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ORIGINAL ARTICLE *Reproductive epidemiology*

Testing the twin testosterone transfer hypothesis—intergenerational analysis of 317 dizygotic twins born in Aberdeen, Scotland

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STUDY QUESTION: Does having a male co-twin influence the female twin's reproductive outcomes?

SUMMARY ANSWER: Women with a male co-twin had the same chances of being pregnant and having children compared to same-sex twin pairs.

WHAT IS KNOWN ALREADY: According to the twin testosterone transfer (TTT) hypothesis, in an opposite-sex twin pregnancy, testosterone transfer from the male to the female co-twin occurs. A large body of literature supports the negative impact of prenatal testosterone exposure on female's reproductive health in animal models; however, evidence from human studies remains controversial.

STUDY DESIGN, SIZE, DURATION: This cohort study included all dizygotic female twins in the Aberdeen Maternity and Neonatal Databank (Scotland) born before 1 January 1979. The 317 eligible women were followed up for 40 years for any pregnancies and the outcome of those pregnancies recorded in the same database.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Fertility outcomes (number of pregnancies, number of livebirths and age at first pregnancy) were compared between women with a male co-twin (exposed group, $n = 151$) and those with a female co-twin (unexposed group, $n = 166$). Population averaged models were used to estimate odds ratios (OR) and 95% CI for all outcomes with adjusting for potential confounders.

MAIN RESULTS AND THE ROLE OF CHANCE: There were no differences in chances of having pregnancies (adj. OR 1.33; 95% CI 0.72, 2.45) and livebirths (adj. OR 1.22; 95% CI 0.68, 2.18) between women from same-sex and opposite-sex twin pairs. Women with a male co-twin were more likely to smoke during pregnancy and, in the unadjusted model, were younger at their first pregnancy (OR 2.13; 95% CI 1.21, 3.75). After adjusting for confounding variables (year of birth and smoking status) the latter finding was no longer significant (OR 1.67; 95% CI 0.90, 3.20).

LIMITATIONS, REASONS FOR CAUTION: The dataset was relatively small. For women without a pregnancy recorded in the databank, we assumed that they had not been pregnant.

WIDER IMPLICATIONS OF THE FINDINGS: Despite the evidence from animal studies concerning the adverse effects of prenatal testosterone exposure on female health, our results do not support the TTT hypothesis. The finding that women with a male co-twin are more likely to smoke during pregnancy highlights the importance of considering post-socialisation and social effects in twin studies.

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Introduction

The twin testosterone transfer (TTT) hypothesis states that, in an opposite-sex twin pregnancy, testosterone transfer from the male to the female co-twin occurs. The effects of intrauterine position, and therefore the passage of hormones between neighbouring foetuses, are extensively described in animals, particularly in litter-bearing species. Female foetuses developing between two males tend to show masculinised anatomical, physiological and behavioural traits (i.e. higher concentrations of testosterone, later vaginal opening, delayed mating and a more pronounced aggressive behaviour) when compared to a female located between two female foetuses (Ryan and Vandenbergh, 2002).

It is unclear whether hormone transfer occurs in human multiple pregnancies due to highly inconsistent findings (Tapp *et al.*, 2011). There is some evidence supporting the behavioural and cognitive masculinisation of females from opposite-sex twins. For instance, females with a male co-twin show higher levels of male pattern experience-seeking behaviours (Resnick *et al.*, 1993; Slutske *et al.*, 2011) and more pronounced aggressive behaviour (Cohen-Bendahan *et al.*, 2005). In contrast, differences in femininity have been variably reported among same-sex and opposite-sex twins (Loehlin and Martin, 2000; Rose *et al.*, 2002; Verweij *et al.*, 2016).

Findings from studies into male–female twin differences in endocrine and reproductive function have also been inconsistent (Kuijper *et al.*, 2013). In the largest study currently available, no indication of higher androgen exposure in females from opposite-sex pairs was found (Kuijper *et al.*, 2015). Similarly, with respect to age at menarche, findings are inconsistent, ranging from earlier menarche (Jahanfar and Walters, 2019), through no difference reported (Rose *et al.*, 2002) and on to delayed menarche (Kaprio *et al.*, 1995). It has been suggested that intrauterine exposure to androgens may lead to polycystic ovary syndrome; however, the prevalence of the disease is not different in women from opposite-sex and same-sex twin pairs (Kuijper *et al.*, 2009).

It remains unclear whether other aspects of female reproductive function are affected by the presence of a male co-twin. Some studies report no significant difference in reproductive outcomes, such as number or pregnancies and number of children (Loehlin and Martin, 1998; Rose *et al.*, 2002; Medland *et al.*, 2008; Korsoff *et al.*, 2014) while others reported that females with male co-twins had a decreased probability of marriage and lower fertility (Lummaa *et al.*, 2007; Bütikofer *et al.*, 2019).

It is difficult to identify the reasons behind the lack of consistency in human studies. We hypothesised that one of the possible explanations could be related to the fact that other prenatal exposures, such as maternal smoking, have not been considered as possible confounding variables. Nevertheless, findings about impacts of *in utero* exposure to maternal smoking on subsequent fertility of the offspring have been inconsistent in singletons (Ye *et al.*, 2010; Tweed *et al.*, 2017). One study evaluating time to first pregnancy in twins who were exposed to maternal smoking *in utero*, reported decreased female fecundity, but unchanged male fertility (Jensen *et al.*, 2006). This suggests that prenatal exposure to cigarette smoke may be an important factor to consider when investigating reproductive function in twins.

In the current study, we compared different fertility outcomes (number of pregnancies, number of children and age at first pregnancy) between females from same-sex with those from opposite-sex twin pairs, born between 1950 and 1978 ($n = 317$), using routinely collected data from a cohort of women in Aberdeen (UK). We also examined the effects of potential confounding factors at both the maternal and twin offspring level (i.e. deprivation and smoking).

Materials and methods

Study population

The Aberdeen Maternity and Neonatal Databank (AMND), initiated in 1950, is an obstetric database, containing information for all pregnancy events occurring in women residing in Aberdeen area (Scotland, UK) (Ayorinde *et al.*, 2016). Twins where at least one was female and who were born between 1 January 1950 and 31 December 1978 were identified in the database ($n = 548$). From these, we selected only dizygotic twins. Zygosity was established by blood groups. All the women were aged over 40 at the time of the analysis, so would be expected to have completed their reproductive life. Female twins were then linked back within the AMND to identify any pregnancy occurring in them.

We used a cohort study design where female–male (FM) twins comprised the exposed cohort while female–female (FF) twins were the unexposed group. This information is recorded directly from the medical case notes at the time of birth. The primary reproductive outcomes analysed were: total number of pregnancies and total number of livebirths. Secondary outcomes, such as total number of miscarriages and age at first pregnancy, were also investigated in female twins who had at least one pregnancy recorded in the database ($n = 232$). Data regarding stillbirths were not analysed, as there were too few events recorded and posed a disclosure risk.

Baseline characteristics of mothers of the twins extracted from the database were: age at delivery, parity, height, smoking status, Carstairs deprivation categories (an area-based measure of deprivation consisting of the indicators social class, overcrowding, car ownership and unemployment). Other baseline variables evaluated were twins' gestational age at delivery and birthweight. For the female twins who then had at least one pregnancy recorded in the AMND, information on their BMI, smoking status and Carstairs deprivation categories were available.

Statistical analysis

Baseline characteristics of mothers were compared between same-sex and opposite-sex twins using appropriate chi-square test for categorical variables, independent *t*-test for continuous variables having normal distribution and Mann–Whitney *U* test for continuous variables having skewed distribution to identify potential confounding variables to adjust for in the final model. Primary and secondary outcomes (number of pregnancies, number of livebirths and number of miscarriages) were grouped into two categories (0/1+), where 0 denotes no event occurring and 1+ denotes one or more event. The secondary outcome age at first pregnancy was divided into two categories; namely age at first pregnancy above 23 years and age at pregnancy below or equal to 23 years. Unadjusted odds ratios (ORs) and adjusted ORs with 95% CI were calculated for each pre-specified outcome. Outcome

variables were dichotomised to allow easier interpretation of odds ratios. A multilevel framework was used to carry out main analyses accounting for the clustering of twins (level 1) nested within the same mother (level 2). A population averaged (PA) model with generalised estimating equations (GEE) was used to estimate the strength of association between twins' pregnancy outcome (having pregnancies, livebirths, miscarriages and age at first pregnancy before 23 years old), specifying binomial distribution and logit link for binary outcomes, and twins' type (FF vs FM) after adjusting for baseline maternal characteristics. The robust SE of the estimate was obtained assuming exchangeable correlation that the reproductive function is same for each member of the twins.

The variables considered for adjustments in the multivariable models were maternal height, maternal smoking status and year of delivery. Smoking status of the mothers of the twins was not available for 48% of the twins, and in particular a larger portion of data was missing for opposite-sex twin pairs. Therefore, a multilevel multiple imputation technique has been used for missing data with the aim to test the robustness of the results obtained with the complete case analysis. In the first model, only the cases for which maternal smoking data were available ($n = 173$) were included (complete case analysis). In the second model, twins for which *in utero* exposure to smoke was unknown were included in the analysis using a separate category. Finally, a model including imputed data was adopted.

For female twins with a pregnancy recorded in the database, baseline variables related to the time of their first pregnancy, were also available and compared between FF and FM, using GEE method, to account for the clustering of twins. For confounder selection, we considered a *P*-value <0.2 to be statistically significant (Dales and Ury, 1978; Harrel, 2001). The higher cut-off was set in order to avoid missing potentially relevant confounders, nevertheless, this approach might potentially lead to the inclusion of non-significant confounders.

Therefore, the variables included for adjustments for secondary outcomes in the multivariable models were both at the mother's level (height and year of delivery—that is the twin's year of birth) and twin's level (own smoking status). All the analysis was performed using SPSS Statistics Version 24 Software (IBM, Chicago, USA). A *P*-value of <0.05 was considered statistically significant for all the other analyses.

Ethics

The AMND is registered as a research database with the North of Scotland Research Ethics Service. For this reason, formal ethical approval from the ethics committee is not required (Ayorinde *et al.*, 2016). Approval to access the data was sought and obtained from the AMND steering committee, and only anonymised extracts of the data items requested were provided to researchers after checking for disclosure risks.

Results

A total of 548 female twins were identified in the database. We selected only dizygotic twins ($n = 326$). The cases of stillbirths and other early neonatal deaths were excluded. Therefore, the final population used for the analysis consisted of 317 female twins (Fig. 1). Of these women, 166 had a female co-twin and 151 had a male co-twin.

Baseline characteristics of the mothers of the twins are summarised and compared in Table I. There were some significant differences in population demographics between mothers of same-sex and of opposite-sex twin pairs (year of delivery and maternal height). These variables were considered as confounders and included in the adjusted model. Furthermore, a significantly larger proportion of data (54.3%) about smoking status was missing for mothers of opposite-sex twin pairs. For primary outcomes (chances of having a pregnancy and a livebirth), in both univariate and adjusted analyses, females from opposite-sex (MF) twin pairs did not differ from same-sex (FF) pairs (Table II). The unadjusted ORs were 1.26 (95% CI 0.74, 2.15) and 1.21 (95% CI 0.73, 2.01), respectively. We included maternal smoking as a confounding variable in different models. This sensitivity analysis revealed that maternal smoking status did not influence the results, as the three models did not show any difference. Maternal smoking status was, therefore, not included in the subsequent analyses of secondary outcomes. Women from both opposite-sex twins and same-sex twins had on average two pregnancies and one child (Supplementary Table SI).

For female twins, with at least one pregnancy recorded in the databank, information about baseline characteristics at the time of their own pregnancy were available (Table III) (Supplementary Table SII). A significantly higher proportion of women from opposite-sex twin pairs (52%) smoked compared to same-sex twins (32%). For this reason, twins' smoking status at the time of their first pregnancy was also included in the final adjusted model.

For women who had at least one pregnancy recorded in the database, secondary outcomes (number of miscarriages and age at first pregnancy) were evaluated (Table IV). No differences were found in miscarriage rates. In univariate analysis, females from opposite-sex twin pairs were more likely to be younger at their first pregnancy (OR 2.13, 95% CI 1.21, 3.75). The median age at first pregnancy was 23.5 for women with a male co-twin and 26.0 for women with a female co-twin. In the fully adjusted model, including the confounding variables, maternal height, twin's year of birth and twin's own smoking status, the finding was no longer significant (OR 1.67, 95% CI 0.90, 3.20).

Discussion

In this study, reproductive outcomes in a total of 317 female twins were examined, investigating the association between the presence of a male co-twin and subsequent fertility outcomes in the female. We found that females from opposite-sex twin pairs did not differ in terms of reproductive outcomes from females from same-sex twins, rather, they had the same chances of getting pregnant and having children compared to women with a female co-twin. In accordance with other studies, baseline characteristics such as birthweight of female twins and their adult BMI were not different between opposite-sex and same-sex twin pairs (Bogl *et al.*, 2017; Jelenkovic *et al.*, 2018). Overall, our findings agree with what Medland *et al.* (2008) reported in a study using three cohorts (Australia, The Netherlands and USA) and evaluating a total of 1979 females from same-sex twin pairs and 913 females from opposite-sex twin pairs. The authors found no differences in the number of children and pregnancies (Medland *et al.*, 2008). Similarly, Korsoff *et al.* (2014) found no differences in number of children and abortions while, Rose *et al.* (2002) reported no difference in fertility of

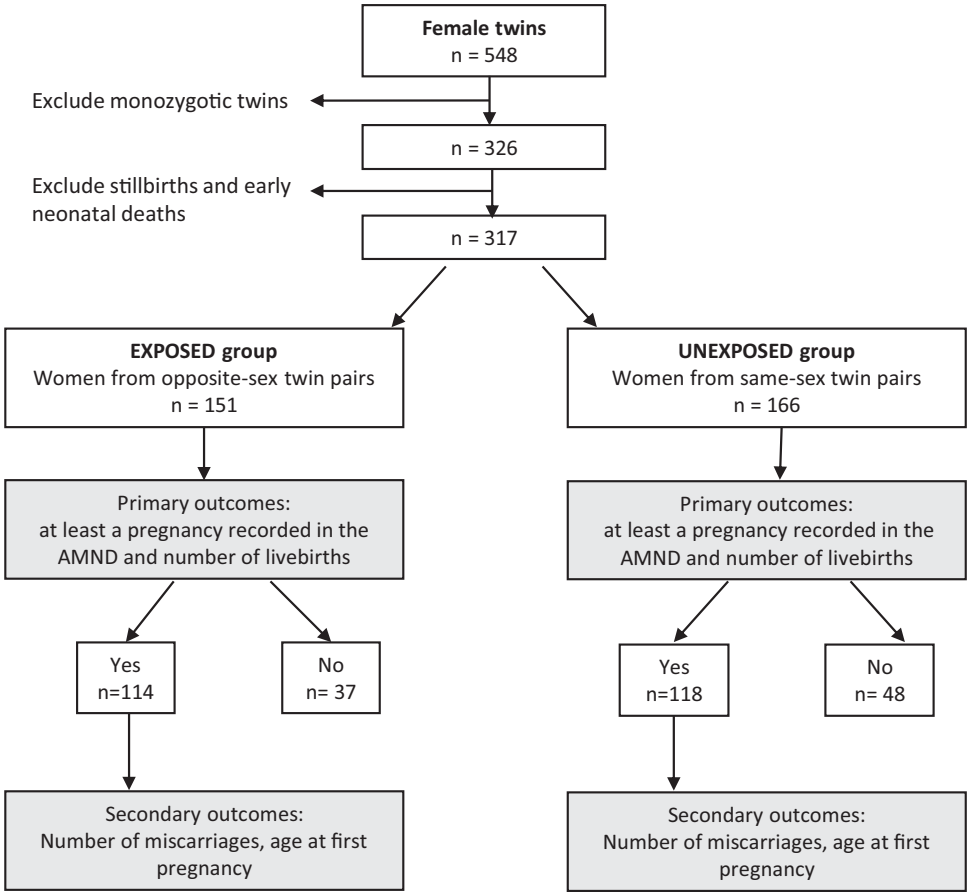

Figure 1 Flowchart of cohort selection for analysis of reproductive outcomes in women from opposite- and same-sex pairs identified in the Aberdeen Maternity and Neonatal Databank (AMND).

females with a male co-twin. On the other hand, two other studies reported a relationship between the presence of a male co-twin and an adverse impact on future fertility of the female twin. In the first, historical data using church registers from pre-industrial Finland were used (Lummaa *et al.*, 2007). However, the population was small (due to very low survival rates), with fertility outcomes compared among 35 females from same-sex twins and 31 females from same-sex twin pairs. Based on all births in Norway, women with a male co-twin had a lower number of children, as reported in a large study (Bütikofer *et al.*, 2019). The findings were no longer significant when the authors included only the females with a deceased male co-twin in the analysis. This represents an important way to exclude observations that are associated with post-natal socialisation effects of being raised with a male co-twin rather than a direct endocrine effect *in utero*. Despite not reaching statistical significance, the coefficients in this subsample were similar to those of the main analysis; therefore, the authors concluded prenatal exposure was the most likely explanation for their findings.

Although we found that females from opposite-sex twin pairs had a significantly higher probability of being younger at first pregnancy compared to females from same-sex pairs in the unadjusted model, adjusting for confounding variables removed significance. Here, we report that females from opposite-sex twin were aged 23.5 on average at first pregnancy, while females from same-sex twin were 26. With regard to age at first pregnancy, our results agree with previous studies. Rose *et al.* (2002) reported the mean age at delivery of the first-born child as 26.05 years for females from same-sex twins and 25.97 years for females from opposite-sex twins. In the study from Medland *et al.* (2008), age at first pregnancy for females with a male co-twin was 25.01 and 25.16 (in the Australian and Dutch cohort, respectively), whereas for females from same-sex pairs, age was 24.4 and 25.8, respectively. Similarly, Korsoff *et al.* (2014) reported no difference in age at first pregnancy.

It is important to note that our cohort differs from the ones used in the aforementioned studies in several ways (Table V). Firstly, the individuals used in our study were born between 1950 and 1978, and all were at least 40 years old at the time of data extraction from the databank. Therefore, they were all highly likely to have completed their reproductive life. Although female twins were born in a similar calendar period (1958–1971) in Rose *et al.* (2002), data were extracted in 1987, when twins ranged in age from 15 to 28. We suggest that evaluating fertility outcomes when the women have not

Table I Baseline characteristics of the mothers of the female twins included in the study.

Variables	Total cohort ($n = 235$)	Female–Female Twin[a] ($n = 84$)	Female–Male twins ($n = 151$)	*P*-value
Year of delivery				
1950–1959	44 (18.7)	9 (10.7)	35 (23.2)	
1960–1969	71 (30.2)	33 (39.3)	38 (25.2)	**0.018**
1970–1979	120 (51.1)	42 (50)	78 (51.7)	
Maternal age at delivery				
<20	19 (8.1)	6 (7.1)	13 (8.6)	0.480
21–25	88 (37.4)	37 (44)	51 (33.8)	
26–35	121 (51.5)	39 (46.4)	82 (54.3)	
>36	7 (3)	2 (2.4)	5 (3.3)	
Maternal parity				
0	79 (33.6)	29 (34.5)	50 (33.1)	0.940
1+	156 (66.4)	55 (65.5)	101 (66.9)	
Maternal height (cm)*	229 (missing $n = 6$)	160.9 (5.4)	159.2 (5.7)	**0.028**
Maternal weight (kg)*	101 (missing $n = 134$)	64.8 (9)	64.4 (11.7)	0.872
Maternal smoking status				**0.036**
Non-smoker	58 (24.7)	26 (31)	32 (21.2)	
Smoker	64 (27.2)	27 (32.1)	37 (24.5)	
Unknown	113 (48.1)	31 (36.9)	82 (54.3)	
Maternal number cigarettes/day^	108 (missing $n = 127$)	0 (0,10)	2.5 (0,15)	0.340
Maternal deprivation				1.000
Least deprived	201 (86.3)	72 (85.7)	129 (86.6)	
Most deprived	32 (13.7) (missing $n = 2$)	12 (14.3)	20 (13.4)	
Gestation at delivery (weeks)^	231 (missing $n = 4$)	38 (35.5,39)	38 (36,39)	0.312
Baby birthweight (g)*[b]	2440.6 (510.2) ($n = 317$)	2480.7 (485.9) ($n = 156$)	2396.5 (533.8) ($n = 151$)	0.335

P-values result from χ^2 test (categorical variables), independent sample *t*-test (continuous variables with normal distribution) and Mann–Whitney *U* test (continuous variables with skewed distribution). Significant *P*-values are in bold. Values are frequencies (percentage %), unless stated otherwise.
*Mean (SD) for normally distributed variables.
^Median (interquartile range) for skewed variables.
[a]Baseline characteristics were evaluated using mothers of the twins, to avoid redundancy for female–female data. Eighty-four women gave birth to female–female pairs, but only 166 female twins were included in the study as two twins died within the first week.
[b]All the female twins were evaluated.

completed their reproductive life will be only partially informative. Secondly, in the cohorts used by Medland *et al.* (2008) the year of birth ranged from 1897 to 1954. While the authors considered several covariates (i.e. education and religion of the twins), it is not clear whether they took birth year into account. It is well known that fertility patterns undergo major changes related to socio-historical context; therefore, considering year of birth is critical when comparing fertility outcomes in individuals born in different decades. Finally, in the analysis of Finnish cohorts used by Korsoff *et al.* (2014), confounding variables have not been investigated.

We did not find any significant effect exerted by *in utero* exposure to maternal smoking. This is contrary to findings by Jensen *et al.* (2006), who reported that female twins exposed *in utero* to maternal smoking had reduced fecundability. One explanation is that the measured outcomes differ in our study. Indeed, Jensen *et al.* used time to first pregnancy, while we used number of pregnancies. Furthermore, in our cohort almost 50% of maternal smoking data was missing, restricting our dataset. This is due to information about smoking being not systematically collected for the pregnancies occurring between 1950 and 1965 (Ayorinde *et al.*, 2016). We partially addressed this limitation using multiple imputation. Nevertheless, we found that females with a male co-twin were more likely to smoke during their own pregnancy. It should be highlighted that for selection of confounders at the level of the twins (such as their smoking status) we set a *P*-value cut-off of

Table II Unadjusted and adjusted odds ratio and 95% confidence interval among female twins from same- and opposite-sex twin pairs.

Variables	Female–female ($n = 166$) Number (%)	Female–male ($n = 151$) Number (%)	Unadjusted OR (95% CI)	Adjusted OR (95% CI)[a]	Adjusted OR (95% CI)[b]	Adjusted OR (95% CI)[c]
Total gravida						
0	48 (28.9)	37 (24.5)	1	1	1	1
1+	118 (71.1)	114 (75.5)	1.26 (0.74, 2.15)	1.13 (0.49, 2.58)	1.33 (0.72, 2.45)	1.28 (0.70, 2.35)
Total livebirth						
0	54 (32.5)	43 (28.5)	1	1	1	1
1+	112 (67.5)	108 (71.5)	1.21 (0.73, 2.01)	1 (0.42, 1.95)	1.22 (0.68, 2.18)	1.16 (0.65, 2.07)

Generalised estimating equations (GEE) method has been used, given the paired structure of the twin data.
[a]Adjusted for Year of Delivery, maternal smoking status and maternal height (complete case analysis, $n = 173$).
[b]Adjusted for Year of Delivery, maternal smoking and maternal height (unknown maternal smoking status included in the analysis as a separate category, $n = 317$).
[c]Adjusted for Year of Delivery, maternal smoking and maternal height. Missing data for maternal smoking has been imputed using a multi-level imputation technique.

Table III Baseline characteristics for female twins who had at least one pregnancy recorded in the database ($n = 232$), separated according to the presence or absence of a male twin.

Variables	Female–female twin (unexposed), $n = 118$	Female–male twins (exposed), $n = 114$	*P*-value
Baby birthweight (g)*	3283 (589)	3265 (474)	0.781
BMI* (missing n=125)	23.87 (4.81)	23.75 (3.66)	0.820
Smoking status			**0.182**
Non-smoker	63 (61.2)	40 (46)	
Smoker	33 (32)	45 (51.7)	
Ex-smoker	7 (6.8)	2 (2.3)	
(missing $n = 42$)			
Deprivation			0.661
Least deprived	65 (57)	64 (56.6)	
Most deprived	49 (43)	49 (43.4)	
(missing $n = 5$)			

P-values result from generalised estimating equations with robust standard error, accounting for the clustering of twins. Significant *P*-values (<0.2) are in bold. Values are frequencies (percentage %), unless stated otherwise.
*Mean (SD) for normally distributed variables.

Table IV Unadjusted and adjusted odds ratio and 95% confidence interval among female twins from same- and opposite-sex twin pairs for secondary outcomes.

Variables	Female–female, $n = 118$ Number (%)	Female–male, $n = 114$ Number (%)	Unadjusted OR (95% CI)	Adjusted OR (95% CI)[a]	Adjusted OR (95% CI)[b]
Miscarriages					
0	100 (84.7)	92 (80.7)	1	1	1
1+	18 (15.3)	22 (19.3)	1.33 (0.7, 2.58)	1.51 (0.69, 3.27)	1.40 (0.68, 2.86)
Age at first pregnancy					
≤23	37 (31.4)	57 (50)	**2.13 (1.21, 3.75)**	1.95 (0.97, 3.92)	1.67 (0.90, 3.20)
>23	81 (68.6)	57 (50)	1	1	1

Generalised estimating equations (GEE) method has been used, given the paired structure of the twin data. Significant ORs are reported in bold.
[a]Adjusted for twin's year of Birth, maternal height and twin's own smoking status (complete case analysis $n = 187$).
[b]Adjusted for twin's year of Birth, maternal height and twin's own smoking status (unknown maternal smoking status included in the analysis as a separate category, $n = 232$).

Table V Relevant literature evaluating fertility outcomes in females from opposite-sex vs same-sex twin pairs.

Study	Number	Population	Findings	Comment
Rose *et al.* (2002)	F from FF= 7528 F from FM= 4767	Finland 1958–1971 Central Population Record	No difference in fertility odds ratios No difference in age at first pregnancy	Data extracted in 1987 (age 15–28)
Lummaa *et al.* (2007)	F from FF= 35 F from FM= 31	Finland 1734–1888 Church records	Reduced probability of reproducing Reduced number of children Reduced probability of being married	Small sample size
Medland *et al.* (2008)	F from FF= 1979 F from FM= 913	Australia, Netherlands, USA 1894-1954 Questionnaires	No difference in number of children No difference in age at first pregnancy	Data collected via questionnaires Year of birth not included in covariates
Korsoff *et al.* (2014)	F from FF= 679 F from FM= 789	Finland 1975–1979 and 1983–1987 Questionnaires	No difference in number of children No difference in age at first pregnancy No difference in number of abortions No difference in fertility	No adjustment for confounding variables
Bütikofer *et al.* (2019)	F from FF= 4533 F from FM= 2568	Norway 1967–1978 Medical Birth Register, Central Population Register	Reduced fecundity (number of children) Lower probability of being married	Significance lost when analysing subset of females with deceased male co-twins

Summary of relevant literature, including sample size with number of females (F) from female–female (FF) and from female–male (FM) twin pairs, population (country, year of birth range and source of data) and main findings. In all studies, females from FF represent the reference category.

<0.2 (Mickey and Greenland, 1989). We consider this to be a suitable approach in order to avoid biased estimates; however, we are aware of the increased risk of selecting non-significant variables. However, the importance of the smoking variable is also supported by the fact that including it in the adjusted model (Table IV) resulted in a change in ORs compared to those obtained with the unadjusted model. This indicates that smoking status was a relevant confounding factor even though statistically non-significant at a *P*-value cut-off of 5%.

The identification of smoking status as a confounder highlights the importance of considering post-socialisation and social effects in twin studies. Interestingly, females from opposite-sex twins are also reported to have higher rates of alcohol use disorder symptoms (Ellingson *et al.*, 2013).

One of the major strengths of our study is that we had data available not only for the twins' own pregnancy but also for their mothers' pregnancy. This allowed the evaluation of potential confounders at the twins' prenatal level. Furthermore, our information came from medical notes of patients, collected at the time of their first antenatal visit (for both the mothers of the twins and for the female twins themselves). Previous studies are based on cohorts with information gathered via questionnaires. For this reason, it is not possible to completely rule out the possibility of recall bias and selection bias, especially given the high level of commitment required from the participants in that study type.

One limitation of our study is the relatively small dataset. However, it is important to point out that the AMND is based on every pregnancy occurring in Aberdeen Maternity Hospital, which is the only maternity hospital of the city of Aberdeen and serves the Grampian region as well (North-East Scotland) (Ayorinde *et al.*, 2016). The AMND population coverage is very high (99% of Aberdeen and 97% of the entire Grampian region), with a very small proportion of home births and deliveries in other peripheral hospitals (Ayorinde *et al.*, 2016). Furthermore, the twinning rate in the database (15 out of 1000 deliveries) is in line with rates reported in the rest of the UK and in developed countries (Pison *et al.*, 2015). Another aspect that needs to be taken into account is that for women without a pregnancy recorded in the databank, it was assumed they had not been pregnant. However, it is possible that some of these women chose not to get pregnant or moved away from the Aberdeen area. Nevertheless, the out-migration rate is very small (3.8%) (Ayorinde *et al.*, 2016).

Overall, our findings do not support the TTT hypothesis. The reasons underlying the multiple disparities in the results of the studies comparing reproductive outcomes among female twins are unclear. For example, delayed onset of reproduction is another feature that has been associated with prenatal androgen exposure (Rhees *et al.*, 1997). However, in the most recent and largest study, females from opposite-sex twins were more likely to experience earlier age at menarche compared to same-sex twins (Jahanfar and Walters, 2019). Results from other studies were also opposite to those expected by the TTT hypothesis. For example, a lower risk of mortality and of developing attention deficit hyperactivity disorder has been reported (Eriksson *et al.*, 2016; Ahrenfeldt *et al.*, 2017).

We found that females with a male co-twin were more likely to smoke during pregnancy. We hypothesised this could be related to post-natal socialisation effect of being raised with a male co-twin and this highlights the importance of considering these aspects in twin studies. Furthermore, as prenatal exposure to cigarette smoke is related

to a variety of adverse birth outcomes (as intrauterine growth restriction) and possibly long-term health consequences, it is important to consider the clinical implications of this finding. Testosterone transfer from the male to the female foetus cannot be ruled out. However, we suggest that mixed findings could be partially explained by the fact that testosterone transfer may indeed occur, but that the interplay between testosterone and other hormones, such as alternative ('back-door') androgens, should be taken into account (O'Shaughnessy *et al.*, 2019). In humans, higher oestriol (E3) level in mothers carrying female–female twins and lower LH concentration in males from opposite-sex twin pairs have been reported, but no indication of higher androgen exposure in females from opposite-sex pairs was found (Kuijper *et al.*, 2015). The information about hormone levels in twin pregnancies and in twins themselves is still very limited. Larger perspective cohort studies, with hormonal measurements (including alternative androgens) at different time points during pregnancy and at birth are needed to confirm the findings by Kuijper *et al.* Follow-up of these twin cohorts later in life (i.e. during pubertal development), ideally until the end of their reproductive life, might also significantly improve our current knowledge. However, it should also be highlighted that the hormonal profile in cord blood might not reflect prenatal hormonal exposure. Unfortunately, prenatal studies, which might provide a deeper mechanistic insight, are very limited for obvious ethical reasons. Furthermore, differences in placental morphology and pathology according to the sexes of twins have been found (Salafia and Maas, 2005; Kalisch-Smith et al., 2017; Jahanfar and Lim, 2018). These aspects highlight that differences in the endocrinology of pregnancy exist between same-sex and opposite-sex twin pairs and that the interaction of testosterone with other hormones could influence future outcomes in terms of reproductive physiology and behaviour. It is also important to consider that the interplay between an individual's genetics and the environment is critical in shaping subsequent life-course decisions, fertility behaviour and outcomes (Mills and Tropf, 2015).

Conclusions

Reproductive outcomes of women with a male co-twin did not differ from those of women with a female co-twin. Therefore, our findings do not support the TTT hypothesis and its adverse impact on female's future fertility. However, further research, with larger cohorts and their endocrine profiles, is needed to elucidate the differences in fertility outcomes, if any, between same-sex and opposite-sex twin pairs. Social characteristics (i.e. deprivation) and post-natal socialisation effects of being raised with a male co-twin (i.e. smoking status) should be considered, along with other prenatal factors, such as exposure to maternal cigarette smoke, that could potentially interfere with hormonal levels during pregnancy.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

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Authors' roles

C.T., S.B. and P.F. helped with the study conception and design. C.T. and E.A.R. were responsible for statistical analysis. C.T. wrote the article. S.B., P.F. and E.A.R. contributed to drafting, revision and approval of the final article.

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Conflict of interest

The authors declare no conflicts of interest regarding this study.

References

Ahrenfeldt LJ, Larsen LA, Lindahl-Jacobsen R, Skytthe A, Hjelmborg JVB, Möller S, Christensen K. Early-life mortality risks in opposite-sex and same-sex twins: a Danish cohort study of the twin testosterone transfer hypothesis. *Ann Epidemiol* 2017;**27**:115–120.

Ayorinde AA, Wilde K, Lemon J, Campbell D, Bhattacharya S. Data resource profile: The Aberdeen Maternity and Neonatal Databank (AMND). *Int J Epidemiol* 2016;**45**:389–394.

Bogl LH, Jelenkovic A, Vuoksimaa E, Ahrenfeldt L, Pietiläinen KH, Stazi MA, Fagnani C, D'Ippolito C, Hur YM, Jeong HU *et al.* Does the sex of one's co-twin affect height and BMI in adulthood? A study of dizygotic adult twins from 31 cohorts. *Biol Sex Differ* 2017;**8**:61.

Bütikofer A, Figlio DN, Karbownik K, Kuzawa CW, Salvanes KG. Evidence that prenatal testosterone transfer from male twins reduces the fertility and socioeconomic success of their female co-twins. *Proc Natl Acad Sci USA*, 2019;**116**:6749–6753.

Cohen-Bendahan CCC, Buitelaar JK, Van Goozen SHM, Orlebeke JF, Cohen-Kettenis PT. Is there an effect of prenatal testosterone on aggression and other behavioral traits? A study comparing same-sex and opposite-sex twin girls. *Horm Behav* 2005;**47**:230–237

Dales LG, Ury HK. Improper use of statistical significance testing in studying covariables. *Int J Epidemiol* 1978;**7**:373–376.

Ellingson JM, Slutske WS, Richmond-Rakerd LS, Martin NG. Investigating the influence of prenatal androgen exposure and sibling effects on alcohol use and alcohol use disorder in females from opposite-sex twin pairs. *Alcohol Clin Exp Res* 2013;**37**:868–876.

Eriksson JM, Lundström S, Lichtenstein P, Bejerot S, Eriksson E. Effect of co-twin gender on neurodevelopmental symptoms: A twin register study. *Mol Autism* 2016;**7**: 8.

Harrel F. *Regression Modeling Strategies*. Springer: Springer International Publishing Switzerland, 2001.

Jahanfar S, Lim K. Is there a relationship between fetal sex and placental pathological characteristics in twin gestations? *BMC Pregnancy Childbirth*, 2018;**18**:285.

Jahanfar S, Walters H. Association of twins' sex discordance and age at menarche. *Arch Gynecol Obstet*, 2019;**299**:1023–1031.

Jelenkovic A, Sund R, Yokoyama Y, Hur Y-M, Ullemar V, Almqvist C, Magnusson PK, Willemsen G, Bartels M, Beijsterveldt CEV *et al.* Birth size and gestational age in opposite-sex twins as compared to same-sex twins: An individual-based pooled analysis of 21 cohorts. *Sci Rep* 2018;**8**:6300.

Jensen TK, Joffe M, Scheike T, Skytthe A, Gaist D, Petersen I, Christensen K. Early exposure to smoking and future fecundity among Danish twins. *Int J Androl* 2006;**29**:603–613.

Kalisch-Smith JI, Simmons DG, Dickinson H, Moritz KM. Review: sexual dimorphism in the formation, function and adaptation of the placenta. *Placenta* 2017;**54**:10–16.

Kaprio J, Rimpelä A, Winter T, Viken RJ, Rimpelä M, Rose RJ. Common genetic influences on BMI and age at menarche. *Hum Biol* 1995;**67**:739–753.

Korsoff P, Bogl LH, Korhonen P, Kangas AJ, Soininen P, Ala-Korpela M, Rose RJ, Kaaja R, Kaprio, J. A comparison of anthropometric, metabolic, and reproductive characteristics of young adult women from opposite-sex and same-sex twin pairs. *Front Endocrinol*, 2014; **5**:28.

Kuijper E, Twisk JWR, Korsen T, Caanen MR, Kushnir MM, Rockwood AL, Meikle AW, Hompes PG, Wit JM, Lambalk CB. Mid-pregnancy, perinatal, and neonatal reproductive endocrinology: a prospective cohort study in twins and singleton control subjects. *Fertil Steril* 2015;**104**:1527–1534.e1–e9.

Kuijper E, Vink JM, Lambalk CB, Boomsma DI. Prevalence of polycystic ovary syndrome in women from opposite-sex twin pairs. *J Clin Endocrinol Metab* 2009;**94**:1987–1990.

Kuijper EAM, Ket JCF, Caanen MR, Lambalk CB. Reproductive hormone concentrations in pregnancy and neonates: a systematic review. *Reprod Biomed Online*, 2013;**27**:33–63.

Loehlin JC, Martin NG. A comparison of adult female twins from opposite-sex and same-sex pairs on variables related to reproduction. *Behav Genet* 1998;**28**:21–27.

Loehlin JC, Martin NG. Dimensions of psychological masculinity-femininity in adult twins from opposite-sex and same-sex pairs. *Behav Genet* 2000;**30**:19–28.

Lummaa V, Pettay JE, Russell AF. Male twins reduce fitness of female co-twins in humans. *Proc Natl Acad Sci USA*, 2007;**104**: 10915–10920.

Medland SE, Loehlin JC, Willemsen G, Hatemi PK, Keller MC, Boomsma DI, Eaves LJ, Martin NG. Males do not reduce the fitness of their female co-twins in contemporary samples. *Twin Res Hum Genet* 2008;**11**:481–487.

Mickey RM, Greenland S. The impact of confounder selection criteria on effect estimation. *Am J Epidemiol* 1989;**129**:125–137.

Mills MC, Tropf FC. The biodemography of fertility: a review and future research frontiers. *Kolner Z Soz Sozialpsychol* 2015; **67**(Suppl 1):397–424.

O'Shaughnessy PJ, Antignac JP, Le Bizec B, Morvan ML, Svechnikov K, Söder O, Savchuk I, Monteiro A, Soffientini U, Johnston ZC et al. Alternative (backdoor) androgen production and masculinization in the human fetus. *PLoS Biol* 2019;**17**:e3000002.

Pison G, Monden C, Smits J. Twinning rates in developed countries: trends and explanations. *Popul Dev Rev* 2015;**41**:629–649.

Resnick SM, Gottesman II, McGue M. Sensation seeking in opposite-sex twins: an effect of prenatal hormones? *Behav Genet* 1993;**23**: 323–329.

Rhees RW, Kirk BA, Sephton S, Lephart ED. Effects of prenatal testosterone on sexual behavior, reproductive morphology and l h secretion in the female rat. *Dev Neurosci* 1997;**19**:430–437.

Rose RJ, Kaprio J, Winter T, Dick DM, Viken RJ, Pulkkinen L, Koskenvuo M. Femininity and fertility in sisters with twin brothers: prenatal androgenization? Cross-sex socialization? *Psychol Sci*, 2002; **13**:263–267.

Ryan BC, Vandenbergh JG. Intrauterine position effects. *Neurosci Biobehav Rev* 2002;**26**:665–678.

Salafia CM, Maas E. The twin placenta: framework for gross analysis in fetal origins of adult disease initiatives. *Paediatr Perinat Epidemiol* 2005;**19(Suppl. 1**):23–31.

Slutske WS, Bascom EN, Meier MH, Medland SE, Martin NG. Sensation seeking in females from opposite-versus same-sex twin pairs: hormone transfer or sibling imitation? *Behav Genet* 2011;**41**: 533–542.

Tapp AL, Maybery MT, Whitehouse AJO. Evaluating the twin testosterone transfer hypothesis: a review of the empirical evidence. *Horm Behav* 2011;**60**:713–722.

Tweed S, Bhattacharya S, Fowler PA. Effects of maternal smoking on offspring reproductive outcomes: an intergenerational study in the North East of Scotland. *Hum Reprod* 2017;**25**:2901–2906.

Verweij KJH, Mosing MA, Ullén F, Madison G. Individual differences in personality masculinity-femininity: examining the effects of genes, environment, and prenatal hormone transfer. *Twin Res Hum Genet* 2016;**19**:87–96.

Ye X, Skjaerven R, Basso O, Baird DD, Eggesbo M, Uicab LAC, , Haug K, Longnecker MP. In utero exposure to tobacco smoke and subsequent reduced fertility in females. *Hum Reprod* 2010;**25**: 2901–2906.