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Chapter 6

Do sex differences in CEOAEs and 2D:4D ratios reflect androgen exposure? A study in women with complete androgen insensitivity syndrome

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ABSTRACT

Studies investigating the influence of perinatal hormone exposure on sexually differentiated traits would greatly benefit from biomarkers of these early hormone levels. Click-evoked otoacoustic emissions (CEOAEs) show sex differences that are thought to reflect differences in early androgen exposure. Women with complete androgen insensitivity syndrome (CAIS), who lack androgen action in the presence of XY-chromosomes, enabled us to study the effect of complete androgen resistance. The main goal was to investigate a possible link between CEOAEs and effective androgen exposure and, thus, whether this can be used as a biomarker. In addition, we aimed to replicate the only previous digit-ratio study in women with CAIS, because regardless of the criticism towards the validity of this measure as a biomarker for prenatal androgen exposure, it is still used for this purpose. CEOAEs and digit ratios from women with CAIS were compared with those from control men and women. The typical sex differences in CEOAEs and digit ratios were replicated in the control groups. Women with CAIS showed a tendency towards feminine CEOAE amplitudes in the right ear and a significant female-typical digit ratio in the right hand. Although these results are suggestive of androgen-related effects, the within-group variability was not reduced in women with CAIS. In line with previous studies, these findings in women with CAIS show that other, non-androgenic, factors also influence the sexual differentiation of digit ratios and CEOAEs. Consequently, use of these measures in adults as retrospective markers for early androgen exposure is not recommended.

INTRODUCTION

Prenatal androgen exposure is thought to play an important role in the development of sex differences in a variety of neural and behavioral characteristics (e.g., reviewed in Beltz et al. 2013). To investigate potentially permanent, i.e. organizational, effects of prenatal androgen exposure in humans, a prospective study design including direct measurements of fetal hormone levels (e.g., obtained from amniocentesis, preferably at several gestational time points) would be ideal. However, this type of study design is expensive, time consuming and, above all, incurs fetal risks. A desirable alternative would therefore be to use retrospective measures as a marker of prenatal hormone levels.

Otoacoustic emissions (OAEs; e.g., reviewed in McFadden 2009) might represent such an indirect marker, because sex differences which potentially reflect sex differences in early androgen levels have been demonstrated. OAEs are sounds measurable in the external ear canal that originate from the cochlea as a by-product of the cochlear amplification mechanism (Kemp 1978, 2008; Davis 1983). They can occur spontaneously (spontaneous OAEs; SOAEs) or as a response to acoustic stimuli such as clicks (click-evoked OAEs; CEOAEs). Previous studies have shown that women have more frequent and stronger SOAEs and larger CEOAE amplitudes than men (McFadden and Pasanen 1998; Snihur and Hampson 2011a; for review see McFadden 2009). These sex differences are not only present in adults, but were also found in neonates (Burns et al. 1992; Morlet et al. 1995; Kei et al. 1997; Newmark et al. 1997; Cassidy and Ditty 2001).

A direct investigation of the proposed link between prenatal hormone levels and OAEs later in life is complicated in humans, because hormonal manipulations are unethical, and the incorporation of direct fetal hormone level measurements in a study design is challenging. To date, OAEs have not been related to fetal hormone levels in typically-developing individuals. Therefore, another approach used in examining the validity of OAEs as a marker of early androgen exposure, is to study disorders/differences of sex development (DSD), in which the development of the chromosomal, gonadal, and/or anatomical sex is atypical (Hughes et al. 2006). In 46,XX women with congenital adrenal hyperplasia (CAH), a condition characterized by excessive prenatal androgen production, the number of SOAEs was lower ("masculinized") compared with control women. However, this was found in the right ear only, and no differences were observed in the CEOAE amplitude of either ear (Wisniewski et al. 2014).

A DSD of particular interest when studying androgenic effects is complete androgen insensitivity syndrome (CAIS). Women with CAIS have a 46,XY karyotype, but no effective androgen exposure due to non-functional androgen receptors caused by genetic mutations in the androgen receptor gene. This leads to a female phenotype despite testosterone levels, produced by their abdominal testes, that are within or above the male range (Oakes et al. 2008; Hughes et al. 2012). Women with CAIS thus provide a unique opportunity to

study whether the reported sex differences in OAEs are likely to originate from differences in androgen exposure or other factors that might influence sexual differentiation, such as sex chromosome configuration or estrogens.

To date, the only OAE study that has been performed in women with CAIS found no differences between control subjects and women with CAIS regarding the number of SOAEs and amplitude of CEOAEs (Wisniewski et al. 2014). It should be noted that in this study the sex difference in CEOAE amplitude was not replicated in the control groups, and that the sample sizes were very small, also for the control groups (7 women with CAIS, 13 control women, 10/11 control men). Therefore, no firm conclusions could be drawn from this study regarding sex differences in CEOAEs and whether or not these can be attributed to differential androgen exposure.

The 2D:4D ratio, which is the length of the 2nd digit (index finger) relative to that of the 4th digit (ring finger), is another measure that has been proposed to reflect prenatal androgen exposure. The 2D:4D ratio is overall larger in women than in men (Manning et al. 1998; for meta-analysis see Hönekopp and Watson 2010). However, the validity of this measure as a marker of prenatal androgen exposure has been criticized. Two digit-ratio studies have measured fetal hormone levels through amniocentesis, and their results were inconclusive. The 2D:4D ratio showed a negative association with fetal testosterone in newborn girls, but not in boys (Ventura et al. 2013), and a negative association with the fetal testosterone-to-estradiol ratio at age two, but not with fetal testosterone or estradiol alone (Lutchmaya et al. 2004). Furthermore, a recent meta-analysis found no associations between functional androgen receptor (AR) gene variants (CAG and GGC repeat-length polymorphisms) and 2D:4D ratio (Voracek 2014), providing evidence against major AR gene-related effects (for a discussion of additional evidence and arguments against AR gene-related effects on 2D:4D, see Voracek 2014).

Digit ratio studies in DSDs suggested a lower, i.e. more "masculine," digit ratio in women with CAH than in control women regarding the right hand (Brown et al. 2002; Ciumas et al. 2009) or in both hands (Okten et al. 2002; Rivas et al. 2014), although a "feminine" digit ratio in CAH was also reported (Buck et al. 2003). Individuals with Klinefelter syndrome, who have 1 Y- and 2 or more X-chromosomes and reduced androgen levels, showed a higher, i.e., "feminized", digit ratio than control men (Manning et al. 2013). A study in 16 women with CAIS showed that their digit ratios were feminized, which, along with the majority of findings in DSDs, is suggestive of an association between androgen exposure and the 2D:4D ratio (Berenbaum et al. 2009). However, some important caveats have been discussed by both the authors of this study (Berenbaum et al. 2009) and in a commentary on this paper (Wallen 2009), such as the moderate effect size of the difference between women with CAIS and control men, and the finding that variability in digit ratios is not smaller in women with CAIS than in both control groups. This has led to the conclusion that the "digit ratio is related to effective androgen exposure but that the relation is too small to use digit ratio as

a marker for individual differences in prenatal androgen exposure" (as cited in Berenbaum et al. 2009). Regardless of this conclusion and other important critical arguments (summarized by Voracek 2014), numerous studies still use the digit ratio for this purpose. The study by Berenbaum et al. (2009) included a sample of only 16 women with CAIS, which is a reasonable sample considering the rareness of the syndrome, but small in terms of power. Therefore, a replication of this finding is warranted.

Because strong evidence for a link between early androgen exposure and CEOAEs is still lacking, the primary objective of the present study was to investigate the proposed association between androgens and CEOAE amplitude by comparing women with CAIS to control men and women. In addition, because many researchers continue to use the digit ratio as a marker of prenatal androgen exposure, regardless of the criticism regarding and evidence arguing against its validity, we measured 2D:4D ratios in the present study in order to attempt to replicate the previous digit ratio study in women with CAIS (Berenbaum et al. 2009).

MATERIALS AND METHODS

Participants

Data from subjects recruited for the present study were included if subjects were Caucasian, did not report having a male twin, and had a heterosexual orientation. A heterosexual orientation was defined as an androphilic orientation, i.e., attraction to men, in control women and women with CAIS, and a gynephilic orientation, i.e., attraction to women, in control men (sexual orientation score >5 on the Dutch version of the Klein Sexual Orientation Grid (Dessens et al. 1999; adapted from Klein et al. 1985)). Not all subjects met all our additional inclusion criteria for both the CEOAE and digit ratio analyses. Therefore, further details of the participant groups will be described for both analyses separately.

For the CEOAE analyses, subjects were divided into four groups: women with CAIS, control men, and, because hormonal contraceptives might influence the CEOAE amplitude (Snihur and Hampson 2012a), two groups of control women; using oral contraceptives (OC-women) and normally cycling (NC-)women not using any hormonal contraceptives. To minimize effects related to handedness (Snihur and Hampson 2011a), all subjects had a right-hand preference for writing and handedness scores of ≥ +7 on the Dutch Handedness Inventory (Van Strien 1992; range -10 = extreme left-handed, +10 = extreme right-handed). All control women were pre-menopausal and not pregnant. NC-women were not tested during the first days of the follicular phase (menses), when sex hormone levels are low, but otherwise could be tested any time during the remainder of their cycle. OC-women also were tested only once and only on a day when they were taking doses of OC, i.e. not during the first week of the cycle when OC is not taken. All OC-women used a monophasic OC, containing ethinylestradiol and levonorg-

estrel (20/100µg (*n* = 2), 30/150µg (*n* left ear = 27, *n* right ear = 30) or 50/125µg (*n* = 2)). The data for both ears were excluded for all subjects exposed to high levels of noise in the 24 hours preceding the measurement and from subjects who reported having a hormonal disorder or taking hormonal medication that might influence sex hormone levels (with the exception of oral contraceptives for OC-women and hormone replacement therapy in women with CAIS). When severe ear problems were reported or measurements were invalid (see CEOAE methods section), the data from that particular ear were excluded from the analyses. These criteria resulted in a total of 125 usable measurements of the left and 132 usable measurements of the right ear in all groups combined. In OC-women there were 31 valid measures of the left ear (Mdn age = 25.31; IQR = 6.2) and 34 of the right ear (Mdn age = 25.43; IQR = 10.7), see Table 1 for Ns and age per group for the other groups.

Table 1 Sample characteristics, CEOAE amplitude and 2D:4D ratio per group

	CAIS	Control Men	Control women	F/χ^2	P
	M/Mdn (SD/IQR)	M/Mdn (SD/IQR)	M/Mdn (SD/IQR)		
CEOAE left					
N	18	33	43		
Age*	27.80 (18.1)	28.30 (19.1)	30.85 (18.0)	0.123	0.940
Amplitude (dB SPL)	10.96 (2.72)	9.42 (4.19)	12.68 (4.08)	6.567	**0.002**
CEOAE right					
N	18	37	43		
Age*	27.80 (18.1)	28.30 (19.6)	31.09 (21.6)	0.319	0.853
Amplitude (dB SPL)	12.48 (2.94)	10.23 (3.77)	12.93 (3.67)	5.986	**0.004**
2D:4D ratio left					
N	21	129	156		
Age*	27.65 (23.9)	26.82 (14.8)	27.00 (14.0)	0.655	0.721
Ratio*	0.965 (0.04)	0.963 (0.04)	0.980 (0.04)	13.565	**0.001**
2D:4D ratio right					
N	20	126	154		
Age*	27.80 (23.0)	26.91 (15.0)	27.00 (12.8)	1.720	0.423
Ratio*	0.978 (0.04)	0.956 (0.04)	0.976 (0.05)	22.819	**< 0.001**

Note: *Non-parametric tests were used for statistical analysis of this variable. Statistics from parametric tests are expressed in M = mean; SD = standard deviation; F. Statistics from non-parametric tests are expressed in Mdn = median; IQR = interquartile range (these are more appropriate statistics for non-parametric tests), and χ^2. Bold P-values represent a significant ($P < 0.05$) main effect of group. For CEOAE data, "control women" refers to NC-women.

For the digit-ratio analyses the use of OCs was not a factor of interest, therefore the data of only 3 groups were used for this part of the study; women with CAIS, control men and NC-women. The latter group will be referred to as "control women". The 2D:4D ratios of 7 left and 13 right hands were excluded from the analyses because of reported digit or hand trauma with a possible influence on digit length, or due to technical problems with the scan of the hand. To increase the size of the control groups, data from 88 control men and 109 control women from a previous digit ratio study, all heterosexual and 18 years and older, were added to the data acquired for the current study (see Wallien et al. 2008 for further details). This resulted in a total of 306 usable left and 300 usable right-hand 2D:4D ratios for all groups combined (see Table 1 for Ns and age per group and per hand).

All women with CAIS had gender identity scores in ranges comparable to reference groups of healthy women, which was assessed with a Gender Questionnaire (Callens et al. submitted; adapted from Hines et al. 2003) with 6 questions added to the original questionnaire to cover the criteria for a gender identity disorder based on the Diagnostic and Statistical Manual of Mental Disorders (American Psychiatric Association 2000). The diagnosis of CAIS was based on clinical characteristics in all 22 women with CAIS included in the analyses. Furthermore, a mutation analysis of the androgen receptor (AR) gene using genomic DNA was performed. In 14 participants the clinical diagnosis was confirmed with a mutation in the AR-gene, in 7 participants an unclassified variant of the AR-gene mutation was found, and in 1 participant the result of the analysis was inconclusive. All women with CAIS were gonadectomized and, with one exception, took hormone replacement therapy (HRT) to compensate for the lack of gonadal sex hormone production. HRT consisted of orally, transdermally or subcutaneously administered estradiol (n = 15), a combination of estradiol and dydrogesteron (n = 3), conjugated estrogens (n = 2), or a monophasic oral contraceptive containing ethinylestradiol and levonorgestrel (n = 1).

Women with CAIS were recruited from the databases of the VU University Medical Center Amsterdam and the Erasmus University Medical Center-Sophia Children's Hospital Rotterdam, as well as from the support group DSDNederland. The majority of these women with CAIS were recruited for a larger neuroimaging study (e.g., described in van Hemmen et al. 2016). Control subjects were recruited from employees of the VU University Medical Center and by using flyers and advertisements in a local newspaper. The study was approved by the Medical Ethics Committee of the VU University Medical Center Amsterdam (application number NL32740.029.10) and the Erasmus University Medical Center (application number MEC-2010-350). All subjects gave their written informed consent according to the Declaration of Helsinki.

CEOAEs

CEOAEs were recorded using the ILO288 Echoport (Otodynamics Ltd., UK) connected to a laptop with EZ-screen software. Prior to a testing session the probe was calibrated using a calibration cavity. For each participant a single-use probe tip was selected with an appropriate size to best fit and seal the external ear canal. Participants were tested in a quiet room while seated and were instructed not to move and particularly to avoid jaw action, swallowing, and vocalizations to prevent cable-rub and ear canal noise during CEOAE recording. CEOAEs always were measured first for the right ear. The QuickScreen (non-linear) mode was used with a 2.5 to 12.5 ms post-click response window. The click stimulus level was set at 80 decibels sound-pressure level (dB SPL) (M = 80.23, SD = 0.80), and 250 responses with a noise level below 6mPa were recorded per ear. CEOAEs were recorded in five frequency bands (1000, 1414, 2000, 2828, and 4000 Hz), but only the absolute CEOAE response, which relates to the total OAE energy recorded across the frequency range, was used for the analyses. A recording was considered valid when showing a minimum amplitude of 1dB SPL and a whole band reproducibility of at least 70% based on two independently recorded waveforms.

Digit ratios

For the present study, high-resolution (600x600dpi) digital scans of the hands were made using a Canon imageRUNNER ADVANCE. The left and right hand were scanned separately. Participants were asked to remove rings and gently press their hand on the glass of the scanning device while straightening and slightly spreading their fingers. Prior to scanning, a photocopy of the hand was made to ensure correct positioning of the hand and quality of the photocopy. Two independent raters, unaware of the participant's group membership, measured the digits using AutoMetric (version 2.2; deBruine, 2006), a program designed to measure hand properties from digital images. The raters manually marked the fingertip and the middle of the basal crease on the ventral surface of the 2nd and 4th digit. The software used these landmarks to automatically calculate the length of the 2nd and 4th digit in pixels and the 2D:4D ratio for each hand. The intra-class correlation coefficient was 0.997 and higher for all four (left and right 2nd and 4th) digits measured by the two raters, indicating a high interrater reliability. Therefore, the average of each digit ratio from the two raters was calculated and used for the analyses. See Wallien et al. (2008) for the methods used for the additional control subjects.

Data analysis

Statistical analyses were performed with IBM SPSS Statistics for Windows, Version 22 (IBM Corp., Armonk, NY, USA). First, we used a t-test to assess whether OC- and NC-women showed differences in CEOAE. Because no differences were found (see results section), we selected the group of NC-women, who have a larger sample size and represents, sex

hormone-wise, a more "natural" control group, to serve as the group of control women for further analyses. We used one-way analyses of variance (ANOVA), followed by post hoc pairwise comparisons, to analyze differences in left and right CEOAE amplitudes between control men, control women (NC-women), and women with CAIS. Effect sizes for pairwise comparisons of the CEOAE data are expressed in Cohen's *d*, with effect sizes of 0.2, 0.5, and 0.8 referring to small, medium, and large differences, respectively (Cohen 1992). Cohen's *d* was calculated as the mean difference of the two groups divided by the pooled standard deviation of these groups. Differences in variance between women with CAIS and the control groups were assessed with pairwise Levene's tests.

Kruskal-Wallis and post hoc Mann-Whitney U-tests were used for the between-group analyses of the left and right 2D:4D ratio, because the right-hand data of women with CAIS and control men were not normally distributed. Effect sizes for pairwise comparisons of the 2D:4D ratio data are expressed in *d*, as calculated from *r* (Z statistic divided by the square root of the combined sample size of the two groups). Differences in variance between women with CAIS and the control groups were assessed with pairwise Levene's tests.

For all analyses, results were considered significant at a $p < 0.05$ threshold. Post hoc pairwise test statistics were corrected for multiple comparisons using a Bonferroni correction.

RESULTS

CEOAEs

The CEOAE amplitudes of NC-women (see Table 1; control women) and OC-women (left ear M = 13.07, SD = 4.26; right ear M = 12.74, SD = 5.16) showed no significant differences (P = 0.690 and 0.859 for left and right ear, respectively). Only NC-women were further used as control women in the ANOVA to assess between-group differences. This ANOVA showed a significant effect of group on CEOAE amplitude in both ears (Table 1). Figure 1A presents the mean left and right-ear CEOAE amplitude for each group with error bars representing the 95% confidence interval. Post hoc pairwise comparisons (Table 2) revealed the expected sex differences between control men and control women (McFadden 2009) in both ears, with greater mean CEOAEs in control women and medium-to-large effect sizes. The greater mean right-ear amplitude in women with CAIS than in control men showed a trend towards significance and a medium-to-large effect size. The other post hoc comparisons did not reveal significant between-group differences. Variances for both the left and right-ear CEOAE amplitudes did not differ between women with CAIS and the control groups (all P's > 0.05, also without applying a Bonferroni correction for multiple comparisons).

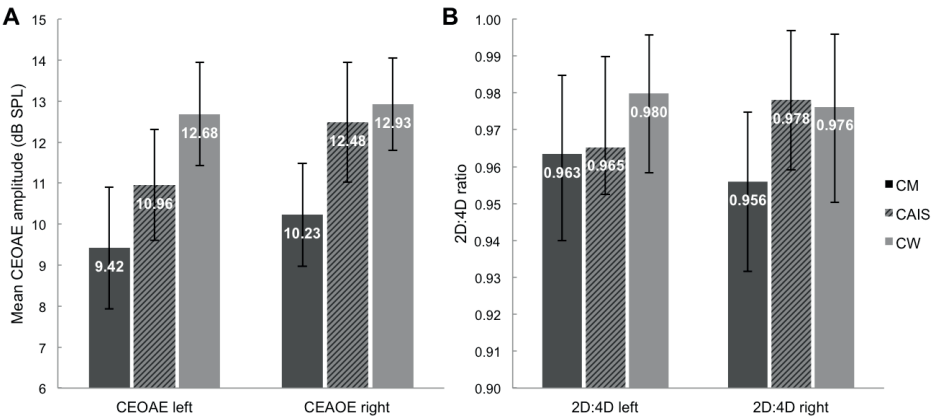

Figure 1 A: Mean (+95% confidence interval) CEOAE amplitude in the left and right ear per group. B: Median (+ IQR) left and right-hand 2D:4D ratio per group. CM = control men, CW = control women

Table 2 Post hoc tests and effect sizes for CEOAE amplitude and 2D:4D ratios

	Left		Right	
	P	Cohen's d	P	Cohen's d
CEOAE amplitude (dB SPL)				
Control men vs control women	**0.001**	-0.789	**0.003**	-0.725
Control men vs CAIS	0.547	-0.436	0.094	-0.666
Control women vs CAIS	0.356	0.498	1.000	0.134
2D:4D ratio*				
Control men vs control women	**0.001**	-0.438	**< 0.001**	-0.574
Control men vs CAIS	1.000	-0.087	**0.043**	-0.414
Control women vs CAIS	0.411	0.225	1.000	-0.002

Note: *Non-parametric tests were used for statistical analysis of this variable. Bold P-values represent a significant ($P < 0.05$) between-group difference after applying a Bonferroni correction for multiple comparisons. For CEOAE data, "control women" refers to NC-women.

Digit ratios

For both the left and right-hand 2D:4D ratios, a main effect of group was found (Table 1). Median digit ratios and the interquartile range (IQR) are displayed in Figure 1B. Post hoc comparisons showed the typical significant sex difference in both hands (Hönekopp and Watson 2010), with higher 2D:4D ratios in control women than men and medium effect sizes. In the right hand, women with CAIS also showed a higher digit ratio than control men, with a small-to-medium effect size. No other comparisons reached significance. Levene's tests showed no differences in variance between women with CAIS and control groups in

digit ratios of either hand (all P's > 0.05, also without applying a Bonferroni correction for multiple comparisons).

DISCUSSION

In the present study we measured CEOAEs and 2D:4D ratios in women with CAIS to investigate a possible link between these measurements and effective androgen exposure. The main objective was to hereby obtain information regarding the validity of CEOAEs as retrospective biomarkers of early androgen exposure and to replicate the previous digit-ratio study in women with CAIS (Berenbaum et al. 2009). The results from both measures will be discussed separately.

CEOAEs

In line with previous studies (e.g., reviewed in McFadden 2009), sex differences with medium-to-large effect sizes were found in both ears, with higher amplitudes in control women (NC-women) than in men. Although none of the comparisons between women with CAIS and the control groups reached statistical significance, there was a trend towards a female-typical CEOAE amplitude in the right ear of women with CAIS. This tendency is further emphasized by the effect sizes, with a medium-to-large effect size for the difference between women with CAIS and control men, whereas the effect size for the comparison with control women is very small (Table 2). A similar tendency for female-typical CEOAEs in women with CAIS was however not found in the left ear.

Although conclusions based on the right-ear tendency towards female-typical CEOAEs in women with CAIS should be drawn with caution, the lack of a significant difference between women with CAIS and control men is likely to be the result of low statistical power caused by the small sample of women with CAIS, along with the strict statistical threshold that was applied, using two-tailed tests and corrections for multiple comparisons. Taking this into consideration, the direction of the effect is in line with the hypothesis that low prenatal androgen levels are associated with stronger, female-typical, CEOAEs.

Because the only previous OAE study in DSDs failed to replicate the typical sex difference in CEOAEs (Wisniewski et al. 2014), an interpretation of those results and comparison with the results from the present study is difficult. However, a sex difference, with medium-to-large effect sizes, was replicated in SOAEs, which in general show a large correlation with CEOAEs (McFadden and Pasanen 1999), suggesting an overlap in mechanisms responsible for the production of these auditory characteristics. In accord with expectation (McFadden 2009), females with CAH showed a masculinized number of SOAEs in the right ear. Although the number of SOAEs in women with CAIS was not significantly different from control men or women, the effect sizes revealed a deviation from control men (d = 0.56) in the direction

of control women in the left ear. This is in the same direction, although with a slightly smaller effect size, as the female-typical tendency found in right-ear CEOAEs in the present study.

The authors of the previous OAE study in DSDs (Wisniewski et al. 2014) suggested that OAEs "may prove useful as bioassays for assessing early brain exposure to androgens" (as cited in Wisniewski et al. 2014), although those authors did emphasize the need for replication. This statement is only statistically supported by the right-ear SOAE results of the CAH group in their study, and it should be noted that a chance finding cannot be ruled out when taking into account the small sample sizes for DSD and control groups in the study by Wisniewski et al. (2014). In the present study we have further assessed this potential association with early androgen levels with regard to CEOAEs by means of the variability of this measure in women with CAIS. If effective androgen exposure, prenatally and/or later in life, would be the prominent factor related to sex differences in CEOAE amplitude, women with CAIS would show a much smaller variability in CEOAE amplitudes due to their complete androgen resistance as opposed to the more variable androgen exposure in both control groups. However, the variability of CEOAE amplitude of both ears was not reduced in women with CAIS. Therefore, although the right-ear results are suggestive of a role for effective androgen exposure in CEOAE amplitude and the left-ear results are inconclusive due to the absence of significant differences, the finding that CEOAEs in women with CAIS are as variable as in both control groups suggests that other factors than androgens alone influence the CEOAE amplitude measured in adulthood.

Previous studies have addressed the potential influence of circulating hormone levels in adulthood on OAEs. CEOAE amplitudes in control men correlated with seasonal variation in testosterone levels (Snihur and Hampson 2012b). However, these effects cannot explain the variability of CEOAE amplitudes in women with CAIS, as they are insensitive to androgens throughout life. In addition, small changes related to the menstrual cycle have been reported for SOAEs (Bell 1992; Haggerty et al. 1993; Penner 1995; Al-Mana et al. 2010), with some of these studies suggesting a positive association with high estrogen phases of the menstrual cycle. Estrogen receptors have been found in the adult human cochlea (Stenberg et al. 2001), and thus, estrogens could affect cochlear function. With one exception, all women with CAIS included in the CEOAE analyses used HRT containing estradiol. Therefore, the tendency towards female-typical CEOAE amplitude in the right ear might result from high estradiol levels in combination with the absence of androgen effects. Estrogen effects could also explain the variability in CEOAE amplitude in women with CAIS, because they have normally functioning estradiol receptors and variable estradiol levels from HRT.

Previous studies have also suggested that oral contraceptives might have a defeminizing or masculinizing influence on CEOAEs (McFadden 2000; Snihur and Hampson 2012a), although only the study by Snihur and Hampson reported significant effects in support of this hypothesis. Because of this proposed influence, both OC- and NC-women were included in the present study. However, because no CEOAE amplitude differences were found

between these groups, both groups would have been appropriate female controls. We have selected the NC-group to serve as the female control group for the comparisons between control men and women with CAIS, because of the larger sample size and the fact that the vast majority of women with CAIS used HRT containing estradiol, and not the synthetic ethinylestradiol as can be found in oral contraceptives.

Although an investigation of OC-related effects in control women was not the primary aim of the present study, we will briefly discuss this issue because the results differ from the study by Snihur and Hampson (2012a). In the present study the sample sizes were larger and the OC formulation more homogeneous, with all women using a monophasic OC, whereas in the study by Snihur and Hampson only 40% used a monophasic OC and 60% used a triphasic OC with varying progesterone dosages over the cycle. In addition, in the present study OC-women were tested at a day of OC use, whereas Snihur and Hampson did not take this factor into account. Based on the typical four week OC cycle, in which during one week no OC is used, in theory a quarter of the OC using women in the Snihur and Hampson study could have been tested on a day without OC use, and thus, could have had much lower estrogen and progesterone levels. Although purely speculative, such a bias could explain the lower CEOAE amplitudes in OC-women in their study. The finding that CEOAEs did not differ between OC- and NC-women in the present study, therefore, argues against the hypothesis proposed by Snihur and Hampson (2012a), that ovarian-derived estrogen, and not the synthetic ethinylestradiol used in OC formulations, acts to facilitate the cochlear amplifiers. Instead, it is at least possible that the differences found in the previous study were the result of differences in estrogen levels, regardless of their origin.

A limitation of the present study is that potential menstrual cycle-related effects were not controlled for in the group of NC-women. Although in a subgroup of NC-women, who were also included in a larger MRI-study, attempts have been made to perform testing only during the high estrogen phase of the menstrual cycle (around ovulation), serum hormone levels that were obtained in this subgroup revealed that these women were tested throughout different phases of their cycle, although not during menses. In the additional subjects that were tested to increase the sample size for the current study, no blood samples were obtained. Therefore, we tested this new group throughout the menstrual cycle, with the exception of the menstrual phase, to match the initial group of NC-women. In addition, testing NC-women throughout their menstrual cycle (except for the menstrual phase) reduced a potential cycle-related bias, and this group would serve as a more typical control group for women with CAIS, because the latter group used hormone replacement in different dosages. Furthermore, exclusion of the menstrual phase would be a better match to women with CAIS, who continuously use their HRT, and OC-women, who were not tested during their week without OC intake.

Although in the present study the sample size of women with CAIS is twice as large as in the study by Wisniewski et al. (2014), thereby increasing statistical power, the sample is still

relatively small. It is challenging to acquire a sufficient amount of data from women with CAIS given the incidence rates of the syndrome, which are estimated between 1:40,800 and 1:99,000 for all subtypes (mild, partial, and complete) of AIS combined (Boehmer et al. 2001). Therefore, this methodological limitation, which also applies to the digit ratio data, is difficult to overcome.

Digit ratios

The previously reported sex difference in the 2D:4D ratio, with larger digit ratios in women than men (Hönekopp and Watson 2010), was replicated in the control groups, with moderate effect sizes, in line with previous studies (Manning et al. 2014). In addition, the findings in women with CAIS relative to the control groups were overall similar to the findings presented by Berenbaum et al. in 2009. The female-typical digit ratios in women with CAIS, which are in line with androgen-related effects on digit ratios, were replicated in the right hand. In the present study this result reached significance while applying a more stringent statistical threshold (using two-tailed tests and correction for multiple comparisons) than what was used in the previous study, thereby reducing the risk of a type I error. The small-to-medium effect size for the difference between control men and women with CAIS was only slightly smaller than in the study by Berenbaum et al., which was 0.61 for the right hand. In the present study, this female-typical effect was not observed for the left hand, which seems to be explained by a lower left-hand 2D:4D ratio in women with CAIS than in the study by Berenbaum et al. However, after careful inspection of the individual digit ratios from this previous study (Fig. 1, Berenbaum et al. 2009), there was one woman with CAIS who had a rather extreme high left-hand digit ratio, which can explain the higher mean left-hand digit ratio of women with CAIS in that study. It is likely that a median for that same data set, which is insensitive to extreme values, would be lower and more similar to the median in our study.

It has been suggested that the sex difference in digit ratio is generally larger in the right hand (Hönekopp and Watson 2010). The current results suggest a slightly larger effect size for the sex difference in the right relative to the left hand. It has been proposed that this could be the result of a greater sensitivity of the right-hand digit ratio to prenatal sex steroids (Manning et al. 1998), which might explain why women with CAIS showed no significant deviations from either of the control groups in the left hand. However, this lack of significant findings prevents us from drawing firm conclusions regarding the left-hand digit ratios in women with CAIS.

The analysis of the variability in digit ratios provides additional information regarding the magnitude of the potential influence of androgens on digit ratios from both hands. Women with CAIS did not show a reduced variability in digit ratios, which would be expected if androgens solely affect this measure. This is also a replication of the findings from the previous digit ratio study in women with CAIS (Berenbaum et al. 2009) and again indicates that variations in digit ratios do not only reflect variations in prenatal androgen levels, but are

also under the influence of other factors. In line with these findings, a meta-analysis of digit ratio studies has found that the magnitude of the digit ratio sex difference is smaller than the difference in amniotic testosterone levels (Hönekopp and Watson 2010), and classification of men and women according to their digit ratios was not very accurate (Berenbaum et al. 2009).

Because estradiol receptors in women with CAIS are functional, estrogens might also play an important role. It has been suggested, for example, that sex differences in digit ratios result from differences in digit adiposity (Wallen 2009). Because both estradiol and androgen receptors have been found in fat tissue, these sex differences might be more strongly related to differences in testosterone-to-estradiol (T:E) ratios, instead of testosterone effects alone (Manning et al. 2014). This view is also in line with the negative association that was found between fetal T:E ratios and digit ratios at age two (Lutchmaya et al. 2004), although it is unknown whether this association is still found in adult digit ratios.

CONCLUSION

The present female-typical findings in women with CAIS in right-ear CEOAE amplitudes, although based on a trend, and right-hand digit ratios, are suggestive of an association between these measures and effective androgen exposure. The absence of similar significant results in the left ear and hand in the present study, prevent us from determining whether androgens affect these measures equally in both sides of the body. Nonetheless, the finding that the variability in both measures was similar across all groups, argues against the notion that differences in digit ratios and CEOAE amplitudes solely reflect differences in prenatal androgen exposure, or androgen exposure later in life. It is therefore not surprising that previous studies have not found an association between digit ratios and CEOAEs or SOAEs (McFadden and Shubel 2003; Snihur and Hampson 2011b). Although both authors have provided several possible explanations for this finding, the explanation stating that "neither measure exhibits a strong relationship with the underlying prenatal processes" (as cited in McFadden and Shubel 2003) is most in line with current knowledge from both the present and previous studies.

Taken together, the present results, along with results from previous studies, are suggestive of an androgenic influence on both digit ratios and CEOAE amplitudes, while additional non-androgenic factors are also reflected in these measures obtained in adulthood. Consequently, both measures cannot be assumed to provide a reliable indication of prenatal androgen levels, and usage of these measures for this purpose is not recommended, as it is likely to result in spurious associations. Further research is needed to clarify which other factors might be involved in the sexual differentiation of digit ratios and CEOAEs and what their exact timing and mechanisms of action would be.

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