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Male microchimerism in females: a quantitative study of twin pedigrees to investigate mechanisms

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STUDY QUESTION: Does having a male co-twin, older brothers, or sons lead to an increased probability of persistent male microchimerism in female members of twin pedigrees?

SUMMARY ANSWER: The presence of a male co-twin did not increase risk of male microchimerism and the prevalence of male microchimerism was not explained by having male offspring or by having an older brother.

WHAT IS KNOWN ALREADY: Microchimerism describes the presence of cells within an organism that originate from another zygote and is commonly described as resulting from pregnancy in placental mammals. It is associated with diseases with a female predilection including autoimmune diseases and pregnancy-related complications. However, microchimerism also occurs in nulliparous women; signifying gaps in the understanding of risk factors contributing to persistent microchimerism and the origin of the minor cell population.

STUDY DESIGN, SIZE, DURATION: This cross-sectional study composed of 446 adult female participants of the Netherlands Twin Register (NTR).

PARTICIPANTS/MATERIALS, SETTING, METHODS: Participants included in the study were female monozygotic (MZ) twins, female dizygotic same-sex twins and females of dizygotic opposite-sex twin pairs, along with the mothers and sisters of these twins. Peripheral blood samples collected from adult female participants underwent DNA extraction and were biobanked prior to the study. To detect the presence of male-origin microchimerism, DNA samples were tested for the relative quantity of male specific Y chromosome gene *DYS14* compared to a common *β-globin* gene using a highly sensitive quantitative PCR assay.

MAIN RESULTS AND THE ROLE OF CHANCE: We observed a large number of women (26.9%) having detectable male microchimerism in their peripheral blood samples. The presence of a male co-twin did not increase risk of male microchimerism (odds ratio (OR) = 1.23: SE 0.40, $P = 0.61$) and the prevalence of male microchimerism was not explained by having male offspring (OR 0.90: SE 0.19, $P = 0.63$) or by having an older brother (OR = 1.46: SE 0.32, $P = 0.09$). The resemblance (correlation) for the presence of microchimerism was similar ($P = 0.66$) in MZ pairs (0.27; SE 0.37) and in first-degree relatives (0.091; SE 0.092). However, age had a positive relationship with the presence of male microchimerism ($P = 0.02$).

LIMITATIONS, REASONS FOR CAUTION: After stratifying for variables of interest, some participant groups resulted in a low numbers of subjects. We investigated microchimerism in peripheral blood due to the proposed mechanism of cell acquisition via transplacental blood exchange; however, this does not represent global chimerism in the individual and microchimerism may localize to numerous other tissues.

WIDER IMPLICATIONS OF THE FINDINGS: Immune regulation during pregnancy is known to mitigate allosensitization and support tolerance to non-inherited antigens found on donor cells. While unable to identify a specific source that promotes microchimerism prevalence within

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pedigrees, this study points to the underlying complexities of natural microchimerism in the general population. These findings support previous studies which have identified the presence of male microchimerism among women with no history of pregnancy, suggesting alternative sources of microchimerism. The association of detectable male microchimerism with age is suggestive of additional factors including time, molecular characteristics and environment playing a critical role in the prevalence of persistent microchimerism. The present study necessitates investigation into the molecular underpinnings of natural chimerism to provide insight into women's health, transplant medicine and immunology.

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Key words: microchimerism / chimerism / twins / female / monozygotic twins / dizygotic twins / pedigree

Introduction

Microchimerism is defined as the presence of a small number of cells within an organism that originate from another genetically distinct zygote. There is an interest in microchimerism and its consequences for health, with a focus on bidirectional foeto-maternal exchange of blood cells during pregnancy (Johnson *et al.*, 2020). Such intrauterine blood exchange can result in long-term persistent microchimerism, suggested to be the result of grafting and proliferation of 'transfused' stem cells in the tissues of the recipient. Early exposure to foreign cells may teach the immune system to develop a tolerance for these cells, supporting long-term persistence of microchimerism (Bianchi *et al.*, 1996; Gammill and Harrington, 2017). These examples of immune tolerance have exacerbated complications for the traditional self versus non-self criterion of immunology (Pradeu and Carosella, 2006). Exposure to foreign cells could contribute to human health and disease (for a review see Johnson *et al.*, 2020), as a contributing factor in the pathogenesis of autoimmune diseases (Nelson, 2012). Interestingly, chimerism has also been associated with paternal care in marmosets, suggesting a role in behavior (Ross *et al.*, 2007).

The presence of male cells in maternal circulation is indicative of microchimerism and has been well documented in women following pregnancy with a male fetus (Lo *et al.*, 1996; Murata *et al.*, 1999; Mosca *et al.*, 2003). The method of delivery, presence of placental complications and hypertensive disorders all can influence the amount of cell trafficking and therefore the exposure to microchimerism (Gammill *et al.*, 2013; Shree *et al.*, 2019). Foetal loss in early pregnancy may also result in microchimerism (Bianchi *et al.*, 2001). However, several studies have reported male microchimerism in women without any history of male pregnancy (Yan *et al.*, 2005; Muller *et al.*, 2015; Peters *et al.*, 2019), leading to a search for natural sources which may result in male microchimerism, such as having older brothers, having a male co-twin, sexual intercourse or unrecognized pregnancies (Dierselhuis *et al.*, 2012). It has even been proposed that every human is born as a microchimera, with a yet undefined source of donor cells (Dierselhuis and Goulmy, 2013).

Intrauterine exchange of cells occurs between twins through foeto-maternal transfer or direct transfer between the twin fetuses via placental transfusion. For many years, twin chimerism was considered to be an exception in humans, with the 30–40 cases reported in literature discovered by coincidence, most commonly by blood typing discrepancies (Tippett, 1983). In 1996, a systematic search by Van Dijk *et al.* showed an 8% prevalence of blood group chimerism in a study of blood samples from 552 dizygotic twin pairs and 24 triplet sets for multiple red cell blood group antigens (van Dijk *et al.*, 1996). This finding placed the concept of twin chimerism in humans in a new light. A recent study of 35 dizygotic twin pairs detected no ABO or D blood group chimerism through serologic assays and additional short tandem repeat (STR) assays also did not detect any blood chimerism (Tavares *et al.*, 2018). Improvements in detecting microchimerism by advanced molecular techniques now allow quantitative real-time polymerase chain reaction (qPCR) to quantify chimerism prevalence in DNA samples (Peters *et al.*, 2019).

The presence of non-inherited maternal antigens via maternal microchimerism in the offspring may be involved in the development of immune tolerance to both the maternal antigens and future overlapping foetal antigen from their own offspring (Kinder *et al.*, 2015). With multiple associations between microchimerism and risk for disease (Johnson *et al.*, 2020), we designed a study to look into the etiology of microchimerism. DNA samples from twin pedigrees provide a unique opportunity to investigate multiple mechanisms for microchimerism, both within and across generations, providing an overview of presence of male microchimerism in women. Here we document the patterns and transmission of microchimerism in multi-generation families. We present rates of male microchimerism as quantified by qPCR in mono- and dizygotic female twins from same- and opposite-sex twin pairs, their singleton (non-twin) sisters and their mothers. The women come from a general population sample and are characterized for the presence of older brothers and male offspring. By studying male microchimerism in female twins and their relatives, we can investigate the effects of having a male co-twin, the prevalence of microchimeris in twins and their non-twin sisters, generation differences, and the degree of shared microchimerism among family members.

Materials and methods

Participants

The participants in this study are enrolled in the Netherlands Twin Register (NTR) Biobank (Willemsen *et al.*, 2010; Ligthart *et al.*, 2019). We included females and identified families with female monozygotic (MZ), female dizygotic same-sex (DZss) or dizygotic opposite-sex (DOS) twins with blood derived DNA samples. Twins were included if a DNA sample was also available from their mother and from at least one singleton sister. Due to the amount of genomic material needed, an additional inclusion criterion was the quantity of genomic material of the sample available in the biobank. The failure to meet this criterion resulted in some incomplete pedigrees. The study included 446 women from 152 families: 62 females from DOS twin pairs, 80 females from MZ twin pairs, 68 females from DZss twin pairs, 106 mothers and 130 non-twin sisters. The median age in the total study population was 34 years (range 18–83), with the mothers of twins having a median age of 59 and their offspring of 32 years. Information on age, the presence of older brothers and the presence of a son was retrieved from the NTR database.

Quantitative real-time PCR

Blood derived DNA samples were tested for the presence of male microchimerism via a qPCR approach for measuring male microchimerism. This approach targets the Y chromosome specific gene *DYS14* as previously described (Peters *et al.*, 2019). In brief, each sample was tested in parallel for *DYS14* in 12 replicates as a measure of male genome mass and for *β-globin* in duplicate to obtain a measure of total genome mass. The PCR cycling and fluorescent measurement was completed on a Quantstudio™ 7 Flex instrument and analyzed by Quantstudio™ Real-Time PCR software (v1.1) (Applied Biosystems, Waltham, MA, USA). Standards were produced using known male and female extracted DNA samples. The *β-globin* standard included 10-fold dilutions of extracted DNA to produce standards from 500 ng to 0.5 ng (Fig. 1A). The standards for *DYS14* were produced by simulating sample conditions by diluting known male extracted DNA into female extracted DNA. The resulting standards maintained a constant 66 ng/µl with 10-fold dilutions of male genome from 66 ng/µl to 0.0066 ng/µl (Fig. 1A). The lower detection limit of the qPCR assay implied a minimum threshold of detection for male microchimerism of one genome equivalent per one million.

A number of precautions were implemented to prevent potential contamination of the samples and qPCR reactions (Peters *et al.*, 2019). All samples from the NTR biobank and reaction preparation were handled in a Class II biosafety cabinet that was rigorously cleaned by chemical and ultraviolet decontamination. Additionally, all pipetting utilized previously established guidelines for PCR reaction preparation (Kwok and Higuchi, 1989). Each reaction plate contained various quality control measures. Six no template control (NTC) wells were included as a true negative for the *DYS14* and *β-globin* assays. Negative control was produced using a known female extracted DNA sample, whereas the positive control was produced by spiking known male extracted DNA sample into female sample. The controls tested as expected for all experiments, such that negative control tested negative for *DYS14* (Fig. 1B) while the NTC tested negative for both assays consistently. The positive control demonstrated consistent detection of both *β-globin* and *DYS14* across reaction plates (Fig. 1C).

Statistical analyses

Participant group male microchimerism status presented as categorical data were compared by Chi-squared (χ^2) tests. Associations of male microchimerism status with age and the presence of sons or older brothers were assessed by generalized estimating equations (GEE) and logistic regression with a correction for family clustering. Concordance for male microchimerism between pairs of relatives was summarized in 2 × 2 contingency tables. Quantitative data for male microchimerism burden were evaluated by a Kruskal–Wallis test. Data on presence of brothers (n = 18) or presence of son (n = 19) were missing in some families; for analyses concerning these variables, these families were excluded. Statistical analyses were performed using SPSS 26.0 (SPSS, Chicago, IL, USA) and R programming language. $P < 0.05$ was considered statistically significant.

To quantify familial resemblance for the presence of male microchimerism, we estimated the tetrachoric correlations for two types of genetic relations: for MZ twin pairs, whose genetic relatedness is ~100% as they derive from the same fertilized egg, and for all others, who are first-degree relatives. All first-degree relatives share either exactly 50% of their segregating genes (mother and daughter), or 50% on average (DZ twin and sister pairs). Tetrachoric correlations represent the relation between variables on the underlying continuous liability scale. Tetrachoric correlations and their SE between family members (Falconer and Mackay, 1996) were estimated and tested for significance in OpenMx (Neale *et al.*, 2016).

Ethics

The Netherlands Twin Register Biobank study was approved by the Central Ethics Committee on Research Involving Human Subjects of the VU University Medical Center, Amsterdam, an Institutional Review Board certified by the U.S. Office of Human Research Protections (IRB number IRB00002991 under Federal-wide Assurance FWA00017598; IRB/institute codes, NTR 03-180) and informed consent was obtained from all participants (Willemsen *et al.*, 2010).

Results

Prevalence

Male microchimerism was detected in 120 of the 446 participating women (26.9%). The prevalence was 16.3% in MZ females and did not differ from the prevalence in DZss twins ($P = 0.27$). In the group of females from DZss twin pairs 23.5% tested positive for male microchimerism, compared to 27.4% of females from DOS twin pairs ($P = 0.61$); OR 1.23 (SE 0.40). Of all 130 singleton sisters included, 33 tested positive for male microchimerism (25.4%). There were 106 mothers of twins, of whom 41 tested positive for male microchimerism (38.7%). Figure 2 summarizes these results for all members of each participant group.

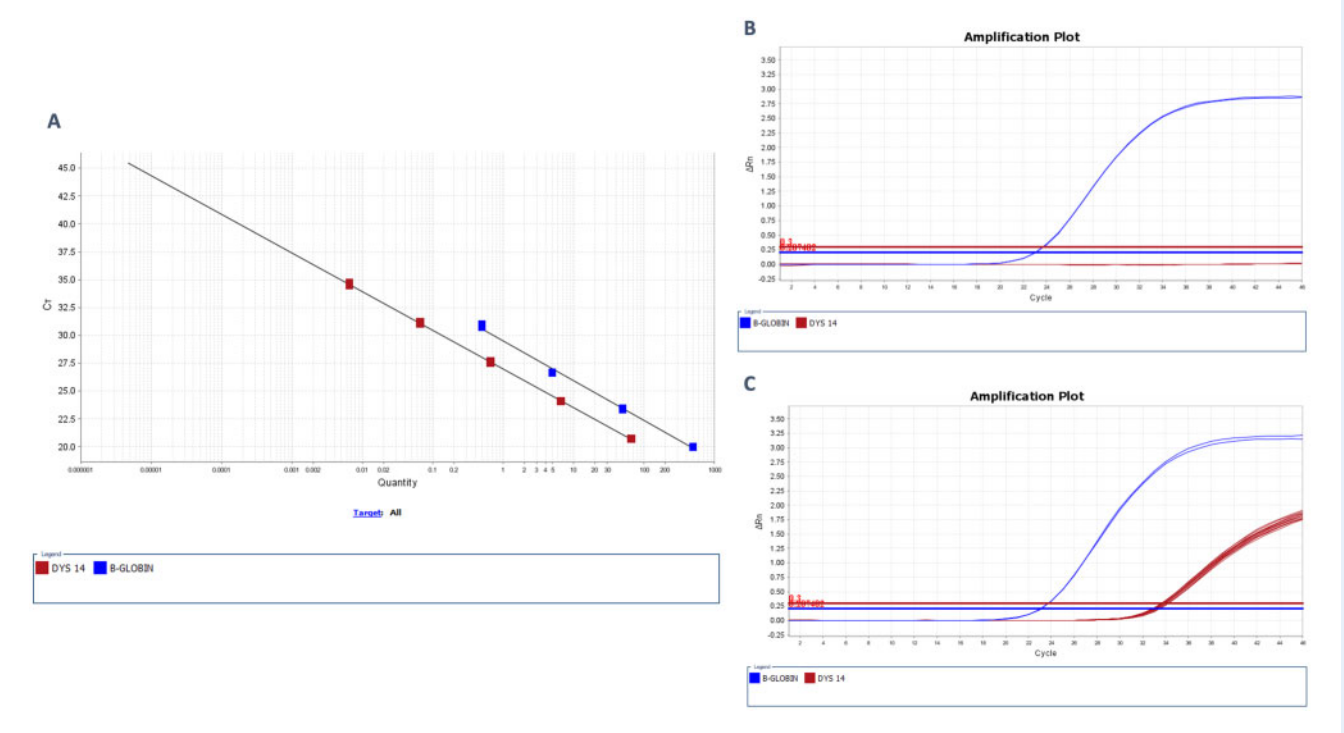

Figure 1 qPCR data for the analysis of male genome equivalents by measure of *DYS14* and *β-globin* targets. (**A**) A plot of standard curves generated using known human genomic samples; (**B**) an amplification plot for a negative control sample with measure of *β-globin* and no detection of *DYS14*. (**C**) An amplification plot for a positive control sample with detectable measurement of both *DYS14* and *β-globin* targets.

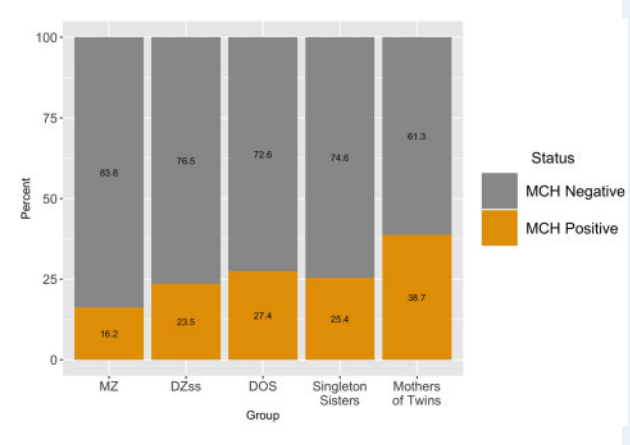

Figure 2 Prevalence of male microchimerism (MCH) in women, stratified by pedigree position. MZ, monozygotic twins; DZss, dizygotic same-sex twins; DOS, dizygotic opposite-sex twins.

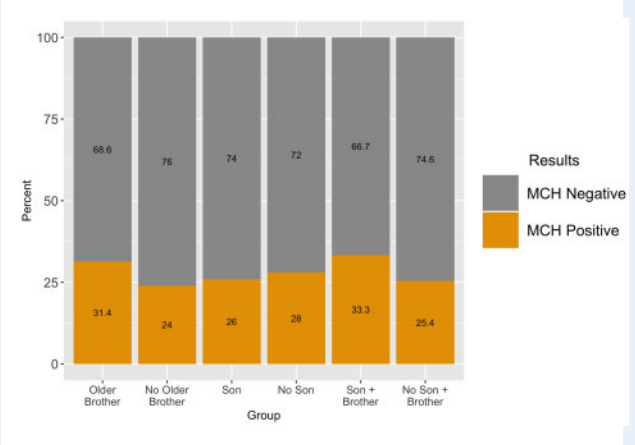

Figure 3 Prevalence of male microchimerism (MCH) in females with and without an older brother, a son, or both an older brother and a son.

Associations with microchimerism

The prevalence of detectable male microchimerism was compared among females who have an older brother, male offspring or both as shown in Fig. 3. The prevalence of male microchimerism tended to be greater in females with an older brother (31.4%) compared to those without (24.0%); OR 1.46 (SE 0.32), $P = 0.09$. Females with and without male offspring had a similar prevalence of male microchimerism (26.0% and 28.0%, respectively); OR 0.90 (SE 0.19), $P = 0.63$. Further assessment of an additive effect of having both an older brother and a male offspring revealed no significant difference among all participants ($P = 0.16$) or in the mother of twins group ($P = 0.50$).

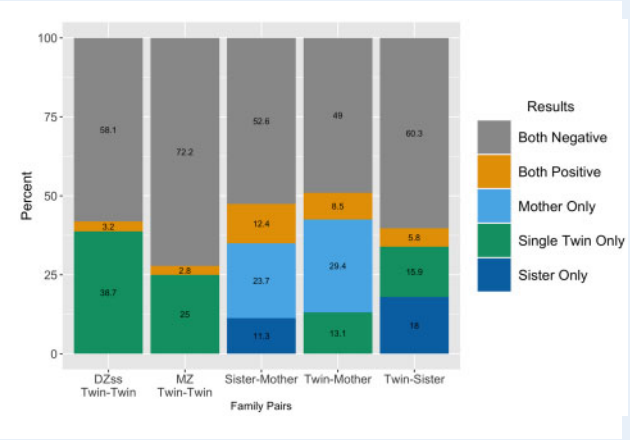

Figure 4 Male microchimerism concordance within families. Data are presented for pairs of relatives including: DZss (dizygotic same-sex) twins, MZ (monozygotic) twins, and mother with singleton sister and twin, and twin-sister.

The mothers of twins group, which showed the largest proportion of detectable male microchimerism, had similar prevalence with and without having male offspring (38.9% and 38.5%); OR 1.018 (SE 0.34), $P = 0.96$. There was a positive relationship between age at the time of biobanking and presence of microchimerism ($P = 0.02$; Nagelkerke $R^2 = 0.017$).

Figure 4 summarizes concordances for DZss and MZ twin, twin-sister, twin-mother and sister-mother pairs for male microchimerism. In the complete MZ twin pairs and DZss twin pairs, twin–twin comparison reveals that 75% and 61% were concordant for microchimerim status ($P = 0.35$). Male microchimerism was more prevalent in mothers ($P = 0.003$ for mother-twin and $P = 0.06$ for mother-non twin offspring comparison). There was no difference between the twins and their sisters for male microchimerism ($P = 0.71$).

Familial resemblance

The offspring (data from twins and their sisters combined) of mothers with male microchimerism presented with male microchimerism somewhat more frequently than female offspring of male microchimerism negative mothers (26.8% and 17.6%, respectively; $P = 0.08$). We investigated the family resemblance by calculating tetrachoric correlations for microchimerism status separately in MZ twin pairs and in all first degree relatives. The correlations were estimated at 0.27 (SE 0.37) for MZ twin pairs and 0.091 (SE 0.092) for all first-degree relative pairs. The two correlations can equated to be the same ($P = 0.66$) and did not differ from zero ($P = 0.25$).

Microchimerism concentration

After selection of the positive samples (i.e. 120 subjects with >1 male GEq per 1 000 000 cells), the median concentration of male microchimerism is similar among twins, their sisters and their mothers ($P = 0.28$) and is summarized in Fig. 5. Outlier samples with elevated levels of detectable male genome (>80 male GEq) were investigated compared to the other participants with detectable male microchimerism revealing this group to have no male offspring or other distinguishing features and a lower age at time of collection ($P = 0.034$) (Fig. 6).

Discussion

We investigated the presence of male chimerism in women and its etiology in members of twin pedigrees. We found that about 27% of the adult women had detectable male microchimerism. The highest prevalence of male microchimerism was detected in the older participants, i.e. mothers of twins (38.7%) and we observed a positive relation between age and presence of microchimerism. This observation indicates that microchimeric cells may persist and stay detectable long-term after they are acquired, although increased exposure to unexplored variables due to age could also be involved in chimerism risk and persistence.

Previous research in twins looked at the presence of blood chimerism via red blood cell antigens and discovered an 8% chimerism prevalence in twins (van Dijk *et al.*, 1996). We expanded upon the previous work by investigating the source of male microchimerism and the familial relationships in two generation twin pedigrees. We found that females from a DOS twin pair do not present with male microchimerism more frequently than females from a DZss twin pair, despite the adjacent in utero presence of the male co-twin. This refutes our hypothesis that a male co-twin promotes persistent male microchimerism. Our data also showed that rates between DZ twins are not different from those among MZ twins and singleton siblings.

Male microchimerism could arise in women having an older brother, which has been suggested in earlier studies (Dierselhuis *et al.*, 2012; Muller *et al.*, 2015). This would support trans-maternal cell flow as an explanation and is supported by a higher prevalence of male microchimerism in female offspring of mothers with male microchimerism. We observed only a tendency of a higher prevalence of male microchimerism in women with an older brother. We also saw no evidence that male microchimerism status was related to the presence of a son. Further, among participants with an exceptionally high level of male microchimerism (>80 male GEq), none had a son at the time of sample collection. Thus, our study indicates that the origin of the microchimeric cells may not necessarily be a close family member. One source is repeated sequential fetal-maternal exchanges across generations (Kinder *et al.*, 2017). Possible further sources are unreported or unrecognized interrupted pregnancies (Bianchi *et al.*, 2001), breastfeeding, placental structure, pregnancy complications including preeclampsia (Bianchi *et al.*, 2001; Gammill *et al.*, 2013; Hassiotou and Geddes, 2015; Peters *et al.*, 2017) and it has also been suggested that sexual intercourse may play a role (Muller *et al.*, 2015; Yan *et al.*, 2005). We did not have these data available for a sufficient number of participants for comprehensive analyses of these alternatives.

There are several limitations that must be considered when interpreting our findings. First, despite obtaining samples from a large register, after stratifying for various analyses we had a relatively low number of subjects within some participant groups. Furthermore, the data do not illustrate the source of the minor cell population beyond that it is of male origin. Due to the suggested mechanism of chimerism acquisition via blood exchange we exclusively investigated blood microchimerism which may not represent global chimerism. Several

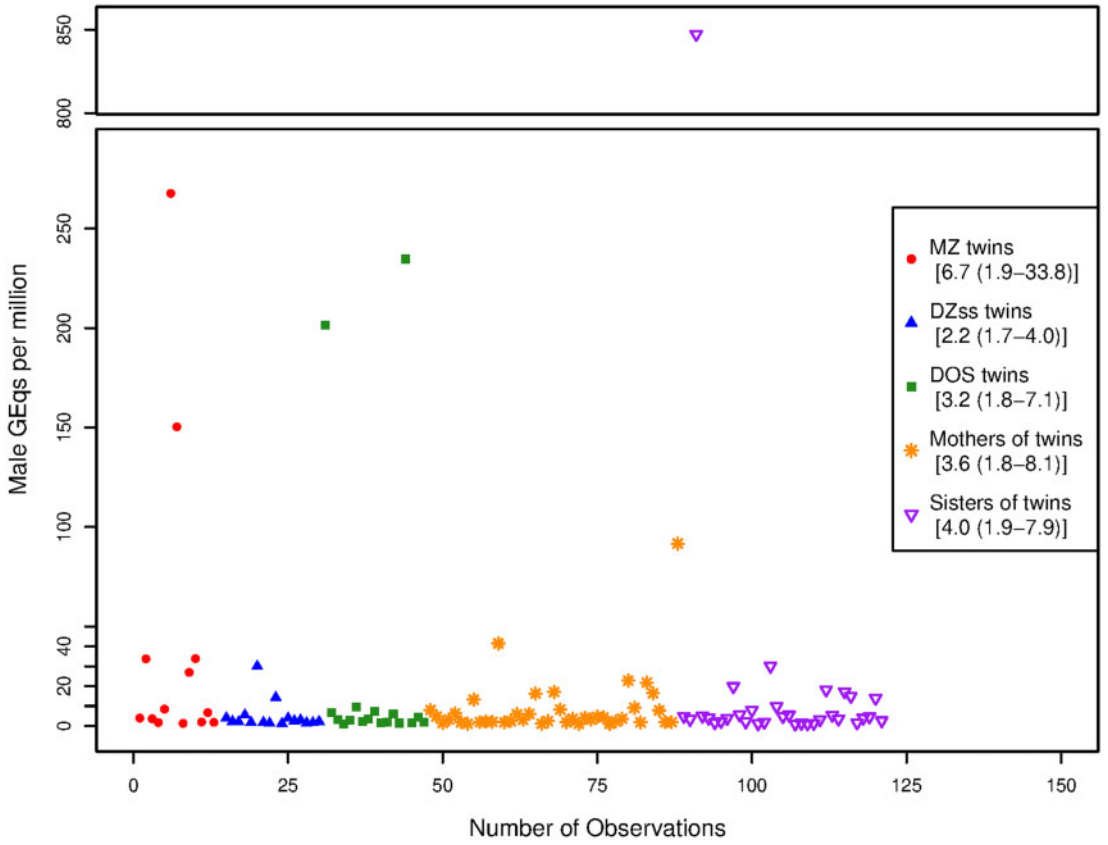

Figure 5 Male genome equivalents (GEq) per one million cells by each participant with detectable male GEq. The data are stratified by participant group. Descriptive statistics presented as Median (IQR). MZ, monozygotic twins; DZss, dizygotic same-sex twins; DOS, dizygotic opposite-sex twins. Male GEqs, male genome equivalents per million.

studies have identified the presence of allogeneic cells in various tissues throughout the body; some of which include the skin (Mahmood and O'Donoghue, 2014), breast (Gadi, 2010; Kamper-Jorgensen *et al.*, 2012; Dhimolea *et al.*, 2013; Nemescu *et al.*, 2016), pancreas (Nelson *et al.*, 2007) and brain (Chan *et al.*, 2012; Broestl *et al.*, 2018) among others. It is probable that cells obtained via blood exchange may localize to any variety of tissues in the host where they may possibly persist, the so-called 'adult stem cell plasticity phenomenon' (Hong *et al.*, 2007; Koopmans *et al.*, 2005). These findings help demonstrate that the temporality and localization of microchimerism in the blood and other tissues may be a factor of exposure, time and environmental influences such as tissue injury which may partially explain the present findings in adult participants.

As with many other studies of microchimerism, the use of a Y chromosome target has been proven effective at identifying low levels of chimerism due to the target specificity. However this technique is limited to the study of females. While other targets, including RBC antigens and HLA typing have been developed, these have a lower sensitivity or increased complexity that often require prior knowledge of the donor's genotype (Johnson *et al.*, 2020). As this work adds to the growing body of knowledge on human chimerism, continued advancements in molecular technologies will provide new opportunities to expand upon our findings presented here. We found that microchimerism occurs frequently, in over one quarter of women. This necessitates research into its etiology and significance.

Others have previously described that exposure to non-inherited maternal antigens improves success of future pregnancies as well as mitigating allosensitization (Kinder *et al.*, 2015). As many changes occur during pregnancy, the complication of immune tolerance is balanced by interaction with non-inherited maternal antigens and is likely to provide insight into other areas of immune sensitization and allotransplantation (Kinder *et al.*, 2017). Already, outcomes in cord blood transplantation have shown improvements when mismatched HLA alleles between donor and recipient include a non-inherited maternal antigen (van Rood *et al.*, 2009) and a beneficial effect against leukemia relapse when graft and donor share inherited paternal HLA antigens, suggesting an effect of maternal microchimerism of T memory cells (Burlingham and Nelson, 2012; van Rood *et al.*, 2012). Our findings provide further support for the remarkable complexity of microchimerism and the challenge it may pose the immune system both during and following pregnancy. Based on our findings of elevated microchimerism concentrations in younger participants (Fig. 6) and a positive relationship between prevalence and age, it is possible that long term conservation of microchimerism is best maintained with low levels of

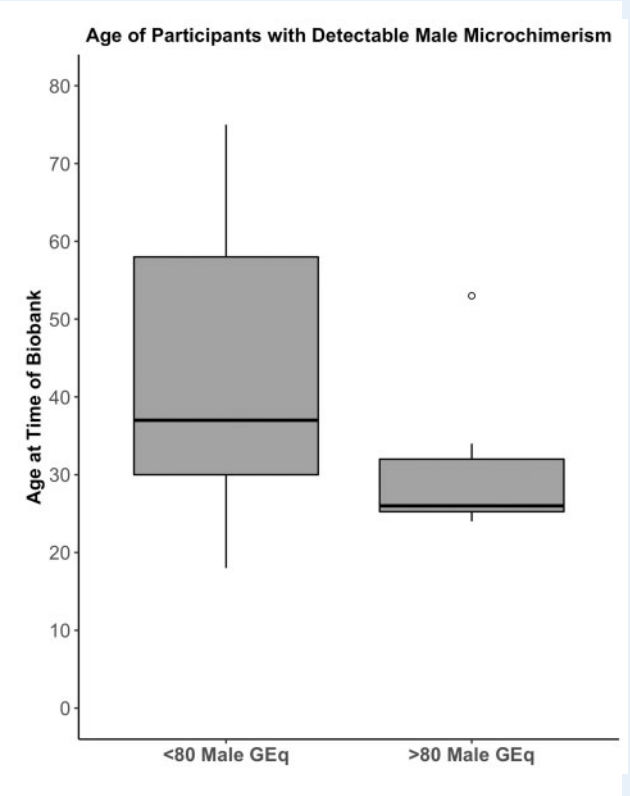

Figure 6 Age distribution of individuals with detectable male microchimerism at a concentration of <80 male genome equivalents per million (GEq) (n = 114) or outliers with >80 male GEq (n = 6).

allogeneic cells. These individual observations of persistent microchimerism may be due to successful evasion of overt sensitization of the immune system. Maintaining balance between microchimerism concentration and immune detection may be essential to long term microchimerism persistence.

Further, there is a growing body of research that has suggested microchimerism to be associated with the pathogenesis of disease. Recently, a study established that women without a protective HLA allele are more likely to develop rheumatoid arthritis when they have microchimerism carrying an HLA protective allele (Kanaan *et al.*, 2019). Similarly, maternal microchimerism has been identified to be associated with Type 1 diabetes and contribute to islet β cells in the offspring (Nelson *et al.*, 2007). Our findings provide further support for the hypothesis that microchimerism sensitizes the immune system. The inverse relationship between quantitative male microchimerism outliers and age (Fig. 6) may illustrate the pressures of age and future pregnancy on maintenance of microchimerism, resulting in lower quantitative levels later in life. Possible mechanisms producing these findings could include immune system responses clearing allogeneic cells or an individual's own cells outcompeting the donor cells over multiple generations of cellular replication. Such studies and findings continue to amplify the need for researching microchimerism prevalence within the general population.

Improvements in future research will require expanding upon current knowledge of human chimerism with additional sample types to further explain chimerism tissue localization and subsequent health implications. Such work will likely necessitate larger consortia driven studies to achieve sample numbers to achieve significance in understanding the underlying complexity of human chimerism. Expanding on the findings presented here requires larger studies of microchimerism in mixed sex populations to evaluate alternative sources of microchimerism and contribution in health and disease. Further, due to the seemingly variable nature of microchimerism over time, longitudinal studies of chimerism presence and concentration are warranted to further understand this phenomenon in human biology.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Authors' roles

Study design, data collection, data analysis and interpretation, and drafting of manuscript were performed by B.N.J. and H.E.P., supervised by E.A.E. and C.B.L. respectively; supervision and contribution to study design by C.B.L., V.M., D.I.B. and E.A.E.; G.W. was responsible for the supervision of the NTR biobank project; assessment of participants and biobank sample selection by G.W. and L.L.; C.V.D. and J.J.H. contributed to data analysis and interpretation; founding of the Netherlands Twin Register (NTR), oversight of NTR participant recruitment and concomitant sample and phenotype collection by D.I.B.; all authors contributed to critical evaluation and approval of final manuscript.

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Conflict of interest

C.B.L. declares a competing interest as editor-in-chief of *Human Reproduction* and his department receives unrestricted research grants from Ferring, Merck and Guerbet. All remaining authors have no conflict of interest to declare in regards to this work.

References

Bianchi DW, Farina A, Weber W, Delli-Bovi LC, Deriso M, Williams JM, Klinger KW. Significant fetal-maternal hemorrhage after termination of pregnancy: implications for development of fetal cell microchimerism. *Am J Obstet Gynecol* 2001;**184**:703–706.

Bianchi DW, Zickwolf GK, Weil GJ, Sylvester S, DeMaria MA. Male fetal progenitor cells persist in maternal blood for as long as 27 years postpartum. *Proc Natl Acad Sci U S A* 1996;**93**:705–708.

Broestl L, Rubin JB, Dahiya S. Fetal microchimerism in human brain tumors. *Brain Pathol* 2018;**28**:484–494.

Burlingham WJ, Nelson JL. Microchimerism in cord blood: mother as anticancer drug. *Proc Natl Acad Sci U S A* 2012;**109**:2190–2191.

Chan WF, Gurnot C, Montine TJ, Sonnen JA, Guthrie KA, Nelson JL. Male microchimerism in the human female brain. *PLoS One* 2012;**7**:e45592.

Dhimolea E, Denes V, Lakk M, Al-Bazzaz S, Aziz-Zaman S, Pilichowska M, Geck P. High male chimerism in the female breast shows quantitative links with cancer. *Int J Cancer* 2013;**133**:835–842.

Dierselhuis MP, Blokland EC, Pool J, Schrama E, Scherjon SA, Goulmy E. Transmaternal cell flow leads to antigen-experienced cord blood. *Blood* 2012;**120**:505–510.

Dierselhuis MP, Goulmy E. We are all born as microchimera. *Chimerism* 2013;**4**:18–19.

Falconer D, Mackay T. *Introduction to Quantitative Genetics*. Essex: Pearson Educational Limited, UK, 1996.

Gadi VK. Fetal microchimerism in breast from women with and without breast cancer. *Breast Cancer Res Treat* 2010;**121**:241–244.

Gammill HS, Aydelotte TM, Guthrie KA, Nkwopara EC, Nelson JL. Cellular fetal microchimerism in preeclampsia. *Hypertension* 2013;**62**:1062–1067.

Gammill HS, Harrington WE. Microchimerism: defining and redefining the prepregnancy context – a review. *Placenta* 2017;**60**:130–133.

Hassiotou F, Geddes DT. Immune cell-mediated protection of the mammary gland and the infant during breastfeeding. *Adv Nutr* 2015;**6**:267–275.

Hong YC, Liu HM, Chen PS, Chen YJ, Lyou JY, Hu HY, Yi MF, Lin JS, Tzeng CH. Hair follicle: a reliable source of recipient origin after allogeneic hematopoietic stem cell transplantation. *Bone Marrow Transplant* 2007;**40**:871–874.

Johnson BN, Ehli EA, Davies GE, Boomsma DI. Chimerism in health and potential implications on behavior: a systematic review. *Am J Med Genet A* 2020;**182**:1513–1529.

Kamper-Jorgensen M, Biggar RJ, Tjonneland A, Hjalgrim H, Kroman N, Rostgaard K, Stamper CL, Olsen A, Andersen AM, Gadi VK. Opposite effects of microchimerism on breast and colon cancer. *Eur J Cancer* 2012;**48**:2227–2235.

Kanaan SB, Sensoy O, Yan Z, Gadi VK, Richardson ML, Nelson JL. Immunogenicity of a rheumatoid arthritis protective sequence when acquired through microchimerism. *Proc Natl Acad Sci U S A* 2019; **116**:19600–19608.

Kinder JM, Jiang TT, Ertelt JM, Xin L, Strong BS, Shaaban AF, Way SS. Cross-generational reproductive fitness enforced by microchimeric maternal cells. *Cell* 2015;**162**:505–515.

Kinder JM, Stelzer IA, Arck PC, Way SS. Immunological implications of pregnancy-induced microchimerism. *Nat Rev Immunol* 2017;**17**:483–494.

Koopmans M, Kremer Hovinga IC, Baelde HJ, Fernandes RJ, de Heer E, Bruijn JA, Bajema IM. Chimerism in kidneys, livers and hearts of normal women: implications for transplantation studies. *Am J Transplant* 2005;**5**:1495–1502.

Kwok S, Higuchi R. Avoiding false positives with PCR. *Nature* 1989;**339**:237–238.

Ligthart L, van Beijsterveldt CEM, Kevenaar ST, de Zeeuw E, van Bergen E, Bruins S, Pool R, Helmer Q, van Dongen J, Hottenga JJ. *et al.* The Netherlands Twin Register: longitudinal research based on twin and twin-family designs. *Twin Res Hum Genet* 2019;**22**:623–614.

Lo YM, Lo ES, Watson N, Noakes L, Sargent IL, Thilaganathan B, Wainscoat JS. Two-way cell traffic between mother and fetus: biologic and clinical implications. *Blood* 1996;**88**:4390–4395.

Mahmood U, O'Donoghue K. Microchimeric fetal cells play a role in maternal wound healing after pregnancy. *Chimerism* 2014;**5**:40–52.

Mosca M, Curcio M, Lapi S, Valentini G, D'Angelo S, Rizzo G, Bombardieri S. Correlations of Y chromosome microchimerism with disease activity in patients with SLE: analysis of preliminary data. *Ann Rheum Dis* 2003;**62**:651–654.

Muller AC, Jakobsen MA, Barington T, Vaag AA, Grunnet LG, Olsen SF, Kamper-Jorgensen M. Microchimerism of male origin in a cohort of Danish girls. *Chimerism* 2015;**6**:65–71.

Murata H, Nakauchi H, Sumida T. Microchimerism in Japanese women patients with systemic sclerosis. *Lancet* 1999;**354**:220.

Neale MC, Hunter MD, Pritikin JN, Zahery M, Brick TR, Kirkpatrick RM, Estabrook R, Bates TC, Maes HH, Boker SM. OpenMx 2.0: extended structural equation and statistical modeling. *Psychometrika* 2016;**81**:535–549.

Nelson JL. The otherness of self: microchimerism in health and disease. *Trends Immunol* 2012;**33**:421–427.

Nelson JL, Gillespie KM, Lambert NC, Stevens AM, Loubiere LS, Rutledge JC, Leisenring WM, Erickson TD, Yan Z, Mullarkey ME. *et al.* Maternal microchimerism in peripheral blood in type I diabetes and pancreatic islet beta cell microchimerism. *Proc Natl Acad Sci U S A* 2007;**104**:1637–1642.

Nemescu D, Ursu RG, Nemescu ER, Negura L. Heterogeneous distribution of fetal microchimerism in local breast cancer environment. *PLoS One* 2016;**11**:e0147675.

Peters HE, Johnson BN, Ehli EA, Micha D, Verhoeven MO, Davies GE, Dekker J, Overbeek A, Berg M, Dulmen-den Broeder EV. *et al.* Low prevalence of male microchimerism in women with Mayer-Rokitansky-Kuster-Hauser syndrome. *Hum Reprod* 2019;**34**:1117–1125.

Peters HE, Konig TE, Verhoeven MO, Schats R, Mijatovic V, Ket JC, Lambalk CB. Unusual twinning resulting in chimerism: a systematic review on monochorionic dizygotic twins. *Twin Res Hum Genet* 2017;**20**:161–168.

Pradeu T, Carosella ED. On the definition of a criterion of immunogenicity. *Proc Natl Acad Sci U S A* 2006;**103**:17858–17861.

Ross CN, French JA, Orti G. Germ-line chimerism and paternal care in marmosets (Callithrix kuhlii). *Proc Natl Acad Sci U S A* 2007;**104**:6278–6282.

Shree R, Harrington WE, Kanaan SB, Forsyth A, Cousin E, Lopez A, Nelson JL, Gammill HS. Fetal microchimerism by mode of delivery: a prospective cohort study. *BJOG* 2019;**126**:24–31.

Tavares L, Da Costa DC, Batschauer APB, Jobim LFJ, Ewald GM, de Mello C, Velazquez ESA, Geraldo A. Blood chimerism in twins. *Immunohematology* 2018;**34**:151–157.

Tippett P. Blood-group chimeras – A REVIEW. *Vox Sang* 1983;**44**: 333–359.

van Dijk BA, Boomsma DI, de Man AJ. Blood group chimerism in human multiple births is not rare. *Am J Med Genet* 1996;**61**:264–268.

van Rood JJ, Scaradavou A, Stevens CE. Indirect evidence that maternal microchimerism in cord blood mediates a graft-versus-leukemia effect in cord blood transplantation. *Proc Natl Acad Sci U S A* 2012; **109**:2509–2514.

van Rood JJ, Stevens CE, Smits J, Carrier C, Carpenter C, Scaradavou A. Reexposure of cord blood to noninherited maternal HLA antigens improves transplant outcome in hematological malignancies. *Proc Natl Acad Sci U S A* 2009;**106**: 19952–19957.

Willemsen G, de Geus EJ, Bartels M, van Beijsterveldt CE, Brooks AI, Estourgie-van Burk GF, Fugman DA, Hoekstra C, Hottenga JJ, Kluft K. *et al.* The Netherlands Twin Register biobank: a resource for genetic epidemiological studies. *Twin Res Hum Genet* 2010;**13**: 231–245.

Yan Z, Lambert NC, Guthrie KA, Porter AJ, Loubiere LS, Madeleine MM, Stevens AM, Hermes HM, Nelson JL. Male microchimerism in women without sons: quantitative assessment and correlation with pregnancy history. *Am J Med* 2005;**118**:899–906.