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Prenatal masculinization of the auditory system in infants: The MIREC-ID study

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ABSTRACT

Sex differences in inner-ear function are detectable in infants, notably through the measurement of otoacoustic emissions (OAEs). Prevailing theories posit that prenatal exposure to high levels of androgens in boys may weaken OAEs, and that this phenomenon may predominantly affect the right ear/left hemisphere (Geschwind-Galaburda (GG) hypothesis). Yet, actual tests of these models have been difficult to implement in humans. Here we examined the relationship between markers of fetal androgen exposure collected at birth (anogenital distances (AGD); penile length/width, areolar/scrotal/vulvar pigmentation) and at 6 months of age (2^{nd} to 4^{th} digit ratio (2D:4D)) with two types of OAEs, click-evoked OAEs (CEOAEs) and distortion-product OAEs (DPOAEs) (n = 49; 25 boys; 24 girls). We found that, in boys, scrotal pigmentation was inversely associated with the amplitude and reproducibility of CEOAEs in the right ear at 4 kHz, with trends also present in the same ear for mean CEOAE amplitude and CEOAE amplitude at 2 kHz. Penile length was inversely associated with the mean amplitude of DPOAEs in both the right and left ears, as well as with DPOAE amplitude in the right ear at 2 kHz and the reproducibility of CEOAEs in the left ear at 2.8 kHz. Finally, AGD-scrotum in boys was positively associated in boys with the amplitude of DPOAEs in the left ear at 2.8 kHz. Unexpectedly, there were no sex differences in the amplitude or reproducibility of OAEs, nor, in girls, any associations between androgenic markers and auditory function. Nonetheless, these findings, reported for the first time in a sample of human infants, support both the prenatal-androgen-exposure and GG models as explanations for the masculinization of auditory function in male infants.

1. Introduction

Sex differences in inner-ear function have been documented for the last several decades, yet many questions remain as to the mechanisms that may underlie this difference (Roche et al., 1978). In comparison with boys, girls generally show lower auditory thresholds and less variability in these thresholds (Roche et al., 1978). Overlaid on the sex differences is an ear asymmetry, with the right ear typically showing more inner activity than the left (Khalfa et al., 1997). Some of these sex and ear differences are present and measurable at birth, and there is evidence that some aspects of inner-ear function remain relatively stable from childhood to adulthood (McFadden, 2008).

Several lines of research point toward prenatal androgen exposure as one of the most likely candidate mechanisms through which masculinization of the mammalian cochlea may occur (McFadden, 2009). Male human fetuses develop embryological testes as early as during the

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8th week of gestation; these testes begin producing androgens, resulting in a masculinization of the prenatal brain and body (Hines et al., 2015). The prenatal-androgen-exposure hypothesis posits that high androgen exposure *in utero* may weaken cochlear amplifiers and, as a result, auditory function of the inner ear (McFadden, 2009, 2011). This model of auditory function partially overlaps with the Geschwind-Galaburda (GG) hypothesis of cerebral lateralization, which postulates that prenatal androgens, in particular testosterone, is a key causal factor in the decreased growth of the left hemisphere, particularly in males, and that this asymmetry may be responsible for sex differences in learning and neuroplasticity (Geschwind and Galaburda, 1985). Taken together, both models thus view prenatal androgen exposure as an explanation for sex differences in inner-ear function as well as higher cortical functions (e.g. speech, language) in humans, linking androgen-related lateralization effects from the peripheral to the central nervous system (CNS). Yet, actual tests of these models have been challenging to implement in humans because of the unacceptability of hormonal manipulations during pregnancy and the difficulty in obtaining reliable measures of fetal androgen exposure.

Further compounding this difficulty is the scarcity of animal models that can test this hypothesis due to a number of differences in inner-ear responses between humans and lower mammals, as described below. Otoacoustic emissions (OAEs) are sounds coming from the inner ear, which can be recorded by a microphone fitted into the ear canal. They are dependent on the motion of the cochlea's sensory hair cells as they energetically respond to auditory stimulation (Kemp, 1978). Because OAEs show many of the useful characteristics outlined above (reasonably constant throughout life, at least prior to the onset of hearing loss, sex and ear differences in humans present at birth and maintained until adulthood), they represent an invaluable tool to study the auditory system in infants (McFadden, 2009, 2011). One important component of OAEs is dependent on the action of the outer hair cells of the inner ear's cochlea, which are the first to be affected by ototoxic damage. As a result, the measurement of OAEs has been recognized as a sensitive measure of hearing loss and is now widely used to test auditory function in newborns (Akinpelu et al., 2014). There are various measurable forms of OAEs - here we will focus on evoked OAEs: click-evoked OAEs (CEOAEs) and distortion-product OAEs (DPOAEs). One advantage of using evoked OAEs is that CEOAEs can be measured in essentially all human ears with normal hearing (Probst et al., 1991), while spontaneous OAEs (SOAEs) can be found in only about 75%–85% of female ears and in about 45%–65% of male ears (Bilger et al., 1990; McFadden and Pasanen, 1999; Talmadge et al., 1993).

CEOAEs are brief sounds that can be recorded in the ear canal immediately after the presentation of an acoustic stimulus (McFadden, 2008). These echo-like sounds depend, in part, upon the stimulus used to elicit them, and the most common stimulus used is a brief sound (1 ms or shorter) having a wide bandwidth. The standard measure of CEOAEs is the root-mean-square (rms) amplitude expressed in decibels, and is usually quite weak, requiring averaging techniques to be detected. CEOAEs are stronger in females than males, and stronger in the right vs. left ears (McFadden, 2008).

Distortion-product OAEs (DPOAEs) are a form of OAEs that may rely on cochlear mechanisms different from those captured by CEOAEs (McFadden, 2009; McFadden et al., 2009, 2006; Shera and Guinan, 1999; Torre and Fowler, 2000). DPOAEs also may show a small sex difference (though less pronounced than the sex differences seen in CEOAEs), being slightly stronger in females than in males, at least in humans and rhesus monkeys (McFadden, 2009; McFadden et al., 2009, 2018; McFadden et al., 2006; Torre and Fowler, 2000).

It is difficult to generalize OAE research based on experimental animal models to human populations, for several reasons: (1) the cochleas of mammals commonly used in auditory research are short, making it difficult to extract measures of CEOAEs; (2) those species typically have no SOAEs; and (3) DPOAEs of lower animals typically do not exhibit sex differences (Siegel et al., 2011). In addition, no prior studies have examined the hypothesis that there is an association between OAEs in infants and physical markers of fetal androgen exposure, such as anogenital distance (AGD), the ratio of the second to the fourth finger (2D:4D ratio), penile length, penile width, and areolar/scrotal/vulvar pigmentation. For all these markers, a rise in androgen 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 (Velez et al., 2016, 2017), based on both molecular and clinical studies of animal and human samples (Ali et al., 2012; Dean and Sharpe, 2013; Diven and Crawford, 2010; Eisenberg et al., 2011; Harlid et al., 2017; Hsieh et al., 2008; Manning and Robinson, 2003; Mitchell et al., 2015; Natale et al., 2016; Tadokoro et al., 1997; Thankamony et al., 2016; van der Laak et al., 2002, 1999; Wilson and Spaziani, 1973).

Thus the present study aimed to examine the relationship between the aforementioned purported markers of *in utero* androgen exposure and inner-ear function in infancy, using data from a Canadian pregnancy cohort, the Maternal-Infant Research on Environmental Chemicals- Infant Development (MIREC-ID). We hypothesize that, as predicted by both the prenatal-androgen-exposure and GG models, markers of higher prenatal androgen exposure will be inversely correlated with auditory function as measured by OAEs, and that this effect will be stronger in the peripheral auditory system connected to the left hemisphere (right ear).

2. Materials and methods

2.1. Study population

The MIREC Study was a cohort of 2000 women from 10 university-affiliated sites, recruited during the 1 st trimester of pregnancy (6 – < 14 weeks) over a 4-year enrollment period (2008–2011) (Arbuckle et al., 2013). To be eligible for MIREC, women had to be able to communicate in English or French, were 18 years or older, and were planning to deliver at one of the research sites. Women whose fetus had a known or suspected chromosomal or major malformation and those who revealed a history of medical complications or drug/alcohol abuse on interview were excluded. The MIREC-Infant Development study (MIREC-ID) was a sub-cohort of the MIREC study. Mothers who delivered healthy singletons were recruited from 6 of the 10 university-affiliated sites (due to limited funding over the recruitment window). 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 malformations, seizures or major neurological disorders during the perinatal period. The MIREC and MIREC-ID studies were reviewed and approved by the ethics committees at Health Canada and all recruitment sites. All mothers provided informed consent for the studies. Sexual development was determined within 1 week of delivery (mean 3.5 days, SD 4.77) for all participants, except for premature newborns, for whom the exam took place within 1 week of the expected date of delivery (n = 195–428 depending on the androgenic marker examined). Testing of auditory function was measured using CEOAEs and DPOAEs around 6 months of age (mean 206.8 days, SD 27.03, n = 66) at two of the MIREC-ID research sites (see section on Auditory Function below for more details). As such, the final sample size included a total of up to 37–49 children (25 boys; 24 girls), depending on the androgenic marker/OAE parameter examined (see Tables 1 and 2 for more details).

2.2. Auditory function

Inner-ear function was measured with CEOAEs and DPOAEs. For

Table 1
Boys: Descriptive data.

Variable	Age	N	Minimum	Maximum	Mean	SD
Androgen markers (based on children who also have DPOAEs available in the right ear)						
Areolar melanin	Birth	24	49.33	128.33	79.25	20.71
Scrotal melanin	Birth	22	4.00	223.00	66.29	60.14
AGD-penis (mm)	Birth	25	39.07	53.07	44.80	3.57
AGD-scrotum (mm)	Birth	25	11.23	29.50	20.63	4.60
Penile length (mm)	Birth	25	14.63	25.37	20.77	2.37
Penile width (mm)	Birth	24	7.50	12.30	10.25	1.29
Right 2D:4D	6 mo	25	0.89	1.03	0.96	0.04
Left 2D:4D	6 mo	25	0.84	1.06	0.94	0.05
Auditory function (based on children who also have penile length)						
CEOAE: amplitude	6 mo	R/L	R/L	R/L	R/L	R/L
Mean (dB SPL)		22/24	9.70/10.3	26.90/27.8	20.54/19.3	4.99/4.40
2 kHz		23/25	1.00/1.00	22.00/22.20	12.14/11.84	5.83/5.29
2.8 kHz		23/25	0.30/1.00	23.60/25.50	15.36/14.40	5.77/6.20
4 kHz		22/24	5.00/4.40	25.00/19.90	14.83/11.88	5.47/4.67
CEOAE: reproducibility (%)	6 mo	R/L	R/L	R/L	R/L	R/L
Mean (%)		22/24	41.00/7.00	97.00/99.00	81.09/76.75	17.13/22.89
2 kHz		24/25	19.10/23.20	99.80/99.90	83.54/85.76	23.55/18.85
2.8 kHz		24/25	19.30/28.30	99.90/99.90	90.40/90.37	19.03/15.64
4 kHz		24/25	37.20/16.40	99.70/99.50	89.60/84.73	18.16/21.09
DPOAE: amplitude	6 mo	R/L	R/L	R/L	R/L	R/L
Mean (dB SPL)		25/25	7.20/7.20	21.10/21.50	15.84/14.19	3.80/3.84
2 kHz		20/21	0.80/3.20	27.50/28.80	12.60/11.07	7.12/6.01
2.8 kHz		19/22	7.50/6.50	25.20/25.80	12.64/12.38	4.82/5.40
4 kHz		20/23	5.40/6.50	27.20/23.60	14.88/12.90	6.91/4.97

both measures, we used Otodynamics ILO equipment (ILO V6 version v6.41.27.33, United Kingdom). This equipment has been validated for newborn hearing screening in multiple populations across the world and as such, is widely used clinically to identify potential hearing losses within hours of birth (https://www.otodynamics.info). Three experienced audiologists were responsible for its maintenance and calibration. The test comprised of a probe tip inserted at the entrance of the external ear canal. Sounds presented through a probe tip went through the middle-ear cavity to reach the inner ear. Otoacoustic emissions are non-linear distortion products generated in the cochlea and transmitted back through the middle ear to the outer ear, where they can be detected as sound using a sensitive microphone.

The ears were tested separately. The first side to be tested (right vs. left) was selected at random. The sound used for the CEOAEs was a 80-microsecond click presented at rate of 50 s^{-1} at around 84 dB peak equivalent between 1 to 4 kHz. The amplitude of CEOAEs at each emitted frequency is commonly believed to be related to auditory threshold. These data were collected using an interleaved sequence of stimuli that yielded two averaged OAE waveforms. The reproducibility of CEOAEs is derived from the correlation between the two waveforms and is informative about another aspect of the integrity of the inner-ear function, i.e. the variability of auditory thresholds. CEOAEs are characterized by good intra-subject stability (Harris et al., 1991; Keppler et al., 2010; Vedantam and Musiek, 1991), high repeatability and test-retest reliability (Kochanek et al., 2015), and high inter-subject variability related to differing distributions of their characteristic components (Blinowska et al., 2005, 2007).

As mentioned above, DPOAEs are another way to measure inner-ear activity and may capture another aspect of cochlear function (Shera and Guinan, 1999). For DPOAEs, two sounds of different frequencies were

Table 2
Girls: Descriptive data.

Variable	Age	N	Minimum	Maximum	Mean	SD
Androgen markers (based on children who also have DPOAEs available in the right ear)						
Areolar melanin	Birth	22	28.00	118.33	73.93	23.16
Vulvar melanin	Birth	22	0.00	177.00	55.70	46.07
AGD-clitoris (mm)	Birth	22	28.70	44.43	34.31	3.88
AGD-fourchette (mm)	Birth	22	10.33	21.40	16.07	3.02
Right 2D:4D	6 mo	22	0.87	1.07	0.94	0.05
Left 2D:4D	6 mo	22	0.82	1.03	0.92	0.06
Auditory function (based on children who also have areolar pigmentation)						
CEOAE: amplitude	6 mo	R/L	R/L	R/L	R/L	R/L
Mean (dB SPL)		24/20	6.50/13.60	28.30/29.20	18.43/19.61	5.82/4.47
2 kHz		23/20	0.50/3.10	18.30/20.10	11.88/12.10	5.22/4.66
2.8 kHz		22/21	4.50/4.10	26.80/28.00	14.96/14.98	5.22/5.64
4 kHz		20/19	2.50/2.90	20.40/20.00	12.67/13.33	5.22/5.01
CEOAE: reproducibility	6 mo	R/L	R/L	R/L	R/L	R/L
Mean (%)		24/21	18.00/29.00	97.00/98.00	73.83/71.71	23.91/23.93
2 kHz		24/23	5.50/17.90	99.60/99.80	85.22/78.52	23.79/26.51
2.8 kHz		24/23	18.40/13.70	99.90/100.00	89.09/85.64	18.68/23.48
4 kHz		24/23	37.20/26.30	99.70/99.70	89.60/83.87	18.16/23.63
DPOAE: amplitude	6 mo	R/L	R/L	R/L	R/L	R/L
Mean (dB SPL)		22/20	0.30/1.00	20.20/22.90	12.92/14.48	6.11/5.48
2 kHz		17/18	6.00/1.30	17.00/24.60	10.34/12.28	3.75/6.48
2.8 kHz		19/17	6.20/6.30	29.20/22.80	14.47/12.12	6.37/5.36
4 kHz		19/19	0.30/4.50	22.60/29.60	12.81/14.33	5.73/6.69

simultaneously presented with a frequency ratio (frequency of the second stimulus/frequency of the first stimulus) of 1.22. The presentation level was 65 dB SPL for the first stimulus and 55 dB SPL for the second stimulus, between 1–6 kHz.

Tympanometric testing also was performed. As for the OAEs, this test consisted of inserting a probe tip in the external ear canal. A slight pressure from 300 to −300 daPa was presented to the external ear canal in order to assess the mobillity of the tympanic membrane and the middle-ear ossicles as well as the middle-ear pressure. The results, illustrated by a graph called tympanogram, show a curve with the mobility of the eardrum/middle-ear ossicles (or static compliance) expressed in mmho on the ordinate axis and the indirect measure of the middle-ear pressure in daPa on the abscissa.

2.3. Anogenital distance and other genital measurements

Measurements for AGD-penis, AGD-clitoris, AGD-fourchette, AGD-scrotum, penile width and penile length each were 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-degree angle to the trunk. For AGD-fourchette distance and AGD-clitoris in female newborns, the distance was measured from the centre of the anus to the posterior convergence of the fourchette (where the vestibule begins), or to the clitoris, respectively. The centre, 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 centre 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). Penile width was measured as the diameter at the base of the penis. Penile 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 penile measurements were made before circumcision. Other genital measurements are not impacted by this procedure and were therefore collected regardless of whether the child had been circumcised or not. All measurements were taken twice, and if there was a difference greater than 2-mm 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 Quality Control).

2.4. 2D:4D Ratio

The length (in mm) of the second and fourth finger of each hand were measured during the 6-month assessment of the infant, in accordance with principles already described in the literature (Manning and Robinson, 2003). These measurements were done with a transparent plastic ruler with millimetre 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, and the mean finger length for each hand was used in any further analyses. All attempts were made to ensure that measurements were made in a standardized manner (see Quality Control).

2.5. Melanin pigmentation

All melanin pigmentation measurements were made at birth using a Mexameter® MX18 (Courage & Khazaka electronic GmbH, Cologne, Germany). The Mexameter uses light absorption/reflectance to measure the two components mainly responsible for the colour of the skin: melanin and hemoglobin (erythema). This instrument has been developed and validated in several human populations across the world (Clarys et al., 2000; Diffey et al., 1984; Hermanns et al., 2001; Kim et al., 2012; Park et al., 2006; Pratchyapruit et al., 2011; Zhao et al., 2017). The probe emits 3 specific light wavelengths, and a receiver measures the light reflected by the skin. Because the quantity of emitted light is a known constant, the quantity of light absorbed by the skin can be calculated. The melanin is measured by specific wavelengths chosen to correspond to different absorption rates by the pigments. For the erythema measurement, specific wavelengths are also used, corresponding to the spectral absorption peak of hemoglobin and to avoid other colour influences (e.g. bilirubin).

Three measurements were made 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. Measurements of baseline pigmentation were made in the posterior buttock area to avoid any areas prone to erythema, in the area 2.54 cm below the horizontal line bisecting the postero-superior iliac spines. Measurements for areolar pigmentation measurements were made by placing the probe vertically just slightly eccentric to the left or right nipple. 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 Quality Control).

2.6. Quality control

The clinical skills of the child testers are of paramount importance for obtaining valid and reliable data. The auditory testing was performed by the same three experienced pediatric audiologists. They have extensive experience with the equipment used in this study and were well trained to detect invalid results (i.e. probe not well inserted or noise level too high, etc.). Similarly, measurement of androgenic indices 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 evaluator assigned to each child, with a second research assistant present to assist the primary evaluator (i.e. to hold the infant in a certain position, to enter the data on the record sheets, etc.). Finally, measurements for androgenic indices were repeated 2–4 times, depending on whether initial measures showed discrepancies exceeding a certain threshold. Intra-rater reliability was high, with intra-class coefficients all greater than 0.93. This threshold varied depending on the specific parameter under examination (see above sections for more details).

2.7. Statistical analyses

Multiple linear regression models were used to examine the relationship between OAEs (outcome) and each androgenic indice (predictor), i.e., anogenital distance (AGD), 2D:4D ratio and areolar pigmentation in boys and girls, penile length/width and pigmentation of the scrotal area in boys, and pigmentation of the vulva in girls. Because most androgenic markers were measured in different ways in boys and girls (e.g. AGD-clitoris in girls vs AGD-penis in boys, no equivalent for penile length/width in girls), models were tested separately in boys and girls. Primary outcomes were the amplitude and reproducibility of CEOAEs averaged across frequency regions, as well as the amplitude of DPOAEs averaged across frequency regions; secondary outcomes were the amplitudes and reproducibility of CEOAEs, as well as amplitudes of DPOAEs by specific frequency (i.e. at 2, 2.8 and 4 kHz). Frequencies lower than 2 kHz were not included in the analyses because the noise

level was too high.

We selected the following control variables:

1) middle-ear factors (static compliance and peak pressure), because of the potential influence of the middle-ear conditions on OAE results (Mcfadden, 1993);
2) research site, because of inter-rater difference;
3) weight-for-length (z-scores of weight-for-length ratios (WFLR): ((Weight/Length) – (mean WFLR))/WFLR standard deviation), due to confounding effects from the conversion of testosterone to estradiol in adipose tissue (Nelson and Bulun, 2001);
4) baseline pigmentation/melanin levels, due to confounding effects of this variable on areolar, vulvar or scrotal pigmentation (Fullerton et al., 1996; Tadokoro et al., 1997; Takiwaki et al., 1994).

All analyses were performed with SPSS 23.0. To limit the number of false positives, a two-step process was implemented: 1) a first screen was conducted with unadjusted analyses (i.e. not including any control variables), in order to screen out predictor variables yielding an unadjusted p-value greater or equal to 0.2, which were considered not significant and were not further considered in any additional analyses; 2) following this first screen, only predictor variables yielding an unadjusted p-value less than 0.2 were entered into adjusted analyses (including control variables). In this second step, only p-values that remained less or equal to 0.05 after adjustment for control variables were considered to be significant. Standardized betas (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.

3. Results

3.1. Sample characteristics

Descriptive data for boys and girls are listed in Tables 1 and 2, respectively. Sample sizes varied between 19–25 for boys and 17–24 for girls depending on the specific androgen and auditory marker. Overall, there were no significant sex differences in the amplitude or reproducibility of OAEs, though trends were present for the mean amplitude of CEOAEs in the left ear and the mean amplitude of DPOAEs in the right ear (one-tailed T-tests, $p > 0.05$, see Table 3 for more details).

3.2. Androgen markers & click-evoked otoacoustic emissions

Significant associations between the amplitude of CEOAEs and androgen markers were present only in boys (see Table 4 for beta coefficients and p-values). Scrotal pigmentation showed trends for inverse associations, both with the mean amplitude of CEOAEs in the right ear as well as with the amplitude of CEOAEs in the right ear at 2 kHz. In addition, scrotal pigmentation showed a significant inverse association with the amplitude of CEOAEs in the right ear at 4 kHz. There were no significant associations between the amplitude of CEOAEs and any other androgen marker in boys (AGD, 2D:4D ratio, penile length/width, and areolar pigmentation), nor were there any significant associations in girls (all p-values˜ > 0.3, see Table 4 for examples).

Significant associations between androgen markers and the reproducibility of CEOAEs emerged only in boys (see Table 4 for beta coefficients and p-values). Scrotal pigmentation was inversely associated with the reproducibility of CEOAEs in the right ear at 4 kHz, and penile length was inversely associated with the reproducibility of CEOAEs in the left ear at 2.8 kHz. There were no significant associations between the reproducibility of CEOAEs and any other androgen marker in boys (AGD, 2D:4D ratio, penile width, and areolar pigmentation), nor were there any significant associations in girls (all p-values˜ > 0.3, see Table 4 for examples).

3.3. Androgen markers & distortion-product otoacoustic emissions

Significant associations between androgen markers and the amplitude of DPOAEs emerged only in boys (see Table 4 for beta coefficients and p-values). Penile length was found to be inversely associated with the mean amplitude of DPOAEs in the right and left ears, as well as with the amplitude of DPOAEs in the right ear at 2 kHz. Unexpectedly, the correlation between AGD-scrotum and the amplitude of DPOAEs in the left ear at 2.8 kHz in boys was in the reverse direction, i.e. there was a positive association between these two variables. There were no significant associations between the amplitude of DPOAEs and any other androgen marker in boys (2D:4D ratio, penile width, and areolar/scrotal pigmentation), nor were there any significant associations in girls (all p-values˜ > 0.3, see Table 4 for examples).

Table 3
Sex differences in OAEs.

1ary outcomes	T-tests	2ary outcomes	T-tests
CEOAE	T = -0.34; p = 0.37	2 kHz	T = 0.19; p = 0.42
Mean amplitude		2.8 kHz	T = 0.68; p = 0.25
Right ear		4 kHz	T = 1.45; p = 0.07
CEOAE	T = 1.49; p = 0.07	2 kHz	T = 1.25; p = 0.11
Mean amplitude		2.8 kHz	T = 0.78; p = 0.22
Left ear		4 kHz	T = 0.63; p = 0.26
CEOAE Reproducibility	T = -0.22; p = 0.41	2 kHz	T = -0.33; p = 0.37
Right ear		2.8 kHz	T = 0.17; p = 0.43
		4 kHz	T = 0.14; p = 0.44
CEOAE Reproducibility	T = 0.88; p = 0.19	2 kHz	T = 1.08; p = 0.14
Left ear		2.8 kHz	T = 0.79; p = 0.22
		4 kHz	T = 0.07; p = 0.47
DPOAE	T = 1.36; p = 0.09	2 kHz	T = 1.30; p = 0.10
Mean amplitude		2.8 kHz	T = -1.00; p = 0.16
Right ear		4 kHz	T = 1.02; p = 0.16
DPOAE	T = -0.01; p = 0.50	2 kHz	T = -1.00; p = 0.16
Mean amplitude		2.8 kHz	T = -0.21; p = 0.42
Left ear		4 kHz	T = -0.68; p = 0.25

*p-values are for one-tailed T-tests.

Table 4
Associations between Androgen Markers and Auditory Function.

Boys		Girls	
Variables	Standardized beta coefficients & p-values *Adjusted	Variables	Standardized beta coefficients & p-values **Adjusted
Scrotal pigmentation		**Vulvar pigmentation**	
CEOAE: amplitude		**CEOAE: amplitude**	
Mean		Mean	
Right ear	b = -0.500, p = 0.025 b = -0.357, p = 0.120*	Right ear	b = -0.156, p = 0.467 b = -0.115, p = 0.594**
2 kHz		2 kHz	
Right ear	b = -0.422,p = 0.057 b = -0.383, p = 0.073*	Right ear	b = -0.121, p = 0.574 b = -0.056, p = 0.794**
4 kHz		4 kHz	
Right ear	b = -0.541, p = 0.011 b = -0.601, p = 0.017*	Right ear	b = -0.006, p = 0.978 b = 0.038, p = 0.838**
CEOAE: reproducibility		**CEOAE: reproducibility**	
4 kHz		4 kHz	
Right ear	b = -0.569, p = 0.007 b = -0.522, p = 0.027*	Right ear	b = 0.062, p = 0.774 b = 0.088, p = 0.625**
Penile length		**AGD-clitoris**	
CEOAE: reproducibility		**CEOAE: reproducibility**	
2.8 kHz		2.8 kHz	
Left ear	b = -0.594, p = 0.002 b = -0.434, p = 0.014*	Left ear	b = -0.151, p = 0.482 b = -0.223, p = 0.382**
DPOAEs		**DPOAEs**	
Mean		Mean	
Right ear	b = -0.652, p < 0.0001 b = -0.519, p = 0.004*	Right ear	b = -0.047, p = 0.821 b = -0.112, p = 0.586**
Left ear	b = -0.569, p = 0.002 b = -0.392, p = 0.019*	Left ear	b = -0.193, p = 0.366 b = -0.073, p = 0.789**
2 khZ		2 khZ	
Right ear	b = -0.335, p = 0.149 b = -0.463, p = 0.035*	Right ear	b = 0.079, p = 0.755 b = 0.239, p = 0.465*
AGD-scrotum		**AGD-fourchette**	
DPOAEs: amplitude		**DPOAEs: amplitude**	
2.8 kHz	b = 0.414, p = 0.055	**2.8 kHz**	b = 0.168, p = 0.505
Left ear	b = 0.414, p = 0.041*	**Left ear**	b = 0.155, p = 0.665**

* Adjusted for control variables (static compliance, peak pressure,research site, weight-for-length and, for analyses including areolar, vulvar or scrotal pigmentation, baseline pigmentation levels).

** Analyses for girls only shown for comparison purposes.

4. Discussion

To our knowledge, this is the first study in human infants supporting a link between purported markers of fetal androgen exposure and inner-ear function. Significant inverse correlations were found only in boys, between inner-ear function and two distinct markers of androgen exposure, penile length and scrotal pigmentation. These relationships tended to be of greater magnitude and significance in the right ear. Unexpectedly, there also was a positive association, in boys, between the mixed androgenic/estrogenic marker AGD-scrotum and the amplitude of DPOAEs in the left ear, but only at one specific frequency (2.8 kHz). These results provide some support for both the prenatal-androgen-exposure hypothesis, which postulates that masculinization of auditory function in humans is linked to an adverse effect of androgen exposure *in utero* on inner-ear responses (McFadden, 2009, 2011), and the GG hypothesis which postulates that androgen-related effects will be lateralized and predominantly affect components linked to the left hemisphere (in this case, the right ear) (Geschwind and Galaburda, 1985).

These findings corroborate those from primate studies supporting the prenatal-androgen-exposure hypothesis (McFadden, 2009, 2011) and the GG hypothesis (Geschwind and Galaburda, 1985). For example, in rhesus monkeys, a sex difference in CEOAEs that fluctuated seasonally according to testosterone levels also has been reported (McFadden et al., 2006). Moreover, exposure to flutamide (which blocks androgen receptors) during early or late gestation strengthened the CEOAEs of males, but had no effect on those of females (McFadden et al., 2006). Androgens received late in gestation weakened CEOAEs in both sexes, but androgens received early in gestation weakened CEOAEs only in females (not males). These findings may suggest that, in males, the effect of androgen exposure on OAEs reaches a peak early in gestation, rendering any additional androgen exposure during later gestational periods extraneous in terms of cochlear development.

Interestingly, no ear asymmetry was observed in rhesus monkeys, suggesting that the ear lateralization process may be unique to humans and relevant to the origins of speech perception and production as well as other cognitive functions (McFadden, 2009). Certainly, this would be consistent with the GG hypothesis, which posits that testosterone plays a prominent role in the lateralization of cognitive functions, through the predominance of its effects on the left hemisphere (Geschwind and Galaburda, 1985). One could speculate that the asymmetries established in the peripheral auditory system as a result of prenatal androgen exposure may have led to the higher-order asymmetries in hemispheric and cortical specialization typically observed in humans. This hypothesis has the benefit of uniting the prenatal-androgen-exposure and GG models.

Other studies in human children have also provided indirect support for the prenatal-androgen-exposure hypothesis. For example, some, although not all, examinations of children suffering from psychiatric disorders with a strong male-to-female bias in the prevalence have shown that, compared to controls, children with autism (Bennetto et al., 2017) or attention-deficit hyperactivity (ADHD) (Martel et al., 2008; McFadden et al., 2005) are more likely to have weaker CEOAEs. Risk for ADHD and autism also has been linked with degree of exposure to androgens *in utero*, supporting a link between fetal androgen exposure, auditory function and the development of male-biased psychiatric

disorders during infancy/middle childhood (Baron-Cohen et al., 2015; Wang et al., 2017). Newborn males already have been shown to have weaker OAEs than newborn females, likely as a result of prenatal differences in androgen exposure (McFadden, 2011). This effect persists beyond infancy, lasting throughout middle childhood, adolescence, and adulthood, supporting the predominance of organizational (i.e. structural changes that are relatively irreversible) vs. activational (i.e. functional and reversible) effects of androgens on OAEs (McFadden, 2011). Further supporting the associations between androgen exposure and OAEs is the demonstrated presence of androgen receptors in the cochlea and the outer hair cells of adult mammals (McFadden, 2011), though the examination of this phenomenon in the human fetus has proved to be challenging.

Both androgen production and inner-ear development occur during the same critical period of gestation. For example, coiling of the human cochlea is complete by about week 9, when testosterone production is just starting in male fetuses (Bezin et al., 1994; McFadden, 2011; Whitlon, 2004). The subsequent fine-tuning of the inner ear then unfolds approximately from week 9 to week 25, when auditory evoked potentials have been recorded at the cortical level in preterm infants, thereby coinciding with the peak of testosterone exposure (Bezin et al., 1994; McFadden, 2011; Whitlon, 2004). In particular, the development of the sensory surface of the basilar membrane, including that of the outer hair cells that modulate the amplitude and reproducibility of OAEs, tends to occur between week 11 and week 14 (Bezin et al., 1994; McFadden, 2011; Whitlon, 2004), overlapping with the specific androgen exposure windows critical to scrotal pigmentation and penile growth.

The question remains as to why certain androgenic markers, such as scrotal pigmentation and penile length, were associated with inner-ear function, while others were not. Two possibilities emerge -the first is that other markers may be responsive to both androgen and estrogen exposure, as recently reported by our group (paper accepted at *Journal of Developmental Origins of Health and Disease*), as well as others (e.g. AGD -(Gerardin et al., 2008; Mitchell et al., 2015) and areolar pigmentation (Fullerton et al., 1996; Tadokoro et al., 1997; Takiwaki et al., 1994)). However, there is also some evidence that penile differentiation and development is also sensitive to estrogens, as demonstrated by penis malformations induced in mice and rats by pre- and early postnatal administration of diethylstilbestrol (Blaschko et al., 2013; Goyal et al., 2007; Zheng et al., 2015).

Another possibility is that associations with hearing sensitivity reflect the different critical windows for the androgen-dependent development and growth of these structures. In particular, scrotal pigmentation may be under androgen control throughout the 1st and 2nd trimesters (gestational weeks 8 to 20) (Wilson, 1983). Penile length is thought to be determined during a similar 'masculinisation programming window', also throughout the 1st and 2nd trimesters (Simon et al., 2012; Welsh et al., 2010). Therefore, both scrotal pigmentation and penile length appear to show a specific developmental plasticity to prenatal androgen exposure over the same period during which the cochlear outer hair cells are undergoing active development (Lavigne-Rebillard and Pujol, 1990). In contrast, sex differences in other markers such as 2D:4D and AGD are detectable from the 1st trimester onward (Martino-Andrade et al., 2016; Simon et al., 2012), with little further variation until adulthood for 2D:4D (Galis et al., 2010; Garn et al., 1972; Malas et al., 2006; Manning and Fink, 2018; Manning et al., 1998; Szwed et al., 2017), and possibly greater cumulative sensitivity to postnatal than prenatal androgens for AGD (Dusek and Bartos, 2012; Mitchell et al., 2015; Thankamony et al., 2016). Thus, the fact that scrotal pigmentation and penile length, but not AGD nor 2D:4D ratio, are associated with OAEs suggests that the influence of androgens on auditory function may be confined to a specific developmental window, namely gestational weeks 8-20. This is supported further by prior evidence that 2D:4D ratio is also not associated with OAEs in adults, regardless of sexual orientation (McFadden and Shubel, 2003).

4.1. Strengths and limitations

To our knowledge, this is the first study in human infants examining the relationship between various anatomical markers of prenatal androgen exposure and OAEs. We included several measurements of markers of androgen exposure collected at birth and 6 months of age, as well as several types of inner-ear responses collected at 6 months of age. Rigorous training and quality control procedures were implemented for each measurement, and repeated measures were taken when discrepancies arose between initial measurements.

On the other hand, we did not find any significant sex differences in the amplitude or reproducibility of OAEs in either ear, though trends favoring girls were present for the mean amplitude of CEOAEs in the left ear and the mean amplitude of DPOAEs in the right ear. In our opinion, these sex differences did not reach significance because of the limited statistical power of our study. Indeed, many prior studies have documented the fact that CEOAEs are stronger in female compared to male infants (Kei et al., 1997; Thornton et al., 2003a, b), although effect sizes have not been as large as for older children or adults (Khalfa et al., 1997). This suggests that the magnitude of sex differences in CEOAEs may increase with age, rendering them less easily detectable at this early stage of development, particularly with a small sample size. Another potential explanation is that differences in gestational age at birth or in race/ethnicity in the male vs. female samples could account for the lack of sex differences in OAEs. However, there are no sex differences in these variables in our study, making this unlikely. Even if we do not report significant sex differences in OAEs, this does not exclude the possibility that the developmental processes underlying inner-ear function are sexually dimorphic. Rather, the current findings highlight the fact that influences other than androgens may play a more important role in the development of the auditory system in girls.

The lack of significant associations between several of the androgenic markers and inner-ear function may be largely attributable to lack of power, itself related to sample size. Conversely, the positive association, in boys, between AGD-scrotum and the amplitude of DPOAEs in the left ear at 2.8 kHz, is particularly at risk of being a false positive given the absence of consistent associations with other parameters of auditory function. Still, this marker may be best described as a mixed androgenic/estrogenic, rather than a 'pure' androgenic marker, given that it could represent a better index of the level of conversion of testosterone to estradiol rather than androgenic activity per se (Gerardin et al., 2008; Mitchell et al., 2015). It is intriguing that this positive correlation only involved the left ear, hinting to the presence of a different, and possibly estrogen-dependent, process underlying the development of the left vs. right cochlea. Nonetheless, this finding requires further confirmation, as it involved a secondary outcome related to a specific auditory frequency. Notwithstanding the above limitations, we still consider the current results to hint at important effects of androgens on auditory function. As such, these findings represent a significant contribution to an under-researched topic, given the current scarcity of data in human infants, and the even smaller sample sizes examined in similar studies of adults.

5. Conclusions

This study describes associations between specific markers of fetal androgen exposure, in particular scrotal pigmentation and penile length with inner-ear function as measured by otoacoustic emissions, reported for the first time in human infants. We find evidence to support both the prenatal-androgen-exposure and GG hypothesis as models for the prenatal masculinization of auditory function in humans.

Declaration of Interest

None of the authors have a conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.psyneuen.2019.02.015.

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