**Association of prenatal exposure to heavy metal mixtures and anogenital distance in newborns**

**BACKGROUND:** Although the association between prenatal exposure to multiple heavy metals and newborns' anthropometric measures has been extensively studied, little is known about the reproductive toxicity and the endocrine disturbance characteristics of these metals.

**METHODS:** We used data of 889 mother-infant pairs, from two major hospitals in Israel. Associations between eight metals (arsenic, cadmium, chromium, mercury, lead, nickel, selenium and thallium) detected in maternal urine samples from day of delivery to anogenital distance index (AGI) at birth were examined. Adjusted estimates were calculated separately for males and females, using single-exposure models, and weights quantile sums (WQS) models accounting for metals mixtures.

**RESULTS:** Females were found more susceptible to prenatal metals exposure as their z-scaled ano-clitoral distance index (AGIac) was positively associated to chromium (β = .158 (95% CI: .061 – .256)), nickel (β = .083 (95% CI: .005 – .161)) and thallium (β = .140 (95% CI: .022 – .258)). Their z-scaled ano-fourchette distance index was positively associated to nickel (β = .079 (95% CI: .001 – .158)). Z-scaled ano-scrotal distance index (AGIas) was the only measure found associated with exposure in the WQS models (β = -.329 (95% CI: -.629 – -.030)) and was highly associated with nickel and selenium. In the single-exposure models, chromium was found positively associated (β = .111 [95% CI: .017 – .206]) to the Z-scored anoscrotal distance index (AGIas) among males.

**CONCLUSIONS:** Our findings suggest that prenatal exposure to chromium, nickel and thallium may be associated to alterations of females AGD, while chromium, nickel and selenium to changes in males AGD. Since AGD alterations could represent wider endocrine interruptions, the effects of these metals on biological and chemical mechanisms during the vulnerable period of pregnancy should be further investigated.

**1. INTRODUCTION**

Newborn's anthropometric measures are commonly used as indicators for fetal growth, and are strongly associated to prenatal conditions in the intrauterine environment1,2. Numerous epidemiological studies have suggested associations between detectable alterations in newborn's anthropometric measures and long-term health outcomes, including: morbidity and mortality3, cognitive abilities 4,5and neurodevelopmental outcomes6. In many cases these anthropometric alterations are considered the 'tip of an iceberg', representing only one of the many possible outcomes of complicated intrauterine biological mechanisms7, involving genetic as well as environmental factors.

One of the fetal measures considered sensitive to intrauterine exposures is anogenital distance (AGD). This dimorphic measure represents the distance from the newborn's anus to the genitals and is longer among males than females8. It was previously suggested that androgens played a time-dependent role in the formation of the perineal growth9 during the masculinization programming window (MPW), fixing the AGD *in utero*. Thus, AGD may serve as a lifelong biomarker of androgen exposure during this window10 (8–14 weeks of gestation), and could shed light on intrauterine endocrine cascades and disruptions11. It was previously suggested that endocrine-disrupting chemicals (EDCs) that interfere the delicate mechanism of perineal formation, could alter the anogenital distance of laboratory animals12 as well as of humans13. These chemicals including a large group of phthalates, plasticizers found in a large number of commonly used consumer products including food, plastics, cosmetics and cleaning products14. Prenatal exposure to these chemicals and their derivates, has been largely associated to alterations in newborn's AGD15–18, timing of delivery19, hypospadias20 and future decrease in fertility21.

While most studies conducted in this field examined the association between prenatal exposure to various known endocrine disruptors22,23 including phthalates, phenols, and persistent organic pollutants to newborn's AGD15–17,24, only few have examined the association between prenatal exposure to heavy metals and newborn's measured AGD25. Since prenatal exposure to heavy metals was previously suggested to associate with various newborn's adverse health outcomes including low birth weight26, birth size27 and various congenital abnormalities 28, their possible association to AGD alterations cannot be neglected, even if not fully understood. This association was previously examined in a large study conducted by Huang et al. (2020) where negative associations between maternal exposure to lead and chromium with anogenital distance of male newborns were found. Yet, the median concentrations of pollutants tested in this study were relatively high and did not necessarily represent a normal daily exposure.

In the current study, we aimed to examine the association between prenatal exposure to mixture of metals as measured in maternal urine, and newborn's AGD. We investigated concentrations of eight metals (arsenic-As, cadmium-Cd, chromium-Cr, mercury-Hg, nickel-Ni, lead-Pb, selenium-Se, and thallium-Tl) in maternal urine samples, and examined their association to AGD, individually and by using Weighted Quantile Sum regression (WQS) approach, which adjusts simultaneously for all exposures to enable the recognition of metals that contribute mostly to the alterations in AGD29,30.

**2. METHODS**

**2.1. STUDY DESIGN**

Beginning in 2016, pregnant women and their newborns were recruited in delivery rooms of two hospitals in Israel: (1) Rambam Medical Center – the largest hospital in the Northern District of Israel, which accounts for around 5500 births annually, and (2) Shamir Medical Center – located in the Central region of Israel and which accounts for around 8000 deliveries annually. Women were considered eligible if they were Hebrew speaking, aged 18 years or older, and pregnant with a singleton. Exclusion criteria included: (1) preterm birth (<37 weeks of gestational age); (2) pregnancies considered by the medical staff to have a high risk of complications (e.g., autoimmune diseases, hypertension, diabetes)31; (3) minor or major congenital malformations as defined by the United States Centers for Disease Control and Prevention (CDC) and the European network of population-based registries for the epidemiological surveillance of congenital anomalies (EUROCAT)32,33. A specialized study coordinator in each hospital obtained written informed consent from each woman prior to her participation and completed a questionnaire covering variables including sociodemographic characteristics, tobacco exposure, health status, pregnancy, and obstetric history. A total of 904 mother-newborn pairs recruited from both hospitals(Figure 1). Maternal urine samples were collected from all participants at the day of delivery, and newborns anthropometric measures as well as AGD examined by specialized neontologists.

**2.2. EXPOSURE MEASUREMENTS**

Each participant was asked to provide a single urine sample. The samples were frozen at −80°C immediately after receiving them and then transported at −20°C for further analysis at the Central Public Health Laboratory of the Israeli Ministry of Health (Abu-Kabir). We measured levels of As, Cd, Cr, Hg, Ni, Pb, Se, and Tl using inductively coupled plasma mass spectrometry (ICP-MS), on an Agilent 7800x ICP-MS instrument equipped with an Integrated Sample Introducing System (ISIS) and High Matrix Introducing mode (HMI). The procedure involved acid dilution of urine and direct injection to the ICP-MS instrument, followed by helium dilution in the HMI instrument. The method followed standard quality assurance and quality control procedures. Urinary metal concentrations were quantified using internal standard calibration procedures and certified analytical standards. Quality control was performed by analyzing aliquots of control material in each series (every ten samples), and accuracy was validated by the annual successful participation in the international proficiency test (G-EQUAS) for all parameters. Urine creatinine was measured using a well-established colorimetric method at the Central Teratology Laboratory at the Shamir Medical Center and was used to standardize the metal concentration detected in the urine samples.

**2.3. CLINICAL MEASUREMENTS**

As part of the routine physical examination, all infants were administered a standard examination. Data concerning birth weight, and AGD was collected and documented under an anonymous number each mother-child pair had received. A total of 904 measures of weight and AGD were conducted (Figure.1). AGDs were measured by a certified pediatrician who specialized at Thankamonys method34 particularly for this study. In this procedure, two measures of AGD were obtained for males; anopenile (AGDap) and anoscrotal (AGDas), and two AGD measures for females: anoclitoris (AGDac) and anofourchette (AGDaf). For this study AGDap and AGDac will be referred as "long" AGD, while AGDas and AGDaf will be referred as "short" AGD. For reliability, each measurement was repeated three times, and mean values were computed. All results were documented in the newborns' medical records.

**2.4. COVARIATES**

Using the comprehensive data collected from each mother via the questionnaires and data collected from maternal medical registries, we were able to adjust our final models to possible confounders including maternal age (continuous, in years), newborn's gender, previous parities (Null puros vs. multiparous) smoking exposure during pregnancy (yes vs. no), sociodemographic status (SES) (standardized score) and geographic area. Maternal standardized SES index was calculated individually using matching of maternal reported zip codes and geographical distribution of SES as reported yearly by the Central Bureau of Statistics35 via geographical information systems (GIS).

Since gestational age could function as a mediator effecting the pathway between exposure and outcome 36, leading to over- or under-estimation of the true effects37 it was excluded from further analysis.

Information on cigarette, cigar, or pipe smoking and the degree to which women were exposed to environmental tobacco smoke during pregnancy was self-reported by participants. Women considered to be smoke exposed if they reported either being an active smoker or were exposed to environmental tobacco smoke for 1 hour or more per week during at least one half of the pregnancy.

**2.5. STATISTICAL ANALYSIS**

To assess the overall effect of correlated metals and their individual effect on AGD, WQS regression, with linear link function connecting outcome mean to a weighted sum of exposure quartiles and covariates, was used. This method was previously described in details29, and was used in the analysis of the effect of prenatal exposure to a mixture of pollutants in previous studies30,38,39. The WQS model aims to address collinearity by examining sets of weights to the metal's variables. For every set of weights, a weighted quartile sum, that is the multiplication of every weight with the quartile level of the metabolite, is calculated and a multivariate linear regression is applied. The set of weights that predicts the AGD measures most accurately is selected, and the coefficients for the weighted quantile and the covariates are calculated. In order to determine the importance of each element in the final weight, it was previously suggested that a weight higher than mean portion of each element (1/8 in our study) would be considered high. In the secondary analysis we evaluated the associations of exposure to individual metals during pregnancy and standardized AGD using multivariate linear regression models.

WQS as well as linear models were conducted separately for males and females for both the 'long' and the 'short' AGD measures. All models were adjusted for maternal age, previous parities, SES, geographic area and maternal tobacco exposure. For sensitivity analysis, WQS models were bootstrapped 10,000 times, and all models were reconducted including gestational age, and after exclusion of newborns defined as small and large for gestational age (SGA and LGA respectively).

All metals concentrations were divided by creatinine to account for urine dilution, and modeled as natural log–transformed and standardized for interquartile range (IQRs), in order to achieve a common scale and account for the positive skewness detected. For further analysis we used the z-scaled anogenital index (AGI = AGD/newborn's weight), as described in previous studies13,24,40. Statistical significance was 2-sided and set at p < .05. All statistical processes were performed using R (version 4.1.1; R Foundation for Statistical Computing) including the R packages: *'data.table'*, '*ggplot2*', '*dplyr*', '*lubridate*' and '*gWQS*'.

**3. RESULTS**

Among 904 mother-newborn pairs recruited to the study, the mean maternal age (SD) was 32.2 (4.5) years, and the mean (SD) gestational age at delivery was 39.51 (1.34) weeks; 478 children (53%) were male, and 426 (47%) were female (Table 1.). Z-scaled AGD measures of 15 newborns were found three or more standard deviations from the mean, thus defined as outliers, and excluded from the analysis.

Characteristics of the 889 newborns included in the final analysis appear in Table.2. The distributions of metals metabolites concentrations were presented in Figure.2. As, Cd, Cr, Pb and Se were detected in 92.5% of urine samples while Ni and Tl were detected in 88.3% and 89.1% respectively. Concentrations of most metals' metabolites were significantly correlated with a maximal Spearman correlation coefficient of .44 (Pb-Cd), and the rest between .05 to .28 (Figure.3).

A significant decrease in 'long' AGI among males (n=471) was observed but not among females (n=417), associated to increase levels of WQS of the metals examined in our study. The covariates adjusted coefficient estimates were β = -.329 (95% CI: -.629 – -.030) for males and β = -.140 (95% CI: -.135 – .416) for females. As shown in Figure.4, among the eight metals, those with the highest weights for anopenile length determination among males were Ni and Se. Levels of WQS were neither associated with the 'short' AGD measures for both males (β = -.07 [95% CI: -.204 – .350]) and females (β = -.04 [95% CI: -.343 – .245]).

Each metal was individually included in a multivariate linear model and its independent association to AGI measures was examined (Figure.5). Increased Cr concentrations were found associated to an increase in the adjusted AGIas (β = .111 [95% CI: .017 – .206]), while no other metal was individually associated to this measure, as well as to the AGIap of male newborns.

Among females increasing levels of Cr, Ni and Tl were positively associated to increased adjusted AGIac with beta coefficients of: β = .158 (95% CI: .061 – .256); β = .083 (95% CI: .005 – .161); and β = .140 (95% CI: .022 – .258) respectively. An increase in the level of Ni was also positively associated the AGIaf adjusted measure β = .079 (95% CI: .001 – .158). No other metal was associated to none of the AGI measures among females while adjusted for the background characteristics. Including gestational age, as well as exclusion of SGA and LGA newborns from the models did not change the significancy of the coefficients obtained from the models.

**4. DISCUSSION**

To our knowledge, this is the first report to examine the association between maternal prenatal exposure to various metals and the anogenital index of both female and male newborns. Although the collinearity among the metals detected in the specimens was not strong, we examined the associations using WQS models with a weighted sum of quartiles of different metals exposure as well as multivariate linear models. The results obtained from both models were inconsistent since the WQS regression showed solely a significant negative association between the whole group of metals and AGIap among males, while the linear models suggested various associations between metals and AGI measures for both genders. These differences highlight the attention one needs to imply before using WQS models since the inclusion of compounds with various structures and biological mechanisms in the same model could result in biases.

During fetal life, the masculinization of genitalia depends on androgens and the production of testosterone by the Leydig cells of the fetal testicles and on its action on target organs41. It is associated with the masculinization of external genitalia42 and elongation of AGD11. Thus, its absence is associated with shorter AGD, and feminization of external genitalia43. It is not unlikely that affected testosterone-mediated pathways could alter the AGD of newborns. Previous studies have shown that newborn AGD could be associated with both; testosterone levels in maternal42 as well as fetal10 bloodstreams. Hence, AGD alterations could reflect interruptions of multiple pathways.

Although previous studies have reported associations between prenatal exposure to various metals and anthropometric measures alternations among newborns26,44–46, very few studies have examined the association between prenatal exposure to heavy metals and AGD measures25. Although the exact biological pathways interrupted by high levels of metals are still a subject of ongoing research, emerging evidence suggests that heavy metals could affect steroid receptor pathways (e.g. estrogen, progesterone, testosterone, corticosteroids, and mineralocorticoids)47, hence could potentially affect the androgen-dependent growth of the anogenital area. Our study did suggest associations between increasing levels of Cr, Ni, Se, and Tl in maternal urine with alterations in AGI among newborns from both genders.

The individual metal-exposure models conducted suggested a positive association between Cr levels in maternal urine and AGIac as well as AGIas in females and males respectively. These findings suggest a dysregulation of the androgen-dependent growth of the anogenital area, whether by a direct effect on androgen receptors, gene regulation, or testosterone production of the fetus or by indirectly affecting the production of the placental androgens. Although a previous study has demonstrated decreased testosterone levels among fetuses of high levels of Cr-exposed rats48, it also showed increased levels of testosterone among offspring of rats exposed to relatively lower levels of Cr. Yet, it is possible that circulating testosterone levels may not be the most important indicator of androgen exposure to the external genitalia49.

Although suggested in our study, the positive associations between maternal urine concentrations of Tl and Ni with AGIac among females were not supported by any other literature, yet few explanations could be suggested; In a study conducted by Ashrap et.al (2019)50 high concentrations of Ni in urine samples of female teenagers were associated with higher levels of testosterone and changes in pubic hair development – these outcomes together suggest possible disruptions of steroids and androgens production pathways51. Since the placenta is permeable to testosterone and androgens, any damage to the placental testosterone-inactivating enzymes (eg. 17βHSD2) can expose the fetus to excess testosterone levels52 and enhance masculinization pathways. Recent studies have associated prenatal exposure to Tl and Ni with placental inflammation and oxidative stress53–55, a condition involving the expression of reactive oxygen species (ROS), highly reactive molecules that can eventually cause structural and physiological damages to DNA, RNA, proteins and lipids56,57. Hence, oxidative stress mediated by heavy metals could potentially affect placental testosterone-inactivating enzymes, thus increasing the amount of testosterone crossing the placental barrier. Yet further research is needed to clarify the mechanisms of action of Tl and Ni in endocrine pathways within the placental and fetal circulation.

While excess activation of testosterone-mediated endocrine pathways is more detectable in female compared to male newborns, an absence or deficiency of testosterone is associated with more adverse outcomes among males58 including; hypogonadism and shorter AGD59. Since testosterone is mainly produced in the Leydig cells of the fetal testicles, its insufficiency is often associated with under masculinization, cryptorchidism, and micropenis60. There is growing evidence to show that several prenatal exposures affect not only the mature Leydig cell function but also their progenitor stem cells, thus affecting Leydig cell development during the fetal and postnatal period61,62. Animal studies have associated postnatal exposure to Pb and Se with spermatogonia and Leydig cells injury63, and Cd exposure to testicular DNA damage and decreased levels of testosterone64,65. The findings obtained from our WQS models, showed a negative association between prenatal exposure to metals-mixture and male newborn's AGIap, suggesting Ni and Se were the most prominent factors. These findings are consistent with a previous study66 associating Ni exposure with testicular damage as well as hypothalamic-pituitary-testis axis disruption in mice. According to Yang et.al (2021)66 Ni was not associated directly with any negative effect on the hypothalamus and pituitary gland but was related to markedly suppressed levels of genes related to testosterone biosynthesis in Leydig cells, a finding that has been well studied in several other studies67,68. The different associations detected between Ni exposure to AGI among males and females could suggest a sex-dependent mechanism, as described previously for several exposures in a study conducted by Thankamony et.al (2016)11.

Interestingly, the findings regarding the effects of Se on Leydig cells were less consistent: while a study conducted by Gan et.al (2019)69 suggested Se could attenuate the Ni-induced testosterone synthesis disturbance, another study found a negative association between Se levels and expression of testicular house-keeping genes70. The protective effect of Se and its role in the maintenance of testicular cells71, as well as reproduction systems of both males and females72 were largely investigated. As a component of selenoproteins, Se plays a structural and enzymatic role in many biological pathways, and it is best recognized for its catalytic and antioxidant activities73,74. Due to its protective characteristics, a sufficient daily Se consumption of 60 µg/day is highly recommended during pregnancy by World Health Organization75 (WHO), yet its application has to be carefully dosed owing to the narrow margin between the recommended level of intake and toxicity76. The findings showed in the study of Shi et.al (2017)71, revealed the double-edged sword nature of Se; while an average exposure to Se was positively associated with expression of testosterone production-related genes in Leydig cells cultures, higher levels were associated with accelerated cells death. Since neither Ni nor Se single-exposure model suggested a significant association between exposure levels and AGD, it is possible that a biological or chemical interaction of the two could affect the AGD setting mechanism; yet these findings require a better understanding of the mechanism involving those metals and should be the subject of further research.

The current study has several strengths; the large sample size; the examination of multiple metals; and the use of classic methods as well as WQS modeling analysis. However, there were also several limitations; Levels of metals observed in our study were relatively low; enabling us to examine the possible effect of daily exposures on one hand but on the other limiting the scope of outcomes associated with high concentrations and wide variance. Since the metals detected in the urine sample exhibited low variance, some associations detected might be spurious and should be further investigated in future epidemiological as well as in-vitro and in-vivo biochemical studies.

Although metals could be measured in urine and were corrected to maternal hydration condition, they had various half-lives with some concentrations reflecting exposure that occurred in the past few days (eg. As, Ni, Pb, Se, Tl), and others reflecting exposures over past weeks and months (eg. Cd, Cr, Hg)77–80. Since maternal urine samples were collected only on the day of delivery, the duration, as well as the critical window of exposure cannot be inferred. Thus, they should be further studied using more frequent examinations of metals in maternal urine throughout the pregnancy in future studies.

**5. CONCLUSION**

Using a large sample size and multi-metal mixture data, we examined the potential association between prenatal maternal exposure and newborn's AGD. Prenatal Ni exposure was positively associated with ano-clitoris and posterior fourchette lengths among females, and along with Se was negatively associated with ano-penile length among males. Cr exposure was positively associated to ano-scrotal length among males, and with ano-clitoral length among females. Tl exposure was positively associated to ano-clitoral length in females. Our findings reflect the necessity of a deeper understanding of the reproductive developmental effects of prenatal exposure to these metals and indicate the possible need to pay more attention to the metal exposures during pregnancy. Since AGD alterations could be only the 'tip of an iceberg' representing other disrupted endocrine pathways that are yet to be detected, newborns should be physically and behaviorally assessed later in their lives. However, our findings need to be confirmed in other populations, and the underlying biological and chemical mechanisms should be clarified.

**FIGURES AND TABLES**

***Figure 1.*** *Flowchart of the population included in our final analysis.*

***Table 1.*** *Participant sociodemographic, current pregnancy characteristics, and newborn's anthropometric measures (N =904).*

***Table 2.*** *Newborn characteristics stratified according to newborn gender (n=889).*

***Figure 2****. Concentrations of metals metabolites (adjusted for urine’s creatinine and log transformed as well as IQR standardized) were measured in maternal urine at admission to the hospital (n = 889).*

***Figure 3****. Correlations between metals metabolites (adjusted for urine’s creatinine and log transformed as well as IQR standardized).*

***Figure 4****.  Weights of all measured metals in association anopenile distance, results from a Weighted Quantile Sum regression.*

***Figure 5****.  Beta coefficients of 'long' and 'short' anogenital indices as function of log transformed IQR standardized metal concentrations (μg/g creatinine) calculated in linear models and adjusted for maternal background characteristics.*

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