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## Perinatal reproductive endocrinology in singletons and twins

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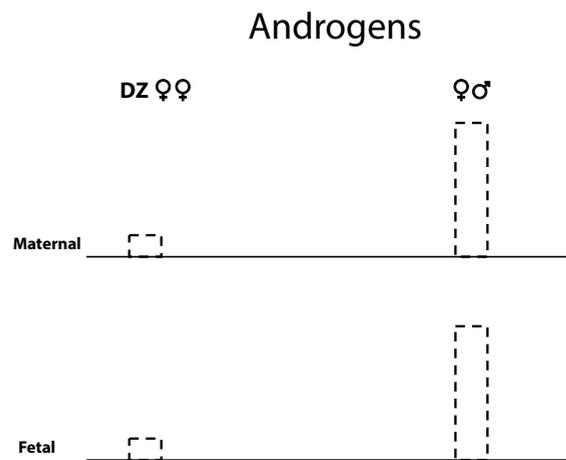
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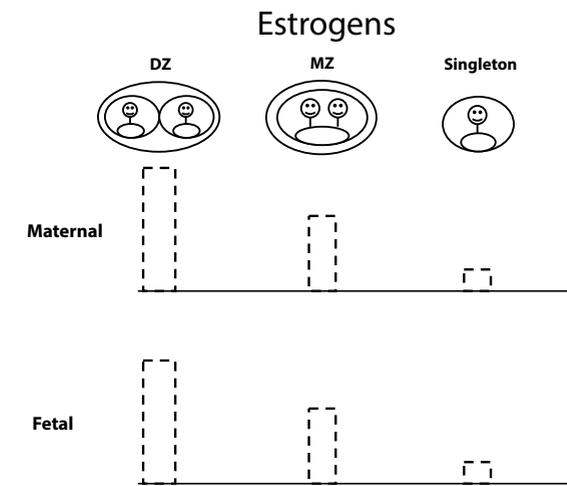


Development of disease in adult life is suggested to, at least partly, have its origin in the womb. For example, under-nutrition resulting in a higher prevalence of coronary heart disease, type 2 diabetes, bronchitis and an altered lipid profile <sup>1</sup>. Furthermore, there are associations between intra-uterine growth restriction or pre-eclampsia and maternal elevated testosterone concentrations during gestation, which is associated with polycystic ovary syndrome, metabolic syndrome and cardiovascular disease in the offspring <sup>1-6</sup>. Not only physical implications but also changes in mental health such as autistic disorders, depression, schizophrenia and changes in gender related play behaviour have been described in this context <sup>1,7,8</sup>. Indirectly, there is evidence suggesting that fetal reproductive hormones in multiple pregnancies seem to influence their co-twin. A study analysing a birth cohort from Finland before the age of contraception, reported a reduced fecundity in women born as part of an opposite-sex twin. Over-exposure to androgens produced by their male co-twin was suggested to be the cause of this loss of reproductive capacity <sup>9</sup> (figure 1). From animal studies we know that over-exposure during gestation to androgens that extremely exceed the physiological range, leads to polycystic ovary syndrome (PCOS) like traits <sup>10</sup>. If this is true for humans as well, girls that are born as part of an opposite-sex twin may be influenced by the androgens produced by their brother, resulting in a higher prevalence of PCOS in this group.



**Figure 1** A visual representation assumed in existing literature, on how girls of opposite-sex twins are influenced by their male co-twin. DZ ♀♀ = girl of a dizygotic girl-girl twin, ♀♂ = girl of an opposite-sex twin. Androgens during gestation and post-partum are suggested to be higher in girls who have a male co-twin compared to girls of DZ girl-girl twins, due to androgens produced by the brother.

Furthermore, intra-uterine exposure to high estrogen concentrations is thought to explain an observed increased risk of developing breast or testicular cancer in dizygotic twins <sup>11</sup> (figure 2).



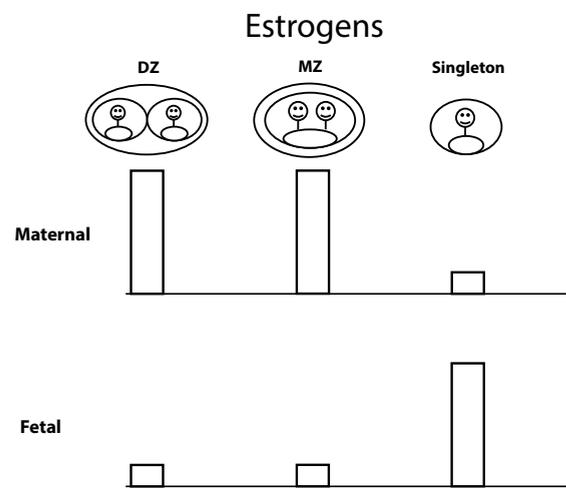
**Figure 2** A visual representation assumed in existing literature of potential estrogen exposure in twin and singleton pregnancies and their offspring. DZ = dizygotic twin, MZ = monozygotic twin. During gestation and post-partum DZ twins are suggested to be exposed to higher estrogen concentrations compared to MZ twins and singletons.

All these observations and assumptions underline the importance to obtain adequate information on reproductive hormone concentrations during gestation. However up until now standardized, longitudinally measured reproductive endocrine data during gestation and in the peri- and postnatal period are lacking, especially for twin pregnancies <sup>12</sup>.

## Main results and conclusions

In this thesis we provided hormonal profiles of twin pregnancies, neonates and singleton controls and analyse aspects of their suggested role in disease development later in life, for example PCOS. To be able to, non-invasively, measure hormones in 6 weeks old neonates we validated the use of urinary gonadotropin measurements against serum concentrations as a gold standard. After correction for creatinin, urinary FSH and LH reflect serum values properly and can be used as a substitute for serum testing. As testicular volume in adult males is an expresses of its function, we measured testicular volume, by means of ultrasound, in 6 weeks old neonates in order to evaluate testicular capacity. It turned out that no referential values were available for ultrasonographically measured testicular volumes in pre-pubertal boys at that time. We provided for the first time these normative data for 0 to 6 year old boys and found that in the first year testicular volume and shape changes significantly. We observed a double hub in testicular growth in the first year which was present in all ethnic

groups indicating a physiological phenomenon. Apparently, apart from the acknowledged mini-puberty around 3-4 months (resulting in increased testicle size at 5 months) there is another hormone peak leading to increased testicular growth around 1-2 months of age. Unfortunately, we did not measure hormones at time of ultrasound evaluation while creating normative data, however, our main study did provide FSH concentrations in cord blood and in urine at 6 weeks of age. In singleton boys we found indeed lower FSH levels at time of birth (median 0.2 IU/L) compared to 6 weeks of age (median 0.6 IU/L). The complete hormonal profiles for pregnant mothers and their neonates provided much information, of which the most important results are discussed here. Against all expectations, although mothers of twins have higher estrogen concentrations during gestation, their children have lower estrogen concentrations at birth (figure 3).

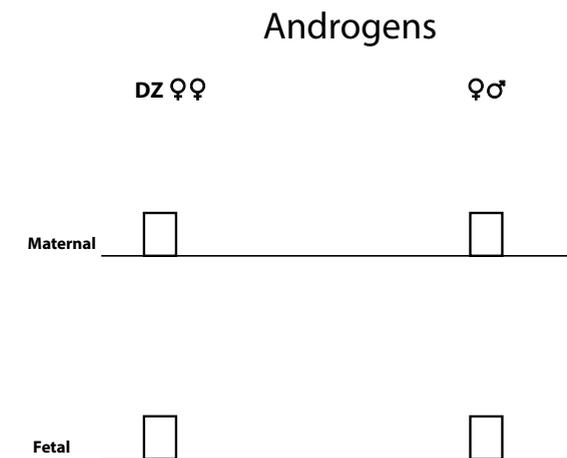


**Figure 3** An overview, based on our results, of estrogen concentrations in twin and singleton pregnancies and their offspring.

We observed the same paradox for progesterone concentrations. Our hypothetical explanation is that with a twin pregnancy there is an increased total mass of placental hormone production and a much larger fold fetal blood volume compared to that of the mother resulting lower concentrations in each fetus and still higher concentrations in the mother. These outcomes challenge the general assumption that maternal serum hormone levels are a reflection of actual fetal hormone exposure. Although no significant differences in concentrations of the various estrogens between mothers of MZ and DZ twins were found, our data do indicate that DZ twins might be exposed to more estrogens, because in cord blood they have higher estradiol concentrations compared to MZ twin neonates. This could

be an explanation for the reported higher chance of developing hormone related tumours (breast, testis) for dizygotic twins compared to monozygotic twins <sup>11</sup>.

Furthermore, opposite-sex twins influence each other however, it seems in a different manner than could be expected based on the existing literature. The emphasis lies mainly on the suggested androgenic effect of the male co-twin on his sister. Remarkably, we found no differences in cord blood androgen concentrations in girls of opposite-sex twins compared to girls of DZ same-sex twins (figure 4). This indicates that either androgens do not have an effect on the female fetus of an opposite-sex twin, probably due to aromatase activity of the placenta which also prevents these girls from virilisation, or androgens do influence these girls but it does not result in an effect on hormone concentrations visible directly after birth. This might be a more epigenetic phenomenon which reveals itself later during childhood or after puberty as, for instance, a PCOS like trait. More prone, male type behaviour in girls from opposite-sex twins is reported <sup>13</sup> however in a large cohort comparing the prevalence of PCOS in women born as part of an opposite-sex twin compared to same-sex twins we found no increased prevalence of PCOS among opposite-sex twins.



**Figure 4** A visual representation based on our results, of androgen concentrations in girls from opposite-sex twins compared to girls from DZ same-sex twins. DZ ♀♀ = girl of a dizygotic girl-girl twin, ♀♂ = girl of an opposite-sex twin. Androgens are not different in girls from opposite-sex and DZ same-sex twins.

Although girls show no signs of direct androgenic effects in cord blood, boys of opposite-sex twins have lower LH and inhibin B concentrations compared to boys of a DZ boy-boy twin. It seems that in opposite-sex twins the girl is able to inhibit the hypothalamic-pituitary-testicular axis of her brother.

Mechanisms through which this inhibition is accomplished are difficult to comprehend. For instance, increased estrogen concentrations could induce central inhibition however we did not find elevated estrogen levels in cord blood of opposite-sex twin boys. This is in line with the assumption that fetal ovaries are virtually inactive with regard to steroid hormone production at time of birth and the fact that fetal testicular tissue produces no significant amounts of estrogens either. Therefore, estrogens do not seem to fit the profile. Since we cannot point to any specific known mechanism it is likely that potentially less known mechanisms act here, such as unknown placental compounds which are capable of inhibiting pituitary GnRH sensitivity. From mid-gestation onwards the hypothalamic-pituitary axis is controlled by kisspeptin regulated GnRH activity<sup>14</sup>. Although kisspeptin seems to predominantly stimulate GnRH secretion<sup>15</sup>, depending on the nucleus that is activated in the brain, it sometimes results in negative feedback<sup>16</sup>. Kisspeptin is produced in large amounts by the placenta and possibly more extensive in case of a twin pregnancy. Unfortunately we did not measure kisspeptin in our study. Feedback systems were more extensively studied in adult male twins. We found that FSH, LH, inhibin B, SHBG and testosterone concentrations are highly heritable but, in contrast to previous data, we observed no differences in hormone feedback mechanisms operating in different types of twins and singletons.

### Future perspectives

The findings of this thesis may contribute to a better understanding of the intra-uterine environment, with regard to reproductive hormone profiles, and the role it plays in singleton and twin pregnancies. On the other hand these findings also lead to questions and implications for future research, some of which are described in the following paragraphs. As a direct result of our findings it could be interesting to measure kisspeptin activity in cord blood samples in order to try and understand more about its possible role in fetal and neonatal feedback mechanisms. Furthermore, longitudinally measured hormone profiles in boys combined with regular ultrasound measurements of their testicle volumes in the first year of life are still unavailable and might shed more light on a period which is important in priming testicles for future reproductive function.

### Substitutes for fetal serum

As a substitute for fetal serum we have used maternal serum for measuring hormones during gestation. Others have used amniotic fluid, only a few studies have data on actual cord blood hormones during gestation as it involves major risks for the pregnancy. Amniotic fluid could be a better reflection of the fetal compartment as it is mostly fetal urine. It would be wise to evaluate the correlation between amniotic fluid (corrected for creatinin) reproductive

hormone concentrations and maternal, and if possible, fetal serum concentrations. Amniotic fluid could be obtained when a regular amniocentesis is done and a maternal serum sample should be taken at the same time for comparison. However, one should take into account that reproductive hormones might not be excreted in a sufficient amount into the amniotic fluid at time of the amniocentesis yet.

### Androgen effects

The origin of androgens in women with PCOS is still debatable. Its origin could be maternal, endogenous production during gestation, endogenous production after puberty as a result of an inherited predisposition or due to imprinting of the fetus. A direct effect of maternal androgens during gestation is highly unlikely, as most steroids are not able to pass the placenta, or when they do, they are metabolized immediately. When focussing on polycystic ovary syndrome (PCOS) we have demonstrated that having a male co-twin does not implicate an increased change of developing PCOS for the female co-twin. This indicates that opposite-sex twin girls are not likely androgenized in utero by their brothers, which is confirmed by the fact that we could not find higher cord blood androgens in these girls. However, PCOS is an inherited disease, affecting about 1 in 20 women in their fertile lifespan. Whether this is purely genetically inherited or whether androgens originating from the mother, male co-twin or the girl herself that circulate during gestation could cause imprinting of the female fetus to eventually demonstrate the PCOS phenotype is an interesting subject which needs further attention.

### Follow up

For now we concluded that in opposite-sex twins the fetuses influence each other, which at birth is more prone in boys compared to girls, resulting in differences in hormone profiles. We believe that these same changes might occur in the girls as well, however they do not become apparent until later stages of life. Therefore, a follow-up study should be conducted of this cohort at the ages of 5 and 10 years. This follow-up study should include at least hormonal screening, behavioural analyses and measurements of the 2D:4D digit ratio, which is an easy indicator of intra-uterine androgen exposure<sup>17,18</sup>.

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