**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 anthropometric measures are commonly used as fetal growth indicators and are strongly associated with prenatal conditions in the intrauterine environment1,2. Numerous studies suggest associations between alterations in newborn anthropometric measures and long-term health outcomes including morbidity and mortality3, cognitive abilities 4,5, and neurodevelopmental outcomes6. These anthropometric alterations are considered to represent one of countless outcomes of complicated intrauterine biological mechanisms7, which involve genetic and environmental factors.

One fetal measure considered sensitive to intrauterine exposures is anogenital distance (AGD). This dimorphic measure represents the distance from the newborn’s anus to its genitals and is longer in males than females8. It was previously suggested that androgens played a time-dependent role in perineal formation9 during the masculinization programming window, fixing the AGD *in utero*. Thus, AGD may serve as a lifelong androgen exposure biomarker during this window10 (8–14 weeks of gestation) and could elucidate intrauterine endocrine cascades and disruptions11. It was also suggested that endocrine-disrupting chemicals that disturb the delicate perineal formation mechanism could alter the AGD of laboratory animals12 and humans13. These chemicals include phthalates—plasticizers found in several common consumer products14. Prenatal exposure to these chemicals and their derivates has been associated with alterations in newborn AGD15–18, delivery timing19, hypospadias20 and future fertility decreases21.

While many studies examined the association between prenatal exposure to various endocrine disruptors22,23 (e.g., phthalates, phenols) and newborn AGD15–17,24, few have examined the association between prenatal exposure to heavy metals and newborn measured AGD25. Since prenatal heavy metal exposure has been associated with adverse newborn health outcomes including low birth weight26, birth size27, and congenital abnormalities 28, their possible association with AGD alterations cannot be ignored. Huang et al. (2020) found negative associations between maternal exposure to lead and chromium and newborn male AGD. However, the median concentrations of pollutants tested in this study were relatively high and did not necessarily represent normal daily exposure.

Here, we examine the association between prenatal exposure to metal mixtures as measured in maternal urine and newborn AGD. We investigated the 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 with AGD individually and using a weighted quantile sum (WQS) regression approach, which adjusts for all exposures to identify metals that strongly influence changes in AGD29,30.

**2. METHODS**

**2.1. STUDY DESIGN**

In 2016, mother-newborn pairs were recruited from two hospitals: (1) Rambam Medical Center – located in the Northern District of Israel (~5500 births annually), and (2) Shamir Medical Center – located in the Central region of Israel (~8000 deliveries annually). Women were eligible if they were 18 years or older, Hebrew-speaking, and had a singleton pregnancy. Exclusion criteria included: (1) preterm birth (<37 weeks of gestational age); (2) pregnancies with possible complications (e.g., hypertension, diabetes)31; (3) minor/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. Written informed consent was obtained from mothers before participation and they completed a questionnaire covering several variables (e.g., sociodemographic characteristics, tobacco exposure). In total, 904 mother-newborn pairs were recruited (Figure 1). Maternal urine samples were collected from participants on the day of delivery and newborn anthropometric measurements and AGD were examined by specialized neontologists.

**2.2. EXPOSURE MEASUREMENTS**

Participants provided a single urine sample. Samples were frozen at −80°C immediately after receipt and transported at −20°C for further analysis at the Central Public Health Laboratory of the Israeli Ministry of Health (Abu-Kabir). We measured As, Cd, Cr, Hg, Ni, Pb, Se, and Tl levels 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 (HMI) mode. The procedure involved acid dilution of urine and direct injection into the ICP-MS instrument, followed by helium dilution in the HMI instrument. This 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 control material aliquots in a series of ten samples, and accuracy was validated with 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 of the Shamir Medical Center and was used to standardize the metal concentration detected in the urine samples.

**2.3. CLINICAL MEASUREMENTS**

All infants were administered a standard physical examination. Birth weight and AGD data was collected and documented under an anonymous number each mother-child pair was assigned. In total, 904 weight and AGD measurements were collected (Figure 1). AGDs were measured by certified pediatricians specializing in Thankamony’s method34. In this procedure, two AGD measures were obtained for males: anopenile (AGDap) and anoscrotal (AGDas); and two for females: anoclitoral (AGDac) and anofourchette (AGDaf). Hereafter, AGDap and AGDac will be called “long” AGD, while AGDas and AGDaf will be called “short” AGD. For reliability, each measurement was repeated three times, and mean values were computed. Results were documented in the newborns’ medical records.

**2.4. COVARIATES**

Using the comprehensive data collected from the questionnaires and maternal medical registries, we adjusted our final models to possible confounders including maternal age (continuous, in years), newborn gender, previous parities (nulliparous vs. multiparous), pregnancy smoking exposure (yes vs. no), sociodemographic status (SES) (standardized score), and geographic area. Maternal standardized SES indices were calculated individually by matching maternally reported zip codes and geographical distribution of SES as reported yearly by the Central Bureau of Statistics35. Since gestational age could function as a mediator affecting the pathway between exposure and outcome 36, leading to over-/under-estimation of true effects37, it was excluded from further analysis.

Information on smoking (e.g., cigarette, pipe) and the degree of exposure to environmental tobacco smoke during pregnancy was self-reported by participants. Women were considered “smoke-exposed” if they reported being an active smoker or exposure to environmental tobacco smoke for 1 hour or more/week during at least one-half of the pregnancy.

**2.5. STATISTICAL ANALYSIS**

To assess the effect of correlated metals and their individual effects on AGD, WQS regression was used with a linear link function connecting outcome mean to a weighted sum of exposure quartiles and covariates. This method was previously described in detail29 and used to analyze the effect of prenatal exposure to a mixture of pollutants30,38,39. The WQS model aims to address collinearity by examining sets of weights to the metal’s variables. For every set of weights, a weighted quantile sum—every weight multiplied by 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 covariates are calculated. To determine the importance of each element in the final weight, it was previously suggested that a weight higher than the mean portion of each element (1/8 in our study) would be considered high. During secondary analysis, we evaluated the influence of individual metal exposure during pregnancy and standardized AGD using multivariate linear regression models.

WQS and linear models were conducted separately for males and females for the ‘long’ and ‘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 excluding newborns defined as small and large for gestational age (SGA and LGA, respectively).

All metal concentrations were divided by creatinine to account for urine dilution and modeled as natural log–transformed and standardized for interquartile range (IQRs) to achieve a common scale and account for positive skew. 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 to p < .05. All statistical processes were performed using R (version 4.1.1; R Foundation for Statistical Computing) and several R packages: *'data.table'*, '*ggplot2*', '*dplyr*', '*lubridate*', and '*gWQS*'.

**3. RESULTS**

Among the 904 mother-newborn pairs recruited, 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 three or more standard deviations from the mean; these were defined as outliers and excluded from analysis. Characteristics of the 889 newborns included in the final analysis appear in Table 2. The metal metabolite concentration distributions 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% of samples, respectively. Most metal metabolite concentrations were significantly correlated with a maximal Spearman correlation coefficient of .44 (Pb-Cd), and the rest between .05 and .28 (Figure 3).

A significant decrease in ‘long’ AGI was observed in males (n=471), but not females (n=417), was associated with increased WQS levels of the metals examined. The covariate-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, Ni and Se had the highest weights for male anopenile length determination. WQS levels were not associated with the ‘short’ AGD measures for either males (β = -.07 [95% CI: -.204 – .350]) or females (β = -.04 [95% CI: -.343 – .245]).

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

Among females, increasing Cr, Ni, and Tl levels were positively associated with 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. Increased Ni levels were also positively associated with the AGIaf adjusted measure β = .079 (95% CI: .001 – .158). No other metal was associated with female AGI measures while adjusted for background characteristics. Including gestational age and excluding SGA and LGA newborns from the models did not change the significance of the models’ coefficients.

**4. DISCUSSION**

To our knowledge, this is the first report examining the association between maternal prenatal metal exposure and the AGI of female and male newborns. Although collinearity among metals detected in the specimens was not strong, we examined the associations using WQS models with a weighted sum of quartiles of different metal exposures and multivariate linear models. The results of both models were inconsistent since the WQS regression only showed a significant negative association between the entire metal group and male AGIap, while the linear models suggested various associations between metals and AGI measures for both genders. These differences highlight the attention one needs to pay when using WQS models since including compounds with various structures and biological mechanisms in the same model could result in biases.

During fetal life, genital masculinization depends on androgens and testosterone production by fetal testicular Leydig cells and its action on target organs41. It is associated with external genitalia masculinization 42 and AGD elongation11. Thus, its absence is associated with shorter AGD and external genitalia feminization 43. It is possible that affected testosterone-mediated pathways could alter newborn AGD. Studies have shown that newborn AGD could be associated with maternal testosterone levels 42 and fetal10 bloodstreams. Hence, AGD alterations could reflect multiple pathway interruptions.

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

The individual metal-exposure models suggested a positive association between Cr levels in maternal urine and female AGIac and male AGIas, respectively. These findings suggest a dysregulation of androgen-dependent anogenital growth, whether by a direct effect on androgen receptors, gene regulation, or fetal testosterone production, or by indirectly affecting placental androgen production. Although another study demonstrated decreased testosterone levels among fetuses of highly Cr-exposed rats48, it also showed increased testosterone levels among rat offspring exposed to lower Cr levels. It is possible that circulating testosterone levels are not a critical indicator of androgen exposure to external genitalia49.

While suggested here, the positive associations between maternal Tl and Ni urine concentrations with female AGIac were not supported by other literature; however, this could be explained. According to Ashrap et al. (2019)50, high Ni concentrations in urine samples of female teenagers were associated with higher testosterone levels and pubic hair development changes– together suggesting possible steroid and androgen production pathway disruptions51. Since the placenta is permeable to testosterone and androgens, any damage to the placental testosterone-inactivating enzymes (e.g., 17βHSD2) can expose the fetus to excess testosterone levels52 and enhance masculinization pathways. Recent studies have associated prenatal Tl and Ni exposure with placental inflammation and oxidative stress53–55, which can cause structural and physiological damage to DNA, RNA, proteins, and lipids56,57. Hence, oxidative stress mediated by heavy metals could affect placental testosterone-inactivating enzymes, increasing the amount of testosterone crossing the placental barrier. Further research is needed to clarify the action mechanisms of Tl and Ni in endocrine pathways within placental and fetal circulation.

While excess activation of testosterone-mediated endocrine pathways is more detectable in female newborns than males, testosterone absence or deficiency is associated with more adverse outcomes in males58 (e.g., hypogonadism, shorter AGD59). Since testosterone is mainly produced in fetal testicular Leydig cells, its deficiency is associated with under-masculinization, cryptorchidism, and micropenises60. Growing evidence shows that several prenatal exposures influence mature Leydig cell function and 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 testosterone levels64,65. Our WQS models showed a negative association between prenatal exposure to metals-mixture and male newborn AGIap, suggesting Ni and Se were vital factors. These findings are consistent with another study66 associating Ni exposure with testicular damage and hypothalamic-pituitary-testis axis disruption in mice. According to Yang et al. (2021)66, Ni was not directly associated with negative effects in the hypothalamus and pituitary gland but was related to markedly suppressed levels of genes associated with testosterone biosynthesis in Leydig cells, a finding thoroughly investigated in previous studies67,68. The different associations detected between Ni exposure to AGI among both genders could suggest a sex-dependent mechanism, as described by Thankamony et al. (2016)11.

Interestingly, the findings regarding the effect of Se on Leydig cells were less consistent; Gan et al. (2019)69 suggested that Se could attenuate the Ni-induced testosterone synthesis disturbance. Another study found a negative association between Se levels and testicular house-keeping gene expression70. Se’s protective effect and its role in testicular cell maintenance71 and male and female reproduction systems72 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, the World Health Organization (WHO) recommends 60 µg/day of Se during pregnancy75; however, Se must be carefully dosed due to the narrow margin between the recommended intake level and toxicity76. Shi et al. (2017)71 revealed the double-edged nature of Se; while average Se exposure was positively associated with testosterone production-related gene expression in Leydig cell cultures, higher levels were associated with accelerated cell death. Since neither Ni nor Se single-exposure models suggested a significant association between exposure levels and AGD, it is possible that a biological or chemical interaction between them could affect the AGD setting mechanism. These findings require a better understanding of the mechanism and further research.

Our study has several strengths: large sample size, examination of multiple metals, and use of classic methods and WQS modeling analysis. However, there were also several limitations: levels of metals observed were relatively low, enabling us to examine the possible effect of daily exposure but also limiting the scope of outcomes associated with high concentrations and wide variance. Since the metals detected in the urine samples exhibited low variance, some detected associations might be weak and should be further investigated in future epidemiological, in-vitro, and in-vivo biochemical studies.

Although metals could be measured in urine and were corrected to maternal hydration conditions, they had various half-lives with some concentrations reflecting very recent exposure (e.g., As, Ni, Pb, Se, Tl), and others reflecting exposures over weeks and months (e.g., Cd, Cr, Hg)77–80. Since maternal urine samples were only collected on the day of delivery, the duration and critical window of exposure are uncertain. Thus, more frequent examinations of metals in maternal urine should occur 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 AGD. Prenatal Ni exposure was positively associated with female anoclitoral and posterior fourchette lengths, and Se was negatively associated with male anopenile length. Cr exposure was positively associated with male anoscrotal length and female anoclitoral length. Tl exposure was positively associated with female anoclitoral length. Our findings reflect the importance of examining the reproductive developmental effects of prenatal metal exposure and the need to further examine metal exposure during pregnancy. Since AGD alterations could represent other disrupted endocrine pathways yet to be detected, newborns should be physically and behaviorally assessed later in life. However, future studies should examine other populations and clarify the underlying biological and chemical mechanisms.

**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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