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1 **Title**

2 Exploration of gene-environment interactions, maternal effects, and parent-of-origin effects in the
3 etiology of hypospadias

4

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22

23 **Runninghead**

24 Gene-environment interactions in hypospadias

25

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49 **Disclosure summary**

50 The authors have nothing to disclose

51 **Abstract**

52 **Purpose** Hypospadias is a common congenital malformation of the male external genitalia.

53 Association studies for single nucleotide polymorphisms (SNPs) in genes encoding steroid-5-alpha-
54 reductase (*SRD5A2*), estrogen receptors 1 (*ESR1*) and 2 (*ESR2*), and activating transcription factor 3
55 (*ATF3*) have been equivocal. The aim of this study was to examine whether non-replication of
56 findings for four SNPs in these genes could be due to interaction with environmental exposures.

57 **Materials and Methods** We genotyped 712 Dutch hypospadias case-parent triads for the four SNPs,
58 used questionnaire information to determine exposures, and performed association tests using the log-
59 linear approach. We studied gene-environment interactions for the four SNPs with exposure to
60 estrogens, cytokines or cigarette smoke, multiple pregnancy, being born small for gestational age, and
61 maternal hypertension or preeclampsia, high BMI, or primiparity. In addition, the presence of maternal
62 genetic and parent-of-origin effects was tested.

63 **Results** Gene-environment interactions were identified for rs523349 in *SRD5A2* with estrogen
64 exposure and maternal hypertension or preeclampsia, as well as for rs11119982 in *ATF3* with
65 exposure to cytokines. Both SNPs only seemed to influence hypospadias risk in exposed cases. For
66 rs6932902 in *ESR1*, only maternally derived alleles appeared to increase hypospadias risk in offspring.

67 **Conclusions** This study shows that interactions between genetic and environmental factors may help
68 to explain non-replication in genetic studies of hypospadias.

69

70 **Introduction**

71 Hypospadias is a congenital hypoplasia of the penis, resulting from developmental arrest of urethral
72 fusion. This leads to displacement of the urethral opening along the ventral side of the penis.

73 Hypospadias is one of the most common birth defects among boys, affecting 0.3-0.7% of newborn
74 boys in Europe¹. It shows familial clustering and segregation analyses suggest that the majority of
75 cases have a multifactorial etiology², involving both genes and environmental factors.

76 Some environmental factors have consistently been associated with hypospadias. Hypospadias
77 occurs more often in children born small for gestational age (SGA), and in first, intracytoplasmic
78 sperm injection (ICSI)-induced, or multiple pregnancies. In addition, maternal hypertension,
79 preeclampsia, high body mass index (BMI), pre-existing diabetes, and use of anti-epileptic drugs
80 increase hypospadias risk, as does maternal intra-uterine exposure to diethylstilbestrol (DES)¹.

81 Genetic associations with hypospadias have also been reported, mainly for single nucleotide
82 polymorphisms (SNPs) in endocrine-related genes, such as those encoding estrogen receptors 1
83 (*ESR1*)³ and 2 (*ESR2*)⁴, activating transcription factor 3 (*ATF3*)⁵, and steroid-5-alpha-reductase
84 (*SRD5A2*)^{6,7}. *ATF3* is an estrogen-responsive gene showing upregulation in hypospadias⁸, while
85 *SRD5A2* encodes an enzyme that converts circulating testosterone in the genital tubercle to the more
86 potent androgen dihydrotestosterone.

87 The numbers of samples analyzed in these genetic studies were relatively small, and most
88 associations could not be replicated in a much larger study by our group⁹.

89 This lack of consistency might reflect differences in environmental exposures between
90 populations⁹. Several reviews have called for studies simultaneously examining genes and
91 environment in relation to hypospadias^{1,10}, but so far, such studies have rarely been performed.
92 Therefore, we set out to examine whether the lack of replication could be due to gene-environment
93 interactions between the four SNPs described above and risk factors for hypospadias.

94 In addition to gene-environment interactions, other (epi)genetic mechanisms may be involved
95 in the etiology of hypospadias. Maternal genotype may affect the intra-uterine environment, thus
96 modulating hypospadias risk, and gene imprinting may cause the copy derived from one parent to be

97 more fully expressed than the copy derived from the other parent¹¹. Therefore, we also examined the
98 maternal genotype and imprinting effects.

99

100 **Materials and methods**

101 *Cases and parents*

102 AGORA (Aetiologic research into Genetic and Occupational/environmental Risk factors for
103 Anomalies in children) is a large data- and biobank at the Radboud University Nijmegen Medical
104 Centre in the Netherlands, in which questionnaire data and DNA samples are collected from patients
105 with congenital malformations or childhood cancer and their parents. For the current study, DNA was
106 available from 796 hypospadias cases born between 1980 and 2008 and 1,422 parents. Medical
107 records of all cases were reviewed to identify syndromic hypospadias cases, collect clinical
108 characteristics, and obtain information about anatomical location of the urethral opening. The regional
109 Committee on Research Involving Human Subjects approved the study protocol and all parents and
110 children over 11 years of age gave written informed consent.

111

112 *Environmental risk factor data*

113 Questionnaires were sent to the parents of all patients, containing a variety of questions on health and
114 lifestyle just before and during pregnancy, which were used to define environmental risk factors.
115 Although exogenous exposure to estrogens is not a known risk factor for hypospadias¹, we included it
116 in the gene-environment interaction analyses, because *SRD5A2*, *ESR1*, and *ESR2* are involved in
117 endocrine processes and *ATF3* is an estrogen-responsive gene. Exogenous exposure to estrogens was
118 defined as continued use of oral contraceptives during early pregnancy or consumption of soy or
119 linseed products, which contain high amounts of phytoestrogens¹², at least once a week in the first 14
120 weeks after conception. Women with a hormonal coil implanted who became pregnant were excluded
121 because of weak estrogen exposure. Women exposed to pesticides at work were also excluded because
122 pesticides can have either estrogenic or anti-estrogenic effects.

123 In addition, we studied interactions with factors associated with hypospadias occurrence: SGA
124 (defined as birth weight < 10th percentile for that gestational age, using Dutch reference curves¹³),
125 mothers with hypertension or preeclampsia, high BMI (defined as BMI > 25 kg/m²), primiparity, and
126 multiple pregnancy.

127 In most tissues, *ATF3* mRNA can be induced by various stress signals, such as cytokines and
128 chemicals from cigarette smoke^{14,15}. Therefore, we also included these exposures in the gene-
129 environment interaction analyses for *ATF3*. Because the placental barrier may be permeable to
130 cytokines^{16,17} and chemicals from cigarette smoke¹⁸, we categorized cases whose mothers smoked at
131 least one cigarette per day during some time in the first 14 weeks after conception as exposed to
132 cigarette smoke, and cases whose mothers reported the presence of an infection and/or inflammation in
133 this period as exposed to cytokines.

134

135 *Genotyping*

136 Blood was collected in EDTA containing tubes (n=1,405) or saliva using Oragene containers (n=687;
137 DNA Genotek Inc., Ottawa, Canada). DNA extraction and genotyping was performed as described
138 previously⁹.

139

140 *Statistical analyses*

141 We used the case-parent triad design. The most frequent homozygous genotypes in parents served as
142 reference genotypes in the log-linear approach¹⁹ that was applied to assess genetic associations. Log-
143 linear models were fitted without assumption of Hardy-Weinberg equilibrium (HWE). Information on
144 families with one missing parental genotype was included in the analyses using the expectation-
145 maximization algorithm²⁰. Likelihood ratio tests (LRT), comparing full models including both mater-
146 nal and offspring genotypes to reduced models including either maternal or offspring genotype only,
147 were computed to determine the relevance of maternal and offspring genotypes for hypospadias risk.
148 We also conducted these analyses separately for the groups of anterior, middle, and posterior
149 hypospadias cases, because different risk factors may be responsible for the different phenotypes^{21,22}.
150 Although the case-parent triad design is robust to population-stratification when testing genetic
151 effects, effects of environmental exposures cannot be estimated.

152 Parent-of-origin analyses were conducted in two steps. As an initial screening, we used the
153 transmission asymmetry test (TAT)¹⁹. This approach provides insights into the data, but is invalid

154 when maternal effects exist. Therefore, the parent-of-origin LRT (PO-LRT), was used to confirm the
155 results²³.

156 Interactions between environmental exposures and offspring genotypes were tested using log-
157 linear models with the LRT comparing a full model including gene-environment interactions to a
158 reduced model including only the offspring genotypic effect²⁴. We used a dominant interaction
159 parameter, assuming that the environmental factor affects carriers with one or two copies of the variant
160 allele similarly. We did not correct the critical *P*-value for multiple testing, as we only tested a limited
161 number of well-founded hypotheses. If the LRT indicated the presence of an interaction ($P_{LRT} < 0.05$),
162 relative risks (RR) and 95% confidence intervals (95% CI) were calculated separately for the different
163 strata of the exposure variable using the variance calculated with the LEM program, which takes into
164 account missing genotypes²⁵. All other analyses were performed using the SAS System for Windows,
165 release 8.02 (SAS Institute, Cary, North Carolina).

166

167 **Results**

168 Of the 796 available hypospadias cases, 38 patients were excluded due to lack of parental DNA. To
169 ensure independence, we excluded the youngest brother from 22 sib-pairs, while from three twin-pairs,
170 one brother was excluded at random. We excluded 19 patients because of syndromic hypospadias,
171 chromosome abnormalities, or a known cause of hypospadias. Finally, two triads were excluded
172 because of Mendelian errors. The final data set consisted of 712 cases. For 668 cases, DNA of both
173 parents was available, while for 44 cases, we only had DNA from one parent. Environmental data
174 were missing for 70 families. The majority of cases were of European Caucasian descent (91%), and
175 the remaining were of non-European (5%) or unknown descent (4%). Almost 60% of cases had an
176 anterior hypospadias, while 20% and 13% had middle and posterior urethral openings, respectively.
177 Table 1 shows the distribution of the environmental risk factors studied. Exogenous exposure to
178 estrogens, multiple pregnancies and fetal exposure to cytokines were relatively rare (<10%), whereas
179 the other factors were more common.

180 Genotyping of the SNPs was completed with a success rate of more than 98.5%. All genotype
181 frequencies in parents were in HWE ($P \geq 0.28$). Genetic association results showed that offspring
182 genotype of the variant in *ESR1* was associated with hypospadias, as reported earlier in a partly
183 overlapping sample⁹, whereas results for the variant in *ATF3* were suggestive of an association.
184 Maternal genotypes were not associated with hypospadias in offspring (Table 2). Repeating the
185 analyses separately for subgroups of anterior, middle, and posterior hypospadias cases showed
186 comparable results.

187 The results of the gene-environment interaction analyses pointed towards interactions between
188 offspring genotype of rs523349 in *SRD5A2* and exogenous estrogen exposure and maternal
189 hypertension or preeclampsia (Table 3). Offspring carrying the variant allele seemed to be at increased
190 risk of hypospadias when estrogen exposure occurred and at decreased risk when the mother had
191 hypertension or preeclampsia. Furthermore, an interaction was observed between rs11119982 in *ATF3*
192 and exposure to cytokines, with an increased risk of hypospadias for offspring carrying the variant
193 allele only when the mother reported an infection and/or inflammation (Figure 1). Due to small
194 numbers of cases with certain exposures, we also considered a reduced model assuming HWE, which

195 handles small sample size situations better. The risk estimates from this model showed the same
196 direction of gene by exposure interaction for the SNP in *SRD5A2* and exogenous estrogen exposure,
197 albeit less strongly (Figure 2). For the other interactions, similar results were obtained as in the full
198 model.

199 The results of the parent-of-origin effects analyses are shown in Table 4. For rs6932902 in
200 *ESR1*, the estimated PO-LRT relative risk for an imprinting effect was 1.61 (95% CI=1.02-2.53),
201 indicating that a maternally derived copy seemed to be associated with a greater risk of hypospadias
202 than a paternally derived copy. The TAT showed that only the maternally derived copy increased the
203 risk of hypospadias (RR=1.8, 95% CI=1.3-2.7).

204

205 Discussion

206 This study is a follow-up to our earlier association study of genetic variants in *SRD5A2*, *ESR1*, *ESR2*,
207 and *ATF3* and hypospadias risk in which 620 cases were included. For the current study, we excluded
208 37 cases because DNA of both parents was not available or a brother was present in the dataset, and
209 included 129 cases not included in the earlier study because of non-Caucasian or unknown ethnicity or
210 because they were collected after 2007. We included gene-environment interactions as well as
211 maternal and parent-of-origin effects in an attempt to reconcile our findings with those of others.

212 The estimated interaction between offspring genotype of the SNP in *SRD5A2* and exogenous
213 estrogen exposure during early pregnancy suggests that offspring carrying the variant have a more
214 than eight fold increased risk of hypospadias only in case of exogenous estrogen exposure in the full
215 log-linear model, and an almost three times increased risk in the reduced model. The interaction
216 between this variant in *SRD5A2* and exogenous estrogen exposure seems biologically plausible, as it
217 causes a valine to leucine substitution (V89L) resulting in an approximately 30% decrease in enzyme
218 activity²⁶ and thus in less dihydrotestosterone. Additional estrogen exposure might cause an androgen-
219 estrogen imbalance in carriers of this variant, resulting in hypospadias. The gene-environment
220 interaction observed could help to explain differences in findings for this SNP between our study and
221 studies from Sweden and China^{6,7,9}. The latter two observed associations with the malformation, not
222 taking environmental parameters into account, while we did not. However, phytoestrogen exposure is
223 known to be higher in Chinese and Swedish populations compared to the Dutch. Chinese people
224 consume more soy products, while in Nordic countries more rye bread and berries are consumed.
225 These food products contain large amounts of isoflavonoids and lignans, respectively, whereas in a
226 typical Western diet, both lignans and isoflavonoids are almost completely lacking²⁷.

227 The SNP in *SRD5A2* seemed to decrease hypospadias risk in case of maternal hypertension or
228 preeclampsia, which may result from placental insufficiency. The latter may also lead to decreased
229 provision of human chorionic gonadotropin (hCG) to the foetus. As hCG stimulates foetal testicular
230 steroidogenesis before the foetus's own pituitary-gonadal axis is established, and the SNP in *SRD5A2*
231 may result in even less DHT being formed, we would expect the SNP to increase hypospadias risk in
232 case of maternal hypertension or preeclampsia.

233 The SNP in *ATF3* seemed to be associated with an increased hypospadias risk only when the
234 mother reported an infection and/or inflammation during pregnancy. *ATF3* shows strong upregulation
235 in hypospadias patients⁸ and is upregulated in response to cytokines¹⁴. While the rs11119982 variant
236 has not been functionally characterized, a working hypothesis could be that the variant underlying the
237 association with hypospadias causes an increased expression of *ATF3* in response to cytokines.
238 However, this finding does not reconcile our results with those reported earlier, describing a decreased
239 hypospadias risk in the presence of the variant⁵.

240 The parent-of-origin analyses indicated that only a maternally derived copy of the variant in
241 *ESRI* seems to be associated with an increased hypospadias risk. This suggests that the maternally
242 derived allele of *ESRI* is more fully expressed than the paternally derived allele. Although *ESRI* is not
243 one of the currently known imprinted genes (www.geneimprint.com), the experimental identification
244 of imprinted genes is challenging, because monoallelic expression of imprinted genes may occur only
245 in particular tissues, at particular stages of development, or in one of the isoforms²⁸. Therefore, it is
246 unlikely that all human imprinted genes are already known. However, the observed parent-of-origin
247 effect could also have arisen from an effect of maternal-fetal genotype interaction. Unfortunately, we
248 do not have enough statistical power to disentangle these two possible effects.

249 We have to acknowledge some limitations. For all factors studied, we relied on information
250 from questionnaires which may result in misclassification due to recall problems, especially since the
251 average time between birth and filling out the questionnaires was 10.2 years, ranging from 0 to 27
252 years. However, most factors studied are relatively easy to remember. Also, this misclassification
253 probably does not depend on genotype and would have resulted in attenuation of the results only,
254 which may have obscured some effects. Misclassification due to measurement error could also account
255 for not finding gene-environment interactions for well-known factors such as SGA or primiparity,
256 which may be proxies for underlying causes that are difficult to measure, such as placental
257 insufficiency. To our knowledge, we investigated the largest sample of hypospadias cases thus far
258 reported in genetic studies, while the power of our study was further increased by including
259 information on families with one missing parental genotype using the expectation-maximization

260 algorithm. Nevertheless, numbers of cases having a specific genotype and being exposed to a
261 particular environmental risk factor were still small, resulting in large confidence intervals for the
262 effect estimates. Our definition of exogenous estrogen exposure, for example, assured a selective
263 group of women experiencing high levels of exposure, but resulted in low numbers of exposed
264 women. This indicates that very large samples are needed to study gene-estrogen-exposure
265 interactions. In addition, the gene-environment interaction test assumes that the SNP under study is a
266 disease-causing mutation. If a marker in linkage disequilibrium with the causative mutation is studied
267 instead, the test is susceptible to exposure-related population-stratification²⁹.
268

269 **Conclusions**

270 We showed that parent-of-origin effects and gene-environment interactions contribute to the etiology
271 of hypospadias, and that environmental factors can explain genetic non-replication between studies.
272 Our results warrant further research directed at elucidating combined effects of genetic and
273 environmental factors for this frequently occurring urological birth defect.

274

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References

1. **van der Zanden LFM, van Rooij IALM, Feitz WFJ et al:** Aetiology of hypospadias: a systematic review of genes and environment. *Hum Reprod Update* 2012; 18: 260
2. **Fredell L, Iselius L, Collins A et al:** Complex segregation analysis of hypospadias. *Hum Genet* 2002; 111: 231
3. **Watanabe M, Yoshida R, Ueoka K et al:** Haplotype analysis of the estrogen receptor 1 gene in male genital and reproductive abnormalities. *Hum Reprod* 2007; 22: 1279
4. **Beleza-Meireles A, Kockum I, Lundberg F et al:** Risk factors for hypospadias in the estrogen receptor 2 gene. *J Clin Endocrinol Metab* 2007; 92: 3712
5. **Beleza-Meireles A, Töhönen V, Söderhäll C et al:** Activating transcription factor 3: a hormone responsive gene in the etiology of hypospadias. *Eur J Endocrinol* 2008; 158: 729
6. **Wang Y, Li Q, Xu J et al:** Mutation analysis of five candidate genes in Chinese patients with hypospadias. *Eur J Hum Genet* 2004; 12:706
7. **Thai HTT, Kalbasi M, Lagerstedt K et al:** The valine allele of the V89L polymorphism in the 5-alpha-reductase gene confers a reduced risk for hypospadias. *J Clin Endocrinol Metab* 2005; 90: 6695
8. **Wang Z, Liu BC, Lin GT et al:** Up-regulation of estrogen responsive genes in hypospadias: microarray analysis. *J Urol* 2007; 177: 1939
9. **van der Zanden LFM, van Rooij IALM, Feitz WFJ et al:** Genetics of hypospadias: are single-nucleotide polymorphisms in *SRD5A2*, *ESR1*, *ESR2*, and *ATF3* really associated with the malformation? *J Clin Endocrinol Metab* 2010; 95: 2384
10. **Kalfa N, Philibert P and Sultan C:** Is hypospadias a genetic, endocrine or environmental disease, or still an unexplained malformation? *Int J Androl* 2009; 32: 187
11. **Pfeifer K:** Mechanisms of genomic imprinting. *Am J Hum Genet* 2000; 67: 777
12. **Thompson LU, Boucher BA, Liu Z et al:** Phytoestrogen content of foods consumed in Canada, including isoflavones, lignans, and coumestan. *Nutr Cancer* 2006; 54: 184
13. **Visser GHA, Eilers PHC, Elferink-Stinkens PM et al:** New Dutch reference curves for birthweight by gestational age. *Early Hum Dev* 2009; 85: 737

14. **Hai T, Wolfgang CD, Marsee DK et al:** *ATF3* and stress responses. *Gene Expr* 1999; 7: 321
15. **Bosio A, Knörr C, Janssen U et al:** Kinetics of gene expression profiling in Swiss 3T3 cells exposed to aqueous extracts of cigarette smoke. *Carcinogenesis* 2002; 23: 741
16. **Zaretsky MV, Alexander JM, Byrd W et al:** Transfer of inflammatory cytokines across the placenta. *Obstet Gynecol* 2004; 103: 546
17. **Dahlgren J, Samuelsson AM, Jansson T et al:** Interleukin-6 in the maternal circulation reaches the rat fetus in mid-gestation. *Pediatr Res.* 2006; 60: 147
18. **Lackmann GM, Salzberger U, Töllner U et al:** Metabolites of a tobacco-specific carcinogen in urine from newborns. *J Natl Cancer Inst* 1999; 91: 459
19. **Weinberg CR, Wilcox AJ and Lie RT:** A log-linear approach to case-parent-triad data: assessing effects of disease genes that act either directly or through maternal effects and that may be subject to parental imprinting. *Am J Hum Genet* 1998; 62: 969
20. **Weinberg CR:** Allowing for missing parents in genetic studies of case-parent triads. *Am J Hum Genet* 1999; 64: 1186
21. **Brouwers MM, van der Zanden LFM, de Gier RPE et al:** Hypospadias: risk factor patterns and different phenotypes. *BJU Int* 2010; 105: 254
22. **van der Zanden LFM, van Rooij IALM, Feitz WFJ et al:** Common variants in *DGKK* are strongly associated with risk of hypospadias. *Nat Genet* 2010; 43: 48
23. **Weinberg CR:** Methods for detection of parent-of-origin effects in genetic studies of case-parents triads. *Am J Hum Genet* 1999; 65: 229
24. **Umbach DM and Weinberg CR:** The use of case-parent triads to study joint effects of genotype and exposure. *Am J Hum Genet* 2000; 66: 251
25. **Vermunt JK:** LEM: a general program for the analysis of categorical data. Tilburg University, Tilburg, the Netherlands, 1997
26. **Makridakis NM, di Salle E and Reichardt JKV:** Biochemical and pharmacogenetic dissection of human steroid 5 alpha-reductase type II. *Pharmacogenetics* 2000; 10: 407

27. **Adlercreutz H:** Epidemiology of phytoestrogens. *Baillieres Clin Endocrinol Metab* 1998; 12: 605
28. **Luedi PP, Dietrich FS, Weidman JR et al:** Computational and experimental identification of novel human imprinted genes. *Genome Res* 2007; 17: 1723
29. **Shi M, Umbach DM, Weinberg CR et al:** Family-based gene-by-environment interaction studies: revelations and remedies. *Epidemiology* 2011; 22: 400

Tables

Table 1. Distribution of environmental risk factors for hypospadias patients.

Exposure	yes		no		unknown ^a	
	n	(%)	n	(%)	n	(%)
Exogenous exposure to estrogens	29	(4%)	580	(81%)	103	(14%)
<i>Use of oral contraceptives during pregnancy</i>	8	(1%)	626	(88%)	78	(11%)
<i>Consumption of soy products during pregnancy</i>	16	(2%)	620	(87%)	76	(11%)
<i>Consumption of linseed products during pregnancy</i>	7	(1%)	626	(88%)	79	(11%)
Known hypospadias risk factors						
Small for gestational age	125	(18%)	505	(71%)	82	(12%)
Hypertension or preeclampsia	107	(15%)	533	(75%)	72	(10%)
BMI > 25 kg/m ²	140	(20%)	468	(66%)	104	(15%)
First pregnancy	361	(51%)	277	(39%)	74	(10%)
Multiple pregnancy	49	(7%)	590	(83%)	73	(10%)
Fetal exposure to cytokines	67	(9%)	499	(70%)	146	(21%)
<i>From a severe cold</i>	15	(2%)	574	(81%)	123	(17%)
<i>From other viral, bacterial or fungal infections</i>	28	(4%)	563	(79%)	121	(17%)
<i>From chronic inflammatory diseases</i>	29	(4%)	605	(85%)	78	(11%)
Fetal exposure to cigarette smoke	117	(16%)	505	(71%)	90	(13%)

Percentages do not add up to 100% due to rounding and overlapping categories; n, number; ^afor 70 patients, environmental data were completely missing because parents did not fill out the questionnaires.

Table 2. Genetic association results for offspring and maternal genotypes of single nucleotide polymorphisms in *SRD5A2*, *ESR1*, *ESR2* and *ATF3* with hypospadias.

Single nucleotide polymorphism	Geno-type	Offspring genotype					Maternal genotype				
		Cases n (%)	RR	95% CI	P- value	P_{LRT}^a	Mothers n (%)	RR	95% CI	P- value	P_{LRT}^b
rs523349 in <i>SRD5A2</i>	CC	333 (48%)	ref			0.87	326 (47%)	ref			0.64
	CG	298 (43%)	1.1	0.9, 1.3	0.60		313 (45%)	1.0	0.8, 1.3	0.69	
	GG	70 (10%)	1.0	0.7, 1.5	0.83		57 (8%)	0.9	0.6, 1.3	0.46	
rs6932902 in <i>ESR1</i>	GG	513 (72%)	ref			3×10^{-3}	523 (75%)	ref			0.42
	AG	177 (25%)	1.5	1.2, 2.0	1×10^{-3}		159 (23%)	1.1	0.8, 1.4	0.61	
	AA	18 (3%)	2.0	1.1, 3.8	0.03		16 (2%)	1.7	0.7, 3.7	0.21	
rs2987983 in <i>ESR2</i>	AA	345 (49%)	ref			0.13	328 (47%)	ref			0.98
	AG	293 (42%)	0.8	0.7, 1.0	0.06		297 (43%)	1.0	0.8, 1.3	0.97	
	GG	67 (10%)	0.8	0.5, 1.1	0.12		72 (10%)	1.0	0.7, 1.4	0.86	
rs11119982 in <i>ATF3</i>	CC	163 (23%)	ref			0.07	180 (26%)	ref			0.09
	CT	354 (50%)	1.2	1.0, 1.6	0.07		332 (48%)	0.9	0.7, 1.1	0.23	
	TT	188 (27%)	1.4	1.0, 1.9	0.02		187 (27%)	1.1	0.8, 1.6	0.37	

Percentages do not add up to 100% due to rounding; CI, confidence interval; LRT, likelihood ratio test; n, number; RR, relative risk; ^aP-value of the likelihood ratio test comparing a full model including maternal and offspring genotypes to a reduced model including only maternal genotypes; ^bP-value of the likelihood ratio test comparing a full model including maternal and offspring genotypes to a reduced model including only offspring genotypes.

Table 3. Results of the tests for gene-environment interactions for single nucleotide polymorphisms in *SRD5A2*, *ESR1*, *ESR2* and *ATF3*.

Single nucleotide polymorphism	Environmental risk factor	P_{LRT}^a
rs523349 in <i>SRD5A2</i>	Exogenous exposure to estrogens	$7 \times 10^{-3*}$
	Small for gestational age	0.92
	Hypertension or preeclampsia	0.04*
	BMI > 25 kg/m ²	0.17
	First pregnancy	0.42
	Multiple pregnancy	0.44
rs6932902 in <i>ESR1</i>	Exogenous exposure to estrogens	0.45
	Small for gestational age	0.11
	Hypertension or preeclampsia	0.06
	BMI > 25 kg/m ²	0.89
	First pregnancy	0.10
	Multiple pregnancy	0.72
rs2987983 in <i>ESR2</i>	Exogenous exposure to estrogens	0.42
	Small for gestational age	0.92
	Hypertension or preeclampsia	0.86
	BMI > 25 kg/m ²	0.28
	First pregnancy	0.27
	Multiple pregnancy	0.11
rs11119982 in <i>ATF3</i>	Exogenous exposure to estrogens	0.78
	Small for gestational age	0.11
	Hypertension or preeclampsia	0.21
	BMI > 25 kg/m ²	0.82
	First pregnancy	0.40
	Multiple pregnancy	0.17
	Fetal exposure to cytokines	0.02*
Fetal exposure to cigarette smoke	0.60	

LRT, likelihood ratio test; ^a*P*-value of the likelihood ratio test comparing a full model including gene-environment interactions to a reduced model including only offspring genotypes; *indication of gene-environment interaction.

Table 4. Results of the parent-of-origin analyses.

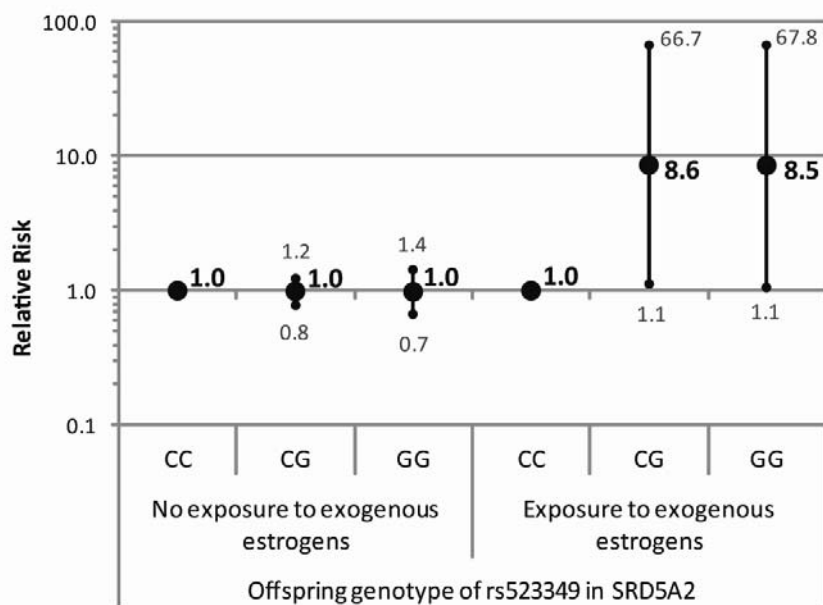
Single nucleotide polymorphism	Minor allele ^a	Transmission asymmetry test (TAT)										PO-LRT		
		Mothers					Fathers					<i>P</i> _{TAT}	RR	<i>P</i> -value
		T	NT	RR	95% CI	<i>P</i> -value	T	NT	RR	95% CI	<i>P</i> -value			
rs523349 in <i>SRD5A2</i>	G	81	80	1.0	0.7, 1.4	0.94	66	81	0.8	0.6, 1.1	0.22	0.34	1.18	0.27
rs6932902 in <i>ESR1</i>	A	79	43	1.8	1.3, 2.7	1×10 ⁻³	58	53	1.1	0.8, 1.6	0.64	0.05	1.61	0.07
rs2987983 in <i>ESR2</i>	G	59	95	0.6	0.5, 0.9	4×10 ⁻³	64	82	0.8	0.6, 1.1	0.14	0.33	0.76	0.27
rs11119982 in <i>ATF3</i>	T	83	71	1.2	0.9, 1.6	0.33	111	70	1.6	1.2, 2.1	3×10 ⁻³	0.17	0.79	0.25

CI, confidence interval; *NT*, minor allele not transmitted; *PO-LRT*, parent-of-origin likelihood ratio test; *RR*, relative risk; *T*, minor allele transmitted; *TAT*, transmission asymmetry test; ^athe least frequent allele in the parents.

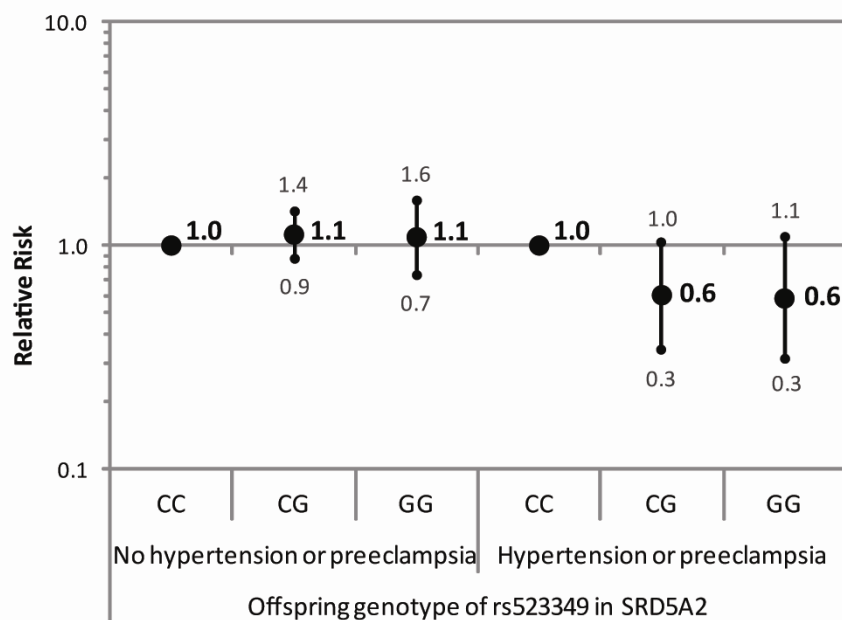
Figures

Figure 1. Relative risks of hypospadias with 95% confidence intervals for genotypes of (a) rs523349 in *SRD5A2* within strata of exogenous estrogen exposure (b) rs523349 in *SRD5A2* within strata of maternal hypertension or preeclampsia and (c) rs11119982 in *ATF3* within strata of fetal exposure to cytokines.

A



B



C

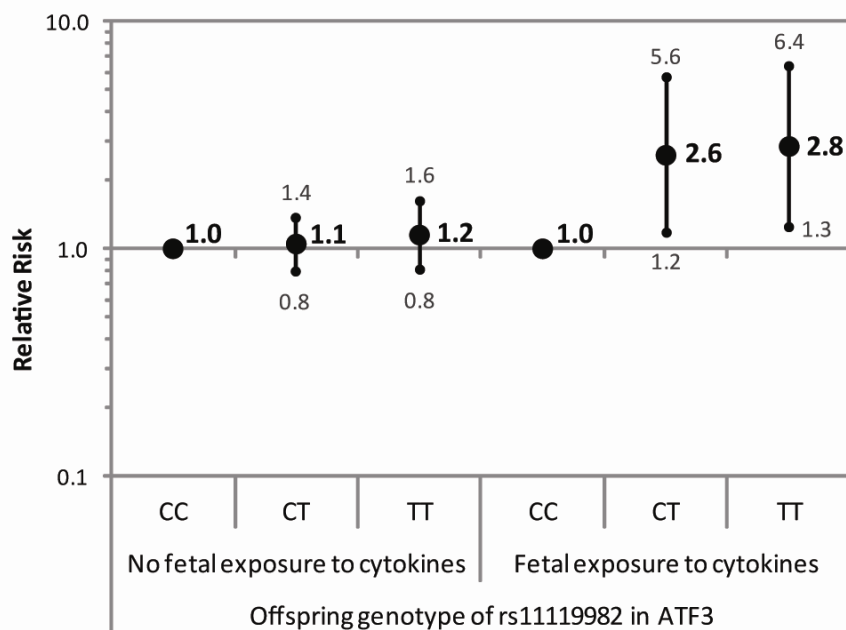


Figure 2. Relative risks of hypospadias with 95% confidence intervals for genotypes of rs523349 in *SRD5A2* within strata of exogenous estrogen exposure using a reduced model assuming Hardy Weinberg equilibrium.

