Androgenic and estrogenic indices in human newborns and infants: the MIREC-ID study

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Abstract

Prenatal sex steroid exposure plays an important role in determining child development. Yet,
measurement of prenatal hormonal exposure has been limited by the paucity of newborn/
infant data and the invasiveness of fetal hormonal sampling. Here we provide descriptive data
from the MIREC-ID study ($n = 173$ girls; 162 boys) on a range of minimally invasive physical
indices thought to reflect prenatal exposure to androgens [anogenital distances (AGDs);
penile length/width, scrotal/vulvar pigmentation], to estrogens [vaginal maturation index
(VMI) – the degree of maturation of vaginal wall cells] or to both androgens/estrogens [2nd-
to-4th digit ratio (2D:4D); areolar pigmentation, triceps/sub-scapular skinfold thickness, arm
circumference]. VMI was found to be associated with triceps skinfold thickness ($\beta = 0.265$,
$P = 0.005$), suggesting that this marker may be sensitive to estrogen levels produced by
adipose tissue in girls. Both estrogenic and androgenic markers (VMI: $\beta = 0.338$, $P = 0.031$;
2D:4D – right: $\beta = -0.207$, $P = 0.040$; left: $\beta = -0.276$, $P = 0.006$; AGD-fourchette –
$\beta = 0.253$, $P = 0.036$) were associated with areolar pigmentation in girls, supporting a role for
the latter as an index of both androgen and estrogen exposure. We also found AGD-penis
(distance from the anus to the penis) to be associated with scrotal pigmentation ($\beta = 0.290$,
$P = 0.048$), as well as right arm circumference ($\beta = 0.462$, $P < 0.0001$), supporting the notion
that these indices may be used together as markers of androgen exposure in boys. In sum,
these findings support the use of several physical indices at birth to convey a more
comprehensive picture of prenatal exposure to sex hormones.

Introduction

Exposure to androgens and estrogens *in utero* is known to play an important role in deter-
mining future development of the child, in terms of both physical and brain maturation.[1,2]
However, obtaining accurate and reliable measurements of fetal exposure to androgens and
estrogens has been limited by ethical and methodological challenges. Such challenges include
the invasiveness and acceptability of hormonal sampling (e.g., amniotic fluid, maternal blood
or umbilical cord blood), and the great variability of hormonal levels depending on fetal sex,
sampling site and window of exposure (e.g., trimester of pregnancy).[3] In addition, hormonal
levels do not necessarily correspond to the magnitude of their actions on target tissues such as
the skin, genitals and brain. Rather, the magnitude of the overall hormonal effect is deter-
mined in great part by local factors such as the degree of activity of genomic and non-genomic
pathways mediated by nuclear and membrane androgen and estrogen receptors at a specific
site.[4–6]

Thus, the search for reliable indicators of androgen and estrogen exposure during preg-
nancy continues to be relevant and critical to our understanding of fetal growth and devel-
opment. Because the sexual maturation process is dependent on exposure to sex steroids,[7,8]
measures of genital and sexual development such as anogenital distances (AGD), penile
length/width and vaginal maturation index (VMI – the degree of maturation of vaginal wall
cells) represent putative androgenic or estrogenic markers.[9–21] Similarly, the pigmentation of
genital areas (e.g., vulva and scrotum), as well as that of other sexualized organs such as the
nipple–areolar complex, represents additional measures of sexual maturation thought to vary
with sex steroid levels.[21–23] Finally, metacarpophalangeal growth, as measured by the 2nd-to-4th

digit ratio (2D:4D), is another process that has been shown to be quite sensitive to intra-uterine sex steroid exposure.[24–30]

For all of the aforementioned parameters, a rise in sex steroid levels is expected to lead to a similar, incremental, linear change in the hormonal marker – either as an increase or a decrease in that specific parameter,[31,32] based on both molecular and clinical studies of animal and human samples.[9–21] One marker may be positively correlated with androgen exposure and negatively correlated with estrogen exposure (e.g., AGD[11,12]), and another positively correlated with estrogen exposure and negatively correlated with androgen exposure (e.g., 2D:4D[12,25,27,30]). Therefore, a given hormonal marker may both be categorized as an androgenic AND estrogenic index.

Different hormonal indices are thought to show specific sensitivity to different windows of exposure during gestation. For example, penile length/width and AGD are androgenic markers that show significant development during the first [8–14 gestational weeks (gw)[12,33]] or second trimester (12–20 gw[12]), respectively. Significant alterations in 2D:4D ratio and VMI may be first detectable at the end of the first (12–14 gw[12,25,27,30]) or second trimester (24 gw onward[34]), respectively. Finally, vulvar/scrotal pigmentation and areolar pigmentation may be most responsive to sex steroid levels throughout the first (8–14 gw[35]) or second trimester (20 gw onward[36]), respectively. Interestingly, human genital melanocytes may be primarily sensitive to androgen exposure, while melanocytes in non-genital regions may also be sensitive to estrogen exposure.[37–39] Thus, vulvar/scrotal pigmentation may primarily represent androgen exposure, while areolar pigmentation may represent both androgen and estrogen exposure.

Some hormonal markers, which have been better studied than others, were shown to be sexually dimorphic traits (AGD: males > > females – Cohen's *d* ~ 1.00; 2D:4D: male < females – Cohen's *d* ~ 0.4–0.6), with the important distinction that the sex difference in AGD can be altered by additional sex steroid exposures at any point during an individual's lifetime,[11,13,40] while the sex difference in 2D:4D ratio is largely determined *in utero* in a narrow time window at the end of the first trimester, with little further variation until adulthood.[24–27,30,41] In contrast to the data available for AGD and 2D:4D ratio, there are little or no newborn/infant data on other putative androgenic or estrogenic indices such as VMI or vulvar/scrotal/areolar pigmentation. Even for those hormonal markers that have been better studied (e.g., AGD), the most reliable and accurate method of measurement is still unclear (i.e., in boys, whether AGD should be measured from the anus to the base of the penis -AGD-penis- or from the anus to the scrotum -AGD-scrotum-; in girls, whether it should be measured from the anus to the clitoris -AGD-clitoris- or from the anus to the rear rim of the vulva -AGD-fourchette-[9,10,42,43]).

To confirm the relevance of these putative hormonal markers during fetal development, it is important to compare them with other, more established anthropometric markers, such as triceps/sub-scapular skinfold thickness, and arm circumference, that primarily reflect overall growth, body mass and fat distribution, and are also sensitive to sex steroid levels. In particular, skinfold thickness may represent an acceptable and safe measure of subcutaneous fat in newborns and infants,[44–46] as opposed to the imaging techniques used in older children and adults such as dual-energy X-ray absorptiometry and computed tomography (CT) scans. Such comparisons between hormonal indices and anthropometric data may provide additional clues as to the

relationship between potential androgen and estrogen exposure *in utero* and overall fetal growth and development.

In sum, this study aimed to provide age- and sex-specific descriptive data on a comprehensive panel of putative hormonal indices (measuring androgen and estrogen *in utero* exposure) collected at birth or 6 months of age in the context of a Canadian pregnancy cohort (Maternal-Infant Research on Environmental Chemicals-Infant Development, MIREC-ID). These indices are less invasive and likely to be more acceptable than hormonal sampling from maternal blood, the umbilical cord or the amniotic fluid. To further establish the usefulness of these measurements, we explored the relationship within these hormonal indices as well as between these indices and anthropometric data.

Materials and methods

Study population

The MIREC study was a cohort of 2000 women from 10 university-affiliated sites, recruited during the first trimester of pregnancy (6 to <14 weeks) over a 4-year enrolment period (2008–2011).[47] To be eligible for MIREC study, women had to be able to communicate in English or French, were 18 years or older and were all planning to deliver at one of the research sites. Women with the following medical history were excluded: those whose fetus had a known or suspected chromosomal or major malformations; and those who had a history of medical complications or drug or alcohol abuse.

The MIREC-ID study was a sub-cohort of the MIREC study that recruited healthy mother-singleton newborn pairs in six of the 10 university-affiliated sites. The sample size was determined by the availability of funding and the delays in obtaining ethics approval at some of the sites. Confirmation of eligibility for participation in MIREC-ID was determined at the time of discharge of the baby from hospital after delivery. In order for newborns to be eligible for MIREC-ID, they were required to result from a singleton birth at ⩾ 28 weeks of gestation. Exclusion criteria for MIREC-ID were newborns with major congenital birth defects, seizures or major neurological disorders during the perinatal period.

The MIREC and MIREC-ID studies were reviewed and approved by the Ethics Committee at Health Canada and all recruitment sites. All mothers provided informed consent for the studies. Data from the current study were extracted from the MIREC-ID study, based on those families who accepted a clinical examination of sexual development at birth (i.e., within the first 24 h, except for premature newborns, for whom the examination took place on the expected date of delivery). As such, androgenic, estrogenic and anthropometric measurements were available for 120–173 girls and 131–162 boys, depending on the measure (see Tables 2 and 3 for more details).

Anogenital distance and other genital measurements

Measurements for anogenital distance (AGD-penis, AGD-clitoris), anus-fourchette distance (AGD-fourchette), ano-scrotal distance (AGD-scrotum), penis width and penis length were each made at birth using skin calipers (modified Vernier caliper), by first laying the newborn supine with the baby's head toward the parent and the bottom toward the examiner. This allowed the mother to look at the newborn's face as well as hold the legs if necessary. The newborn was to be in the lithotomy position with legs flexed at hips

and pushed back at a 60° angle to the trunk. For AGD-fourchette distance and AGD-clitoris in female newborns, the distance was measured from the center of the anus to the posterior convergence of the fourchette (where the vestibule begins), or to the clitoris, respectively. The center, as opposed to the edge, of the anus was selected as the starting point for AGD because this facilitates repeated measurements and optimizes the reliability of measurements from one baby to another. For AGD-penis and AGD-scrotum in male newborns, distance was calculated from the center of the anus to the cephalad base of the penis, or to the base of the scrotum (junction of the smooth perineal skin and the rugated skin of the scrotum). Penis width was measured as the diameter at the base of the penis. Penis length was measured by compressing gently the suprapubic fat pad to position one end of the caliper along the dorsum of the penis, then stretching the penis to the tip of the glans along the length of the caliper, using the longest stretch length as the final measurement. All measurements were measured twice and if there was a >2 mm difference between the two measures, then a third measurement was taken. The mean of the two closest measures was used as the final measurement if three measurements were completed on a particular child. All attempts were made to ensure that measurements were made in a standardized manner (see the section 'Quality control of child assessments').

2D:4D ratio

The length (in cm) of the second and fourth finger of both the left and right hand were measured during the 6-month assessment of the infant. These measurements were done with a transparent plastic ruler with millimeter increments, ensuring the bottom of the ruler was aligned with the basal crease of each finger. Measurements were taken twice for each finger length for each hand. For the purpose of this analysis, the mean finger length for each hand was employed. All attempts were made to ensure that measurements were made in a standardized manner (see the section 'Quality control of child assessments').

Vaginal cytology

Examination of vaginal wall cells was made at birth, only from newborns seen at one research site (CHU Ste-Justine, Montreal) to avoid technical differences between sites. Vaginal wall cells were taken from the introitus using the Papanicolaou smear technique, with the newborn laying supine with feet in stirrups and knees bent outwards. A sterile cotton swab was first used to clean the baby's introitus to remove secretions. A second sterile cotton swab was then applied gently for 10–15 s to collect cells from the baby's introitus. The swab was then spread horizontally and longitudinally on the surface of a slide with no superposition, in order to allow for the maximum number of cells. As a rule, caution was taken not to touch the blade to avoid contaminating the surface with skin cells from external sources that could confound the pathological examination. A CytoPrep fixative was pulverized toward the slide from a distance of 15 cm to fix the organic material. The slide was dried 4–6 min at room temperature, then placed in the slide mailer. Two slides were collected per newborn.

VMI was calculated as the percentage of superficial cells added to half the percentage of intermediate cells. The slides were evaluated manually by two independent pathologists. If there was a disagreement between these two original raters, a third, independent pathologist would evaluate the slides again, and the two closest measurements were averaged to yield the final result.

Melanin pigmentation

All melanin pigmentation measurements were made at birth using a Mexameter® MX18 (Courage & Khazaka electronic GmbH, Cologne, Germany). Erythema measurements were also made using the same method, to rule out effects related to skin vasodilation. Three measurements were to occur in a dark room, far from a direct light source, such as a lamp or a window, with a fourth measurement done if there was a major discrepancy between the first three measurements. We averaged the three closest measurements to obtain the final pigmentation or erythema value for each specific site. Baseline pigmentation measurements were made in the posterior buttock area to avoid any areas prone to erythema. Four measurements were made for both melanin and erythema, in the area 1 inch below the horizontal line bisecting the postero-superior iliac spines. Breast/areolar pigmentation measurements were made, either on the left or the right, by placing the probe vertically just slightly eccentric to the nipple, in order to not block the probe. Measurements for scrotal pigmentation were made on either the left or right scrotum. Measurements for vulvar pigmentation were made on the internal face of either the left or right labium majora. All attempts were made to ensure that measurements were made in a standardized manner (see the section 'Quality control of child assessments').

Anthropometric measurements

All anthropometric measurements were made at birth. Newborn weight was measured with a seca® 727 baby scale with fine 1 g graduation, and length was measured with the baby supine using a seca® 416 infantometer (seca Deutschland, Hamburg Deutschland) and measured from the crown of the head to the heel while both legs were stretched simultaneously (with one hand on the baby's knees) and the chin supported perpendicular to the surface. Right arm circumference was measured by finding the mid-point between the tip of the acromion and the tip of the olecranon and measuring the circumference of the arm at that mid-point. Right arm skinfold measurement was made with the elbow extended and the arm relaxed, measuring the fold in the posterior midline of the upper arm, over the triceps muscle, halfway between the acromion process and olecranon process. Back skinfold thickness was measured by taking a fold on the diagonal line coming from the vertebral border between 1 and 2 cm from the inferior angle of the scapulae. From the lower edge of the scapulae, the fold was taken diagonally from the paraspinous line. Measurements were taken at least twice (and a third time if there was a discrepancy of more than 2–3 mm between the first two measurements, depending on the parameter). All attempts were made to ensure that measurements were made in a standardized manner (see the section 'Quality control of child assessments').

Quality control of child assessments

Newborn and infant testing as well as maternal interviews were conducted by trained research professionals. Examination techniques were monitored prospectively through direct observation by the research coordinator who was responsible for the initial training session. There was one rater assigned to each child, with a second person present to assist the evaluator (to hold the infant

Table 1. Intra-rater reliability: intra-class coefficients (ICCs)

Variable	ICCs
Baseline melanin	0.999
Areolar melanin	0.986
Vulvar melanin	0.951
Scrotal melanin	0.988
AGD-penis	0.949
AGD-scrotum	0.952
AGD-clitoris	0.929
AGD-fourchette	0.968
Penis width	0.927
Penis length	0.965
Right 2D:4D	0.968–0.961
Left 2D:4D	0.958–0.980
Arm skinfold	0.975
Back skinfold	0.688
Arm circumference	0.972

in a certain position, to enter the data on the record sheets, etc.). Finally, measurements were repeated 2–4 times, depending on whether initial measures showed discrepancies exceeding a certain threshold (see Table 1 for intra-class coefficients as a measure of intra-rater reliability). This threshold varied depending on the specific parameter under examination (see above sections for more details about each hormonal/anthropometric measure).

Statistical analyses

SPSS 23.0 was used to perform all analyses. Linear regression models were used to test the relationship within androgenic and estrogenic indices, with the expectation that these indices would be highly correlated within each category (androgenic, estrogenic, anthropometric indices). In addition, linear regression models were used to test the relationship between hormonal indices and anthropometric data, with the expectation that higher androgen exposure will be associated with lower adiposity (i.e., lower skinfold thickness and arm circumference) and higher estrogen exposure will be associated with higher adiposity (i.e., higher skinfold thickness and arm circumference). Because there is very little data supporting potential prediction models, we selected to use linear regression models as the most efficient model to test associations while also controlling for selected confounding variables (see below). Because of the limited sample size, use of cubic or quadratic models would be associated with unacceptable decreases in power while not being supported by any previous investigations. Nonetheless, for comparison purposes, we are including curve estimation parameters as Supplementary Material. Control variables included research site to account for inter-rater differences, weight-for-length (z-scores of weight-for-length ratios, calculated by dividing weight by length then subtracting the mean weight-for-length ratio from the resulting variable and dividing by the standard deviation); to account for overall effects

of growth on hormonal and anthropometric indices, and baseline melanin levels and to account for the confounding effects of skin color on areolar, vulvar or scrotal pigmentation. A P-value of $\leqslant 0.05$ was considered significant. Standardized betas, that is, the estimates of the regression coefficients that have been standardized so that the variances of dependent and independent variables are 1, are reported. Standardized coefficients refer to how many standard deviations a dependent variable will change, per standard deviation increase in the predictor variable. Reporting standardization of the coefficient is useful to determine the magnitude of the effect of each independent variable on the dependent variables, when the variables are measured in different units of measurement.

Results

Girls

Descriptive data are listed in Table 2. Curve fit models showed that linear models were a significantly better fit for our overall data than cubic and quadratic models. Curve estimation parameters and raw correlational data are included as Supplementary Material. The estrogenic marker VMI was positively associated with areolar pigmentation, when controlled for baseline melanin indices, weight-for-length scores and research site ($\beta = 0.338$, $P = 0.031$), while the androgenic/estrogenic marker 2D:4D ratio was negatively associated with areolar pigmentation, when controlled for baseline melanin indices, weight-for-length scores and research site (right 2D:4D: $\beta = -0.207$, $P = 0.040$; left 2D:4D: $\beta = -0.276$, $P = 0.006$).

The androgenic marker AGD-fourchette was also positively associated with areolar pigmentation, when controlled for baseline melanin indices, weight-for-length scores and research site ($\beta = 0.253$, $P = 0.036$). As expected, androgenic markers AGD-clitoris and AGD-fourchette were positively associated, when controlled for weight-for-length scores and research site ($\beta = 0.295$, $P < 0.0001$).

As for anthropometric markers, triceps and sub-scapular skinfold thickness were positively associated, when controlled for weight-for-length scores and research site ($\beta = 0.356$, $P < 0.0001$), as expected. The estrogenic marker VMI was also positively associated with triceps skinfold thickness, when controlled for weight-for-length scores and research site ($\beta = 0.265$, $P = 0.005$). Finally, AGD-clitoris was positively associated with right arm circumference, when controlled for weight-for-length scores and research site ($\beta = 0.176$, $P = 0.012$).

Boys

Descriptive data are listed in Table 3. Curve fit models showed that linear models were a significantly better fit for our overall data than cubic and quadratic models. Curve estimation parameters and raw correlational data are included as Supplementary Material. The androgenic marker AGD-penis was positively associated with scrotal pigmentation, when controlled for baseline melanin indices, weight-for-length scores and research site ($\beta = 0.290$, $P = 0.048$). As expected, androgenic markers AGD-penis and AGD-scrotum were positively associated, when controlled for weight-for-length scores and research site ($\beta = 0.362$, $P < 0.0001$).

As for anthropometric parameters, right arm and back skinfold thickness were positively associated, when controlled for

Table 2. Girls: descriptive data

Variable	Age	*n*	Minimum	Maximum	Mean	Median	Std. deviation
Hormonal indices							
Baseline melanin	Birth	159	34.33	475.67	104.63	95.00	53.41
Baseline erythema	Birth	157	325.67	859.33	608.90	612.00	110.70
Areolar melanin	Birth	156	5.33	302.33	69.24	66.00	37.61
Areolar erythema	Birth	156	283.00	852.00	563.65	558.17	98.58
Vulvar melanin	Birth	154	0.00	494.00	71.11	58.83	64.67
Vulvar erythema	Birth	158	265.67	992.67	742.01	740.67	123.58
AGD-clitoris	Birth	159	23.07	44.43	33.36	33.73	4.03
AGD-fourchette	Birth	160	7.57	33.03	14.60	13.93	4.12
Right 2D:4D	6 mo	152	0.71	1.07	0.95	0.96	0.05
Left 2D:4D	6 mo	151	0.82	1.09	0.94	0.94	0.05
Vaginal MI	Birth	120	32.00	72.50	52.64	59.00	5.65
Anthropometric indices							
Length	Birth	163	43.05	59.10	49.80	49.55	2.45
Weight	Birth	157	2.02	4.58	3.21	3.19	0.45
Arm skinfold	Birth	173	2.00	16.00	5.16	5.00	1.88
Back skinfold	Birth	166	2.00	14.00	4.73	4.50	1.69
Arm circumference	Birth	173	8.55	13.80	10.95	10.90	1.04

AGD: anogenital distance (mm); VMI: vaginal maturation index; length (cm); weight (kg); skinfold/circumference (mm)

weight-for-length scores and research site ($\beta = 0.572$, $P < 0.0001$), as expected. The androgenic marker AGD-penis was also positively associated with right arm circumference ($\beta = 0.462$, $P < 0.0001$), when controlled for weight-for-length scores and research site.

Discussion

This study provides detailed age- and sex-specific reference data on a number of indices related to androgen and estrogen exposure. We found novel relationships within markers of androgen and estrogen exposure, as well as between hormonal indices and anthropometric data: (1) VMI, an index of estrogen exposure, was found to be associated with triceps skinfold thickness in girls; (2) both estrogenic and androgenic markers, including VMI, 2D:4D ratio, and AGD-fourchette, were associated with areolar pigmentation in girls; and (3) the androgenic marker AGD-penis was associated with scrotal pigmentation in boys. In addition, several of the expected associations were demonstrated: (1) a significant association between AGD-clitoris and AGD-fourchette, both markers of androgen exposure, in girls; (2) a significant association between AGD-penis and AGD-scrotum, both markers of androgen exposure, in boys; (3) a significant association between right arm/triceps and back/sub-scapular skinfold thickness in both sexes.

We found positive associations between VMI and areolar pigmentation in girls, supporting the notion that both measures are responsive to estrogen exposure.[15,19,39,48,49] This further serves to position the VMI as a reliable index of estrogen exposure, as has been shown in several prior studies examining differences in the vaginal maturity index of infant girls in response to soy (rich in phyto-estrogens) *v.* breastmilk.[14,48,50] Greater exposure to estrogens, most commonly related to pregnancy, has been previously associated with increased pigmentation in several non-genital locations, including in the ring of pigmented skin surrounding the nipple (areola), as well as the face and midline of the abdomen (*linea alba*).[39] There are also several reports of nipple and areolar hyper-pigmentation following the use of estradiol spray,[19,20] supporting the notion that areolar pigmentation may be sensitive to estrogen exposure.[39]

Interestingly, smaller 2D:4D ratios and higher AGD-fourchette measurements (both hypothesized to represent higher androgen exposure) were associated with areolar pigmentation in girls. This suggests that pigmentation in non-genital areas in girls may also be responsive to androgen exposure, similar to the trend seen in girls between vulvar pigmentation and AGD-clitoris, a known androgen marker.[12] Associations between areolar pigmentation in girls and estrogenic as well as androgenic markers could be explained by the concomitant presence of androgen and estrogen receptors in the nipple–areolar complex.[18,21] While care was taken to measure pigmentation of the areola (and not the nipple) in this study, measurement error or differences across research sites could have led to the inclusion of nipple melanocytes that are sensitive to androgen exposure. Future studies examining the effect on areolar pigmentation of various androgenic and

Table 3. Boys: descriptive data

Variable	Age	*n*	Minimum	Maximum	Mean	Median	Std. deviation
Hormonal indices							
Baseline melanin	Birth	159	29.00	610.67	108.49	100.67	61.78
Baseline erythema	Birth	158	342.67	914.33	615.57	620.50	127.72
Areolar melanin	Birth	156	4.50	426.33	75.69	68.83	43.95
Areolar erythema	Birth	159	316.00	775.67	560.84	567.00	94.50
Scrotal melanin	Birth	136	4.00	777.33	77.67	49.17	95.67
Scrotal erythema	Birth	152	174.00	1121.67	650.69	663.33	138.24
AGD-penis	Birth	162	30.33	56.97	43.61	43.68	5.14
AGD-scrotum	Birth	162	11.23	38.77	22.34	21.60	4.46
Right 2D:4D	6 mo	131	0.80	1.08	0.95	0.95	0.04
Left 2D:4D	6 mo	132	0.80	1.12	0.95	0.95	0.05
Penis length	Birth	161	9.97	34.70	21.32	21.13	4.48
Penis width	Birth	162	0.90	24.80	10.41	10.35	2.00
Anthropometric indices							
Length	Birth	154	43.60	57.55	51.05	51.00	2.31
Weight	Birth	129	1.95	4.40	3.33	3.33	0.45
Arm skinfold	Birth	162	2.00	11.50	4.98	5.00	1.47
Back skinfold	Birth	161	2.00	8.50	4.58	4.33	1.39
Arm circumference	Birth	160	7.75	13.15	10.78	10.80	1.08

AGD: anogenital distance (mm); penis length/width (mm); length (cm); weight (kg); skinfold/circumference (mm)

estrogenic disruptors, as measured in this cohort, will help in parsing out the respective impact of androgen and estrogen receptors on this parameter.

The estrogenic marker VMI was positively associated with right arm/triceps skinfold thickness in girls, underlining the previously documented role of estrogen exposure on adipose tissue differentiation and lipid homeostasis.[46,51] Predictive relationships have been established in prior studies between skinfold thickness, thought to represent subcutaneous adipose tissue, and clinically relevant measures such as abdominal adiposity, itself predictive of cardio-metabolic complications in adulthood.[52] Reliable sex differences in skinfold thickness have also been previously found in infants and children, suggesting that body fat development in humans is particularly responsive to sex steroid exposure during pregnancy, similar to findings from the molecular and animal literature.[44–46] Estrogenic effects on fat distribution may be mediated by a variety of mechanisms and estrogen receptors (estrogen receptor alpha, beta, g-protein coupled estrogen receptor), though a comprehensive discussion of the exact mechanisms through which these receptors may interact in order to maintain lipid homeostasis is beyond the scope of this manuscript.

Another significant finding is the positive association between AGD-penis and scrotal pigmentation in boys. A similar trend was present between AGD-clitoris and vulvar pigmentation in girls but did not survive when controlling for all confounding variables. AGD was previously shown to be a marker of androgen

exposure, as supported by previous molecular, animal and clinical studies.[42] Thus, an association between AGD and scrotal pigmentation provide *in vivo* evidence that human genital melanocytes may be androgen target cells, as reported by prior *in vitro* investigations. Indeed, previous molecular and animal studies have demonstrated the following: (1) the presence of a high density of androgen receptors in human genital melanocytes; (2) a decrease in melanin content, cell volume, dendritic branching and tyrosinase activity of scrotal epidermal melanocytes following castration; followed by (3) a recovery of these parameters to normal levels following testosterone replacement.[17,21] However, it is important to note that these relationships were only documented in male samples.[17,21] Thus, taken together with prior evidence from molecular and animal models, our current findings support the notion that human genital melanocytes in boys may be more sensitive to androgen exposure than vulvar pigmentation in girls.

Positive associations were also seen between right arm circumference and androgenic markers, that is, AGD-penis in boys and AGD-clitoris in girls. Right arm circumference is a gross measure that varies mainly a result of differences in muscle bulk and adipose tissue.[53] However, neither AGD-penis nor AGD-clitoris were significantly associated with other proxies for subcutaneous fat, such as skinfold thickness of the triceps or subscapular areas. Thus, the significant correlations seen here between AGD indices and right arm circumference do not appear to be related to underlying differences in fat distribution but may

instead reflect the simultaneous impact of androgen exposure on genital and muscle development.[53]

Notably, there were no associations between 2D:4D ratio and any androgenic index in boys, nor did we find any reverse relationships between 2D:4D ratio and any estrogenic or anthropometric indices in boys. Similarly, there were no significant associations between penile length or width and any other androgenic, estrogenic or anthropometric indices in boys. This suggests that neither 2D:4D nor penile length or width at birth may be reliable or predictive markers of androgen exposure, at least in newborn and infant boys.

Finally, no significant associations emerged between any of the androgenic or estrogenic markers and back/sub-scapular skinfold thickness, despite the strong correlation between right arm/triceps and back/sub-scapular skinfold thickness. This may be due to the challenging nature of measuring skinfold thickness in the sub-scapular area in newborns, leading to measurement errors. Alternatively, right arm/triceps skinfold thickness may be a better marker of subcutaneous adipose tissue than back/sub-scapular skinfold thickness in newborns because of the unique and unstable fat distribution in this population, which differs from that of older children or adults.[46,51]

Strengths and limitations

Strengths of this study include high-quality measurements of several hormonal and anthropometric indices as well as high intra-rater reliability. Indeed, rigorous training and quality control procedures were implemented for each measurement, and repeated measures were taken when discrepancies arose between initial measurements. In particular, one strength of this study is that we have measured digit lengths directly on the newborns (using a ruler rather than indirectly from scans or photocopies or photographs). Indirectly measured finger lengths produce 2D:4Ds that are lower than those directly measured, and this effect is stronger in males than females, though it remains unclear why this is the case.[28,29] Though there is ongoing debate as to whether direct or indirect measurements are preferable, direct measurements, in our opinion, are more likely to be accurate representations of the newborn's actual fingers than indirect measurements, which may include artifacts of the scanning process. The study sites were also selected to represent different geographical regions of the country, and as such, sought to be as representative as possible to the general population of women giving birth in Canada. Few examinations of these parameters have been completed in newborns, and therefore age-, sex-specific descriptive data from this study represent a significant contribution to the literature. In addition, we were able to control partly for race and ethnicity by measuring baseline pigmentation, and in doing so, indirectly account for the effect of racially- or ethnically-based confounding factors on the observed associations between vulvar/scrotal/areolar pigmentation and other hormonal/anthropometric indices. As a result, these relationships can be interpreted as part of a universal sexual maturation process that occurs in all individuals, regardless of race or ethnicity. Finally, identifying relationships within hormonal indices and between hormonal and anthropometric measures is critical because of the challenges of determining which covariates should be considered when these sexual maturation outcomes are examined. Thus, findings from this study advance our current understanding of these relationships, with the ultimate goal of including these indices in comprehensive statistical models to evaluate newborn/infant hormonal exposure.

On the other hand, it is important to note that we cannot directly address questions of causality due to the correlational nature of the present study, although inferences can be made on the general direction of associations based on previous molecular or animal data. In addition, any of the relationships found between parameters measured at birth may be due to prenatal maternal hormonal exposure, between-individual prenatal variation in the fetus, or a combination or both, and based on our methods, we cannot confirm/infirm any of these possible scenarios. Still, given the stability in 2D:4D ratios until adulthood, the relationship between 2D:4D ratios measured at 6 months of age and areolar pigmentation in girls is likely to reflect an association in the fetus at the end of the first trimester. Finally, the sample size of this study represents another limitation, as it is relatively modest for some of the hormonal markers and may have limited our power to test non-linear models (e.g., cubic, quadratic). This is especially important in light of prior evidence suggesting that several hormonal effects may follow non-linear trajectories (e.g., U-shaped or inverted U-shaped).[5,6] However, the ability to fully address this issue is not only limited by sample size, but further compounded by the need to control for several confounders such as research site, weight-for-length scores and, in analyses related to areolar/vulvar/scrotal pigmentation, baseline melanin pigmentation. As a result, findings should be interpreted as preliminary rather than confirmatory due to the novelty of several of the selected hormonal indices in being used for the quantification of androgenic or estrogenic exposures *in utero*.

Conclusions

In sum, we provide descriptive and correlational data about a range of putative androgenic and estrogenic indices at birth. We found evidence to support the use of areolar pigmentation in girls as an index of both androgen and estrogen exposure, as well as the use of scrotal pigmentation in boys as an index of androgen exposure. In addition, we show that VMI is an estrogenic index that may particularly be sensitive to the circulating levels of estrogens produced by adipose tissue. These findings support the utility of measuring several minimally invasive physical indices at birth to convey a more comprehensive picture of androgen and estrogen exposure *in utero*.

References

1. Abbott DH, Padmanabhan V, Dumesic DA. Contributions of androgen and estrogen to fetal programming of ovarian dysfunction. *Reprod Biol Endocrinol*. 2006; 4, 17.
2. Hines M. Prenatal endocrine influences on sexual orientation and on sexually differentiated childhood behavior. *Front Neuroendocrinol*. 2011; 32, 170–182.
3. Hollier LP, Keelan JA, Hickey M, Maybery MT, Whitehouse AJ. Measurement of androgen and estrogen concentrations in cord blood: accuracy, biological interpretation, and applications to understanding human behavioral development. *Front Endocrinol (Lausanne)*. 2014; 5, 64.
4. Keelan JA, Mattes E, Tan HW, et al. Androgen concentrations in umbilical cord blood and their association with maternal, fetal and obstetric factors. *PLoS One*. 2012; 7, e42827.
5. Foradori CD, Weiser MJ, Handa RJ. Non-genomic actions of androgens. *Front Neuroendocrinol*. 2008; 29, 169–181.
6. Luconi M, Forti G, Baldi E. Genomic and nongenomic effects of estrogens: molecular mechanisms of action and clinical implications for male reproduction. *J Steroid Biochem Mol Biol*. 2002; 80, 369–381.
7. Reyes FI, Winter JS, Faiman C. Studies on human sexual development. I. Fetal gonadal and adrenal sex steroids. *J Clin Endocrinol Metab*. 1973; 37, 74–78.
8. Reyes FI, Boroditsky RS, Winter JS, Faiman C. Studies on human sexual development. II. Fetal and maternal serum gonadotropin and sex steroid concentrations. *J Clin Endocrinol Metab*. 1974; 38, 612–617.
9. Eisenberg ML, Hsieh MH, Walters RC, Krasnow R, Lipshultz LI. The relationship between anogenital distance, fatherhood, and fertility in adult men. *PLoS One*. 2011; 6, e18973.
10. Hsieh MH, Breyer BN, Eisenberg ML, Baskin LS. Associations among hypospadias, cryptorchidism, anogenital distance, and endocrine disruption. *Curr Urol Rep*. 2008; 9, 137–142.
11. Mitchell RT, Mungall W, McKinnell C, et al. Anogenital distance plasticity in adulthood: implications for its use as a biomarker of fetal androgen action. *Endocrinology*. 2015; 156, 24–31.
12. Dean A, Sharpe RM. Anogenital distance or digit length ratio as measures of fetal androgen exposure: relationship to male reproductive development and its disorders. *J Clin Endocrinol Metab*. 2013; 98, 2230–2238.
13. Thankamony A, Pasterski V, Ong KK, Acerini CL, Hughes IA. Anogenital distance as a marker of androgen exposure in humans. *Andrology*. 2016; 4, 616–625.
14. Harlid S, Adgent M, Jefferson WN, et al. Soy formula and epigenetic modifications: analysis of vaginal epithelial cells from infant girls in the IFED study. *Environ Health Perspect*. 2017; 125, 447–452.
15. van der Laak JAWM, de Bie LMT, de Leeuw H, de Wilde PCM, Hanselaar AGJM. The effect of Replens (R) on vaginal cytology in the treatment of postmenopausal atrophy: cytomorphology versus computerised cytometry. *J Clin Pathol*. 2002; 55, 446–451.
16. van der Laak JAWM, Schijf CP, Kerstens HMJ, et al. Development and validation of a computerized cytomorphometric method to assess the maturation of vaginal epithelial cells. *Cytometry*. 1999; 35, 196–202.
17. Wilson MJ, Spaziani E. Testosterone regulation of pigmentation in scrotal epidermis of the rat. *Z Zellforsch Mikrosk Anat*. 1973; 140, 451–458.
18. Natale CA, Duperret EK, Zhang JQ, et al. Sex steroids regulate skin pigmentation through nonclassical membrane-bound receptors. *Elife*. 2016; 5, e15104.
19. Ali A, Berens P, Siddiqui G, Ali V. Nipple and areolar hyperpigmentation secondary to the use of estradiol spray on the ipsilateral forearm skin: a report of two cases. *J Womens Health*. 2012; 21, 363–365.
20. Diven DG, Crawford JM. Ipsilateral areolar hyperpigmentation following unilateral application of estradiol spray. *Am J Obstetr Gynecol*. 2010; 203, E8.
21. Tadokoro T, Itami S, Hosokawa K, Terashi H, Takayasu S. Human genital melanocytes as androgen target cells. *J Invest Dermatol*. 1997; 109, 513–517.
22. Fullerton A, Fischer T, Lahti A, et al. Guidelines for measurement of skin colour and erythema – a report from the standardization group of the European society of contact dermatitis. *Contact Dermatitis*. 1996; 35, 1–10.
23. Takiwaki H, Overgaard L, Serup J. Comparison of narrow-band reflectance spectrophotometric and tristimulus colorimetric measurements of skin color - 23 anatomical sites evaluated by the dermaspectrometer(R) and the chroma-meter-Cr-200(R). *Skin Pharmacol*. 1994; 7, 217–225.
24. Garn SM, Hertzog KP, Poznanski AK, Nagy JM. Metacarpophalangeal length in the evaluation of skeletal malformation. *Radiology*. 1972; 105, 375–381.
25. Galis F, Ten Broek CM, Van Dongen S, Wijnaendts LC. Sexual dimorphism in the prenatal digit ratio (2D:4D). *Arch Sex Behav*. 2010; 39, 57–62.
26. Manning JT, Fink B. Sexual dimorphism in the ontogeny of second (2D) and fourth (4D) digit lengths, and digit ratio (2D:4D). *Am J Hum Biol*. 2018; 30, e23138.
27. Malas MA, Dogan S, Evcil EH, Desdicioglu K. Fetal development of the hand, digits and digit ratio (2D:4D). *Early Hum Dev*. 2006; 82, 469–475.
28. Fink B, Manning JT. Direct versus indirect measurement of digit ratio: new data from Austria and a critical consideration of clarity of report in 2D:4D studies. *Early Hum Dev*. 2018; 127, 28–32.
29. Ribeiro E, Neave N, Morais RN, Manning JT. Direct versus indirect measurement of digit ratio (2D:4D): a critical review of the literature and new data. *Evol Psychol-Us*. 2016; 14, 1–8.
30. Szwed A, Kosinska M, Manning JT. Digit ratio (2D:4D) and month of birth: a link to the solstitial-melatonin-testosterone effect. *Early Hum Dev*. 2017; 104, 23–26.
31. Velez MP, Arbuckle TE, Monnier P, Fraser WD. Is maternal periconceptional smoking associated with 2D:4D digit ratio in their children? *J Dev Orig Hlth Dis*. 2017; 8, 597–603.
32. Velez MP, Arbuckle TE, Monnier P, Fraser WD. Female digit length ratio (2D:4D) and time-to-pregnancy. *Hum Reproduct*. 2016; 31, 2128–2134.
33. Welsh M, MacLeod DJ, Walker M, Smith LB, Sharpe RM. Critical androgen-sensitive periods of rat penis and clitoris development. *Int J Androl*. 2010; 33, e144–152.
34. Fritsch H, Hoermann R, Bitsche M, Pechriggl E, Reich O. Development of epithelial and mesenchymal regionalization of the human fetal uterovaginal anlagen. *J Anat*. 2013; 222, 462–472.
35. Wilson MJ. Inhibition of development of both androgen-dependent and androgen-independent pigment-cells in scrotal skin dermis of the rat by anti-androgen treatment during fetal growth. *Endocrinology*. 1983; 112, 321–325.
36. Javed A, Lteif A. Development of the human breast. *Semin Plast Surg*. 2013; 27, 5–12.
37. Wilson JD, Leihy MW, Shaw G, Renfree MB. Androgen physiology: unsolved problems at the millennium. *Mol Cell Endocrinol*. 2002; 198, 1–5.
38. Fritsch M, Orfanos CE, Zouboulis C. Sebocytes are the key regulators of androgen homeostasis in human skin. *J Invest Dermatol*. 2001; 117, 785–785.
39. Thornton MJ. The biological actions of estrogens on skin. *Exp Dermatol*. 2002; 11, 487–502.
40. Dusek A, Bartos L. Variation in ano-genital distance in spontaneously cycling female mice. *Reprod Domest Anim*. 2012; 47, 984–987.
41. Manning JT, Scutt D, Wilson J, Lewis-Jones DI. The ratio of 2nd to 4th digit length: a predictor of sperm numbers and concentrations of testosterone, luteinizing hormone and oestrogen. *Hum Reprod*. 1998; 13, 3000–3004.
42. Sathyanarayana S, Beard L, Zhou C, Grady R. Measurement and correlates of ano-genital distance in healthy, newborn infants. *Int J Androl*. 2010; 33, 317–323.
43. Swan SH, Main KM, Liu F, et al. Decrease in anogenital distance among male infants with prenatal phthalate exposure. *Environ Health Persp*. 2005; 113, 1056–1061.
44. Santos S, Gaillard R, Oliveira A, et al. Associations of infant subcutaneous fat mass with total and abdominal fat mass at school-age: the generation R study. *Paediatr Perinat Ep*. 2016; 30, 511–520.
45. Gale C, Logan KM, Jeffries S, et al. Sexual dimorphism in relation to adipose tissue and intrahepatocellular lipid deposition in early infancy. *Int J Obesity*. 2015; 39, 629–632.
46. Bluher M. Importance of estrogen receptors in adipose tissue function. *Mol Metab*. 2013; 2, 130–132.

47. Arbuckle TE, Fraser WD, Fisher M, *et al.* Cohort profile: the maternal-infant research on environmental chemicals research platform. *Paediatr Perinat Ep.* 2013; 27, 415–425.

48. Bernbaum JC, Umbach DM, Ragan NB, *et al.* Pilot studies of estrogen-related physical findings in infants. *Environ Health Perspect.* 2008; 116, 416–420.

49. Farage M, Maibach H. Lifetime changes in the vulva and vagina. *Arch Gynecol Obstet.* 2006; 273, 195–202.

50. Adgent MA, Umbach DM, Zemel BS, *et al.* A longitudinal study of estrogen-responsive tissues and hormone concentrations in infants fed soy formula. *J Clin Endocrinol Metab.* 2018; 103, 1899–1909.

51. Pedersen SB, Bruun JM, Hube F, Kristensen K, Hauner H, *et al.* Demonstration of estrogen receptor subtypes alpha and beta in human adipose tissue: influences of adipose cell differentiation and fat depot localization. *Mol Cell Endocrinol.* 2001; 182, 27–37.

52. Bhasin S. Effects of testosterone administration on fat distribution, insulin sensitivity, and atherosclerosis progression. *Clin Infect Dis.* 2003; 37, S142–S149.

53. Bhasin S, Taylor WE, Singh R, *et al.* The mechanisms of androgen effects on body composition: Mesenchymal pluripotent cell as the target of androgen action. *J Gerontol a-Biol.* 2003; 58, 1103–1110.