Intrauterine mix-up:
chimerism and endocrinology

Henrike Peters

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Intrauterine mix-up: chimerism and endocrinology

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TABLE OF CONTENTS

GENERAL INTRODUCTION AND THESIS OUTLINE

John Hunter 1786: 'Account of the freemartin'

"It is a fact known, and I believe almost universally understood, that when a cow brings forth two calves, and one of them a bull calf the other to appearance a cow, that the cow-calf is unfit for propagation, but the bull-calf grows up into a very proper bull. Such a cow-calf is called in this country a freemartin."

GENERAL INTRODUCTION

Embryonic development genitalia

The hormonal milieu of the intrauterine environment is an essential factor in embryonic development. It is a unique setting in which the presence—or absence—of certain hormones will have definitive results for genital sex differentiation. The genetic sex of the fetus itself plays the most important role.

In mammals, genetic sex is determined by the sex chromosomes: XX for female and XY for male. In the first six weeks of development, the female and male embryo are similar. After this period, the testis-determining factor on the Y-chromosome in males causes the undifferentiated gonads to develop into testes. In females, the absence of the Y-chromosome results in the formation of ovaries.

Then, in the 6th week of embryonic development, the fetus becomes endocrinologically active. The testes in the male fetus start to secrete testosterone and anti-Müllerian Hormone (AMH) resulting in masculinization of the embryo. The Wolffian duct (mesonephric duct) develops and AMH causes regression of the Müllerian duct (paramesonephric duct). The androgen stimulation also results in masculinization of the external genitalia.

In females, in absence of AMH, the Müllerian duct does not go in regression, forming a uterus and upper part of the vagina. The Wolffian duct regresses. The absence of (dihydro) testosterone prevents the fusion of the labioscrotal fold and external female genitalia are formed. So, feminization of the genitalia actually reflects an absence of testes-secreted hormones.[1]

Intrauterine position effect

When the uterus, and thus the intrauterine environment, is shared by multiple offspring of different sex this means a complex situation can occur. In animals, it has been shown that hormones can diffuse across amniotic membranes, or be transported by vascular exchange via the placenta.[2,3] When there are offspring of opposite-sex, inter-fetal transfer of androgens can result in masculinization of the female embryo.[4]

The effect of the hormonal transfer is dependent on the proximity of the opposite-sex fetus, this is referred to as the intrauterine position effect. This effect has been studied in detail in mice. Due to the anatomy of the uterus, with two elongated uterine horns, a female fetus can be located between zero (0M), one (1M) or two (2M) male fetuses as depicted in Figure 1. Concentrations of testosterone have been reported to be significantly increased in amniotic fluid and blood serum in 2M mice compared to 0M mice. Estradiol is decreased in 2M versus 0M.[5] The impact of this intrauterine position has been measured by behavioral and morphological studies. Masculinized genitalia, decreased offspring production and more male-typical behavior, as aggression, have been reported in female mice with male littermates.[4]

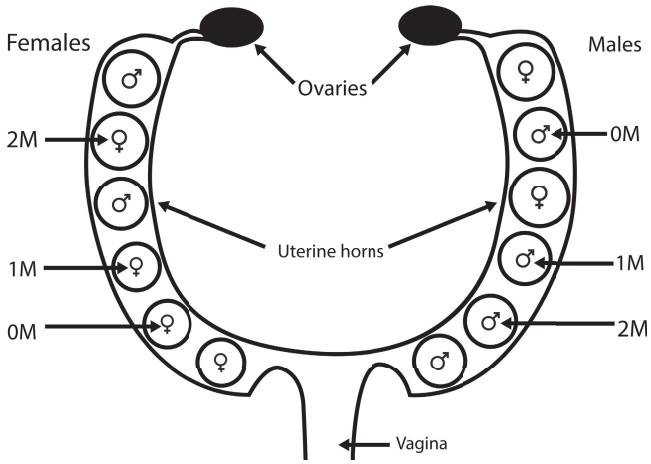

Figure 1. Fetus positions in a womb. The 0M-2M classification refers to the number of males flanking the fetus.
From: Ryan & Vandenbergh, 2002[4]

In cattle, the intrauterine position effect is reflected by the Freemartin syndrome. An opposite-sex twin pregnancy can result in the birth of an infertile female cow: a Freemartin.[6] As the result of a combined placenta with vascular connections between the opposite-sex twins, AMH secreted by the testes of the bull is transferred to the cow. See Figure 2. This inter-fetal hormonal exchange of AMH is indicated to be responsible for the regression of the Müllerian duct in the Freemartin, leading to the absence of uterus and vagina.[7]

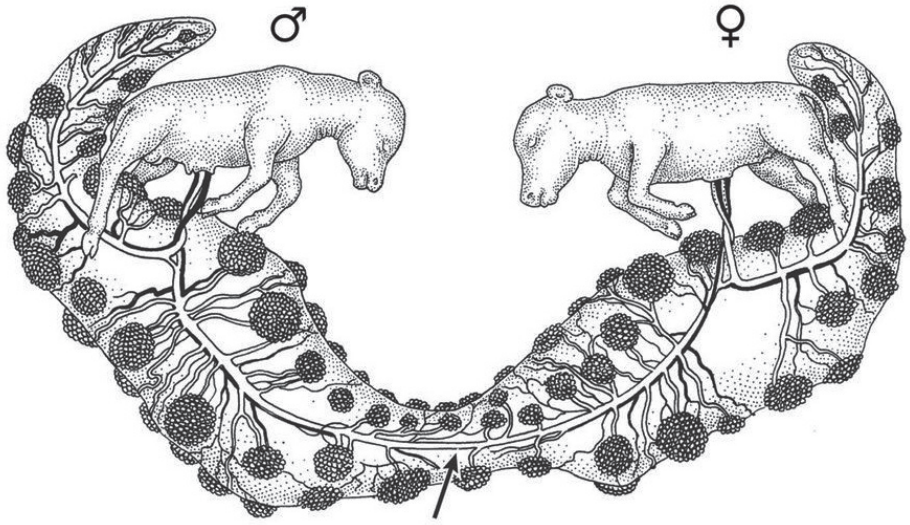

Figure 2. Vascular connections in opposite-sex twin pregnancy in cattle
From: Frank Lillie. 1916 [6]

Chimerism

According to Greek mythology, a chimera (Χίμαιρα), is a creature composed of more than one animal. It has the head of a lion, the tail of snake, and a head of a goat at his back. In medical terms, chimerism can be defined as the presence of a genetically distinct cell-line that originates from another individual. Artificial chimerism can be present following bone marrow transplantation or blood transfusion. Natural chimerism originates during pregnancy and fetal development. It can occur through bidirectional maternal-fetal exchange,[9] referred to as microchimerism due to the limited amount of chimeric cells. During fetal development, chimerism can also occur when two separately fertilized oocytes admix to form one single embryo which results in the birth of a singleton with two different cell lines in different cells of the body ('tetragametic chimerism').[10]

In this thesis I focus on twin chimerism, which refers to the phenomenon that dizygotic twins are chimeric for the cells of their co-twin. This may arise by direct blood exchange via vascular anastomoses in the placenta or through maternal blood by trans-placental trafficking. Most cases of twin chimerism are found by coincidence.[11] In one systematic search in 1996, blood group chimerism was found in as many as 8% of the dizygotic twin pairs and even 21% of the triplet pairs.[12] Whether it has clinical consequences is unknown.

Hormonal transfer in human twins?

The possible effect of hormone transfer between opposite-sex human twins has been studied extensively over the last decades. Masculinization of the female fetus by her male co-twin has been suggested by many researchers, referred to as the twin testosterone transfer hypothesis. Studies for behavioral changes and mental health[13,14] in opposite-sex twins show some evidence supporting this in humans. However, the current body of research is controversial.[15] Social effects of being raised with a twin brother may possibly contribute to the reported associations. Also, in females with a male co-twin, a later age at menarche has been reported[16] and it has been suggested that females with a male co-twin are less likely to reproduce than females with a female co-twin[17]. However, these findings have not been replicated.[18-20] In humans, there is no direct evidence for hormonal transfer between twins.[21]

There is no general consensus about testosterone transfer in human twins. To investigate possible hormonal transfer in twins we should, however, also focus on the other testes-secreted hormone: AMH. An essential and important question should be whether inter-fetal transfer of AMH occurs and if this, like in Freemartins, has clinical consequences in human twins.

Mayer-Rokitansky-Küster-Hauser syndrome

A phenotypically similar syndrome to Freemartinism in humans, is Mayer-Rokitansky-Küster-Hauser (MRKH) syndrome. This congenital disorder in females is characterized by embryonic underdevelopment of the Müllerian duct, resulting in aplasia of the uterus and

upper part of the vagina. It is also referred to as Müllerian aplasia or Müllerian agenesis. The syndrome occurs in 1:5000 women.[22] Occasional familial cases of MRKH syndrome have been reported, but most cases are isolated.[23]

Diagnosis is often made in puberty during gynaecological examination for primary amenorrhoea with normal female development and normal secondary sex characteristics. Karyotype is XX and endocrine analysis shows normal female results. Ultrasonography can be used to assess the absence of a uterus. Magnetic resonance imaging (MRI) will show rudimentary Müllerian structures in 90% of patients.[24] During diagnostic work-up, evaluation of other congenital malformations is important because around 50% of the women with MRKH have urinary tract malformations or skeletal deformities as well. Management requires psychosocial counselling, vaginal elongation—1st choice—or surgical creation of a neovagina can be considered.

Freemartin-effect in humans?

The etiology of MRKH is still largely unknown.[25] A potential role for AMH has been implicated—considering its important function in Müllerian duct development—but without much empirical supporting evidence, although the hypothesis of AMH involvement seems supported by experiments in mice. 'Overexpression' of AMH results in a phenotype resembling MRKH syndrome.[26,27]

In this thesis, I explore the hypothesis that Freemartinism in humans causes the MRKH syndrome. We sought to explain whether uterine development is inhibited by exposure to AMH during gestation, for which a male co-twin is the identified source.

The critical time window of uterine development in which the Müllerian duct is sensitive to AMH, starts in the 6th week of embryonic development. See Figure 3. So hypothetically, twin-to-twin transfusion of AMH from a male to a female co-twin could occur in early pregnancy, even before ultrasound has confirmed a (twin) pregnancy. Vanishing twin syndrome describes a spontaneous reduction of one fetus of a twin pregnancy. This occurs in 18–36% of twin gestations[28-30] and can occur in the first trimester without any presenting symptoms such as vaginal bleeding.[31] Consequently, in the presence of a male vanishing twin, AMH exchange could hypothetically occur without ever identifying a twin pregnancy.

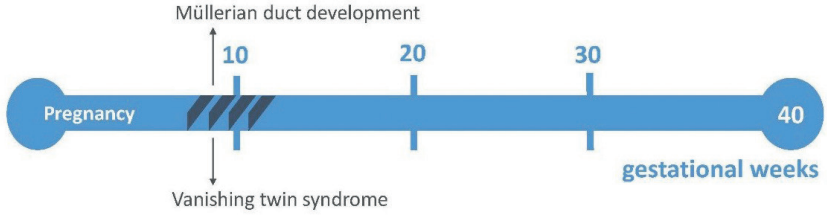

Figure 3. Overlapping time windows for uterine development and occurrence of vanishing twin syndrome in pregnancy

AIM AND OUTLINE OF THE THESIS

The primary goal of this thesis is to study whether Freemartinism exists in humans and whether the Freemartin-effect is present with the MRKH syndrome.

The first part of this thesis, reports on the presence of twin chimerism in humans. Firstly, we investigate an unusual type of twinning which results in chimerism: monochorionic dizygotic twins. In a systematic review on cases of monochorionic dizygotic twins, we study conception, obstetric data, clinical presentation, and chimerism. This is described in **chapter 2**.

In **chapter 3**, we aim to investigate if having a male co-twin leads to an increased prevalence of male microchimeric cells. By studying male microchimerism in females of opposite-sex and same-sex twin pairs and their relatives (their non-twin sisters and mothers), we can investigate the effects of having a male co-twin, the prevalence of microchimerism in twins and their non-twin sisters and generation differences. Also we study if the presence of older brothers and sons could explain microchimerism and if there is evidence for familial resemblance in the presence of microchimeric cells.

In the second part of this thesis, I evaluate MRKH syndrome as a possible consequence of intrauterine AMH transfer between opposite-sex twins. We hypothesize that a male (possibly vanished) co-twin was present during early embryological development in MRKH, resulting in the exchange of AMH via placental vascular connections—similar as in the Freemartins. We investigate the presence of male microchimerism as a result of cell trafficking from male twin to female twin in women with MRKH syndrome, and compare these results with control women. This is described in **chapter 4**.

In **chapter 5**, we investigate whether exposure to testes-secreted hormones could have been of influence in MRKH. Therefore, we study two biomarkers for prenatal androgen exposure: the anogenital distance and the ratio between the 2nd and 4th digit. These anthropometric biomarkers are measured in women with MRKH syndrome, and compared to other patient groups.

In the third part of this thesis we focus on therapeutic options for the infertility associated with the MRKH syndrome: absolute uterine factor infertility. **Chapter 6** reports on the gestational surrogacy program in the VU Medical Center over a 10-year period, including pregnancy outcomes and maternal complications.

In **chapter 7** we describe the results of the feasibility study for performing uterus transplantations in our hospital. This procedure involves surgical removal of a uterus from a brain-dead donor or a live donor (for example a family member or friend) followed by transplantation. In the Netherlands, no uterus transplantations have been carried out yet. In this study, we investigate the possibility to perform this procedure in our hospital by searching for ethical, medical and financial support for this new technique. Additionally,

we study the patient support by performing a questionnaire study in women with the MRKH syndrome.

Finally, **chapter 8** summarizes the research described in this thesis, discusses the findings, and gives recommendations for further research.

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UNUSUAL TWINNING RESULTING IN CHIMERISM: A SYSTEMATIC REVIEW ON MONOCHORIONIC DIZYGOTIC TWINS

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ABSTRACT

Traditionally, it is understood that dizygotic (DZ) twins always have a dichorionic placenta. However, with 8% blood chimerism in DZ twins, placental sharing is probably more common than previously has been recognized. In this article, we will review all available cases of monochorionic dizygotic (MCDZ) twins. A total of 31 twins have been described in literature. A monochorionic diamniotic placenta is reported in all cases. Assisted reproductive technology is responsible for the origin of the pregnancy in 82.1% of the cases. In 15.4% of the sex-discordant twins, a genital anomaly was reported in one of the twins. Chimerism is demonstrable in 90.3% of the twins, leading to various diagnostic difficulties. As this review shows that most MCDZ twins are discovered by accident, it can be argued that it is far more common than has been assumed until now. However, the prevalence is still unclear. Awareness of MCDZ twinning is important, with subsequently correct medical strategies. Similarly, the resulting (blood) chimerism is essential to consider in diagnostic procedures, pre- and postnatally. More research on the effect of placental transfusion between sex-discordant twins is required.

INTRODUCTION

Traditionally, it is understood that monochorionic twins are monozygotic (MZ), and dizygotic (DZ) twins always have a dichorionic placenta. In recent years, researchers have become increasingly interested in atypical ways of twinning.[1] In 2000, the first well-documented case of monochorionic DZ (MCDZ) twins was reported.[2] Subsequently, several studies have reported this unusual way of twinning, and an association between assisted reproductive technology (ART) and MCDZ twinning has been suggested.[3]

Well-known consequences of a monochorionic pregnancy include the higher obstetric risks, such as twin-to-twin transfusion syndrome (TTTS), intrauterine growth restriction, congenital malformations, and intrauterine fetal death. But this monochorionicity also means that vascular anastomoses, present in nearly all monochorionic pregnancies,[4] connect the two different fetal circulations. In MZ twins, the admixture of two blood cell lines with the same DNA remains unrecognized. But in DZ twins this results in intrauterine cell trafficking between the two different zygotes, resulting in blood chimerism. Blood chimerism means that in one organism two blood cell lines exist, from two genetically different zygotes.

The first report of human blood chimerism dates back to 1953.[5] Even then, this was already a well-known phenomenon in cattle. The intrauterine blood exchange between male and female calves is recognized as the origin of the freemartin. This infertile female calf is lacking the Müllerian duct derivatives.[6] Vigier et al.[7] stated that anti-Müllerian hormone (AMH) originating from the male co-twin arrives via vascular placental connections in the female calf-twin and is responsible for the freemartinism.

In humans, however, little attention has been devoted to clinical consequences of twin chimerism. With 8% blood chimerism in DZ twins,[8] this 'unusual' way of twinning is probably more common than previously has been recognized. Therefore, it seems important to assess clinical consequences, such as possible freemartinism in humans. By reviewing the available cases of MCDZ twins in literature, our aim is to create awareness of this relatively unknown twinning event. In addition, we attempt to formulate recommendations for clinicians dealing with MCDZ twin pregnancies and chimerism.

METHODS

Search strategy

This systematic review was performed in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines.[9] The literature search was conducted (by H.P. and J.K.) using PubMed, Embase.com and ISI/Web of Science. These

databases were searched from inception to 29 April 2016, with use of the following terms (including synonyms and closely related words): 'DZ twins and monochorionic', 'twins and chimerism' and 'freemartinism'. The full search strategies for all databases can be found in the supplementary material.

Study selection

We considered all case reports and case series of MCDZ twins. We selected only papers in which a specific description of the placenta was available. Eligibility assessment of the retrieved articles was performed by one author (H.P.).

Data collection and analysis

From all included case reports, the following data were extracted: baseline data, method of conception, pregnancy data, birth outcomes, testing for chimerism, and physical examination of the twins. Relative frequencies were calculated and expressed in percentages.

RESULTS

Through the literature search we identified 1,877 records. After removal of the duplicates, 1,219 records were screened based on title and, when unclear, on abstract. Figure S1 shows the flow diagram of the study selection following the PRISMA guidelines (see the supplementary material).

We included 31 unique cases of MCDZ twins, reported in 27 articles. The main characteristics of the cases are reported in Table 1. In all of the 31 cases, a monochorionic diamniotic placenta was documented by ultrasound during pregnancy or by macroscopic evaluation after birth. In 71% of cases, this was also confirmed by histological examination of the placenta.

Conception

In 28 cases, the method of conception was documented. These data are presented in Table 2. ART was responsible for the origin of the MCDZ twin pregnancy in 82.1% of the cases. More than half of the pregnancies were conceived after in vitro fertilisation/intracytoplasmic sperm injection (IVF/ICSI) treatment. Three cases report assisted hatching during ICSI procedure. In 17.9% (five cases) the MCDZ twins arose from natural conception. Three of the naturally conceived MCDZ twins were sex-discordant and two pairs were male twins; the dizygosity in these male twins was in one case discovered after invasive prenatal testing and in the other case the parents arranged zygosity testing because of significant discordant features in the twins at the age of 14 months.[10]

Table 1. Case reports of monochorionic dizygotic twin pregnancies

	Year	Maternal characteristics	Conception	Obstetric information	Delivery (GA in weeks)	Chimerism	Phenotype	Follow-up
Vietor[2]	2000	29 yr G1P0	-	-	34	Blood(+) Skin(-)	M / V normal	7 months
Souter[11]	2003	48 yr	IVF (donor oocytes)	Spontaneous reduction triplet	37+0	Blood(+) Skin(-)	M / V normal	5 months
Johannsen[12]	2003	-	IUI (donor sperm)	-	-	Blood(+) Skin(-) Ovaries(-)	M / V normal	15 months
Quintero[13]	2003	28 yr G1P0	-	TTTS, laser	Miscarriage 2nd trimester	-	M / V normal	-
Williams[14]	2004	38 yr G3P1	ICSI (AH+)	Pre-eclampsia	CS 28+0	Blood(+) Skin(-) Ovaries(-)	V: clitoromegaly M: normal	7 weeks
Ginsberg[15]	2005	35 yr	clomid	-	Immature - 22	Amniotic fluid(+)	M / V normal	Both twins expired
Yoon[16]	2005	34 yr G1P0	ICSI	-	-	-	M: 47XXY + BWS M: normal	-
Miura & N.[3] -Nishio	2005	-	IVF	-	-	Blood(+) Skin(-)	M / V normal	-
-Tsuruta		-	IUI	-	-	-	M / V normal	-
-Yamaguchi		-	ICSI	Spont reduction triplet, TTTS	-	Blood(+) Skin(-)	M / V normal	-
-Niikawa		-	IVF	-	-	Blood(+)	M / V normal	-
-Niikawa		-	IVF	-	-	Blood(+)	M / V normal	-
Aoki[17]	2006	27 yr G1P0	clomid	-	CS 34+0	Blood(+)	M / M normal	3 months
Shalev[18]	2006	39 yr G4P2	natural	-	Termination 2nd trimester	Blood(+)	M: normal M: Down syndrome	-
Walker[19]	2007	30 yr G2P1	IVF	Hypertension, IUGR	CS 36	Blood(+)	M / M: normal	15 months
Ekelund[20]	2008	38 yr	ICSI (AH+)	TTTS	CS 32	Blood(+) Buccal(-)	M / V normal	6 months

continuation table 1.

	Year	Maternal characteristics	Conception	Obstetric information	Delivery (GA in weeks)	Chimerism	Phenotype	Follow-up
Hackmon[21]	2009	33 yr G3P2	natural	-	SVD 37	Blood(+) Buccal(-)	M / V normal	18 months
Shaikh[22]	2009	-	IVF	-	CS 34+1	Blood(+) Buccal(-)	M / V normal	-
Bogdanova[23]	2010	32 yr	IUI	Premature contractions	CS 32	Blood(+) Skin(-)	M: normal V: absence of uterus + Down syndr	2 years
Assaf[24]	2010	28 yr G1P0	ICSI	AFT: M: 46XY, TTTS, laser	CS 37+4	Blood(+) Buccal(-)	M / V normal	28 months[25]
Hawcutt[26]	2011	-	IVF	PPROM 24+2,	CS 25+1	Blood(+) Buccal(-)	M / V normal	10 months
Loriaux[27]	2011	30 yr	-	TTTS, PPROM, IUFD boy	29+5	Blood(+) Skin(-)	M (IUFD) / V normal	3 years
Umstad[10]	2012	36 yr	natural	TTTS stage 1, no intervention	CS 36	Blood(+) Buccal(-)	M / M normal	14 months
Choi[28]	2013	31 yr G3P0	ICSI (AH+)	IUFD girl AD 33+5	CS	Blood(+) Skin(-)	M: testicular hypoplasia V (IUFD): normal	39 months
Kanda[29]	2013	28 yr G1P0	natural	Discordant growth	SVD 36+1	Blood(+) Skin(-)	M / V normal	1 year
Smeets[30]	2013	37 yr G4P1	IUI	-	SVD 37	Blood(+) Buccal(-)	M / V normal	3 weeks
H. Lee[31]	2014	34 yr G1P0	IVF	-	SVD 38+0	Blood(+) Skin(-)	M / V normal	-
Fumoto[32]	2014	32 yr	ICSI	TTTS	CS 35	Blood(+) Buccal, m(+)v(-)	M / V normal	11 months
O. Lee[33]	2014	-	IVF	-	-	Blood(+) Buccal(-)	M / V normal	23 months
Rodriguez-B[34]	2015	21 yr G4P2	natural	-	CS 39	Blood(+) Skin, m(+)v (-)	M: hypospadias, aortic stenosis V: normal	3 months
Le Bras[35]	2016	32 yr G2P0	clomid	PPROM, umbilical cord prolapse	CS 36+1	Blood(+) Buccal(-)	M / V normal	5 months

AFT = amniotic fluid test, AH = assisted hatching, CVS = chorionic villus sampling, CS = caesarean section, ICSI = intracytoplasmic sperm injection, IUFD = intrauterine fetal death, IUI = intrauterine insemination, IVF = in vitro fertilisation, PPROM = premature prelabour rupture of membranes, PUBS = percutaneous umbilical cord blood sampling, SVD = spontaneous vaginal delivery, TTTS = twin-to-twin-transfusion syndrome.

Table 2. Conception of MCDZ twins

Type of conception		
OI	10.7%	ART total 82.1%
IUI	14.3%	
IVF	32.1%	
ICSI	25%	
Natural	17.9%	
Total	*100%*	

ART = assisted reproductive technology, OI = ovulation induction, IUI = intrauterine insemination, IVF = in vitro fertilization, ICSI = intracytoplasmic sperm injection.

Obstetric data

In all cases, an ultrasound in early pregnancy reported absence of the lambda sign, confirming a monochorionic diamniotic placenta. However, when sex-discordance was noted on a following ultrasound, 7.4% of these pregnancies were nonetheless assumed to be dichorionic. In 16.1% of pregnancies, invasive prenatal testing was conducted (see Table 3). In two cases, the reason for invasive prenatal testing was the discrepancy between a monochorionic placenta and unexpected sex-discordance on ultrasound. TTTS was reported in 16.1% of the pregnancies, in two cases followed by laser therapy. Pregnancy loss under 24 weeks occurred in 6.5% of cases. Most (83.9%) of the reported MCDZ twin pregnancies led to a live birth of twins.

Table 3. Prenatal testing in MCDZ twin pregnancies

	AFT	**CVS**	**Cordocentesis**	**Conclusion after PND**
Johannsen[12]	GA 18: XX/XY 'mosaicism'	-	-	XX cell line judged as maternal cells. Monozygotic males were suspected.
Ginsberg[15]	GA 20: 1. 46XY 2. 46XX	GA 11: 2x similar result, 46XY	-	AFT performed after conflicting result of CVS and ultrasound (discordant sex).
Shalev[18]	GA 18: 1. 46XY 2. 47XY+21	-	GA 20: 1. 35%46XY/ 65%47XY+21 2. 20%46XY/ 80%47XY+21	Parents decided termination of the pregnancy.
Ekelund[20]	GA 25: 1. 46XX 2. 46XY	-	-	Pregnancy was assumed to be dichorionic.

AFT = Amniotic fluid testing, CVS = chorionic villus sampling, GA = gestational age in weeks, PND = prenatal diagnosis

Clinical presentation

In 71% of the presented cases, two healthy twins were born without any described abnormalities. In 83.9% of the reported DZ twins a sex-discordance was noted. In 15.4% of the sex-discordant twins, a genital anomaly was reported in one of the twins (see Table 4), including one girl with aplasia of the Müllerian derivatives —possibly resembling the freemartinism in animals.[23]

Chimerism

In 90.3% of all cases, chimerism was demonstrable. Cytogenetic analysis of peripheral blood lymphocytes was performed by standardized G-banding technique and/or using FISH analysis. In four cases, chimerism was already revealed prenatally, but not always recognized. In 28 cases, the chimerism was discovered postnatally by cytogenetic analysis. In 32.1% of these cases, the chimerism was discovered by coincidence, and in 67.9%, further investigations were initiated because of a sex-discordance and a monochorionic placenta.

In most cases, confined blood chimerism was reported: only present in hematopoietic tissue. The degree of blood chimerism altered from 1 to 99%. In all cases in which the chimerism was tested after the postnatal period (3 months-3 years), the chimerism was still detectable. In 21 cases, non-hematopoietic tissue was also tested for chimerism. Two cases reported tissue chimerism besides blood chimerism in one of the twins, namely buccal and skin chimerism.

Johannsen et al.[12] report a case wherein non-recognized chimerism led to a gonadectomy in a healthy girl. A male karyotype in this phenotypical girl was interpreted as 46,XY gonadal dysgenesis. However, after removal, the ovaries were completely normal, with a normal female karyotype. The diagnosis was changed to blood chimerism.

Table 4. Genital anomalies in sex-discordant MCDZ twins

·	Ambiguous external genitalia female [14]
·	Absence of uterus/fallopian tubes [23]
·	Testicular hypoplasia [28]
·	Glanular hypospadias [34]

DISCUSSION

Main findings

In this review, we present 31 cases of MCDZ twins, of which most were conceived after ART. Especially double embryo transfer in IVF/ICSI treatment seems to be a risk factor. Based on

this review, it appears that there is a similar obstetric risk as for monozygotic monochorionic pregnancies; therefore it is important to identify the MCDZ twin pregnancies. In 15.4% of the sex-discordant twins, a genital anomaly was reported in one of the twins. Confined blood chimerism was reported in most cases and was persistent into young childhood. The finding of chimerism can cause diagnostic difficulties, pre- and postnatally.

Strengths and limitations

A potential limitation with case reports is that this method can involve publication bias. In particular, a publication bias regarding ART pregnancies and clinical consequences of chimerism must be considered. Nevertheless, due to the rarity, it was inevitable that case reports would be reviewed. This systematic review gives the first complete inventory of reported cases of MCDZ twins and shows what already is known about this unusual way of twinning. With 31 existing cases in literature since 2000, it is certain that MCDZ twinning is a topic that deserves attention. The strength of this review is the description of different outcomes of MCDZ twin pregnancies, with or without clinical consequences. This is important in medical decision making and counseling for the parents. Intrauterine cell trafficking between DZ twins and the long-term effects of chimerism are subjects that need further exploration. Follow-up studies of MCDZ twins would be of value.

Interpretation

Conception

A monochorionic diamniotic twin pregnancy suggests formation of the placenta between the 4th and 8th day after conception. Schiewe et al.[36] have described the occurrence of MCDZ twin formation in vitro: "Two prematurely hatching day-5 blastocysts had merged together. These blastocysts had completely fused to form a single blastocoel cavity with two distinct inner cell masses" (pp. 418-419). This event supports the hypothesis posed by Souter et al.,[11] that trophoblasts from two embryos fuse before implantation, producing MCDZ twins. In 1970, the first case of chorionic fusion in a human placenta was reported, resulting in a partly monochorionic placentation in heterosexual twins. In one half of the placenta a ridge with "two layers of chorionic tissue between two layers of amnion" was found (like in dichorionic placentas), while the other half of the placenta was considered to be monochorionic after histologic examination.[37] Furthermore, when taking into account sporadic reports of fused dichorionic placentas (also resulting in chimerism), there may exist a sliding scale based on the time of fusion.[38,39]

In the literature, other explanations have been discussed. It could be the presence of binovular follicles, in which two oocytes are present within a single zona pellucida. It is credible that this leads to close contact between embryos, whereby monochorionic

placentation forms.[17] The most complex hypothesis contains an oocyte and second polar body surrounded by one zona pellucida, which is penetrated by more than one sperm.[40]

While the precise explanation of MCDZ twin formation is uncertain, ART as a risk factor has been proposed before.[3] The various hypotheses also support the idea that with ART, the chance of MCDZ twins is increased. The chance of cell fusion seems smaller in a natural pregnancy, in which two blastocysts would not be likely to co-exist extremely close to each other. Possibly, assisted hatching could increase the chance of cell fusion. Moreover, binovular follicles appear more often in FSH-stimulated cycles.[41] These observations together support the idea that ART increases the risk of MCDZ twin formation.

Chimerism

The confined blood chimerism present in the majority of the MCDZ twins is a direct result of intrauterine blood sharing via the placenta. However, in two cases, tissue chimerism was also present. This is more difficult to interpret. Fumoto et al.[32] discusses two different explanations: one consists of "ectopic differentiation of chimeric hematopoietic stem cells" (p. e1099). Another possibility is that the chimera was generated at an early stage of embryogenesis, with double inner cell masses in one fused blastocyst (as described before) where "cells derived from one fetus could have migrated to the ectoderm of the other fetus" (p. e1100). It is possible this could result in chimerism in any organ, with still unknown consequences.

This review shows that the finding of chimerism is often unexpected and can cause diagnostic difficulties. Moreover, it can result in uncertainty for the caretaker and of course the parents, resulting in more diagnostic testing and confusion about the sex differentiation of the fetus/child. Therefore, it is important to consider the possibility of chimerism pre- and postnatally. Chorionic villus sampling and cordocentesis are prenatal diagnostic tests that reflect only the DNA in hematopoietic tissue. This means that blood chimerism can be the cause of a false test result, or the reason that a result is difficult to interpret. In amniotic fluid testing mostly fibroblasts will be analyzed, which makes it the most reliable test for chromosomal analysis in twins - without detecting blood chimerism.

Also postnatally, it is important to consider chimerism, illustrated by the case of Johannsen et al.[12] Diagnosing blood chimerism involves at least comparing the karyotype in blood with other tissues (e.g., through buccal smear or skin biopsy). Furthermore, this review demonstrates that chimerism is still detectable in young childhood. This could point towards a permanent state. Several reports of coincidentally discovered chimerism in adult twins support this, with detectable chimerism until the age of 70.[42,43,44,45]

Chimerism (in specific feto-maternal microchimerism) is also a known subject in immunology, as it has been proposed that microchimerism may play a role in the etiopathogenesis of some autoimmune diseases.[46,47,48] A monochorionic placenta in DZ

twins is, however, not the only possible cause for twin chimerism. Vascular anastomoses in a dichorionic placenta can also result in chimerism.[49] Even in singletons there are reports of (micro)chimerism, with a possible vanishing twin as the source for chimerism.[50] Consequently, the 8% prevalence of chimerism in DZ twins (according to van Dijk et al.[8]) is probably only partially caused by monochorionic pregnancies. A challenging task for further research is to identify the different causes and long-term consequences of twin chimerism.

Clinical consequences

Perinatal mortality and morbidity is considerably higher in monochorionic twin pregnancies than in dichorionic twin pregnancies.[51] The higher obstetric risk seems similar for MCDZ twin pregnancies. This is illustrated by the prevalence of TTTS: 16.1% in MCDZ twin pregnancies versus 10-20% in 'normal' monochorionic pregnancies.[52] Also, the risk of pregnancy loss under 24 weeks (6.5%) is comparable with the reported risk in monochorionic pregnancies of 7.8%.[53] However, a substantial part of the reported MCDZ twin pregnancies was incorrectly assumed to be dichorionic. It is important to identify MCDZ twins timely and correctly to take appropriate measures during pregnancy.

The prevalence of genital anomalies (15.4%) in sex-discordant twins can suggest an association with intrauterine cell trafficking. This would be comparable with the freemartin phenomenon in cattle.[6,7] The case described by Bogdanova et al.[23] could be the first published case of possible freemartinism in human. Also the case of testicular hypoplasia[28] is comparable with reports of testicular hypoplasia in bulls with confined blood chimerism.[54] This means that close observation of genital anomalies is recommended in chimeric twins. During pregnancy, counseling should be provided to parents expecting MCDZ twins with discordant sex. It is important to discuss the risk of a genital anomaly, but also to emphasize the possibility of healthy twins with normal development.

CONCLUSION

Awareness of MCDZ twinning is important, with subsequently correct medical strategy in prenatal testing, pregnancy measures, and parental counseling. Similarly, the resulting (blood) chimerism is essential to consider in pre- and postnatal testing.

Most MCDZ twins are discovered by accident, and it can be argued that it is far more common than has been assumed until now. However, the prevalence is still unclear. Various hypotheses are posed for the origin of MCDZ twins; most point in the direction of fusion of two trophoblasts. An association of MCDZ twinning and ART is confirmed in this review; whether there is a causal effect remains uncertain. Additionally, it is possible that placental transfusion between sex-discordant twins can result in genital anomalies. More research is required for the effect of placental transfusion between DZ twins.

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SUPPLEMENTARY FILES

Supplementary file 1
Search strategy

PubMed (887 articles):
("Twins"[Mesh] OR twin[tiab] OR twins[tiab]) AND ("Chimerism"[Mesh] OR "Chimera"[Mesh] OR chimer[tiab] OR chimaer*[tiab] OR microchimer*[tiab] OR microchimaer*[tiab]) OR ("Freemartinism"[Mesh] OR free martin*[tiab] OR freemartin*[tiab]) OR (("Twins, Dizygotic"[Mesh] OR dizygo*[tiab]) AND monochor*[tiab])*

Embase (539 articles):
('twins'/exp OR twin:ab,ti OR twins:ab,ti) AND ('chimera'/exp OR chimer:ab,ti OR chimaer*:ab,ti) OR ('freemartinism'/exp OR (free NEXT/1 martin*):ab,ti OR freemartin*:ab,ti) OR (('dizygotic twins'/exp OR dizygo*:ab,ti) AND monochor*:ab,ti) AND [embase]/lim*

Web of science (451 articles):
((twin OR twins) AND (chimer OR chimaer*)) OR "free martinism" OR freemartin* OR (dizygo* AND monochor*)*

Supplementary file 2

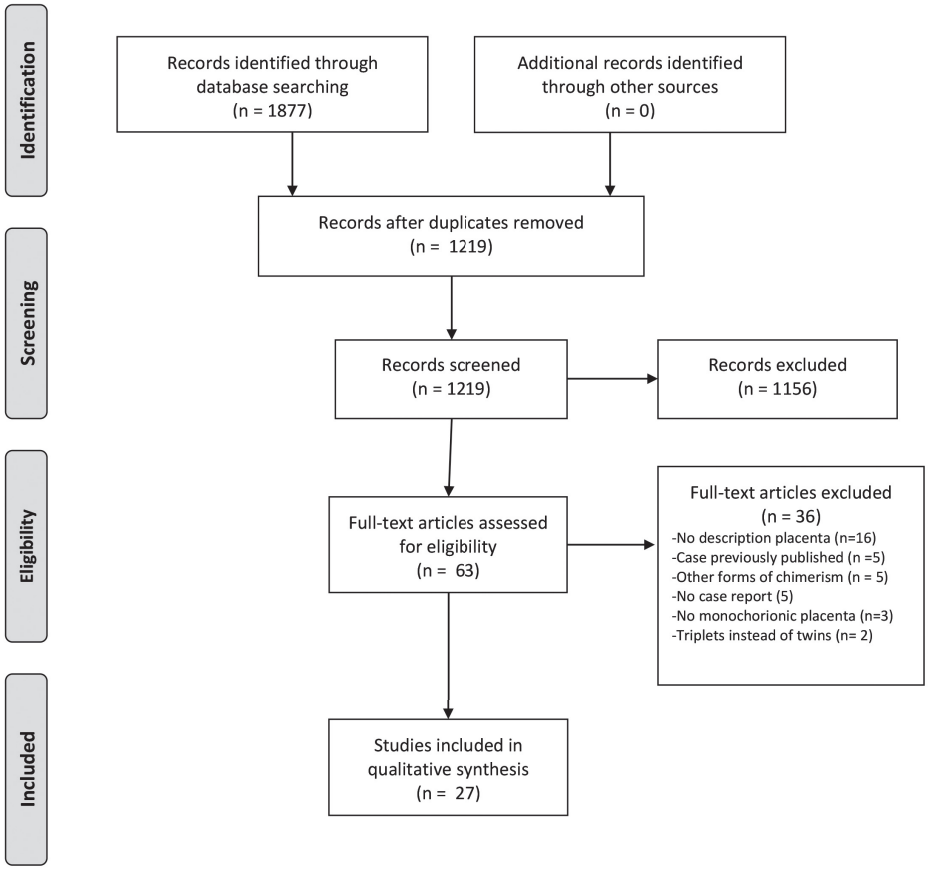

Figure – Flow diagram of inclusion of articles

MALE MICROCHIMERISM IN WOMEN: A QUANTITATIVE STUDY IN FEMALE TWINS, THEIR SISTERS AND MOTHERS

B.N. Johnson*
H.E. Peters*
C.B. Lambalk
C.V. Dolan
G. Willemsen
L. Lighthart
V. Mijatovic
J.J. Hottenga
G.E. Davies
E.A. Ehli^
D.I. Boomsma^

** The authors consider that the first two authors should be regarded as joint first authors*

^ The authors consider that the last two authors should be regarded as joint last authors

Submitted

ABSTRACT

The occurrence of microchimerism has been described as a consequence of pregnancy in placental mammals, including humans. There is substantial interest in the role of microchimerism in disease pathology, most notably those diseases with a female predilection, e.g. autoimmune disorders. Here we present a novel study investigating male microchimerism among female members from twin pedigrees. We set out to test if a male co-twin is a source for male microchimeric cells in women, if the presence of older brothers and sons is associated with microchimerism and if the data from mono- and dizygotic twins, their mothers and sisters show evidence for familial resemblance in microchimerism. DNA samples from 446 adult female participants of the Netherlands Twin Register were tested for the presence of male microchimerism via sensitive quantitative real-time PCR of Y chromosome gene DYS14. We observed a high prevalence of microchimerism with 26.9% of participants having detectable male microchimerism in their peripheral blood samples. Age had a positive relationship with the presence of male microchimerism. The presence of a male co-twin did not increase risk of male microchimerism as female twins with and without a male co-twin did not differ in presence of microchimerism. The prevalence of male microchimerism was not explained by having male offspring or by having an older brother. The twin correlation for the presence of microchimerism was 0.27 (SE 0.37) in monozygotic pairs and not significantly different from zero in either monozygotic twins or first-degree relatives. These findings show a high prevalence of male microchimerism in adult women, which is more prominent with increasing age, but which is not explained by the presence of brothers or sons, highlighting the need for further study of the origins of microchimerism.

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 feto-maternal exchange of blood cells during pregnancy. 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.[1,2] These scenarios have exacerbated complications for the traditional self versus non-self criterion of immunology.[3] Exposure to foreign cells could contribute to human health and disease (for a review see Johnson et al., 2020[4]), as a contributing factor in the pathogenesis of autoimmune diseases.[5] Interestingly, chimerism has also been associated with paternal care in marmosets, suggesting a role in behavior.[6]

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.[7-9] The method of delivery, presence of placental complications and hypertensive disorders all can influence the amount of cell trafficking.[10,11] Fetal loss in early pregnancy may also result in microchimerism.[12] However, several studies have reported male microchimerism in women without any history of male pregnancy[13-15], leading to a search for other sources which may result in male microchimerism, such as having older brothers, having a male co-twin, sexual intercourse or unrecognized pregnancies.[16] It has even been proposed that every human is born as a microchimera, with a yet undefined source of donor cells.[17]

Intrauterine exchange of cells occurs between twins through feto-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 in literature found by coincidence.[18] In 1996, a systematic search by Van Dijk et al. showed an 8% prevalence of blood group chimerism in dizygotic twins.[19] This finding placed the concept of twin chimerism in humans in a new light, although a recent study of dizygotic twins using blood typing and short-tandem repeat assays did not reveal evidence for chimerism.[20] Improvements in detecting microchimerism by advanced molecular techniques now allows quantitative real-time polymerase chain reaction (qPCR) to quantify chimerism prevalence in DNA samples.[15]

The presence of non-inherited maternal antigens via maternal microchimerism in the offspring may be involved in the development of immune tolerance to maternal antigens and future overlapping fetal antigen.[21] With multiple associations between microchimerism and risk for diseases[4], 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 aim to 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 microchimerism in twins and their non-twin sisters, generation differences, and the degree of shared microchimerism among family members.

MATERIAL AND METHODS

Participants

The participants in this study are enrolled in the Netherlands Twin Register (NTR) Biobank.[22,23] 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 (Figure 1). 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 with a twin brother, 80 females from monozygotic (MZ) twin pairs, 68 females from dizygotic (DZ) same-sex (ss) 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.[15] In brief, each sample was tested in parallel for *DYS14* in 12 replicates as a measure of male genome mass and for *b-globin* in duplicate to obtain a measure of total genome mass. The PCR cycling and fluorescent measurement was completed on a Quantstudioä 7 Flex instrument and analyzed by Quantstudioä Real-Time PCR software (v1.1) (Applied Biosystems, Waltham, MA, USA). Standards were produced using known male and female extracted DNA samples. The *b-globin* standard included ten-fold dilutions of extracted DNA to produce standards from

500ng-0.5ng. 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 66ng/mL with ten-fold dilutions of male genome from 66ng/mL-0.0066ng/mL. 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.[15] 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.[24] Each reaction plate contained various quality control measures. Six no template control (NTC) wells were included as a true negative for the *DYS14* and *b-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 *b-globin* while the NTC tested negative for both assays consistently. The positive control demonstrated consistent detection of both *b-globin* and *DYS14* across reaction plates.

Statistical analysis

Categorical data were compared by chi-squared (c^2) tests. Associations of male microchimerism status with age and the presence of sons or older brothers were assessed by using generalized estimating equations (GEE) logistic regression. Concordance for male microchimerism between pairs of relatives was summarized in 2x2 contingency tables and the associations were evaluated by McNemar's tests. 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 a few 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 presence of male microchimerism we estimated the tetrachoric correlations for 2 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 standard errors (SE) between family members[25] were estimated and tested for significance in OpenMx.[26]

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.[23]

RESULTS

Prevalence

Male microchimerism was detected in 120 of the 446 participating women (26.9%). In the group of DZ female twins with a twin brother 27.4% tested positive for male microchimerism, compared to 23.5% of females with a dizygotic twin sister (P = 0.61). Prevalence was 16.3% in MZ females and did not differ from the prevalence in DZ same-sex twins (P = 0.27). 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 1 summarizes these results.

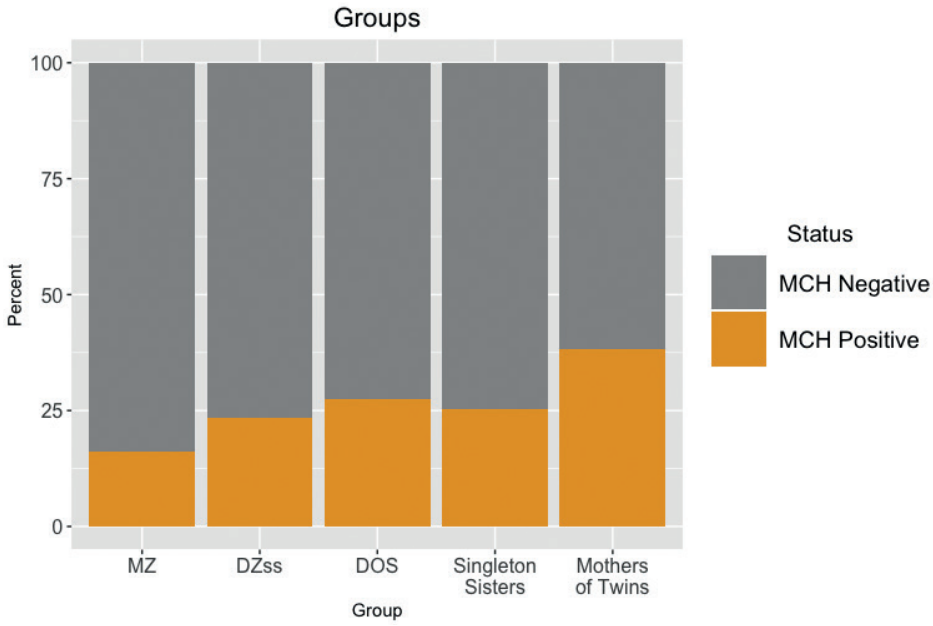

Figure 1. Prevalence of male microchimerism (MCH) in women. MZ; monozygotic twins, DZss; dizygotic same-sex twins, DOS; dizygotic opposite-sex twins.

Associations with microchimerism

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. There was a positive relationship between age and presence of microchimerism (P = 0.02; Nagelkerke R^2 = .017). Figure 2 summarizes concordances for female-female DZ 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% are concordant for microchimerism 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).

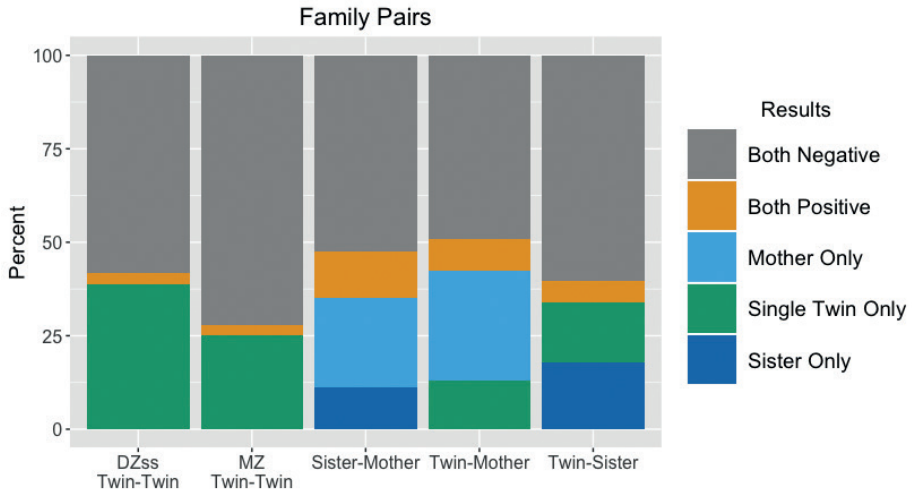

Figure 2. Male microchimerism concordance within families. Data are presented for pairs of relatives including: DZss (dizygotic same-sex) twins, MZ (monozygotic) twins, and mother with singleton sister and twin, and twin-sister.

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).

Concentration microchimerism

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 Figure 3.

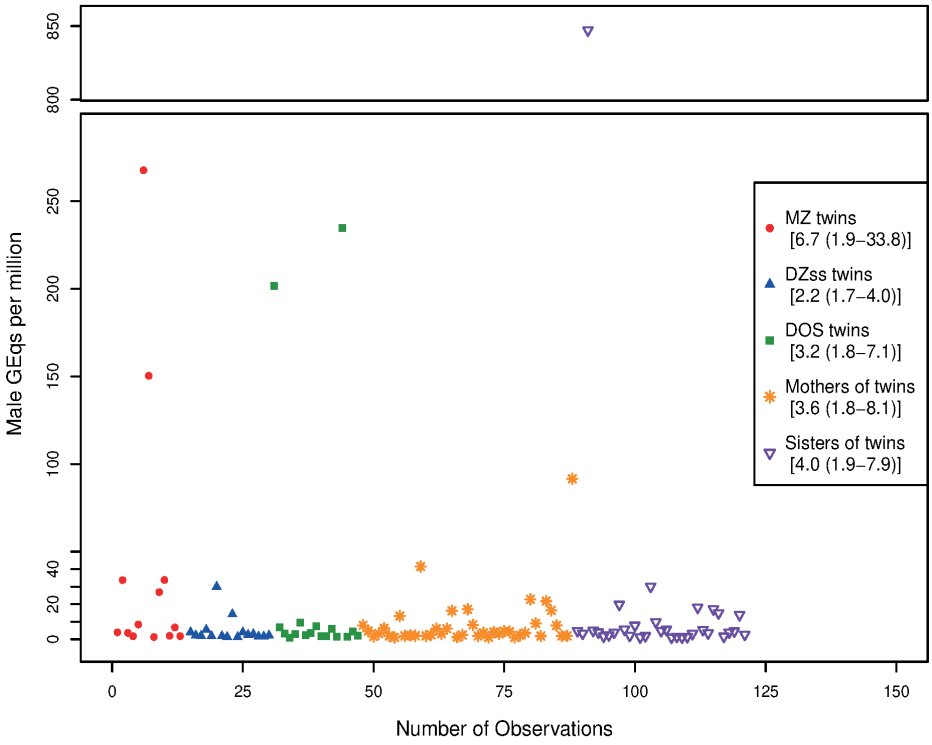

Figure 3. Male genome equivalents (GEq) per one million cells by each participant with detectable male GEq. The data are stratified by participant group. Descriptive statistics presented as Median (IQR). MZ; monozygotic twins, DZss; dizygotic same-sex twins, DOS; dizygotic opposite-sex twins. Male GEqs; male genome equivalents per million.

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.

Previous research in twins looked at the presence of blood chimerism via red blood cell antigens and discovered an 8% chimerism prevalence in twins.[19] 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 with a male co-twin do not present with male microchimerism more frequently than DZ females with a female co-twin, despite the adjacent in utero presence of the male. 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.[13,16] 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. 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.[27] Possible further sources are unreported or unrecognized interrupted pregnancies[12], breastfeeding, placental structure, pregnancy complications including preeclampsia[10,12,28,29] and it has also been suggested that sexual intercourse may play a role.[13,14] 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 but rather only that it is of male origin. In addition, due to the suggested mechanism of chimerism acquisition via blood exchange we exclusively investigated blood microchimerism which may not represent global chimerism. 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'.[30,31]

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.[4] 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 future pregnancies as well as mitigating allogeneic sensitization.[21] 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.[27] Already, outcomes in cord blood transplantation have shown improvements when mismatched HLA alleles between donor and recipient include a non-inherited maternal antigen[32] 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.[33,34] 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.[35] Similarly, maternal microchimerism has been identified to be associated with type 1 diabetes and contribute to islet b cells in the offspring.[36] Such studies 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. 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.

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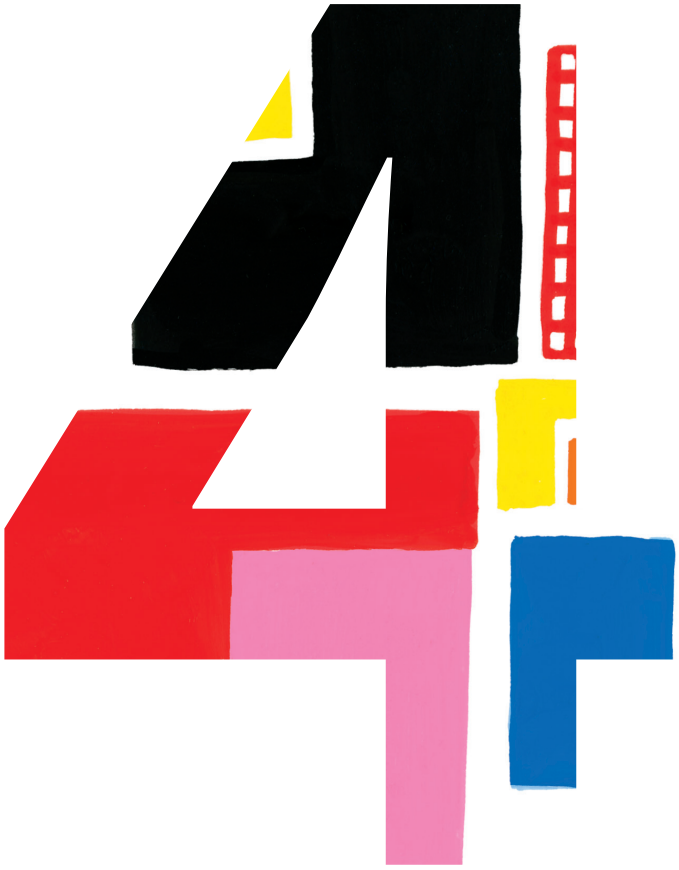

LOW PREVALENCE OF MALE MICROCHIMERISM IN WOMEN WITH MAYER-ROKITANSKY-KÜSTER-HAUSER SYNDROME

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ABSTRACT

Study question

Is there an increased prevalence of male microchimerism in women with Mayer-Rokitansky-Küster-Hauser (MRKH) syndrome, as evidence of foetal exposure to blood and anti-Müllerian hormone (AMH) from a (vanished) male co-twin resulting in regression of the Müllerian duct derivatives?

Summary answer

Predominant absence of male microchimerism in adult women with MRKH syndrome does not support our hypothesis that intrauterine blood exchange with a (vanished) male co-twin is the pathophysiological mechanism.

What is known already

The etiology of MRKH is unclear. Research on the phenotype analogous condition in cattle (freemartinism) has yielded the hypothesis that Müllerian duct development is inhibited by exposure to AMH *in utero*. In cattle, the male co-twin has been identified as the source for AMH, which is transferred via placental blood exchange. In human twins a similar exchange of cellular material has been documented by detection of chimerism, but it is unknown whether this has clinical consequences.

Study design, size, duration

An observational case-control study was performed to compare the presence of male microchimerism in women with MRKH syndrome and control women. Through recruitment via the Dutch patients' association of women with MRKH (comprising 300 members who were informed by email or regular mail), we enrolled 96 patients between January 2017 and July 2017. The control group consisted of 100 women who reported never having been pregnant.

Participants/material, setting, methods

After written informed consent, peripheral blood samples were obtained by venipuncture, and genomic DNA was extracted. Male microchimerism was detected by Y-chromosome- specific real-time quantitative PCR, with use of DYS14 marker. Possible other sources for microchimerism, for example older brothers, were evaluated using questionnaire data.

Main results and the role of chance

The final analysis included 194 women: 95 women with MRKH syndrome with a mean age of 40.9 years and 99 control women with a mean age of 30.2 years. In total, 54 women (56.8%) were identified as having typical MRKH syndrome and 41 women (43.2%) were identified as having atypical MRKH syndrome (when extra-genital malformations were present). The prevalence of male microchimerism was significantly higher in the control group than in the MRKH group (17.2% versus 5.3%, $P = 0.009$). After correcting for age, women in the control group were 5.8 times more likely to have male microchimerism (odds ratio 5.84 (CI 1.59 – 21.47), $P = 0.008$). The mean concentration of male microchimerism in the positive samples was 56.0 male genome equivalent per 1,000,000 cells. The prevalence of male microchimerism was similar in women with typical MRKH syndrome and atypical MRKH syndrome (5.6% versus 4.9%, $P = 0.884$). There were no differences between women with or without microchimerism in occurrence of alternative sources of XY cells, such as older brothers, previous blood transfusion, or history of sexual intercourse.

Limitations, reason for caution

We are not able to draw definitive conclusions regarding the occurrence of AMH exchange during embryologic development in women with MRKH syndrome. Our subject population includes all adult women and therefore is reliant on long-term prevalence of microchimerism. Moreover, we have only tested blood, and, theoretically, the cells may have grafted anywhere in the body during development. It must also be considered that the exchange of AMH may occur without the transfusion of XY cells and therefore cannot be discovered by chimerism detection.

Wider implications of the findings

This is the first study to test the theory that freemartinism causes the MRKH syndrome in humans. The study aimed to test the presence of male microchimerism in women with MRKH syndrome as a reflection of early fetal exposure to blood and AMH from a male (vanished) co-twin. We found that male microchimerism was only present in 5.3% of the women with MRKH syndrome, a significantly lower percentage than in the control group (17.2%). Our results do not provide evidence for an increased male microchimerism in adult women with MRKH as a product of intrauterine blood exchange. However, the significant difference in favor of the control group is of interest to the ongoing discussion on microchimeric cell transfer and the possible sources of XY cells.

INTRODUCTION

Mayer-Rokitansky-Küster-Hauser (MRKH) syndrome is characterized by congenital aplasia of the uterus and the upper part of the vagina. It affects around 1 in 5000 females.[1] The diagnosis is usually made in adolescence after the presentation of primary amenorrhea. Further examination reveals vaginal agenesis with absence of the uterus, normal secondary sex characteristics and normal female 46,XX karyotype.[2] It may also be associated with renal and/or skeletal malformations, classified as atypical MRKH syndrome.[3,4]

The etiology of the MRKH syndrome is unclear.[5] Embryological evidence supports the hypothesis that this syndrome occurs as a result of failure of Müllerian duct development. In the normal male embryo, the Sertoli cells of the testes produce anti-Müllerian hormone (AMH), resulting in regression of the Müllerian duct. This inhibitory action of AMH on the Müllerian duct starts during the fifth week of development, and is progressive during the critical time window of uterine development.[6] Genetic activation of AMH or its receptor has been implicated as a cause of MRKH syndrome but without any supporting results.[7,8]

In cattle, a similar phenotypical syndrome to MRKH exists: a so-called freemartin. In this infertile female calf, the absence of Müllerian structures occurs due to intrauterine AMH exposure, originating from a male co-twin.[9] Vascular connections in the placenta transport AMH from the male to the female calf.[10] As a result of this intrauterine blood exchange via the placenta, the freemartins are *'chimeras'*: in addition to the normal XX cells, they have an extra XY cell line, originating from their co-twin. The term chimerism means that two genetically different cell lines are present in one individual, originating from more than one zygote.

In humans, intrauterine cell trafficking between twins can also result in chimerism.[11,12] It is unknown whether this has clinical consequences. Bogdanova et al. reported a possible case of 'human freemartinism' in a female twin with aplasia of the uterus. In this female, blood exchange via the placenta with her brother had resulted in male chimerism (the presence of XY cells).[13] In addition, a vanishing twin can leave its traces in the form of microchimerism, in which a second cell population is present at variable concentrations in the surviving fetus.[14]

It should also be mentioned that fetal-maternal exchange in a normal pregnancy is a well-known source for chimeric cells.[15] Fetal microchimerism refers to the phenomenon of fetal cells entering the maternal circulation during pregnancy. As the exchange between fetus and mother is bidirectional, the presence of maternal cells in the circulation of their children is called maternal chimerism. Chimerism can also occur following transplantation or blood transfusion.[16]

There have been no studies to date to examine the presence of male chimerism in women with the MRKH syndrome. The main purpose of the research reported here was to study whether the etiology associated with freemartinism could be the cause of the

MRKH syndrome in humans. We hypothesized that a male—possibly vanished—co-twin was present during the early embryological development of women with MRKH syndrome, resulting in exchange of AMH via placental vascular connections. Therefore, we investigated the presence of male microchimerism (as a result of cell trafficking from male twin to female twin) in MRKH patients and compared these results with control women.

MATERIALS AND METHODS

Study subjects

This observational case-control study compared the presence of male microchimerism in women with the MRKH syndrome and control women. Through recruitment via the Dutch patients' association of women with MRKH ('Stichting MRK-vrouwen'), we enrolled 96 women diagnosed with the MRKH syndrome (in total, the 300 members were informed by email or regular mail and the participation rate was 32%). All participants were 18 years or older and provided written informed consent prior to enrollment. Between January 2017 and July 2017, blood samples were obtained by venipuncture, by one researcher (HP). The blood samples were collected in EDTA vacutainer® tubes. All participants completed a questionnaire, comprising questions about demographic information, medical history, MRKH diagnosis, and family history.

The control group comprised women who volunteered to participate in an earlier study at our hospital. This study evaluated reproductive functioning in female childhood cancer survivors (DCOG LATER-VEVO study, NL15106.029.06).[17,18] For this study, women who did not have a history of cancer in the past were included as control subjects. Phenotypic data were collected between 2008 and 2014 by questionnaires, and biological material was collected via blood sampling. Out of the 390 women who provided biological material for the study, we selected 100 women who reported to have never been pregnant for the control group.

MRKH diagnosis

The MRKH diagnosis of the participants was confirmed by contacting the general practitioner or gynecologist, to retrieve the detailed information about the diagnosis based on physical examination, imaging or laparoscopy results. The patients were identified as having typical MRKH syndrome (also referred to as type 1) in case of no known other malformations, and as atypical (type 2) MRKH syndrome when renal and/or skeletal malformations were present (Oppelt, et al., 2006).

Preparation of samples

Genomic DNA was extracted from peripheral venous blood according to standard procedures. The extracted DNA was stored at -80°C until further processing. The DNA concentration ranged from 2.48 to 261 ng/µL (mean 77.47 ng/µL). The A260/A280 ratio ranged from 1.33 to 2.46 (mean 1.85).

Analysis of male microchimerism

Real-time quantitative polymerase chain reaction (qPCR) was performed using a QuantStudio™ 7 Flex system (Applied Biosystems, Waltham, MA, USA). The DNA sequence utilized for detection of male genome targets a Y-chromosome specific region, *DYS14*. Amplification primers and probe sequences for *DYS14* have been described previously.[19] Also, the rationale for using this region as a Y-chromosome target is detailed.[19] As a measure of the total input quantity of genomic mass per reaction, qPCR of the *b-globin* gene was measured in parallel. Primers and probes for *b-globin* are also previously described.[20] All genome equivalent (GEq) conversions utilized a conversion factor of 6.6 pg DNA per cell.[21] Male GEq was reported per 1 million cells and calculated using the following equation:

$$C = \frac{Q_{DYS14}}{6.6pg} \times \frac{6.6pg \times 10^6}{Q_{\beta\text{-globin}}}$$

where C = concentration of male GEq per 1 million cells, Q_{DYS14} = mean mass of *DYS14* (ng) determined by qPCR, and $Q_{\beta\text{-Globin}}$ = mean mass of *β-globin* (ng) determined by qPCR.

Amplification measurement was accomplished using a dual-labeled minor groove binder probe with a 5'-bound fluorescent dye reporter [FAM (6-carboxyfluorescein)] and a 3'-bound non-fluorescent quencher. While both are bound to the probe, the fluorescent signature of the reporter is quenched. Taq DNA polymerase 5'-3' exonuclease activity during the extension phase of PCR causes cleavage of the probe, releasing the FAM reporter.[22,23] Once separated from the quencher, the reporter will emit its fluorescence.[19,23] This fluorescent emission is detected by the Quantstudio™ 7 Flex system and analyzed by Quantstudio™ Real-Time PCR software (v 1.1) (Applied Biosystems, Waltham, MA, USA).

Calibration standards for the *DYS14* assay were produced by diluting known male DNA in female DNA to a total concentration of 66ng/mL, with subsequent 10-fold dilutions ranging from 66ng to 0.0066ng male genome (mean $r^2 = 0.994$, n = 11). Calibration standards for the b-globin assay were produced by 10-fold serial dilution of DNA, ranging from 500ng-0.5ng (mean $r^2 = 0.997$, $n = 11$). All standards were measured in triplicate on each reaction plate simultaneously with subject reactions. Also included on each reaction plate were positive and negative control subjects for male genome diluted in female DNA and a no template control (NTC) for each assay. The negative controls and NTC tested consistently negative during all experiments.

Performance of the assay was measured for reproducibility and linearity by using female samples spiked with male DNA. A series of 10-fold dilutions of male DNA in female DNA demonstrated target specificity of the *DYS14* assay. The measured male GEq of our validation controls demonstrated excellent linear regression with a correlation coefficient of 0.998. Reproducibility was measured using three samples with mean male GEqs per million of 1594.64 ± 137.41 (coefficient of variation (CV) = 8.62), 10.50 ± 2.31 (CV = 22.02), and 6.72 ± 1.82 (CV = 27.14).

The qPCR reactions were set up in a 10uL reaction on 384-well reaction plates. The target genome input of blood-extracted DNA was 66 ng per reaction, thus achieving 10,000 genomes per reaction. Each reaction also included 5.0 mL TaqManâ Fast Advanced Master Mix (Applied Biosystems, Waltham, MA, USA), 0.5 mL 20X custom assay (Applied Biosystems, Waltham, MA, USA) including a dual labeled probe as well as forward and reverse primers. Each sample simultaneously tested 12 aliquots for *DYS14* and in duplicate for *b-globin* on the same reaction plate. The amplification thermal cycling initiated with 50°C for 2 minutes then denaturation at 95°C for 2 minutes then 46 cycles of 95°C for 15 seconds and 56°C for 1 minute.

All qPCR reactions and analyses were performed by a scientist who was blinded to MRKH status. Results were reported as male GEq per 1 million cells, calculated by using the mean measure of *DYS14* and *b-globin* for each subject. Male microchimerism was defined as a quantifiable measure of *DYS14* in any of 12 aliquots tested per sample.

Precautions against contamination

We utilized strict precautions while handling these samples to prevent contamination. Standard precautions for a PCR reaction set up were followed.[24] Separate pre-amplification and post-amplification areas were strictly adhered to. All handling of samples was done inside a class II biosafety cabinet, which pulls contaminated air from the work surface and exterior environment through a HEPA filter to sterilize before returning to the work surface. All pipetting was carried out using aerosol-resistant filtered pipette tips. Each reaction plate included multiple NTC wells for each assay. After each use and preparation of each plate, the biosafety cabinet was thoroughly cleaned using a system of 10% bleach, cleaned with deionised water, followed by DNAZapä (Invitrogen, Waltham, MA, USA) as per manufacturer's directions, two cycles of deionised water cleaning and 1 h of an ultraviolet lamp.

Statistical analysis

For sample size calculation, we assumed 25% microchimerism in the control group [according to results in previous studies[25]]: and we assumed that in at least 50% of the adult women with MRKH syndrome, male microchimerism would be detectable in the blood. With a significance level (alpha) of 5% and a power of 95%, we calculated a sample size of 91 women in both groups. Anticipating a 5% margin of error, in total, 192 women needed to be included.

Not normally distributed variables were compared using the Mann-Whitney U test. For categorical variables, the chi-squared (χ^2) or Fisher's exact test was used as appropriate. We used logistic regression to examine the association between microchimerism and MRKH syndrome by calculating the odds ratio (OR). Age (years) was included as a covariate as it may affect the presence of microchimerism. $P < 0.05$ was considered statistically significant. The statistical analysis was performed using SPSS 22.0 (SPSS, Chicago, IL, USA).

Ethics

The study protocol has been approved by the Institutional Review Board of the VU Medical Center, Amsterdam, the Netherlands, on 5 January 2017 (METC VUmc 2016.374). The trial was registered in the Dutch National Trial Registry (trial registration number NTR5961).

RESULTS

For this study, 96 women were included in the MRKH group, and 100 control women were selected who reported to have never been pregnant. After collection of all available information, we excluded one woman in the patient group because, in retrospect, she did not have the MRKH syndrome. In the control group, one subject was excluded due to the limited amount of extracted DNA available for the microchimerism analysis. The final analysis included 194 women.

Patient characteristics

All women in the patient group reported having been diagnosed with the MRKH syndrome, with a mean time since diagnosis of 25.5 years (range 2-64 years). The mean age at the time of diagnosis was 16.5 years (range 6-26 years). In five women (5.3%), confirmation of the MRKH diagnosis by medical records could not be achieved. In total, 54 women (56.8%) were identified as having typical MRKH syndrome, and 41 women (43.2%) were identified as having atypical MRKH syndrome. For the women with atypical MRKH syndrome, 28 out of 41 (53.7%) had a renal malformation, 18 (24.4%) had a skeletal malformation, and 9 (22.0%) had combined malformations (Table I). Of the participants in the patient group, three (3.2%) reported a positive family history for MRKH syndrome: two sisters were both participants in this study, and one woman reported an affected cousin (daughter of sister of father of participant). Two women with MRKH syndrome were part of dizygotic twin pairs, one with a twin brother and one with a twin sister, the latter twin sister being unaffected for the MRKH syndrome. All pregnancies resulting in the birth of a woman with MRKH syndrome were conceived naturally. The mean age of the mother at the time of birth of the women with MRKH syndrome was 28.7 years (range 18-42). All women in the control group reported having reached menarche and had never been pregnant. No women in the control group were part of a twin pair.

Male microchimerism

In total, 194 women were included in the analyses concerning male microchimerism (Table II). In the total group, 22 out of 194 (11.3%) women tested positive for male microchimerism. The prevalence of male microchimerism was significantly higher in the control group than in the MRKH group (17.2% versus 5.3%, $P = 0.009$) (Fig. 1). After correcting for age, women in the control group were 5.8 times more likely to have male microchimerism (OR 5.84 (95%CI 1.59 – 21.47), $P = 0.008$). The mean concentration of male microchimerism in the positive samples was 56.0 male GEq per 1,000,000 cells (Fig. 2). The mean concentration of male microchimerism was significantly higher in the control group than in the MRKH group ($P = 0.007$). The prevalence of male microchimerism was similar in women with typical MRKH syndrome and atypical MRKH syndrome (5.6% versus 4.9%, $P > 0.999$).

To account for a possible older brother as a source for chimerism[26], we made a subgroup analysis. From the total group, 134 women (69.1%) had no older brother (see Table II). Also in this subgroup, the prevalence and concentration of male microchimerism was higher in the controls than in the MRKH group.

Table I. Reported malformations in 41 women with atypical MRKH syndrome (n)

Renal malformations (28/41)*	
– Unilateral renal agenesis	15
– Pelvic kidney	3
– Hypoplastic kidney	3
– Horse shoe kidney	2
– Abnormal position	2
– Malrotation	1
– Cirrhotic kidney	1
– Duplex kidney	1
Skeletal (18/41)*	
– Scoliosis	13
– Cervical ribs	1
– Arm agenesis	1
– Scoliosis and fusion of vertebrae	1
– KFS, SD and scoliosis	1
– Spina bifida, KFS, SD and scoliosis	1
Other malformations (4/41)*	
– Hearing loss	2
– Scaphoid hypoplasia and hearing loss	1
– Clubfoot	1

** Combined malformations in nine women. KFS: Klippel-Feil syndrome (cervical vertebral fusion), SD: Sprengel's deformity (high scapula).*

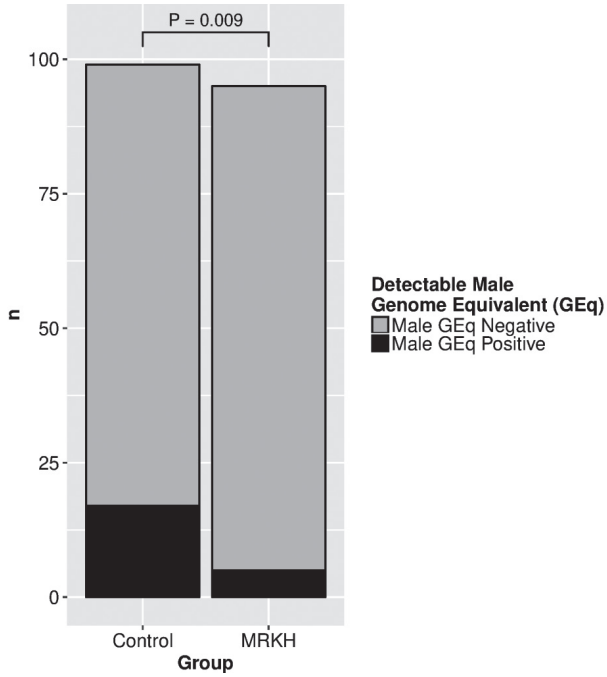

Figure 1. Number of subjects with male microchimerism in blood in control women and women with Mayer-Rokitansky-Küster-Hauser syndrome.
MRKH: Mayer-Rokitansky-Küster-Hauser; Statistical test used: Chi square test.

Table II. Male microchimerism in study subjects

Total group (n=194)	**MRKH (n=95)**	**Control (n=99)**	**P-value**
Age (years)	40.9 (15.5)	30.2 (6.6)	-
BMI (kg/m^2)	24.3 (5.3)	22.8 (3.5)	-
Presence of male microchimerism	5 (5.3%)	17 (17.2%)	0.009 [a]
	OR [c]: 0.27 (CI 0.10 – 0.76)		
	OR [d]: 0.17 (CI 0.05 – 0.63)		
Concentration of male microchimerism (GEq*)			
– Positive samples (n=22)	2.3 (1.5)	71.8 (127.0)	0.055 [b]
– Total group	0.1 (0.6)	12.3 (58.1)	0.007 [b]
Selection of study subjects with no older brother	**MRKH (n=63)**	**Control (n=71)**	**P-value**
Age (years)	38.1 (12.9)	30.5 (6.5)	-
Presence of male microchimerism	3 (4.8%)	10 (14.1%)	0.069 [a]
	OR [c]: 0.31 (CI 0.08 – 1.16)		
	OR [d]: 0.18 (CI 0.03 – 0.90)		
Concentration of male microchimerism (GEq*)			
– Positive samples (n=13)	1.3 (0.3)	27.3 (67.1)	0.018 [b]
– Total group	0.1 (0.3)	3.9 (25.9)	0.053 [b]

*Data are mean (SD) or n (%). MRKH: Mayer-Rokitansky-Küster-Hauser syndrome. *Male genome equivalents (GEq) per 1,000,000 female cells. [a] Chi square test [b] Mann-Whitney U test*
[c] Unadjusted odds ratio (95% confidence interval (CI)) [d] Odds ratio (95% CI), adjusted for age.

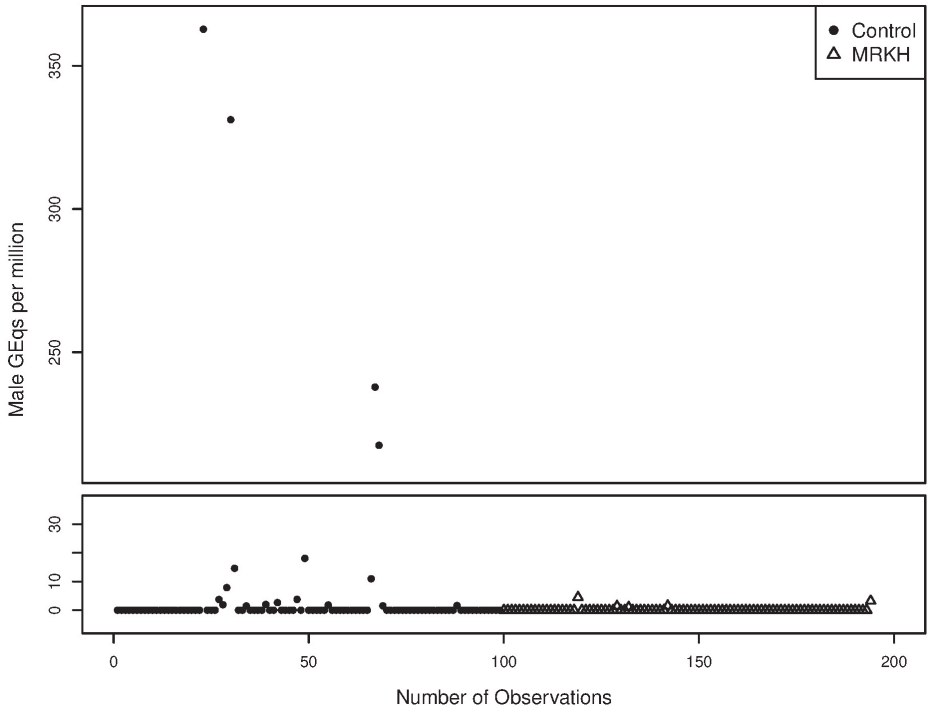

Figure 2. Calculated male GEq per 1 million cells in blood, for control women and women with Mayer-Rokitansky-Küster-Hauser (MRKH) syndrome

Table III presents possible sources for male microchimerism. No significant difference was found for the prevalence of male microchimerism in the group with an older brother as opposed to the group without ($P = 0.263$). In the MRKH group, five women reported having received a blood transfusion; these women all tested negative for male microchimerism. The prevalence of male microchimerism was similar when comparing the group with and without blood transfusion ($P > 0.999$). In the control group, eight women reported never having had sexual intercourse; of these women, one tested positive for male microchimerism. The prevalence of male microchimerism was similar when comparing the group with and without sexual intercourse ($P > 0.999$).

Table III. Identifying additional sources for male microchimerism

	Male microchimerism positive (n=22)	Male microchimerism negative (n=172)	P-value
Age, years	35.3 (11.6)[+]	35.4 (13.1)	-
Study group			
– MRKH	5 (22.7%)	90 (52.3%)	0.009 [a]
– Control	17 (77.3%)	82 (47.7%)	
Older brother			
– yes	9 (40.9%)	50 (29.1%)	0.263 [a]
– no	13 (59.1%)	121 (70.3%)[#]	
Blood transfusion *			
– yes	0	5 (5.6%)	>0.999 [b]
– no	5 (100%)	85 (94.4%)	
Sexual intercourse **			
– yes	16 (94.2%)	75 (91.5%)	>0.999 [b]
– no	1 (5.9%)	7 (8.5%)	

*Data are mean (SD) or n (%). *Data missing for control women, this question was only asked in questionnaire for MRKH women; ** data missing for MRKH women, this question was only asked in questionnaire for control women; [+]mean (SD) age in women with MRKH syndrome who tested positive for microchimerism = 49.9 (10.9) years, age in control women = 31.0 (7.9) years; [#]1 MRKH woman with a twin brother tested negative for male microchimerism. Statistical analyses: [a] Chi square test; [b] Fisher's exact test.*

DISCUSSION

MRKH is a condition that has been well characterized yet the etiology has remained elusive.[27] Research of the phenotype analogous condition in cattle, freemartinism, has yielded the hypothesis that uterine development is inhibited by exposure to AMH *in utero*.[9] In cattle, the male co-twin has been identified as the source of AMH, which is transferred via placental blood exchange. In humans, a similar exchange of cellular material has been documented between twins by detection of blood chimerism.[11,12] We sought to explain fetal exposure to blood and AMH from a male (vanished) co-twin in women with MRKH by measure of male blood microchimerism.

The use of a male genome target has been previously implemented in other studies of chimerism including fetal microchimerism.[19] However, to our knowledge, there are no studies to date that have explored the presence of male microchimerism in women with MRKH. Our approach utilized a qPCR technique that was able to demonstrate exceptional target specificity and sensitivity for male genome. In this study of 194 women, 5 of 95 women (5.3%) with MRKH demonstrated male microchimerism, while the control group demonstrated male microchimerism in 17 of 99 (17.2%). This difference shows a significantly

lower frequency in detection of male microchimerism for women with MRKH ($P = 0.009$). Similarly, when subjects with older brothers are removed, the percentage of male microchimerism in controls and cases is 14.1% and 4.8%, respectively. Our results do not support increased male microchimerism in adult women with MRKH as a consequence of intrauterine blood exchange.

Although these results contradict our underlying hypothesis of inadvertent AMH exposure in early pregnancy as the pathophysiological mechanism, it is too early to definitively conclude that occurrence of AMH exchange during early embryologic development in MRKH was not involved. There are several limitations to our approach that must be considered. First, this method relies on the detection of male genome in these subjects and is therefore contingent on the occurrence of cell grafting as a consequence of this exchange. There are several factors that are essential to effective grafting of cellular material in a host in order to evade immune targeting and thrive.[28] It is possible for intrauterine cellular exchange to occur passively between twins and not result in persistent chimerism. Similarly, our subject population includes all adult women and therefore is reliant on long-term prevalence of the male (XY) cell population to be detected at this stage of life. These XY cells may be eliminated or never graft in the host body, thereby shortening the timespan where detection is achievable. This technique exclusively examines the blood-derived genome, resulting in a limited scope of microchimerism detection and is not a global representation of chimerism throughout the individual. Owing to the proposed transfusion mechanism, it is most plausible that a male cell population may be localized in the blood, however, the cells may have grafted anywhere in the body during development.[29] It must also be considered that the exchange of AMH may occur without the transfusion of XY cells and therefore cannot be discovered by chimerism detection.

Our finding of male microchimerism in a larger percentage of women (17.2%) in the control group without a history of pregnancy is in line with findings from the literature. Prior studies demonstrated male microchimerism being present in 13.6% of adolescent girls[30] and 13.3% of healthy null gravid women.[25] Potential sources for the male cells are considered to be transfusion,[31] older brothers (or discontinued male pregnancies from their mother),[26] unrecognized male miscarriages,[25] vanishing twins,[14] or possibly sexual intercourse without pregnancy.[30] The significant difference in our study, in favor of the control group, suggests that a substantial proportion of the microchimerism could be explained by unrecognized pregnancies or the harbouring of microchimeric cells after sexual intercourse. Moreover, the higher concentration in the control group could reflect a larger transfusion of microchimeric cells by these sources. In our study, there were no differences in occurrence of older brothers, previous blood transfusion, or history of sexual intercourse between women with or without microchimerism, both in participants and

controls. We are unable to rule out the potential for microchimerism resulting from an unrecognized pregnancy or a vanishing twin.

The findings in women with MRKH illustrate that this population has a decreased prevalence of male microchimerism relative to others. This significant difference demonstrates that women with MRKH may serve as a suitable control group in chimerism research, in part due to the certainty that they have no history of pregnancy. Still, three women with MRKH and no older brother had detectable microchimerism, which may have been obtained through several mechanisms including a vanishing twin or miscarriage in their mother.

Considering the several potential sources of XY cells, our results from the control population demonstrate that there may be a larger prevalence of microchimerism in the general population, which requires further investigation. In general, there is increasing interest in the clinical consequences of microchimerism.[32,33] It has been associated with various autoimmune disorders,[34] but possible beneficial consequences have also been described. Microchimeric cells could replace injured cells in diseased tissues.[32] Recently it has been suggested that naturally acquired microchimerism has "protective effects in promoting success of future pregnancies".[35]

In summary, we hypothesized that women with MRKH syndrome had an unrecognized male co-twin that was lost early in gestation (a vanishing twin) and have investigated possible fetal exposure to male blood by measurement of male microchimerism. We are led to the conclusion that women with MRKH syndrome are not evident (micro)chimeras. However, we are not able to draw definitive conclusions regarding the occurrence of AMH exchange. Further research should involve the presence of MRKH syndrome in a large cohort of girls with a twin brother and in girls born after a pregnancy complicated by vanishing twin syndrome. Additional research on twin chimerism is needed to study the true prevalence of intrauterine blood exchange between dizygotic twins.

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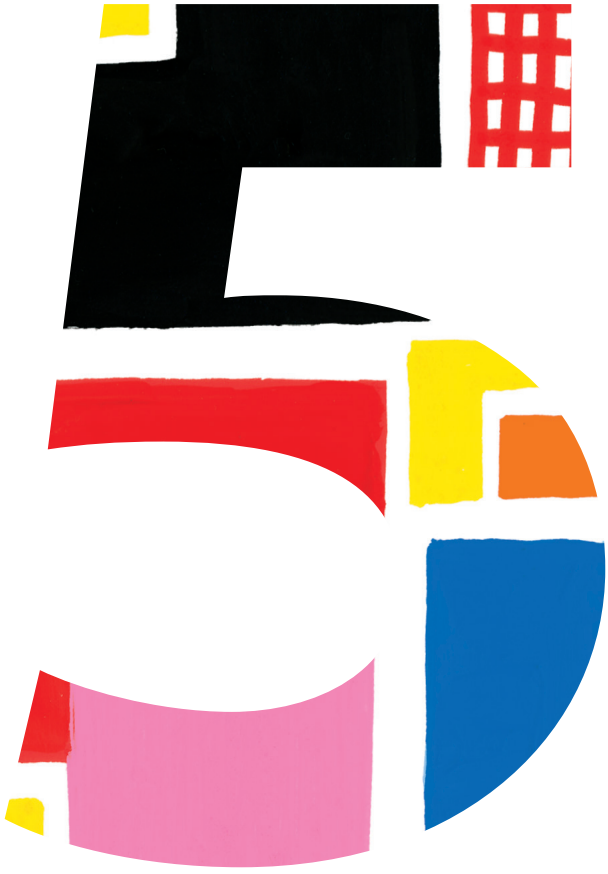

ANTHROPOMETRIC BIOMARKERS FOR ABNORMAL PRENATAL REPRODUCTIVE HORMONE EXPOSURE IN WOMEN WITH MRKH, PCOS AND ENDOMETRIOSIS

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ABSTRACT

Objective: To study whether markers of prenatal exposure to reproductive hormones are related to Mayer-Rokitansky-Küster-Hauser (MRKH) syndrome, polycystic ovary syndrome (PCOS) and endometriosis.

Design: Case-control study. Comparison of sex hormone-related external genital and digital characteristics in cases and controls.

Setting: University hospital.

Patients: We enrolled 172 women in four groups: MKRH, PCOS, endometriosis and controls (43 in each group).

Interventions: Measurement of two anthropometric biomarkers: anogenital distance and digit ratio.

Main Outcome Measures: Anogenital distance was measured from the anus to the anterior clitoral surface (AGDac) and from the anus to the posterior fourchette (AGDaf). For the digit ratio we used a direct, as well as a computer-assisted graphical measurement to measure the length of the 2nd and 4th digit.

Results: After adjustment for BMI and age, AGDac was the shortest in endometriosis and the longest in PCOS, with a mean difference of 10mm (95%CI 3.1-16.8, P < 0.001). AGDaf but not AGDac measures were found to be significantly larger in MRKH, with a mean difference compared to controls of 2.6mm (95%CI 0.1-5.2, $P = 0.045$). The digit ratio was not significantly different between the groups.

Conclusion: In this study we did find limited but first evidence for androgen exposure during the development of MRKH. This is compatible with the hypothesis that the utero-vaginal agenesis may have been the result of temporary prenatal exposure to altered gonadal hormone concentrations. For endometriosis and PCOS we confirm previously observed associations for anogenital distance reflecting possible estrogen- and androgen-based intrauterine origins, respectively.

INTRODUCTION

The Mayer-Rokitansky-Küster-Hauser (MRKH) syndrome is characterized by congenital aplasia of the uterus and upper part of the vagina, resulting from embryonic underdevelopment of the Müllerian duct. The etiology of the MRKH syndrome is still largely unclear.[1] In male embryos, regression of the Müllerian duct occurs in early embryonic development after exposure to anti-Müllerian hormone (AMH) produced by the fetal testes from the 6th week of development. In this study we hypothesize that exposure to testes-derived hormones, among which AMH, may cause uterine and vaginal agenesis in MRKH syndrome. A method for the measurement of prenatal exposure to AMH is not available. However there has been wide interest in the measurement of biomarkers reflecting exposure to androgens in early gestation.[2]

There is increasing evidence that the measurement of the anogenital distance (AGD) can be used as biomarker for intrauterine androgenic influence[3]. Prenatal exposure to higher androgen levels results in a longer AGD, while lower androgen levels lead to a shorter AGD.[4] In most mammals including humans, AGD is approximately 2 times longer in males than in females.[5] Cross-sectional studies in humans have reported an association between measures of AGD and reproductive function.[6-9] Moreover, the reports of a longer AGD in women with polycystic ovary syndrome (PCOS) have contributed to the idea that PCOS has an intrauterine origin and is influenced by prenatal exposure to androgens.[10,11] In women with severe endometriosis the opposite has been hypothesized, the presence of a shorter AGD possibly reflects prenatal estrogenic influence.[12]

Additionally, the ratio between the 2nd and 4th digit (2D:4D ratio) is hypothesized to be an indicator of androgen exposure during fetal development.[13-15] Generally, women have a higher 2D:4D ratio compared with men. A decreased 2D:4D ratio in women with PCOS reflects a possible prenatal androgenic milieu.[16] However, this has not been confirmed.[17]

To date, no studies have examined these anthropometric biomarkers in women with MRKH syndrome. The objective of this paper is to assess the measures of the AGD and 2D:4D ratio, as biomarkers of the intrauterine hormonal milieu, in MRKH syndrome. By comparing the measures to control women and to women with PCOS and endometriosis, we can verify our measurement method and determine whether there is evidence for prenatal exposure to hormones in MRKH syndrome. We hypothesize that exposure to androgens results in a longer AGD and a decreased 2D:4D ratio in women with PCOS and MRKH syndrome. Women with endometriosis are hypothesized to have a shorter AGD and an increased 2D:4D ratio. This paper reports on the measurement of both biomarkers in these three groups of patients when compared to control women.

METHODS

Study subjects

This observational case-control study compared the measurements of the biomarkers in four groups: women with MRKH syndrome, PCOS, endometriosis and control women. Participants were included at the hospital Amsterdam UMC - location VUmc between October 2018 and June 2019. Through recruitment via the Dutch patients' association ('Stichting MRK-vrouwen') we enrolled women with MRKH syndrome for a previous study.[18] The women who gave consent to contact them for participation in follow-up studies were informed about this study. Eligible patients with PCOS or endometriosis were women attending the outpatient clinic of reproductive medicine or the Endometriosis Center Amsterdam UMC and included prevalent and newly diagnosed cases. PCOS was diagnosed using the Rotterdam Criteria, when a minimum of 2 out of 3 criteria were present: (1) oligo- or amenorrhoea; (2) polycystic ovaries; (3) clinical or biochemical hyperandrogenism PCOS consensus workshop group, 2004[19]. As result of the standard clinical work-up, the endocrine pathologies such as thyroid dysfunction, congenital adrenal hyperplasia and hyperprolactinemia were ruled out in the PCOS group. Endometriosis was diagnosed using pelvic ultrasound, surgery and/or MRI. Only women with deep infiltrating endometriosis (infiltrating the peritoneum by 0.5 mm) and/or ASRM grade >3 were included. Patients with PCOS or endometriosis were allowed to continue hormonal therapies. The control group comprised women with regular cycles who attended the IVF clinic for intracytoplasmic sperm injection (ICSI) treatment and no history of PCOS or endometriosis. We selected this group as controls because these women undergo a fertility treatment in our centre because of severe male subfertility (VCM < 1 x 10^6), for which the standard treatment is ICSI. We considered this to be a group of women without any fertility disturbing factors. Exclusion criteria in all groups were age < 18 years, pregnancy and history of vaginal delivery. For the PCOS women, diagnosis of endometriosis was an exclusion criterion and for the endometriosis women, diagnosis of PCOS was an exclusion criterion. All participants provided written informed consent prior to measurement of the biomarkers.

Measurement of biomarkers

A stainless steel digital calliper (DIGI-MET, Helios Preisser®, Germany) was used to measure the AGD. The AGD measurements were performed by three trained researchers (H.P, C.L and C.T). For measuring AGD, the women were asked to lay down on the gynaecological chair in the lithotomy position. AGDac (anus-clitoral hood) was measured from the centre of the anus to the anterior labial commissure (or anterior clitoral surface). AGDaf (anus-fourchette) was measured from the centre of the anus to the posterior fourchette (or posterior labial commissure).[6,10] See figure 1. Two researchers performed the same measurements in

a subsample of 63 patients. For the 2D:4D ratio, there is currently no consensus on how to measure the digit lengths and there has been some discussion on what is the best technique.[20] Therefore we used a direct (d2D:4D), as well as an computer-assisted graphical measurement (indirect measurement, i2D:4D). For the direct measurement a digital calliper, similar to the one used for AGD measurement, was used to measure the length of the 2nd and 4th finger. We also made digital scans (200 dpi) of the hands using a Hewlett Packard scanner (HP Colour Laser Jet). The left and right hand were scanned separately. The digits were measured in the scanned image using Adobe Photoshop CS6. The digit lengths were measured on the ventral surface of the hand, from the basal crease of the digit to the tip of the finger in the midline. The digit ratio was calculated by dividing the length of the 2nd finger by the length of the 4th finger. The computer-assisted graphical measurements were performed by two researchers (C.L. and C.T.). Two researchers performed the same measurement for i2D:4D in a subsample of 30 patients. Three measurements were taken per researcher for all the measures (AGD, d2D:4D and i2D:4D) and the average was used in analysis.

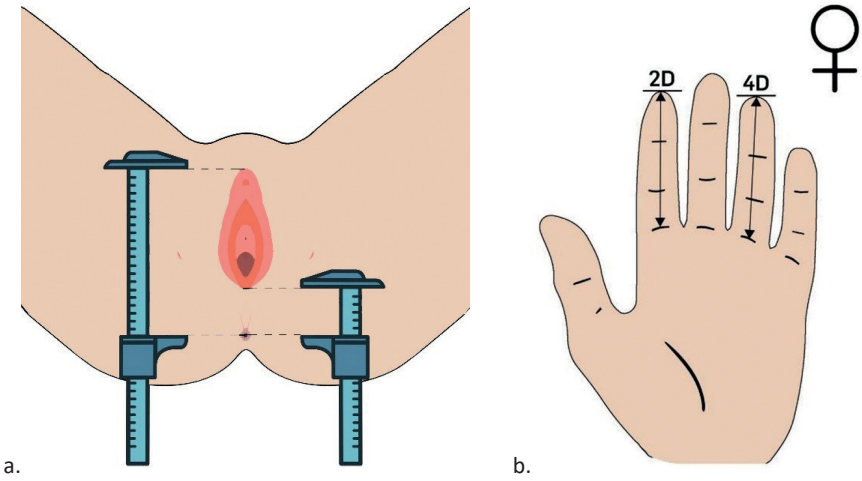

Figure 1. Measurement of anthropometric biomarkers
a. Anogenital distance (AGD), left: AGDac (anus-clitoral hood) and right: AGDaf (anus-fourchette)
b. Ratio between 2nd and 4th digit (2D:4D ratio)

Clinical information

The MRKH diagnosis was confirmed by contacting the general practitioner or gynecologist to retrieve the detailed information about the diagnosis. With this information and the information retrieved from the questionnaire the patients were identified as having typical MRKH syndrome (also referred to as type 1) in case of no known other malformations, and as atypical (type 2) MRKH syndrome when renal and/or skeletal malformations were

present.[21] When vaginal reconstruction made it possible, transvaginal ultrasound was performed for antral follicle count (AFC).

The women with PCOS, endometriosis and controls underwent complete gynaecological examination in our hospital according to regular care, including transvaginal ultrasound. The mean duration of the menstrual cycle without the use of hormonal contraceptives was used for analyses. AFC ≥12 on transvaginal ultrasound in one or both ovaries was classified as polycystic ovary morphology (PCOM). During standard work-up in women with PCOS, blood serum levels of testosterone, androstenedione and Free Androgen Index (FAI) were determined using LCMS. Biochemical hyperandrogenism was defined as testosterone >2 nmol/L, androstenedione >6 nmol/L and/or FAI >4.4.

All participants completed a questionnaire comprising questions about demographic information, handedness (right or left), serious trauma to the digits, clinical signs of hyperandrogenism, and in the case of MRKH syndrome about vaginal reconstruction. To assess clinical hyperandrogenism we asked two questions in our questionnaire: 1) Do you suffer from excessive hair growth? If yes, a simplified Ferriman Gallwey score,[22] was obtained by self-assessment. Hirsutism was defined as simplified Ferriman Gallwey score > 3. The second question was: 2) Do you or have you ever suffered from severe acne? We defined clinical hyperandrogenism as the self-reported presence of hirsutism or severe acne.

Statistical analysis

The primary outcome measure was the AGDac. Group sizes of 43 were selected based on power calculations informed by previous work on AGD in women with PCOS.[10]

Normally distributed variables were compared between groups using one-way ANOVA and not normally distributed variables using the Kruskal Wallis test. For categorical variables the chi-squared ($\chi 2$) test was used. Strength of association between biomarkers and baseline variables was quantified by means of Pearson's correlation. Given the exploratory nature of this study, the small sample sizes, as well as the a priori hypothesis, we did not perform corrections for multiple comparisons. For the digit ratio the i2D:4D of the right hand was used in correlation analyses. The paired T-test was used to compare right versus left hand and direct versus indirect measurement. By linear regression (using general linear models) we estimated the mean differences in biomarkers in the four research groups and studied the association between the biomarkers and various reproductive characteristics. We included age and BMI as possible confounding factors in the linear regression analyses because earlier studies reported associations with AGD.[10] *P*-value < 0.05 was considered statistically significant. A two-way random intra-class correlation coefficient (ICC) analysis, for absolute agreement, was performed to assess intra-observer reliability and inter-observer reliability for measurement of all biomarkers. For calculating the inter-observer reliability, the mean of the three measurements per observer was used. Coefficients of

variation (%CV) were used to assess intra- and inter-examiner variability in AGD and digit measurements.Statistical analyses were performed using SPSS 24.0 (SPSS, Chicago, IL, USA).

Ethics

The study protocol has been approved by the Institutional Review Board of the VU Medical Center, Amsterdam, the Netherlands, date: September 14th 2018 (METC VUmc 2018.306). The trial was registered in the Dutch National Trial Registry (trial registration number NTR7492).

RESULTS

Study population

A total of 172 women: 43 women with MKRH syndrome, 43 women with PCOS, 43 women with endometriosis and 43 control women, were recruited for this study. Table 1 shows the general characteristics of the participants. The median age in the total study population was 35 years, with the PCOS group being significantly younger compared to the women with endometriosis ($P = 0.004$) and MRKH ($P = 0.003$). Significantly more women in the MRKH group were Caucasian compared with the endometriosis group. Other groups did not differ in ethnicity.

In the MRK group, 74.4% was identified as having typical MRKH syndrome. The women with atypical MRKH syndrome had a renal malformation in 16.3%, a skeletal malformation in 4.7% and combined malformations in 4.7%. The most commonly used method for vaginal dilation was the Frank method (using a vaginal mold as a dilator) in 44.2%. A surgical method for vaginal construction was used in 25.6%. In 9.3% a functional vagina was created by natural dilation by sexual intercourse. 20.9% of the women did not use any therapy for creation of a vagina.

The PCOS women in our study were diagnosed according to the Rotterdam criteria, 69.8% were classified as 'frank' PCOS, having all three criteria. In the other women two criteria were present: 14.0% oligomenorrhea and PCOM, 9.3% (biochemical and/or clinical) hyperandrogenism and PCOM, 7.0% hyperandrogenism and oligomenorrhea. Of all PCOS women, 41.9% had biochemical hyperandrogenism (elevated serum levels of Testosterone, androstenedione and/or FAI). All women in the endometriosis group were diagnosed with severe and/or deep endometriosis, 58.1% underwent 'therapeutic' endometriosis surgery. In 67.4% the presence of ovarian endometrioma was reported in medical history or current imaging diagnosis. The control group consisted of women currently undergoing ICSI treatment because of severe male subfertility. Mean number of oocytes obtained after ovarian stimulation was 12 (±5.5). Based on the number of oocytes, 18.6% was classified as high responders (>15 oocytes).

Table 1. Baseline characteristics

	MRKH (n=43)	PCOS (n=43)	Endometriosis (n=43)	Controls (n=43)	Overall *P*-value
Age (years)	40.0 [29-55]	32.0 [29-35]	36.0 [32-41]	35.0 [33-37]	0.001[1]
BMI (kg/m2)	24.1 [21.6-28.7]	26.4 [21.8-29.2]	23.9 [21.1-30.0]	23.2 [21.0-27.2]	0.71
Caucasian	42 (97.7%)	35 (81.4%)	34 (79.1%)	40 (93.0%)	0.02[2,3]
Pregnancy in med. history	0	8 (18.6%)	11 (25.6%)	17 (39.5%)	<0.001[4,5]
- Live birth (by CS)	-	3 (7.0%)	9 (20.9%)	7 (16.3%)	0.178
- Pregnancy <12 weeks	-	5 (11.6%)	2 (4.7%)	10 (23.3%)	0.036[6]
PCOM	1 (2.3%)	40 (93.0%)	0	9 (14.0%)	<0.001[1]
Regular menstrual cycle	-	2 (4.7%)	23 (53.5%)[#]	43 (100%)	<0.001[1,6]
Clinical hyperandrogenism	7 (16.3%)	28 (65.1%)	9 (20.9%)	8 (18.6%)	<0.001[1,4]
- Acne	2 (4.7%)	15 (34.9%)	8 (18.6%)	7 (16.3%)	0.004[2,5]
- Hirsutism	5 (11.6%)	17 (39.5%)	2 (4.7%)	1 (2.3%)	<0.001[1]

Data are presented as median [IQR] or number (%). CS: Caesarean section. [#]in 18 (41.9%) cycle duration was unknown due to the long-term use of hormonal therapy. Significant differences were found when comparing: [1]PCOS with other 3 groups; [2]PCOS with MRKH; [3]MRK with endometriosis; [4]MRK with other 3 groups; [5]PCOS with controls; [6]endometriosis with controls.

Measurement of biomarkers

ICC analysis showed >0.97 degree of intra-observer reliability for both AGD measurements and analysis of inter-observer reliability showed an ICC of 0.98 for AGDac and 0.94 for AGDaf. Intra- and inter-examiner coefficient of variation for AGDac was respectively 1% and 5% and for AGDaf, respectively 4% and 7%. Intra- and inter-examiner coefficient of variation were 1% for all digit measurements. ICC analysis showed > 0.97 degree of intra-observer reliability for digit ratio in both hands and both measurement methods and > 0.92 degree of inter-observer reliability for both hands in the indirect measurement.

AGDac and AGDaf were positively correlated (Pearson's correlation (r) = 0.50 , P < 0.001). AGDac and AGDaf were both correlated with BMI (respectively, (r) = 0.52, P < 0.001 and (r) = 0.32, P < 0.001), but not with age (respectively, (r) = 0.06, P = 0.43 and (r) = 0.11, P = 0.17). AGD measures were not correlated with any 2D:4D ratio. 2D:4D ratio was not correlated with age ((r) = 0.07, P = 0.39) and BMI ((r) = 0.07, P = 0.34). d2D:4D and i2D:4D were strongly correlated for both hands, (left (r) = 0.85, P <0.001, right (r) = 0.79, P <0.001).

Table 2 shows the unadjusted analysis of the anthropometric biomarker measurements in the four groups. AGDac was significantly different between groups, with an increased AGDac in women with PCOS and a decreased AGDac in women with endometriosis (mean

difference: 9.97 mm, $P = 0.006$). After adjusting for BMI and age, AGDac measures were still found to be different in the groups, again with posthoc tests showing PCOS and endometriosis to differ. Overall, AGDac was the largest in the PCOS women (although not significantly different compared with controls) and the smallest in women with endometriosis. AGDaf measures were found to be significantly larger in women with MRKH syndrome, compared to the other three groups. The mean difference between MRKH and controls was 2.6 mm ($P = 0.045$). See table 3.

Table 2. Overview of anthropometric biomarker measurements in four patient groups

	MRKH (n=43^)	**PCOS (n=43)**	**Endometriosis (n=43)**	**Controls (n=43#)**	**Overall *P*-value***
Anogenital distance					
AGDac (mm)	108.2 ±11.3	113.8 ±16.9	103.9 ±12.6	111.4 ±13.7	0.007[1]
AGDaf (mm)	24.6 ±6.2	22.0 ±5.8	21.9 ±6.2	21.7 ±6.2	0.10
Digit ratio					
d2D:4D –right	0.981 ±0.027	0.983 ±0.032	0.986 ±0.030	0.980 ±0.029	0.76
d2D:4D –left	0.987 ±0.029	0.987 ±0.032	0.986 ±0.035	0.977 ±0.033	0.44
i2D:4D –right	0.964 ±0.025	0.963 ±0.031	0.971 ±0.033	0.965 ±0.029	0.59
i2D:4D –left	0.969 ±0.032	0.979 ±0.032	0.971 ±0.032	0.964 ±0.028	0.19

*Data are presented as mean ±SD. AGD was measured from centre of anus to anterior clitoral surface (AGDac) and from centre of anus to posterior fourchette (AGDaf). Digit ratio was measured using direct measurement (d2D:4D) and computer assisted -indirect- measurement (i2D:4D). ^One woman refused AGD measurement.# Due to severe digit trauma, data of two control women are missing for the right hand. *one-way ANOVA test. Post-hoc testing shows significant differences for: [1]PCOS group with endometriosis, p = 0.006.*

The digit ratios did not differ between groups, with no differences in both the direct and indirect measurements for the right and the left hand. Comparing the digit ratios of the dominant hand did also not reveal any differences. The resulting digit ratio of the direct measurement was significantly higher than of the indirect measurement for both hands (mean difference right: 0.017 ± 0.02, $P < 0.001$, mean difference left: 0.014 ± 0.018 $P < 0.001$). In the PCOS group there was a significant difference for the i2D:4D between right and left hand (right < left) (mean difference of ratio 0.02, $P < 0.001$). In the MRKH, endometriosis and control group no differences were found comparing the right with the left hand, in indirect and direct measurement.

The relationship between reproductive characteristics with biomarker measurements, examined by linear regression analyses, is presented in supplementary table S1. In the total study population, presence of hirsutism was associated with an increased AGDac;

with the presence of hirsutism resulting in an average AGDac of 5.6mm longer ($P = 0.03$). In the MRKH group, this association was present for the AGDaf ($P < 0.01$). Additionally the method of vaginal dilation was associated with AGDaf in the MRKH group, with natural dilation—by sexual intercourse—resulting in an increased AGDaf ($P = 0.01$). The women with PCOS without PCOM, but with oligomenorrhea and hyperandrogenism, had the longest ADGac; 17.2 mm longer than the women with other PCOS phenotypes. In the PCOS group a marginally significant positive association was found between the presence of biochemical hyperandrogenism and AGDac. No associations were found between patient characteristics and i2D:4D ratio.

Table 3. Mean differences on anogenital distance (AGD) and digit ratio (2D:4D) between various patient groups after adjustment for BMI and age as possible confounding factors

	AGDac			**AGDaf**			**2D:4D***		
	Mean diff (mm)	**95%CI**	***P*-value**	**Mean diff (mm)**	**95%CI**	***P*-value**	**Mean diff**	**95%CI**	***P*-value**
MRKH vs. controls	−4.8	−10.0, 0.4	0.07	2.6	0.1, 5.2	0.05	−0.002	−0.015, 0.011	0.78
PCOS vs. controls	0.8	−4.3, 5.8	0.77	−0.2	−2.7, 2.3	0.88	−0.002	−0.015, 0.011	0.80
Endometriosis vs. controls	−9.2	−14.2, −4.2	<0.001	−0.2	−2.7, 2.3	0.88	0.006	−0.007, 0.019	0.38
PCOS vs. endometriosis	10.0	4.9, 15.0	<0.001	0.0	−2.5, 2.5	1.00	−0.007	−0.020, 0.006	0.26
MRKH vs. PCOS	−5.6	−10.9, −0.2	0.04	2.8	0.2, 5.5	0.04	0.000	−0.014, 0.013	0.98
MRKH vs. endometriosis	4.4	−0.7, 9.4	0.09	2.8	0.3, 5.4	0.03	−0.008	−0.020, 0.005	0.25

*AGD was measured from centre of anus to anterior clitoral surface (AGDac) and from centre of anus to posterior fourchette (AGDaf). * indirect measurement of right hand.*

DISCUSSION

The cause of the utero-vaginal aplasia in MRKH syndrome is unclear. Embryonic Müllerian duct development is influenced by AMH, a testes-secreted hormone. We generated the hypothesis that androgens—as other testes-secreted hormones—could be of influence during the development of MRKH. In this study, we explored our hypothesis by measuring the AGDac, AGDaf and 2D:4D ratio as biomarkers of prenatal androgen exposure. We

observed longer AGDaf in women with MRKH syndrome compared to controls. None of the other biomarkers differed between the two groups.

In women with MRKH syndrome the AGDaf was significantly larger compared to the other three groups. This reveals for the first time some evidence for prenatal androgen exposure in MRKH syndrome, reflecting possible intrauterine origin related to reproductive hormonal environment. Our results suggest possible inadvertent androgen exposure during a critical window of embryonic development and by proxy could be considered as first evidence for exposure to the full range of male gonadal hormones of which AMH is the known suppressor of uterine development.

However there are some points to be considered. AGDac is generally considered to be the strongest measure for prenatal exposure to androgens[11]—although previous studies have also shown strong associations for AGDaf.[23,24] It has been suggested that the AGDaf measurement is more difficult, showing high variability in different centers.[25] This measurement is likely to be more subject to inconsistency, because of uncertainty for the accurate point to measure the posterior fourchette. Moreover, it could be possible that the AGDaf is influenced by *a priori* changed anatomy related to MRKH or subjective to the applied vaginal dilation techniques, since this measurement includes the posterior fourchette of the vagina. Of note, we did not perform corrections for multiple comparisons, yet after doing so the difference in AGDaf became statistically insignificant.

Additionally, in women with MRKH, the presence of hirsutism was positively associated with AGDaf. In recent years a number of cases have been presented describing failure of Müllerian duct formation combined with hyperandrogenism.[26,27] This may represent a clinical disorder distinct from the MRKH syndrome, and is associated with mutations in the WNT4-gene. Possibly some of the hirsute women in our MRKH cohort fit this profile. We do not have data on biochemical hyperandrogenism in this group. Possibly WNT4 mutations lead to a prenatal androgen environment influencing the AGD. By excluding the small proportion of the women in our MRKH cohort with clinical signs of hyperandrogenism, the difference in AGDaf becomes insignificant. So it may be too premature to conclude that differences in AGDaf in MRKH were definitively the result of altered prenatal androgen exposure.

This study provides limited support for our general hypothesis that the Müllerian duct abnormalities with the syndrome may have originated from overexposure to AMH, along with androgens, early in pregnancy. Potentially, with the origin of MRKH, there was only minimal androgen exposure but strong AMH exposure, and/or minimal effects of androgen exposure, but strong AMH effect. This could be related to placental aromatase activity. It must also be considered that the methodology has been insufficient to trace evident elevated androgen exposure, for instance as a result of very subtle hormonal influence.

By measuring the biomarkers in four different patient groups, our goal was to provide a perspective for assessment of these anthropometric biomarkers. Between the four groups, the AGDac was significantly different. We showed that the AGDac was the longest in women with PCOS and the shortest in women with endometriosis. This is consistent with the concept of the AGD measurement and follows existing literature on this subject, suggesting a prenatal androgenic environment in PCOS and an estrogenic prenatal environment in endometriosis. Although the cause of endometriosis remains not yet elucidated, an intrauterine origin due to prenatal exposure to estrogens has been described as a risk factor for endometriosis.[28] By confirming earlier findings that endometriosis is associated with a shorter AGD,[23] we provide additional evidence with respect to intrauterine hormonal influence in the early onset development of endometriosis.

In our study cohort the AGDac was longer in women with PCOS compared to the other groups, however we could not confirm earlier studies that show significant differences. In retrospect a power issue could be present. We performed a sample size calculation assuming a relatively large difference in AGD of 7mm (using data from a recent study comparing PCOS with controls[10]) and more recent studies revealed smaller differences in AGDac.[11,29] Would this difference have been used in our calculations, a much larger sample size would have been necessary to detect a significant difference. Also, considering the overall study population, our control group had a relatively long AGDac. Note the difference in AGDac between endometriosis and controls, which is larger than presented in earlier studies.[30] The AGD has recently been reported to be positively associated with ovarian response in ovarian stimulation.[6] Our control group comprises 20% hyperresponders, so possibly this influenced the outcome as well. Furthermore, it has been demonstrated that the AGDac was associated with the severity of the phenotypic subtypes of PCOS.[11] In our cohort the non-PCOM phenotype showed the largest AGDac, however this phenotype had a low prevalence ($n = 3$).

Another important question may be: does our technique indeed allow the measurement of hyperandrogenism? Our results show that current hyperandrogenism, represented by presence of hirsutism, was positively associated with AGDac in the overall study cohort. Moreover, in women with PCOS, biochemical hyperandrogenism was also associated to AGDac. A positive association of testosterone levels and AGD has been reported in previous studies.[10,31] Many studies have revealed that the AGD is fixed in early gestation.[2] Experimental studies in animals have demonstrated that AGD is influenced by androgen exposure during a specific window, so-called masculinization programming window, between 8-14 weeks of gestation.[4]

For the digit ratio, we did not find any differences between groups and we did not observe an association between digit ratio and hyperandrogenism. Our study is unique in measuring both anthropometric biomarkers, AGD and 2D:4D ratio, in a human study

population consisting of three patient groups and one control group. Two indicators for the same, namely prenatal hormonal environment, should reasonably be correlated. However, this is not the case. One report in mice studying both biomarkers, similarly did not find a correlation between AGD and digit ratios.[32] Conversely, Abbot et al. have reported a positive association between right hand 2D:4D ratio and anogenital distance in a non-human primate study.[33] The current body of research of the 2D:4D ratio as biomarker for prenatal androgen exposure is controversial.[34] The present study also shows that digit ratio may represent an insufficient or weak measure reflecting prenatal androgen exposure. In consideration of this, interestingly, comparing right and left hand we found a significant difference only in the PCOS group, with the right hand being shorter and thus more 'androgenized'. Since it has been suggested that the right hand is more sensitive to androgens, this might be an indication for exposure to fetal androgens in PCOS.[16]

There are limitations to our study. Measurement bias could have occurred, and should be taken into account. For practical reasons it was not possible to blind the researchers for the gynaecological status of the participant. To reduce the variability, only three researchers did all measurements; both biomarkers show a good reliability index. It is unknown whether ovarian stimulation or other hormonal treatment affect the AGD. However, AGD has been reported to be stable during menstrual cycle.[35] There is some data that suggests a decrease in AGD in females with age,[36] while in our study age was not associated with AGD measurements. Furthermore, information on family history, obstetric complications and specific details on intrauterine life were unknown. Also, we may have chosen a non-optimal control group. The presence of low-grade endometriosis is not excluded in our control group, since laparoscopic visualization, as gold standard for diagnosing endometriosis, has not been performed in these women prior to starting IVF/ICSI treatment.[37] However the impact of possible occult endometriosis seems to be minimal, as women in the control group seem to show a somewhat androgenized profile which could be reflected by the relatively large proportion of hyperresponders during ovarian stimulation.[6] Moreover, it is important to note that there are two ways of measuring the AGD, from the centre of the anus—as we did—or from the upper verge of the anus, resulting in a shorter distance. When also taking into account different statures due to ethnicity, and possibly different measurement techniques it is difficult to compare AGD results between studies.

In conclusion, the present study does suggest for the first time some evidence for prenatal exposure to androgens in MRKH syndrome. Our results together with earlier studies suggest that prenatal reproductive hormonal environment contributes to the development of PCOS and endometriosis. Moreover, we propose not to use the 2D:4D ratio as reliable marker for prenatal androgen exposure. Further research is needed to clarify the etiology of the MRKH syndrome and study the possible prenatal influence of male gonadal hormones.

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SUPPLEMENTARY TABLE S1.

Table S1. Associations of patient characteristics with anogenital distance (AGD) and 2D:4D ratio after adjustment for BMI and age

	AGDac		AGDaf		i2D:4D (right hand)*	
	Beta (95% CI)	***P*-value**	**Beta (95% CI)**	***P*-value**	**Beta**	***P*-value**
Total study population						
- Caucasian	−1.6 (−7.2, 4.0)	*0.58*	−0.7 (−3.4, 2.0)	*0.60*	−0.001	*0.89*
- Pregnancy in medical history	0.4 (−4.1, 4.9)	*0.86*	1.1 (−1.1, 3.3)	*0.33*	0.004	*0.43*
- Ongoing pregnancy^	−2.7 (−8.5, 3.2)	*0.37*	1.6 (−1.2, 4.4)	*0.26*	0.008[1]	*0.25*
- PCOM	3.4 (−0.9, 7.8)	*0.12*	-0.9 (−3.0, 1.1)	*0.37*	-0.003	*0.58*
- Hirsutism+	5.6 (0.4, 10.9)	*0.04*	2.4 (−0.1, 4.9)	*0.06*	-0.008	*0.23*
- Severe acne	−2.4 (−7.4, 2.5)	*0.33*	−2.4 (−4.7,−0.03)	*0.05*	0.010	*0.11*
Subgroup specific: MRKH syndrome						
- Type 1	2.3 (−6.5, 11.0)	*0.60*	0.2 (−4.8, 5.1)	*0.95*	−0.001	*0.91*
- Vaginal dilation with sexual intercourse	5.9 (−7.7, 19.5)	*0.38*	9.3 (2.2, 16.4)	*0.01*	−0.016	*0.23*
- Hirsutism+	7.2 (−3.5, 18.0)	*0.18*	8.7 (3.2, 14.3)	*<0.01*	−0.010	*0.44*
Endometriosis						
- Endometriosis surgery	-1.8 (−9.2, 5.7)	*0.63*	−2.3 (−6.2, 1.7)	*0.25*	−0.007	*0.50*
PCOS						
- PCOM	-17.2 (−35.6, 1.2)	*0.07*	−1.5 (−9.2, 6.2)	*0.70*	0.021	*0.33*
- Hirsutism+	0.1 (−9.2, 9.4)	*0.99*	1.0 (−2.7, 4.7)	*0.60*	0.000	*1.00*
- Oligomenorrhea	10.0 (−10.1, 30.2)	*0.32*	−1.6 (−9.7, 6.6)	*0.70*	-0.030	*0.20*
- Biochemical hyperandrogenism*	8.0 (−0.5, 16.5)	*0.06*	−0.3 (−3.8, 3.2)	*0.86*		
Controls						
- High ovarian response	1.7 (−5.5, 9.0)	*0.64*	2.9 (−1.2, 7.1)	*0.16*	−0.010	*0.41*

*[#] indirect measurement of right hand ^Only deliveries by Caesarean section, women with history of vaginal delivery were excluded from participation in the study; +Hirsutism: simplified Ferriman Gallwey score ≥ 3; *Elevated serum levels testosterone, androstenedione and/or Free Androgen Index.*

GESTATIONAL SURROGACY: RESULTS OF 10 YEARS OF EXPERIENCE IN THE NETHERLANDS

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ABSTRACT

Research question

What are the reproductive and obstetric outcomes of the gestational surrogacy treatment in the Netherlands?

Design

This retrospective cohort study reports all data of gestational surrogacy treatment in the VU University Medical Centre over a period of 10 years. Data was collected from 60 intended parents and 63 gestational carriers, including reproductive and obstetric outcomes.

Results

All intended mothers had a medical indication for gestational surrogacy and used autologous oocytes, and semen of the intended father. Ninety-three IVF cycles were initiated in 60 intended mothers, with subsequent 184 single embryo transfers in 63 gestational carriers. This resulted in 35 ongoing singleton pregnancies. At least one live birth was achieved for 55.0% of intended couples. Pregnancy was complicated in 20.6% by a hypertensive disorder. Labour was induced in 52.9%, and the Caesarean section rate was 8.8%. None of the pregnancies was complicated by preterm birth. Post-partum haemorrhage (>500 ml) occurred in 23.5%.

Conclusions

This study shows the effective results of the non-commercial gestational surrogacy programme in the Netherlands, in a multidisciplinary team setting. An increased risk for adverse obstetric outcomes in surrogate mothers is noted for hypertensive disorders and post-partum haemorrhage compared with the incidence in non-surrogacy pregnancies.

KEY MESSAGE

This retrospective cohort study shows an effective non-commercial gestational surrogacy program in the Netherlands. Gestational carriers show an increased risk for adverse obstetric outcomes, including hypertensive disorders and post-partum hemorrhage. This requires extensive counselling during intake and careful perinatal monitoring by obstetric caregivers.

INTRODUCTION

Surrogacy in combination with in vitro fertilization (IVF) is a treatment first reported in the USA in 1985.[1] Oocytes are retrieved from the intended mother after ovarian stimulation and are fertilized *in vitro* with semen of the intended father. The resulting embryo is transferred into the uterus of the gestational carrier. This treatment enables couples to have their own genetic offspring when a woman is not able to carry a pregnancy due to absence or non-functioning of the uterus.

Gestational surrogacy treatment is controversial. It is not allowed by law in a number of countries in Europe.[2] In the USA, Australia, Canada and Finland it is practised substantially.[3-6] In the Netherlands it has been allowed since 1997. The law changed from a complete prohibition of surrogacy, to allowing surrogacy while prohibiting mediation between intended parents and surrogate mothers. The first results in the Netherlands (between 1997 and 2004) showed that non-commercial gestational surrogacy is feasible, with good results in terms of pregnancy outcome and psychological outcome in the absence of legal problems.[7] Since 2006 the VU University Medical Centre in Amsterdam has been the only hospital in the Netherlands performing IVF treatment in gestational surrogacy.

In recent literature, perinatal outcomes have been well documented.[8] However, there are few data on obstetric outcomes after surrogacy pregnancies. This report summarizes the legal and ethical aspects concerning this treatment in the Netherlands. Moreover, all data will be presented from our gestational surrogacy programme in a tertiary centre over a 10-year period, including pregnancy outcomes and maternal complications.

PATIENTS AND METHODS

This retrospective cohort study reports all data of gestational surrogacy treatment in the VU University Medical Centre between October 2006 and March 2017. In this period, 60 couples attempted one or more IVF surrogacy cycles, with 63 gestational carriers. The data on the IVF treatment and resulting pregnancies were derived from medical records, of both the intended parents and the gestational carriers. In case of an ongoing pregnancy the obstetric caregiver was contacted, after consent of the patient, to retrieve the detailed information about pregnancy and labour, including maternal and neonatal complications.

This study has been formally exempted from ethical approval granted by the Institutional Review Board of the VU University Medical Centre (reference 2017.288, dated 16 June 2017).

Intake procedure

Various medical conditions indicate the need for a gestational carrier and are as such described in the Dutch guideline on surrogacy (NVOG guideline no 18, Jan 1999): (i) congenital or acquired (post-hysterectomy) absence of the uterus; (ii) a serious medical condition that contra-indicates for a pregnancy; (iii) a non-functioning uterus (e.g. untreatable Asherman syndrome). To evaluate whether the intended parents are suitable for a gestational surrogacy treatment, the indication and the medical history is in our center verified by a specialized gynecologist (RS), and if necessary by a multidisciplinary team of obstetric experts and other specialists. Due to the regulations in the Netherlands, the intended parents are themselves responsible to find a suitable gestational carrier. A detailed history and physical examination, including a pelvic ultrasound, are performed in both the intended mother and the gestational carrier. A semen analysis of the intended father is performed. The intended parents and the gestational carrier (and possible partner) are screened for transmissible diseases, such as HIV, hepatitis B virus, hepatitis C virus and syphilis. See Table 1 for inclusion criteria for intended parents and gestational carriers.

Table 1. Inclusion criteria for accepting gestational surrogacy treatment

Intended parents	- Medical indication for gestational surrogacy - Intended mother ≤40 years - Expected sufficient supply of oocytes and semen - Good biopsychosocial health - Successful recruitment of surrogate mother (no commercial surrogacy allowed) - Meet legal requirements
Gestational carrier	- One or more uncomplicated term pregnancies and vaginal deliveries (exclusion criteria are e.g. hypertensive disease, post-partum haemorrhage >2 l, previous Caesarean section) - Good biopsychosocial health (exclusion criteria: high risk of obstetric complications) - Age 25-45 years - Meet legal requirements

Psychological counselling

After medical acceptance, psychological screening is started. A specially trained team of psychologists (JLS, IPP) evaluate the motivation for gestational surrogacy and the psychological well-being. Parents and gestational carrier are seen separately by the psychologist and in a joint session with all parties. If the gestational carrier has a partner, explicit approval is necessary in the process and he/she will be included in the psychological counselling. Part of the work-up is psycho-education concerning expectations about the procedures and possible pregnancy. After completion of the psychological screening, an

extensive consent form is signed addressing mutual commitments of intended parents and surrogate parents, including for example arrangements about the voluntary character of the treatment, pregnancy care and responsibility of care for the child. However, the legal value of this form is limited, the aim is to obtain a clear mutual agreement. Continuation of the procedure is followed only after approval by the gestational surrogacy team.

Legal and financial aspects

The earlier mentioned informed consent also addresses the financial aspects. Costs resulting from the surrogacy procedure and/or pregnancy must be covered by the intended parents. Medical insurance usually reimburses three IVF cycles and the medical costs of pregnancy and labour. It is obligatory to obtain life insurance for the gestational carrier during the pregnancy, and it is advised to draft a will concerning the custody of the child should the intended parents pass away. For legal parenthood the intended parents have to follow a formal adoption procedure after birth. Dutch law assigns the woman who gives birth to a baby as the mother (*mater semper certa est*). For permission to have the responsibility to take care of the child immediately after birth, individual permission is required from the Dutch Child Care and Protection Board (in Dutch: *Raad voor de Kinderbescherming).*

Ovarian stimulation and IVF treatment

Cycle synchronization between gestational carrier and intended mother is achieved using oral contraceptives. For the IVF procedure in the intended mother, a standard GnRH agonist protocol is used. Ovarian stimulation is performed with recombinant FSH

(Gonal-F®; Merck, Germany or Puregon®; MSD, USA). Ovulation trigger is achieved with 10,000 IU of human chorionic gonadotropin (HCG; Pregnyl®; Organon, The Netherlands) or 6,500 IU recombinant chorionic gonadotropin (Ovitrelle®; Merck, Germany). Oocyte retrieval is performed 36 h after administration of HCG, under conscious sedation. In case of successful synchronization during the stimulation phase, a fresh single embryo transfer (SET) is performed and good quality supernumerary embryos are cryopreserved. In case of failed synchronization, primary cryopreservation of all good quality embryos is performed.[9] Embryo transfer is carried out 3-5 days after oocyte retrieval. The frozen thawed (cryo) cycles are preferably performed in a natural cycle.[10]

Analysis

The data are presented with use of different outcomes: spontaneous miscarriage was defined as the absence of a gestational sac (by ultrasound) after previous detection of a clinical pregnancy; ongoing pregnancy was defined as pregnancy >12 weeks of gestation; implantation rate was defined as the number of pregnancies per transfer, and as the

number of pregnancies per transfer per patient. The study compared the treatment factors and outcomes between the indication groups (described in 'Intake procedure'), because the underlying reason for gestational surrogacy is very diverse in these three groups. One aim was to study if this results in different outcomes, which would be interesting for research purposes and also useful for correct patient counselling. The data were analyzed in SPSS® version 22.0 (IBM Corp., USA). Chi-squared, one-way ANOVA or non-parametric Kruskal-Wallis tests were performed where appropriate. P <0.05 was considered as evidence of significant group difference.

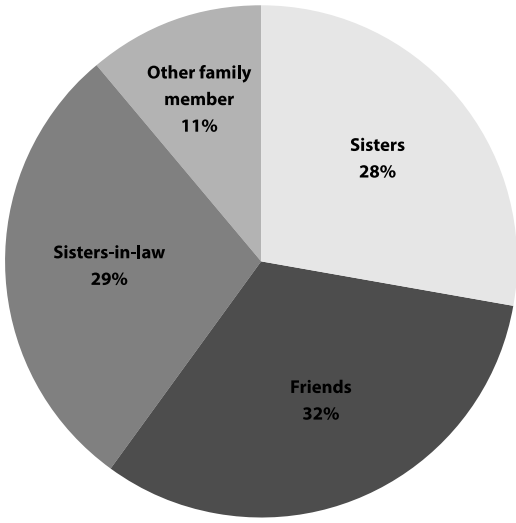

Figure 1. Relationship between surrogate mother and intended mother.

RESULTS

The gestational surrogacy treatment of 60 intended parents and 63 gestational carriers was reviewed. Three intended mothers had more than one gestational carrier. The mean age of the intended mothers was 33.5 years (range 24-39) and the mean age of the gestational carriers was 35.3 years (range 27–44). The relationships of the intended mothers with the gestational carriers are shown in Figure 1. In 31.7% of the cases there was a genetic relation between the women. The medical indications are shown in Table 2. The various maternal medical conditions posing a contraindication for pregnancy were: Alport syndrome (hereditary nephritis), B-cell lymphoma in the kidney with renal transplantation, chronic heart failure (NYHA class 2), congenital malformation of urogenital system with renal failure, cystic fibrosis with lung transplantation, haemolytic uremic syndrome with renal failure

(*n* = 2), paroxysmal nocturnal hemoglobinuria (*n* = 2), and systemic lupus erythematosus with antiphospholipid syndrome.

In total, 93 IVF stimulation cycles were initiated. Five of the intended mothers had already undergone an IVF stimulation followed by embryo cryopreservation prior to the start of the surrogacy procedure: one patient with cancer and four patients in whom, during infertility treatment, an indication for gestational surrogacy emerged. A cycle was defined as one stimulation cycle with fresh transfer and/or any subsequent frozen-thawed embryo transfers from the same cycle. Six cycles were cancelled, four because of poor response, one because of premature ovulation during stimulation and one because of failed cycle synchronization between the intended mother and gestational carrier. Seventy-four transvaginal and 13 transabdominal oocyte retrievals in 59 intended mothers were performed. An average of 10.5 oocytes (range 0-30) were collected. In one case no oocytes were obtained. Ovarian hyperstimulation occurred in 2.2% of the cycles: both were classified as mild due to the absence of laboratory findings and hospitalization was not required. In 76 treatments, fertilization was achieved by IVF, in 10 treatments fertilization was achieved by ICSI. In 41 stimulation cycles a fresh embryo transfer in the gestational carrier was performed, and in 42 cycles the embryos were primarily cryopreserved. In three cases there were no embryos suitable for transfer. A total of 142 cryopreserved SET were performed (95.8% in natural cycle, 4.2% with hormone substitution therapy). One intended couple transported the cryopreserved embryos formed after IVF treatment to another country, for use in commercial surrogacy. In this case after double-embryo transfer an ongoing singleton pregnancy followed. In total, 184 embryo transfers were performed in 61 gestational carriers.

Table 2. Indication categories for gestational surrogacy

	Number	**Percentage**
Indication 1: Absence of the uterus	36	60.0%
Acquired: obstetric complication	8	
Acquired: oncological disease	9	
Acquired: benign gynecological disease	5	
Congenital: Mayer-Rokitansky-Küster (MRK) syndrome	14	
Indication 2: Maternal medical condition	10	16.7%
Indication 3: Non-functioning uterus	14	23.3%
Endometrium failure	4	
Uterus myomatosus	4	
Uterus anomaly	4	
Other	2	
Total	**60**	**100**

In 60 patients, 93 initiated IVF/ICSI cycles yielded 52 pregnancies; see Table 3 (results per cycle) and Table 4 (results per couple). The mean implantation rate per patient was 40.1%. The ongoing pregnancy rate (OPR) per intended couple was 56.7% (for a first child). One couple had two ongoing pregnancies. In Table 5 the results categorized per indication are shown. Comparing the chance of an ongoing pregnancy in women with an absence of uterus versus women with a medical contra-indication for a pregnancy shows an odds ratio of 3.00 (confidence interval (CI) 95% 0.71–12.69). Comparing women with an absence of uterus versus a non-functioning uterus shows an odds ratio of 2.67 (CI 95% 0.75–9.45). In a subgroup of women with a congenital absence of the uterus (all women with Mayer-Rokitansky-Küster (MRK) syndrome): 78.6% reached an ongoing pregnancy. Comparing the chance of an ongoing pregnancy (per couple) in women with MRK syndrome versus all other women in our cohort shows an odds ratio of 3.67 (CI 95% 0.90–14.89). From the 52 pregnancies, 35 were ongoing pregnancies >12 weeks gestation. One pregnancy was terminated at a gestational age of 22 weeks, due to the presence of Down's syndrome combined with a severe cardiac anomaly.

Table 3. Pregnancy results of gestational surrogacy treatment, per initiated cycle (*n* = 93 cycles)

		Number	Percentage
Live births		34	36.6%
Pregnancies		52	55.9%
-	Ongoing pregnancies >12 weeks	35[a]	37.6%
-	Spontaneous abortions	5	5.4%
-	Biochemical pregnancies	12	12.9%

[a] *One pregnancy was terminated at a gestational age of 22 weeks.*

Table 4. Pregnancy results of gestational surrogacy treatment, per couple (*n* = 60 couples)

		Number	Percentage
Couples with live birth		33 [a]	55%
Couples with pregnancy		40	66.7%
-	Couples with ongoing pregnancy >12 weeks	34 [b]	56.7%
-	Couples with only spontaneous abortion	2	3.3%
-	Couples with only biochemical pregnancy	4	6.7%

[a] *One couple had two ongoing pregnancies, both resulting in a live birth.*
[b] *One pregnancy was terminated at a gestational age of 22 weeks.*

Thirty-four singleton pregnancies were reported with a gestational age of >24 weeks. These pregnancies all resulted in a live birth, see Table 6. Hypertensive disorders occurred in

20.6% of the gestational carriers (*n* = 7), in three of these cases there was a genetic relation with the intended mother (all sisters). In 91.2% a spontaneous vaginal birth occurred. The Caesarean section rate (CSR) was 8.8%, in two cases a Caesarean section was performed because of a transverse position of the fetus and in one case because of a failed (elective) induction. In 23.5% of the pregnancies blood loss of >500 ml was reported, including three cases of post-partum haemorrhage (PPH) >1 l. One patient needed surgery and a blood transfusion after a total blood loss of 4 l, due to retention of placental tissue. In one case blood loss of >500 ml was reported directly post-partum, and due to heavy blood loss on day 10 and 25 post-partum, repeated readmission was necessary, for dilatation and curettage (late PPH).

There were no cases of preterm birth. The mean gestational age was 39.2 weeks. The mean birth weight was 3591 gram (range 2465–4550). No congenital malformations were reported in the live born babies. Labour was induced in 52.9% of the pregnancies (without any preterm induction of labour). In nine cases the reasons for induction were elective; in six cases labour was induced due to a hypertensive disorder; in two cases induction was due to fetal macrosomia; and in one case induction was due to hydronephrosis.

Table 5. Results of gestational surrogacy in the three indication groups

	Indication 1: Absence of the uterus (*n* = 36) Total 48 cycles, 1.3 cycle per patient (range 1-3)	**Indication 2: Maternal medical condition (*n* = 10)** Total 17 cycles, 1.7 cycle per patient (range 1-3)	**Indication 3: A non-functioning uterus (*n* = 14)** Total 28 cycles, 2.0 cycle per patient (range 1-4)
Age (years) intended mother [a, b]	32.8 (3.3)	32.9 (2.4)	35.6 (1.9)
Age (years) gestational carrier [a]	35.5 (4.6)	33.6 (4.3)	35.9 (4.4)
No. of oocytes retrieved per cycle [c]	10.5 (7.3)	8.8 (6.8)	9.1 (8.3)
No. of embryo transfers	95	29	60
- per initiated cycle [a]	1.9 (1.6-2.3)	1.7 (1.1-2.4)	2.1 (1.5-2.8)
- per patient [a]	2.6 (2.0-3.2)	2.9 (1.5-4.3)	4.3 (2.1-6.4)
No. of implantations	32	9	11
- IR[d] per transfer [a] %	34 (24–43)	31 (13-49)	18 (8–28)
- IR[e] per patient [a] %	44 (31-57)	38 (9-67	31 (11-52)
No. of ongoing pregnancies	24	4	6
- OPR per couple [f] (%)	66.7%	40.0%	42.9%

Data are presented as mean (SD) or mean (95% confidence interval). IR = implantation rate; OPR = ongoing pregnancy rate. [a] One-way ANOVA test. [b] P = 0.011. [c] Kruskal-Wallis test. [d] Calculated as the number of pregnancies divided by the number of embryos transferred, multiplied by 100. [e] Calculated as the number of pregnancies per patient, divided by the number of transferred embryos per patient, multiplied by 100. [f] Pearson chi-squared test.

Table 6. Pregnancy complications in 34 ongoing pregnancies >24 weeks gestation

	n (%)
Obstetric complications	
Multiple pregnancies	0
Hypertensive disorders	7 (20.6)
- PIH	5 (14.7)
- PE	1 (2.9)
- HELLP	1 (2.9)
Labour	
Caesarean section	3 (8.8)
Mild PPH > 500mL	5 (14.7)
Early PPH > 1L	3 (8.8)
Late PPH >1L	1 (2.9)
Fetal complications	
Prematurity	0
LBW <2500 gram	1 (2.9)
Macrosomia	3 (8.8)
Shoulder dystocia	1 (2.9)

PIH = pregnancy induced hypertension (blood pressure ≥140 mmHg (systolic) and/or ≥90 mmHg (diastolic)); PE = pre-eclampsia (hypertension + proteinuria); HELLP = haemolysis, elevated liver enzymes and low platelets; PPH = post-partum haemorrhage; LBW = low birth weight.

DISCUSSION

The reported live-birth rate (LBR) per initiated cycle of 36.6% is comparable with other recent reports on gestational surrogacy performed in Canada (34.8%) and the USA (34.0%).[3,11] Earlier results of gestational surrogacy treatment in the Netherlands (between 1997 and 2004) show a LBR per couple with an initiated IVF cycle, of 44.8%.[7] The LBR (for a first child) per intended couple in the current report cohort was 55.0%. When comparing the gestational surrogacy results (OPR per cycle 37.6%) with non-surrogacy IVF treatments in our centre (OPR of 39.8%, unpublished results of 2010-2015) it can be concluded that the gestational surrogacy program is successful. Moreover, in this study cohort of 60 intended parents there are couples that still have cryopreserved embryos available for transfers and couples who will start fresh cycles, so the pregnancy rate can only increase.

When focusing only on the women with an absence of the uterus, the OPR per couple is 66.7%. Especially women with MRK syndrome have a high chance on a successful treatment, with an OPR per couple of 78.6%. This is in line with a recent review on gestational surrogacy in MRK patients, showing LBR per patient of 56.8%.[12] It can be concluded that in a large proportion of the women with MRK syndrome the oocyte quality is good and oocyte retrieval is feasible. Gestational surrogacy is a good reproductive option for this patient population. Furthermore, the women with an acquired absence of the

uterus also show good IVF results, in contrast to the belief that blood flow to the ovaries is compromised after hysterectomy resulting in a lower ovarian reserve.[13]

After 93 initiated IVF/ICSI cycles, in 83 cycles embryos suitable for transfer were obtained. However, in 51% no fresh embryo could be transferred and the embryos were primarily cryopreserved. It turned out to be challenging to synchronize the cycle of two women following the use of oral contraceptives. Possibly, the preparation of the gestational carrier for the embryo transfer with the use of hormone substitution therapy (oestradiol and progesterone) can increase the fresh embryo transfer rate, but it also means the administration of hormones to the gestational carrier, while the starting point of this protocol was to administer the gestational carrier no more hormones than absolutely necessary.

In the ESHRE taskforce it has been recommended since 2005 to perform SET in gestational surrogacy.[14] However, this is still often not the case, for example in a surrogacy programme in the USA in which 78.5% of the embryo transfers comprised two or more embryos.[4] In the IVF surrogacy programme in the Netherlands, only SET was performed. This programme is designed to have the lowest risk for the gestational carriers. This is also in accordance with Dutch regulations, in which SET is mandatory. There were no twin pregnancies. This likely contributed to the absence of preterm birth (0%) and low incidence of low birth weight (2.9%). Still, the incidence of hypertensive disorders in our study was 20.6%, a higher rate than previously reported (incidence between 4.3 – 10%).[15] This incidence is also considerably higher than that in assisted reproductive technology pregnancies without the use of a surrogate, where an incidence of 10% for pregnancy induced hypertension and 5% for pre-eclampsia is reported.[16] However, rates of hypertensive disorders in oocyte donation pregnancies are even higher (16-40%).[17] This supports the idea of a connection between nulliparity and hypertensive disorders. In the placenta of oocyte donor pregnancies, histological and immunohistochemical reactions have been described, which could represent a host-versus-graft-rejection-like phenomenon.[18] For gestational carriers in a surrogacy procedure, it has been speculated that "a healthy carrier with a normal reproductive background might somehow compensate for atypical immunological reactions related to a foreign embryo".[15] Moreover, it has been reported that the incidence of a hypertensive disorder is higher if a oocyte donor is genetically unrelated to the recipient (20% versus 8%).[17]

The CSR in the cohort of this study is higher than in low-risk multipara patients in the Netherlands (8.8% versus 1%).[19] However, in other countries a CSR of 21.3–70% is reported for gestational surrogacy pregnancies.[3,5,20] This difference could be explained by the low rate (0%) of multiple pregnancies in this study cohort, the absence of preterm pre-eclampsia, or is possibly partly due to normal topographic variation in CSR. In other cohorts no details are supplied about reasons for Caesarean section. We advocate that an elective Caesarean

section should not be offered routinely for gestational surrogacy pregnancies. Besides, a Caesarean section also results in the impossibility of being a gestational carrier again.

The incidence of PPH is often not reported in literature about gestational surrogacy. Oocyte donation pregnancies are reported to be associated with PPH, with an incidence between 0–17.3%.[21-24] In the cohort from this study, PPH (>1000 ml blood loss) occurred in 8.8% of the cases. Furthermore, 23.5% of the deliveries had a total blood loss of more than 500 ml (mild PPH). Compared with an incidence of PPH (>1000 ml) of 3% in low-risk multipara women[19] it can be cautiously suggested that the risk of PPH is increased after gestational surrogacy.

Although this is a complete series of all gestational surrogacy treatments as far as we know in the Netherlands over a period of 10 years, it is a limited number of patients and these data are retrospective. A strength of this study is that follow-up data were available on birth outcomes and obstetric complications. The long-term follow-up data on surrogate mothers and intended parents, with special attention to the psychological effects, was unfortunately not available to us at the time of publication. In the literature, there are not enough scientific reports about the psychological consequences of a gestational surrogacy treatment. More research is required to get a better image of the long-term consequences and impact on the surrogate mother, intended parents and their family.

We often encounter patients with a solid medical indication for surrogacy, for which finding a suitable gestational carrier seems to be very difficult. Dutch law penalizes commercial mediation or the public search or public offer for a gestational carrier ('Wetboek van Strafrecht', art 151b). It is a fact that an unknown number of couples with or without solid medical indication go abroad for surrogacy, and this is even increasing.[4] We feel that regulations concerning mediation for surrogacy should be a subject for discussion nowadays. In our opinion, a government agency should manage a database of surrogacy mothers that could be consulted by intended parents.

In summary, gestational surrogacy is a suitable treatment for couples in which the woman is not able to carry the pregnancy herself. Using an extensive intake procedure in our centre, including medical and psychological counselling and testing, this study shows an effective, non-commercial gestational surrogacy program. However, gestational carriers seem to have an increased risk for adverse obstetric outcomes, such as hypertensive disorders and post-partum haemorrhage. This requires extensive counselling during the intake procedure and careful perinatal monitoring by the obstetric caregivers.

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FEASIBILITY STUDY FOR PERFORMING UTERUS TRANSPLANTATION IN THE NETHERLANDS

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ABSTRACT

Study question

Is it feasible to perform uterus transplantations (UTx) in a tertiary center in the Netherlands?

Summary answer

Considering all ethical principles, surgical risks and financial aspects, we have concluded that at this time, it is not feasible to establish the UTx procedure at our hospital.

What is known already

UTx is a promising treatment for absolute uterine factor infertility. It is currently being investigated within several clinical trials worldwide, and has resulted in the live birth of 19 children so far. Most UTx procedures are performed in women with the Mayer-Rokitansky-Küster-Hauser (MRKH) syndrome, a congenital disorder characterized by absence of the uterus. In the Netherlands, the only possible option for these women for having children is adoption or surrogacy.

Study design, size, duration

We performed a feasibility study to search for ethical, medical and financial support for performing UTx at the Amsterdam UMC, location VUmc.

Participants/materials, setting, methods

For this feasibility study, we created a special interest group, including gynaecologists, transplant surgeons, researchers and a financial advisor. Also, in collaboration with the patients' association for women with MRKH, a questionnaire study was performed to research the decision-making in possible recipients. In this paper, we present an overview of current practices and literature on UTx and discuss the results of our feasibility study.

Main results and the role of chance

A high level of interest from the possible recipients became apparent from our questionnaire amongst women with MRKH. The majority (64.8%) positively considered UTx with a live donor, with 69.6% having a potential donor available. However, this 'non-life-saving transplantation' requires careful balancing of risks and benefits. The UTx procedure includes two complex surgeries and unknown consequences for the unborn child. The costs for one UTx are calculated to be

around €100,000 and will not be compensated by medical insurance. The Clinical Ethics Committee places great emphasis on the principle of non-maleficence and the 'fair distribution of health services'.

Limitations, reasons for caution

In the Netherlands, alternatives for having children are available and future collaboration with experienced foreign clinics that offer the procedure is a possibility not yet investigated.

Wider implications of the findings

The final assessment of this feasibility study is that there are not enough grounds to support this procedure at our hospital at this point in time. We will closely follow the developments and will re-evaluate the feasibility in the future.

INTRODUCTION

With uterus transplantation (UTx), the uterine graft of a donor is removed and transplanted in a woman who has no uterus due to congenital or surgical reasons. The incentive for this procedure is a desire to have children in women with ovaries but no uterus. The first human UTx was performed in Saudi Arabia in 2000, but was rejected within 3 months.[1] In 2014, the first child after a successful UTx was born in Sweden[2] after years of animal research with successful transplantations in mice, rats, sheep and baboons.[3-8] The UTx procedure in humans is currently being investigated by several clinical trials worldwide. At present, around 60 human UTx have been performed, resulting in the birth of 19 children.[9-16]. In the Netherlands, no uterus transplantations have been performed so far.

Most UTx procedures are performed in women with Mayer-Rokitansky-Küster-Hauser (MRKH) syndrome. These women are born without a uterus and upper part of the vagina. They have normal- functioning ovaries and normal secondary sexual characteristics. MRKH occurs in 1:5000 women.[17] Based on Dutch population statistics, around 1500 women in the Netherlands are affected, with 20 new cases of MRKH each year.

Due to absence of the uterus, these women have absolute uterine factor infertility. In the Netherlands, possible options for having children are adoption, and traditional and gestational surrogacy. Gestational surrogacy means an *in vitro* fertilization (IVF) procedure is performed and the resulting embryo is placed in a gestational carrier. Surrogacy, in general, is a controversial treatment and is not allowed by law in various countries in Europe, for example in Sweden. The only hospital in the Netherlands to perform this treatment is the Amsterdam UMC, location VU University Medical Center (VUmc). In the past 10 years, 34 live births were achieved by gestational surrogacy.[18]

To investigate the possibility of performing uterus transplantations in the Amsterdam UMC, we performed a feasibility study to search for ethical, medical and financial support for this innovative, yet ethically and morally challenging, technique. Additionally, we performed a questionnaire study in women with the MRKH syndrome to study the support in patients. In this paper, we present an overview of current practices and literature on UTx and discuss the results of our feasibility study for performing UTx in the Netherlands.

METHODS

For this feasibility study, we created a special interest group; this was initiated by the gynaecologists from Amsterdam UMC, location VUmc. The UTx team included gynaecologists, transplant surgeons, researchers and a financial advisor. We pointed out relevant stakeholders: the patients association of women with MRKH syndrome (in Dutch:

'Stichting MRK-Vrouwen') and the possible patients, the executive board of the hospital, the Netherlands Association for Obstetrics and Gynaecology (NVOG), the VUmc Clinical Ethics Committee and the potential future UTx team itself for discussing medical technical and ethical aspects.

Moreover, in collaboration with the patients association of women with MRKH syndrome, a questionnaire was designed consisting of questions about UTx, associated risks and financial aspects. A comprehensive information letter was attached to the questionnaire regarding the UTx procedure, financial aspects and possible risks. The questionnaire was sent by the patients association to all its members with diagnosis of MRKH syndrome, age between 18 and 36 years and Dutch nationality (n=108). This study has been formally exempted from ethical approval, granted by the Institutional Review Board of the VUmc (reference 2019.151, date 20th March 2019).

UTx PROCEDURE

Recipient and donor selection

Potential recipients for UTx are women with absolute uterine factor infertility caused by congenital absence of the uterus (MRKH syndrome) or when acquired after hysterectomy, due to obstetric complications or gynaecological disease. Also, women with a non-functioning uterus can be potential recipients, for example after therapy resistant Asherman syndrome (severe intrauterine adhesions). Prior to the UTx procedure an extensive multidisciplinary medical and psychological screening is essential (see Table I).

The donor uterus can originate from living (LD) or deceased donors (DD). Living donors are commonly a family member or friend. The recipients are themselves responsible for finding a donor. The donor will then be screened for suitability. They should at least have a fulfilled child wish with an uncomplicated obstetric history. The most important concerns in using LD-uterine grafts are the surgical and psychological risks for the donor. DD-uterine grafts can principally be retrieved from brain-dead women, who are formally registered as organ donor. The procedure of uterus retrieval from deceased donors is more simple than from living donors.[19,20] However, the logistic organization is complex, with multiple surgical teams to be mobilized when a deceased donor becomes available. The increased transit time for the graft and the removal of vital organs prior to non-vital organs increases the ischemia time. This possibly reduces quality of the uterine graft.[21] Moreover, for a DD-graft, a less comprehensive assessment of the uterus prior to transplantation is possible.[20]

Most successful transplantations have been performed with living donors, as 17 out of 19 babies are born after LD-UTx. In 2018 the first birth from a deceased donor was reported.[11] In this report we focus on UTx from living donors.

Table I. Schematic summary of the program of uterus transplantation.

Recipient	Living donor
Screening (12-18 months) Congenital or acquired absence of uterus, good mental and physical health, stable relationship, age 21-36 years.	**Screening (12-18 months)** Good mental and physical health, good relation to recipient, non-smoking, ≥1 uncomplicated term pregnancy, complete family, age <60-65y.
IVF treatment (3-6 months) Before start of UTx, an IVF treatment with successful cryopreservation of embryos should be performed.	
Transplantation Abdominal surgery, duration 6-7 hours. Followed by immunosuppressive therapy to reduce the risk of repulsion.	**Transplantation** Abdominal surgery, duration 8-9 hours. Uterus will be removed, including vascular pedicle. Ovaries will stay in situ.
Embryo transfer *(1 year after transplantation)* Single embryo transfer.	
Pregnancy Intensive prenatal care and medical control for possible repulsion. Continuation immunosuppressive therapy. Delivery via caesarean section.	
Follow-up After 1 or 2 successful pregnancies the uterus will be removed.	
Duration for recipient: 3-4 years	*Duration for donor: 1-2 years*

Surgical procedure

UTx is a complex procedure demanding major abdominal surgery in the donor and the recipient. The surgical isolation of the donor-uterus involves the delicate dissection of the uterine arteries and veins. Bilateral long vascular pedicles are required to allow for adequate anastomoses to the external iliac vein and artery in the recipient. Details have been extensively described by Brännström et al.[22] After removal of the uterus, it is flushed and prepared at the back table and then transplanted in the recipient. The procedures in recipient and donor should be carried out synchronally, by teams consisting of a specialized gynaecologist and transplant surgeon.

Most donors reported in the literature had an uncomplicated post-operative course, with a hospital stay of around 6 days. Two major post-operative complications have been described: one ureterovaginal fistula requiring a nephrostomy catheter and a vaginal cuff dehiscence that was surgically repaired.[22-24]

Follow-up

For the recipient, a combination of immunosuppressive drugs is administered to prevent rejection. This is initiated prior to surgery and is maintained until removal of the graft, i.e. also in the event of pregnancy. Post-operatively, the recipient will be closely monitored through clinical examination and the graft is monitored via imaging and cervical biopsies to detect histopathological signs of rejection. In the first clinical trial in Sweden, two out of nine grafts had to be removed in the first year after transplantation due to post-operative complications. The causes were severe uterine infection and acute thrombosis.[22,25]

IVF and possible pregnancy

Prior to performing the transplantation, an IVF procedure has to be completed with successful cryopreservation (freezing) of embryos. Six to twelve months post-transplantation, a first transfer of a cryo-embryo can be planned with no signs of rejection. In case of pregnancy, this will be closely monitored. In pregnancies following transplantations of solid organs such as kidney, the neonatal outcomes are well-studied and immunosuppressive therapy is deemed safe.[26] In the Swedish UTx trial, the main obstetric complications were hypertensive disorders. The mean gestational age reached was 35 weeks, and all babies were delivered via elective caesarean section. No birth defects have been reported so far. After one or two pregnancies, hysterectomy is performed to remove the graft, in some at the same time as the caesarean section.

ALTERNATIVES

Alternatives for having children for these patients are adoption and surrogacy. Both options are established and allowed by law in the Netherlands. The adoption process is however a complex and lengthy procedure, and obviously the parents do not have a genetic relation with the child. In case of traditional surrogacy, the child is genetically related to the intended father, but not to the intended mother (as the gestational carrier is both genetic and birth mother). A procedure mentioned as an alternative for UTx to achieve a genetically related child is gestational surrogacy, in which an IVF treatment is performed with the intended mother and the resulting embryo is placed in a gestational carrier. With this procedure in the Netherlands, according to legal regulations, the patient herself is responsible for recruiting a woman as gestational carrier. For many women finding a suitable gestational carrier is very difficult to impossible. Moreover, absence of specific regulation for surrogacy in civil right is a source for legal uncertainty. This treatment also involves complex ethical considerations, since the gestational carrier takes the medical risks of pregnancy. The psychological implications for gestational carriers and parents have not

been studied in detail. In children born after surrogacy, good psychological adjustment is reported although psychological follow-up is currently very limited.[18,27]

SUPPORT FOR UTX IN THE NETHERLANDS

Professional support

The Netherlands Association for Obstetrics and Gynaecology (NVOG) had declared support for this feasibility study. The executive board of the hospital also supported the initiative. Ethical issues brought up by the local Clinical Ethics Committee will be discussed separately (see paragraph 'Ethical considerations').

Patient support

An important aspect in our feasibility study was the rate of support of the possible recipients. The questionnaire was anonymously completed online by 71 women with MRKH syndrome (response rate 66%), with a mean age of 26.7 years (range 18–36). The majority (64.8%) of the respondents would consider UTx with a live donor (see Figure 1a, depicted per age group). In this group, 69.6% indicated having a potential donor available, with 75% of those being their mother.

We also asked a question about available alternatives to fulfil the desire to have children. If uterus transplantations, gestational surrogacy and adoption were all to be reimbursed by health insurance, still 60.6% would prefer a uterus transplantation, 28.2% preferred gestational surrogacy, 7.0% chose adoption and the remaining 4.2% chose 'none of the above' (Figure 1b).

With regard to financial aspects, it was asked how much they would be willing to contribute, with the options allowing: € 0, € 5,000, € 20,000, € 50,000 and € 100,000 (Figure 1c). The most frequently given answers were € 5,000 (32.2%) and € 20,000 (35.6%). The two younger groups of women were generally willing to contribute more: 13.6% (18-23 years) and 15% (24-29 years) were willing to contribute € 100,000 compared with 0% in the group of 30-36 years.

In addition, we asked the question of which complications they would be willing to accept for themselves and for their potential donor. The majority of the respondents indicated to accept the chance of a post-surgical haemorrhage for both herself (73.2%; figure 2a) and her potential donor (66.2%; figure 2b). The risk of rejection of a newly acquired uterus was accepted by 36.6%. The risk of dying during the procedure was unacceptable for all respondents. The women tended to accept more risks with their own surgery than the donor surgery.

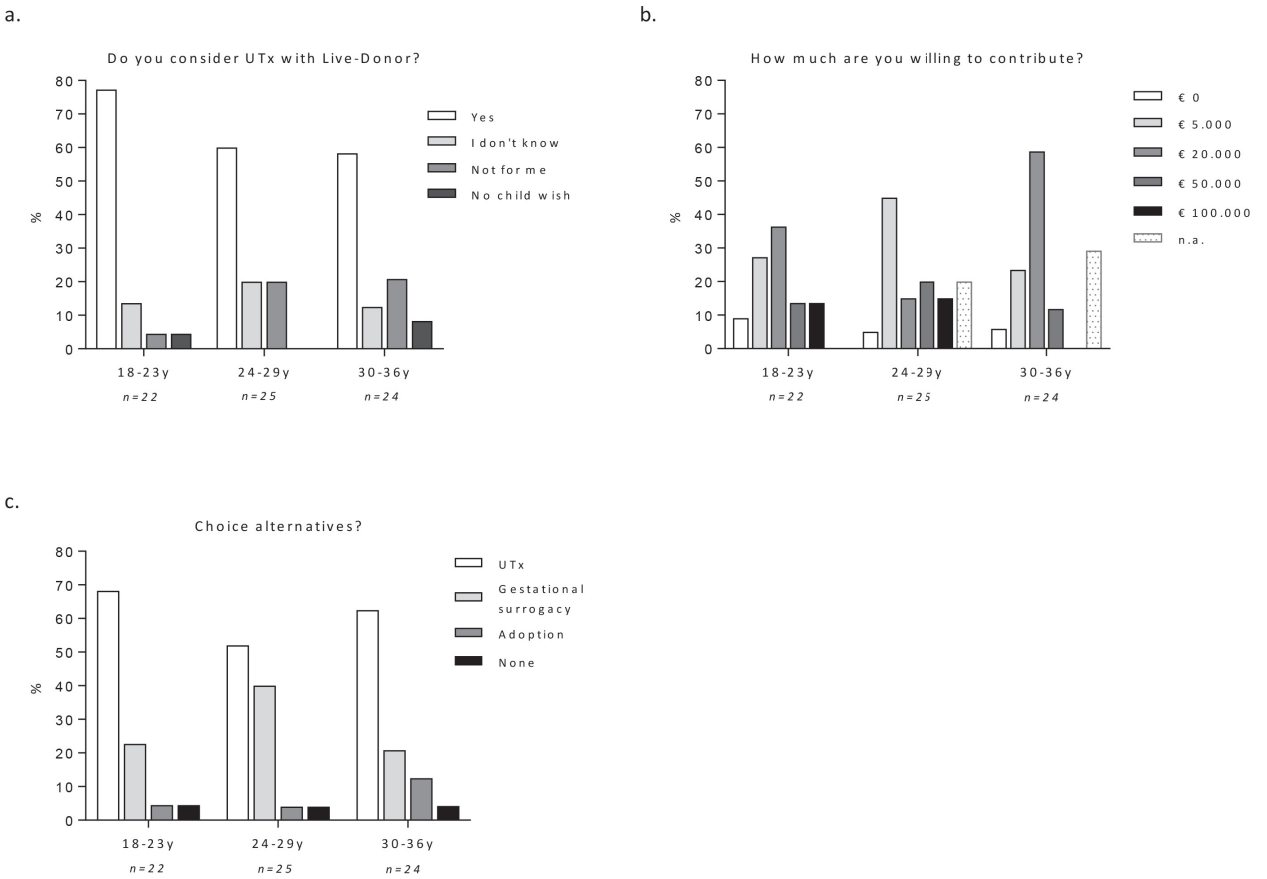

Figure 1. Results of questionnaire study amongst 71 women with MRKH syndrome. Questions were (a) 'Do you consider UTx with a live donor?'; (b) 'How much are you willing to contribute?'; and (c) 'Which of the options, UTx, gestational surrogacy or adoption would you choose?'. Answers are depicted as percentage per age group.

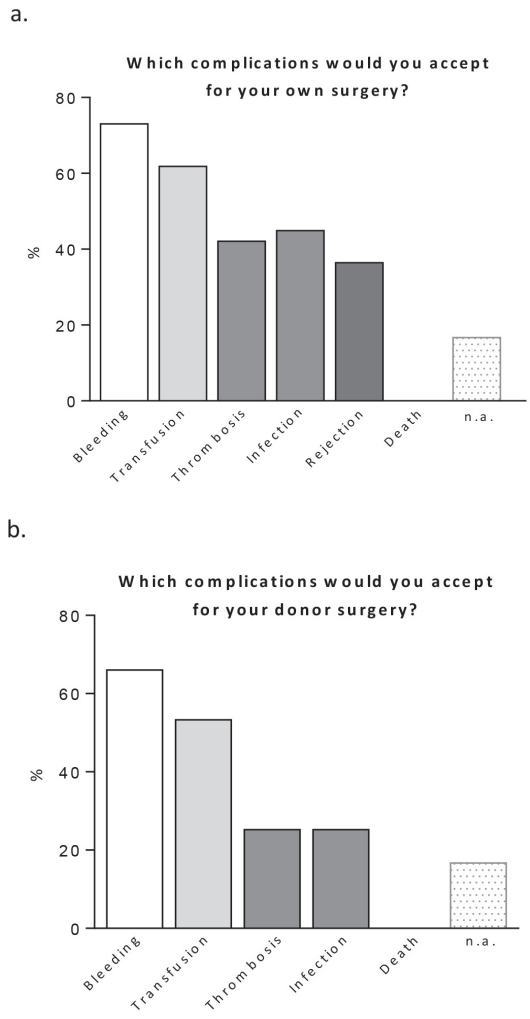

Figure 2. Results of questionnaire study amongst 71 women with MRKH syndrome.
The questions were regarding potential surgical complications for their own surgery (a) and the surgery of their potential donor (b). Answers are depicted as percentage of the women accepting the proposed complication.

LEGAL AND FINANCIAL ASPECTS

Legal aspects

Organ transplantations are filed under the Act on Special Medical Procedures (in Dutch: *Wet bijzondere medische verrichtingen*); this means that the organ transplant must be performed in a facility with a special license for this type of transplantation from the Dutch Ministry

of Health, Welfare and Sport (in Dutch: *Ministerie van Volksgezondheid, Welzijn en Sport*). No licenses have been acquired for uterus transplantation in the Netherlands yet.

Financial aspects

The costs for one UTx are calculated in Table II, resulting in total costs of nearly € 100,000. This table does not include the costs for a possible pregnancy and/or labour, because this is covered by medical insurance. The costs for UTx however, so far, are not reimbursed. Therefore, funding by research grants would initially be the only way to finance such a program.

Table II. Calculated costs for UTx in the Netherlands (for the year 2018).

		Costs	Total
UTx	Screening donor and recipient	€ 8.750	€ 93.850
	IVF treatment (3 cycles)	€ 13.650	
	Surgery donor (incl. robot, 8h surgery) and recipient (7h surgery)	€ 54.750	
	Follow-up and immunosuppressive therapy (2 years)	€ 14.200	
	Removal donated uterus	€ 2.500	

ETHICAL CONSIDERATIONS

Along with medical and scientific progress on UTx, worldwide ethical questions have risen. It is clear that this 'quality of life transplant' requires balancing of risks and benefits.[28-30] In the light of UTx, the ethical principles of beneficence, non-maleficence, autonomy and justice have been extensively discussed.[31,32] To translate this into clinical practice, 'the Montreal Criteria for ethical feasibility of uterine transplantation' were constructed.[33] These criteria define the acceptable conditions in which UTx could be performed in human.

The Clinical Ethics Committee of the Amsterdam UMC has considered the feasibility of UTx, in view of various ethical principles. They place great emphasis on the principle of non-maleficence, as UTx implicates potentially physical and psychological risks for both recipients and donors. Moreover, it involves the health of the (unborn) child. The 'fair distribution of health services' is also an important point in their discussion; by performing UTx in our hospital, it will potentially be at the expense of another treatment or patient population due to the high financial costs. Another essential issue raised is the availability of alternatives such as adoption or gestational surrogacy, in our country. They advised to follow and evaluate the international evolution of the UTx procedure and scientific reports concerning the development of the children born after UTx and reconsider this issue in the future.

DISCUSSION

UTx is a promising experimental treatment for absolute uterine factor infertility. There have been 19 live births after UTx reported so far, and several clinical trials are currently being performed worldwide showing a high level of interest for this technique. In this feasibility study, we considered the various aspects of performing UTx in our hospital. A high level of interest from the possible recipients became apparent from our questionnaire amongst Dutch women with MRKH. However, strict screening regulations for both donor and recipient would apply, allowing only a small portion of women to be eligible.[34] The UTx procedure puts two women at major health risk because of the extensive surgical procedures and, for the recipient, the immunosuppressive treatment. Moreover, emergency uterine graft removal is reported to be 28.6%, because of complications in the first 6 months after transplantation.[15] Also, high costs apply and we do not know the long-term consequences for children born after these procedures. In the Netherlands, alternatives are available and future collaboration with experienced foreign clinics that offer the procedure is a possibility not yet investigated. The final assessment of this feasibility study is that that there are not enough grounds to support this procedure at our hospital at this point in time.

Patients' perspective

It is important to acknowledge the wish for a genetically related child and the possibility to take a role in the child's health prior to birth by carrying a pregnancy.[35] Our questionnaire study confirms earlier studies in Europe, that the majority of women with MRKH would consider UTx and have a preference for UTx over gestational surrogacy and adoption.[36-38] After evaluation of the results, it appears that the younger women tend to be more positive towards UTx and are willing to contribute more financially. We hypothesize that younger women might have less insight in the risks and high financial burden. The participants in our study only received written comprehensive information on UTx, and we did not discuss this face-to-face. However, a previous study shows that after more extensive counselling, the majority of the women still see UTx as a good treatment option.[38] By collaborating with the national patient organization we have reached a broad range of MRKH patients, although our questionnaire study did not include possible recipients with other indications for UTx, such as a post-hysterectomy condition. Saso et al.[38] reported no differences in perspective on UTx between MRKH and non-MRKH women. In addition, MRKH syndrome is by far the leading indication for requesting UTx.[39]

Ethical concerns

Despite the fact that UTx is already conducted elsewhere in the world, the narrative on ethical aspects of uterus transplantations is currently still ongoing with contradictory

opinions and views.[40,41] In our feasibility study, ethical considerations played a major role. Yet, medical ethics is not straightforward science; for example the principle of non-maleficence is difficult to judge. Concerning the uterus donor, a healthy woman is subjecting herself to a high-risk surgical procedure. The risks of the hysterectomy that has to be performed in a living uterus donor are different from that of regular hysterectomy. The dissection of the connected blood vessels is more delicate, the duration of the surgery is longer and there is a higher risk of ureteral injury. Mortality rates in donors have been estimated to be similar to that for live kidney donation. In living kidney donors, there is no increased risk for mortality,[42] although there is some discussion about a possible increased long-term risk for all-cause mortality.[43] This is not likely to apply to uterus donors as the uterus is not an essential organ after reproductive age. Most problems are to be expected perioperative; there is no uterus donor mortality reported as far as we know.

Also, the recipient is subjecting herself and the unborn child to a high-risk pregnancy following transplantation. The most experience with post-transplant pregnancies comes from kidney transplant recipients. Although pregnancy following renal transplantation is considered to be safe, obstetric complications (affecting both mother and child) occur relatively often.[44]

Mortality and morbidity risk for the donor would be eliminated when using deceased donors. We feel it is important to consider this option when DD-UTx is proved to be equally successful to LD-UTX. It has even been mentioned that 'living uterus donation would no longer be ethically appropriate' if DD-UTx appears to be effective.[28]

Future considerations

Evidently, should uterus transplantation be carried out in our center in the future, sufficient and extensive surgical training is required for both the procurement of the uterine graft and the recipient operation. It has been suggested by the Swedish team to perform first training on sheep, followed by training on bodies donated to science. Moreover, in many countries the experienced Swedish team was present during the first transplantation.
If UTx is going to be performed in the Netherlands, this should always be in the context of a clinical trial. Consequently, research grants are then needed for funding because of the high costs without insured reimbursement and to avoid UTx from becoming a privileged treatment for the wealthy. Further research should provide information on psychological and general health consequences for all parties involved, including the child. Also newer surgical techniques should be investigated. At present, a clinical trial in Sweden investigates the possibility for UTx using robot surgery in the donor, with the aim to minimize surgical risks.[45] Minimizing surgical risks also reduces the ethical principle of 'non-maleficence'. The results of this study are expected in the near future.

In addition, we consider the possibility of an international collaboration with more experienced clinics in UTx to refer patients. The centralization of care for UTx would preserve the best medical program and a concentrated experience in specialized hospitals in a limited number of countries. Recently, a clinical trial for uterus transplantation from deceased donors is initiated in Ghent, Belgium, where they performed their first, and latest, UTx in October 2018. The continuity of performing the procedure is hampered by the limited availability of suitable donors. This was also shown by a screening program in Germany where more than 50% of the potential patients were excluded after screening as a result of self-withdrawal, unavailability of a donor and incompatibility between donor and recipient.[34]

Donor availability is an important aspect of the discussion on UTx. In this article, we have focused on related living donors (family members or friends). Non-directed living organ donation is allowed by law in the Netherlands for kidney and (part of the) liver. Evidently, this is voluntary, and financial compensation is prohibited *(*in Dutch: *'Wet op orgaandonatie'*). In the USA, the first four non-directed living UTx were performed in 2016. The women who volunteered to be a donor all expressed the desire for another woman to be able to carry her own child.[46] However, it remains a controversial subject, especially in the case of non-life-saving donation. So-called 'cross-over' donation can be a solution for donor-recipient incompatibility. In the Netherlands, cross-over kidney transplantation has been introduced as an extra option in the living kidney donation program in 2004. With this program, patients who cannot receive their own partner's kidney for immunological reasons receive a kidney from the partner of another patient in exchange for a kidney from their own partner.[47] This might also be an option for future clinical trials with uterus transplantations. Female-to-male transgenders have also been suggested as potential donor candidates, because of the voluntary hysterectomy at sex-reassignment surgery.[48] As the Amsterdam UMC has a special focus for transgender care, we want to consider and investigate this unique patient group as possible donors in the future when the technique to harvest the uterus during laparoscopic robot surgery is well-developed.

In summary, considering all medical, ethical and financial aspects we conclude that it is currently not feasible to establish the UTx procedure at the Amsterdam UMC. We will follow the impending developments concerning this treatment and will re-evaluate its feasibility in the future.

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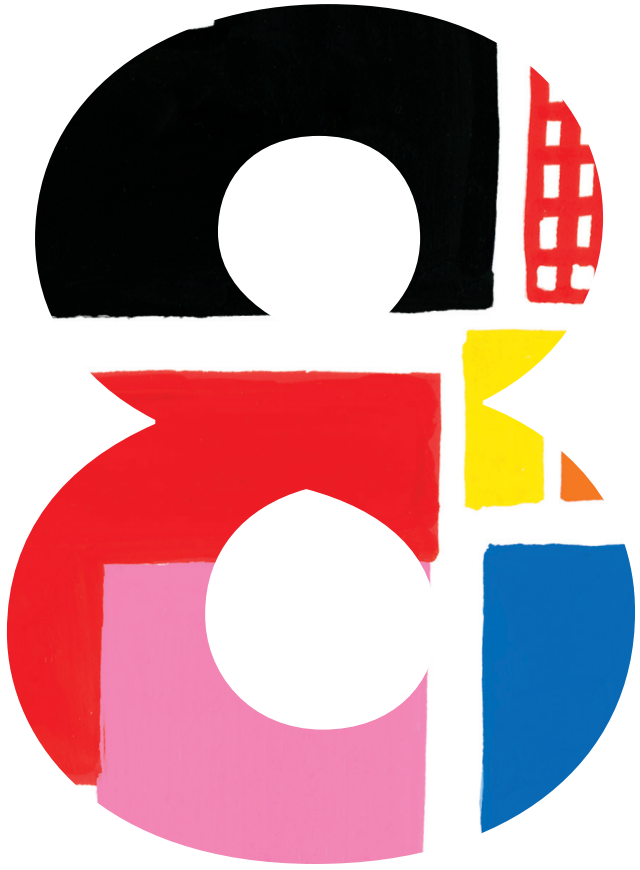

SUMMARY AND GENERAL DISCUSSION

SUMMARY

In this thesis, I sought to explain whether the ´Freemartin-effect` is present in humans by investigating whether uterine development in Mayer-Rokitansky-Küster-Hauser (MRKH) syndrome is inhibited by exposure to AMH in utero, for which a male co-twin is the identified source. MRKH syndrome is a congenital disorder which is characterized by aplasia of the uterus and upper part of the vagina. The etiology is yet unknown. The general introduction of this subject is provided in **chapter 1.** In dizygotic cattle twin pairs, placental connections between the twins are often present, resulting in placental blood exchange. In opposite-sex twins this results in severe congenital malformation of the female internal genitalia. Anti-Müllerian hormone (AMH) is produced by the testes of the bull embryo during early embryonic development. Because vascular anastomoses in the placenta allow blood exchange and hormonal transfer between the male and female embryo, AMH can be transferred from bull to cow. AMH exposure in the cow results in regression of the Müllerian duct.[1] This means the uterus and upper part of the vagina in the cow are not developed. The cow is born infertile and is called a Freemartin.[2] Besides AMH, also blood stem cells are exchanged through the vascular placental anastomoses. This results in XX/XY twin chimerism in the Freemartin, the manifestation of a genetically distinct cell-line originating from their co-twin.

In part one of this thesis, we have evaluated the presence of twin chimerism in humans. **Chapter 2** describes a systematic review of all cases reported in literature of monochorionic dizygotic twins. Traditionally, it is understood that human dizygotic ('fraternal') twins always have a dichorionic placenta, and monochorionic twins are always monozygotic ('identical'). In a dichorionic placenta, both twins have their own placenta, but with a monochorionic placenta vascular anastomoses connect the two different fetal circulations. In our systematic review, we report 31 cases of dizygotic twins with a monochorionic placenta, in which blood chimerism was demonstrable in 90% of the twins. The reported prevalence of genital anomalies (15.4%) in opposite-sex twins may suggest an association with intrauterine hormonal transfer between twins. This means that close observation of genital anomalies is recommended in chimeric twins. In the review, we show that assisted reproductive technology (ART) was responsible for the origin of the monochorionic dizygotic pregnancy in 82% of the cases. While the precise explanation of monochorionic dizygotic twin formation is uncertain, ART is proposed as a risk factor. Moreover, most monochorionic dizygotic twins are discovered by accident and it can be argued that it is more common than has been assumed until now. However, the prevalence is still unclear. Awareness of this unusual twinning resulting in chimerism is important, with subsequently correct medical strategy in prenatal testing, pregnancy

measures, and parental counseling. Similarly, the resulting (blood) chimerism is essential to consider in pre- and postnatal testing.

A limited amount of chimeric cells is referred to as microchimerism, which can have various sources. It can occur for example through bidirectional maternal-fetal exchange. The presence of some male cells in a female circulation is called male microchimerism, which is well documented in women following pregnancy with a male fetus. Some other sources for microchimeric cells are hypothesized to be having a twin or an older brother, sexual intercourse or unrecognized pregnancies. In **chapter 3**, we assessed male microchimerism in female twins from same- and opposite-sex twin pairs, their singleton sisters and their mothers. We set out to test if a male co-twin is a major source for male microchimeric cells and compared females from oppose-sex twin pairs to others in twin family pedigrees. With this study we also could explore various mechanisms for microchimerism exposure (e.g. having sons, older brothers and genetics). The participants for this study came from the Netherlands Twin Register (NTR) Biobank. The study included 446 adult women with a median age of 34 years: 62 females with a twin brother, 80 females of monozygotic twin pairs, 68 females of dizygotic female twin pairs, 130 singleton sisters and 106 mothers. From all subjects, blood derived DNA samples were tested for the presence of male microchimerism by a highly sensitive Y-chromosome-specific real-time quantitative PCR, with use of DYS14 marker.

We found a high prevalence of microchimerism with 26.9% of participants having detectable male microchimerism in their peripheral blood samples. Of the dizygotic females with a co-twin brother, 27.4% tested positive for male microchimerism, compared to 23.5% of dizygotic females with a twin sister (P = 0.61). The prevalence was highest in the mothers of twins (38.7%). Age had a positive relationship with the presence of male microchimerism. Overall, there was a tendency that prevalence of male microchimerism is greater in women with an older brother (31.4%), compared to those without (24.0%), P = 0.09. Women with or without male offspring had a similar prevalence of male microchimerism (26.0% and 28.0%, respectively; P = 0.63). Despite the presence of a male co-twin in utero, females with a twin brother have a similar prevalence of male microchimerism compared to females with a female twin sister or to their singleton sisters, implying that the presence of a male co-twin does not increase risk of male microchimerism.

In the second part of this thesis, I evaluated MRKH syndrome as a possible consequence of intrauterine AMH transfer between opposite-sex twins. We hypothesized that intrauterine blood exchange with a (vanished) male co-twin, and subsequent AMH exposure, influenced regression of the Müllerian duct in MRKH syndrome. In **chapter 4,** we described the results of an observational case-control study to compare the presence of male microchimerism in women with MRKH syndrome and control women, as evidence of fetal exposure to male blood. The MRKH women were enrolled through recruitment

via the Dutch patients' association of women with MRKH. The control group comprised women who volunteered to participate in an earlier study at our hospital and reported never having been pregnant. In all study subjects, peripheral blood samples were obtained by venipuncture, and genomic DNA was extracted. Male microchimerism was detected by Y-chromosome- specific real-time quantitative PCR, with use of DYS14 marker.

The study included 194 women: 95 women with MRKH syndrome, with a mean age of 41 years, and 99 control women, with a mean age of 30 years. The prevalence of male microchimerism was significantly higher in the control group than in the MRKH group (17.2% versus 5.3%, P = 0.009). There were no differences between women with or without microchimerism in occurrence of alternative sources of XY cells, such as older brothers, previous blood transfusion or history of sexual intercourse. Predominant absence of male microchimerism in adult women with MRKH syndrome does not support our hypothesis that intrauterine blood exchange with a (vanished) male co-twin is the pathophysiological mechanism. The significant difference in our study, in favor of the control group, may suggest that a substantial proportion of the microchimerism could be explained by other sources, such as unrecognized pregnancies or the harboring of microchimeric cells after sexual intercourse.

In **chapter 5** we hypothesized that testes-secreted hormones may have been present in early embryonic development in MRKH syndrome. We investigated two anthropometric biomarkers for prenatal androgen exposure. The measurement of the anogenital distance (AGD) can be used as biomarker for intrauterine androgenic influence. The reports of a longer AGD in women with polycystic ovary syndrome (PCOS) have contributed to the idea that PCOS has an intrauterine origin and is influenced by prenatal exposure to androgens.[3,4] In women with severe endometriosis the opposite has been hypothesized, the presence of a shorter AGD possibly reflects prenatal estrogenic influence.[5] Also the ratio between the 2nd and 4th digit (2D:4D ratio) is considered a potential indicator of androgen exposure during fetal development.[6-8]

We performed an observational case-control study in which a total of 172 women were recruited: 43 women with MKRH syndrome, 43 women with PCOS, 43 women with endometriosis and 43 control women. Anogenital distance was measured from the anus to the anterior clitoral surface (AGDac) and from the anus to the posterior fourchette (AGDaf). For the digit ratio, we used a direct, as well as a computer-assisted graphical measurement to measure the length of the 2nd and 4th digit. By comparing measures of the AGD and 2D:4D ratio to control women, and to women with PCOS and endometriosis, we could verify our measurement method and determine whether there is evidence for prenatal exposure to hormones in MRKH syndrome.

In women with MRKH syndrome, the AGDaf was significantly longer compared to the other three groups. The other biomarkers showed no association with the presence of

MRKH syndrome. This reveals some evidence for prenatal androgen exposure in MRKH syndrome. After adjustment for BMI and age, AGDac was the shortest in endometriosis and the longest in PCOS. This is consistent with the concept of AGD measurement and follows existing literature on this subject, suggesting a prenatal androgenic environment in PCOS and an estrogenic prenatal environment in endometriosis. For the 2D:4D ratio no associations were found.

In the third part of this thesis, I describe therapeutic possibilities for having children in women with the MRKH syndrome. **Chapter 6** reports on the gestational surrogacy program in the VU University Medical Center over a 10-year period. Various medical conditions can indicate the need for a gestational carrier, such as (i) congenital (MRKH) or acquired (post-hysterectomy) absence of the uterus, (ii) a serious medical condition that contra-indicates for a pregnancy or (iii) a non-functioning uterus. Due to the regulations in the Netherlands, the intended parents are themselves responsible to find a suitable gestational carrier (e.g. sister or friend). It requires the presence of functional ovaries in the intended mother; oocytes are retrieved after ovarian stimulation. The oocytes are then fertilized *in vitro* (IVF) with semen of the father. The resulting embryo is transferred into the uterus of the gestational carrier. Gestational surrogacy treatment is controversial and not allowed by law in a number of countries in Europe. Since 2006 the VU University Medical Centre in Amsterdam has been the only hospital in the Netherlands performing IVF treatment in gestational surrogacy. From 2006 to 2016, 93 IVF cycles were initiated in 60 intended mothers, with subsequent 184 single embryo transfers in 63 gestational carriers. This resulted in 34 live births. At least one live birth was achieved for 55.0% of intended couples. Pregnancy was complicated in 20.6% by a hypertensive disorder. None of the pregnancies was complicated by preterm birth. Labor was induced in 52.9% and the Caesarean section rate was 8.8%. Post-partum hemorrhage (>500 ml) occurred in 23.5%. Using an extensive intake procedure in our center, including medical and psychological counselling and testing, this study shows an effective non-commercial gestational surrogacy program. However, the observed risk for adverse obstetric outcomes in gestational carriers, who had previous non-complicated pregnancies and deliveries, requires extensive counselling during the intake procedure and careful perinatal monitoring.

Another therapeutic possibility, uterus transplantation (UTx), is currently being investigated worldwide in several studies. This procedure involves that a uterus from a brain-dead donor or a live-donor (for example a family member of friend) is surgically removed and transplanted. Sweden started with experimental UTx in mice more than 20 years ago and developed this technique into a clinical procedure with successful transplantations and the first live birth in 2014. At the time of writing, five years after the first live birth, worldwide 19 children have been reported born after UTx. In the Netherlands,

no UTx have been carried out yet. In **chapter 7** we performed a feasibility study to search for ethical, medical and financial support for performing this procedure at the Amsterdam UMC, location VUmc. Furthermore, in collaboration with the patients' association we asked women with the MRKH syndrome to fill out a questionnaire. We found that the majority (64.8%) of these women thought positively about uterus transplantation with live-donor, with 69.6% already having a potential donor available. To investigate the feasibility of the procedure, we pointed out important stakeholders and discussed the ethical principles, surgical risks and financial aspects of the procedure. It includes two complex surgeries with yet unknown consequences for the unborn child. The costs are calculated to be around €100.000 and will not be compensated by medical insurance. This 'non-life-saving transplantation' requires careful balancing of risks and benefits. Moreover, alternatives for having children are available in the Netherlands, by adoption or surrogacy. We have concluded that at this time, it is not feasible to establish the uterus transplantation procedure at our hospital. We will closely follow the developments and will re-evaluate the feasibility in the future.

GENERAL DISCUSSION

One of the major goals of this thesis was to study whether the Freemartin-effect is present in MRKH syndrome. Here we will discuss our findings in relation to this aim. The reported association of a longer anogenital distance in MRKH provides some support for our general hypothesis that the Müllerian duct abnormalities with the syndrome may have originated from female overexposure to AMH, along with androgens, early in pregnancy. However, most of the results of our studies are not in support of our hypothesis on AMH transfer coming from a male (vanished) co-twin. This means we only found limited evidence for the Freemartin-effect in MRKH syndrome. We discuss our findings by answering the following questions:

- Why do opposite-sex twin pregnancies in humans not result in evident Freemartinism?
- Is the presence of Freemartinism excluded in humans?
- What is the prevalence of twin chimerism in humans?
- What can we say about the etiology of the MRKH syndrome?
- What are the future perspectives on maternal desires for women with MRKH syndrome?

Why do opposite-sex twin pregnancies in humans not result in evident Freemartinism?

In male embryonic development, Sertoli cells in the testes secrete AMH from the time of testicular differentiation. This results in regression of the Müllerian duct in early pregnancy. In female embryonic development, AMH levels are very low. Only after birth AMH is produced by ovarian follicles, when the Müllerian duct is no longer sensitive to the hormone.[9] Subsequently, high levels of AMH (median 148 pmol/liter (20.7 µg/L)) are present in umbilical cord blood of males, while AMH is undetectable or very low (median <2 pmol/liter (<0.3 µg/L)) in umbilical cord blood of females.[10,11]

So, in the situation of a shared intrauterine environment with a male and female co-twin, in which the male embryo secretes significant hormones during embryonic sex differentiation, why does this not influence Müllerian duct development in the female? Why do (the majority of) opposite-sex twin pregnancies in humans not result in Freemartinism?

The first obvious reason is that in the large majority of human dizygotic twins there are no placental vascular connections, so direct blood exchange—with transfer of AMH—between opposite-sex twins does not occur. Most dizygotic twins have a dichorionic placenta and vascular anastomoses are uncommon in a dichorionic placenta.[12-14] Prevalence of monochorionic dizygotic twinning is unclear, yet is considered to be an exception with only several cases in literature reported, usually found by coincidence. This is in contrast to the twinning situation in cattle where placental fusion frequently occurs. Frank Lillie, who made the first detailed description of the Freemartin in 1916, showed that both arterial systems in dizygotic cattle twins 'light up' after injecting ink in the umbilical artery of one of the twins.[2,15] However, the anastomosis of blood vessels of the placenta is rare in humans.

Besides direct blood contact via vascular connections, another possibility for the exchange of AMH could be via the blood of the mother. It should then pass the placenta from fetal to maternal side and again in reverse from maternal to fetal side to the other twin. AMH is a polypeptide with large molar mass of 140 kDa, with a biologically active form comprising two identical monomers of 12.5 kDa.[16] It is known that hormones larger than 12 kDa are not able to pass the placenta barrier. There are few reports on maternal AMH levels during gestation in humans, the majority of which have not investigated the possible influence of fetal sex on maternal AMH levels.[17] However, the study of Santillan reveals that maternal AMH levels in the first trimester are positively associated with male fetal sex.[18] An experiment in cattle showed that cows carrying a male fetus have significantly greater increase in serum AMH level during pregnancy than cows carrying a female fetus, supporting that fetal sex alters maternal AMH during pregnancy.[19] It is however unclear if such an increase in maternal AMH level is the result of direct transfer from male fetus to mother or that possibly ovarian AMH production in the mother is influenced by fetal sex.

A recent experimental study in mice, in which fluorescent AMH was injected in a pregnant mouse after which the placenta was studied, shows there is no AMH transfusion over the placenta from maternal to fetal side.[20](*suppl 2H)* This may show that AMH exchange via the mother is excluded.

It should be noted that, although rare, there are several cases reported in literature of females with a twin brother *with* a monochorionic placenta. The majority of these females are born with a normally developed uterus. It is possible that the vascular connections are not yet present, or active, during the window of uterine development. As has been described in the book entitled 'Hormones' by Norman (pp 148): "By the end of the 8th week, the fate of the Müllerian ducts has already been determined: If no AMH has been produced by then, the uterus and fallopian tubes will develop, where withdrawal of AMH after the 8th week will not stop Müllerian duct regression".[16] Moreover, in relation to a vanishing twin, the timing of the demise can be essential. An early vanishing twin, before AMH production, could prevent AMH transfer. This has been shown during experimental Freemartin-studies in cows. When the placental anastomoses between the male and female twin were interrupted before 45 days of gestation, this prevented regression of the Müllerian duct and therefore the occurrence of Freemartinism.[21]

Another explanation for the absence of freemartinism in humans may be that there *is* indeed placental AMH transfer between the twins, but with no effect in the female. This effect has been studied in the marmoset monkey.[22] These animals possess interesting reproductive features, namely that singleton pregnancies rarely occur, and the majority of offspring consists of dizygotic twins in which a fused placenta with vascular anastomoses is formed. This results in the presence of blood chimerism in almost 100% of marmoset twins. However, the 'intrauterine position effect', as described in the introduction of this thesis, is minimal in the marmoset. A female marmoset with a male co-twin is known to be born with XX/XY chimerism, but also with a uterus. In this monkey, mutations in AMH-related genes have been discovered, possibly protecting the female from the male hormones.[23]

Is the presence of Freemartinism excluded in humans?

So, taking abovementioned facts into consideration, must we conclude that the presence of Freemartinism is excluded in humans? No, we do not definitively refute the hypothesis.

The case report describing the absence of a uterus in a girl born as an XX/XY chimera after monochorionic placentation with a male co-twin, could represent the Freemartin-effect in humans.[24] Of the total cohort of dizygotic opposite-sex monochorionic twins, this is the only report of absence of the uterus. However, since monochorionic placentation is rare in dizygotic twins, it means that in literature now reported, freemartinism exists in 3.8% of the opposite-sex dizygotic twins. A publication bias should be considered. Still, this could suggest intrauterine hormonal transfer in a small portion of the opposite-sex twins.

Also, by measuring the anogenital distance (AGDaf; anus—posterior fourchette) as biomarker, we found limited but first evidence for inadvertent prenatal androgen exposure in MRKH syndrome. This might be an indication for temporary exposure to altered gonadal hormone concentrations, including AMH. The source of these hormones is to be hypothesized, evidently the Freemartin-effect should be considered. The intrauterine exposure to hormones produced by a male co-twin has been reported to result in an increased anogenital distance in female cattle[25]. Other sources for androgenic influence should be also considered. It might be from internal production related to WNT4-gene mutation, possibly present in our patient group. This mutation is present in a clinical disorder in which failure of Müllerian duct formation is combined with hyperandrogenism. Possibly this condition is associated with an androgenic environment also influencing the anogenital distance.

Our finding of an increased AGDaf in MRKH syndrome provides some support for our general hypothesis that the Müllerian duct abnormalities with the syndrome may have originated from female overexposure to AMH. However, the male microchimerism study and the other anthropometric biomarkers did not reveal an association with MRKH syndrome. It must be considered that our methodology in the microchimerism study was insufficient to trace possible AMH exchange, as we used an indirect measurement method. AMH transfer via diffusion through fetal membranes or amniotic fluid may still have been a possibility. A challenging task for further research is to identify whether AMH exchange does occur in opposite-sex twins, for instance by measurement of AMH in amniotic fluid or in umbilical cord blood during opposite-sex twin pregnancy.

Studying large datasets of twins may allow to identify a possible relation of congenital malformations with hormonal transfer in opposite-sex dizygotic twinning. By collaborating with large international twin registries, the incidence of MRKH syndrome in females with a twin brother compared to the general population could provide more insight. Also, the impressive birth registries in some European countries could provide the unique opportunity to study clinical consequences of (vanishing) twin pregnancies.

What is the prevalence of twin chimerism in humans?

Despite the presence of a male co-twin in utero, adult women with a twin brother have a similar prevalence of male microchimerism compared to women with a twin sister or compared to their singleton sisters, implying that the prevalence of persistent twin chimerism in humans is low. This was also concluded in a recent study in which adult twin pairs were analyzed for chimerism using STR assays, in this study no chimerism was detected.[26] The absence of evident twin chimerism contributes to the earlier statement that opposite-sex twin pregnancies in humans do not result in evident Freemartinism. However, a study in

4-year old children detected blood group chimerism in 8% of dizygotic twins.[27] We do have to consider that twin chimerism is temporary, and can decrease with age.

However, the prevalence of microchimerism in a general adult population is significant. We detect male microchimerism in around one in four adult women. This is in line with findings from literature.[28-30] The high prevalence of male microchimerism in a general population of adult women highlight a need for further study of microchimerism origin. Potential natural sources for the male cells are hypothesized to be older brothers (or discontinued male pregnancies from their mother), unrecognized male miscarriages, vanishing twins, or possibly sexual intercourse without pregnancy. Our study shows a relationship between age and presence of microchimerism. It must be considered that increased exposure to unexplored variables due to age may be involved in chimerism risk. We also show a tendency of higher prevalence of male microchimerism in women having an older brother, supporting trans-maternal cell flow as source for microchimeric cells. It has been suggested that the origin may not necessarily be a close family member due to the possibility of sequential fetal-maternal exchanges resulting in chimerism shared across generations.[31] It has even been proposed that every human is born as microchimera, with a yet unidentified source of donor cells.[32] Longitudinal studies of chimerism presence and concentration are warranted to further understand this phenomenon in human biology.

What can we say about the etiology of the MRKH syndrome?

MRKH is a heterogeneous disorder. This is illustrated by the presence of extra-genital malformations, then described as atypical (or type 2) MRKH syndrome. This includes mainly renal defects and various skeletal malformations. This atypical form is reported in about half of the women in our study cohort, which is in agreement with larger MRKH cohorts.[33] A small portion of women with this atypical form are diagnosed with the MURCS association, this represents the most severe form which is characterized by uterine (Müllerian duct), Renal and Cervico-thoracic Somite malformations. The uterine anomaly with MRKH is also heterogeneous. This can be differentiated as the classical form, with symmetric uterine buds and normal fallopian tubes, or the partial form, with aplasia of one or both uterine buds and dysplasia of one or both tubes.[34]

Experiments in mice support our hypothesis of AMH involvement.[35] Overexpression of AMH results in a phenotype resembling MRKH syndrome.[36,37] The clinical heterogeneity of the internal genitalia with MRKH, could be a reflection of timing and duration of exposure to AMH. Furthermore, it could be hypothesized that other Müllerian duct anomalies, i.e. uterus didelphys or arcuate uterus, may possibly reflect a sliding scale of AMH exposure or AMH effect with yet unknown source. Moreover, the association with renal malformations suggests that defects occur during embryonic development of the intermediate mesoderm, as this is the structure from which internal genitalia and kidneys derive.

The etiology of MRKH syndrome is complex and presumably heterogeneous. A comprehensive review concerning the genetics of MRKH, suggests some evidence for "an autosomal dominant inheritance pattern, with incomplete penetrance and variable expressivity".[38] This is based on the heterogeneous inheritance pattern. Most cases are isolated, but occasional familial cases of MRKH syndrome have been reported and even within one family, the MRKH phenotype may differ.[39] Multifactorial inheritance is also supported by the absence of uterine malformations in biological daughters of women with MRKH syndrome, born through surrogacy.[40,41] Although in literature there have been no reports of daughters with MRKH syndrome to a mother with MRKH syndrome, counseling regarding recurrence risk remains complex. The recurrence risk is estimated to be 1 - 5%, since MRKH is assessed as a disorder with multifactorial etiology.[42] Therefore, genetic counseling should be offered in women with MRKH prior to surrogacy treatment.

To further clarify the role of (epi)genetics in the development of MRKH syndrome, extensive techniques, such as whole genome sequencing and genome-wide epigenetic studies should be considered in affected families, including investigating genital tract tissue to account for tissue-specific epigenetic features.

What are the future perspectives on maternal desires for women with MRKH syndrome?

Generally, the diagnosis of MRKH syndrome is made in adolescence, when a young woman with a normal female development and karyotype (46,XX) presents with primary amenorrhoea at the age of 16 years or older. The diagnosis can be accompanied by uncertainty about the female role and femininity and psychological distress.[43] Sexual consequences should proactively be discussed to help women develop sexual self-confidence.[44] Psychological support at critical times may be helpful.[45] In the Netherlands, a semi-structured group program is available for women with MRKH syndrome which has positive effects on the general feeling of wellbeing.[43]

The maternal desires are also an important subject during the counselling of women with MRKH syndrome. Gynecologists should discuss this subject in an early stage and explain the possibilities for having a biological or non-biological child. Explanation of, and clarity on their reproductive potential may help women to cope with concerns regarding fertility. At time of writing this thesis, MRKH women in the Netherlands can only have their own biological children through gestational surrogacy. A significant hurdle in this treatment is that the intended mother is responsible for finding another woman as gestational carrier. For many women finding a suitable gestational carrier is very difficult to impossible. Possibly an agency that mediates between intended parents and possible surrogates, as existing in the UK, can be a solution. Since recently, there has been a proposal

for a new law in which mediation between intended parents and surrogate parents is possible with a government license on a non-profit base.

The new technology of uterus transplantation is a ground-breaking surgical procedure. We believe that international collaboration with more experienced clinics in UTx may provide access to this special and advanced technology for Dutch women. It is important to realize this technique is still under scientific evaluation and cannot be regarded as routine fertility treatment. Furthermore, the ethical aspects are essential, but are also expected to evolve over time and advancement of the technique.

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NEDERLANDSE SAMENVATTING

Dit proefschrift is het resultaat van het promotieonderzoek van H.E. Peters, met de naar het Nederlands vertaalde titel: 'Intra-uteriene uitwisseling: chimerisme en hormonen'.

In dit proefschrift heb ik onder andere onderzoek gedaan naar een mogelijke oorzaak van het Mayer- Rokitansky-Küster-Hauser (MRKH) syndroom. Het MRKH-syndroom is een aangeboren aandoening bij vrouwen waarbij de baarmoeder en het bovenste gedeelte van de vagina (van de schede) niet is aangelegd. Het is onbekend waardoor dit wordt veroorzaakt. In koeien bestaat er een zelfde soort aandoening, deze koeien worden ook zonder baarmoeder geboren. Zo'n koe wordt een 'kwee' genoemd, in het Engels een 'freemartin'. Deze koeien worden meestal samen geboren met een tweelingbroer. Bij twee-eiige tweelingparen in koeien bestaat er vaak een gedeelde placenta (de moederkoek) met vaatverbindingen, waarbij er bloeduitwisseling tussen de tweeling kan plaatsvinden. In de mannelijke stier-embryo wordt al vroeg in de zwangerschap het anti-Muller hormoon (AMH) geproduceerd door de testes van de stier. Dit hormoon zorgt ervoor dat tijdens de embryonale ontwikkeling de buis van Müller in regressie gaat. Dit zorgt ervoor dat de stier, zoals gebruikelijk, zonder baarmoeder wordt geboren. Echter de bloeduitwisseling via de placenta zorgt ervoor dat het hormoon AMH kan worden overgedragen naar de vrouwelijke koe-embryo. Blootstelling aan dit hormoon zorgt ervoor dat er in de koe geen baarmoeder en geen vagina wordt aangelegd. Naast de uitwisseling van AMH worden ook stamcellen uitgewisseld via het bloed, dit leidt tot *chimerisme* in deze koeien. Chimerisme betekent dat er een genetisch aparte cellijn te vinden is naast het eigen genetische materiaal. Freemartin-koeien hebben naast de vrouwelijke XX cellijn, ook mannelijke XY-cellen in hun bloed. In dit proefschrift heb ik onderzocht of het ´Freemartin-effect´ aanwezig is bij mensen. Onze hypothese was dat de baarmoederontwikkeling bij het MRKH syndroom op dezelfde manier wordt geremd door blootstelling aan AMH tijdens de embryonale ontwikkeling.

In deel één van dit proefschrift hebben we de aanwezigheid van chimerisme, in het bijzonder als gevolg van een tweelingzwangerschap, bij mensen geëvalueerd. In **hoofdstuk 2** wordt een systematisch overzicht gegeven van alle twee-eiige tweelingen met een gedeelde placenta die in de wetenschappelijke literatuur gerapporteerd zijn in mensen. In principe was bij mensen lange tijd het idee dat twee-eiige tweelingen altijd twee aparte placenta's hebben en dat tweelingen met één gedeelde placenta dus altijd identieke tweelingen moeten zijn (in tegenstelling tot bij koeien). Het is belangrijk om te bedenken dat er als gevolg van een gedeelde placenta in de baarmoeder bloeduitwisseling mogelijk is tussen de tweeling, en bij twee aparte placenta's in principe niet. In ons literatuuroverzicht rapporteren we 31 twee-eiige tweelingparen mét een gedeelde placenta. In 90% van deze tweelingen was er sprake van chimerisme. Een groot gedeelte van deze tweelingen waren van verschillend geslacht, en in 15% van deze tweelingparen werd er een genitale afwijking gevonden bij één van de tweeling. Dit zou verband kunnen

houden met uitwisseling van hormonen via de placenta. Er wordt aanbevolen dat hier aandacht voor moet zijn bij chimerische tweelingen. Ook laten we zien dat geassisteerde voortplantingstechnieken (zoals een IVF-behandeling) in 82% ten grondslag lagen aan het ontstaan van deze tweelingzwangerschappen. Hoewel de precieze oorzaak niet duidelijk is, worden geassisteerde voorplantingstechnieken beschreven als risicofactor. De exacte prevalentie van dit soort tweelingzwangerschappen is niet duidelijk. Echter blijkt uit ons review dat deze tweelingen meestal per toeval worden ontdekt en er kan worden gesteld dat het vaker voorkomt dan tot nu toe werd aangenomen. Bewustzijn van dit zeldzame type tweelingzwangerschap en het resulterende chimerisme is essentieel tijdens de beoordeling van prenatale testen en zwangerschapscomplicaties bij tweelingen.

Microchimerisme is de term die gebruikt wordt indien er een kleine hoeveelheid chimerische cellen aanwezig is. Dit kan verschillende bronnen hebben, bijvoorbeeld door uitwisseling van bloed tijdens de zwangerschap, van moeder naar foetus en andersom. Ook een tweelingbroer of zus, een oudere broer of zus of miskramen worden verondersteld mogelijke bronnen voor microchimerisme te zijn. De aanwezigheid van een kleine hoeveelheid mannelijke cellen in vrouwelijk bloed wordt mannelijk microchimerisme genoemd. Dit is voornamelijk bestudeerd bij vrouwen die zwanger zijn geweest van een jongen. In **hoofdstuk 3** hebben wij mannelijk microchimerisme bestudeerd bij vrouwelijke tweelingen met een tweelingbroer of een tweelingzus, hun eenling zussen en hun moeders. We hebben onderzocht of een tweelingbroer een bron is voor mannelijke chimerische cellen. Met deze studie konden we ook verschillende mechanismen voor het ontstaan van microchimerisme onderzoeken (bijvoorbeeld het hebben van zonen of oudere broers en genetica). De deelnemers aan dit onderzoek waren afkomstig van de Nederlands Tweeling Register (NTR). De studie omvatte 446 volwassen vrouwen met een gemiddelde leeftijd van 34 jaar: 62 vrouwen met een (twee-eiige) tweelingbroer, 68 vrouwen met een twee-eiige tweelingzus, 80 vrouwen met een eeneiige tweelingzus, 130 eenling zussen en 106 moeders. Van alle proefpersonen werd DNA uit bloed getest op de aanwezigheid van mannelijk microchimerisme. Hiervoor werd een kwantitatieve PCR-methode uitgevoerd gebruikmakend van een Y-chromosomale marker (DYS14).
We vonden een prevalentie van mannelijk microchimerisme van 26.9% in de hele groep vrouwen. Van de vrouwen met een twee-eiige tweelingbroer was 27.4% positief voor mannelijk microchimerisme, vergeleken met 23.5% van de vrouwen met een twee-eiige tweelingzus (P = 0.61). De prevalentie was 16.3% in vrouwen van eeneiige tweelingparen, en 25.3% in de eenling zussen. De prevalentie was het hoogst bij de moeders van een tweeling (38.7%). Leeftijd had een positieve relatie met de aanwezigheid van mannelijk microchimerisme. De prevalentie van mannelijk microchimerisme werd niet verklaard door het hebben van een zoon of een oudere broer. Er kan dus geconcludeerd worden dat,

ondanks de directe aanwezigheid van een mannelijke foetus in de baarmoeder, vrouwen met een tweelingbroer een vergelijkbare prevalentie van mannelijk microchimerisme hebben in vergelijking met vrouwen met een tweelingzus of hun eenling zussen. Dit impliceert dat de aanwezigheid van een tweelingbroer het risico op mannelijk microchimerisme niet verhoogt. Ook tonen deze bevindingen een hoge prevalentie van mannelijk microchimerisme aan bij volwassen vrouwen, die prominenter is naarmate ze ouder worden, maar niet verklaard wordt door de aanwezigheid van broers of zonen, wat de noodzaak van verder onderzoek naar de oorsprong van microchimerisme benadrukt.

In het tweede deel van dit proefschrift heb ik onze hypothese over het ontstaan van het MRKH-syndroom bestudeerd. We hebben onderzocht of intra-uteriene bloeduitwisseling met een tweelingbroer (mogelijk een 'vanished twin') de regressie van de buis van Müller heeft veroorzaakt door blootstelling aan AMH. De term 'vanished twin' wordt gebruikt om een zwangerschap te beschrijven die is begonnen als tweelingzwangerschap, waarbij er uiteindelijk toch een eenling geboren wordt doordat er een miskraam optreedt bij één foetus, meestal in het eerste trimester. In **hoofdstuk 4** hebben we de resultaten beschreven van een observationele case-control studie waarin we de aanwezigheid van mannelijk microchimerisme bij vrouwen met MRKH-syndroom en controle-vrouwen hebben vergeleken. Het mannelijk microchimerisme wordt gebruikt als bewijs van foetale blootstelling aan mannelijk bloed (eventueel afkomstig van een tweelingbroer). Via de Nederlandse patiëntenvereniging van vrouwen met MRKH hebben we de vrouwen gevonden voor deelname in de MRKH groep. De controlegroep bestond uit vrouwen die zich vrijwillig hadden aangemeld om deel te nemen aan een eerdere studie in ons ziekenhuis, en hadden gerapporteerd nooit zwanger te zijn geweest. Bij alle vrouwen werd er DNA verkregen uit bloed. Mannelijk microchimerisme werd onderzocht met een kwantitatieve PCR-methode, gebruikmakend van een Y-chromosomale marker (DYS14). De studie omvatte 194 vrouwen: 95 vrouwen met het MRKH-syndroom, met een gemiddelde leeftijd van 41 jaar, en 99 controle vrouwen, met een gemiddelde leeftijd van 30 jaar. De prevalentie van mannelijk microchimerisme was significant hoger in de controlegroep dan in de MRKH-groep (17.2% versus 5.3%, $P = 0.009$).
Afwezigheid van mannelijk microchimerisme bij vrouwen met het MRKH-syndroom ondersteunt niet onze hypothese, dat intra-uteriene bloeduitwisseling met een (verdwenen) tweelingbroer de verklaring is voor het ontstaan van dit syndroom. Het onverwacht gevonden hoge percentage microchimerisme in de controlegroep, kan erop wijzen dat een aanzienlijk deel van het microchimerisme kan worden verklaard door andere bronnen, zoals niet-herkende zwangerschappen of het opslaan van microchimere cellen na geslachtsgemeenschap.

In **hoofdstuk 5** veronderstelden we dat mannelijke hormonen mogelijk aanwezig waren in de vroege embryonale ontwikkeling bij het MRKH-syndroom. We hebben twee

biomarkers van prenatale blootstelling aan androgenen onderzocht: de meting van de anogenitale afstand (AGD) en de verhouding tussen de lengte van de wijs- en ringvinger (2D: 4D ratio). Eerdere resultaten van een langere AGD bij vrouwen met polycysteus ovariumsyndroom (PCOS) hebben bijgedragen aan het idee dat PCOS een intra-uteriene oorsprong heeft en mogelijk wordt beïnvloed door prenatale blootstelling aan androgenen. Bij vrouwen met ernstige endometriose is juist het tegenovergestelde beschreven, de aanwezigheid van een kortere AGD zou de prenatale oestrogene invloed weerspiegelen. We voerden een observationele case-control studie uit waarbij in totaal 172 vrouwen werden geïncludeerd: 43 vrouwen met MKRH-syndroom, 43 vrouwen met PCOS, 43 vrouwen met endometriose en 43 vrouwen in de controlegroep. De anogenitale afstand werd gemeten van de anus tot de clitoris (AGDac) en van de anus tot de commissura posterior (AGDaf). Voor de 2D:4D ratio hebben we een directe meting met liniaal en een computer-meting gebruikt om de lengte van de wijs- en ringvinger te meten. Onze onderzoeksresultaten laten zien dat bij vrouwen met het MRKH-syndroom de AGDaf significant langer is in vergelijking met de andere drie groepen. De andere biomarkers vertoonden geen associatie met MRKH. Dit laat enig bewijs zien voor prenatale blootstelling aan androgenen bij het MRKH-syndroom. De AGDac was het kortste in vrouwen met endometriose en het langste bij vrouwen met PCOS. Dit volgt de bestaande literatuur en suggereert een prenatale androgene omgeving in PCOS en een prenatale oestrogene omgeving bij endometriose. Voor de 2D: 4D-ratio werden geen associaties gevonden.

In het derde deel van dit proefschrift beschrijf ik de therapeutische mogelijkheden voor het krijgen van kinderen voor vrouwen met het MRKH-syndroom. **Hoofdstuk 6** rapporteert over de hoogtechnologisch-draagmoederschap (HTDM) behandelingen in het VU medisch centrum over een periode van 10 jaar. Deze behandeling houdt in dat er een IVF behandeling wordt uitgevoerd bij de beoogde moeder, waarbij er eicellen worden verkregen die buiten het lichaam worden bevrucht met zaadcellen van de vader. Het resulterende embryo wordt geplaatst in de baarmoeder van een draagmoeder. Verschillende medische aandoeningen kunnen leiden tot de noodzaak van deze behandeling, zoals aangeboren (MRKH) of verworven (na een operatie) afwezigheid van de baarmoeder, een ernstige medische aandoening die een contra-indicatie is voor een zwangerschap of een niet-functionerende baarmoeder. Door de wetgeving in Nederland zijn de beoogde ouders zelf verantwoordelijk voor het vinden van een geschikte draagmoeder (bijvoorbeeld een zus of een vriendin), die gezond is en zelf een zwangerschap en vaginale bevalling heeft doorgemaakt die zonder grote problemen is verlopen. De HTDM-behandeling is maar in een aantal Europese landen wettelijk toegestaan. Van 2006 tot en met maart 2017 was het VU medisch centrum in Amsterdam het enige ziekenhuis in Nederland dat deze HTDM behandelingen uitvoerde. Wij hebben de uitkomsten van al deze HTDM behandelingen onderzocht.

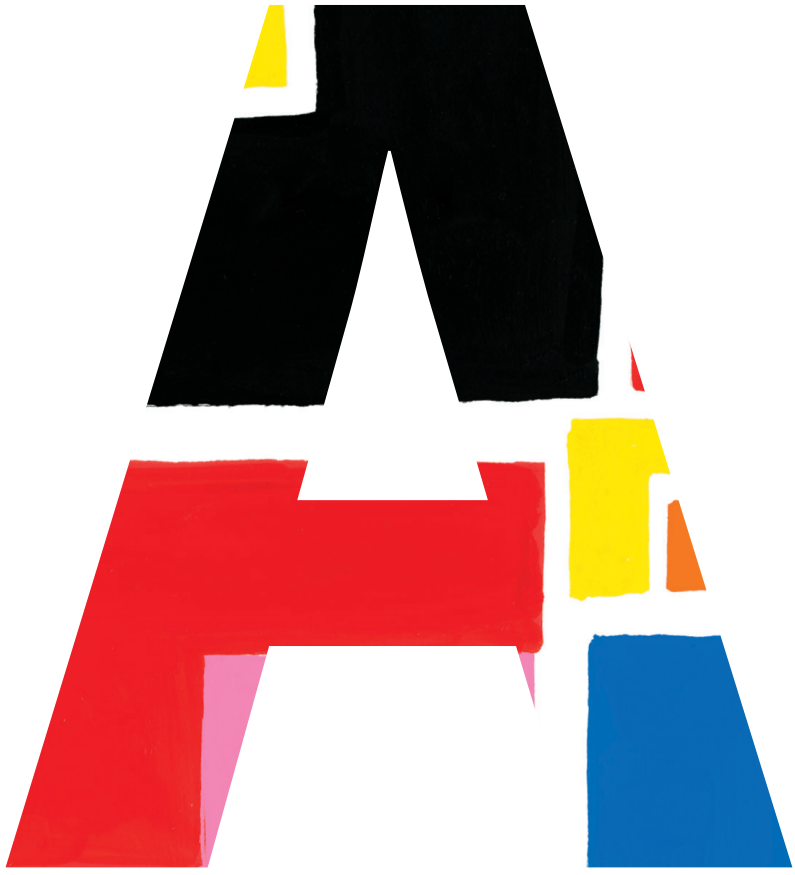
Na een uitgebreide medische en psychologische screening zijn er in deze periode 60 wensouders gestart met de behandeling, met 63 draagmoeders (3 ouders hadden verschillende draagmoeders). In deze groep zijn er 93 IVF behandelingen gestart, en hebben er 184 embryo-terugplaatsingen plaatsgevonden. Er zijn 34 kinderen geboren, waarbij 55.0% van de koppels die aan deze behandeling begon, ten minste één baby kreeg. In 26% van de zwangerschappen bleek de draagmoeder een hoge bloeddruk te hebben ontwikkeld aan het einde van de zwangerschap, waardoor de bevalling moest worden ingeleid. Geen van de zwangerschappen werd gecompliceerd door vroeggeboorte. Daarnaast had 23.5% van de draagmoeders ruim bloedverlies (>500ml) na de bevalling, in de meeste gevallen was hier geen bloedtransfusie of extra operatie voor nodig. 8.8% draagmoeders zijn bevallen met een keizersnede. In dit onderzoek laten we zien dat, met behulp van een uitgebreide intakeprocedure in ons centrum inclusief medische en psychologische beoordeling, de HTDM behandeling in Nederland goede resultaten geeft. Er is wel een iets verhoogd risico op complicaties tijdens de zwangerschap en bevalling. Dit vereist goede counseling tijdens de intakeprocedure en zorgvuldige begeleiding tijdens de zwangerschap en bevalling.

In **hoofdstuk 7** beschrijven wij een haalbaarheidsonderzoek naar de baarmoedertransplantatie. Deze behandeling wordt momenteel wereldwijd onderzocht. De procedure houdt in dat een baarmoeder van een hersendode donor of een levende donor (bijvoorbeeld van een familielid) operatief wordt verwijderd en getransplanteerd. Zweden begon meer dan 20 jaar geleden met experimentele baarmoedertransplantaties bij muizen en ontwikkelde deze techniek tot een succesvolle behandeling, waarbij de eerste baby in 2014 werd geboren uit een getransplanteerde baarmoeder. Circa vijf jaar nadien zijn er wereldwijd 19 kinderen gerapporteerd in de medische literatuur die geboren zijn na baarmoedertransplantatie. In Nederland is deze behandeling nog nooit uitgevoerd. We hebben een onderzoek uitgevoerd om de ethische, medische en financiële ondersteuning voor deze procedure te onderzoeken in het Amsterdam UMC, locatie VUmc. Ook hebben we, in samenwerking met de patiëntenvereniging, vrouwen met het MRKH-syndroom gevraagd een vragenlijst in te vullen over deze experimentele behandeling. Hieruit blijkt dat de meerderheid (64.8%) van deze vrouwen positief staat tegenover een baarmoedertransplantatie met levende donor, en bijna 70% van deze vrouwen wisten zelfs al een potentiële donor die haar baarmoeder zou willen afstaan. Om de haalbaarheid van de procedure te onderzoeken bespraken we de ethische principes, de chirurgische risico's en de financiële aspecten van de procedure. Het omvat twee complexe operaties met nog onbekende gevolgen voor het ongeboren kind. De kosten zijn rond de €100.000 en worden niet vergoed door een ziektekostenverzekering. Deze 'niet-levensreddende transplantatie' vereist een zorgvuldige afweging van risico's en voordelen. Bovendien zijn er in Nederland alternatieven voor het krijgen van kinderen,

zoals adoptie of draagmoederschap. We hebben geconcludeerd dat het op dit moment niet haalbaar is om de procedure van een baarmoedertransplantatie in ons ziekenhuis uit te voeren. We blijven de ontwikkelingen volgen en evalueren de haalbaarheid in de toekomst opnieuw.

In **hoofdstuk 8** van dit proefschrift worden alle resultaten bediscussieerd. Het onderzoek beschreven in dit proefschrift is begonnen met onze 'Freemartin'-hypothese over het ontstaan van het MRKH-syndroom. De beschreven langere anogenitale afstand bij vrouwen met MRKH biedt enige ondersteuning voor onze algemene hypothese dat dit syndroom mogelijk is ontstaan door overmatige blootstelling aan AMH, samen met androgenen, vroeg in de embryonale ontwikkeling. De meeste resultaten van onze studies ondersteunen onze hypothese over AMH-overdracht van een tweelingbroer echter niet. Dit betekent dat we slechts beperkt bewijs hebben gevonden voor het Freemartin-effect bij het MRKH-syndroom. Ook laten we zien dat mannelijk microchimerisme even vaak voorkomt in vrouwen met een tweelingbroer, vergeleken met vrouwen met een tweelingzus of vergeleken met hun eenling zussen, wat impliceert dat de prevalentie van tweeling-chimerisme bij mensen laag is. Echter de prevalentie van microchimerisme in een algemene populatie is ongeveer 25%. Deze hoge prevalentie onderstreept de noodzaak van verder onderzoek naar de oorsprong en mogelijke bronnen van microchimerisme.

Tijdens de begeleiding van vrouwen met het MRKH-syndroom speelt een kinderwens een belangrijke rol. Op dit moment kunnen MRKH-vrouwen in Nederland alleen hun eigen biologische kinderen krijgen door hoogtechnologisch draagmoederschap. Een belangrijke hindernis bij deze behandeling is dat de MRKH vrouw zelf verantwoordelijk is voor het vinden van een draagmoeder. Recent is er een voorstel gedaan voor een nieuwe wet waarin er een bemiddeling mogelijk is tussen wensouders en draagmoeders. Dit kan de behandeling voor een breder publiek toegankelijk maken. Daarnaast is de nieuwe techniek van baarmoedertransplantatie een baanbrekende chirurgische techniek. Wij geloven dat internationale samenwerking met meer ervaren klinieken mogelijk ook Nederlandse vrouwen toegang zou kunnen geven. Het is belangrijk om te beseffen dat deze techniek nog steeds wordt onderzocht en niet kan worden beschouwd als reguliere vruchtbaarheidsbehandeling. Bovendien spelen de ethische aspecten een belangrijke rol, welke mogelijk zullen veranderen bij de ontwikkeling en vooruitgang van de techniek.



APPENDICES

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- Oral presentation: 'Anti-Mullerian hormone levels and features of PCOS in adolescence'

2017 33nd Annual Meeting of ESHRE, Geneva, Switzerland
- Poster presentation: 'Unusual twinning resulting in chimerism: a systematic review on MCDZ twins'

2017 The Joint 4th World Congress on Twin Pregnancy & 16th Congress of the ISTS, Madrid, Spain
- Oral presentation: 'Chimerism in dizygotic twins: an overview and clinical relevance'

2019 35th Annual Meeting of ESHRE, Vienna, Austria
- Oral presentation: 'Anti-Müllerian hormone levels in adolescence in relation to long-term follow up for presence of polycystic ovary syndrome'

2019 19th Congress of the International Society of Psychosomatic Obstetrics and Gynaecology (ISPOG), The Hague, the Netherlands
- Oral presentation: 'Low prevalence of male microchimerism in women with MRKH syndrome'

National presentations

2017 & 2019 Jaarlijkse bijeenkomst patiëntenvereniging 'Stichting MRK-vrouwen'

2017 Wetenschappelijke discussiedag Voortplantingsgeneeskunde, UZ Gent
- Session: 'Vroege zwangerschap' & 'Chimerisme'

2018 FC Noord Holland
- Oral presentation: MEASURE studie

2018 Refereeravond Verloskunde & Gynaecologie, Amsterdam UMC
- Oral presentation: case study of a patient (award for best presentation)

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OVER DE AUTEUR

Henrike Emma Peters werd op 14 april 1987 geboren in Nieuwegein, als tweede dochter van ouders Jos en Maria. In de kindertijd werd er veel gedanst, getennist en braaf huiswerk gemaakt. Na het behalen van haar gymnasiumdiploma aan het Oosterlicht College in Nieuwegein startte zij met de studie Geneeskunde aan het AMC te Amsterdam. Een drukke en gezellige studietijd volgde, met o.a. een bestuursjaar voor studievereniging MFAS. Na een stage in het ziekenhuis in Jakarta gecombineerd met een lange reis, startte ze in 2010 met haar coschappen.

Tijdens haar coschappen ontstond haar passie voor de gynaecologie. Na haar afstuderen begon zij in 2013 als arts-assistent Verloskunde & Gynaecologie in het Diakonessenhuis te Utrecht. In 2015 startte zij als protocollen-arts in het IVF centrum en op de afdeling voortplantingsgeneeskunde van het VU Medisch Centrum. Onder begeleiding van promotoren Nils Lambalk, Velja Mijatovic, Dorret Boomsma en copromotor Marieke Verhoeven werd er in 2016 een protomotietraject opgestart leidend tot deze dissertatie.

Per september 2019 is zij met veel enthousiasme gestart met de opleiding tot gynaecoloog aan het VU Medisch Centrum. Momenteel volgt zij het eerste deel van haar opleiding in het Diakonessenhuis te Utrecht.

Henrike woont samen met Jeroen en dochter Suus in Amsterdam. Binnenkort volgt er nog meer gezinsuitbreiding.