-15 y)	2017-2018	1.05	Present study
	BE	196 (14-15 y)	2008-2009	2.21	(Geens et al., 2014)
	EU ^a	653 (5-12 y)	2011-2012	1.96	(Covaci et al., 2015)
	US	462 (12-19 y)	2013-2014	1.20	(Lehmler et al., 2018)
	BR	300 (6-14 y)	2012-2013	1.66	(Rocha et al., 2018)
	CN	213 (8-11 y)	2015	0.25	(Chen et al., 2018)
	CA	524 (12-19)	2016-2017	0.96	(Health Canada, 2019)
	DE	100 (19-30)	2017	1.24	(Frederiksen et al., 2020)
	JP	396 (7 y)	2012-2017	0.89	(Gys et al., 2020a)
BPF	BE	423 (14-15 y)	2017-2018	0.14	Present study
	US	462 (12-19 y)	2013-2014	0.40	(Lehmler et al., 2018)
	BR	300 (6-14 y)	2012-2013	<LOQ	(Rocha et al., 2018)
	CN	213 (8-11 y)	2015	0.19	(Chen et al., 2018)
	DE	100 (19-30)	2017	0.30	(Frederiksen et al., 2020)
	JP	396 (7 y)	2012-2017	0.07	(Gys et al., 2020a)
BPS	BE	423 (14-15 y)	2017-2018	0.12	Present study
	US	462 (12-19 y)	2013-2014	0.40	(Lehmler et al., 2018)

BR	300 (6-14 y)	2012-2013	<LOQ	(Rocha et al., 2018)
CN	213 (8-11 y)	2015	0.03	(Chen et al., 2018)
DE	100 (19-30)	2017	0.17	(Frederiksen et al., 2020)
JP	396 (7 y)	2012-2017	0.11	(Gys et al., 2020a)

317 N: number of participants in study population; y: years of age. ^aData from Belgium, Denmark, Luxembourg,
318 Slovenia, Spain and Sweden.

319

320 **3.4. Analysis of exposure determinants**

321 We investigated whether demographic, dietary and other variables available from questionnaires
322 were associated with urinary concentrations of BPA, BPF and BPS. Results of the univariate regression
323 analyses of the potential determinants of exposure are shown in Table SI-4. Results of the multiple
324 regression models for BPA and BPF are summarized in Table 4. For BPS, none of the investigated
325 variables remained significant in the multiple model.

326 Urinary BPF and BPS levels did not differ between male and female participants. Concentrations of
327 BPA were significantly higher in female participants ($p = 0.010$). Contradictory results have been
328 reported in the literature on gender differences in urinary BPA. Most studies report no significant
329 association between gender and BPA levels in children and adolescents (Frederiksen et al., 2013;
330 Geens et al., 2014; Covaci et al., 2015; Hoffman et al., 2018). The relationship between gender and
331 BPA might also depend on the age of the participants, as one American study found slightly higher
332 levels in young girls compared to boys (6-19 years old) as well, but for adults, the relation was reversed
333 and significant (Lehmler et al., 2018). For the other bisphenols, fewer studies investigated the
334 influence of gender on urinary levels. The available data so far suggests no significant association exists
335 between gender and levels of BPA-alternatives (Liao et al., 2012a; Lehmler et al., 2018).

336 The age of the adolescents was not significantly associated with bisphenol concentrations. In FLEHS II,
337 a positive association was found with age, despite the very narrow range (Geens et al., 2014). In this
338 specific period in life, lifestyle habits, such as food consumption and use of cosmetics and personal
339 care products, may change substantially and quickly, which can add to the variability. Moreover,
340 adolescents' development and habits may have changed over the past ten years. BMI was significantly
341 associated with BPA ($p = 0.027$) and BPF ($p = 0.029$) concentrations in univariate analysis: participants
342 in the overweight/obese group presented higher urinary levels. However, BMI did not remain
343 significant in the multiple regression model (available in Table 4). Urinary BPS was also higher in the
344 overweight/obese group but was not significantly related to BMI class (univariate analyses). During
345 previous analyses of urinary BPA in Flemish adolescents, no association was found with BMI class
346 (Geens et al., 2014). It must be noted that the current FLEHS IV included a relatively higher share of
347 overweight adolescents compared to earlier cycles, which might have had an influence on this

348 outcome. A case-control study on Indian children found no significant difference in BPA levels between
349 the overweight/obese and non-obese group (Xue et al., 2015), but other studies found significantly
350 higher BPA levels in overweight or obese adults (Geens et al., 2015; Do et al., 2017). For alternative
351 bisphenols, literature on their relation with BMI is scarce. A few studies report higher levels for BPS in
352 obese individuals as well (Liu et al., 2017; Jacobson et al., 2019). Studies in mice indicated that BPS
353 might be obesogenic (Ivry Del Moral et al., 2016; Ahn et al., 2020), but more research is needed to
354 examine the potential association between emerging bisphenols and BMI.

355 Migrant background of the participants or their parents (univariate analysis, $p = 0.037$) and the home
356 language ($p > 0.05$) appeared to influence BPF concentrations but did not remain significant in the
357 final multiple regression model (Table 4). In American children (6-19 years old), significantly different
358 BPF and BPS concentrations were reported depending on the ethnicity of the participant (Lehmle et
359 al., 2018). School type of the adolescent did not appear to influence urinary bisphenol concentrations
360 significantly. Bisphenol levels were consistently higher in participants following a vocational
361 education, but not significantly. The highest educational level of the parents seemed to have an
362 influence on the bisphenol exposure of the adolescent: BPA levels in the adolescents differed
363 significantly depending on the highest educational level of their mother, while BPF levels were
364 significantly influenced by that of their father. Interestingly, BPA levels were highest in the group with
365 a secondary maternal educational level, but BPF concentrations were highest in the group with a
366 primary paternal educational level. In our study population, a relatively high percentage of parents
367 had a tertiary educational level (even more among mothers), which might have an effect on the
368 outcome. The size of the household income was not significantly associated with any bisphenol levels
369 in our study population. Inconsistent results considering household income have been reported in
370 literature, with some studies reporting statistically significant inverse associations (Lakind and
371 Naiman, 2011; Geens et al., 2014; Gys et al., 2020a) while other studies reporting no association (Kim
372 et al., 2011) and some even a positively significant association (Ye et al., 2008). Additionally, the
373 questionnaire included a question about the level of difficulty experienced by the household in making
374 ends meet. Participants in the category reporting difficulty or great difficulty in making ends meet,
375 showed the highest median BPA levels, but also this association was not statistically significant. It is
376 likely that the socio-economic status (comprising e.g. household income and educational level) has an
377 effect on the purchasing and consumption behavior of a household. Furthermore, these variables
378 might have different implications on lifestyle habits in different countries and categorization of socio-
379 economic status might not be standardized between studies.

380 Smoking habits and exposure to environmental tobacco smoke (secondhand or passive smoking) were
381 examined as well. Active smokers showed higher levels of all three investigated bisphenols, but not

382 significantly. This might be due to a lack of sufficient statistical power, as only 18 participants reported
383 being active smokers. Recent smoking (i.e. within the last three days) resulted in significantly higher
384 levels for BPF ($p = 0.013$), but not for the others. Exposure to passive smoking increased BPA and BPF
385 levels significantly and lead to slightly higher BPS concentrations as well (univariate analysis). In FLEHS
386 II, similar relations were reported for BPA (Geens et al., 2014) and in our study on Japanese children,
387 we found a comparable association (Gys et al., 2020a). However, the opposite correlation has also
388 been reported for BPA (Lakind and Naiman, 2011) and it is possible that smoking might be an
389 intermediary for other factors associated with higher exposure to bisphenols (Lehmle et al., 2018).
390 The prevalence of exposure to secondhand smoke in the house was significantly higher (Chi-square; p
391 < 0.001) in households with primary (24%) and secondary (29%) educational levels, compared to
392 tertiary (10%) education. The same relation was observed between in-house passive smoke exposure
393 and household income ($p < 0.001$) and between active smoking of the adolescent and household
394 income ($p = 0.031$); the prevalence of passive or active smoking decreased as the income increased.
395 These findings indicate that there is a relation between smoking habits and socio-economic status and
396 that these variables could have a synergistic effect on bisphenol concentrations, or that they are
397 proxies for another, unidentified, variable.

398 Because food intake is considered the major human exposure route to BPA (European Food Safety
399 Authority, 2015), potential associations of measured concentrations with questionnaire data on food
400 consumption and various other product use parameters were tested. Interestingly, consumption of
401 canned fish within three days before sampling did not have a significant impact on bisphenol levels,
402 despite reports of presence of bisphenols (mostly BPA, also BPB and BPF) in canned foodstuffs and
403 correlations between canned food consumption and higher bisphenol levels (Carwile et al., 2011;
404 Russo et al., 2019; Gonzalez et al., 2020). However, the same absence of association between canned
405 food consumption and BPA levels was reported in a large cross-sectional American study (Lakind and
406 Naiman, 2011). Recent consumption of barbecued or grilled foods was related to a higher urinary BPA
407 concentration. The use of insecticide by the participant three days before sampling resulted in
408 significantly higher levels for BPA and BPF. Consumption of locally caught fish was related to higher
409 BPA concentrations and consuming shellfish in the last year was associated to higher BPF levels. From
410 the univariate analysis, it appeared that recent use of haircoloring was also significantly associated
411 with higher urinary BPF levels, as did the recent consumption of fried food. All these associations are
412 likely related to the packaging of the food or product or the utensils used to cook or apply them, as
413 bisphenols are widely applied in many consumer products (Geens et al., 2011; von Goetz et al., 2017).
414 BPS concentrations seemed to be less influenced by food consumption or product use variables, which
415 might indicate that it is being used as a BPA-alternative in other applications that were not surveyed

416 in our study. A significant association was also found between dental braces and BPF concentrations
417 in our study population. However, BPF levels were higher in urine of participants who reported not to
418 have braces, which might indicate that this variable is a proxy for other factors and should be
419 investigated further.

420

421 The season of sample collection had a significant influence on the measured BPA concentration in
422 univariate analysis ($p = 0.023$) and higher levels were detected in autumn. However, this variable did
423 not remain significant in the final multiple model (Table 4). In a study on Japanese school children,
424 BPA levels were higher in autumn compared to other seasons as well (Gys et al., 2020a). BPF levels
425 were associated with the season of urine collection and appeared to be higher in autumn and spring.
426 Urinary BPS was not subject to significant seasonal variation. Various factors may account for this
427 observation, e.g. seasonal changes in food consumption, amount of time spent indoors/outdoors
428 (Geens et al., 2014). As mentioned, no urine samples were collected during summer, meaning this
429 result should be interpreted with caution. In general, analysis of a single spot urine sample might also
430 not represent exposure accurately due to within-individual variation as a consequence of short half-
431 life of bisphenols (Vernet et al., 2018; Wang et al., 2019; Gys et al., 2020b).

432 Overall, the proportion of variance in urinary bisphenol concentrations explained by the multiple
433 regression models was low ($R^2 = 0.066$ for BPA and $R^2 = 0.095$ for BPF). For BPS, no significant
434 determinants were retained in the final model. These findings suggest that major predictors of
435 exposure to bisphenols could not be identified and that the questionnaires should be refined for
436 future studies. Additionally, bisphenols are compounds that are quickly metabolized but used in
437 numerous, heterogenous applications. Moreover, in comparison to FLEHS II, the GM of urinary BPA in
438 FLEHS IV is lower, and its variation is smaller, which might also partially explain why certain
439 associations reported in FLEHS II were not confirmed in FLEHS IV.

440 **Table 4** Multiple regression analysis for the assessment of determinants of exposure for bisphenols,
 441 normalized for specific gravity.

Compound	(n) variable	B (95%CI)	p-value
BPA R ² = 0.066	Sex		0.010
	(184) boy	0.772 (0.635, 0.939)	0.010
	(211) girl	reference	
	Highest education level of the mother		0.045
	(37) primary	0.752 (0.539, 1.063)	0.108
	(137) secondary	1.165 (0.948, 1.432)	0.147
	(221) tertiary	reference	
	Consumption of barbecued/grilled food in last 3 days		0.007
	(276) no	0.749 (0.607, 0.923)	0.007
	(119) yes	reference	
	Consumption of local fish in last year		0.017
	(374) no	0.598 (0.392, 0.913)	0.017
	(21) yes	reference	
Use of insecticide by participant		0.013	
(384) no	0.480 (0.271, 0.853)	0.013	
(11) yes	reference		
BPF R ² = 0.095	Highest education level of the father		0.010
	(48) primary	0.564 (0.190, 0.938)	0.003
	(149) secondary	0.036 (-0.224, 0.295)	0.788
	(161) tertiary	reference	
	Smoking in last three days		0.013
	(350) no	0.356 (0.158, 0.804)	0.013
	(8) yes	reference	
	Season of urine collection		0.015
	(116) winter	0.696 (0.497, 0.974)	0.035
	(163) spring	1.036 (0.759, 1.414)	0.825
	(0) summer	N/A	N/A
	(179) autumn	reference	
	Consumption of shellfish in last year		0.008
	(175) no	0.721 (0.566, 0.919)	0.008
	(183) yes	reference	
Use of insecticide by participant		0.020	
(349) no	0.405 (0.189, 0.867)	0.020	
(9) yes	reference		
Time between urine collection and previous toilet visit		0.015	
(40) ≤ 2 h	0.530 (0.342, 0.822)	0.005	
(144) 2-4 h	0.966 (0.712, 1.310)	0.823	
(83) 4-6 h	0.769 (0.545, 1.085)	0.134	
(91) > 6 h	reference		

442 N/A: not available. Only variables showing p < 0.05 were retained in the model. For BPS, no significant
 443 multiple regressions model could be constructed.

444

445 3.5. Comparison with guidance values

446 A preliminary risk assessment was carried out by 1) by direct comparison of measured urinary
 447 bisphenol levels with available HBM-values and 2) calculating estimated daily intakes (EDI) based on

448 the urinary concentrations and comparing them with available reference doses (tolerable daily
449 intakes; TDI). The calculation of estimated daily intakes (EDIs) for BPA, BPF and BPS in this study
450 population was carried out according to the following equation:

$$451 \quad EDI = \frac{C_U \times V_U}{BW} \times 1000$$

452 where the EDI is expressed in ng/kg bw/day, C_U is the measured urinary concentration of the
453 respective bisphenol (in ng/mL), V_U is the daily urine excretion rate (L/day) and BW is the measured
454 body weight of the participant expressed in kg (Lakind and Naiman, 2011; Geens et al., 2015; Chen et
455 al., 2018; Zhang et al., 2020). The urine excretion rate is estimated to be 1.2 L/day for 15-year-olds
456 (Valentin, 2002; Lakind and Naiman, 2008). Calculated EDIs are presented in Table 5. As these EDI
457 values are calculated based on the measured internal exposure and bisphenols are short-lived
458 chemicals ($t_{1/2} < 7$ h) completely excreted in urine, these numbers represent the intake from all
459 exposure sources (Völkel et al., 2002; Dekant and Völkel, 2008; Thayer et al., 2015). Pharmacokinetic
460 data for alternative bisphenols are scarcer than for BPA, but so far, research indicates that total urinary
461 levels can be considered robust measurements for internal exposure (Koch et al., 2012; Lehmler et al.,
462 2018; Oh et al., 2018). Guidance values such as the HBM-I values define the concentration of a
463 chemical in a biological matrix which is consistent with existing noncancer health-based exposure
464 guidance values such as the TDI calculated by the European Food Safety Authority (EFSA) (Apel et al.,
465 2017). These values allow for a direct comparison of the measured biomonitoring concentration and
466 are intended as a screening tool to assess which contaminants are near or above risk assessment
467 values.

468 A TDI of 4 $\mu\text{g}/\text{kg}$ bw/day was established by the EFSA for BPA (European Food Safety Authority, 2015).
469 Other institutions such as USEPA and Health Canada provide higher TDI values for BPA: 50 and 25
470 $\mu\text{g}/\text{kg}$ bw/day, respectively (Huang et al., 2017). As expected from the measured urinary levels in
471 adolescents and in accordance to other studies, the EDI is highest for BPA (Zhang et al., 2020).
472 However, even in a high-exposure scenario (95th percentile), the EDI is much lower (factor 45) than
473 any of the established TDI values, indicating that there are no expected health concerns for this study
474 population (Table 5). For other bisphenols, no TDI values are available yet. An HBM-I value was also
475 only available for BPA: 0.1 mg/L in urine for children (Apel et al., 2017). In accordance with the TDI-
476 EDI comparison, no participants showed a urinary concentration (see Table 2) above the HBM-I value,
477 which additionally indicates a low risk potential for the adolescents. However, this preliminary risk
478 assessment is based on the current knowledge on single compounds, which neglects the potential
479 cumulative effects of bisphenols and other environmental chemicals on human health. This implies
480 that continued monitoring is recommended, preferably of multiple classes of contaminants.

481 Several international studies have calculated EDI values for BPA based on biomonitoring data. Most
 482 of these studies report higher EDI values (Lakind and Naiman, 2011; Zhang et al., 2011; Lakind et al.,
 483 2012; Huang et al., 2017). Important to note is that EDI values greatly depended on the period of
 484 sample collection, since levels of BPA are decreasing during recent years in various countries. The
 485 intake of alternative bisphenols is less investigated. A recent study on children from South-China
 486 reported lower EDIs for BPA and BPS compared to our results, but a similar value for BPF, which is
 487 consistent with their and our reported urinary levels (Chen et al., 2018). EDIs were calculated for
 488 Chinese university students and were consistently higher for BPA, BPF and BPS (Zhang et al., 2020).
 489 Liao *et al.* calculated EDIs for bisphenols based on measured concentrations in indoor dust.
 490 Expectedly, they reported substantially lower values for BPA, BPF and BPS in teenagers compared to
 491 our calculations, most probably because the contribution of dust ingestion to the total bisphenol
 492 intake is rather small (Liao et al., 2012b). When calculating EDIs based on specific environmental
 493 measurements (e.g. bisphenol levels in (canned) food or dust), values will be lower, as bisphenols are
 494 used in numerous applications (Geens et al., 2010). Although dietary ingestion is considered the main
 495 exposure route for BPA, it is likely that not all non-food sources have been elucidated yet (Geens et
 496 al., 2011; European Food Safety Authority, 2015; von Goetz et al., 2017). For alternative bisphenols,
 497 the major exposure route has not been established yet, but as they are meant to serve as
 498 replacements for BPA, it can be expected that sources are comparable. As the collected urine samples
 499 in this study were spot samples rather than 24-h pooled urine, the calculated EDIs of these rapidly
 500 excreted chemicals need to be interpreted with caution (Lakind and Naiman, 2008).

501

502 **Table 5** Selected percentiles for estimated daily bisphenol intake (ng/kg BW/day).

Analyte	25 th	Median	75 th	95 th	TDI ^a	% > TDI	Ratio TDI/95 th
	ng/kg BW/day						
BPA	11.6	22.4	39.5	88.8	4000	0	45
BPF	1.4	2.9	6.0	22.7	N/A		
BPS	1.3	2.5	4.8	17.0	N/A		

503 TDI: tolerable daily intake; N/A: not available; ^avalue as provided by EFSA.

504

505 3.6. Strengths and limitations

506 Reliable performance of the analytical method applied in the present study was ensured, both by
 507 excellent QA/QC results and by successful participation in various proficiency tests. Our study is, to
 508 the best of our knowledge, the first exposure assessment of bisphenol analogues, other than BPA, in
 509 a large Flemish study population and the first large European study in adolescents. Because BPA was
 510 measured in a previous cycle of this study, comparison between concentrations at the two time points

511 was possible. Control samples showed good agreement between both cycles, although measured with
512 different analytical methods. A limitation of the study is the lack of multiple urine sampling from the
513 same adolescent because bisphenols are short-lived compounds and can vary considerably within an
514 individual (Gys et al., 2020b). This might have influenced the comparison between FLEHS II and FLEHS
515 IV and the exposure determinant analysis. Given the low proportion of variance in urinary bisphenol
516 concentrations that could be explained by the multiple regression models, it is clear that information
517 on major determinants of exposure was lacking and that questionnaires for future studies should be
518 modified. EDI was calculated based on measured internal exposure, thus accounting for all sources,
519 and measured body weight. The accuracy of this value might have been more accurate if a 24 h pooled
520 urine sample had been collected.

521

522 **4. Conclusions**

523 In the framework of the 4th Flemish Environment and Health Study (FLEHS IV), BPA and 5 alternative
524 bisphenols were measured in 423 Flemish adolescents. This study was the first to measure other
525 bisphenols in a large Flemish study population and the first in a European study population of this age
526 category. All included compounds were detected in the urine samples of the study population, with
527 BPF, BPA and BPS showing high detection frequencies, indicating extensive and simultaneous
528 exposure. Despite still being the predominant bisphenol, showing highest levels, BPA concentrations
529 had decreased significantly compared to previous measurements during FLEHS II in 2008. Levels of
530 BPA, BPF and BPS were generally in the same range as those reported in literature. Both active and
531 passive smoking were associated with higher bisphenol levels. Some food consumption and product
532 use variables showed significant associations with higher levels of BPA and BPF. EDIs were calculated
533 based on measured internal exposure and were in the same range as or lower than other reported
534 values. Even in a high-exposure scenario, preliminary risk assessment showed that BPA stays below
535 the available health-based guidance values. The exposure data presented in this work are
536 representative for Flanders, Belgium.

537

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546

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