

The central aim of this thesis was to gain an understanding into the complex network of genetic and environmental factors that contributes to DZ twinning in humans. In this thesis I therefore first present an extensive review on the current knowledge of the epidemiology, genetics and endocrinology of DZ twinning. Next, I describe three studies completed with data collected in a large mailed questionnaire. Finally, I describe a pilot study to the feasibility of biological sample and interview data collection in Dutch families in which both sisters have had DZ twins.

In this final chapter, I first summarize the conclusions from the main results from the three questionnaire studies presented in this thesis and the pilot study. I then discuss the methodology and results obtained from the questionnaire studies. Finally, I briefly discuss the research done on the genetics of DZ twinning and the implications for future studies. I discuss the recruitment of the Dutch part of the study “Genetics of DZ twinning” and describe the results for future linkage and association studies.

Summary

Chapter 4 looked at the willingness to answer to questions about mode of conception of twin pregnancies in a large survey study that was completed by mothers of twins from the Netherlands Twin Registry (NTR). The amount of missing data was examined and by using data from earlier survey studies, responders and nonresponders were compared with respect to their answers to questions on assisted reproduction techniques. In contrast to the findings from an earlier study carried out in Australia, we found no indication that mothers of twins were not prepared to answer questions on mode of conception. In the survey, only a small number of mothers did not fill in the question on mode of conception and the amount of missing data did not differ from the amount for more neutral questions, such as height of the mother. In addition, we assessed the reliability of the question on mode of conception by comparing the survey data with hospital records in a subsample of 80 mothers of twins. This comparison of the survey data with the hospital records showed that mothers can accurately report on the mode of conception of their twins.

In chapter 5 and 6 two studies are described using the questionnaire data set. In chapter 6 data from 19,357 surveys were analyzed and in chapter 5, data from 20,150 surveys were used (including 793 surveys that were returned at a later date). Data from mothers who were not the biological mother of the twin pair were excluded; surveys with missing data on ART and zygosity were also excluded. In chapter 5 mothers without data on the question regarding familial twinning were excluded from data analysis.

Chapter 5 looked at familial twinning and fertility indices in mothers with spontaneous MZ and DZ twins and in mothers who conceived their twin offspring after the use of ART. Participants in this study consisted of 8,222 probands with spontaneous DZ twins, 5,505 probands with spontaneous MZ twins, 4,164 probands with ART DZ twins and 250 probands with ART MZ twins. Of these probands with spontaneous DZ offspring, 61 mothers had had multiple sets of spontaneous DZ twins. This study showed that probands with spontaneous DZ twins more often reported female relatives with twins than probands with spontaneous MZ twins. Also the proportion of DZ versus MZ familial twinning in probands with spontaneous DZ offspring was larger than in probands with MZ offspring. The first group also reported a shorter time to conceive. Probands with ART twins had fewer siblings and less often reported relatives with twins than probands with spontaneous twins. There was a trend for probands with multiple sets of DZ twins to report more familial twinning than probands with a single set of DZ twins. I did not observe that DZ twinning was more familial in probands who had their twins before age 36 years. Familial DZ twinning is clearly present in mothers of spontaneous DZ twins. The mechanisms underlying

spontaneous and non-spontaneous DZ twinning are different and fertility treatment should be taken into account in any study of twinning. Twinning is not more familial in women who have their twins at a younger age.

Chapter 6 examined differences in body composition and smoking between mothers of spontaneous MZ and DZ twins, while taking into account maternal age, gravidity and educational attainment. Participants in this study were mothers of spontaneous DZ twins (N=8,515) and mothers of spontaneous MZ twins (N=5,663) registered with the NTR. This study showed that spontaneous DZ twinning is significantly associated with increasing height (odds ratio=1.6, CI 1.5 - 1.8 for the tallest versus the shortest height quartile), and increased BMI (odds ratio=1.3, CI 1.1 - 1.4 for overweight vs. normal weight) and with smoking before the twin pregnancy (odds ratio=1.4, CI 1.3 - 1.5 for smoker vs. non-smoker). Maternal age and gravidity, but not educational attainment, had to be included in the model. It can be concluded that spontaneous dizygotic twinning is associated with body composition and smoking.

The study described in chapter 7 has shown that, in the Netherlands, it is feasible to collect blood and urine samples, by making home visits to participants. The samples were collected in the morning after overnight fasting. After the home visit, blood samples were immediately transported by car to the laboratory. Women with a natural cycle were visited on days 2-4 of menstrual cycle and women on oral contraceptives were visited during their pill-free week. All mothers of DZ twins are interviewed by telephone. The effects of transportation and storage on blood quality, lipids, RNA with and without challenge, lymphocytes and other parameters were examined. Genomic DNA was isolated from blood and cells were immortalized using Epstein-Barr virus. In 78.6 % of the women with a natural cycle blood samples were collected on the second, third or fourth day of the menstrual cycle. I concluded that the pilot study demonstrated the feasibility of this protocol to collect good quality of plasma, DNA, RNA and lymphocyte samples by home visits.

Discussion

Willingness to reply to survey items

Chapter 4 shows that mothers who participated in the questionnaire study correctly filled out questions regarding the mode of conception of their twins. Also, mothers who conceived their twins after assisted reproductive techniques were as likely to return the questionnaire as mothers who spontaneously conceived their twins. These findings indicate that the mailed questionnaire provides an accurate reflection of reality, with respect to questions regarding the mode of conception and most likely also to other less sensitive issues as well.

Familial twinning

It has long been known that genetic background contributes significantly to variation in DZ twinning both between and within populations. In chapter 3 of this thesis I describe in detail the studies done on the inheritance of DZ twinning (Weinberg, 1900; Greulich, 1934; Wyshak and White, 1965; Bulmer, 1970; Parisi et al., 1983; Meulemans et al., 1996; Lewis et al., 1996). The main conclusion from these studies is that DZ twinning runs in families and that female relatives of mothers of DZ twins have an increased risk of having DZ twins themselves. The trait can be carried by both father and mother, but can only be expressed in women.

In our study of familial twinning (chapter 5), familial DZ twinning was clearly demonstrated in mothers of spontaneous DZ twins. This association predominantly applied to the female relatives, but

equally to the female relatives on the fathers and on the mothers' side of the probands' family. Our study further extends the current understanding on familial twinning because I also examined familial DZ twinning in mothers of twins conceived after assisted reproductive techniques. Iatrogenic factors might interact with genetic factors causing DZ twinning or these factors might affect twinning independently. I found that, at least with respect to the genetic susceptibility of having DZ twins, it is necessary to treat spontaneous and artificially conceived twins as two separate groups. This study provides researchers the scientific ground to do so.

Body composition smoking and spontaneous DZ twinning

In the last 10 years, there have been a number of new developments in our understanding of mechanisms contributing to spontaneous DZ twins. For example, it was found that body composition (Basso et al., 2005; Reddy et al., 2005) and smoking (Morales-Suarez-Varela et al., 2007) also seems to contribute to the variation in DZ twinning frequency. The study presented in chapter 6 confirmed these associations. I found that overweight and smoking prior to the pregnancy of the twins are associated with spontaneous DZ twinning. In addition to previous studies I took into account maternal age, gravidity and educational attainment. Another advantage of our study is that I found that increased bodyweight and smoking are independently associated with DZ twinning. Interestingly, these factors are also associated with decreased fertility, while DZ twinning is associated with high fecundity. The question arises how fertility decreasing factors can also be associated with mothers of DZ twins, who are thought to be very fertile? Although the answer remains unclear, a possible explanation might be that women prone to conceive DZ twins are less likely to be affected by factors which normally decrease fertility. In other words, it is not that more DZ twins are born due to for example smoking or overweight, but the explanation is that fewer singletons and MZ twins are born to mothers who are less resistant to the fertility decreasing effects of smoking, overweight and maternal age.

General approach

The major part of this thesis is based on the data obtained from a 2 page survey mailed to all mothers of twins registered with the NTR. A major strength of this kind of data collection is the large attainment and high response in a short period of time. This study was done within the setting of the NTR and therefore I had additional data on mothers participating in previous NTR surveys. This gave us the opportunity to examine the possibility that nonresponse bias affected the results of the studies done with data from the questionnaire. The analyses on nonresponse in this questionnaire demonstrated small, but significant differences between responders and nonresponders. However, the same differences were seen in mothers of spontaneous DZ and MZ twins. It is therefore unlikely that response bias influenced the comparisons between DZ and MZ twin mothers.

The questionnaire was initially designed to select eligible families for the DZ twinning project. I therefore explicitly asked for other biological family members with twins, specified by sex and zygosity. I also asked for additional pre-pregnancy information, such as age, parity, weight and height, family size of the mother, smoking history, and mode of conception, oral contraceptive use and folic acid use. I specifically limited the questionnaire to two pages, so mothers would not be discouraged from filling out the questionnaire by the number of questions. Because of the limited space, I choose to phrase some questions and answer possibilities in a shorter way than may be ideal. The questionnaire for example asked about smoking prior to the twin pregnancy, but did not specify if this was immediately prior to or ever

smoking prior to the twin pregnancy. However, analyses with data from previous surveys showed that in the majority of the cases women who indicated that they smoked prior to the pregnancy also indicate that they smoked the year before the twin pregnancy.

Finally, this questionnaire was sent to all mothers of twin registered with the NTR, mothers of spontaneous DZ, spontaneous MZ and artificially conceived twins replied. Results from the studies described in this thesis are therefore limited to multiple births. I assume that spontaneous MZ twin pregnancies are a random subset of all spontaneous pregnancies. It is generally found that MZ twinning is not influenced by genetic, maternal or environmental factors. If, as proposed, MZ twinning is a randomly occurring event, MZ twin mothers are just as appropriate for controls as singleton mothers. In fact, they may form an even better control group. Although the mechanisms leading to a MZ twin pregnancy are very different from the mechanisms leading to a DZ twin pregnancy, both MZ and DZ twin mothers have to carry the twin pregnancy to full-term.

Future

The search for genes associated with DZ twinning

As described in chapter 3, the genetic basis for DZ twinning has already been discovered in sheep. Three genes from the TGF β signalling pathway (GDF9, BMP15 and BMPR1B) involved in intra-ovarian signalling have been identified and result in variation in twinning frequency in sheep (Galloway et al., 2000; Hanrahan et al., 2004; Wilson et al., 2001; Mulsant et al., 2001). BMP15 and GDF9 are closely related growth factors expressed specifically in the oocyte (Galloway et al., 2000; Hanrahan et al., 2004). The third gene (BMPR1B) is the receptor for BMP15 expressed on multiple cells in the ovary (Wilson et al., 2001; Mulsant et al., 2001).

In humans, common variation in BPM15, GDF9 and BMPR1B does not seem to contribute to spontaneous DZ twinning (Derom et al., 2006; Duffy et al., 2001; Zhen Zhen Zao et al., 2008). Rare variants in GDF9 are associated with DZ twinning in a small number of families (Montgomery et al., 2004; Palmer et al., 2006). These variants account for only a small proportion of variation in DZ twinning. In addition, association between other candidate genes and twinning, FMR1 (Vianna-Morgante., 1999; Marozzi et al., 2000), FSHR (Al Hendy et al., 2000) and the protease inhibitor locus (Pi) (Boomsma et al., 1992) have also been reported but not replicated, although the Pi locus was suggested by numerous studies before the 1992 paper.

No strong association has been found between twinning and genes from the TGF β signalling pathway in the ovary, though they have an evident relationship with human fertility. Several recent studies showed that variants in BMP15 and GDF9 are associated with premature ovarian failure (POF) (Dixit et al., 2005, 2006; Di Pasquale et al., 2006; Laissue et al., 2006). Four of these POF associated variants of GDF9 are also reported in mothers of spontaneous DZ twins (Kovanci et al., 2007; Palmer et al., 2006; Zhen Zhen Zhao et al., 2008). In addition, three POF associated variants in BMP15 are also found in mothers of spontaneous DZ twins, though the frequency of these genes is very low (Zhen Zhen Zhao et al., 2008).

There are controversial findings about the association of one variant of BMP15 with POF (p.Leu263_Arg264insLeu). One study found an association (Dixit et al., 2006), while two other studies did not (Di Pasquale et al., 2006; Laissue et al., 2006).

It can be concluded that DZ twinning in humans is probably more complex than was originally thought. The search for likely candidate genes (as described above) gave some understanding of pathways

involved and not involved in spontaneous DZ twinning. Further study, for example, on the nature of the relationship between DZ twinning and POF is necessary. These studies will provide us with more knowledge of the genes responsible for spontaneous DZ twinning and will identify key mechanisms controlling ovarian function, providing a greater understanding of female fertility and infertility. Very large samples are probably needed to identify the genetic contribution to human DZ twinning.

In collaboration with the QIMR in Australia I recruited a large sample of informative families in the Netherlands and in Australia. Blood, urine samples and interview data of mothers of twins and blood samples for DNA of additional family members were collected. In the Dutch part of this study I also collected samples for RNA, hormone concentrations of e.g. LH, FSH, inhibin A and B. In order to conduct linkage with endocrine parameters I collected blood samples on the third day of the natural menstrual cycle or during the pill free week in fertile women. Likewise, once the genome-wide markers become available, other parameters, which have been assessed during the interview and home visit (e.g. height, weight) and in plasma (e.g. lipids and apolipoproteins) can be used for sib-pair based linkage analyses. At this point linkage scans are running for 125 families, including 523 individuals.

In the near future we aim to ascertain blood samples from additional informative families from the Netherlands. We eventually want to complete a 10 cM genome scan (400 markers) in 500 families from Australia and New Zealand and 500 Netherlands families and analyze linkage with the twinning phenotype.

Spontaneous DZ twinning in humans is a complex trait, influenced by environmental, maternal and genetic factors. This thesis shows that in addition to maternal age and gravidity, being overweight and smoking are significantly associated with DZ twinning. Although these factors play a role in DZ twinning their joint effect is not large. Genetic factors also play an important role and might interact with environmental and maternal factors increasing the risks of having DZ twins. Future studies of the genetic basis of spontaneous DZ twinning are needed to further unravel this complex phenomenon.