Metals, hormones and sexual maturation in Flemish adolescents in three cross-sectional studies, (2002-2015)

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Abstract

Sex hormone levels and timing of sexual maturation are considered important markers for health status of adolescents in puberty, and previous research suggests they might be influenced by metal exposure. In three campaigns of the Flemish Environment and Health Study (FLEHS I 2002-2006; FLEHS II 2007-2011 and FLEHS III 2012-2015), data were collected on internal exposure to metals (Cd, Cu, Pb, Cr, Mn, Tl, Ni, Sb, Hg, As and As species) and sexual maturation in 2671 14-15 years old adolescents. All metals were measured in blood and/or urine, except total- and methylmercury which were measured in hair samples. Sex hormone levels were measured in blood serum of adolescent males of the cohorts of FLEHS I and FLESH II. The use of a uniform methodology in successive campaigns allows to confirm associations between exposure and health in different cohorts and over time. Furthermore, mathematical and statistical density correction methods using creatinine or specific gravity were tested for urinary markers.

Significant associations between sex hormones and maturity markers were observed in the FLEHS I and II campaigns, when both were assessed together. Regardless of the applied correction method, creatinine correction systematically introduced bias due to associations of creatinine with sex hormones and maturation markers, especially in adolescent males, while this is not the case for specific gravity. A series of exposure-response associations were found, but several involving Cd, Pb, As, Tl and Cu persisted in different FLEHS campaigns. The effects of Pb and Cu on luteinizing hormone, (free) testosterone, (free) oestradiol and maturation support a xenoestrogenic agonistic action on the feedback of oestradiol to the hypothalamus-pituitary-gonadal axis.

Our results suggest that specific care should be taken when selecting urine density correction for investigating associations with hormonal and maturation markers in adolescent adolescent males. Furthermore, the possibility of xenoestrogenic effects of certain metals in environmentally exposed adolescents warrants further investigation.

1. Highlights

- Creatinine correction confounds associations with hormones and maturity markers
- Specific gravity seems to be a better option for density correction
- Multiple metals show consistent effect over different studies
- Results suggest xenoestrogenic effect of Cu and Pb at environmental exposure levels

2. Keywords

FLEHS, xenoestrogen, exposure-response, creatinine, multiple regression, metals

3. Introduction

Puberty is suspected to be a period where an individual is especially vulnerable to environmental exposures to pollutants. Since the body and especially the brain is under development, external substances that influence this maturation could have long lasting effects on an individual (Sato et al., 2008). An important step in the onset of puberty is the activation of the hypothalamus-pituitary-gonadal (HPG) axis, which leads to increased formation of gonadal hormones involved in sexual maturation (Foster et al., 2006; Sisk and Foster, 2004). Epidemiological research has also reported correlations between sex hormone levels and the progression of puberty, reflecting the link between these processes (Den Hond et al., 2011; Kletter et al., 1993). It follows that external disturbances on the normal functioning of the HPG axis could affect puberty (including sexual and brain development) through influencing concentrations of hormones (Sato et al., 2008).

Exposure to certain metals has been observed to influence concentrations of sex hormones and/or maturation (positively or negatively) in tests on animals (Al-Hamood et al., 1998; Cheng et al., 2003; Dearth et al., 2002; Liu et al., 2013; Srivastava et al., 2004), clinical tests on humans (Ayala et al., 2008) or in epidemiological research (Interdonato et al., 2015; Meeker et al., 2010; Schell and Gallo, 2010; Zawatski and Lee, 2013). Some of the effects of metals have been linked to

69 xenoestrogenic actions in vitro (Darbre, 2006). Adolescents have potential exposure pathways to
70 several of these metals, so from a health perspective it would be interesting to check associations in
71 this age group between exposure to metals on one hand, and maturity markers and levels of HPG
72 related hormones on the other hand. However, metals other than Cd and Pb were considered in
73 very few studies.
74
75 In three successive Flemish Environment and Health Studies (FLEHS I, FLEHS II and FLEHS III)
76 data were collected on exposure and effect markers through human biomonitoring and
77 questionnaires (Schoeters et al., 2012). Adolescents aged 14-15 years were one of the focus groups
78 during all three campaigns of FLEHS (2002-2006; 2007-2011; 2012-2015). The gathered information
79 includes concentrations of several metals and maturity markers in all adolescents for all three
80 campaigns, and information on sex hormone concentrations in adolescent males for the first and
81 second campaign (FLEHS I and II). As a first step, the datasets were used to explore significant
82 associations between exposure and effect markers. Subsequently, those associations that were
83 supported by the literature or several FLEHS campaigns were further investigated and discussed.
84 Where possible, suggestions are made for the mechanism by which the exposure markers affect sex
85 hormones and/or maturity.
86
87 Another goal of this study was to determine a reliable urine density correction factor for
88 urinary concentrations. Therefore, we compared the use of creatinine and specific gravity, as
89 evidence is emerging that creatinine correction is less reliable in adolescents since it is significantly
90 affected by skeletal muscle mass and diet, and has been shown to increase during
91 maturation(Martin et al., 2008; Weaver et al., 2015).

4. Methods

4.1. Recruitment, sampling and questionnaires

Recruitment, sampling and use of questionnaires have been described in the literature for FLEHS I (Den Hond et al., 2011), FLEHS II (Croes et al., 2014b) and FLESH III (De Craemer et al., 2016). In studies were sex hormones were measured in adolescent males (FLEHS I and II), samples were taken around the same time in the morning (8:00 to 12:00) to limit the influence of diurnal variability of hormones. The number of participants in each study for whom metal exposure were measured was 1659, 606 and 406 respectively, although not all exposure and effect markers were measured in each individual. The total number of observations for each association investigated can be found in the relevant tables in sections 6, 7 and 8 of the supplementary material. An informed consent was signed by both the participants and their parents, and the biomonitoring studies were approved by the Ethical Committee of the University of Antwerp, Belgium (FLEHS I and II) and of the University hospital of Antwerp (FLEHS III).

The biomarkers of effect considered in this study are presented in Table 4. The questionnaires served to get personal information on lifestyle, education, living environment and other factors that could have an influence on metal concentrations or effect markers in the individual. Assessment of sexual development has been previously described for FLEHS I and II (Croes et al., 2014b; Den Hond et al., 2011). Briefly, development of genitals in adolescent males, breasts in adolescent females and pubic hair in both sexes was scored using the international scoring criteria of Marshall and Tanner, where stage 1 corresponds to the start of puberty and stage 5 to the adult stage. Information on menarche was obtained through self-assessed questionnaires.

4.2. Description and measurement of exposure and effect markers

4.2.1. Exposure markers

Table 1 indicates which markers were measured in which study, and whether they were measured in blood (B), urine (U) and/or hair (H). Trace element analysis in blood and urine in FLEHS I

(Schroijen et al., 2008) and II (Baeyens et al., 2014; Vrijens et al., 2014) has been previously described, induced coupled plasma mass spectrometry (ICP-MS) was used for most markers, except As-metabolites, toxicologically relevant arsenic (TRA), total mercury (THg) and methylmercury (MeHg). Trace element analysis in FLEHS III was identical to the analysis in FLEHS II for the reported elements, except for arsenic species in urine. In FLEHS II, TRA was measured directly using flow injection –hydride generation atomic absorption spectrometry (Baeyens et al., 2014). In FLEHS III, trivalent As(III), pentavalent As(V), monomethyl arsenic acid (MMA), dimethyl arsenic acid (DMA) were measured using High Performance Liquid Chromatography-ICP-MS, with a Dynamic Reaction Cell, and TRA was calculated as the sum of these markers. The measurement of total and methylmercury in hair samples in FLEHS II was described by Croes et al. (2014a).

In urine specific gravity and creatinine were determined respectively by densitometer and the Jaffe method, and used to correct for urine density.

Table 1: Description of exposure markers.

Exposure marker	unit	N	%>LOD	Geometric mean (95%CI)	P95 (95%CI)	Min	Max
				FLEHS I			
B-Cd	µg/l	1659	86.2	0.337 (0.320-0.355)	1.64 (1.52-1.72)	0.045	3.23
B-Cu	µg/l	1658	100	699 (692-706)	971 (959-996)	182	1760
B-Pb	µg/l	1659	99.6	21.3 (20.7-22.0)	57.6 (53.7-62.1)	1.00	212
				FLEHS II			
B-As	µg/l	210	100	0.616 (0.549-0.689)	2.95 (2.45-4.02)	0.121	8.1
B-Cd	µg/l	606	99.5	0.212 (0.202-0.223)	0.583 (0.502-0.86)	0.03	22.9
B-Cr	µg/l	387	100	0.291 (0.275-0.308)	0.648 (0.61-0.794)	0.019	2.32
B-Cu	µg/l	606	100	821 (810-831)	1040 (1010-1070)	357	1870
B-Ni	µg/l	386	100	1.2 (1.16-1.24)	1.91 (1.74-2.48)	0.528	9.38
B-Mn	µg/l	606	100	9.86 (9.64-10.1)	15.4 (14.5-16.4)	3.9	39.2
B-Pb	µg/l	606	100	13.8 (13.3-14.3)	28 (25.5-31)	5.3	76.9
B-Tl	µg/l	605	100	0.0303 (0.0297-0.0309)	0.045 (0.044-0.048)	0.013	0.099
U-Cd	µg/l	533	100	0.344 (0.331-0.358)	0.724 (0.655-0.787)	0.0592	1.24
U-Tl	µg/l	533	100	0.269 (0.26-0.278)	0.487 (0.461-0.539)	0.0791	1.1
U-Cr	µg/l	533	98.7	0.345 (0.322-0.369)	1.46 (1.14-1.85)	0.000149	4.31
U-Ni	µg/l	533	99.2	2.67 (2.54-2.81)	6.92 (6.45-8.05)	0.195	24.4
U-Cu	µg/l	531	100	13 (12.6-13.4)	22.7 (21.4-24.7)	3.98	148
U-Sb	µg/l	533	80.1	0.0885 (0.0834-0.0938)	0.277 (0.235-0.297)	0.0169	3.32
U-As	µg/l	605	99.8	13.6 (12.5-14.6)	93.3 (75.3-121)	2.78	1030
U-TRA	µg/l	605	97	6.06 (5.71-6.43)	17.1 (15.4-20.7)	0.15	46.1
H-THg	µg/g	594	100	0.169 (0.157-0.181)	0.639 (0.538-0.853)	0.012	4.27
H-MeHg	µg/g	580	100	0.112 (0.104-0.121)	0.47 (0.401-0.539)	0.004	1.74
				FLEHS III			
B-As	µg/l	406	100	0.78 (0.719-0.846)	3.11 (2.66-5.26)	0.131	11.3
B-Cd	µg/l	406	100	0.185 (0.175-0.197)	0.513 (0.391-1.17)	0.065	4.66
B-Cu	µg/l	406	100	888 (873-903)	1270 (1150-1390)	491	1760
B-Mn	µg/l	406	100	10.4 (10.1-10.7)	17.5 (16.3-19.3)	4.14	25.7
B-Pb	µg/l	406	100	9.26 (8.89-9.64)	18.7 (17.4-21.2)	2.7	38.6
B-Tl	µg/l	406	100	28.6 (28-29.3)	41.8 (40.1-44.5)	12.5	71.6
U-As(III)	µg/l	407	44.2	0.143 (0.13-0.157)	0.707 (0.6-0.87)	0.04	4.2
U-As(V)	µg/l	407	62.2	0.179 (0.164-0.197)	0.754 (0.65-0.94)	0.03	2.74
U-DMA	µg/l	407	99.5	3.45 (3.22-3.7)	11.3 (9.24-15.1)	0.06	60.2
U-MMA	µg/l	407	88.2	0.569 (0.523-0.618)	1.8 (1.46-2.1)	0.05	4.49
U-TRA	µg/l	407	-*	4.69 (4.41-4.98)	13.3 (11.4-17.9)	0.49	63

* U-TRA in FLEHS III was calculated by summing other As species instead of directly measured, as such no LOD is defined.

In general, urinary metal concentrations were corrected for urine density by specific gravity using equation 1, with SG as specific gravity and C and C_{SG}.as uncorrected and corrected biomarker concentrations respectively.

$$C_{SG} = C\frac{1.024\text{-}1}{SG\text{-}1} \quad \textbf{(1)}$$

136 Creatinine correction was done by dividing the metal concentrations by the concentration of

137 creatinine of that sample.

4.2.2. Effect markers

Table 2: Description of continuous effect markers

Effect marker	unit	N	Geometric mean (95%CI)	P95 (95%CI)	Min	Max
FLEHS I						
T/E2 (aromatase)	ratio	814	21.8 (20.9-22.7)	38.6 (37.7-40.2)	0.125	50.6
E2	pg/ml	813	14.5 (14.2-14.9)	23.4 (22.4-24.8)	5.82	51.8
fE2	pg/ml	813	0.250 (0.243-0.258)	0.457 (0.437-0.480)	0.0624	0.859
T	ng/dl	814	318 (302-335)	691 (664-714)	13.0	1050
fT	ng/dl	814	6.09 (5.70-6.51)	15.8 (150.1-17)	0.0894	24.8
SHBG	nmol/l	814	32.8 (31.7-33.9)	85.6 (77.1-90.4)	6.40	133
LH	mU/ml	811	2.61 (2.51-2.71)	6.16 (5.82-6.65)	0.316	21.6
Age menarche	years	595	12.6 (12.5-12.7)	14.5 (14.4-14.7)	7.83	16.0
FLEHS II						
T/E2 (aromatase)	ratio	321	16.8 (15.9-17.7)	35.2 (28.6-40.4)	1.49	80.6
E2	pg/ml	322	21.2 (20-22.3)	37.8 (35.9-42.2)	6	71.2
fE2	pg/ml	303	0.31 (0.285-0.338)	0.763 (0.696-0.833)	0.03	1.25
T	ng/dl	321	356 (331-383)	706 (672-780)	13.4	1000
fT	ng/dl	321	5.17 (4.66-5.74)	15.6 (13.8-16.5)	0.11	20.1
SHBG	nmol/l	321	38.9 (37.1-40.9)	86.5 (78-93.1)	5	137
LH	mU/ml	322	3.06 (2.87-3.26)	6.63 (6.09-7.29)	0.1	23.9
FSH	mU/ml	322	3.76 (3.53-4)	9.48 (8.13-10.6)	0.34	59.7
Age menarche	years	224	12.9 (12.7-13)	14.5 (14.3-14.6)	9.76	15
FLEHS III						
Age menarche	years	248	12.8 (12.6-12.9)	14.6 (14.2-15)	8.58	16.2

Table 3: Description of binary effect markers (maturation markers), as percentage reaching indicated Tanner stages

Effect marker	unit	N	Geometric mean (95%CI)
FLEHS I			
Breast development (adolescent females)	%B5	626	49.2 (45.3-53.1)
Pubic hair (adolescent females)	%P5	636	52.8 (49.0-56.7)
Genital development (adolescent males)	%G4-G5	769	63.2 (60.0-66.6)
Pubic hair (adolescent males)	%P4-P5	765	60.4 (56.9-63.9)
FLEHS II			
Breast development (adolescent females)	%B5	152	63.2 (55.5-70.9)
Pubic hair (adolescent females)	%P5	145	62.1 (54.2-70)
Genital development (adolescent males)	%G4-G5	154	83.8 (78-89.6)
Pubic hair (adolescent males)	%P4-P5	155	82.6 (76.6-88.6)
FLEHS III			
Breast development (adolescent females)	%B5	283	60.1 (54.4-65.8)
Pubic hair (adolescent females)	%P5	266	56 (50-62)
Genital development (adolescent males)	%G4-G5	272	68.8 (63.3-74.3)
Pubic hair (adolescent males)	%P4-P5	272	68.8 (63.3-74.3)

Sex hormones investigated in this study were oestradiol (E2), testosterone (T), free oestradiol and testosterone (fE2 and fT), sex hormone binding globulin (SHBG), luteinizing hormone (LH) and follicle stimulating hormone (FSH). Hormone levels in adolescent males were measured in blood serum using commercial immunoassays as previously described for FLEHS I and II (Croes et al., 2014b; Den Hond et al., 2011), FSH as described in Dhooge et al. (2006). Aromatase was calculated as the ratio of testosterone to oestradiol (T/E2).

The effect markers for sexual development were binary markers based on the Tanner stage reached. For genital and pubic hair development in adolescent males, the value 1 was assigned if they were at stage 4 or 5. For breast and pubic hair development in adolescent females, the value 1 was assigned if they were at stage 5. Otherwise, the value 0 was assigned to these markers. These cut-offs were chosen in order to have reasonably large subgroups below and above the cut-off in each campaign of FLEHS. The percentage at or above these cut-offs were around 70% in adolescent males, and around 60% in adolescent females.

4.3. Statistical analysis

Table 4: Assessed effect markers and their confounders, accepted covariates and scale (normal or ln-transformed). Sex hormones were only measured in adolescent males and not assessed in FLEHS III. Supplementary table 1 lists which covariates were retained in the final models.

Effect	Confounders	Covariates*	Scale	Campaigns
Age menarche	Age, BMI	FLEHS III: -Passive smoking at home	normal	all
Pubic hair (adolescent females)	Age, BMI, Contraceptive pill usage	FLEHS III -Urbanisation	normal	all
Breast development (adolescent females)	Age, BMI, Contraceptive pill usage		normal	all
Pubic hair (adolescent males)	Age, BMI		normal	all
Genital development (adolescent males)	Age, BMI		normal	all
T/E2 (aromatase)	Age, hour of blood collection, BMI, smoking status		ln	I, II
E2 (pg/mL)	Age, hour of blood collection, BMI, smoking status		ln	I, II
fE2 (pg/mL)	Age, hour of blood collection, BMI, smoking status	FLEHS II: -Season -Illness in last 14 days	ln	I, II
T (ng/dL)	Age, hour of blood collection, BMI, smoking status		ln	I, II
fT (ng/dL)	Age, hour of blood collection, BMI, smoking status	FLEHS II: -Illness in last 14 days	ln	I, II
SHBG (nmol/L)	Age, fasting, BMI, smoking status, hour of blood collection	FLEHS II: -Alcohol weekly	ln	I, II
LH (mU/mL)	Age, BMI, smoking status		ln	I, II
FSH (mU/mL)	Age, BMI, smoking status		ln	II

*Accepted covariates for FLEHS II and III. For covariates of sex hormones in FLEHS I, see (Dhooge et al., 2009). No covariates were accepted for markers of sexual development in FLEHS I since none were significant in univariate regression models.

Since not all exposure and effect markers were measured in all campaigns of FLEHS, and study designs differed slightly between the campaigns, it was decided not to work on a pooled dataset. Instead, we used identical statistical methods for the datasets of each campaign separately. Continuous effect or exposure markers below the limit of detection (LOD) were replaced by LOD/2.

To assess exposure-response associations, multiple regression was performed by linear regression models for continuous effect markers (hormones in adolescent males and age at menarche), and logistic regression models for binary effect markers (pubertal staging). Confounders were selected a priori based on experience in previous studies (Den Hond et al., 2002; Dhooge et al., 2009; Staessen et al., 2001; Van Den Heuvel et al., 2002) and a literature search in Pubmed, and incorporated into the model for each study, regardless of significance. Covariates were accepted for each FLEHS study when they showed reasonably significant associations with the effect markers ($P<0.20$) in univariate analysis (shown in Table 4), but they were only retained if they were significant in the multiple regression model ($P<0.05$). (shown in supplementary table 1)

Regression analyses were done using R version 3.3.0 and RStudio version 0.99.902, including use of the packages car and lmtest for all regression models, and logistf for firth logistic regression for associations where exposure variables showed quasi separation in normal logistic regression. Variance inflation factors for each independent variable were checked, and none were higher than 2, well below usual thresholds (O'brien, 2007). Binary effect markers were by definition on the normal scale, while continuous effect markers were ln-transformed if this normalized the distribution of the regression residuals. For linear and logistic regression, estimates and odds ratios (OR) were calculated respectively. Estimates, OR and their 95% confidence intervals are reported for increases of exposure from the 25^{th} percentile to the 75^{th} percentile. For estimates of continuous effect markers on the normal scale, this is the additive difference in effect due to the increase of exposure, while for estimates of ln-transformed continuous markers or odds ratios of binary effect markers, this is the multiplicative difference due to increase of exposure.

Regression analyses are sensitive to influential cases, which are outliers with high leverage . Outliers are cases with y values, in this case effect marker values, deviating from the trend. Cases have high leverage if they have extreme or unusual combinations of predictor values, here exposures, confounders or covariates. Although these are not necessarily faulty data, they lie "far" from the other cases and are too few to have a good model of their behaviour. In order to get a

model that was correct for the general population, it was decided to test exposure-response associations without these influential cases, employing two methods for their identification. The first was to identify outliers as cases with Studentized residuals with an absolute value larger than 3, and check which of these also had high leverage by determining if their hat value was higher than twice the average hat value of all cases in the regression. The second method was to plot the Cook's distance of each case. We decided not to work with a cut-off value for the Cook's distance since the traditional cut-off values identified either no influential cases or too many (often 5% or more). Instead, cases that had a Cook's distance clearly higher than the rest of the cases and/or higher than one of the influential cases identified using the first method were also identified as influential cases (Chatterjee and Hadi, 1986; Wang et al., 2005). The supplement also contains regression results without removal of influential cases, but these are not considered in this article. No significant associations were observed for which removing influential cases resulted in an opposite significant trend.

The effect of urine density correction, either by creatinine (CRT) or specific gravity (SG) was examined in 2 ways: dividing each marker by CRT or SG (mathematical correction) before introducing it into the regression model, or by including the uncorrected exposure marker along with the urine density marker as a covariate in the model (statistical correction). In addition, we investigated the associations between hormone concentrations and puberty stages of adolescent males. In all of these regression models, the confounders and covariates of each effect marker were included. It should be noted that all associations in this article are intrinsically sex-stratified, as hormonal markers were only measured in adolescent males, whereas maturation markers are defined separately for each sex.

4.4. Exposure-response associations in different campaigns

To assess the re-appearance of significant associations ($P<0.05$) between metals and maturation parameters, we checked which associations were significant in at least one campaign of

FLEHS. Then we considered and evaluated weaker criteria for these associations: a non-significant association with $P<0.5$ from one campaign that has the same trend as the significant association is considered a confirmation. In section 9 of the supplement, this method is compared considering an association as returning only if it is significant in multiple studies.

5. Results

5.1. Associations between male maturity and sex hormones

Associations between male maturity markers (genital and pubic hair development) and sex hormones are shown in supplementary table 2. Results from FLEHS I and II are very similar, showing many highly significant associations with the two markers of male sexual development. Furthermore, associations of the two markers with the same effect marker often have a similar level of significance and if both are significant in one campaign they always show the same trend (both negative or both positive).

5.2. Effect of urine density correction

Urinary correction was studied on exposure- effect associations in FLEHS II that were significant using at least one of the different correction methods mentioned in section 4.3. Supplementary section 8 contains detailed results pertaining to this comparison, which can be summarized as follows:1) Corrections using CRT resulted in more negative (or less positive) associations between exposure and effect markers compared to SG, an effect which was most pronounced when correcting the exposure markers mathematically. See supplementary tables 23 and 24.

2) 27 significant association between urinary makers and effect markers were found using mathematical CRT correction (supplementary table 23) and 15 when using statistical CRT correction (supplementary table 24). Of these, 13 significant associations were found regardless of CRT correction method. 10 significant associations were found using mathematical SG correction

(supplementary table 23) and 8 when using statistical SG correction. Of these, 8 associations were found regardless of SG correction method. Clearly, the choice of the method has a much more dramatic impact in the case of CRT.

3) 19 significant associations between exposure and effect markers were observed when using statistical correction for CRT or SG (supplementary table 24). The associations between the density markers and the effect markers in complete models containing the exposure marker were studied in these 19 cases (supplementary tables 25 and 26). The association of CRT with the effect marker is always positive and significant in 14 of the 19 cases, while for SG the associations are negative in 5 cases and significant in 3. CRT has thus a much greater influence on the associations found with the effect markers. Based on these results and further consideration in section 6.3 of the discussion, mathematical correction using SG was applied for urinary markers.

5.3. Exposure-response associations

Due to the results from the previous section, urinary metal concentrations were corrected using specific gravity. Significant ($P<0.05$) exposure-response associations between metals and hormones are presented in Table 5, while those with maturity markers are presented in Table 6. These tables only include those associations that return in different FLEHS campaigns. One exception that is included although it is found only once is the associations between B-Pb and hormones because we found a convincing body of evidence for this association in the literature, as discussed in section 6.4.1. Significant associations with limited evidence are included in section 5 of the supplement. As can be seen, some associations with binary maturity markers have very large estimates with large uncertainties. This might be caused by exposure markers nearing quasi-separation for these effect markers in our regression models. While this renders the estimate itself less useful, it does not compromise the significance of the results, which are significant despite large uncertainties.

Associations of B-Cu in with hormones FLEHS I and II are very similar, and are generally decrease negative. The same association is found between B-Pb and fT and fE2.

Regarding maturation markers, B-Cu is associated with a delay in maturation in both sexes, B-Tl only in adolescent females B-Cd is associated with a delay in adolescent females. Like B-Cu, B-Pb delays maturation in both sexes, except that it accelerates menarche. B-As is associated with an acceleration in both sexes.

Table 5: Significant (*P*<0.05) associations between metals and sex hormone levels in male adolescents of FLEHS I and II. The sign indicates the direction of the trend. Regression results for non-significant associations can be found in supplementary tables 8 and 13.

Exposure	Effect	N	Sign	Estimate* (95% CI)	P25-P75 (µg/l)	P
			FLEHS I			
B-Cu (µg/l)	T/E2	800	-	0.91 (0.878 ; 0.944)	617 - 745	<0.001
	E2	802	-	0.967 (0.948 ; 0.986)	617- 746	0.001
	fE2	802	-	0.949 (0.924 ; 0.975)	617- 746	<0.001
	T	803	-	0.878 (0.837 ; 0.921)	617 - 745	<0.001
	fT	803	-	0.853 (0.804 ; 0.906)	617 - 745	<0.001
	SHBG	796	+	1.034 (1.004 ; 1.065)	618- 745	0.024
	LH	800	-	0.941 (0.908 ; 0.975)	617 - 745	0.001
			FLEHS II			
B-Cu (µg/l)	T/E2	317	-	0.923 (0.867 ; 0.982)	737 - 858	0.01
	E2	321	-	0.88 (0.833 ; 0.93)	737 - 858	<0.001
	T	315	-	0.845 (0.785 ; 0.909)	737 - 856	<0.001
	fT	312	-	0.886 (0.793 ; 0.989)	737 - 858	0.029
	SHBG	314	+	1.047 (0.999 ; 1.098)	738 - 858	0.049
	LH	320	-	0.868 (0.813 ; 0.926)	737 - 858	<0.001
B-Pb (µg/l)	fE2	296	-	0.908 (0.839 ; 0.983)	12.2 - 19.1	0.015
	fT	317	-	0.909 (0.828 ; 0.997)	12.2 - 19.1	0.041

*Estimated multiplicative increase in effect due an increase in exposure from the 25th to the 75th percentile.

Table 6: Significant (*P*<0.05) exposure-response associations of metals with maturity markers stratified by sex. Also included are suggestive recurring associations as defined in section 4.4, which are indicated by brackets around the sign of the association. Regression results for other non-significant associations can be found in supplementary tables 6, 8, 10, 13, 16 and 18.

Exposure	Effect	N	Sign	Estimate/Odds ratio* (95% CI)	P25-P75	P
			FLEHS I			
B-Cd (µg/l)	Female pubic hair	589	-	0.798 (0.635 ; 0.999)	0.16 - 0.795	0.049
	Female breast development	583	-	0.796 (0.644 ; 0.979)	0.16 - 0.79	0.03
B-Cu (µg/l)	Age menarche	556	(-)	-0.065 (-0.181 ; 0.05)	643 - 817	0.261
	Male pubic hair development	754	(-)	0.919 (0.78 ; 1.081)	629 - 774	0.306
	Male genital development	758	-	0.82 (0.692 ; 0.967)	621 – 749	0.019
B-Pb (µg/l)	Age menarche	557	(+)	0.039 (-0.072 ; 0.15)	12.2 - 27.6	0.482
	Male pubic hair	754	-	0.808 (0.686 ; 0.949)	14.6 - 31.3	0.009
	Male genital development	758	-	0.843 (0.717 ; 0.99)	17.5 - 34.8	0.037
			FLEHS II			
B-As (µg/l)	Female pubic hair	73	(+)	1.4 (0.921 ; 2.37)	0.336 - 0.861	0.118
	Female breast development	72	(+)	1.191 (0.804 ; 1.865)	0.34 - 0.861	0.386
	Male pubic hair	94	(+)	1.499 (0.936; 3.731)	0.369-0.887	0.134
	Male genital development	92	+	14.56 (1.68 ; 255.20)	0.369-0.987	0.003
B-Cu (µg/l)	Age menarche	181	-	-0.264 (-0.387 ; -0.142)	747.78 - 909	<0.001
	Male pubic hair	149	(-)	0.631 (0.361 ; 1.124)	728 - 858	0.115
	Male genital development	149	(-)	0.743 (0.427 ; 1.339)	728-858	0.314
B-Pb (µg/l)	Age menarche	180	+	0.257 (0.091 ; 0.424)	8.866 - 15.269	0.002
	Male pubic hair	150	(-)	0.849 (0.563 ; 1.365)	13.1 - 20	0.477
	Male genital development	149	-	0.697 (0.462 ; 0.998)	12.9 - 20.1	0.049
B-Tl (µg/l)	Female pubic hair	137	-	0.546 (0.334 ; 0.862)	0.024 - 0.03	0.009
	Female breast development	143	-	0.592 (0.374 ; 0.906)	0.024 - 0.03	0.015
			FLEHS III			
B-As(µg/l)	Female pubic hair	176	+	1.363 (1.036 ; 1.975)	0.452 - 1.288	0.023
	Female breast development	190	+	1.688 (1.188 ; 2.56)	0.449 - 1.256	0.001
	Male pubic hair	175	+	1.375 (1.051 ; 2.025)	0.393 - 1.219	0.017
	Male genital development	175	(+)	1.219 (0.981 ; 1.602)	0.404 - 1.27	0.075
B-Cd (µg/l)	Female pubic hair	172	(-)	0.807 (0.596 ; 1.015)	0.125 - 0.23	0.07
	Female breast development	187	(-)	0.819 (0.633 ; 1.005)	0.126 - 0.231	0.057
B-Cu (µg/l)	Male pubic hair	174	-	0.376 (0.23 ; 0.591)	776.7 - 914.1	<0.001
	Male genital development	178	-	0.411 (0.251 ; 0.649)	776.7 - 913.5	<0.001
B-Pb (µg/l)	Age menarche	180	(+)	0.126 (-0.021 ; 0.273)	6.01 - 9.27	0.088
	Male pubic hair	176	-	0.515 (0.327 ; 0.774)	8.27 - 13.8	0.001
	Male genital development	174	-	0.621 (0.388 ; 0.967)	8.28 - 13.77	0.035
B-Tl (ng/l)	Female pubic hair	173	(-)	0.702 (0.448 ; 1.066)	23.6 - 31.3	0.098
	Female breast development	191	(-)	0.844 (0.597 ; 1.169)	23.9 - 31.6	0.31

*Age menarche: Estimate: additive increase due to increase of exposure by IQR. Other effect markers: odds ratio due to increase of exposure by IQR.

[a] Due to separation in exposure marker using regular logistic regression, firth regression was used

6. Discussion

6.1. Summary

Links between maturity markers and hormone concentrations were confirmed. Creatinine correction tended to introduce bias to the exposure-response associations due to correlations with the effect markers, while this tendency was much less for specific gravity correction . Some exposure-response associations with effect markers were repeatedly observed in different FLEHS studies, especially those involving B-Pb and B-Cu. The effects of these associations on both hormones and maturity markers are investigated, and possible links between these effects are explored.

6.2. Associations between hormones and maturation of adolescent males

The rise of LH, FSH and sex steroids, and the decrease of SHBG during puberty is a well-known phenomenon, and (strong) correlations with Tanner stages have been reported (Hammond, 2011; Kletter et al., 1993; Nottelmann et al., 1987). These trends are also observed in the FLEHS studies, which strengthens confidence in the assessment of hormone levels and maturity stages. All hormones showed positive (higher hormones linked to earlier maturity)associations with maturity markers, whereas a negative association was observed for SHBG. For the data from FLEHS I, this was already reported by Den Hond et al. (2011). The strong associations between these markers in models adjusted for age support the link between hormones and male maturation, and that perturbations in one could affect the other (Sisk and Foster, 2004). As the literature generally follows

the theory that rising steroid levels initiate development of the secondary sexual characteristics on which the Tanner stages are based, it is also adopted for the rest of the discussion (Richmond and Rogol, 2007; Root, 1973; Sisk and Foster, 2004; Ulijaszek et al., 1998; Zawatski and Lee, 2013).

6.3. Bias due to creatinine correction

From the results in section 5.2, it is clear that the results are very sensitive to which correction is employed in the case of CRT, whereas in the case of SG the difference is limited. When looking at the results in supplementary tables 25 and 26, we can see that only CRT has a tendency for significant associations with the effect markers.

To the best of our knowledge, this is the first study reporting associations of sex hormones in serum with urinary creatinine in adolescents. Only one study reported a similar finding for T but not E2, although in a population of adult males aged 17 to 97 (Yeap et al., 2014). A cross sectional study in school children aged 4 to 16 also reported that CRT is significantly positively correlated with age and puberty stage.(Skinner et al., 1996). This is in line with recent studies, which suggest differences in muscle mass to cause differences in CRT unrelated to hydration status, and cause CRT to increase during maturation (Martin et al., 2008; Weaver et al., 2015). As such, a possible explanation for our results could be that not only sex hormones, but also CRT in urine are linked to the progression of maturation in adolescent males. Including CRT as a covariate in a model with these dependent variables could thus have a negative impact when trying to assess the influence of other covariates. The links between different markers that may result in confounding on the association between the exposure marker and the effect marker are shown in Figure 1.

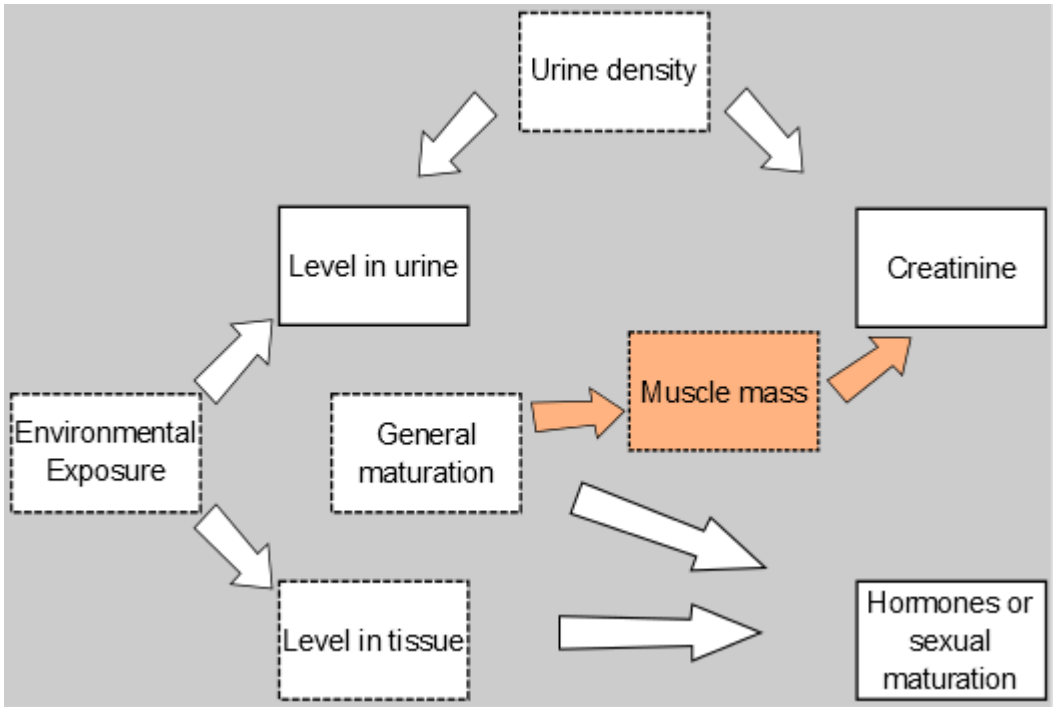

Figure 1: Directed acyclic graph of possible cause of confounding by CRT correction. Variables that were quantified in this study (markers) have solid outlines, others have dashed outlines. Arrows point from cause to effect, variables (in)directly sharing a cause are associated. The white variables and arrows show the ideal situation where CRT correction could be used, while the coloured figures shows the confounding effect that might explain our problems with CRT.

Regardless of the validity of this hypothesis, the high proportion of significant associations between effect markers and CRT compared to SG suggests the latter is a better alternative for density correction in this context. There is, however, no preference for either mathematical or statistical correction using SG based on our calculations or on existing literature. In the end we opted for mathematical correction.

6.4. Exposure-response associations

6.4.1. Recurring trends and associations with limited evidence

With the available data, it was possible to determine which associations were observed in multiple of our studies. The criteria by which this tendency is evaluated are described in paragraph

4.4. Concerning associations with hormones, only those with B-Cd, B-Pb and B-Cu were considered more than once (FLEHS I and II). B-Cd showed no associations in either study, while B-Cu showed very similar associations in both. B-Pb, B-Cu and B-As had associations with both male and female maturity markers that returned in multiple of our studies, whereas B-Cd and B-Tl only had recurring associations with female maturity markers.

It should be noted that none of the reported associations imply causality. Furthermore, some associations could be tested or were significant in only one campaign of FLEHS. Due to lack of information in the current literature, these results have a high risk of being false positives, but could still be of value in the future if similar associations are also found in other studies. Therefore, these results are reported and discussed in section 5 of the supplement. This concerns all associations with Mn, Cr, Ni, Sb, Hg, MeHg and metabolites of As, associations of hormones with B-As, B-Tl, U-Tl and U-Cu and associations of some maturity markers with B-Cu, B-Pb, B-Cd, U-Cd, U-Tl and U-Cu. However, the association of B-Pb with hormones that was only found in the FLEHS II campaign is reported here, as it is conform mechanistic information in the literature as discussed (see section 6.4.2).

6.4.2. Associations with hormones

Blood Pb is negatively associated with fE2 and fT in FLEHS II, but not in FLEHS I, although both the population (roughly 800 versus 300 observations) and the interquartile range of B-Pb concentrations (roughly 17-36 versus 12-19 µg/l) were much larger in FLEHS I. A possible explanation would be that an association exists which is linear in the lower concentration range measured in FLEHS II, but not in the higher range measured in FLEHS I (eg. above a certain concentration of lead further increase has no or an opposite effect). Due to insufficient overlap of the concentration range of these datasets, it was not possible to test this hypothesis. Concerning reported effects in animals, a study on rats by Dearth et al. (2002) suggested a negative association between plasma concentrations of lead and concentrations of oestradiol and LH. Research suggests that ROS

formation and suppression of LH caused by lead inhibits steroidogenesis (Pandya et al., 2012; Rana, 2014; Srivastava et al., 2004).

Over different campaigns, B-Cu is significantly negatively associated with aromatase, E2, fE2, T, fT and LH, and positively with SHBG. Meeker et al. (2010) confirms the negative association between B-Cu and LH, but found no significant association with T or E2. Another study reported decreased serum testosterone levels with high hair copper content in adult males (Chang et al., 2011). Prolonged administration of copper sulphate to adult males and females has been shown to have a non-monotonous effect on SHBG concentrations (Ayala et al., 2008). Unlike Pb, Cu is an essential nutrient that is only toxic at high concentrations(Gaetke and Chow, 2003). Cu is subject to a complex homeostasis in the body, since the efficiency of both absorption of Cu and its endogenous excretion through the biliary tract depend on dietary intake of copper (Araya et al., 2006; Turnlund, 1998) and also competes with other metals like zinc and iron for the same transport mechanism in the brush border. As such, differences in Cu levels in blood might not only be caused by a higher exposure, but also by individual differences in the regulation of the homeostasis. Factors that impair copper homeostasis could thus also have an effect on hormone levels and sexual maturation, perhaps more than the level of exposure.

Some literature was found that suggests a mechanism for the effects of Pb and Cu on hormone concentrations. It was reported that Cd, Pb and Cu have xenoestrogenic properties, and might even increase expression of specific genes in the presence of oestradiol beyond the normal level of expression when only oestradiol is present (Darbre, 2006). Moreover, treatment of hypogonadic men with E2 has been shown to reduce LH production (Bagatell et al., 1994). These observations may explain the effects of B-Pb and B-Cu as xenoestrogenic activity on the feedback mechanism of oestradiol by binding and activating oestrogen receptors involved in this process, thought to be located at the hypothalamus but possibly also at the pituitary gland(Shaw et al., 2010). Increased activation of these receptors would result in a negative feedback to the HPG axis, reducing

the production of LH, T and E2, which is the observed effect in our analysis, although a negative association with LH is not observed for B-Pb.

Although B-Cd was reported to have xenoestrogenic properties and inhibit steroidogenesis (Darbre, 2006; Rana, 2014), it shows no associations with hormones in our study. Possibly, there is a lack of statistical power, and/or the effect is only noticeably at higher Cd concentrations than those in our cohorts. In FLEHS I, Dhooge et al. (2009) concluded the same using similar statistical techniques, although they found associations of hormones with U-Cd corrected for creatinine. Other studies also found associations of hormones with U-Cd when correcting for creatinine (Ciarrocca et al., 2013; Lewis and Meeker, 2015). As described in the previous paragraph and introduction, creatinine correction in adolescents could be biased and since SG was not measured in FLEHS I U-Cd results of that campaign are not included in this study

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6.4.3. Associations with maturity markers

B-Cd is associated with a delay of development of pubic hair and breasts for adolescent females. This agrees with the results of Gollenberg et al. (2010) that show that cadmium might be linked to pubertal delays in girls.

B-Pb is significantly associated with delayed development of pubic hair and genitals for adolescent males and delayed menarche for adolescent females. The negative associations of B-Pb with hormone levels and male maturity markers are consistent with the link between both effect markers. Pb is known to delay maturation and growth in humans and animals at different life stages (Dearth et al., 2002; Naicker et al., 2010; Shukla et al., 1991). Other studies in humans associated Pb with a delay of the menarche (Schell and Gallo, 2010; Selevan et al., 2003; Wu et al., 2003).

B-Cu is associated with a delay of genital and pubic hair development in adolescent males, but also with an accelerated menarche in adolescent females. No literature was found reporting effects of copper on maturation.

B-As shows no association with menarche, but seems related to an acceleration of all other maturity markers. No prior studies are found that investigated these associations.

B-Tl is associated with delayed development of pubic hair and breasts of adolescent females. No literature could be found to explain the observed associations.

6.4.4. Associations with both hormones and maturity markers

The returning associations of B-Pb and B-Cu with maturity markers in adolescent males are also in line with their associations with hormones, considering the positive associations between sexual development and hormone concentrations. A suggestion for the mechanism that links hormones and maturation markers has been made in the case of Pb, based on several studies in rats. The idea is that by suppression of the secretion of sex steroid hormones (Pb- specific pathways mentioned in section 6.4.2) involved in initiation of puberty, sexual maturation is delayed (Iavicoli et al., 2009). While this has not been tested for Cu, the its associations with hormones and sexual development might have a similar basis. An alternative hypothesis, taking into consideration reversed causality, would be that certain stages of maturation coincide with an altered metabolism that results in lower B-Cu and B-Pb levels. Such an effect may exist, as it is known that absorption of some metals in the gastro-intestinal tract varies with age (Philip et al., 1998). However, an earlier publication shows that the FLEHS II adolescent dataset does not support this explanation, as there was no association of B-Cu with age, and B-Pb actually increased with age (Baeyens et al., 2014).

For B-Cd no association with hormones was found, but it could possibly still have an effect on maturation by interacting with hormone receptors not included in the HPG axis or feedback mechanism, mimicking the actions of hormones without affecting their concentrations (Toppari and Juul, 2010).

7. Strengths and weaknesses

A weakness of this study is that not all exposure and effect markers were measured in each of the 3 campaigns, due to budget constraints, practicality of field work and scientific progress. For

example,the lack of specific gravity measurements in FLEHS I prevented us from using U-Cd data from that campaing. Furthermore, most of the selected markers have relatively short half-lives of less than a month, with the exception of U-Cd. Therefore, exposure to these metals should remain relatively constant to find meaningful correlation with longer term effects like maturation. However, this assumption is (implicitly) made in all cross-sectional epidemiological studies reporting effects of pollutants on timing of maturation, a great deal of which have been included in the review by Zawatski and Lee (2013).

In spite of this, the design of this research allowed to asses exposure-response associations from different periods in comparable cohorts, and to compare the results in order to find trends. This strengthens confidence in those associations that return in different studies.

8. Conclusions and implications

From the analyses presented in this article, several conclusions can be drawn. Significant associations between sex hormones and maturation in adolescent males that could be expected based on the literature were confirmed. When studying associations of sex hormones or sexual development with urinary markers (including but not limited to metals) in adolescent males, creatinine correction is to be avoided since it shows associations with these effect markers. Possibly, creatinine and sex hormones are indirectly associated because both are associated with maturation. Specific gravity shows no significant associations with the effect markers, so urine density corrections based on this marker are preferable. A variety of associations of sex hormones and maturity markers with blood and urinary metals were found, several of which are supported by the literature. The reproducibility of associations assessed in multiple campaigns of FLEHS is limited if only significant ($P<0.05$) associations are considered. When using the weaker criteria for those associations that are significant at least once, several associations with Cd, Pb, Cu, Tl and As return in different campaigns. Associations reported in literature support the observed trends.

Of the recurring associations, B-Cu and B-Pb are of specific interest, as their effects on sex hormones and maturity markers are perfectly in line with the associations between hormones and maturity markers. In case of B-Cu and B-Pb, evidence exists for xenoestrogenic agonistic effects for these metals, which could explain the effects observed in this study through an effect on oestrogen receptors involved in the feedback mechanism of oestradiol to the HPG axis. To the best of our knowledge, this is the first epidemiological study with findings supportive of xenoestrogenic effects of B-Cu and B-Pb in humans at environmental levels. Contrary to expectations, B-Cd was not associated with hormones, although this could be due to the low exposure in our cohorts.

It would be interesting to see how creatinine and sex hormones are related in other demographics, especially adult males, to see if the associations found only occur around puberty, or have a more general occurrence. The possibility of a xenoestrogenic effect of certain metals in environmentally exposed adolescent males also warrants further exploration, especially for B-Pb, as B-Cu is involved in a complex homeostasis and external exposure at concentrations found in our study is expected to have a smaller effect on internal exposure, and thus possibly on hormones and maturation, than for B-Pb.

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