

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 opposite-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 MRKH 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 or 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.