Dizygotic Twinning

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Chapter 1

General introduction

Twinning has fascinated human beings over the centuries. Most people find it interesting to see siblings who are obviously of the same age and (partly) look alike. For scientists twins also incur a scientific interest. Twins are used in a "natural experiment" to estimate the importance of genetic factors on trait variance. Moreover, the twinning process itself is of great interest. The knowledge of the processes involved in twinning might lead to the identification of key mechanisms controlling ovarian function and provide a greater understanding of female fertility and infertility.

There are two types of twins, identical or monozygotic (MZ) twins and non-identical or dizygotic (DZ) twins (Hall, 2003). The different types of twins arise through different pathways (figure 1). The mechanism(s) leading to MZ twins affect the early development of the embryo immediately after fertilization resulting in the separation of the cells into two or more embryos (Bomsel-Helmreich et al., 2005). The mechanism(s) leading to DZ twins operate on the selection of developing follicles within the ovary so instead of one egg being released, two eggs are released ready for fertilization (Bulmer, 1970). The difference in the mechanisms leading to MZ and DZ twins is also evident in differences in the occurrence of MZ and DZ twins across ethnic groups, in the familial and genetic inheritance and in the contribution of maternal factors such as age and parity. In fact, MZ twinning seems to be a random event, while DZ twinning is not. In this thesis the focus is on the etiology of DZ twinning. To understand processes leading to DZ twinning, it is necessary to consider the events leading to fertilization and early embryo development. Fertilization is the process where a sperm penetrates a mature egg starting the development of an embryo. This is the beginning of the process of new life, but the end of a series of events required for the development and maturation of an egg.

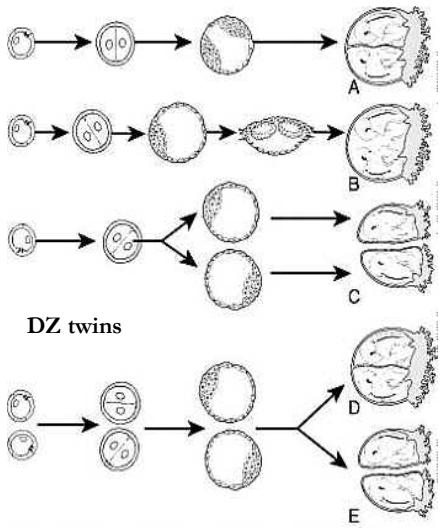


Figure 1: The two different types of twins; MZ twins A: separation of the cell between days 7-14. B: separation of the cells between days 4-7. C: separation of the cells between days 0-3. And DZ twins D: the twins share 1 placenta, E: the twins have separate placentas.

The release of the egg (ovulation) takes place in the middle of each menstrual cycle sending the egg off on the journey down the reproductive tract where it may encounter a sperm and be fertilized. One and sometimes two or more eggs are released at each menstrual cycle. This event only occurs 400 to 500 times during a women's lifetime. Therefore, only a small fraction of the original 400,000 to 600,000 egg cells a woman has in stock at birth, will make it through to ovulation and most are destined to die (Speroff et al., 2005). There are many critical steps and selection events during the growth and maturation of ovarian follicles, constituting a tightly controlled process so only one mature follicle ovulates in most cycles.

Chemical messengers or hormones play a big part in the control of reproduction. There is a small critical gland called the pituitary gland located at the base of the brain. Two hormones called Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH), released by the pituitary gland are essential for reproductive function (McGee and Hsueh, 2000). These hormones ensure that the cycle progresses through each phase; regulate the later stages of the growth of ovarian follicles (FSH) and control the process of ovulation (LH) when the egg is released for fertilization.

Co-ordination of ovarian function requires a complex regulation with signal exchange between tissues and between cells within tissues. Recent studies have demonstrated a complex signaling network within the ovarian follicle that responds to the external signals received from the hormones (Roy and Matzuk, 2006; Shimasaki et al., 2004) and that ensures coordinated growth and development of the egg cell and its surrounding support cells. It has become clear that in the follicle, it is mainly the egg cells that control this process. The egg cells send out

growth factors to the support cells to stimulate cell division and cell growth. Important in this process are two closely related growth factors which are expressed specifically in the egg and known as bone morphogenetic protein 15 (BMP15) and growth differentiation factor 9 (GDF9) (Shimasaki et al., 2004). Mechanisms involved in DZ twinning find their origin in these regulatory processes of follicle development. Also, mothers of twins are able to successfully bear twins to full term. The possibility exists that mechanisms such as implantation of the fertilized ovum in the endometrium (nidation) and embryo quality play an additional role in twinning. The question is why do some women have twins and others do not? Which environmental and genetic maternal factors contribute to an increased risk of having DZ twins?

This thesis

This thesis aims to understand aspects of the complex network of genetic and environmental factors that contribute to DZ twinning in humans. To achieve this aim I used a variety of data collection procedures. I collected data on familial twinning, pre-pregnancy information and maternal characteristics by sending out a survey to all mothers of twins registered with the Netherlands Twin Register (NTR), this data collection is described in chapter 2.1. In addition, a large-scale biological sample collection was undertaken in Dutch families in which two or more sisters gave birth to spontaneous DZ twins. This data collection is described in detail in chapter 2.2.

In chapter 3 an extensive review is presented of the current knowledge regarding the epidemiology, genetics and endocrinology of DZ twinning.

Chapters 4 through 6 focus on the survey study in which data on familial twinning and genetic and environmental factors that contribute to DZ twinning were collected. Chapter 4 reports on the willingness of twin mothers to reply to questions about mode of conception of twin pregnancies. 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 addition, the reliability of the question on mode of conception was assessed by comparing the survey data with hospital records in a subsample of twin mothers.

Chapter 5 explores the genetic contribution to DZ twinning by studying whether mothers of spontaneous DZ twins have more other family members with a DZ twin than mothers of spontaneous MZ twins and mothers of twins conceived after fertility treatment. For genetic studies it is important to know if mothers of twins conceived after fertility treatments have to be treated differently than mothers of spontaneous DZ twins in studies of familial twinning. In addition, this chapter examines whether "super mothers" (mothers who have multiple sets of DZ twins and mothers of spontaneous DZ twins from families with multiple sets of DZ twins) become pregnant in less time than mothers with a MZ twin and mothers with one set of DZ twins.

The frequency of DZ twining has dramatically increased over the last 30 years. Maternal age and the increased use of fertility treatments are considered to be the primary cause for this increase in DZ twinning. However, DZ twinning has also been associated with other factors that are quite often also associated with decreased fertility, like smoking and obesity. In chapter 6 I sought to explore the contributions of these factors to spontaneous DZ twinning.

Chapter 7 describes the biological sample and interview data collected for the study "Genetics of dizygotic twinning". This study is a collaboration between the VU University Amsterdam and the Queensland Institute of Medical Research (QIMR) in Brisbane, Australia. The goal of this project is to collect biological samples and interview data in 1000 sister-pairs who both have given birth to spontaneous DZ twins. The parents of these sister-pairs are also asked to take part in the study. These data will be used in the near future to localize

and identify gene(s) that contribute to variation in DZ twinning through linkage and association studies. Chapter 7 investigates the feasibility of the Dutch part of this large-scale sample collection.

Chapter 8 offers a summary and a general discussion of the results presented in the previous chapters. A number of recommendations and future perspectives are given based on the findings of this thesis especially with regard to the findings obtained from the study "Genetics of dizygotic twinning".

Chapter 2.1

General research design

Study 1: Mailed survey study

To study factors contributing to DZ twinning, I used two different approaches. I collected questionnaire data in a large sample of mothers of twins described in this chapter and obtained detailed interview data and biological materials in a sample of families selected to be informative for genetic linkage and association studies, described in chapter 2.2.

Mailed survey study

In April 2005, all mothers registered with the NTR (N= 33,528 women) were mailed a 2 page survey to collect information on familial twinning and the pregnancy of their twins.

The NTR obtains longitudinal data on twins and their family members in two samples: 1) in newborn and young twins (YNTR) who are registered at birth by their parents and whose parents and teachers complete questionnaires about the children and 2) in adolescent and adult twins (ANTR). In the ANTR, twins as well as their parents, siblings, spouses and more recently, children are asked to participate. Most (>90%) of the participants are born in the Netherlands (Boomsma et al., 2006; Bartels et al., 2007). A survey about twinning and fertility was sent out to all mothers who are registered with the YNTR (i.e. mothers of twins born in 1986 or later) and mothers of ANTR twins (i.e. twins born before 1986). Table 1 shows the number of proband mothers from the ANTR and the YNTR who received and returned the questionnaire. Especially in the ANTR there is a group of probands who had never before taken part in any of the NTR studies, and who were seen as "passively" registered. Although they were sent the survey, I did not expect that many mothers in this group would respond. This was indeed the case.

All probands were asked about the zygosity of their twin offspring; if they had other relatives with twin offspring; how many brothers and sisters they had themselves; about their own birth weight; and the birth weight of the father of the twins, their age at menarche and about height and weight prior to, during and after the twin pregnancy. I also asked them to report on the number of pregnancies the mother had had, including singleton births, twin- and, higher multiple births and miscarriages. A series of questions about the period before and during the twin pregnancy included the use of folic acid, oral contraceptives and whether the mother had smoked. With respect to the twin pregnancy, there were questions about spontaneous versus assisted pregnancy. Appendix II presents the complete version of the questionnaire (in Dutch).

Table 1 Number of surveys send and received and the number of participants by NTR sample.

	ANTR	YNTR	Total
Questionnaires sent	**4,839**	**28,689**	**33,528**
Actively registered mothers	2,629	22,991	25,620
Passively registered mothers	2,210	5,698	7,908
Questionnaires received	**1,858**	**17,499**	**19,357**
Received from actively registered mothers	1,662	16,021	17,683
Received from passively registered mothers	196	1,478	1,674
Cohort			
Mother born before 1960	1,828	2,905	4,733
Mother born between 1960 and 1965	23	4,708	4,731
Mother born between 1965 and 1969	1	4,598	4,599
Mother born in/after 1969	6	5,288	5,294
Total	1,858	17,499	19,357

Response and nonresponse

Some of the proband mothers registered with the NTR are passively registered, which means that they never participated in previous research projects. Although it may be an active decision not to return the survey, it is also likely that the addresses for some of these individuals are incorrect. From the group of actively registered proband mothers (N=25,620), I received 17,683 completed questionnaires. I also received 1,674 completed questionnaires from mothers who never participated before. In total 19,357 completed questionnaires were returned. Within the group of actively registered mothers, I was able to compare responders (N=17,683) and nonresponders (N=7,937) by using data from earlier NTR survey studies. This should provide some insight into possible response bias. An overview of characteristics of mothers from the ANTR and the YNTR that did or did not return the survey is listed in table 2.

Table 2 Characteristics of nonresponders and responders in the survey study in the YNTR and ANTR. Comparisons are based on earlier NTR survey studies.

		Nonresponders		Responders	
		YNTR	**ANTR**	**YNTR**	**ANTR**
Zygosity of the twin	% MZ	23.0	39.4	31.8	46.1
	% DZ	77.0	60.6	68.2	53.9
Maternal age at birth twin	% < 28	26.2	54.0	18.7	53.6
	% 28 – 30	17.8	16.4	17.5	18.0
	% 30 ≥ 32	18.1	11.3	20.2	13.3
	% 32 ≥ 35	21.9	10.1	25.6	9.6
	% > 35	16.0	8.2	18.0	5.4
Height	%< 165 cm	28.2	39.9	24.7	38.5
	% 165 ≥170 cm	20.7	27.9	21.4	26.4
	% 170 ≥ 174 cm	26.8	18.2	27.7	18.8
	% ≥ 174 cm	24.3	14.0	26.3	16.3
BMI	% < 20	14.5	6.1	13.3	7.2
	% 20 – 24,99	57.0	53.5	58.4	57.8
	% 25- 29.99	19.3	31.1	20.2	27.3
	% > 30	9.2	9.3	8.0	7.7
Use of fertility techniques to conceive the twin	% yes	16.6	-	16.6	-
	% no	83.4	-	83.4	-
Educational attainment level	% low	31.9	71.6	19.1	45.7
	% intermediate	41.0	13.6	42.8	25.3
	% high	27.1	14.8	38.1	29.0
Smoking during pregnancy	% yes	28.9	30.8	18.8	26.1
	% no	71.1	69.2	81.2	73.9
Number of sibs mother of twin	% no other sibs	4.4	0.4	3.8	1.1
	% 1 or 2 sibs	55.2	29.9	59.8	31.5
	% 3 or more sibs	40.4	69.7	36.3	67.5
Parity	% no others kids	53.9	48.7	51.0	45.3
	% 1 or more kids	46.1	51.3	49.0	54.7
The mother herself is a twin	% yes	2.5	3.1	2.4	2.8
	% no	97.5	96.9	97.6	97.2
Familial Twinning	% no other twins in family	45.8	42.3	45.7	31.6
	% other twins in family	54.2	57.7	54.3	68.4
Religion	% not religious	36.7	27.2	34.5	24.8
	% religious, not active	44.3	37.3	45.2	32.1
	% religious and active	19.0	35.5	20.3	43.1
Urbanization level	% very heavy	10.1	13.8	6.9	12.0
	% heavy	20.2	16.0	18.5	19.2
	% moderate	23.4	21.7	23.4	19.2
	% low	23.6	22.3	25.0	24.5
	% very low	22.8	26.2	26.2	25.2

A general test for response bias has been carried out and is described in chapter 6 of this thesis. Table 2 additionally shows the difference in response for the YNTR and the ANTR separately. This makes it possible to see trends in time. For example the distribution of MZ and DZ twins in the YNTR and the ANTR is different. Since the twin offspring of the ANTR mothers are born before 1986, it can be assumed that fewer women used fertility treatments to conceive the twins, probably explaining the lower number of DZ twins.

Table 2 shows that MZ twin mothers were more likely to return the questionnaire than DZ twin mothers. Also, nonresponse is associated with lower educational attainment, smoking during the twin pregnancy and having more brothers and sisters. Being younger, shorter and living in an urbanized area was also related to

nonresponse. There was no difference in response regarding BMI, parity and whether the mother herself was a twin.

Zygosity

Of all mothers who returned the survey, zygosity data were available for 18,837 their twin offspring and were missing for 520 twin pairs (in 349 cases the mother indicated that she did not know the zygosity of her twins and in 171 cases the mother did not answer the question or her answer was unclear). For these twin pairs no other information on zygosity (e.g. from earlier NTR surveys) was available.

For same-sex pairs, zygosity was based on DNA polymorphisms obtained in participants in previous NTR studies (N = 1,801) or from previous survey questions (N = 7,978). When DNA and previous survey data were not available, zygosity was based on the answers of the proband in my own survey study (N = 2,748).

Previous questions regarding offspring zygosity asked whether the twins were alike in eye-, hair- or face color and in face form and whether the twins were often mistaken for each other by their parents, other relatives and by strangers. Based on the answers to these questions, zygosity was determined in same-sex twin pairs. The association between DNA and questionnaire zygosity is 93% in the YNTR sample (Rietveld et al. 2000) and 97% in the ANTR sample (Willemsen et al., 2005). A comparison of the zygosity of same-sex twin pairs provided by the mothers in the current survey and the zygosity obtained from previous questionnaires also showed a high degree of agreement (91 %). There were 6,141 mothers with opposite-sex twin offspring and 169 mothers of di- and tri-zygotic triplets. In total 12,799 mothers of DZ twins and 6,038 mothers of MZ twins returned the questionnaire.

Chapter 2.2

General research design

Study 2: The genetics of dizygotic twinning

Genetics of dizygotic twinning

This thesis also covers the Dutch part of a collaboration with the Queensland Institute of Medical Research (QIMR) in Australia. The objective of this study is the selection and recruitment of a large sample (N=1000 pairs) of sister pairs who have both given birth to spontaneous DZ twins. If possible the parents of these sister pairs are also recruited, and in some cases (if parents were not available) additional siblings are recruited.

Several approaches can be employed to carry out molecular genetic studies aimed at localizing and identifying genes for complex traits such as DZ twinning. Localization of genes is possible by affected sister-pair linkage analysis. Candidate gene association and whole genome association studies can be used to identify genes in candidate regions or across the genome. Several designs are possible, e.g. case-control or transmission disequilibrium tests (TDT) when large groups of DZ twin mothers and their parents participate. One reason for the lack of success so far to identify the genetic contribution to human DZ twinning may be the small sample size of many studies. I therefore in collaboration with the QIMR aimed to recruit large numbers of affected sister pairs, their parents and other informative family members in the Netherlands and in Australia and collect biological samples in these participants.

So far, in the Netherlands 368 families, including 394 sister pairs and 430 families in Australia have been recruited through the NTR and Australian Twin Register (ATR) respectively. In the Netherlands, biological samples have been collected in a total of 1,393 family members.

Selection of informative families

For linkage studies I recruited informative families from the NTR in which there were multiple female relatives (usually sisters) with spontaneous DZ twins and their parents. If parents were not available, I recruited the additional siblings of the sisters. Linkage studies of affected sibling pairs test for excess sharing of marker alleles identical by descent in affected-affected sibling pairs. This method is often described as a nonparametric and model-free alternative to the parametric LOD score method. Figure 1 gives an example of a pedigree.

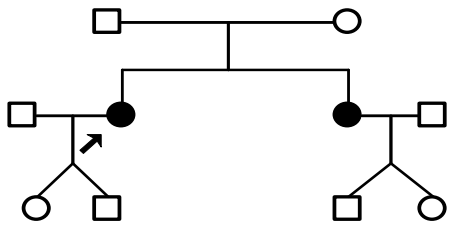


Figure 1 Pedigree of family with two twin pairs. The affected sisters with twin offspring are colored black. One of the sisters is registered with the NTR (proband) and reported that her sister is a mother of a twin pair as well. The proband mother is indicated with an arrow. Spouses of the mothers are indicated by squares. The generation above represents the parents of the sister pair and the generation below indicates the twins.

The affected sister pairs are also informative for association studies. However, association can also be carried out with case-control designs. For case-control designs, all mothers of DZ twins (regardless of whether or not they have a sister or another female relative who is a mother of a DZ twin) can take part. In case-control studies, the DNA of mothers of DZ twins is compared to the DNA of women who are not mothers of twins. In association studies that employ a TDT, DNA of a mother of a DZ twin and her two parents is required and the test involves examining the over-transmission of parental alleles.

To select mothers from informative families for linkage I first (November 2003) used data obtained from previous NTR survey studies. Because some mothers answered the question on familial twinning in previous survey studies some years ago, it was possible that her sister did not have children at that time. I therefore obtained recent information on familial twining in all mothers registered with the NTR by my own survey described above (in April 2005, see appendix II).

In earlier survey studies mothers of twins were asked to report on other women with multiples or twins in their family. From these data, a total 517 probands were selected.

In the YNTR the mothers were asked "Do you have other multiples in your family?" The mothers were asked this question when their twins were 2 years old. If they answered this question with a yes they were asked to indicate which family member: my sister, mother, sister of my mother, sister of my father, grandmother on mother's side, grandmother on father's side has multiples/twins. I selected mothers who crossed the first, second or third possibility.

In the ANTR, participants were asked questions about familial twinning at several time points, in 1991, 1995 and 2005. They were asked "Are there other twins or multiples in the family?" When they answered yes, they were asked to describe which family member this was and whether this concerned a monozygotic, dizygotic or a higher order multiple. I selected mothers that crossed yes and described they had a sister with multiples/twins. I also selected mothers that crossed yes and had no clear descriptions.

My own survey contained a series of items regarding familial twinning. Mothers were asked "which of your own biological family members are also parents of twins/multiples?" I selected all mothers who replied that she had a sister with multiples/twins and all mothers that said her mother and the sister of her mother (aunt) had multiples/twins. In addition to the 517 probands selected from previous survey studies, 658 probands were selected by using the new survey information.

The recruitment of informative families

All probands (N=1,175) were sent a letter and information brochure to invite her and her family for the study "The genetics of DZ twinning" (see appendix III and IV). This was followed by a phone call to obtain permission to approach the probands' sister, her parents and/or other siblings. All family members were initially informed about the study by the proband. After giving their permission to her to be contacted by us, they also received a letter and information brochure about the study.

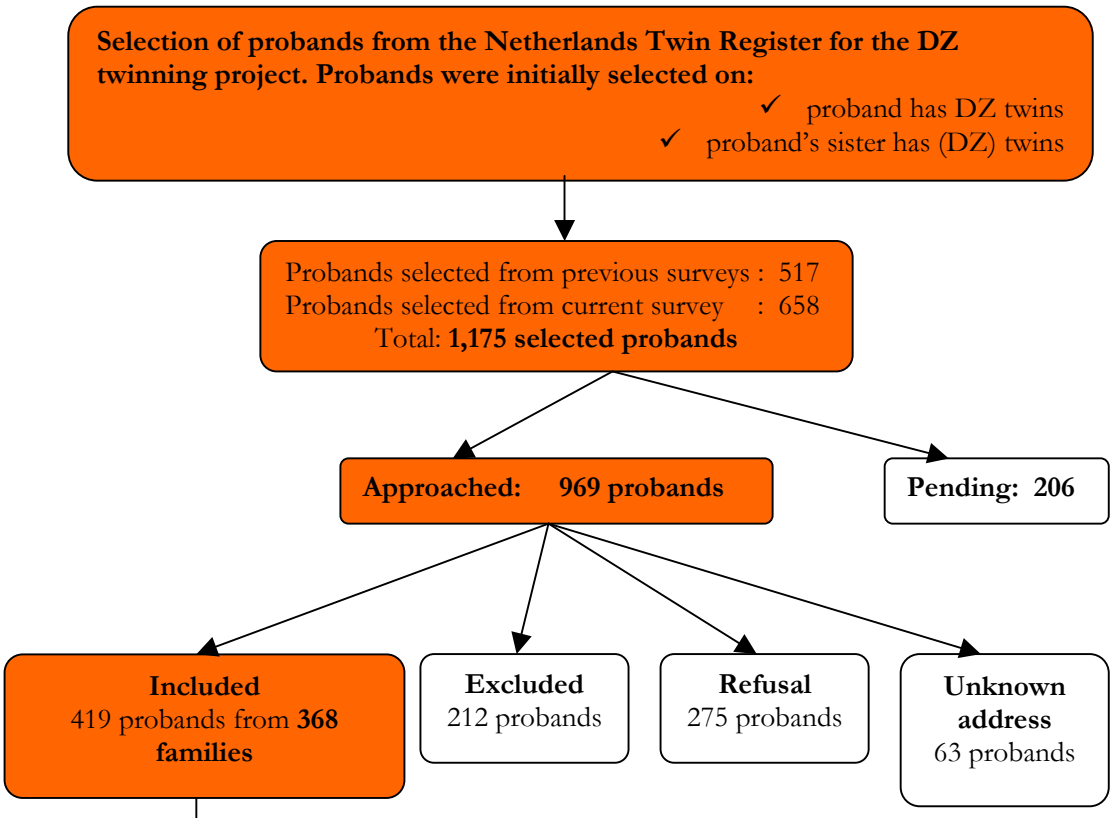
If one or both parents of the proband were unavailable or deceased, other siblings were asked for their participation. When a family agreed to participate, all members received a confirmation letter with an informed consent form. After informed consent was obtained, I made an appointment for blood collection. In addition to the blood collection all mothers of twins were interviewed by telephone (see appendix I).

Of the 1,175 selected probands, 969 probands were contacted and 206 probands were recruited later. Of those 1,175 probands, 419 probands agreed to participate. However, 51 probands were probands from families in which both sisters with twins were registered with the NTR. Both sisters were initially approached as

probands. I decided to refer to the first approached mother as the proband and to the second approached mother as the sister. In other words there were 368 participating families out of 419 selected probands. 212 probands were excluded. The main reasons for exclusion were because the probands had no sister or other family members with twins or because the probands had a sister but the sister did not have twins (N=152). Another reason for exclusion was that the proband did not had contact with her sister (N=29) or the proband or her sister was deceased (N=20). Finally, 11 probands were excluded because they or their sister did not speak fluently Dutch or lived abroad. In addition, 275 approached probands did not want to participate, 180 probands had no interest and time for the project and of 95 probands the sister had no interest and time. Finally, of 63 probands the current address was unknown, and their invitation letters were returned to us.

In total 368 families agreed to participate. Families in which at least two sisters have had spontaneous DZ twins are eligible for linkage studies. This was the case in 175 families. There were 165 families that did not have two mothers of spontaneous DZ twins, excluding them from the linkage studies, though these families are still eligible for association studies. Families eligible for association studies are for example families with one mother of spontaneous DZ twins and one mother of spontaneous MZ twins, or families with one mother of spontaneous DZ twins and one mother of twins conceived after fertility treatments. There were for example 48 families with mothers of spontaneous DZ twins (DZ) and artificially conceived DZ twins (DZ ART). In addition to the families eligible for future association and linkage studies to locate genes responsible for DZ twinning, there were 28 incomplete families. These families consisted of mothers who had a twin pregnancy and lost one or both twins due to miscarriage or stillbirth. Because it is not possible to determine the zygosity of these twins I consider this group of families as incomplete families (see figure 2).

Figure 2 Overview of the selection and recruitment of probands for the study "Genetics of DZ twinning".



N = 175 families for linkage studies

N = 165 families for association studies
N = 57 MZ-DZ pairs
N = 48 DZ- DZ ART pairs
N = 2 DZ- MZ ART pairs
N = 13 MZ-MZ pairs
N = 14 DZ ART- DZ ART pairs
N = 19 MZ- DZ ART pairs
N = 3 MZ- MZ ART pairs
N = 5 (1mother with DZ twins)
N = 4 (1 mother with MZ twins)

N = 28 incomplete families
N = 7 (1 mother with MZ twins and 1 mother with miscarriage)
N = 4 (1 mother with DZ ART twins and 1 mother with miscarriage)
N = 17 (1 mother with DZ twins and 1mother with miscarriage)

On the left side of table 3 the participating mother pairs of each family are presented. For example, there are 130 families with two mothers of spontaneously conceived DZ twins and 23 families with 3 mothers of spontaneously conceived DZ twins. In addition, one family consists of 3 mothers of spontaneously conceived DZ twins and 1 mother who had a miscarriage. In total there were 175 families with at least two mothers with spontaneous DZ twins. Because there were families with more than 2 mothers of DZ twins it was possible to create 250 DZ-DZ mother pairs out of 175 families. One family for example consisted of 9 mothers of spontaneous DZ twins on the mothers side of the proband mother and of 3 mothers of DZ twins on the fathers side of the proband mother. In total 6 mothers of this family agreed to participate (all on the mothers side) (see figure 3).

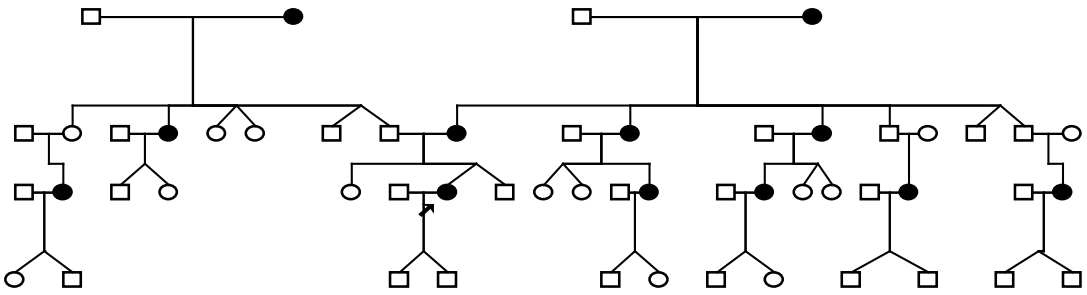


Figure 3 Participating family with in total 12 mothers of twins. The proband mother is indicated with an arrow. The affected mothers with DZ twin offspring are colored black.

On the right side of table 3 the total number of mothers of spontaneous DZ/MZ and artificially conceived DZ/MZ twin offspring are presented. For example, there are 130 families with 2 mothers of spontaneous DZ twins; this makes 260 mothers of spontaneous DZ twins. In total there were 627 (515 + 112) mothers of DZ twins and 138 (133+5) mothers of MZ twins and 43 mothers that had a miscarriage or stillborn twins.

Table 3 The number of recruited families specified by reported twin offspring zygosity and reported mode of conception obtained in the telephonic interview.

	Mothers of					Total number of mothers of				
	Spontaneous		Artificial		Miscarriage	Spontaneous		Artificial		Miscarriage
N	DZ twins	MZ twins	DZ twins	MZ twins	Unknown zygosity	DZ twins	MZ twins	DZ twins	MZ twins	Unknow zygosity
1	6	-	-	-	-	6	-	-	-	-
1	4	-	1	-	-	4	-	1	-	-
2	4	-	-	-	-	8	-	-	-	-
2	3	-	1	-	-	6	-	2	-	-
1	3	-	-	-	1	3	-	-	-	1
23	3	-	-	-	-	69	-	-	-	-
1	2	2	-	-	-	2	2	-	-	-
1	2	1	1	-	-	2	1	1	-	-
1	2	1	-	-	2	2	1	-	-	2
3	2	1	-	-	-	6	3	-	-	-
6	2	-	1	-	-	12	-	6	-	-
3	2	-	-	-	1	6	-	-	-	3
130	2	-	-	-	-	260	-	-	-	-
1	1	3	-	-	1	1	3	-	-	1
1	1	2	-	-	1	1	2	-	-	1
6	1	2	-	-	-	6	12	-	-	-
1	1	1	1	-	-	1	1	1	-	-
1	1	1	-	-	1	1	1	-	-	1
47	1	1	-	-	-	47	47	-	-	-
1	1	-	2	-	-	1	-	2	-	-
2	1	-	1	-	1	2	-	2	-	2
45	1	-	1	-	-	45	-	45	-	-
2	1	-	-	1	-	2	-	-	2	-
17	1	-	-	-	1	17	-	-	-	17
5	1	-	-	-	-	5	-	-	-	-
1	-	3	-	-	-	-	3	-	-	-
1	-	2	-	-	1	-	2	-	-	1
11	-	2	-	-	-	-	22	-	-	-
1	-	1	1	-	1	-	1	1	-	1
18	-	1	1	-	-	-	18	18	-	-
1	-	1	-	1	1	-	1	-	1	1
2	-	1	-	1	-	-	2	-	2	-
7	-	1	-	-	1	-	7	-	-	7
4	-	1	-	-	-	-	4	-	-	-
1	-	-	3	-	-	-	-	3	-	-
1	-	-	2	-	1	-	-	2	-	1
12	-	-	2	-	-	-	-	24	-	-
4	-	-	1	-	1	-	-	4	-	4
368						**515**	**133**	**112**	**5**	**43**

Zygosity

The mothers of twins or the twins themselves when they were 18 years or older were asked permission to conduct zygosity typing in DNA samples obtained from buccal swabs. This was used to determine information on offspring zygosity of 489 same sex twin pairs. Opposite sex twins were not typed (N=276) and in addition there were 43 mothers of stillborn/miscarriage twins.

Zygosity packages were sent to the mother of the twins when the twins were younger than 18 years old and when they still lived in their parental home. When the packages were not returned within one month the mothers were phoned and asked again.

Of the 489 packages 339 were returned. 14 mothers did not want to cooperate because they thought it was too hard for her twins, 4 twins did not want to cooperate because they thought it would be too much work, 45 mothers did not want to cooperate because they were certain about the zygosity of the twins and could not be convinced that DNA typing is necessary. For 87 twin pairs the DNA package was not sent back, without reason (mothers told us they would).

Interview

In the 368 participating families the mothers of twins were interviewed. In total 808 mothers of twins were interviewed. These included 368 probands, 339 of their sisters, 37 mothers, 33 aunts, 27 nieces and 4 daughters. The interview took between 20-30 minutes and consisted of five parts. Appendix I gives a detailed description of all variables asked in the interview and also presents the descriptive statistics for these variables.

Table 4 Number of mothers of twins (by zygosity of first twin and relation) who were interviewed to obtain pedigree data and data on fertility.

Participant	N	Offspring zygosity						Mean age (SD)	
		MZF	MZM	DZF	DZM	DZO	?		
Proband	368	48	22	87	74	137	0	43.9	(8.3)
Sisters	339	24	28	75	68	112	32	45.0	(8.9)
Mothers	37	6	1	12	4	7	7	68.6	(7.3)
Aunts	33	1	1	7	11	10	3	63.5	(10.5)
Nieces	27	3	3	6	6	8	1	41.3	(9.0)
Daughters	4	1	0	0	1	2	0	38.5	(7.9)
Total	**808**	**83**	**55**	**187**	**164**	**276**	**43**	**46.2**	**(10.6)**

Part A (zygosity and pre-pregnancy health)

Part A consisted of 19 items regarding; present age of the mother, birth weight of the mother, the number of children the mother had and mother's judgment of zygosity of the twins. The mothers were only asked to judge the zygosity of their twin if they were of the same sex. Information was asked regarding similarity of the children and experiences of mistaking one for another (eight items). The last three questions in part A asked about fertility treatments in any pregnancy.

Part B (pregnancies)

In this part I asked information on every separate pregnancy the mother had, including miscarriages. Information on birth history was asked in 11 items.

Part C (familial twinning)
Part C asked about family history of twinning and fertility. Pedigree data were obtained for the family of the proband mother, for her mother's family, her father's family and for the family of her husband.

Part D (health of mother)
In part D 19 items regarding health and medical history were asked. Data were collected on height, weight (before, during and after the twin pregnancy), menstrual cycle, menopause (of proband and her mother), use of contraception and health problems.

Part E (ancestry)
This part contained 4 items about ancestry of the parents and grandparents of the interviewed mothers.

Blood and urine collection

Probands who took part on the interview study, their sisters, parents and additional siblings who agreed to donate blood and urine samples to study the genetics of DZ twinning (N=1,393) were contacted by phone to make an appointment for the biological sample collection. Venous blood samples and morning urine samples were taken between 7.00 a.m. and 10.00 a.m. and after overnight fasting. A total of seven blood tubes were collected from all participants for DNA, lymphocytes, RNA (basal and challenged) serum and plasma. A complete description of the blood sampling is presented in chapter 7.

To assess hormone levels at the time of follicle recruitment in fertile women, blood and urine samples of women with a natural cycle were taken on the second, third or fourth day of the menstrual cycle. In women who used oral contraceptives (OC) blood and urine samples were taken in the week the women did not take OC pills. All of these women used combination OCs. Menopausal women, did not have to meet these criteria. Table 5 gives an overview of the families' visited.

Table 5 Blood collection in the participating families.

Status of biological sample collection	Number of families	Number of persons bled
Entire family bled	330	1,231
Partly bled	30	71
No one bled	8	0
Total	**368**	**1,302**

Of the initial 1,393 participants 94% was bled. Of the 91 participant who were not bled 4 were deceased and 87 did not want to participate any longer.

The data collection resulted in several studies. Chapters 4 through 6 use data from the large survey study. In chapter 7 I describe the pilot study for a large scale biobank in informative families. Appendix I summarizes the results from the interview study.

Chapter 3

Dizygotic twinning

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Abstract

The tendency to conceive spontaneous dizygotic twins is a complex trait with important contributions from both environmental factors and genetic disposition. Twins are relatively common and occur on average 13 times per 1000 maternities, though the twinning frequency varies over time and geographic location. This variation is mostly attributed to differences in dizygotic twinning rate, since the monozygotic twinning rate is relatively constant. Dizygotic twinning is in part under genetic control, with mothers of dizygotic twins reporting significantly more female family members with dizygotic twins than mothers of monozygotic twins.

Maternal factors such as genetic history, advanced age and increased parity are known to increase the risk of dizygotic twins. Recent research confirmed that taller mothers and mothers with a high BMI (30 >) are at greater risk of dizygotic twinning. Seasonality, smoking, oral contraceptive use and folic acid show less convincing associations with twinning. Genetic analysis is beginning to identify genes contributing to variation in twinning. Mutations in one of these genes (growth differentiation factor 9) are significantly more frequent in mothers of DZ twins. However, the mutations are rare and only account for a small part of the genetic contribution to twinning.

Introduction

Of the 30 people you meet in Europe or the USA, one of them is likely to have a twin brother or sister. The lowest chance of meeting a twin is in Asia, where 1 in 70 persons is a member of a twin and the highest chance in Nigeria where 1 in 12 persons is a member of a twin pair. In most countries the twinning rate has steadily increased since the 1980s following a long term decline in twinning rates from 1900 onwards (Eriksson et al., 1995; Derom et al., 1995).

There are two types of twins, dizygotic (DZ) and monozygotic (MZ) twins (Hall, 2003), and their etiology is very different. DZ twinning occurs when two separate oocytes are released during the same menstrual cycle (a multiple ovulation event) and fertilized by two sperm. DZ twins therefore have the same genetic relationship as ordinary brothers and sisters and share on average 50% of their genes. They can be same sex (boy-boy or girl-girl) or opposite sex (boy-girl) twin pairs. In contrast, MZ twins arise when an embryo splits soon after fertilization. MZ twins carry essentially identical genetic instructions (share 100% of their genes) and are always of the same sex (boy-boy or girl-girl) (Bomsel-Helmreich and Al Mufti, 1995).

The mechanism(s) leading to DZ twins operate on the selection of developing follicles within the ovary where instead of one ovum being released mid-cycle, two follicles mature and both oocytes are released ready for fertilization. For MZ twins, unknown mechanism(s) affect the early development of the embryo immediately after fertilization leading to separation of the cells into two or more embryos. The reasons for the relatively high incidence of MZ twins in humans remain unclear. There are no clear associations between MZ twinning and maternal, environmental or genetic factors and the mechanisms have not been identified, although families with a history of MZ twinning have been reported (Hamamy et al., 2004) This review will focus on DZ twinning and recent insights into this intriguing phenomenon. We will discuss the epidemiology of DZ twinning including recent secular trends in twinning rate, the factors affecting DZ twinning in relation to natural selection, the genetics and endocrinology of DZ twinning in humans.

Epidemiology

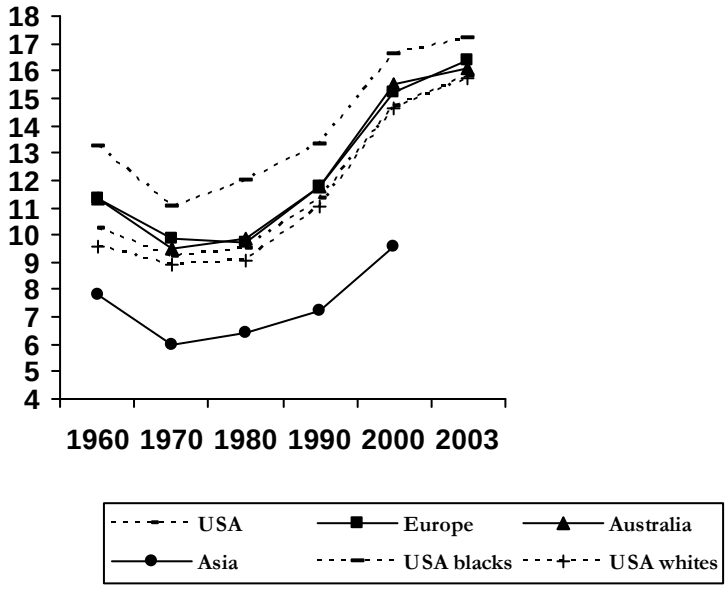
Prevalence of Twinning

Descriptions of variation in the prevalence of twinning for the Nordic Countries were made well before the 19th century. The registration of births began in the 17th century in church registries and was officially introduced in 1749 in Sweden and Finland (Eriksson, 1962). The twinning rate is defined as the number of twin maternities per 1000 maternities and includes still births (≥ 28 weeks) as well as live births. Thus the total number of children is twice that of the twin maternities. In the 1970s, Bulmer (1970) reviewed the geographical distribution of twinning for three large human racial groups from different regions: Europe/North-Africa, Sub-Saharan-Africa and Asia. He described differences in twinning frequencies between these groups, with the highest rate for Sub-Saharan-Africa (about 23 per 1000 maternities) and the lowest for Asia (about 5-6 per 1000 maternities). Little (1988) divided the international variation in twinning rate into three groups; low prevalence, intermediate prevalence; high prevalence. The low prevalence group included countries with twinning rates between 2 and 7 per 1000 maternities, such as Hawaii, Japan and Taiwan. Intermediate twinning rates between 9 and 20 per 1000 maternities were seen for most countries in North Africa, America, Asia, Oceania and Europe. The high prevalence group consisted of countries from Africa, especially Nigeria, Seychelles, Transvaal and Zimbabwe, with twinning rates of 20 per 1000 and higher (Little, 1988; Nylander, 1979).

Fluctuations in twinning rates for the USA, Western Europe, Australia and Asia are illustrated in figure 1. Data are restricted to the total twinning rate, because twinning rates by sex were not always available. Twinning rates were obtained from literature searches

(PubMed, http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) and by consulting relevant books and Vital Statistics Reports for twinning rates between 1960 and 2003. Actual data were available for the USA, Australia and The Netherlands. When actual data were not available we used the twinning rates as reported in the literature. All rates were reported per 1000 maternities allowing the comparison of twinning rates between different countries regardless of the size of their population (Table 1). In most European countries and in the USA, twinning rates started to decline around 1900. However, as shown in figure 1 from the 1970s onwards, twinning rates have increased steadily in most countries including the USA, Europe, Australia and Asia (Derom et al., 1995; Imaizumi, 1997; Imaizumi, 1998; Taffel, 1995).

Figure 1 Twinning rates in the USA (total/white/blacks), Europe, Australia and Asia. NB. Twinning rates are presented in terms of twin maternities per 1000 maternities from sources listed in Table 1.



In western European countries twinning rates rose from 9-11 per 1000 in the 1970s to 15-18 per 1000 by 2001 (Macfarlane et al., 2005). Likewise, in Japan, Hong Kong and Singapore the twinning rate rose from 5 to 6 per 1000 births in 1972 to 9 per 1000 births by 2001 (Imaizumi, 2005). Similar increases in twinning rate were also seen for the USA and Australia. The total twinning rate in the USA in 2003 was 15.8 per 1000 maternities. In addition to the total twinning rate, we described the twinning rates for black and white Americans separately because of the heterogeneity of the population in the USA. For black Americans the twinning rate was 17.2 and for white Americans the twinning rate was 15.7 (compared with twinning rates of 16.1 in Australia and 16.4 in Europe). Twinning rates of Sub-Saharan African countries are not included in Figure 1 because few data are available. However, Nigeria is often cited in the literature with twinning rates of 40 per 1000 maternities (Little, 1988).

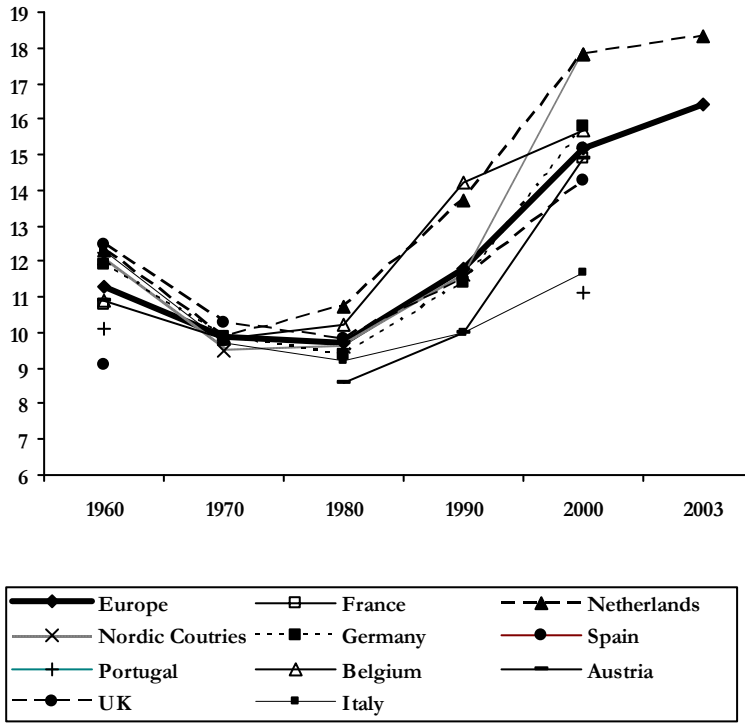
Table 1 Summary of studies of twinning rates 1960-2003.

Europe	**Period**	**Area studied & Remarks**
(Bulmer, 1970)	1950-1960	Spain, Portugal, France, Belgium, Austria, Luxembourg, Switzerland, The Netherlands, Germany, Norway, Sweden, Italy, UK (standardized for maternal age)
(Derom *et al.*, 1995)	1960-2000	Denmark, Netherlands, W-Germany, UK, Luxembourg, Belgium
(Eriksson and Fellman, 1967; Eriksson and Fellman, 1973)	1950-1960	Finland, Sweden
(Little, 1988)	1950-1970	Bulgaria, Ireland
(James, 1995)	1960-1980	UK, Belgium
(Imaizumi, 1997)	1972-1996	Austria, Finland, Norway, Sweden
(Astolfi et al., 2003)	1975-1995	Italy, Norway, Sweden, Austria, UK
(Centraal Bureau voor de Statistiek, 2004)	1950-2003	The Netherlands
(Macfarlane and Blondel, 2005)	1998-2002	Austria, Belgium, Denmark, Finland, France, Germany, Greece, Ireland, Italy, Luxembourg, The Netherlands, Portugal, Spain, Sweden, UK
USA	**Period**	**Area studied & Remarks**
(Bulmer, 1970)	1950-1960	USA (American whites and blacks)
(Terry, 1962)	1960	USA, birth records of NCHS
(Little, 1988)	1978-1979	USA, not specified
(Jewell and Yip, 1995)	1980-1989	USA, birth records of NCHS
(Martin and Park, 1999)	1980-1997	National Vital Statistics Report (NCHS)
(Kiely and Kiely, 2001)	1971-1998	USA, birth records of NCHS
(Martin et al., 2002)	2000	National Vital Statistics Report (NCHS)
(Mathews and Hamilton, 2002)	2002	Mean age of mother, 1970-2000 (NCHS)
(Martin et al., 2005)	2003	National Vital Statistics Report (NCHS)
(Markovitz J. and Hershlag A., 2005)	1997-2001	SART-CDC Registry
Australia	**Period**	**Area studied & Remarks**
(Australian Bureau of Statistics, 2001)	1980-2000	Births, Australia, 2000, No. 3301.0
(Umstad and Lancaster, 2005)	1983-1999	Australia
(Australian Bureau of Statistics, 2005)	1985-2005	Births No. 3301.0
Asia	**Period**	**Area studied & Remarks**
(Bulmer, 1970)	1960-1970	Japan, China
(Little, 1988)	1960-1980	Japan, Hong Kong, Singapore
(Imaizumi, 2005)	1970-2001	Japan, Hong Kong, Singapore
Africa	**Period**	**Area studied & Remarks**
(Bulmer, 1970)	1960	Bantu, West-Africa, South-Africa, Ghana
(Nylander, 1969;1979)	1970	Nigeria

Nylander reported twinning rates of 33 to 66.5 per 1000 maternities in Yoruba women in Western and Eastern Nigeria (Nylander, 1971; Nylander, 1978) and a twinning rate of 19.4 in Hausa women in the Northern part of Nigeria (Nylander, 1971).

Within Europe, twinning rates vary considerably (Figure 2). In 2003, the average twinning rate in Europe was 16.4 per 1000 maternities, but rates varied widely from country to country from 11 per 1000 maternities in Luxembourg and Portugal to 20 per 1000 in Denmark, Greece and the Netherlands (Centraal Bureau, 2004; Hall, 2003; Macfarlane and Blondel, 2005).

Figure 2 Twinning rates in Europe. Twinning rates are presented in terms of twin maternities per 1000 maternities from sources listed in Table 1.



As shown above, twinning rates vary considerably across time and place. As MZ twinning generally occurs at a constant rate of approximately 4 per 1000 maternities around the world (Tong et al., 1997) the variation in twinning rate is generally accepted as being the result of variation in DZ twinning rates.

Contribution of iatrogenic twins in the rising prevalence of twinning

The increased use of fertility treatments such as in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI), intra uterine insemination (IUI) and ovulation induction (OI) is commonly cited as the main cause of the steep increase in twin births in the past two decades (Martin et al., 2005; Fauser et al., 2005). Lambalk and colleagues (2004) showed however that the increase in opposite sex twins (i.e. DZ twins) in the period 1995-2002 was mainly caused by natural conception (56%) and not by ICSI/IVF (35%) or OI/IUI (9%). Maternal reproductive age was significantly higher in mothers who conceived their twins after natural conception and in mothers who conceived their twins after OI/IUI. In contrast, maternal reproductive age remained the same for mothers who conceived their twins after the use of ICSI or IVF over the period 1995-2002. Lambalk and colleagues (2004) therefore argued that the increase in twin births in the Netherlands was due not only to the

use of ART, but also to the increase in maternal reproductive age. It is difficult to say whether maternal age or ART causes the increase in multiple births in the OI/IUI group because maternal age also increased significantly in this group. Tandberg and colleagues (2007) agrees with the general argument that most of the increase in twinning frequency comes from maternal age, but also suggests age and ART does not account for all the increase. Recently Jones (2007) showed that in the USA in 2003 the greatest number of twin births resulted from natural conception (60.1%), but a very large number of twins are also born after OI/IUI (31.6%). However he did not control for maternal age which means that the percentages of spontaneous twin births could be underestimated and the percentages of OI/IUI births overestimated.

Factors affecting twinning

Major factors influencing twinning are maternal age, parity and genetic inheritance. An important early contribution to the study of twinning in relation to maternal characteristics was made in 1865 by the Scottish physician Matthews Duncan, who reported an increase in twin pregnancies with an increase in maternal age (Bulmer, 1970). Duncan also described an increased risk of twinning with increased parity, the number of children born to the mother prior to the twin pregnancy. Although maternal age and parity are highly correlated the effects are independent of each other (Bulmer, 1970). It was later discovered that these factors predominantly influence the DZ twinning rate, and not the MZ twinning rate (Bulmer, 1970; MacGillivray et al., 1988). DZ twinning rate increases four-fold from age 15 to 35 (Bulmer, 1970). The decline in the DZ twinning rate in the early 20th century reflected a decrease in mean maternal age and a lower number of maternities. The increase in twinning rate reported in the late 1970s has been mainly associated with an older age at childbearing (Derom et al., 1995; Lambalk et al., 2004). The increased use of fertility treatments such as in vitro fertilization (IVF) in the late 1980s further added to the rising incidence in twin births (Fauser et al., 2005; Martin et al., 2005).

Body composition has been linked to twinning. Nylander showed that for tall women (164 cm and over), the relative risk of having twins was 1.5 to 2.0 times higher than for short women (under 155 cm), after standardization for age and parity (Nylander, 1981). Other research found a direct, but somewhat inconsistent association between increased maternal height and DZ twinning (Bortolus et al., 1999). Recently, Basso and colleagues found a similar trend (Basso et al., 2004). Twin mothers were taller and had a higher body mass index (BMI) compared to mothers of singleton children. A BMI of less than 20 was associated with a lower risk of twinning and a BMI of 30 or more was associated with a higher risk of twinning. These results remained after correcting for maternal age and parity (Basso *et al.*, 2004).

Significantly higher multiple birth rates have been reported in mothers who smoke (Olsen et al., 1988; Parazzini et al., 1996), although insufficient control for covariates (Olsen *et al.*, 1988) and limited power (Parazzini et al*.,* 1996) suggest caution in interpretation of these findings (Kallen, 1998). A recent prospective study in Denmark supports a small effect of smoking on twinning (Morales-Suarez-Varella et al., 2007).

Associations with socio-economic status (SES) have also been reported (MacGillivray and Campbell, 1978; MacGillivray et al., 1988) but findings on differences in twinning by SES are often difficult to interpret. This may be due to ambiguity of definition and because of its interrelation with factors associated with SES, like height, weight, smoking behavior, and age of the mother at birth of the children (Campbell, 2005).

Seasonal variation influences DZ twinning. Higher rates of DZ twinning are reported for conceptions during summer and autumn in several countries (Eriksson and Fellman, 2000; Fellman and Eriksson, 1999; Dionne et al., 1993; Sharma, 1997), although this trend is not seen in all studies (Bonnelykke et al., 1987; Krieger et al., 1996). The seasonal variation appears stronger for data from the 19th century compared with recent trends (Fellman and Eriksson, 1999; Eriksson and Fellman, 2000). Seasonal variation in day length may influence

hormonal concentrations driving ovarian activity and influence fertility and multiple ovulation (Dionne et al., 1993). Seasonal changes in food supply may also have contributed to stronger effects in the past (Eriksson and Fellman, 2000).

Two iatrogenic factors; the use of oral contraceptives and folic acid are also reported to influence DZ twinning (Ericson et al., 2001; Li et al., 2003; Lumley et al., 2001). Some studies found an increase in twin births associated with the consumption of folic acid around the time of conception (Ericson et al., 2001; Kallen, 2004; Vollset et al., 2005), while others were not able to replicate these findings (Berry and Kihlberg, 2005; Li et al., 2003). The issue is important since large scale preconception use of folic acid has been introduced for the prevention of neural tube defects (Botto et al., 1996) and folic acid may increase the chance of successful implantation and survival of two embryos (Lumley et al., 2001). No clear relationship has been established and data may be confounded by fertility treatment and maternal age (Levy and Blickstein, 2006). Recently a systematic review evaluated 12 studies to examine if the preconception use of folic acid increases the risk of twinning (Muggli and Halliday, 2007). They concluded there is some evidence for a positive relationship between preconception folic acid use and twinning. However, the relationship is tentative and additional well designed studies are required that focus on the dose relationship and obtain accurate data on infertility treatments (Muggli and Halliday, 2007).

Earlier studies investigating a possible association between oral contraceptives and twinning found a decrease in DZ twinning rates after the use of oral contraception and sometimes an increase in MZ twinning rates (Macourt et al., 1982). In 1977, Rothman evaluated the effect of oral contraceptives on reproduction (Rothman, 1977). Twinning was more frequent when mothers became pregnant soon after they stopped taking oral contraceptives, and the increase was mainly caused by DZ twins. A large study in Aberdeen found no association between the use of oral contraception and either MZ or DZ twinning (Campbell et al., 1987). In this study zygosity was determined from blood samples and placentation, and data regarding oral contraceptive use was collected prior to the twin pregnancy with three control groups. A more recent study however did find an increased risk of twinning after discontinuation of the oral contraceptive pill (Murphy et al., 1989).

A theoretical reason for an increased risk of DZ twinning after taking oral contraceptives is that the hypothalamic-pituitary-ovarian axis (HPO-axis) has to recover from the effects of exogenous steroids, causing a temporary increase of FSH levels (Jernstrom et al., 1995). A clinical situation that mimics such a condition is when women with hypothalamic amenorrhea are treated with GnRH to induce ovulation where in the first treatment only this leads to higher FSH levels in association with multiple follicle growth and an increased risk of multiple pregnancies (Lambalk et al., 1998).

Genetic factors

The initial link between body composition and twinning was made by Tchouriloff in 1877. He argued that taller women are more likely to bring a twin pregnancy to full-term because of their body size, and are therefore predisposed to conceive twins (MacGillivray et al., 1988). Because height was already known to be an inherited characteristic this was the first attempt to link twinning with heredity. Later Weinberg (1901) discovered familial clustering of DZ twin pregnancies. He found that mothers, sisters and daughters of mothers with multiples, had increased risks of conceiving a multiple by 39%, 95% and 30% respectively. However, he could not find this increased risk in relatives on the paternal side. Furthermore he compared the twinning rate among the relatives of mothers of opposite sex and same sex twins, using his differential method (Weinberg, 1934). This method is based on the numbers of same sex and opposite sex twins. According to this method, the DZ twinning rate (DZr) can be estimated by doubling the number of opposite sex twins (OS) and dividing the number by the total number of maternities (N) (DZr= 2OS/N). The MZ twinning rate (MZr) can be estimated

by subtracting the number of OS twins from the number of same sex (SS) twins and dividing the number by the total number of maternities (N) (MZr = (SS-OS)/N). He found twinning rates of 22.0 and 11.1 respectively in contrast to a twinning rate of 11.9 in the general population. Weinberg therefore suggested that the inheritance of DZ twinning was restricted to the female line. In 1934, Greunlich disputed this as he found that the paternal side was as important as the maternal side in determining the twinning rate in siblings of fathers and mothers of twins (Greunlich, 1934). Later, Wyshak and White however analyzed the inheritance of twinning using data obtained from the archives of the Genealogical Society of the Mormon Church in Salt Lake City and concluded that DZ twinning is determined by recessive genes, and limited to the female side (White and Wyshak, 1964; Wyshak and White, 1965). Further support was obtained from a study by Bulmer (1970) showing that only relatives of mothers of twins report significantly higher twinning rates in other family members. In this study the risk of conceiving a twin was 1.7 times higher in female relatives than in male relatives. Looking in more detail at the DZ twinning rate among female relatives, he showed that the risk of having a DZ twin is 2.5 times higher than the twinning rate in the general population for women with a sister with DZ twins. For mothers and daughters of a mother with a DZ twin this was about twice as high as the risk for the general population (Bulmer, 1970). Studies in Italy reported significantly increased twinning in maternal relatives and some evidence for an increase among paternal relatives of DZ twins (Parisi et al., 1983).

More recently, Meulemans and colleagues (1996) investigated the inheritance of DZ twinning in 1422 Dutch and Flemish pedigrees of mothers of spontaneous DZ twins by formal pedigree analysis. Analysis showed that the phenotype of "having DZ twins" is consistent with an autosomal monogenic dominant model. This finding means that the phenotype of giving birth to a twin is expressed in women, but can be inherited from both the maternal and the paternal side. In the same period, Lewis and colleagues (1996) investigated frequencies of other twins in families of 6596 twin pairs from the Australian Twin Registry. They found a relative risk of 1.7 and 2.5 respectively for sisters of mothers of DZ twins and offspring of female DZ twins. The evidence strongly suggests that genetic contribution to DZ twinning is a trait expressed by the mother that may be inherited from either parent. Limited evidence for a paternal effect was recently supported by a small study showing semen quality of the father may play some role in twinning (Asklund et al., 2007). DZ twinning is influenced by many genes (see below) and unlikely to be a simple dominant or recessive trait.

Natural selection for twinning

Twinning is often associated with complications during pregnancy and delivery for both the mother and the twins (Conde-Agudelo et al., 2000; Gabler and Voland, 1994; Elster, 2000). So why do women have twins, when the costs are so high? Two hypotheses have been put forward to explain the human twinning rate; the "the insurance ova hypothesis" (Anderson, 1990) and the "natural selection hypothesis" (Lummaa et al., 1998). The insurance ova hypothesis states that producing more then one ovum increases the probability that at least one will be fertilized and survive to term. Conceiving twins is therefore a by-product of selection for increased fertility (Anderson, 1990). The second hypothesis views twinning as either adaptive, or maladaptive, depending on the environment (Lummaa et al., 1998). Lummaa and colleagues investigated their natural selection hypothesis by comparing the lifetime reproductive success between singleton and twin mothers living on the archipelago of Åland and Åboland and the mainland of Finland. In the food rich archipelago, lifetime reproductive success was maximized for mothers having twins. In contrast, in the poorer mainland environment, lifetime reproductive success was maximized by having singletons (Lummaa et al., 1998). The fall in DZ twinning rate associated with malnourishment in World War II and subsequent increase in DZ twinning rates when food availability became stable again (Bulmer, 1970) provide further support for this 'natural selection' hypothesis.

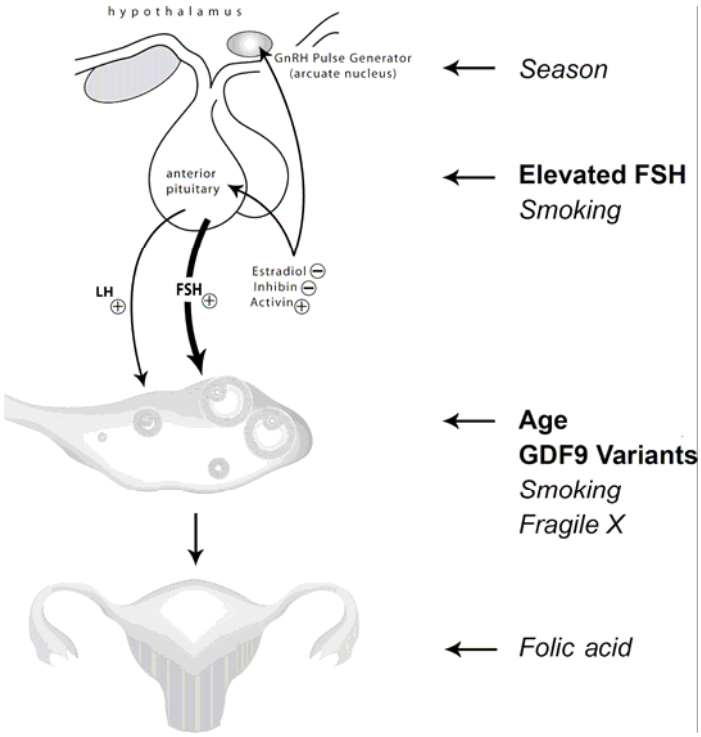
Helle and colleagues contrasted both hypotheses in three historical Sami populations from Northern Scandinavia. They found that twin mothers had a higher overall reproductive success compared with singleton mothers. Mothers of twins started reproduction at a younger age, and continued for longer; they had higher lifetime fecundity and raised more offspring to adulthood in all studied populations. However, if the mother begun to reproduce at a very late age (> 37 years), or if she had a long reproductive life span (>20 years), it was more favorable to produce singletons (Helle et al., 2004). This finding indicates that women predisposed to having twins are more likely to produce twins in a healthy reproductive environment (Helle et al., 2004).

Mechanisms controlling DZ twinning

It is generally accepted that hereditary DZ twinning results from the fertilization of two separate oocytes by two sperm (Hall, 2003). The increase in multiple ovulation (and twinning frequency) resulting from improved nutrition or specific genetic mutations is well documented for other species (Hunter et al., 2004; Montgomery et al., 1993; Montgomery et al., 1988). Direct observations of increased multiple ovulation in human twinning have not been made. However, increased follicle recruitment has been observed in mothers of spontaneous twins (Martin et al., 1991) and similar genome wide allele sharing for DZ twins and sibs (Montgomery et al., 2006) supports the view that DZ twins arise from multiple ovulation and subsequent fertilization and survival.

Growth and selection of follicles destined for ovulation is controlled by a complex regulatory network within the hypothalamic-pituitary-ovarian axis (Figure 3).

Figure 3 Regulatory factors in the growth and selection of follicles GDF9 = growth differentiating factor 9.



Two hormones from the pituitary gland are essential for reproductive function. These are the two gonadotrophins, follicle stimulating hormone (FSH) and luteinizing hormone (LH) and the concentrations of both FSH and LH change in characteristic patterns during each menstrual cycle. FSH concentrations begin to

increase at the beginning of each cycle starting a new wave of follicle growth among the larger follicles in the growing pool. In turn, the growing follicles secrete hormones that signal back to the brain and pituitary gland causing FSH concentrations to fall. Few follicles can sustain growth in a time of falling FSH concentrations (McGee and Hsueh, 2000). Usually, development of one dominant follicle takes place when FSH concentrations reach a required threshold early in each cycle (Baird, 1987; Brown, 1978). Multiple follicle growth results when greater FSH concentrations occur at the time of follicle selection or when FSH concentrations exceed the threshold for too long (Baird, 1987; Schoemaker et al., 1993).

Recent studies have defined a complex signaling network within the ovarian follicle itself (Roy and Matzuk, 2006; Shimasaki et al., 2004). This pathway responds to the external FSH and LH signals and ensures coordinated growth and development of the oocyte and other follicle cell types. Major players include two closely related growth factors expressed specifically in the oocyte, namely bone morphogenetic protein 15 (BMP15) and growth differentiation factor 9 (GDF9) (Shimasaki et al., 2004). These growth factors bind to specific receptors expressed on multiple cells in the ovary ensuring the oocyte is supported during growth and development.

Mothers of DZ twins have elevated FSH concentrations (Lambalk et al., 1998; Martin et al., 1984; Nylander, 1974) suggesting that factors influencing twinning may operate through an increase in secretory drive from the hypothalamic-pituitary system. However, increased FSH concentrations have not been observed in all studies (Gilfillan et al., 1996). There is evidence for an increase in the number of FSH pulses in the early follicular phase in mothers of DZ twins (Lambalk et al., 1998). These FSH pulses occur without a concurrent LH pulse. LH secretion and both the LH and the FSH responses from the pituitary to gonadotrophin releasing hormone (GnRH) were not different (Lambalk et al., 1998).

FSH release is regulated by feedback from inhibin peptides in the ovary (Fig. 3). Inhibin produced by the ovary acts as a classic hormone at the level of the pituitary by inhibiting pituitary FSH synthesis and release (Baird and Smith, 1993). There are two forms of inhibin, inhibin A and inhibin B; both forms consist of two sub-units, a common α-subunit (INHA) and either β_a or β_b (INHBA and INHBB respectively) (Burger et al., 1995; Martin et al., 1991). No differences in concentrations of inhibin A or B were detected across the menstrual cycle between mothers of DZ twins and controls (Gilfillan et al., 1996; Lambalk et al., 1998) suggesting no difference in the mode of ovarian feedback on FSH concentrations. The data indicate that neuroendocrine mechanisms may be involved in the generation of higher FSH levels in hereditary twinning (Lambalk et al., 1998; Martin et al., 1984).

The age related increase in the frequency of DZ twins

The chance of having DZ twins increases approximately four-fold with increasing maternal age (Bulmer, 1970). The reason for the increase in twinning frequency with age is thought to lie in the dynamic interplay of hormonal signals between the pituitary gland and the ovary (Lambalk et al., 1998). In younger women there is usually a pool of growing follicles ready to respond immediately to the rise in FSH at the beginning of each menstrual cycle. The immediate response is to send hormonal signals back to the brain and pituitary gland to turn down the FSH signal (Baird, 1983; Baker and Spears, 1999; Macklon and Fauser, 2000; Zeleznik, 2001). This stops the growth of other follicles and the one dominant follicle usually goes on to ovulate at mid-cycle.

With age, the pool of ovarian follicles available to grow and respond to the hormonal signals diminishes (Lambalk et al., 1998). Consequently, when FSH rises at the beginning of each cycle, large follicles may not be available to respond rapidly to this signal. Sometimes hormonal feedback from two smaller follicles needs to be combined before the message gets through to turn down the FSH signal. When this happens both follicles mature and ovulate increasing the chance of having twins. As the pool of growing follicles decreases further, the

feedback signal diminishes and the background concentration of FSH rises, further increasing the chance of two follicles ovulating and giving rise to twins (Lamballk et al., 1998). Thus the increase in DZ twinning with age is thought to be due to rising FSH concentrations driving the selection of more ovarian follicles.

Genetic variants contributing to DZ twinning

One approach to better understand mechanisms contributing to variation in twinning is to identify the gene or genes that account for the genetic variation in DZ twinning. Given our current understanding of mechanisms contributing to ovarian follicle selection and twinning, an obvious place to look is within the complex regulatory network of the hypothalamic-pituitary-ovarian axis (Fig. 3). Candidate genes include the genes coding for FSH and the specific transmembrane receptor for FSH (FSHR). Al-Hendy and colleagues suggested that two linked mutations (Thr307Ala and Asn680Ser), causing a higher sensitivity of the FSH receptor to FSH, may contribute to variation in twinning (Al Hendy et al., 2000). However, other researchers could not agree with the conclusions of these authors, stating that it is very doubtful the mutations are responsible for variation in DZ twinning (Derom et al., 2001; Gromoll and Simoni, 2001; Liao et al., 2001). Montgomery and colleagues sequenced the transmembrane region of the FSHR in 21 unrelated mothers of DZ twins and found no association. Furthermore a linkage study of 183 sister pairs with spontaneous DZ twins excluded linkage to the region on chromosome 2 where FSHR is located which suggest that mutations in FSHR are not a common cause of hereditary twinning (Montgomery et al., 2001).

Polymorphisms in the INHA gene located on chromosome 2 in humans were typed in DZ twinning families (Montgomery et al., 2001). There was no evidence for association between variation at the α-inhibin locus and dizygotic twinning. Although the inhibin B form of inhibin could be an interesting candidate to investigate for association with DZ twinning, the authors also suggest that mutations in other candidates on chromosome 2, like the β_b -inhibin subunit cannot be major contributors to risk for DZ twinning (Montgomery et al., 2001).

Lessons from Animal Studies

The number of offspring is an important economic trait in farmed animals and factors influencing variation in twinning and litter size have been studied extensively. Sheep provide a valuable model because most breeds have singles or twins, but some strains have a high incidence of triplets and higher order multiples (Montgomery et al., 2001; Montgomery et al., 1992). This high frequency of twins and triplets is influenced by genetic background. Studies of one of these strains thirty years ago showed that the high litter size resulted from the actions of a single gene (Montgomery et al., 1992). At the time this was a surprise because genetic effects on twinning were thought to result from the actions of many genes with small effects rather than the actions of one or two genes (or variants) of large effect. The discovery sparked a worldwide search to find the gene responsible and to look for other sheep strains with single genes affecting twinning (Montgomery et al., 2001; Moore et al., 2004).

Both searches were successful. Mutations have been found in three different genes that increase the frequency of twinning (Galloway et al., 2000; Hanrahan et al., 2004; Mulsant et al., 2001; Souza et al., 2001; Wilson et al., 2001). Two of these genes are closely related growth factors expressed specifically in the oocyte and known as bone morphogenetic protein 15 (BMP15) (Galloway et al., 2000) and growth differentiation factor 9 (GDF9) (Hanrahan et al., 2004). The third gene is the receptor for BMP15 expressed on multiple cells in the ovary and known as bone morphogenetic protein receptor 1B (BMPR1B) (Wilson et al., 2001; Mulsant et al., 2001). The mutations increasing twinning in sheep are all found in these three genes within the ovary suggesting,

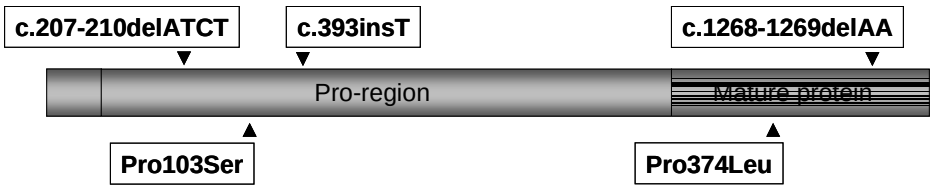
at least for this species, the primary control of the number of ovulated follicles and hence twinning frequency resides within the ovary itself (Moore et al., 2004).

Some of these mutations are also associated with infertility (Galloway et al., 2000; Hanrahan et al., 2004), when individuals carry two copies of the mutations. Infertility in sheep for some mutations in BMP15 and GDF9 is recessive. Carriers of one copy of the mutation have more twins, while carriers with two non-functional copies of the gene exhibit a failure of normal ovarian development and are infertile.

The role of genes from ovarian regulatory pathways in human DZ twinning

GDF9 and BMP15 are both expressed in human oocytes and play important roles in folliculogenesis. The role of common variation in GDF9 influencing DZ twinning was tested by genotyping markers across the gene in families with spontaneous DZ twins (Montgomery *et al.*, 2004). Six common alleles of human GDF9 were identified with no evidence for association with DZ twinning. DNA sequencing in twenty mothers of DZ twins found a loss of function mutation on one copy of the GDF9 gene in one family (Montgomery *et al.*, 2004). The mutation was present in two sisters with spontaneous DZ twins and inherited from their father. A further search for GDF9 variants in mothers of spontaneous DZ twins was carried out in additional families (Palmer *et al.*, 2006). Two novel deletions and four missense alterations in GDF9 were identified in mothers of DZ twins. One deletion mutation was identified in two families and in each family was carried by two sisters with DZ twins including one individual with three sets of DZ twins. Taken together, the frequency GDF9 variants were significantly higher in mothers of DZ twins compared with controls. Variants contributing to increased twinning are summarized in Figure 4 and appear to double the chance of having twins for women who carry the variants. However, the frequency of the variants is low (less than 4% for all variants) and so the contribution of these variants to the overall incidence of twinning is small.

Figure 4 Insertion/deletion and missense mutations reported in GDF9 that are associated with an increase in twinning frequency (Montgomery et al., 2004; Palmer et al., 2006).



The search for other genes

Several groups are collecting families with a high incidence of twinning to identify genes contributing to twinning variation. The standard method to search for genes is to follow the inheritance of fragments across all chromosomes in the families and look for chromosome segments more often inherited together in mothers of twins. One candidate for multiple ovulation and increased fertility is the protease inhibitor locus (Pi). Analysis of Pi types in twins suggested that intermediate antitrypsin deficiency was more common in twins and parents of twins and increased fertility may provide a selective advantage to maintain polymorphism at this locus (Lieberman et al., 1978). In a sample of 160 Dutch twin families, mothers of DZ twins carried the S and the Z allele at the protease inhibitor (Pi) three times more frequently than a control sample (Boomsma et al., 1992). It was argued that the S allele might increase the risk of multiple ovulations and increase multiple gestations, because mothers of MZ twins also had an increased frequency on the S allele.

Almost 20 years ago, female carriers of the fragile X (FRAXA) syndrome were found to have an increased risk of DZ twin pregnancies (Kenneson and Warren, 2001; Fryns, 1986). Three classes of the gene (FMR1) are defined by the number of CGG repeats. Individuals with less than 60 CGG repeats have a normal gene. Individuals with 55-200 CGG repeats have a premutation which means they carry an unstable mutation which can expand in future generations. Individuals with over 200 repeats have a full mutation which causes fragile X syndrome. Carriers of the FMR1 premutation are at risk for POF, early menopause, ovarian dysfunction and an increase in DZ twinning (Turner et al., 1994; Vianna-Morgante, 1999; Welt et al., 2004; Schwartz et al., 1994). However, Healey (1997) and colleagues found no premutations or full mutations in mothers of spontaneous DZ twins and concluded that FMR1 can play no more than a minor role in the inheritance of DZ twinning. Associations with twinning may relate to earlier ovarian aging in premutation carriers (Welt et al., 2004)

Recently, Busjahn and colleagues (2000) reported that a C→T substitution allele in the gene PPRAG on chromosome 3p25 encoding peroxisome proliferator-activated receptor (PPARγ) is associated with DZ twinning. This gene might also be involved in the intra-uterine selection and thus survival of the unborn twins (Busjahn et al., 2000). Duffy and colleagues (2001) could not replicate linkage at the PPRAG gene region for either survival of twin pregnancy or multiple ovulation.

Recently a genome-wide linkage scan for natural DZ twinning was conducted in 14 Flemish families. The observed peaks were highest under a dominant model of inheritance, with LOD scores of 1.51, 1.36 and 1.99 found on chromosome 2, 7 and 18 respectively (Derom et al., 2006). These chromosomes may contain genes contributing to variation in DZ twinning, but clarification awaits further study. The results also allowed examination of evidence for contributions to DZ twinning from common variation at many of the candidates discussed above, including genes from the pituitary-ovarian axis implicated in control of DZ twinning (Figure 3). A role for many of these genes was excluded (Derom et al., 2006). These conclusions agree with previous studies for inhibin alpha (INHA; (Montgomery et al., 2000)), FSH receptor (FSHR) (Montgomery et al., 2001), bone morphogenetic protein receptor 1B (BMPR1B) (Duffy et al., 2001) and methylenetetrahydrofolate reductase (MTHFR) (Montgomery et al., 2003). There was no evidence for linkage in the region of GDF9 (Derom et al., 2006), despite the recent evidence for mutations in GDF9 increasing the chance of having twins (Palmer et al., 2006). However, the frequency of these rare GDF9 mutations is low. They contribute only a small proportion to the variation in twinning and the signal would not be picked up in the linkage scan. It is therefore possible that rare mutations in other candidates could still contribute to twinning in some families.

Relationship between twinning and premature ovarian failure

GDF9 and BMP15 are critical genes for normal human fertility (Di Pasquale et al., 2004; Di Pasquale et al., 2006; Dixit et al., 2005; Dixit et al., 2006). A dominant-negative mutation in BMP15 identified in Italian sisters causes ovarian dysgenesis (Di Pasquale et al., 2004) and recent studies found higher frequencies of rare mutations in both GDF9 and BMP15 in patients with premature ovarian failure (POF) when compared with controls (Di Pasquale et al., 2006; Dixit et al., 2005; Dixit et al., 2006; Laissue et al., 2006; Kovanci et al., 2007). POF is diagnosed when women younger than 40 years have unexplained amenorrhea for longer than 6 months, have high FSH levels and low estrogen levels (Coulam et al., 1986).

Mutations in GDF9 in mothers of DZ twins and in patients with POF imply a possible direct relationship between twinning and POF. Mothers of DZ twins reach menopause significantly earlier than mothers of MZ twins (Martin and Park, 1999; Gosden et al., 2007). The small increase in the frequency POF in mothers of DZ twins could be explained by mutations in GDF9 and BMP15 influencing both aspects of ovarian function. The variants detected in GDF9 in mothers of twins (Palmer et al., 2006) were different from variants in

POF patients in India (Dixit et al., 2005), although this may reflect the different populations used for the studies. Future studies should examine whether the same variants in GDF9 and BMP15 affect both twinning and POF via common mechanisms or whether different variants affect these two aspects of human fertility.

Summary

MZ and DZ twins arise through different mechanisms. Mechanisms for MZ twinning remain obscure, but both types of twinning probably have their origins in the complex regulation of growth and development of the ovarian follicle and early embryo. More is known about the factors involved in DZ twinning. Maternal age has played a major role in fluctuations in twinning frequency during the last 100 years following changing demographic trends. Other factors including body composition, height, seasonality and smoking also seem to contribute to the variation in DZ twinning frequency.

Genetic background contributes significantly to variation in DZ twinning both between and within populations. The known effects of maternal age and genetic variation on DZ twinning suggest that in women the number of follicles that ovulate is controlled by both external hormonal drive and growth factor signaling within developing follicles. Genetic mapping studies in humans and other species are beginning to identify the genes and pathways contributing to the variation in DZ twinning. Associations with alpha-1-antitrypsin and FMR1 should be regarded as tentative and effects of FMR1 may be indirect through accelerated ovarian aging. Recent studies have shown that mutations in the oocyte specific growth factor GDF9 contribute to both DZ twinning and to premature ovarian failure. Future studies will examine whether variants in GDF9 and BMP15 affect both twinning and POF via common mechanisms or whether different variants affect these two aspects of human fertility. Greater understanding of the causes of DZ twinning may provide new insights into mechanisms influencing both fertility and infertility.

Chapter 4

Mode of conception of twin pregnancies: Willingness to reply to survey items and comparison of survey data to hospital records

Toos van Beijsterveldt, Chantal Hoekstra, Roel Schats, Grant W. Montgomery, Gonneke Willemsen and Dorret I. Boomsma

Twin Research and Human Genetics (2008) 11(3) 1-3.

Abstract

Based on results from a survey study in a sample of Australian parents of twins, Raj and Morley (2007) reported that questions concerning the mode of conception of twins may be offensive to parents. We looked at the willingness to reply to questions about mode of conception of twin pregnancies in a large survey study that was completed by 20,150 mothers of twins from the Netherlands Twin Registry. Data collection took place in 2005/2006. 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 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. We found no indication that mothers of twins were not prepared to reply to questions on mode of conception. Only a small number of mothers did not fill in the question on mode of conception (0.8%). Also, the use of artificial fertility techniques did not differ between mothers who returned and mothers who did not return the 2005/2006 survey. The comparison of the survey data with the hospital records showed that mothers can accurately report on the mode of conception of their twins.

Introduction

According to Raj and Morley (2007) questions about mode of conception can be offensive to some parents of twins. In an anonymous survey they examined the willingness of parents of twins to report about themode of conception of their twins. Participants were members of the local branch of the Australian Multiple Birth Association. About 6% of the twin parents indicated that they were not prepared to inform the researchers about the mode of conception when asked for this information in studies about twinning and twin offspring. The authors suggested one should include questions about the mode of conception only when there is a need for it. We looked at the willingness to reply to questions about the mode of conception by mothers of twins registered with the Netherlands Twin Register (NTR; Bartels et al., 2007; Boomsma et al., 2006) who participated in a large questionnaire survey of fertility and twin pregnancies (Hoekstra et al., 2008) that was carried out in 2005/2006. In addition to looking at the willingness to respond to this question, we also examined the reliability of reporting about the mode of conception in a subsample by comparing the responses to the survey question to data from hospital records. In the 2005 survey mothers of twins were asked how their twins were conceived. In the same questionnaire we also asked for permission to link the survey data to other registration systems in The Netherlands, such as hospital records. For a subgroup of 80 mothers of twins, whose children were born in the VU University Medical Centre Amsterdam, we retrieved information on whether the twin pregnancy was assisted or unassisted and whether assisted reproduction had involved in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI) or intrauterine insemination (IUI).

Methods

A two-page survey with questions about familial twinning, fertility and twin pregnancy was sent to all mothers of twins who were registered with the NTR (N = 33,528; Hoekstra et al., 2008). From the mothers who had participated in NTR studies before (N = 25,620), we received 17,683 completed questionnaires, and 1,674 completed questionnaires were received from mothers who had never previously participated. In subsequent years, the survey was also sent to newly registered mothers of the NTR. In total 20,150 surveys were available. Of this group, 8% of the twin pairs were born before 1980, 10% between 1980 and 1989, 49% between 1990 and 1999, and 33% after 1999. The survey contained one question about mode of conception. Possible answers were (1) naturally conceived (with a specification of the time it took to conceive the twins), (2) IVF, (3) ICSI, (4) IUI, (5) ovulation induction, or (6) other, with additional space for comments. In addition, the survey also asked whether the mother gave permission to link the data to other registration systems in The Netherlands. A total 94.3% of mothers gave a positive reply to this question, 4.4% said no and 1.3% did not reply to this question (their answers were treated as *no* responses). Mothers of young twins (twins born after 1986) who registered their children with the NTR also completed a questionnaire about the twin pregnancy shortly after registration (average age of the twins when this first questionnaire was completed = 8.4 months). From this database we could retrieve information for the mothers who took part in the present study (N = 14,423) as well as for 6,062 nonresponders to the 2005 survey. We compared responders and nonresponders with respect to the use of assisted reproduction techniques to see if there was a response bias in the 2005 survey.

A subgroup of twins whose mothers gave permission to link survey data to hospital records, was born in the VU Medical Centre between 1999 and 2006. From the hospital records information on IVF, ICSI, and IUI was retrieved. By comparing the survey data with the hospital records, we first investigated whether the two databases differed in the reporting of assisted versus spontaneous conception, and secondly in the method of conception (IVF or ICSI).

Results

In the survey, only a small number of mothers did not fill in the question on mode of conception (*N* = 159, 0.8%). This amount of missing data does not differ from the amount on a more neutral question, such as height of the mother (*N* = 168). For those who responded, 75% of the mothers reported a natural conception, 16.9% reported IVF/ICSI/IUI, 6.4% a conception after ovarian stimulation, and 0.7% indicated other ways of conception. Part of the sample had also replied to questions about mode of conception in previous surveys. We compared the frequency of the use of assisted reproduction techniques from previous surveys in the responders and nonresponders of the 2005 survey. We found no differences in use of artificial fertilization techniques between the responding and nonresponding group ($\chi^2 = 0.002$, $p = .969$). Comparison to hospital records in a small subset showed that most mothers reported accurately on the mode of conception. The agreement between maternal report and hospital records was 94% — only five out of 80 responses differed between the survey and hospital records. There was a spontaneous pregnancy for these five twin pairs according to the hospital records, but in the survey two mothers reported an assisted conception (IVF/ICSI), two mothers reported an IUI, and one mother reported conception by donor eggs. Agreement was found for 36 twin pairs conceived spontaneously, 31 twin pairs conceived by IVF/ICSI, and eight twin pairs conceived by IUI. Mothers of the assisted conception group did not always distinguish correctly between the methods of assisted reproduction. Of the 31 mothers there were four mothers who reported ICSI while the hospital records indicated IVF. Inspection of the comments field of the 'other' option of our survey question revealed that the meaning of the five answer categories was not always clear and that the comments field was used also by those who marked one of the five answer categories. Table 1 gives an overview of some of the most frequent comments. One of the most frequent comments related to the 'ovulation induction' category suggesting that the term was not clear to the participants. In addition, a large number of comments were from mothers with naturally conceived twin pairs, and included comments such as 'after birth of first child', 'after stopping contraception', 'normal way', 'on holiday', 'was an accident', or 'was not planned'. A large group also mentioned falling pregnant while using contraception or whilst still breastfeeding.

Table 1 Selection of comments from survey question on mode of conception of twin pregnancy.

	Frequency
Spontaneous, accident, not planned, during holiday	202
Ovulation induction, hormones, hormones injections, hormones pills	168
During contraceptive use, during breast feeding	100
Artificial insemination (with and without hormones)	64
After medical treatment, like rinsing oviduct; removing oviduct	48
Shortly after miscarriage	38
Artificial insemination with donor sperm	28
Medical help for conception but not in the cycle of the twin pregnancy	24
Egg donation	14
Cryo frozen eggs	8
Alternative medical help	8

Discussion

The response pattern in a large survey study completed by mothers of twins does not indicate that mothers of twins were not prepared to reply to questions on mode of conception. We observed less than 1% of missing answers. The comparison of hospital records in a small subgroup with the NTR survey data showed that parents can quite accurately report on the mode of conception of their twins. In a study from Australia, Raj and Morley (2007) reported that about 6% of the parents were not be prepared to tell researchers in twin studies about the mode of conception of their twins, while in our study only a small number of mothers did not complete the question effectively. There may be cultural differences between the two countries in the willingness to reply to this question, but it may also reflect differences in the organization of the approach of the parents. In the case of the Australian sample, parents were approached through a branch of the Australian Multiple Birth Association, whose main interest does not involve research participation. In the case of the NTR, however, participants agree to take part in scientific research projects and expect to be sent questionnaires on their twins and on twinning. This may have influenced the rates of willingness to answer the question on mode of conception. Our results demonstrate that within a research group, parents of twins are prepared to answer questions on mode of conception. Unwillingness to answer these questions may be linked to unwillingness to participate in research per se. It is possible that nonresponse to the questionnaire was influenced by questions on mode of conception. If this influenced response rate, results from Raj and Morley (2007) would predict a higher proportion of assisted conceptions in nonresponders. To address this question we compared the replies of responders and nonresponders in this survey on the answers to similar questions from earlier survey data. There was no difference between responders and nonresponders with respect to the frequency of assisted conception of the twin pregnancy. In this Dutch sample, including a question on mode of conception seems unlikely to affect the participation rate. On the basis of the comments from the survey question on the mode of conception, we would advise the incorporation of a field for comments in the survey.

Chapter 5

Familial twinning and fertility in Dutch mothers of twins

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Abstract

We studied twinning and fertility indices in mothers with spontaneous monozygotic (MZ) and dizygotic (DZ) twins and in mothers who conceived their twins after the use of assisted reproduction techniques (ART). Participants in this study consisted of 8,222 and 5,505 women with spontaneous DZ and MZ offspring and 4,164 and 250 women with ART DZ and MZ twin pairs, respectively. Women were compared with respect to the number of sibs and offspring, the presence of other relatives with twins and the time it took to conceive the twins. We also compared familial twinning between a younger and an older age group.

Women with spontaneous DZ twins more often reported female relatives with twins than those with spontaneous MZ twins. The proportion of DZ versus MZ twin offspring in relatives was also larger in women with spontaneous DZ offspring than in women with MZ offspring. The first group of women reported a shorter time to conceive. Women with ART twins had fewer sibs and offspring and less often reported relatives with twins. We did not observe that DZ twinning was more familial in women who had their twins before age 36 years compared to older women.

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.

Introduction

It has long been known that dizygotic (DZ) twinning runs in families (e.g., Hoekstra et al., 2008a). In 1901, Weinberg described familial clustering of DZ twin pregnancies. He observed that mothers, sisters, and daughters of women who have given birth to multiples, had an increased risk of conceiving twins or multiples by 39%, 95%, and 30%, respectively. This increased risk was not found in relatives on the paternal side of the family. Some 60 years later, Wyshak and White came to the same conclusion when analyzing the inheritance of twinning using data obtained from the archives of the Genealogical Society of the Mormon Church in Salt Lake City (White and Wyshak, 1964; Wyshak and White, 1965). Further support was obtained by Bulmer (1970) who found that the risk of conceiving twins was 1.7 times higher in female relatives than in male relatives of index mothers. Examining the DZ twinning rate among female relatives in greater detail, Bulmer showed that the twinning rate in women who had a sister with DZ twins was 2.5 times higher than the twinning rate in the general population. The risk of having twins for mothers and daughters of a woman with DZ twins was about twice as high as the risk in the general population (Bulmer, 1970). Additional studies support evidence for familial DZ twinning. Lewis et al. (1996) investigated familial twinning in 6,596 twin pairs from the Australian Twin Registry and found a relative risk of 1.7 for sisters and mothers of DZ twins and of 2.5 for the offspring of female DZ twins. Meulemans et al. (1996) investigated the inheritance of DZ twinning in 1,422 Dutch and Flemish families by formal segregation analysis. The phenotype of "having DZ twins" was consistent with an autosomal dominant monogenic model with incomplete penetrance. Thus, the genetic contribution to DZ twinning is observed in women, but the trait may be inherited from either parent (Greulich, 1934; Parisi et al., 1983).

Additional factors influencing DZ twinning, which may or may not interact with genotype, are maternal age, parity and the use of fertility treatments (Bulmer, 1970; Fauser et al., 2005). The decline in the DZ twinning rate in the early 20th century reflected a decrease in mean maternal age and a lower number of maternities. The increase in twinning rate reported in the late 1970s has been mainly associated with an older age at childbearing (Derom et al., 1995; Lambalk et al., 2004). The use of fertility treatments such as ovulation induction (OI), in vitro fertilization (IVF), intro cytoplasmatic sperm injection (ICSI), and intra-uterine insemination (IUI) has further added to the rising incidence in twin births (Fauser et al., 2005; Martin et al., 2005).

Monozygotic (MZ) twinning has a very different etiology than DZ twinning (Hall, 2003). MZ twinning does not seem to be influenced by genetic factors, maternal age and parity, though families with a history of MZ twinning have been reported (Bulmer, 1970; Hamamy et al., 2004). Recently, an increase in MZ twin births has been reported after IVF and OI (Steinman, 2003; Derom et al., 2006). If MZ twinning is the result of a random event while DZ twinning is influenced by a genetic predisposition, the comparison of mothers who gave birth to DZ twins with mothers who gave birth to MZ twins may provide valuable clues concerning the processes involved in fertility and subsequently infertility. Such a comparison was undertaken by Lewis et al. (1996), who obtained information on familial twinning through the twins themselves. The prevalence of additional DZ twins in the family of DZ twins was much higher than the prevalence of additional MZ twins in the family of MZ twins, confirming a stronger familial component for DZ twinning.

We collected data on familial twinning from mothers of twins. Familial twinning was examined in mothers with spontaneous MZ and DZ twin offspring and in mothers of MZ and DZ twins who conceived their offspring after assisted reproduction techniques (ART).We compared these groups on the following familial and fertility related variables (1) the number of brothers and sisters of the index mother and the number of offspring of the index mother (2) the presence of other family members with twin offspring (3) the ratio of DZ and MZ familial twinning and (4) the time it took to become pregnant with the twins.

It is still unclear if maternal and iatrogenic factors affect twinning independently or interact with genetic factors causing DZ twinning. It is possible that women who conceived DZ twins after ART also had a genetic susceptibility for DZ twinning, which may be expressed as providing the embryos with an optimal uterine milieu or having embryos of high quality (Lambers et al., 2007). If mothers of ART DZ twins have a genetic predisposition, they should have similar numbers of additional family members with twins as mothers of spontaneous DZ twins. We therefore compared women with spontaneous and with ART DZ twins on the proportion of other family members with twins. We also compared mothers of ART MZ twins with mothers of ART DZ twins. If having DZ twins after fertility treatment is influenced by genetic factors, while having MZ twins after ART is not, we would expect fewer mothers of ART MZ pairs to report a family history of twinning than ART DZ.

Women with multiple sets of spontaneous DZ twin pairs may be considered as an even more genetically predisposed subgroup of DZ twin mothers. We compared women with multiple sets of spontaneous DZ twins to those with one set of DZ twins on familial twinning and the time it took to become pregnant. DZ twinning rates increase with maternal age; the chance of having DZ twins increases approximately fourfold up to the age of 36 years (Bulmer, 1970). This means that any genetic predisposition to DZ twinning may be more apparent in younger women, so we examined whether women who had DZ pairs at a younger age (<36 years) reported more familial twinning than women who had DZ pairs at an older age.

Finally, in women who are twins themselves we examined whether their cotwin was also a mother of twins and if so, whether they were concordant for twin offspring zygosity.

Methods

Participants. The Netherlands Twin Register (NTR) collects longitudinal data on twins and their family members in two samples: (1) in mothers of newborn or young twins (YNTR) who are registered at birth by their parents and (2) in adolescent and adult twins (ANTR) and their family members. Over 90% of the participants are born in the Netherlands (Boomsma et al., 2002; 2006; Bartels et al., 2007).

In 2005, a survey was mailed to all mothers of twins and multiples (N = 33,528) registered with the NTR (referred to as index mothers). Index mothers were asked to complete a series of questions on maternal characteristics prior to the birth of the twins, familial twinning and mode of conception. From the mothers who participated in NTR studies before (N = 25,620), we received 17,683 completed questionnaires, and 1,674 completed questionnaires were received from mothers who had never previously participated. In 2006, the survey was also sent to newly registered mothers. In total, data from 20,150 surveys were available; 260 surveys were returned by mothers of triplets. We previously reported on the absence of response bias by comparing data from women who returned this survey with data from mothers who did not return the survey, but who had taken part in earlier NTR studies (Hoekstra et al., 2008b; Van Beijsterveldt et al., 2008).

Data were excluded when the index mother was not the biological mother of the twins or when the relation with the twins was unknown (N = 94); when data on fertility treatment were lacking or when the twins were conceived by other methods than IVF, ICSI, IUI and OI (N = 294), such as egg donation; when data on familial twinning were missing (N = 406); or when the response to the familial twinning questions was unclear (N = 585). There were 134 women, who completed a similar questionnaire designed for mothers with multiple sets of twins. If the zygosity and/or mode of conception was different for the two pairs, e.g. one twin pair was spontaneous and one twin pair was artificially conceived, data were excluded (N = 53). Data of 577 twin mothers were excluded due to missing information on zygosity of the twin offspring (in 388 cases the index mother indicated that she did not know the zygosity of her twins and in 189 cases the index mother did not answer the

question or her answer was unclear). For these twin pairs, no other information on zygosity (e.g., from earlier NTR surveys) was available.

Zygosity data were available on 18,141 twin pairs. For same-sex pairs, zygosity was based on DNA polymorphisms obtained in participants in previous NTR studies (N = 1,656) or from previous survey questions (N = 7,403). When DNA and previous survey data were not available, zygosity was based on the answers of the mother in the current survey (N = 2,983). Previous questions regarding offspring zygosity asked whether the twins were alike in eye-, hair- or face color and in face form and whether the twins were often mistaken for each other by their parents, other relatives and by strangers. Based on the answers to these questions, zygosity was determined in same-sex twin pairs. The association between DNA and questionnaire zygosity is 93% in the YNTR sample (Rietveld et al., 2000) and 97% in the ANTR sample (Willemsen et al., 2005). A comparison of the zygosity of same-sex twin pairs based on information from the current survey and the zygosity obtained from previous questionnaires also showed a high degree of agreement (90.7%). The sample included 5,943 (32.8%) index mothers with opposite-sex twin offspring which is comparable with the general Dutch population (34.4%) (Statistics Netherlands, 2007). The sample included 78 mothers of sponteneous di- and tri-zygotic triplets, who were included in the mothers of DZ twins group and 24/6 mothers of spontaneous/ART MZ triplets.

The final sample consisted of 5,505 women with spontaneous MZ twin offspring (including 8 mothers of 2 sets of twin pairs), 8,222 women with spontaneous DZ twin offspring (including 61 mothers of multiple sets of twin pairs), 4,164 women with ART DZ twins (including 12 mothers of multiple sets of twins) and 250 women with ART MZ twins.

Family size. Mothers of twins were asked how many sibs they had with the same biological mother and father. They could indicate 0-8 or more brothers and 0-8 or more sisters.

Number of children. The number of own offspring is obtained by summing the number of twins, number of triplets and the number of single children.

Relatives with twin offspring. The survey contained a series of items regarding familial twinning. Women were asked "Which of your own biological family members are also parents of twins/multiples?". Familial relationships and the number of index mothers who reported a relative with twin offspring are described in Table 1. There were 5,520 index mothers who reported 1 additional type of relative with twins/multiples and 2,058 index mothers who reported more than 1 type of relative with multiples. The categories listed in Table 1 are used to describe the relationship of the index mother with her relatives in all analyses. When family members with twins were indicated, the index mother could report if the twins of these family members were girls (FF), boys (MM) or a girl and boy (FM). The index mother was also asked to report whether the twins of her relatives were monozygotic (MZ) or dizygotic (DZ). She could also report that she did not know the zygosity of the twins of her relatives.

Familial twinning was defined as follows: if an index mother indicated that she had a female relative with twins, regardless of zygosity and the number of family members that were reported, then the index mother was given a yes for familial twinning. Similarly, if an index mother reported a female relative with DZ twins she received a yes for DZ familial twinning. If an index mother had female relatives with MZ twins, she received a yes for MZ familial twinning. If both female relatives with MZ offspring and female relatives with DZ offspring were present (e.g., an aunt with DZ twins and a sister with MZ twins), she received a yes for both DZ and MZ familial twinning.

Table 1 Number of index mothers with family members who have twin/multiple offspring (by family relation).

Relationship	Category	N of probands with 1 type of relative with multiples	N of probands with > 1 type of relative with multiples
My daughter(s) has/have multiples	Daughter	15	29
My son(s) has/have multiples	Son	9	11
My sister(s) has/have multiples	Sister	415	382
My brother(s) has/have multiples	Brother	265	257
My mother has multiples	Mother	594	532
Sister(s) of my mother has/have multiples	Aunt (M)	686	671
Brother(s) of my mother has/have multiples	Uncle (M)	511	456
The parents of my mother has/have multiples	Grandma (M)	951	661
The sister(s) of my father has/have multiples	Aunt (P)	604	573
The brother(s) of my father has/have multiples	Uncle (P)	489	427
The parents of my father has/have multiples	Grandma (P)	981	642
Number of families with twins/multiples		**5,520**	**2,058**

Note: P = paternal; M= maternal

Use of assisted reproduction techniques (ART) and Time to become pregnant. Whether the twins were conceived spontaneously or not and the time it took to become pregnant with twins were asked in a combined question. The index mother was asked "How did you become pregnant with your first twin pregnancy?" The answer categories were: (1) It was a spontaneous pregnancy, and I became pregnant in (a) 0-2 months (b) 3-5 months (c) 6-12 months (d) more than 12 months; (2) IVF; (3) ICSI; (4) IUI; (5) OI and (6) other, specify. Based on the answers to these questions, we created a variable with 3-categories to indicate whether the birth was (1) spontaneous or the result of (2) IVF, ICSI, IUI or (3) OI. Mothers who ticked the category "other specify" in the original question were either classified as a mother of spontaneous twins or a mother of an ART twin pair depending on the answer. Data from women which did not fall within one of these classes of conception were excluded (N=71). A 4-category variable was created to indicate for those who had a spontaneous pregnancy the time it took to conceive (0-2 months, 3-5 months, 6-12 months, more than 12 months).

Maternal age. Maternal age at time of the twin birth was obtained by subtracting the birth date of twins from the birth date of the mother. Maternal age was recoded into two categories; younger than 36 years and 36 years or older at twin birth.

Maternal twin status. All index mothers were asked if they were twins themselves and, if yes, about their zygosity. If a woman was a twin herself and indicated that she had at least one sister who was a mother of twins, we tried to contact her by phone to obtain information on which of her sisters was the mother of twins.

Analysis. We tested whether the number of sibs and the number of own offspring differed between women with spontaneous DZ twins, women with spontaneous MZ twins and women with ART DZ and ART MZ twins using Chi-squared tests. Next, we compared the frequency of familial twinning (both MZ and DZ) in women with spontaneous DZ twins and women with spontaneous MZ twins. We also examined whether women with spontaneous DZ twins differed from women with spontaneous MZ twins in the proportion DZ

versus MZ twin offspring in relatives. This was done for each familial relationship (e.g., sister, parents) as well as for familial twinning (having at least one female relative with twins). We repeated these analyses by using only relatives with DOS twins as zygosity is certain for DOS twins. There were 797 index mothers with a sister who also was a mother of twins. In some families (N = 249) both sisters were registered with the NTR and returned the questionnaire. As these sisters pairs were ascertained independently, all data were used in the analyses. However, repeating the analyses, with the data from one sister removed, did not change the results.

At the second step, women with spontaneous DZ twins were compared to women with ART DZ twins on the frequency of familial twinning and on the proportion of DZ versus MZ twin offspring in their relatives. The frequency of familial twinning was also compared between ART MZ and ART DZ group. At the third step, the frequency of familial twinning in women who had multiple spontaneous DZ twin pairs was compared to the frequency of familial twinning in women who had one spontaneous DZ twin pair. We also examined whether women with spontaneous DZ twins differed in the time it took to conceive the twin pregnancy from women with spontaneous MZ twins. To investigate if genotype interacts with age, we examined whether women who had their DZ twins at an older age (36 or older) less often reported relatives with twins than women who had their DZ twins when they were younger. Finally, we looked at the women who were twins themselves and at the concordance with their twin sister for being a twin mother.

Results

Table 2A shows the number of sibs of the index mothers. There was no difference in the number of sibs reported by index mothers with spontaneous DZ twins and spontaneous MZ twins ($\chi^2 = 3.710$, df=8, $p = 0.882$). This was also the case when examining the number of brothers ($\chi^2 = 9.326$, df=8, $p = 0.316$) and sisters ($\chi^2 = 6.760$, df=8, $p = 0.563$) separately. However, women with spontaneous DZ twins reported significantly more sibs than women with ART DZ twins ($\chi^2 = 96.628$, df=8, $p < 0.001$). This was the also the case when looking at the number of brothers ($\chi^2 = 59.978$, df=8, $p < 0.001$) and sisters ($\chi^2 = 49.961$, df=8, $p < 0.001$) separately. We found no difference in number of sibs between women with spontaneous MZ twins and ART MZ twins ($\chi^2 = 6.472$, df=8, $p = 0.595$).

Table 2 Number and percentage of own siblings (part A) and own children (part B) for women with spontaneous DZ twins, spontaneous MZ twins, ART DZ twins and ART MZ twins.

A.	Proband with spontaneous				Proband with ART			
Number of siblings	DZ twin offspring		MZ twin offspring		DZ twin offspring		MZ twin offspring	
	N	%	N	%	N	%	N	%
0	373	4.6	256	4.7	218	5.3	10	4.0
1	2,485	30.9	1,687	31.3	1,518	37.2	82	33.2
2	2,154	26.7	1,437	26.6	1,098	26.9	68	27.5
3	1,217	15.1	845	15.7	564	13.8	43	17.4
4	696	8.6	441	8.2	300	7.4	22	8.9
5	392	4.9	254	4.7	146	3.6	9	3.6
6	281	3.5	166	3.1	80	2.0	5	2.0
7	174	2.2	115	2.1	63	1.5	1	0.4
8 or more	282	3.5	195	3.6	89	2.2	7	2.8
Total	7,990	100.0	5,396	100.0	4,068	100.0	263	100.0
B. Number of own children								
	N	%	N	%	N	%	N	%
2	2,271	28.9	1,577	30.1	2,108	54.4	117	49.6
3	3,553	45.3	2,472	47.1	1,419	36.6	91	38.6
4	1,451	18.5	879	16.8	274	7.1	18	7.6
5	395	5.0	200	3.8	54	1.4	7	3.0
6 or more	177	2.3	119	2.3	18	.5	3	1.3
Total	7,847	100.0	5,247	100.0	3,873	100.0	236	100.0

Note: ART = assisted reproduction technologies

In Table 2B the number of own children is given for the women with spontaneous MZ and DZ twins, ART DZ and ART MZ twins. Women with spontaneous DZ twins had three or more children more often than women with ART DZ twins (χ^2 = 865,741, df=4, $p < 0.001$). Also, women with spontaneous MZ twins had three or more children more often than women with ART MZ twins (χ^2 = 44.322, df=4, $p < 0.001$). Women with spontaneous DZ twins had more families of four or more children than women with spontaneous MZ twins (χ^2 = 19.306, df=4, $p = 0.001$).

We first compared the frequency of familial twinning (at least 1 female relative with twins, irrespective of zygosity) between women with spontaneous DZ and MZ twins. Women with spontaneous DZ twin offspring had a female relative with twins significantly more often than women with spontaneous MZ twin offspring (χ^2 = 37.122, df=1, $p < 0.001$). Of all women with MZ twins, 21.9% (1,204 of 5,505) had female relatives with twins, while of all women with DZ twins, 26.4% (2,174 of 8,222) had female relatives with twins.

Table 3 shows the information on the proportion of DZ versus MZ twin offspring in relatives of women with spontaneous DZ and MZ twins. Because of the small numbers reported, we excluded the categories "my daughter has twins" (N = 44, 36 with known zygosity) and "my son has twins" (N = 20, 16 with known zygosity) from the analyses. With respect to familial twinning, the proportion of DZ versus MZ twin offspring in relatives was greater in women with spontaneous DZ offspring than in those with spontaneous MZ offspring (χ^2 = 53.409, df=1, $p < 0.001$). Of the twin offspring in relatives of women with DZ twins 81.9% were DZ (1,873 of 2,288), while this percentage was 71.3% (912 of 1,279) in the relatives of women with MZ twins. The same direction of effect was found for all individual female relatives, but not for male relatives. One exception was the proportion of DZ offspring in brothers. The brothers of women with spontaneous MZ twins had significantly more DZ offspring (113 of 144) than the brothers of women with spontaneous DZ twins (139 of 202).

Since index mothers may overreport the zygosity of their relatives' twin based on their own twin offspring' zygosity (i.e., mothers of DZ twins may be more likely to report twins of relatives as dizygotic), we repeated the analyses including DOS twin offspring instead of all DZ twin offspring. We found similar results to the comparison with all DZ twins. The proportion of DZ offspring in female relatives was higher in women with DZ twins (72.8%) than in the female relatives of women with MZ offspring (59.3%).

Table 3 Number and percentage of relatives with MZ, DZ or DZ opposite-sex twin offspring in index mothers with spontaneous DZ and MZ twin offspring.

	Index mothers with DZ twin offspring (N= 8,222)								Index mothers with MZ twin offspring (N=5,505)					
	Relative with MZ offspring		Relative with DZ offspring			Relative with DOS offspring			Relative with MZ offspring		Relative with DZ offspring		Relative with DOS offspring	
	N	%	N	%		N	%		N	%	N	%	N	%
Sister	57	15.9	301	84.1	**	157	73.4	**	51	27.0	138	73.0	54	51.4
Brother	63	31.2	139	68.8	*	72	53.7		31	21.5	113	78.5	44	58.7
Mother	87	17.1	421	82.9	**	201	69.8	**	90	32.3	189	67.7	104	53.6
Aunt (M)	99	17.5	468	82.5	**	250	71.6	**	73	25.5	213	74.5	107	59.4
Uncle (M)	90	25.7	260	74.3		155	63.3		66	24.8	200	75.2	109	62.5
Grandmother (M)	90	15.3	497	84.7	**	271	75.1	**	96	29.1	234	70.9	134	58.3
Aunt (P)	87	18.0	396	82.0	**	212	70.9	**	91	32.9	186	67.1	98	51.9
Uncle (P)	75	24.8	227	75.2		112	59.9		79	31.1	175	68.9	105	57.1
Grandmother (P)	88	15.0	499	85.0	*	267	75.2	**	79	25.1	236	74.9	135	63.1
At least 1 female relative with twins	415	18.1	1,873	81.9	**	1,110	72.8	**	367	28.7	912	71.3	543	59.3

Note: M= maternal; P = paternal; DZ offspring includes opposite sex twins (DOS) offspring. Percentages in the 2nd and 4th columns (relative with MZ offspring / relative with DZ offspring) sum to 100% across rows. Percentages in column 6 (relatives with DOS offspring) are calculated as percentages of relatives with MZ + DOS offspring. * p<.05, ** p<.01; significance levels are given for the comparison of the proportion of relatives with DZ versus MZ offspring in index mothers with spontaneous DZ and MZ twin offspring.

Next, we examined differences in familial twinning between index mothers with spontaneous DZ and ART twins. We first tested whether DZ mothers who conceived their twins after OI (N = 1,103) and DZ mothers who conceived their twins after IVF, ICSI or IUI (N = 3,061) differed in familial twinning (MZ or DZ) and found no differences (χ^2 = .308, df=1, p = 0.579). We therefore treated these groups of mothers as one group.

Table 4 shows the frequency of familial twinning in women with spontaneous DZ twins compared to women with ART DZ and ART MZ twins. Women with spontaneous DZ twins had a female family member with twins (MZ or DZ) significantly more often than women with DZ twins conceived after fertility treatment (χ^2 = 55.777, df=1, p < 0.001). Of the spontaneous DZ group, 26.4% (2,174 of 8,222) had at least one female relative with twins, compared to 20.3% (847 of 4,164) of the ART DZ index mothers. Differences in familial

twinning between women with spontaneous DZ offspring and women with ART DZ offspring were found for all female relatives, but not in the male relatives with the exception of the uncle on mothers' side.

With respect to DZ familial twinning, women with spontaneous DZ twins had a female relative with DZ twin offspring more often than women with ART DZ twins (81.9% versus 73.3%, $\chi^2 = 28.607$ df=2, $p < 0.001$). The pattern of results regarding the ratio of DZ-MZ twinning was also found for both maternal and paternal aunts ($\chi^2 = 14.266$, df=1, $p < 0.001$ and $\chi^2 = 8.209$, df=1, $p = 0.004$, respectively) and both maternal and paternal grandmothers ($\chi^2 = 6.634$, df=1, $p = 0.01$ and $\chi^2 = 9.688$, df=1, $p = 0.002$, respectively). The proportion of brothers with DZ twins did not differ significantly between women with spontaneous DZ twins (139 of 202) compared to women with ART DZ twins (74 of 95).

The last columns in Table 4 present the data for familial twinning for index mothers with ART MZ offspring. We only analyzed the data for familial twinning, as the numbers of women with ART MZ offspring were too small to compare the ratio for individual relationships. Women with ART DZ offspring did not differ from women with ART MZ offspring with respect to the presence of relatives with a twin ($\chi^2 = 0.876$, df=1, $p = 0.349$). In addition, the groups did not differ in the proportion of DZ twins; 73.3% of the ART DZ mothers reported DZ familial twinning, compared to 77% of the ART MZ mothers ($\chi^2 = 0.404$, df=1, $p = 0.525$).

Table 4 Number and percentage of relatives with twin offspring and relatives with MZ / DZ twin offspring in index mothers with spontaneous DZ twin offspring and probands with ART DZ or ART MZ twins.

	Index mothers with DZ twin offspring (N= 8,222)								Index mothers with ART-DZ twin offspring (N=4,164)								Index mothers with ART-MZ twin offspring (N=250)							
	Relative with twins			Relative with MZ offspring		Relative with DZ offspring			Relative with twins		Relative with MZ Offspring		Relative with DZ offspring				Relative with twins		Relative with MZ offspring		Relative with DZ offspring			
	N	%		N	%	N	%		N	%	N	%	N	%			N	%	N	%	N	%		
Sister	353	4.3	**	57	15.9	301	84.1	*	120	2.9	30	24.6	92	75.4			11	4.4	3	25.0	9	75.0		
Brother	202	2.5		63	31.2	139	68.8		95	2.3	21	22.1	74	77.9			9	3.6	2	22.2	7	77.8		
Mother	507	6.2	**	87	17.1	421	82.9	**	150	3.6	43	28.5	108	71.5			6	2.4	1	16.7	5	83.3		
Aunt (m)	555	6.8	**	99	17.5	468	82.5	**	218	5.2	66	29.6	157	70.4			14	5.6	2	14.3	12	85.7		
Uncle (m)	346	4.2	*	90	25.7	260	74.3		137	3.3	43	30.9	96	69.1			12	4.8	4	30.7	9	69.3		
Grandmother (m)	580	7.1	**	90	15.3	497	84.7	*	235	5.6	55	22.8	186	77.2			22	8.8	6	27.3	16	72.7		
Aunt (f)	473	5.8	**	87	18.0	396	82.0	**	154	3.7	45	28.7	112	71.3			14	5.6	5	35.7	9	64.3		
Uncle (f)	300	3.6		75	24.8	227	75.2	*	137	3.3	50	36.0	89	64.0			13	5.2	4	30.8	9	69.2		
Grandmother (f)	582	7.1	**	88	15.0	499	85.0	**	241	5.8	58	24.1	183	75.9			15	6.0	2	13.3	13	86.7		
At least 1 female relative with twins	2,174	26.4	**	415	18.1	1,873	81.9	**	847	20.3	237	26.7	652	73.3			57	22.8	14	23	47	77		

Note: The total number of female relatives with twins also includes families in which both MZ and DZ offspring occur (114 probands with DZ offspring had relatives with both MZ and DZ offspring); DZ offspring includes DOS offspring; percentages in the 4th and 6th columns (relative with MZ offspring /relative with DZ offspring) sum to 100% across rows. * Significant ($p<.05$), ** Significant ($p<.01$)

Table 5 shows the frequency of familial twinning in women with one pair of spontaneous DZ twins and women with multiple sets of spontaneous DZ twins (N = 61). Women with MZ and ART DZ offspring were not included (N = 20). For the comparison of these two groups, we only examined familial twinning (having a female relative with twins) because there were too few observations to study each familial relationship separately. We found that women with multiple sets of spontaneous DZ twins had a female relative with twins (MZ or DZ) significantly more often than women with one set of spontaneous DZ twins (χ^2 = 10.081, df=1, p = 0.001). However, women with multiple sets of spontaneous DZ twins did not more often have a relative with DZ twin offspring (Fisher Exact test, p = 0.458).

Table 5 The proportion of relatives with twins, relatives with DZ twin offspring and MZ twin offspring and relatives with only DOS offspring in women with one set of spontaneous DZ twins and women with multiple sets of spontaneous DZ twins.

	Index mothers with one DZ twin (N= 8,012)								Index mothers with multiple sets of spontaneous DZ twins (N= 61)							
	Relative with twins		Relative with MZ offspring		Relative with DZ offspring		Relative with DOS offspring		Relative with twins		Relative with MZ offspring		Relative with DZ offspring		Relative with DOS offspring	
	N	%	N	%	N	%	N	%	N	%	N	%	N	%	N	%
All relatives	2,105	26.3	403	18.2	1,813	81.8	1,071	72.7	27	44.3	3	10.7	25	89.3	12	80.0

Note: DZ offspring includes DOS offspring; percentages in the 4th and 6th columns (relative with MZ offspring / relative with DZ offspring) sum to 100% across rows.

The data for time to conceive for index mothers with spontaneous DZ and MZ twins are shown in Table 6. Women with spontaneous DZ twin offspring became pregnant more quickly with their twins than women with spontaneous MZ twin offspring ($\chi^2 = 31.873$, df=3, $p < 0.001$). We did not find a difference in the time it took to become pregnant between women with one spontaneous DZ twin pair and women with multiple sets of spontaneous DZ twin pairs ($\chi^2 = 2.311$, df=3, $p = 0.510$).

Table 6 Time to conceive in mothers of spontaneous twins for women with DZ twins, MZ twins and for women with one set and multiple sets of DZ twins.

Time it took to conceive twins	All DZ twin* offspring		MZ twin offspring		One set of DZ twins		Mothers with multiple sets of DZ twins	
	N	%	N	%	N	%	N	%
0-2 months	3,705	50.2	2,234	45.2	3,616	50.2	30	54.5
3-5 months	1,883	24.8	1,298	26.3	1,790	24.9	12	21.8
6-12 months	1,060	14.4	817	16.5	1,034	14.4	10	18.2
> 12 months	784	10.6	593	12.0	760	10.6	3	5.5
Total	7,432	100.0	4,942	100.0	7,200	100.0	55	100.0

* All DZ offspring includes mothers with one set of DZ twin offspring and mothers with multiple sets of DZ twin offspring.

When comparing index mothers who gave birth to their twin at a younger (N = 7,353) versus a later age (N = 815), we found no differences in female familial twinning ($\chi^2 = 0.814$, df=1, $p = 0.367$). Of the women who were younger than 36 years at the time of the twin birth, 26.3% had a female relative who had given birth to twins. Of the women of 36 years or older at the time of twin birth, 27.7% had a female relative who had given birth to twins. If only females relatives with DZ offspring were considered these percentages were 23.5% for women < 36 years and 24.8% for the older group ($\chi^2 = 0.419$, df=1, $p = 0.654$).

There were 482 index mothers who reported to be a twin themselves. Of these, 112 women indicated they were MZ, 323 were DZ and 47 mothers did not know their zygosity. In the group of 112 index mothers who were MZ twins themselves, there were 7 women with a sister who was also the mother of twins. Of these women, four women represent two twin pairs; one pair was concordant for having MZ twins, one pair was concordant for having DZ twins. In one family, the sister with twins was not the co-twin and in two families it was not clear if it was the co-twin or another sister; two of these families were discordant for twin offspring zygosity.

In the group of 323 index mothers with a DZ twin sister there were 23 women who had a sister with twins. In five cases the co-twin was also a mother of twins; two of them were concordant having DZ offspring and three were discordant for twin offspring zygosity. In nine cases the sister with twins was not the co-twin (six were concordant for DZ twinning, one for MZ twinning and two were discordant) and in the other cases it was not clear whether the sister with a twin was the co-twin.

Discussion

Family and fertility related characteristics were compared in women with spontaneous DZ and MZ twins and in women with ART MZ and DZ twins. For spontaneous twinning, we found that women with DZ twin offspring reported female relatives with DZ twins significantly more often than women with MZ twin

offspring. This association predominantly applied to the female relatives, but equally to the female relatives on the fathers and on the mothers' side of the family. Thus, these results are consistent with the current understanding that DZ twinning is a trait which is passed on from both father and mother, which can only be expressed in women and for which men can be carriers (Meulemans et al., 1996). Women with spontaneous MZ and DZ twins did not differ in the number of brothers and sisters, so this result is not biased because of a larger number of sibs in DZ twin mothers.

It is likely that the larger presence of DZ familial twinning (i.e., relatives with DZ twins) reported by women with DZ twins indicates a genetic predisposition to have DZ twins. A possible mechanism may be a higher rate of ovulation (Martin et al., 1991; James, 2007). Martin et al. (1991) found that mothers of DZ twins had increased rates of ovulation compared to mothers of MZ twins. We found that women with spontaneous DZ twins conceived more quickly than women with spontaneous MZ twins, supporting the notion that mothers of spontaneous DZ twins can be viewed as being more fertile than mothers of spontaneous MZ twins. A special group consists of women who have had multiple sets of spontaneous DZ twins. These women may have a stronger genetic predisposition for DZ twinning than women with a single set of DZ twins. If this is the case, we expect to see a higher frequency of familial DZ twinning and a shorter time to conceive in the women with multiple spontaneous DZ twins. Women with multiple sets of DZ twins indeed had significantly more familial twinning (DZ or MZ) than women with one set of DZ twins. Probably due to the small sample size, there was no significant difference with regard to DZ twinning only. There was a trend, however, for women with multiple sets of DZ twins to have more relatives with DZ twins than women with one set of DZ twins. Analysis of the time it took to conceive showed no significant difference between women with one set and more than 1 set of DZ twins. Again, the reason for not finding such a difference might be that the sample of women with multiple sets of DZ twins was small. Alternatively, these women have had access to contraceptives, and it therefore may be difficult to pick up differences in genetic susceptibility as women (and their husbands) may have decided after having had one set of twins that they would not further increase their number of offspring. A true comparison of familial twinning in mothers of one and of more spontaneous DZ twin pairs may only be obtained through designs such as that of Lummaa et al. (2007) who had access to extensive family data through church records in a time that contraceptives were not available or, alternatively, in present times by examining pedigrees in communities in which the use of contraceptives is restricted.

One of the earliest reported factors influencing DZ twinning is maternal age, with a fourfold increased risk of having DZ twins after up to the age of 36 years (Bulmer, 1970). The etiology of having twins at an older age might be different from that of younger mothers (Lambalk et al., 1998). Our findings do not support this idea; women who had spontaneous DZ twins at a young age (<36 years) reported relatives with DZ twins as often as women who had their twins at a later age.

Little is known about family size and familial twinning in families of twins conceived after fertility treatment. Iatrogenic factors might interact with genetic factors causing DZ twinning or these factors might affect twinning independently. In our study we found a significant difference in the proportion of female relatives with DZ twins between women with spontaneously conceived DZ twins and women with ART DZ twins. Women with ART DZ twins reported female relatives with DZ twins less often than women with spontaneous DZ twins. This confirms that the mechanisms underlying spontaneous and non-spontaneous DZ twinning are different. Some studies hypothesize that embryo quality plays an important role in the maintenance of a multiple pregnancy after IVF (Zegers-Hochschild et al., 2004). This might be an inherited maternal characteristic in addition to a double ovulation in women with spontaneous DZ twins. If good embryo quality is also inherited in mothers of artificially conceived DZ twins one would expect to see an increased number of relatives with DZ twins in these mothers, compared to women with ART MZ twins. However, we did not find

any differences for DZ familial twinning in mothers of ART DZ and ART MZ twins. This suggests that spontaneous DZ twinning is largely a function of double ovulation. In women with ART DZ twins, embryo's are selected from a better cohort of embryo's which increases the change of double implantation and continuation of the multiple pregnancy (Tummers et al., 2003; Lambers et al., 2007) and this process does not seem to be related to a genetic predisposition to have DZ twins.

Women with ART DZ twins came from smaller families. This should be kept in mind when comparing this group with spontaneous twin mothers with regard to familial twinning. Alternatively, the finding that mothers who had infertility treatment came from smaller families than mothers who had their twins spontaneously, may imply that mothers of ART DZ twins come from families that are less fertile, though we do not know whether the reason for fertility treatment was because of fertility problems in the mother or the father of the twins. Still, there is a well documented positive association in family size between parents and children (Axinn et al., 1994; Pouta et al., 2005) and while it is very likely there is a significant genetic component to this association, it is difficult to estimate the true contribution of genes to this association due to the large contribution of social or cultural circumstances (Pluzhnikov et al., 2007). The lower number of sibs in women with ART DZ twins compared to women with spontaneous DZ twins seems in line with a genetic contribution to reproduction, at least with regard to decreased fertility.

The present study demonstrated that, at least with respect to the genetic susceptibility of having DZ twins, it is necessary to treat spontaneous and ART twins as two separate groups. This not only refers to the study of the mechanisms involved in DZ twinning, but may also apply to the study of twins in general as ART DZ twins come from smaller families, with fewer brothers and sisters.

A limitation of this study is that index mothers from "twinning" pedigrees may be more likely to report on familial twinning than others. This bias is likely to be small because the item about familial twinning was one among many others in the survey. We also have a relatively high proportion of mothers of spontaneous MZ (N=5,505) compared to mothers of spontaneous DZ twins (N=8,222) while population frequencies are closer to 2/3 (Bulmer, 1970).

A possible bias may have occurred in the form of an interaction between the zygosity of the offspring of the index mother and the zygosity of the offspring of her relatives. It is possible that an MZ twin mother is more likely to judge the twins of her relatives as MZ, while a DZ mother is more likely to judge them as DZ, thereby increasing the difference in the prevalence of DZ and MZ familial twinning between the two groups. However, the difference in the DOS twinning rate, which can be determined with certainty, between relatives of women with spontaneous MZ and DZ twins was 3.7%, while it was 6.2% when examining DZ twinning rate. Even when correcting for this additional difference due to reporter bias in MZ twinning rates, familial twinning is still increased in mothers of DZ twins.

In conclusion, familial DZ twinning in index mothers with spontaneous DZ twin offspring is clearly demonstrated in this study. With respect to the genetic predisposition to have twins, the mechanisms underlying spontaneous and non-spontaneous DZ twin births are very different and these two groups should be treated differently in studies of the genetics of DZ twinning.

Chapter 6

Body composition, smoking and dizygotic twinning

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Abstract

We examined differences in body composition and smoking between mothers of spontaneous monozygotic (MZ) and dizygotic (DZ) twins, while taking into account maternal age, gravidity and educational attainment. Mothers of twins (N=19,357) registered with the Netherlands Twin Register were selected. The final sample consisted of 5,663 mothers of spontaneous MZ twins and 8,515 mothers of spontaneous DZ twins.

Compared with spontaneous MZ twinning, spontaneous DZ twinning is significantly associated with increasing height (odds ratio=1.6, 95% CI 1.5 - 1.8 for the tallest versus the shortest height quartile), an increased BMI (odds ratio=1.3, 95% CI 1.1 - 1.4 for overweight vs. normal weight) and with smoking before the twin pregnancy (odds ratio=1.4, 95% 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.

Introduction

Twinning rates vary across the world, from six per 1000 births in Asia to 40 per 1000 births in Nigeria (Nyander, 1981; Bulmer, 1970, Derom et al., 1995; Hoekstra et al., 2008). Twinning rates also vary over time. For example, over the last 30 years Dutch twinning rates increased from 10 per 1000 pregnancies to 18 per 1000 pregnancies (Hoekstra et al., 2008; Statistics Netherlands., 2007). The regional and time-dependent differences are mainly due to differences in dizygotic (DZ) twinning rates (Tong et al., 1997). Monozygotic (MZ) twinning occurs around the world at a constant rate of around three to four per 1000 births and presents a random process that may happen to any woman having children (Bulmer, 1970; Hoekstra et al, 2008; Tong et al., 1997; Bortulus et al., 1999; Hall., 2003; Obi-Osius., 2004). In contrast, DZ twinning is influenced by genetic, maternal and environmental factors. Family history and increased parity or gravidity are known to increase the risk of spontaneous DZ twinning (Nylander, 1981; Bulmer, 1970; Hoekstra et al., 2008; Bortulus et al., 1999; MacGillivray et al., 1988; Eriksson and Fellman, 2000). The increase in DZ twin births seen in the Netherlands and other Western countries in the last decades can be attributed to increases in the number of fertility treatments but also to increases in maternal age (Martin et al., 2005; Fauser et al., 2005; Lambalk et al., 2004).

Established risk factors do not explain all variation in DZ twinning and additional factors are likely to be involved. These may include body composition and smoking. In a study by Basso et al. (2004), mothers of twins (MZ and DZ) were taller and had a higher body mass index (BMI) than the mothers of singletons. Information on the zygosity of the same-sex twin pairs was not available, but the association became more pronounced when singleton mothers were compared with opposite-sex twin mothers (i.e. DZ twin mothers). In accordance with these findings, Reddy (2005) compared MZ and DZ twin mothers to singleton mothers and found that increasing height and BMI were related to an increased risk of spontaneous DZ twinning (adjusted odds ratios for being in the tallest quartile for body height and for being overweight were 1.66 and 2.07, respectively). Such an association was not found for MZ twinning. With respect to smoking, significantly higher multiple birth rates have been reported for mothers who smoke (Yerushalmy, 1964; Olsen et al., 1988; Kallen, 1998; Morales-Suarez-Varella, 2007), although this association is not always significant (Parazzini et al., 1996).

Additional research is needed to verify the role of body composition and smoking in DZ twinning. We collected data from mothers of spontaneous DZ twins and mothers of spontaneous MZ twins on body composition and smoking history. Rather than using singletons as the control group, as done in earlier studies, we compared mothers with spontaneous DZ twins to mothers with spontaneous MZ twins. Since MZ twinning is likely a random event, MZ twin mothers are as appropriate for controls as are singleton mothers. They may actually be the preferred controls as both MZ and DZ twin mothers need to be able to carry the twin pregnancy to full-term.

In the analyses of the data we took the established DZ twinning risk factors maternal age and gravidity into account. In addition, we took into account the possible association between socioeconomic status and DZ twinning. Smoking behavior and body composition are associated with socioeconomic status (Conrad et al., 1992; Ericson et al., 1991; Laaksoonen et al., 1998; Willemsen et al., 2002; Ball et al., 2002; Martinez et al., 1999) and socioeconomic status has been suggested to be associated with DZ twining (Nylander, 1981; MacGillivray et al., 1988; Bonnelykke, 1990), although the direction of the association is as yet unclear. We thus compared DZ twin mothers to MZ twin mothers for body composition and smoking before the twin pregnancy, after controlling for effects of maternal age, gravidity and educational attainment.

Methods

Participants

The Netherlands Twin Registry (NTR) collects longitudinal data on twins and their family members in two subsamples: (1) newborn or young twins (YNTR) who are registered at birth by their parents and (2) adolescent and adult twins (ANTR) who self-register, along with their parents, siblings and spouses (Boomsma et al., 2006; Bartels et al., 2007). About 90% of the participants are born in the Netherlands. A questionnaire was mailed to all mothers of twins (N=33,528) registered with the NTR. Some of the mothers are passively registered, which means that they never participated in previous research projects. This was more often the case within the ANTR since initially addresses for these twin families were received through civil service administrations and may not always have been correct (Boomsma et al., 2006). From the group of actively registered mothers (N=25,620), we received 17,683 completed questionnaires and 1,674 completed questionnaires were received from mothers who had never participated before. In total, 19,357 twin mothers participated in this study. The average time between the birth of the twins and the survey was 10.4 years (SD=7.7).

Body height, BMI at time of twin pregnancy and smoking prior to twin pregnancy

The mothers were asked about their height and to indicate their weight just before the twin pregnancy. Data on height were divided into four quartiles (141-165, 165-169, 170-173, 174-195 cm). BMI before the pregnancy was calculated as BMI=weight in kilograms/height in meters squared and categorized according to the same categories used by Basso et al. (2004) and Reddy et al. (2005): less than 20 (15-19.99, low), 20-24.99 (normal), 25-29.99 (overweight), and 30 or more (30-47.99, obese).

The mothers were also asked about their smoking behavior before and during the twin pregnancy. They could report their smoking behavior as (1) no; (2) yes, but only before the pregnancy; (3) yes, but only during the twin pregnancy; (4) yes before and during the twin pregnancy; (5) I don't know. Smoking at any time before the twin pregnancy was divided into two categories, non-smoker and smoker before the twin pregnancy. Mothers who reported having smoked only during the twin pregnancy (N=31) were also classified as smokers.

Maternal age, gravidity and educational attainment

Maternal age at time of the twin pregnancy was obtained by subtracting the birth date of twins from the birth date of the mother and was recoded into the same 4 categories as reported by Basso et al. (2004); 25 or younger (17-25), 26 - 30, 31 - 35, 36 or older (36-45).

Gravidity was defined as the number of pregnancies, including miscarriages, the mother had before the birth of the twins. Gravidity was dived into two categories: null (no pregnancies before the twin pregnancy) and ≥ 1 (one or more pregnancies before the twin pregnancy).

Information on educational attainment was not available from the questionnaire, but was obtained from previous ANTR and YNTR survey data. To obtain a proxy for socioeconomic status of the family the highest educational attainment level achieved within a family (by the father or the mother of the twins) was used. Educational attainment was classified into three categories: low (primary or lower secondary education), intermediate (higher secondary education), and high (college or university education).

Sample selection

Of the 19,357 women who returned questionnaires, all mothers who were not the biological mothers of the twins (N=97) were excluded.

In the questionnaire, the twin mothers were also asked about conception of the twin/multiple pregnancy. Answers were (1) spontaneously conceived (with a request for specification of the time it took to become pregnant; 0-2, 3-5, 6-12 or more than 12 months), (2) IVF, (3) intracytoplasmic sperm injection, (4) IUI, (5) ovulation induction, (6) or other (with a request for specification). We excluded mothers who conceived their twins after the use of fertility techniques (N=4,451) or if data about mode of conception were missing (N=108).

Finally, we excluded twin mothers when the zygosity of their twin offspring was unknown (N=523), resulting in a sample of 14,178 mothers with twin offspring.

Based on sex of the twin offspring, 3,777 twins were classified as opposite sex DZ twins. In the case of same-sex twin offspring (10,401), we used zygosity based on DNA polymorphism, when available (for 16% of the same-sex twins). When this information was not available, zygosity from previous survey questions (for 65% of the same-sex twins) was used. These survey questions regarding offspring zygosity asked whether the twins were alike in eye color, hair color, face color and face form and whether the twins were sometimes mistaken for each other by their parents, other relatives and by strangers. Based on the answers to these questions, twin zygosity was determined. Previous studies have shown the correspondence between DNA and questionnaire zygosity determination to be high in the NTR: 93% in the YNTR sample (Rietveld et al., 2000) and 97 % in the ANTR sample (Willemsen et al., 2005). When data from previous studies were not available, we used the zygosity as reported in the current questionnaire by the mother (for 19% of the same-sex twins), who was asked to indicate whether the zygosity of the twin pair was MZ, DZ or unknown. A comparison of the zygosity of the same sex twin pairs provided by the mothers in the current questionnaire and the zygosity obtained from previous questionnaires showed a high degree of agreement (91%, N=5,953).

The final sample consisted of 5,663 mothers of spontaneous MZ twins and 8,515 mothers of spontaneous DZ twins. The dataset consists of 13,966 mothers with one twin pair and 212 mothers with two or more sets of multiples. In the case of multiple twin pregnancies, we only included the data of one twin pregnancy. In most cases this meant the data for the firstborn twin were used. In the case where data were available for both twins and the firstborn twin was MZ while the second-born twin was DZ, we used data for the second-born twin pregnancy (N=25).

Information on nonresponders

Nonresponse can limit the interpretation of data collected in questionnaire studies (Barchielli and Balzi, 2002; Goyder et al., 2002; Korkeila et al., 2001; Van Loon et al., 2003). As indicated, we approached 25,620 twin mothers who had participated in earlier NTR surveys. This information was used to compare responders (N=17,683) and nonresponders (N=7,937) to the present questionnaire. In this analysis, we included both mothers of spontaneous twins and mothers of twins conceived after fertility treatment to determine whether this was a factor influencing nonresponse. In responders and nonresponders we compared zygosity (MZ or DZ), height (141-165, 165 - 169, 170 - 173, 174-196 cm), BMI (15-19.99, 20-24.99, 25-29.99, 30-48.99 kg/m^2), smoking during the twin pregnancy (yes or no), age at the time of the twin pregnancy (17-25, 26 - 30, 31 - 35, 36-47) and highest educational attainment level of the mother and her spouse (low, intermediate, or high). Responders and nonresponders were also compared on the use of artificial fertility techniques to conceive the twins (yes or no); sib-ship size, that is, the number of siblings of the mother herself (no other siblings, one or two siblings and three or more siblings); whether the mother herself is a twin (yes or no); whether other female family members who were mothers of twins were present (familial twinning, yes or no); religious status (not religious, religious but not active or religious and active); and the urbanization level of the residence of the mother (very heavy to heavy, moderate, low to very low). The information for the comparison of responders and nonresponders came from earlier surveys that were collected over a 20-year period. The questions contained in

the surveys sometimes changed over time, or were only asked of YNTR or of ANTR mothers. The number of observations therefore differs for each of these variables (see Table 1).

Statistical analyses

To compare responders and nonresponders, we performed Pearson χ^2-tests. When significant differences were found, we conducted a binary logistics regression analysis to test whether these significant associations applied to both zygosity offspring groups, that is, whether the response bias was the same for mothers of MZ and mothers of DZ twins.

To compare spontaneous DZ twin mothers with spontaneous MZ twin mothers, we used binary logistic regression. We tested a model with DZ twinning (defined as yes/no) included as the dependent variable and as independent variables for height, BMI, and smoking as well as age, gravidity and educational attainment. The reference categories in this model were age at twin birth of 25 years or younger, low education, being nulliparous before the twin pregnancy, height less than 165 cm , normal BMI (20-24.9 kg/m^2) before twin pregnancy and not having smoked before the twin pregnancy. In these analyses, data were only included when information was present for all variables entered in the model. Since educational attainment was obtained from previous surveys, this reduced the sample size substantially. We therefore also ran the model without educational attainment.

Results

Comparison of responders and nonresponders

Table 1 summarizes the results for the comparison of responders and nonresponders to the questionnaire. Nonresponse status was associated with twin offspring zygosity (more MZ mothers participated), with being shorter, smoking during the twin pregnancy, being younger at twin birth, and having a lower educational attainment. In addition, nonresponse was associated with having more brothers and sisters and living in an urbanized area. However, the response bias was similar in mothers of spontaneous MZ and DZ twins.

Table 1 Characteristics of responders and nonresponders obtained from previous surveys (number of observations and % for each trait) and test results for response bias (χ^2 and P-value) and, when significant, for differences in response bias between mothers of MZ and DZ twins (logistic regression; OR, 95% CI, and P-value).

	Nonresponders		Responders		Pearson Chi square		Logistic Regression DZ vs. MZ comparison		
					χ^2	*P*	OR	CI	*P*
Zygosity of the twins:									
MZ	1,505	25.6%	5,106	33.3%	117.4	.00			
DZ	4,379	74.4%	10,240	66.7%					
Missing	2,053		2,337						
Height, cm:									
141 165	2,008	29.8%	4,066	26.1%	36.7	.00	ref		
165 - 169	1,461	21.7%	3,410	21.9%			1.0	0.8-1.2	.85
170 - 173	1,726	25.6%	4,171	26.8%			0.9	0.7-1.1	.20
174 - 196	1,541	22.9%	3,928	25.2%			1.0	0.8-1.3	.73
Missing	1,201		2,108						

	Nonresponders		Responders		Pearson Chi-square		Logistic regression DZ vs. MZ comparison		
					χ^2	*P*	OR	CI	*P*
BMI, kg/m²:									
15 – 19.99	885	13.4%	1,949	12.7%	13.2	.00	ref		
20 – 24.99	3,735	56.5%	8,977	58.3%			1.1	0.7-1.5	.79
25 - 29.99	1,383	20.9%	3,226	21.0%			1.1	0.8-1.4	.63
30 – 48.99	610	9.2%	1,228	8.0%			0.8	0.6-1.1	.22
Missing	1,324		2,303						
Smoking during the twin pregnancy:									
yes	2,211	29.1%	3,292	19.3%	291.8	.00	1.0	0.8-1.1	.69
no	5,386	70.9%	13,771	80.7%			ref		
Missing	340		620						
Maternal age at twin birth:									
17 - 25	1,231	16.1%	1,788	10.3%	185.3	.00	ref		
26 – 30	3,052	39.3%	6,819	39.1%			1.1	0.8-1.4	
31 – 35	2,649	34.4%	6,837	39.2%			1.1	0.8-1.4	
36 - 45	787	10.2%	1,981	11.4%			1.0	0.8-1.3	
Missing	232		226						
Educational attainment:									
Low	1,694	39.6%	3,046	22.1%	547.2	.00	ref		
Intermediate	1,527	35.7%	5,631	40.9%			1.0	0.3-1.4	.58
High	1,059	24.7%	5,106	37.0%			1.1	0.7-1.1	.57
Missing	3,657		3,900						
Use of artificial fertility techniques:									
Yes	1,004	16.6%	2,392	16.6%	0.0	.97			
No	5,058	83.4%	12,031	83.4%					
Missing	1,875		3,260						
Sib-ship size mother:									
No other siblings	164	3.6%	485	3.5%	52.1	.00	ref		
One or two siblings	2,281	50.7%	7,786	56.7%			0.7	0.4-1.1	.08
3 or more siblings	2,060	45.7%	5,459	39.8%			1.1	0.9-1.2	.51
Missing	3,432		3,953						
Self part of twin:									
Yes	129	2.6%	354	2.4%	0.6	.45			
No	4,837	97.4%	14,354	97.6%					
Missing	2,971		2,975						
Familial twinning:									
No other twins	2,063	45.3%	6,173	44.5%	1.1	.30			
Other twins	2,487	54.7%	7,713	55.5%					
Missing	3,387		3,797						
Religious status:									
Not religious	1,357	35.2%	4,517	33.4%	4.8	.09			
Religious, not active	1,664	43.2%	5,916	43.8%					
Religious and active	833	21.6%	3,076	22.8%					
Missing	4,083		4,174						
Urbanization level:									
(Very) heavy	2,351	30.2%	4,495	25.9%	57.2	.00	ref		
Moderate	1,804	23.2%	3,990	23.0%			1.0	0.8-1.1	.57
(Very) low	3,627	46.6%	8,844	51.0%			1.0	0.9-1.2	.90
Missing	155		354						

Data on (non) responders were available from earlier surveys. Not all variables were collected in all surveys.

Association of body composition and smoking prior to the twin pregnancy with DZ twinning

Table 2 shows the distribution (% and number) of the variables of interest in mothers of spontaneous MZ and mothers of spontaneous DZ twins who took part in the questionnaire study. Because data on education came from earlier surveys the sample size reduces to 10,719 for this variable.

Table 2 Prevalence (number and %) of maternal characeristics of mothers of spontaneaus MZ and DZ twins.

		Mothers of spontaneous MZ twins		Mothers of spontaneous DZ twins	
Height, cm:	**N**	**N**	**%**	**N**	**%**
141 – 164	3,659	1,646	29.3%	2,013	23.8%
165 – 169	3,089	1,313	23.4%	1,776	21.0%
170 – 173	3,832	1,480	26.3%	2,352	27.9%
174 - 195	3,485	1,183	21.0%	2,302	27.3%
missing	113	41		72	
BMI before twin pregnancy, kg/m²:					
15 – 19.99	2,004	872	15.8%	1,132	13.7%
20 - 24.99	8,625	3,566	64.4%	5,059	61.1%
25 - 29.99	2,431	829	15.0%	1,602	19.3%
30 – 47.99	758	266	4.8%	492	5.9%
missing	360	130		230	
Smoking before twin pregnancy:					
Nonsmoker	9,158	3,829	67.9%	5,329	63.0%
Smoker	4,949	1,813	32.1%	3,136	37.0%
Missing	71	21		50	
Maternal age at twin birth:					
17 - 25	1,519	745	13.2%	774	9.1%
26 – 30	5,522	2,359	41.8%	3,163	37.2%
31 – 35	5,539	2,011	35.6%	3,528	41.5%
36 - 45	1,565	535	9.5%	1,030	12.1%
Missing	33	13		20	
Gravidity:					
Null	5,360	2,330	42.2%	3,030	36.7%
≥ 1	8,412	3,189	57.8%	5,223	63.3%
Missing	406	144		262	
Educational attainment:					
Low	2,328	975	21.2%	1,353	22.1%
Intermediate	4,334	1,893	41.1%	2,441	39.9%
High	4,057	1,740	37.8%	2,317	37.9%
Missing	3,459	1,055		2,404	

Table 3 presents the odds ratios (ORs) and 95% confidence intervals (CIs) for changes in the DZ/MZ twinning proportion. Results for the full model are presented at the left side of the table (N=10,234). DZ twinning was associated with body composition. Mothers in the tallest quartile for height were 1.6 times as likely to have DZ twins versus MZ twins when compared with mothers who were in the shortest height quartile. Compared with mothers with a normal BMI, mothers who were overweight or obese were 1.3 and 1.2 times more likely to have DZ twins than MZ twins. Having a BMI lower than 20 reduced the chance of having DZ twins. Smoking before pregnancy was also associated with DZ twinning; twin mothers who had smoked before the twin pregnancy had an increased chance of being a DZ twin mother (OR = 1.4). In these comparisons, the possible effects of maternal age, gravidity and educational attainment on DZ twinning are accounted for. As can

be seen in Table 3, maternal age and gravidity were, as expected, associated with DZ twinning, but educational attainment was not. Since educational attainment was not available for all participants and was not significantly associated with DZ twinning, we reran the analysis excluding educational attainment from the model (N= 13,346). The results of this analysis, presented at the right side of Table 3, are similar to those for the model including educational attainment.

Table 3 ORs and 95% CIs for the comparison of the proportion of DZ to MZ mothers in each age, gravidity, height, BMI, smoking and educational attainment category. Left side and right side of the table give results for models including and excluding educational attainment.

	N	OR	95% CI	*P*	N	OR	95% CI	*P*
Height, cm:								
141 - 165	2,699	Reference			3,446	Reference		
165 – 169	2,318	1.2	1.0 - 1.3	.00	2,934	1.1	1.0 - 1.2	.02
170 – 173	2,745	1.3	1.2 - 1.5	.00	3,640	1.3	1.2 - 1.5	.00
174 - 195	2,472	1.6	1.4 - 1.8	.00	3,326	1.6	1.5 - 1.8	.00
BMI before twin pregnancy, kg/m²:								
15 – 29.99	1,523	0.9	0.8 – 1.0	.09	1,935	0.9	0.8 – 1.0	.08
20 - 24.99	6,521	Reference			8,331	Reference		
25 - 29.99	1,707	1.3	1.1 - 1.4	.00	2,352	1.4	1.2 - 1.5	.00
30 – 47.99	483	1.2	1.0 - 1.5	.03	728	1.3	1.1 - 1.5	.00
Smoking before twin pregnancy:								
Nonsmoker	6,599	Reference			8,637	Reference		
Smoker	3,635	1.4	1.3 - 1.5	.00	4,709	1.3	1.2 - 1.4	.00
Maternal age at twin birth:								
17 - 25	1,098	Reference			1,408	Reference		
26 – 30	4,220	1.4	1.2 - 1.6	.00	5,249	1.3	1.2 - 1.5	.00
31 – 35	3,890	1.7	1.5 - 2.0	.00	5,244	1.7	1.5 - 1.9	.00
36 – 45	1,026	1.9	1.5 - 2.2	.00	1,445	1.8	1.6 - 2.1	.00
Gravidity:								
Null	4,042	Reference			5,220	Reference		
≥ 1	6,192	1.1	1.0 - 1.2	.00	8,126	1.2	1.1 - 1.3	.00
Educational attainment:								
Low	2,176	Reference					Excluded	
Intermediate	4,153	0.9	0.8 - 1.0	.12			Excluded	
High	3,905	0.9	0.8 - 1.0	.12			Excluded	

Note: The analysis was based on data for 10,234 persons for the model including educational attainment and based on data for 13,346 persons for the model excluding educational attainment.

Discussion

This study investigated whether DZ twinning is associated with maternal body composition and maternal smoking history. After taking into account maternal age at twin birth, gravidity and educational attainment, mothers of DZ twins were significantly taller, had a higher BMI and smoked more often before the twin pregnancy than mothers of MZ twins. As expected, increased maternal age and increased gravidity were significantly associated with spontaneous DZ twinning, but there was no association between educational attainment and spontaneous DZ twinning.

This study replicates previous findings on height, BMI and smoking in relation to DZ twinning (Nylander, 1981; Bortolus et al., 1999; MacGillivray et al., 1988; Basso et al., 2004; Reddy et al., 2005; Olsen et al., 1988; Morales-Suarez-Varella et al., 2007; Parazzini et al., 1996). Our study further extends these findings by taking into account possible influences of socioeconomic status, which was operationalized as educational

attainment. Educational attainment did not alter the association of body composition and smoking before the twin pregnancy with DZ twinning. In fact, while both positive (Bonnelykke, 1990) and negative (Nylander, 1981) associations have been reported for socioeconomic status and twinning, our study did not show any association between educational attainment and DZ twinning.

We looked at the possibility that nonresponse bias affected the results. Nonresponse may influence results based on data collected in questionnaire research, although generally only small differences between responders and nonresponders have been found (Van Loon et al., 2003; Vink et al., 2004; Distel et al., 2007; Heath et al., 2001). Because data from earlier survey collections were available for a large proportion of nonresponders in this study, we could compare characteristics of both groups as a function of the zygosity of their offspring. These analyses demonstrated small, but significant differences between responders and nonresponders. However, the same differences were seen in mothers of MZ and DZ twins. It is therefore unlikely that response bias influenced the comparisons between MZ and DZ twin mothers.

The present study reports on the comparison of mothers of spontaneous DZ twins with mothers of spontaneous MZ twins. The results may therefore be limited to multiple births, and results may be more pronounced when comparing DZ twin mothers with singleton mothers. However, MZ twinning is generally not found to be 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 an MZ twin pregnancy are very different from the mechanisms leading to a DZ twin pregnancy (the focus of our study), both MZ and DZ twin mothers have to carry the twin pregnancy to full term.

We analyzed data from mothers after a spontaneous twin pregnancy. Raj and Morley (2007) suggested that parents of twins are not very willing to answer questions regarding the mode of conception. However, in the present study, the percentage of missing data on this question was low (< 1%) and van Beijsterveldt and colleagues (2008) showed that the pattern of missing for this variable is not different from the pattern of missing for other traits such as height (Van Beijsterveldt et al., 2008). Also, if women who received fertility treatment are less likely to respond to surveys including this question, we would have expected to find a response bias for this variable. As shown in Table 1, women who received fertility treatment were as likely to participate in the survey as women who conceived spontaneously. A related issue is whether women accurately report on the mode of conception. Using a subset of the NTR sample, van Beijsterveldt and colleagues compared data on self-reported mode of conception with the data from hospital records (Van Beijsterveldt et al., 2008) and found that mothers accurately report on the mode of conception.

The data were obtained through self-report after the twin pregnancy, on average 10 years later. Although height in this age group is a trait that remains stable across this time period, weight and smoking behavior may have changed in this time period and recall may have influenced the results. Should either the actual change or the recall be different in MZ and DZ twin mothers, this would bias the results. However, when we performed the analysis separately for the ANTR and YNTR (older and younger cohort) the results for BMI and smoking were in the same direction. In the ANTR (N=1,486) being overweight increased the chance of having DZ twins by 1.9 (95% CI, 1.3-2.8), and having smoked before the pregnancy increased the chance by 1.1 (95%CI, 0.9-1.4), while in the YNTR (N=11,860) these odds were 1.4 (95% CI, 1.2-1.6) and 1.4 (95% CI, 1.3-1.5), respectively.

For smoking behavior before the twin pregnancy, the way the question was phrased may have influenced the results. Smoking status was defined based on a four-category variable, which roughly identified persons as having smoked or not having smoked before the twin pregnancy. The option 'yes, smoked during the pregnancy, but not before' was answered by 31 twin mothers, and they were classified as smokers before the pregnancy. It is possible that these mothers were misclassified, though due to the small number the effect would

be negligible. No information was obtained on smoking duration or number of cigarettes smoked. In addition, there was no specification of the time period between the twin pregnancy and prior smoking. When we compared our classification as smoker/non-smoker prior to twin birth with the data on smoking prior to the twin pregnancy (yes/no) from the questionnaire that mothers completed within the first year after the twin birth (available for 11,971 twin mothers), a correspondence of 93.5% was found.

Similarly, educational attainment may reflect the education level obtained after the twin pregnancy. However, the vast majority of the sample was 26 years or older at the time of the twin pregnancy and therefore most mothers will have reached their highest educational attainment. The average age at which women in the Netherlands have their first child has increased over the last 30 years predominantly owing to the fact that women will wait to have their first child until they have finished their education (Van Agtmaal-Wobma and Van Huis, 2008). Here too, a comparison with an earlier survey in the younger cohort that was nearer to the birth of the twins (YNTR data collection took place when twins were 3 years) showed similar results to those for the total sample. Although some bias has occurred, it is likely to be small and to occur in both MZ and DZ mothers, and is thereby unlikely to influence the observed differences between MZ and DZ twin mothers.

Overall, our results extend previous findings that smoking and body composition are related to DZ twinning. Surprisingly, the same factors we observed to be associated with spontaneous DZ twinning are also associated with fertility problems. Maternal age, obesity and smoking are all associated with an increased risk of infertility (Flegal et al., 2002; Goumenou et al., 2003; Hedley et al., 2004; Lash and Armstrong, 2008). The relationships between these factors are likely to be complex and it is unclear whether effects on twinning or fertility act through the same or different mechanisms.

In conclusion, DZ twinning is moderately but significantly associated with body composition and smoking. Other mechanisms including genetic factors are likely to play a role, and it is also possible that these factors interact with body composition and smoking in increasing the risk of having DZ twins.

Chapter 7

Genetics of dizygotic twinning: A feasibility study for a biobank

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Abstract

To locate the genes that make a substantial contribution to variation in natural dizygotic twinning in human, large-scale studies are needed. New studies should not stop at DNA genotyping, but collect material that allow gene expression analysis, transcriptomics, proteomics and endocrinology.

In this paper we describe a pilot study to examine the feasibility, effectiveness and logistics of large-scale nationwide sample collection in Dutch families in which two or more sisters have given birth to spontaneous dizygotic twins.

Pedigree data and addresses from family members of proband mothers were collected by phone. Blood and urine samples were collected during a home visit, and handled in the afternoon. All subjects were bled between 7 and 10 a.m. after overnight fasting. Blood samples of fertile women with a natural cycle were colleted on the second, third or fourth day of their menstrual cycle.

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. This percentage is likely to increase with the more dense geographical distribution of subjects in the larger population. We conclude that the pilot study demonstrated the feasibility of this protocol to collect good quality of plasma, DNA, RNA and lymphocyte samples by home visits.

Introduction

It has been firmly established that variation in dizygotic (DZ) twinning has a genetic component (Bulmer, 1970). Family studies found significantly higher frequencies of dizygotic (DZ) twins in female relatives of DZ probands compared with monozygotic (MZ) probands. Both autosomal monogenic dominant and recessive models of inheritance have been described (Lewis et al., 1996; Meulemans et al., 1996). However, the genetic architecture of twinning in humans remains unclear.

There are good animal models of twinning in mammals, especially in sheep. An important question is whether natural variation in human twinning is controlled by some of the same mechanisms as have been identified in sheep, or through other pathways. For example, in sheep mutations in three members of the intra-ovarian transforming growth factor beta (TGFb) signaling pathway (BMP15, BMPR1B and GDF9) increases the frequency of twins and higher order multiples (Galloway et al., 2000; Hanrahan et al., 2004; Wilson et al., 2001). Mutations in BMP15 and GDF9 are associated with both increased ovulation rate and sterility in Cambridge and Belclare sheep (Hanrahan et al., 2004).

One reason for the lack of success so far to identify the genetic contribution to human DZ twinning may be the small sample size of many studies. We are therefore recruiting a large sample of sister pairs who have both given birth to spontaneous DZ twins. Affected sister pairs their parents and other informative family members will be recruited in the Netherlands, in Australia and New Zealand. In The Netherlands, 500 families will be identified through the Netherlands Twin Registry (NTR) and in Australia and New Zealand, 500 families will be recruited through appeals in the media. The proposed study aims to extend our previous studies of the endocrinology, epidemiology and molecular genetics of twinning in humans (Martin et al., 1991; Martin et al., 1984; Boomsma et al., 1992; Boomsma et al., 1992; Lambalk et al., 1998; Montgomery et al., 2003).

The collection of biological material other than DNA samples that enables the study of the endocrinology of twinning, the identification of candidate genes through expression studies and possibly the characterization of affected women through system biology approaches requires the establishment of large biobanks. Biobanks can be defined as stored collections of genetic samples and biological materials from participants coming, from selected groups or from random population. Biological materials include genomic DNA, RNA, lymphocytes serum and/or plasma which enables investigators to perform long term genomic, transcriptomic, or proteomic studies (Nederhand R.J et al., 2003; Kaiser, 2002; Austin et al., 2003). At the moment, characterization of individuals and families, using these techniques, including large-scale genotyping of microsatellites and SNPs, represents a major cost. Therefore, the aim is to collect multiple phenotypes in such studies, so that linkage and association analysis can be conducted for multiple phenotypes within the same families. To be cost-effective and to minimize the future burden for participants, a broad range of biomaterials should be collected in a single visit that allows future study of multiple (disease) traits and endophenotypes. Ideally, the multiple phenotypes reflect variation in physiological parameters, which are related to affection status, but with appropriate sample collection, large-scale studies will also provide a valuable resource for many traits that can be assessed in plasma or serum. Some of these traits require standardized protocols. For example, to assess lipid levels, glucose and insulin subjects ideally are bled after overnight fasting and at a fixed time of the day. For other parameters, following a certain circadian pattern (such as cortisol) subjects need to be bled at a fixed time of the day. To study the endocrinology of twinning blood samples in fertile women need to be collected around the time of follicle recruitment.

In this paper, we describe a pilot study to examine the feasibility, effectiveness and logistics of large-scale nationwide biomaterials collection in the Netherlands to locate genes responsible for DZ twinning. We first describe the procedure for recruitment of families of affected sister pairs and next of phenotype and biological

sample collection. The logistics of transport and blood handling and the procedures for blood handling and results of RNA isolation are described.

Methods

Subjects

The study was approved by the Central Ethics Committee on Research Involving Human Subjects of the Vrije Universiteit (Amsterdam). We approached 24 mothers of dizygotic twins (MODZT) registered with the Netherlands Twin Registry (NTR). These mothers reported in survey studies of the NTR having one or more sisters with a twin or multiple births. Most of them had responded to an article in the yearly newsletter of the NTR about this study.

The proband mother was contacted by letter, followed by a phone call to collect pedigree data and to obtain permission to approach her sister, her parents and/or other siblings. All family members were initially informed about the study by the proband mother and after giving their permission to be contacted, were sent a letter and information brochure about the study. To be eligible for the study, two or more sisters had to have given birth to spontaneous dizygotic twins. If one or both parents were unavailable or deceased, other siblings were asked for their participation.

To assess hormone levels at the time of follicle recruitment in fertile women, blood and urine samples of women with a natural cycle were taken on the second, third of fourth day of the menstrual cycle. In women who used oral contraceptives (OC) blood and urine samples were taken in the week the women did not take OC pills. All of these women used combination OCs. Menopausal women, fathers and brothers did not have to meet these criteria. All subjects gave written informed consent prior to participation in the study.

Interview

Pedigree data about DZ twinning and fertility were obtained from a telephone interview with the proband mother and her sister. The interview time was between 20-30 minutes. The interview for MODZT consisted of five parts. Part A contained 20 items regarding age, birth weight, parity and mother's judgment of zygosity of the twins. In part B, 11 items about birth history, use of hormone treatment or IVF in the conception of the twins and in other conceptions were asked. Part C asked about family history of twinning and fertility. Pedigree data were obtained for the family of the proband mother, for her mother's family, her father's family and for the family of her husband. Part D contained 19 items regarding health and medical history. Data were collected on height, weight (before, during and after the twin pregnancy), menstrual cycle, menopause (of proband and her mother), use of contraception and health problems. The last part of the questionnaire part E, contained 4 items about ancestry of the parents and grandparents of the MODZT.

Blood sampling

Venous blood samples and ´morning` urine samples were taken from the MODZT, their parents and/or additional siblings between 07.00 and 10.00 a.m. and after overnight fasting. A total of 7 blood tubes was collected from all participants for DNA, lymphocytes, RNA (basal and after dexamethasone challenge) and plasma. The order of sample draw was 2 x 9 ml EDTA, 3 x 9 ml Heparin, 1 x 4.5 ml citrate and 1 x 2 ml EDTA. For collection, we used a safety-lock butterfly needle. With this system, tubes can be quickly and hygienically changed. To prevent clotting all tubes were inverted gently 8-10 times immediately after collection.

For DNA isolation and EDTA plasma, blood was collected in 2 x 9 ml EDTA anticoagulant tubes. The tubes were stored in melting ice (0-2˚C) during transport. For RNA isolation, we used 2 x 9 ml lithium heparin coagulant tubes. Within 1 hour after collection one heparin tube for basal RNA was divided in 3 x 3ml aliquots.

The aliquots were snap frozen in acetone with dry ice and stored at -80˚C during transport. Dexamethasone (dex) was added to the second heparin tube to a final concentration of 10^{-7} M within 1 hour after collection. During transport and for exactly 6 hours, the tube was stored in an insulated box with a constant temperature of 37˚ C.

After six hours incubation the tube was inverted gently 8-10 times and subsequently divided in 3 x 3 ml aliquots. The aliquots were snap frozen in acetone with dry ice and stored at -80˚C.

For future coagulation studies, blood was collected in a 4.5 ml sodium citrate tube, which during transport was stored in melting ice (0-2˚C). Blood variables including levels of lipids, apolipoproteins, HbA1c (haemoglobin A1c: an index of average blood glucose levels over a 2-3- month period) and CRP (C-reactive protein) were measured in samples collected in a 2 ml EDTA tube, which was stored at room temperature (RT) during transport (see Table 1 for blood picture overview).Urine samples were collected in 2 x 10 ml vacutainers, and stored in melting ice during car transport.

Table 1 Overview of traits assessed in blood/plasma in all participants.

Blood variable		N	Mean	SD	Range
HbA1c	HbA1c (%)	69	5.6	0.44	4.5 – 7.1
CRP	CRP (mg/L)	73	5.45	12.10	0.14 - 83.80
Hematocrit	Ht (%)	49	40.54	3.54	29.2 – 52.3
White blood cell counts	WBC*	49	6.04	1.65	3.1 – 11.0
Red blood cell count	RBC**	49	4.47	0.40	3.83 – 5.71
Red cell distribution width	RDW (%)	48	12.80	1.75	10.9 – 23.3
Haemoglobin concentration	Hb (g/dl)	49	13.31	1.12	10.0 – 15.8
Mean corpuscular haemoglobin	MCH (pg)	49	29.81	1.95	24.5 – 33.6
Mean corpuscular Hb concentration	MCHC (g/dl)	49	32.92	2.04	29.3 – 43.2
Mean corpuscular volume	MCV (fl)	49	90.70	5.51	71.5 – 99.6
Platelet count	TROM*	49	270.49	114.65	73.0 – 532.0
Mean platelet volume	MPV (fl)	49	8.60	0.86	7.1 – 12.6
Total cholesterol	TC (mmol/L)	86	5.48	0.92	3.46 – 7.4
Triglycerides	TGs (mmol/L)	86	1.26	0.52	0.48 – 3.01
High density lipoprotein	HDL (mmol/L)	86	1.70	0.51	0.54 – 3.31
Low density lipoprotein (calculated)	LDL (mmol/L)	86	3.19	0.84	1.69 – 4.83

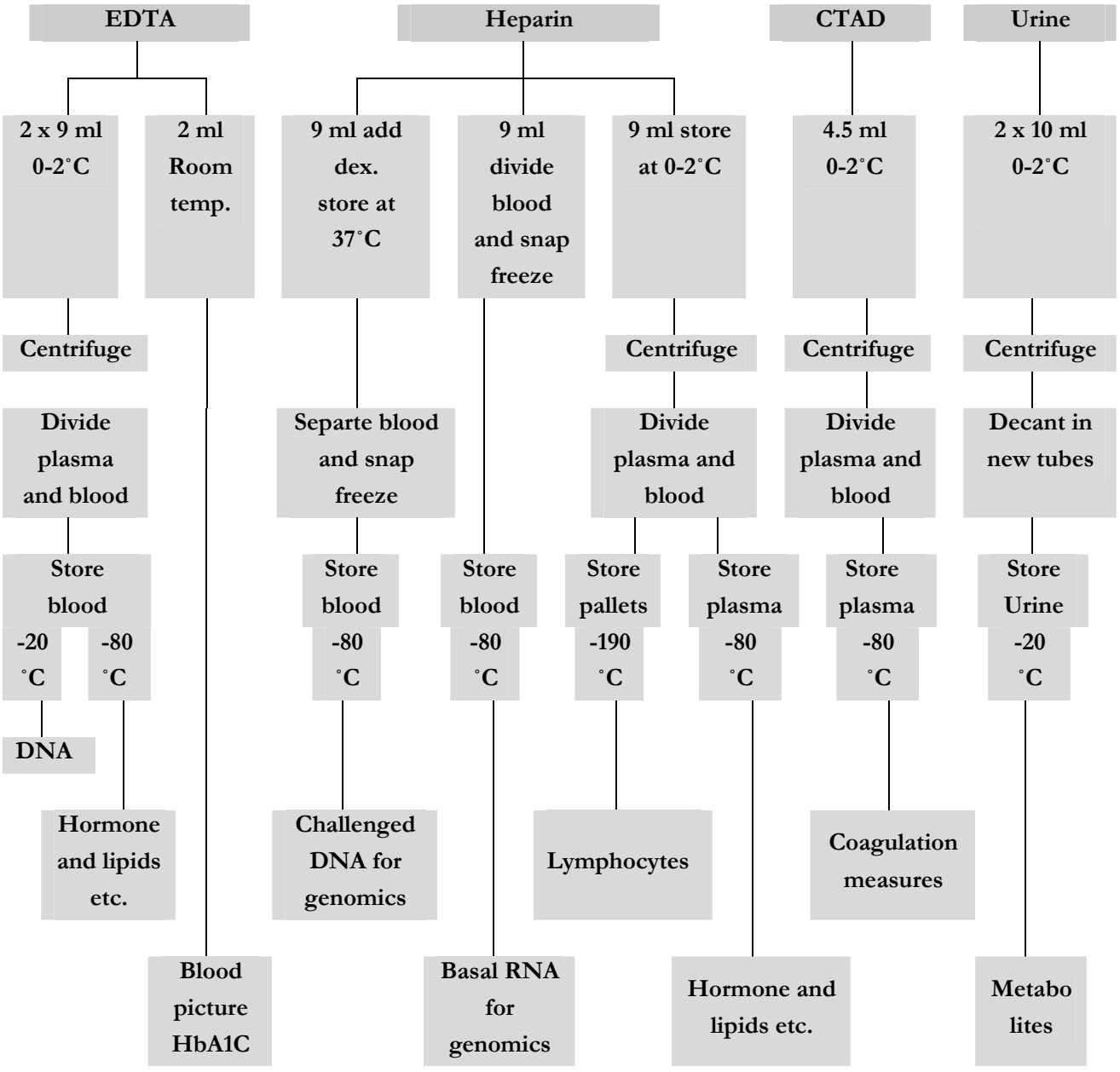
*** 10^3/uL**
**** 10^6/uL**

All samples were transported to the laboratory in Leiden. Average transport time was 4 hours and all samples were centrifuged within 6 hours after collection. The degree of haemolysis was determined by observing the color of the plasma after the centrifugation step.

The EDTA, citrate tube and the urine samples were centrifuged 20 minutes at 2000xg 4˚C. EDTA and citrated plasma was harvested from the buffy coat and red blood cells, aliquoted (0.5ml), snap frozen in acetone and dry ice, and stored at -80˚C. The urine was decanted carefully into new plastic tubes without the debris pellet. The EDTA vacutainers with buffy coat and red cells, and the urine samples were stored at -20˚C. The heparin vacutainer for lymphocyte isolation was centrifuged for 15 minutes at 1000xg at 4˚C. After the centrifugation step, heparin plasma was divided into 8 sub samples of 0.5ml, snap frozen and stored at -80˚C. For lymphocyte isolation 14ml phosphate-buffered saline (PBS) was added to the heparin blood, buffycoat and remaining plasma, then the diluted blood was added to 10 ml Ficoll.

This was centrifuged 25 minutes at 800xg at RT. The lymphocytes are pipetted into a new tube and 45 ml PBS was added. The solution was centrifuged 10 minutes at 250xg RT. This washing step was repeated after discarding the supernatant. The lymphocyte pellet was then taken up into 1ml RPMI: 20% Fetal Calf Serum (FCS) 10% and dimethyl sulfoxide (DMSO). Finally, pellets were frozen and stored overnight at -80˚C and then transferred to liquid nitrogen for long-term storage. An overview of blood collection, transport and blood handling is listed in Figure 1.

Figure 1 Overview of blood collection and handling for EDTA, heparin, citrate and urine tubes.



To test the viability of frozen lymphocyte pellets we transfected 8 samples, originating from 4 individuals, with Epstein Barr virus B95-8. Cells were centrifuged for 10 minutes at 1200 rpm. Supernatant was removed and the cell pellet washed in 10.5 ml RPMI with 1 % FCS. After a second centrifugation of 10 minutes at 1200 rpm ~10^6 cells were suspended in 2 ml RPMI plus 15% FCS and 1 mM Na-pyruvate and PenStrep and co-cultured with EBV virus. After 24 hours the medium with virus was removed and replaced by 2.5 ml RPMI, 15% FCS 1mM Na-pyruvate and 1 μg/ml cyclosporine A. This medium was refreshed regularly according to standard procedures.

All samples are handled in the central laboratory in Leiden where labeling of samples and distribution of samples is centralized. The samples obtain a label with double coding: one code represents the individual according to the twin register ID (code of family and position in the family, e.g. father, mother, sister or twin); the other code is sequential and is used to identify the storage position of the sample. The different types of samples are identified by the specific tube or specific cap of the tube or storage box. Labeled isolated samples of DNA are transported to Amsterdam for storage. Labeled cryo- tubes with lymphocytes are sent for storage in liquid nitrogen to Amsterdam. Tubes challenged for gene expression are labeled in addition as A (basal control) and B (challenged) and stored in PAX tubes sequentially in separate boxes in Leiden. Urine is stored in 15 ml tubes sequentially in separate boxes in Leiden. Plasma samples from EDTA blood (purple cap), from heparin blood (green cap), form CTAD blood (blue cap) are stored in boxes containing material from 4 patients in sequential order. The content from each box and the position from each box in trays and the position of each tray in a freezer cache is recorded in an excel file.

Zygosity testing

Twins born to mothers recruited for the study included 22 opposite sex (OS) twin pairs and 24 same-sex (SS) twin pairs. DNA typing from buccal swabs determined information on zygosity of 24 SS twins (Meulenbelt et al., 1995). OS twin pairs were not typed. Zygosity packages were given to the mothers of the twins during the home visit. One SS twin pair was not typed for zygosity, because the mother of the twins was missed for blood collection during this pilot study. This twin pair and their mother will participate with the follow up study. Subjects returned the packages by mail. Zygosity testing was performed essentially as described (Rietveld et al., 2000).

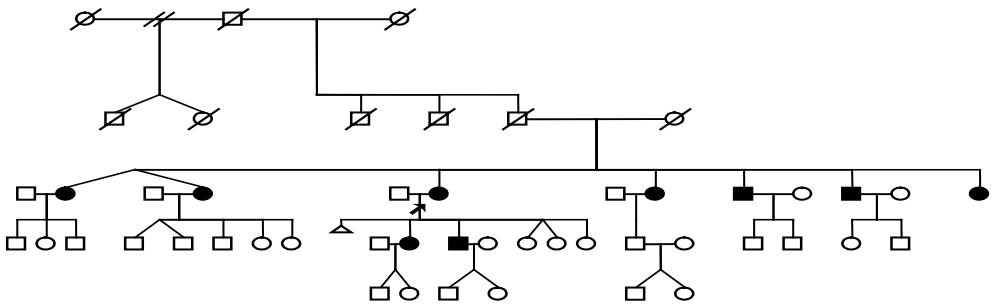
Results

Subjects

Twenty-three of the 24 MODZT participated in the pilot study. During the telephone interview, one of the MODZT was excluded, because she did not meet the criteria of having a sister with DZ twins. A total of 96 subjects agreed to participate. Eleven participants were not bled (3 MODZT, 7 parents, 1 sister). We missed 3 MODZT who had the 3rd day of their natural cycle at the same time as other participants, due to the large distances between their homes. The parents of the mothers we missed will be visited for blood collection at the same time as their daughters. Three parents of the MODZT, were not bled due to health problems. One sister of a MODZT lived in Canada and was therefore not bled. These 4 subjects were asked to participate in DNA collection via buccal swabs. One subject agreed to participate and returned the packages by mail.

In 10 families, two sisters with twins and their parents were bled. In 8 families only one of the parents of the ASPs participated (all women), in 2 of them additional siblings participated. In 5 families both parents were deceased, we therefore asked siblings of the ASPs to participate. A total of 18 additional siblings (11 male and 7 female) participated. In a few informative and interesting families, other family members were also asked to participate (e.g. grandparents and offspring of the MODZT who had also given birth to DZ twins, see Figure 2.

Figure 2 Pedigree of a participating family. Participating family members are shown with black circles.



The mean age of the proband was 44 years (range 28-70 years, Table 2) and the mean age of the sister with twins was 46 years (range 25-74 years, Table 2). Fourteen women had a natural cycle, 10 used oral contraceptives, 16 were in menopause, and 6 had had a hysterectomy. Of the proband mothers 39 % had a natural cycle, two of them also had a sister with a natural cycle.

Table 2 Mean age of proband mothers and participating family members. Number of women with natural menstrual cycle, menopause, oral contraceptive (OC) use or other.

Relative	**N**	**Mean age**	**Range**	**Cycle**			
				Natural	Menopause	OC	Other
Proband	23	44	28-70	9	7	5	2
Sister with twin	23	46	25-74	5	9	5	4
Sisters	7	65	59-74				
Brothers	11	49	37-65				
Mother	17	69	50-80				
Father	10	68	54-78				
Grand mother with twin	1	80					
Grand father	1	82					
Daughter with twin	1	41					
Son with twin	1	43					
Total	96						

Interview

All MODZT (N=46) were interviewed by telephone. The average interview time was between 20-30 minutes. Table 3 gives an overview of the characteristics of the MODZT obtained from the telephone interview.

Before determination of zygosity by DNA typing from buccal swabs, questions regarding similarity of the twins and experiences mistaking one for another were asked together with the mothers' judgment on the zygosity of the twins. Eighteen of the 24 SS twin pairs were judged by the mother as DZ. Five were judged as probably DZ and one SS twin pair was judged as probably MZ.

The mean age of the twins of the interviewed mothers was 14.5 years. The youngest twin was 8 months old and the oldest nearly 40. The twin mothers gave birth to between two and seven children (mean 3.3). None of the pregnancies was assisted by IVF or a similar treatment. Clomiphene citrate (Clomid) was used to assist a pregnancy in one twin mother, she was one of a sister pair, but not excluded for the pilot study.

Table 3 Interview: Characteristics mothers of dizygotic twins (obtained from the telephone interview).

	N	Mean	SD	Range
Age mother conception of twins	46	30.29	5.02	19 – 40
Age mother conception first child	46	26.28	4.91	14 – 39
Parity	46	3.33	1.19	2 – 7
Birth weight MODZT(gram)	38	3544	553.44	2500 – 5000
Height (cm)	46	171.33	6.56	152 – 185
Weight (current)	46	69.48	11.96	55 – 104
Minimum weight since 18 years old (kg)	46	59.20	6.71	45 – 78
Maximum weight since 18 years old (kg)	46	74.70	14.07	55 – 118
Weight during twin pregnancy	42	82.38	14.00	65 – 119
BMI (current)	46	23.71	4.14	17.9 – 35.6
Menstrual cycle (days)	35	25.5	3.32	19 – 30

Twelve MODZT reported that they had female family members with fertility problems (7 first-degree family members and 5 second degree). None of the mothers reported current medical problems.

Transport and Logistics

All subjects (N = 85) were visited for blood collection within a period of 2 months in Dutch wintertime. The average traveling distance was 445 kilometers per day and the average time between collection of the samples and arrival in the laboratory was 4 hours. We collected blood on the second, third or fourth day of the menstrual cycle in 11 of the 14 women with a natural cycle. We missed 3 women with a natural cycle due to the broad dispersion of these mothers' homes. Subjects were awake on average 1.4 hours before the blood collection and they got out of bed between 5.30 a.m. and 8.55 a.m. Two subjects were visited before 7.00 a.m., before they went to work, and in 4 subjects we collected blood after 10.00 a.m. (but before 11.00 a.m.) due to traffic jams.

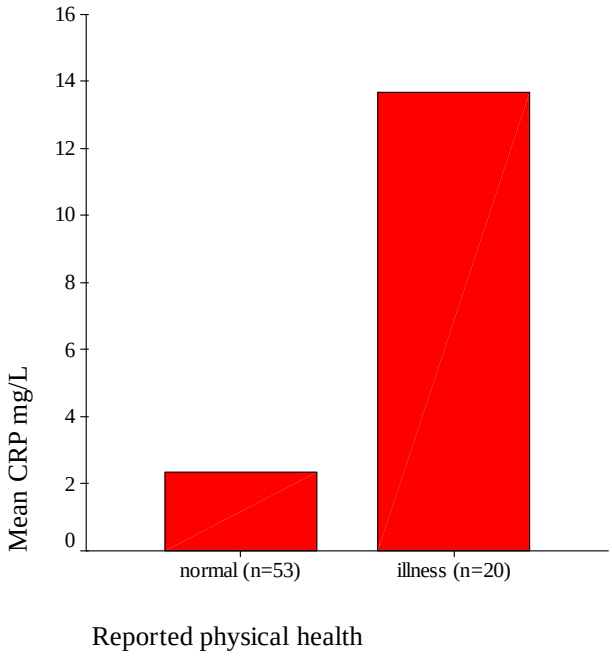
Blood sampling

Before blood collection subjects were asked the following questions. How is your current physical condition? When was the last time you were ill (fever)? What did you have for breakfast this morning, and did you smoke this morning? We also assessed height and weight. Five of the 85 subjects reported that they smoked before blood collection, and 7 subjects reported not to have fasted.

Sixty-two subjects reported that their physical condition was good. Ten subjects had a cold, 11 said they did not feel well (no fever) and 2 were really ill (fever). C- reactive protein (CRP) was measured as an

inflammatory marker in 73 subjects. Figure 3 shows the difference in mean CRP levels and reported physical health, divided in two categories, normal health and discomfort/illness. Of the 53 individuals reporting normal health (score 1), only one person showed an increased CRP level above 10 mg/l. Among persons reporting some kind of discomfort or illness (score 2-4), six of the 20 subjects showed a CRP level above10 mg/l. We concluded that the questionnaire is mainly effective in excluding acute phase reactions.

Figure 3 Reported physical condition and CRP-level (N=85).



Evaluation of the platelet product beta-Thromboglobulin in citrated plasma revealed a value generally above 150 IU/ml which is well above the expected 50 IU/ml and indicative of release of platelet products or activation of platelets during transport and handling (Kluft and Meijer, 1996). This precludes assay of some haemostatic variables including PAI-1. To reduce this effect in the future we will use CTAD anticoagulant mixture, which exerts platelet stabilisation (Kluft and Meijer, 1996).

Discussion

This pilot study has shown that, in the Netherlands, it is feasible to collect blood and urine samples for research purposes by making home visits to participants. We collected samples in nuclear families 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. In this pilot study both the proband mother and her sister were interviewed, in future research all mothers of DZ twins will participate in the telephone interview.

Lymphocytes and RNA can be extracted with sufficient yield and quality. For lymphocytes blood was frozen at - 80° C during transport and then transferred to liquid nitrogen for long-term storage. For RNA blood was frozen at – 80 ° C and for a maximum of 1 month. RNA isolated from whole blood showed no degradation of transcripts and it should be feasible to perform gene expression profiling studies with these samples. The effects of prolonged storage (> 1 month) on the amount of extracted RNA in polyethylene aliquots is currently not known (see accompanying paper Spijker et al.). In future research, we may also challenge blood cells with

immune systems related lipopolysaccharide (LPS), since greater shifts in gene-expression were observed during our pilot study than after a dexamethasone challenge.

To obtain representative plasma variables, the materials have to be stable for all individuals. We performed our pilot in the winter period with a significant number of flu cases in the Netherlands. We observed an increased CRP > 10 mg/l in 7 individuals raising the question whether some plasma variables for these individuals are in a stable phase. The presence of high CRP indicates an acute phase reaction, which may influence multiple quantitative traits and affect the analysis. We observed that a questionnaire is insufficient to predict high CRP, but excellent in predicting CRP < 10 mg/l. In future, we have to decide whether we will exclude cases with CRP > 10 mg/l or re-sample. The number of high values is expected to be less in other periods of the year.

In future research we will modify the sampling of citrate tubes, by using CTAD tubes in an attempt to stabilize the platelets better. In a preliminary study with lithium heparin we observed that an interaction with platelets occurs which dictates we first prepare platelet-rich plasma and separate white cells from platelets. This is not required for Natrium heparin and would reduce the complexity of the procedures. However, the lymphocyte harvest is better with lithium heparin.

This study focused on families with affected sister pairs (both sisters are mothers of dizygotic twins). It is a pilot project for a larger study which aims to localize and identify the genes causing variation in human DZ twinning. Families from the Netherlands, Australia and New Zealand participate in the study. Outside the Netherlands participants are bled by their local general practitioner (GP) and the samples are returned with courier service to the Queensland Institute for Medical Research. This procedure is an efficient and cheap way of collecting DNA samples, but limited, when blood samples need special handling after collecting for RNA, lymphocytes and other blood parameters. Another advantage of our approach is that transport and blood handling, with a special team of motivated trained nurses and analysts will minimize error and ensure high quality of the blood samples.

Of the 96 participants, 85 were bled. We could not visit 11 subjects, because distances between these subjects' homes were too great to be feasible to cover in 1 day. This was a relatively small pilot group and we do not expect these logistic problems with larger study designs. Home visits for blood and urine collection resulted in high response rates and cooperation. We expect that cooperation and response rates will decline when participants have to be active in providing blood samples (e.g. leaving their homes), especially when participants are older (e.g. the parents of the affected sister pairs). We will therefore use this sample protocol in studies of other complex traits. The protocol is currently implemented in the genetic study of migraine, cardiovascular disease of attention and memory and of depression. These projects, including the twinning study, will approach twin families registered with Netherlands Twin Register. Initially we will invite 8000 participants. In this feasibility study, we focused on twinning because it presented the most complex logistics. The nation wide standardized blood sampling and laboratory organization makes it possible to create a large, informative biobank.

In combination with the advances in molecular technologies, such a biobank will help to broaden the knowledge of the endocrinology and of the genes involved in human DZ twinning and other complex traits. In the study of DZ twinning hormone concentrations of e.g. LH, FSH, inhibin A and B will be determined so that genome scans can be conducted for linkage with endocrine parameters. 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.

In conclusion, the pilot study showed that nation-wide sample collection is feasible in Dutch families with two or more sisters who have given birth to spontaneous dizygotic twins. Large-scale blood and urine sample

collection for this project will be combined with similar sample collection in several other linkage projects will start in the fall of 2004.

In July 2004, we started recruiting families for the proposed DZ twinning study. Of the families approached around 70% agreed to participate. Sample collection, transport, blood handling and laboratory organization will be based and standardized on the results from this pilot study.

Chapter 8

General summary and discussion

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 twining

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 twining (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 twining 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-Varella 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 TGFb 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 TGFb 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 enviromental, 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.

Samenvatting

Dutch summary

Dit proefschrift beoogt meer inzicht te bieden in het samenspel van genetische factoren en omgevingsinvloeden die de kans op het krijgen van twee-eiige (DZ) meerlingen vergroten. In hoofdstuk 1 en 2 worden de basis van dit proefschrift en de dataverzameling uitvoerig beschreven. In hoofdstuk 3 wordt een uitgebreid overzicht gegeven van de eerdere onderzoeken naar de epidemiologie, de genetica en de endocrinologie van de DZ tweelingzwangerschap. Zowel de meest recente onderzoeken als het onderzoek dat in het verleden gedaan is, komen hierbij aan bod.

In hoofdstuk 4 tot en met 6 worden drie studies besproken die gebaseerd zijn op een schriftelijke vragenlijst die door moeders van meerlingen is ingevuld. Ten slotte beschrijft hoofdstuk 7 een onderzoek naar de haalbaarheid van het verzamelen van biologisch materiaal en interviewgegevens in een groep moeders van tweelingen, die een zus hebben die ook moeder van een tweeling is.

In dit hoofdstuk zal ik eerst de onderzoeken, waarover in dit proefschrift gerapporteerd wordt, samenvatten en bediscussiëren. Omdat deze onderzoeken merendeels berusten op gegevens die zijn verzameld met een schriftelijke vragenlijst, zal ik hiervan de methodologie bespreken.

Tot slot geef ik een beknopt overzicht van het onderzoek dat tot op heden is gedaan naar de genetica van het krijgen van DZ tweelingen en bespreek ik de implicaties voor de toekomst. In dit kader bespreek ik ook de dataverzameling van het Nederlandse deel van de studie "Genetics of DZ twinning".

Samenvatting en discussie

In hoofdstuk 4 wordt met een grootschalig vragenlijstonderzoek getoetst of moeders van meerlingen bereid zijn om vragen te beantwoorden over eventuele vruchtbaarheidsbehandelingen voorafgaand aan de conceptie van hun tweeling. Hiervoor zijn eerder verkregen vragenlijstgegevens gebruikt, waarbij we keken naar het aantal keren dat de vraag niet beantwoord is. We vinden dat maar een klein groepje moeders geen antwoord geeft op de vraag naar de wijze waarop de tweelingzwangerschap is ontstaan. De hoeveelheid missende gegevens voor deze vraag is vergelijkbaar met de hoeveelheid missende gegevens voor andere, neutralere, vragen zoals vragen over lichaamslengte. Daarnaast is gevonden dat moeders die een vruchtbaarheidsbehandeling bij de conceptie van hun tweeling ondergingen, net zo vaak de vragenlijst terugstuurden als moeders bij wie de tweelingzwangerschap op natuurlijke wijze tot stand kwam. Tot slot blijkt dat de vraag over het ontstaan van de tweelingzwangerschap betrouwbaar beantwoord wordt. Bij een subgroep van 80 moeders zijn de antwoorden die zij op de vragenlijst hebben gegeven, vergeleken met ziekenhuisgegevens. Deze uitkomsten tonen aan dat de vragenlijst een realistisch beeld geeft over het gebruik van vruchtbaarheidsbehandelingen bij de conceptie van de tweeling en daarom waarschijnlijk ook van andere, minder gevoelige, kwesties.

In hoofdstuk 5 wordt gekeken naar specifieke aspecten van fertiliteit en het familiaal voorkomen van tweelingen bij moeders van spontane eeneiige (MZ) tweelingen en DZ tweelingen en moeders van MZ of DZ tweelingen die geboren zijn na een vruchtbaarheidsbehandeling (artificiële technieken: ART). Uit dit onderzoek blijkt dat er in de families van moeders van spontane DZ tweelingen significant vaker andere vrouwelijke familieleden met DZ tweelingen voorkomen dan in families van moeders met MZ tweelingen en moeders van ART tweelingen. Moeders van spontane DZ en MZ tweelingen blijken uit een even groot gezin te komen; ze hebben ongeveer evenveel broers en zussen. Moeders van ART tweelingen blijken daarentegen uit kleinere gezinnen te komen. Uit dit onderzoek blijkt ook dat moeders van spontane DZ tweelingen sneller zwanger zijn van hun tweeling dan moeders van MZ tweelingen. We vinden geen verschil in het familiaal voorkomen van DZ tweelingen tussen moeders die hun spontane DZ tweeling op jonge leeftijd hebben gekregen (jonger dan 36) en moeders die hun spontane DZ tweeling op oudere leeftijd hebben gekregen (36 of ouder). De mechanismen die ten grondslag liggen aan het krijgen van een spontane DZ of een ART DZ tweeling zijn dus duidelijk verschillend van aard. Aangezien in de familie van moeders van ART DZ tweelingen niet vaker DZ tweelingen

voorkomen, is bij deze moeders geen sprake van een genetische aanleg, terwijl deze aanleg wel zichtbaar is voor moeders van spontane DZ tweelingen. Het is dus belangrijk om in genetisch onderzoek deze groepen van elkaar te onderscheiden.

In hoofdstuk 6 wordt gekeken of moeders van spontane MZ en DZ tweelingen van elkaar verschillen met betrekking tot lengte, 'body-mass index' (BMI , lichaamsmassa-index) en rookgedrag voor de tweelingzwangerschap. Hierbij wordt rekening gehouden met factoren als leeftijd, graviditeit (het aantal zwangerschappen voor de tweelingzwangerschap) en opleidingsniveau. Uit de resultaten blijkt dat de kans op het krijgen van spontane DZ tweelingen geassocieerd wordt met langer zijn, het hebben van een hogere BMI en roken. Eerdere studies tonen ook een verband aan tussen roken en het krijgen van DZ tweelingen en tussen lengte en BMI en het krijgen van DZ tweelingen. Deze studie heeft als additionele waarde dat wij getoetst hebben of dit verband beïnvloed wordt door sociaal economische status gedefinieerd als opleidingsniveau. Uit de resultaten blijkt dat de sociaal economische status van de moeder geen invloed heeft op de associatie tussen lichaamslengte, BMI en DZ tweelingen, en roken en DZ tweelingen. Leeftijd en graviditeit blijken wel significant geassocieerd met het krijgen van DZ tweelingen; de kans op het krijgen van een DZ tweeling neemt toe met de leeftijd en met het aantal zwangerschappen. Het is interessant om te zien dat factoren die doorgaans geassocieerd worden met verminderde vruchtbaarheid zoals roken en overgewicht ook geassocieerd zijn met 'super' vruchtbaarheid (het krijgen van tweelingen). De vraag is natuurlijk hoe dit kan. Een mogelijkheid is dat vrouwen die de genetische aanleg hebben voor het krijgen van tweelingen zo vruchtbaar zijn dat zij geen hinder ondervinden van factoren die normaal gesproken de vruchtbaarheid verminderen. Vrouwen die deze genetische aanleg missen, zijn wel verminderd vruchtbaar als ze bijvoorbeeld roken. Onder rokende vrouwen die geen aanleg hebben voor het krijgen van DZ tweelingen vermindert het aantal eenlingzwangerschappen en MZ tweelingzwangerschappen, terwijl het aantal zwangerschappen onder rokende vrouwen met een genetische aanleg voor DZ tweelingen niet vermindert.

Hoofdstuk 7 beschrijft een studie naar de haalbaarheid van het verzamelen van bloed- en urinemonsters bij een groep moeders die een zus hebben die ook moeder van een tweeling is en bij hun familieleden. Het doel van dit onderzoek was om te bepalen of het verzamelen van biologische monsters via een vast protocol tijdens een huisbezoek binnen heel Nederland haalbaar is en of de kwaliteit van de monsters na transport goed blijft. De deelnemers werden tussen 7 uur en 10 uur 's ochtends thuis of op hun werk bezocht. Vrouwen met een natuurlijke menstruele cyclus werden op dag 2 tot 4 van hun cyclus geprikt en wanneer zij de pil gebruiken werden zij in de pilvrije week geprikt. Tevens werden alle moeders van tweelingen ook telefonisch geïnterviewd. Dit interview duurde ongeveer 20 tot 30 minuten. Uit deze studie blijkt dat het mogelijk is om op een efficiënte manier bloed- en urinemonsters te verzamelen. Het transport van het bloed naar het laboratorium is niet nadelig voor de kwaliteit van het bloed, cholesterol, RNA met en zonder toediening van een activerende stof, lymfocyten en andere parameters.

Voor en nadelen van de vragenlijst

De drie studies, beschreven in de hoofdstukken 4, 5 en 6 zijn gebaseerd op een 2 pagina's tellende vragenlijst. De vragenlijst is in april 2005 verstuurd naar alle moeders van meerlingen (N=33.528) geregistreerd bij het Nederlands Tweelingen Register (NTR). In de vragenlijst werden vragen gesteld over het familiaal voorkomen van meerlingen en over het aantal broers en zussen van de moeder van de tweeling. Ook werd het geboortegewicht van de moeder en de vader van de tweeling gevraagd. Daarnaast werd de leeftijd bij menarche, het aantal zwangerschappen, miskramen, en het aantal geboren kinderen (meerlingen, eenlingen) uitgevraagd. Ook werden vragen gesteld met betrekking tot het tot stand komen van de tweelingzwangerschap en het gebruik

van de anticonceptiepil. Lengte en gewicht op het moment van invullen van de vragenlijst en gewicht voor en op het einde van de tweelingzwangerschap werden gevraagd. Ten slotte werd gevraagd of de moeder rookte en foliumzuur gebruikte voor en tijdens de tweelingzwangerschap.

Met dit vragenlijstonderzoek hebben we binnen een relatief korte tijd veel moeders van tweelingen kunnen bereiken. Sommige moeders die bij ons ingeschreven staan zijn passief geregistreerd, dit betekent dat zij nooit eerder aan vragenlijstonderzoek bij het NTR hebben meegedaan. Hoewel iemand actief kan beslissen niet mee te doen aan vragenlijstonderzoek lijkt het aannemelijk dat de adressen van een aantal van deze moeders niet correct zijn. Van de groep actief geregistreerde moeders (N=25.620), zijn 17.683 (69.0%) vragenlijsten geretourneerd. Daarnaast zijn er ook nog 1.674 (21.2%) vragenlijsten teruggestuurd door passief geregistreerde moeders. In totaal werden 19.357 (57.7%) vragenlijsten geretourneerd.

Zoals aangegeven wordt deze studie uitgevoerd binnen het kader van het NTR. Van alle moeders die ingeschreven staan, zijn al gegevens aanwezig. In het minste geval betreffen dit inschrijvingsdata maar meestal zijn ook gegevens uit eerdere (vragenlijst)onderzoeken beschikbaar. Hierdoor is het mogelijk om een non-respons onderzoek te doen. We toetsen in hoofdstuk 6 of moeders die de vragenlijst wel hadden teruggestuurd op een aantal eigenschappen, zoals roken, lengte en opleidingsniveau, verschillen van moeders die de vragenlijst niet terugstuurden. De resultaten van dit onderzoek wijzen op een kleine response bias. Echter deze bias is gelijk in moeders van MZ en DZ tweelingen, en het is dus zeer onwaarschijnlijk dat deze bias van significante invloed is op de studies waarin we moeders van MZ en DZ tweelingen met elkaar vergelijken.

De vragenlijst is in eerste instantie verstuurd om tweelingmoeders te kunnen selecteren die ook nog een zus hebben die moeder is van een tweeling. Dit is gedaan in het kader van de studie "Genetics of DZ twinning". Moeders van een tweeling, die aangeven dat hun zus ook een tweeling heeft, worden voor dit onderzoek uitgenodigd. Moeders worden ook uitgenodigd voor de studie als zij aangeven dat hun eigen moeder en hun tante (zus van moeder) een tweeling hebben. Om deze reden zijn de vragen naar het familiaal voorkomen van tweelingen in de vragenlijst vrij uitvoerig en nemen relatief veel ruimte in beslag. Een consequentie hiervan is dat sommige andere vragen door ruimtegebrek mogelijk minder ideaal uitgevraagd zijn.

Tot slot moet opgemerkt worden dat deze vragenlijst is verstuurd naar moeders van meerlingen en de resultaten uit de studies beschreven in dit proefschrift betreffen vergelijkingen tussen groepen van meerlingmoeders, zoals de vergelijking tussen spontane DZ moeders en spontane MZ moeders. Men zou kunnen beargumenteren dat eenlingmoeders een betere vergelijkingsgroep vormen dan bijvoorbeeld MZ tweelingmoeders. De algemene opvatting navolgend gaan wij er van uit dat het krijgen van een MZ tweeling een random gebeurtenis is. Deze gebeurtenis wordt niet beïnvloed door genetische en/of omgevingsfactoren en kan dus iedere vrouw overkomen. MZ tweelingmoeders zijn daarom een even goede vergelijkingsgroep als eenlingmoeders. In feite, omdat MZ tweelingmoeders ook een meerlingzwangerschap hebben doorgemaakt, zou men kunnen beargumenteren dat MZ tweelingmoeders juist een betere vergelijkingsgroep vormen als het gaat om het onderzoeken van de genetische invloeden voor het krijgen van DZ tweelingen.

Implicaties voor de toekomst

De zoektocht naar genen die geassocieerd worden met het krijgen van DZ tweelingen

Bij schapen is de genetische basis voor het krijgen van meerlingen voor een groot deel in kaart gebracht. In drie verschillende genen zijn mutaties gevonden die tot een hogere meerlingfrequentie leiden. Twee van deze genen betreffen de nauw gerelateerde groeifactoren, BMP15 (bone morphogenetic protein 15) en GDF9 (growth differentiation factor-9). Het derde gen is een receptor (BMPR1B, bone morphogenetic protein type IB receptor). Deze genen komen allemaal tot expressie in de eierstokken en hebben invloed op de reactie van individuele follikels op externe hormoonsignalen. Er is onderzocht of deze genen bij mensen ook een rol spelen

bij het krijgen van tweelingen. Er zijn een aantal families gevonden waarin vrouwen deze varianten dragen. Deze vrouwen lijken twee keer zoveel kans te hebben om een tweeling te krijgen dan andere vrouwen. De frequentie van deze varianten is echter zo laag (minder dan 4% voor alle varianten) dat de bijdrage van deze varianten aan de totale incidentie van DZ tweelingen klein is. In onderzoeken zijn ook associaties gevonden tussen andere kandidaatgenen, zoals FMRI (fragile X mental retardation 1), FSHR (follicle stimulating hormone receptor) en Pi (protease inhibitor locus) en het krijgen van DZ tweelingen. Deze bevindingen zijn echter niet gerepliceerd in recente onderzoeken.

Er blijken dus geen sterke associaties te zijn tussen de groeifactoren BMP15 en GDF9 en het krijgen van DZ tweelingen bij de mens, ook al zijn deze genen bij de mens wel gerelateerd aan de vruchtbaarheid. Meerdere recente onderzoeken naar vrouwen met een vroege menopauze (premature ovarian failure) hebben uitgewezen dat sommige van deze vrouwen ook mutaties van zowel GDF9 als BMP15 dragen. Opnieuw is echter de frequentie van deze genvarianten in vrouwen met vervroegde overgang laag. Het is tot dusver niet bekend of dezelfde mutaties zowel verantwoordelijk zijn voor een verhoogde kans op tweelingen als voor de vervroegde overgang.

Uit bovenstaande bevindingen kan geconcludeerd worden dat de genen die worden geassocieerd met het krijgen van DZ tweelingen, bij mensen moeilijker te lokaliseren zijn dan in eerste instantie werd gedacht. De zoektocht naar kandidaatgenen heeft ons enig inzicht gegeven, maar nadere studie is nodig. Een interessante focus is daarbij het onderzoek naar de aard van de relatie tussen vervroegde overgang en het krijgen van DZ tweelingen. Deze onderzoeken zullen ons inzicht geven in de sleutelprocessen van vrouwelijke vruchtbaarheid.

In samenwerking met het Queensland Institute of Medical Research (QIMR) in Australië hebben wij een grote groep moeders van tweelingen gerekruteerd voor de studie 'Genetics of DZ twinning'. Moeders van DZ tweelingen met een zus die ook moeder is van een DZ tweeling en hun beide ouders worden gevraagd om mee te doen aan het project. Meedoen houdt in dat een bloed- en een urinemonster wordt afgenomen bij de deelnemers en daarnaast nemen de moeders van tweelingen ook deel aan een telefonisch interview. In het Nederlandse deel van deze studie is bloed verzameld voor DNA, RNA en hormoonconcentraties zoals LH, FSH en inhibine A en B. Om koppelingsonderzoek met endocriene parameters te kunnen doen is het bloed bij vruchtbare vrouwen op de 2e tot 4e dag van de menstruele cyclus verzameld. Wanneer vrouwen de anticonceptiepil gebruiken is in de pilvrije week geprikt. Op het moment dat genome-wide markers beschikbaar zullen zijn, kunnen we ook andere parameters gebruiken voor zusterpaar koppelingsonderzoek. Het gaat hierbij om variabelen die verkregen zijn tijdens het interview (zoals lengte en gewicht) en parameters uit plasma (zoals cholesterol en glucose).

Op dit moment wordt er bij 523 personen uit 125 families koppelingsonderzoek gedaan. Het doel van dit project is om uiteindelijk koppelingsonderzoek uit te voeren voor het DZ tweelingen fenotype door middel van een 10cM genome scan (400 markers) in 500 Nederlandse families en in 500 Australische families.

De vraag waarom vrouwen DZ tweelingen krijgen blijkt niet eenvoudig te beantwoorden. Het spontaan krijgen van een DZ tweeling wordt beïnvloed door omgevingsfactoren, eigenschappen van de moeder en genetische aanleg. De onderzoeken die in dit proefschrift staan beschreven laten zien dat naast leeftijd en graviditeit ook overgewicht, lengte en rookgedrag van invloed zijn op het krijgen van DZ tweelingen. Hoewel deze factoren een significante rol spelen is hun gezamenlijke effect echter niet erg groot. Genetische factoren spelen ook een belangrijke rol en kunnen in samenwerking met omgevingsfactoren het risico op het krijgen van DZ tweelingen vergroten. Meer onderzoek naar de genetische basis van het krijgen van DZ tweelingen is nodig om dit complexe fenomeen te ontrafelen.

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Appendices

Appendix I

Telephone interview: protocol (A) and descriptive results (B)

DZ TWINNING INTERVIEW
(Mother's of DZ twins)

NTRID	
Mother's ID number	
Date of birth	
Interviewers ID	
Date of interview	

A1.	How old are you now?	
A2	What is your birth weight?	
A3	Are you currently pregnant?	
A3.a	A How many times have you been pregnant?	
A4.	How many children have you had?	
A5.	How many of your pregnancies where twin pregnancies?	

A5a. How did you know it was a twin pregnancy (how was it diagnosed?

1 Ultrasound
2 Miscarriage
3 Live birth
4 Other

Specify other

A5b. If more than one twin pregnancy:
How did you know it was a twin pregnancy (how was it diagnosed?

1 Ultrasound
2 Miscarriage
3 Live birth
4 Other

Specify other

A6. How many sets of twins do you have?

1 One
2 Two
3 Three

A7. Do you have twin girls, twin boys of one of each sex?

ASK FOR EACH SET OF TWINS SEPARATELY
IF ONLY ONE SET CODE UNDER 'FIRST SET'

A7.1 First Set		**A7.2 Second Set**		**A7.3 Third Set**	
MZF (identical girls)	**1**	MZF (identical girls)	**1**	MZF (identical girls)	**1**
DZF (non-identical girls)	**2**	DZF (non-identical girls)	**2**	DZF (non-identical girls)	**2**
MZM (identical boys)	**3**	MZM (identical boys)	**3**	MZM (identical boys)	**3**
DZM (non-identical boys)	**4**	DZM (non-identical boys)	**4**	DZM (non-identical boys)	**4**
DZO (one boy, one girl)	**5**	DZO (one boy, one girl)	**5**	DZO (one boy, one girl)	**5**

ATTENTION!
QUESTION A8. TO A16. HAS TO BE ASKED FOR ALL TWIN SETS

How much does the twin look alike regarding?

A8. Facial appearance
1 Exactly
2 More or less
3 Absolutely not

A9. Hair colour
1 Exactly
2 More or less
3 Absolutely not

A10. Complexion
1 Exactly
2 More or less
3 Absolutely not

A11. Eye colour
1 Exactly
2 More or less
3 Absolutely not

IF TWINS ARE OS (GIRL/BOY) SKIP TO B.

A12. How often do you have difficulty telling the twins apart?

1 Always
2 Usually
3 Sometimes
4 Rarely
5 Never

A13. How often do/did teachers or other family members have difficulty telling the twins apart?

1 Always
2 Usually
3 Sometimes
4 Rarely
5 Never
9 Don't know

A14. How often do strangers have difficulty telling the twins apart?

1 Always
2 Usually
3 Sometimes
4 Rarely
5 Never
9 Don't know

A15. As children were/are the twins as alike as "two peas in a pod", or only of normal family physical likeness. That is, no more alike physically than ordinary sisters/brothers?

1 Two peas in a pod
2 Only family likeness

A16. In your opinion, are the twins….

1 Definitely identical
2 Probably identical
3 Probably non-identical
4 Definitely non-identical

A16a How did you reach that conclusion?

1 Blood test
2 DNA test
3 Physical appearance
4 Told at birth because of number of placentas
5 Told at birth, other or reasons unknown
6 Told later by doctor (no blood tests)
7 Personality
8 Other (please specify)

SPECIFY "OTHER _____

A17. Did any of your pregnancies (including those that may have ended in miscarriage or stillbirth), involve hormone treatment prescribed by your doctor?

1 No
2 Yes

A18. Did any of your pregnancies (including those that may have ended in miscarriage or stillbirth), involve IVF?

1 No
2 Yes

A19. Did any of your pregnancies (including those that may have ended in miscarriage or stillbirth), involve alternative or natural fertility treatments?

1 No
2 Yes, specify

SPECIFY _____

B. BIRTH HISTORY

Now I'd like to ask you some further questions about the history of your pregnancies and the births of your children. Starting with your first pregnancy.

PREGNANCY	**FIRST**	**SECOND**	**THIRD**
B1. How old where you at the beginning?			
B2. Was that pregnancy planned?	NO 1 YES 2	NO 1 YES 2	NO 1 YES 2
B3. How long did you try for this pregnancy?	_____ MONTH	_____ MONTH	_____ MONTH
B4. Did IVF or a similar program assist this pregnancy?	NO 1 YES 2	NO 1 YES 2	NO 1 YES 2
B5. Was this pregnancy assisted by alternative or natural fertility treatments?	NO 1 YES 2	NO 1 YES 2	NO 1 YES 2
B6. What was the outcome of this pregnancy? 1. A live birth (vaginal) 2.A live birth (caesarean) 3. A miscarriage 4. A termination 5. A still birth			
B7. How many weeks did the pregnancy continued for?			
B8. Did you need drugs such as hormones to sustain the pregnancy? **If yes, specify** _____ _____	NO 1 YES 2	NO 1 YES 2	NO 1 YES 2
B9. LIVE BIRHT ONLY….. What is/are the first names of this/ these children? Oldest of twins first than youngest.			
B10. What is/are his/her/their date of birth?	___/___/___ dd mm yy	___/___/___ dd mm yy	___/___/___ dd mm yy
B11.What sex is/are this/these child/children?	FEMALE 2 MALE 1	FEMALE 2 MALE 1	FEMALE 2 MALE 1

PREGNANCY	**FOURTH**	**FIFTH**	**SIXTH**
B1. How old where you at the beginning?			
B2. Was that pregnancy planned?	NO 1 YES 2	NO 1 YES 2	NO 1 YES 2
B3. How long did you try for this pregnancy?	_____ MONTH	_____ MONTH	_____ MONTH
B4. Did IVF or a similar program assist this pregnancy?	NO 1 YES 2	NO 1 YES 2	NO 1 YES 2
B5. Was this pregnancy assisted by alternative or natural fertility treatments?	NO 1 YES 2	NO 1 YES 2	NO 1 YES 2
B6. What was the outcome of this pregnancy? 1. A live birth (vaginal) 2.A live birth (caesarean) 3. A miscarriage 4. A termination 5. A still birth			
B7. How many weeks did the pregnancy continued for?			
B8. Did you need drugs such as hormones to sustain the pregnancy? **If yes, specify** _____ _____	NO 1 YES 2	NO 1 YES 2	NO 1 YES 2
B9. LIVE BIRHT ONLY….. What is/are the first names of this/ these children? Oldest of twins first than youngest.			
B10. What is/are his/her/their date of birth?	___/___/___ dd mm yy	___/___/___ dd mm yy	___/___/___ dd mm yy
B11.What sex is/are this/these child/children?	FEMALE 2 MALE 1	FEMALE 2 MALE 1	FEMALE 2 MALE 1

C. HEALTH AND MEDICAL

Now I am going to ask you some questions about your health and medical history.

C1.	How tall are you?	**cm**	_____
C2.	How much do you currently weigh?	**Kg**	_____
C3.	What is the most you have weighed since you where 18 years old, not including when you where pregnant	**Kg**	_____
C3a.	What is the most you have weighed since you, where 18 years old, including when you where pregnant	**Kg**	_____
C3b.	What is the least you have weighed since you where 18 years old, don't count any period of physical illness	**Kg**	_____
C4.	How old where you when you had your first menstrual period?	**Age**	_____

******* Before any pregnancy *******

C5.	On average, how many days of bleeding do you have each natural period?	**Days**	_____
C6.	Where your natural periods	**1**	**Heavy**
		2	**Moderate**
		3	**Light**
		4	**Don't know**
C7.	Where your natural periods	**1**	**Very painful**
		2	**Moderately**
		3	**No trouble**
		4	**Don't know**
C8.	Where your natural periods	**1**	**Very limiting**
		2	**Moderately**
		3	**Not at all**
		4	**Don't know**

C9.	What is the average time between the start of one period and the start of the next?	**Days**	_____
C9a.	Days between periods too variable to specify	**9**	

Smoking

C10. Where you a smoker before the twin pregnancy?

1 No

2 Yes, but I quit directly when I new I was pregnant. I stopped smoking in the ___ week of my pregnancy. Before I became pregnant I smoked on average ___ cigarettes/cigars per day for ___ years.

3 Yes, before my pregnancy. I quit smoking ____ weeks ____ months _____ years before I became pregnant. I smoked on average ____ cigarettes/cigars per day for _____ years.

4 Yes, but only during my pregnancy. I smoked for ___ weeks and I smoked on average ____ cigarettes/cigars per day.

5 Yes, before my twin pregnancy and during the pregnancy. Before my pregnancy I smoked on average____ cigarettes/cigars per day for _____ years and during my pregnancy I smoked ____ cigarettes/cigars per day.

Oral contraceptives

C11.	Did you use oral contraceptives prior to the twin pregnancy?	**1**	**No**
		2	**Yes**
	If yes, which Pill did you use?		
	If yes, how many weeks before the twin pregnancy did stopped taking the oral contraceptives	**Weeks**	_____

Folic acid

C12.	Did you use folic acid before/during the twin pregnancy?	**1**	**No**
		2	**Yes**
	Yes, but only before the pregnancy	**Weeks**	_____
	Yes, but only during the pregnancy	**Weeks**	_____
	Yes before and during the pregnancy	**Weeks**	_____
C13.	Are you using contraception at present?	**1**	**No**
		2	**Yes**

C13a. If Yes, which method are you currently using?

1 **Combination pill**
2 **Progesterone only pill**
3 **Hormone patches**
4 **Nuvaring**
5 **Injection, Depo-provera**
6 **IUD**
7 **Sterilization**
8 **Other, specify**

C14.	Have you reached menopause?	**1**	**No**
		2	**Yes**
C14a.	How old were you when your periods stopped?	**Age**	_____
C14b.	Did you use oral contraceptives until you reached menopause?	**1**	**No**
		2	**Yes**
C14c	Did your periods stopped directly after you stopped taking oral contraceptives?	**1**	**No**
		2	**Yes**
C15.	Did you have problems with menopause?	**1**	**No**
		2	**Yes**
C15a.	Where these problems?	**1**	**Very severe**
		2	**Severe**
		3	**Moderately**
		4	**Light**
C16.	Has your mother reached menopause?	**1**	**No**
		2	**Yes**
		3	**Don't know**
C16a.	How old was your mother when her periods stopped?	**Age**	_____
C16b.	Did she use oral contraceptives until you reached menopause?	**1**	**No**
		2	**Yes**
		3	**Don't know**
C16c.	Did here periods stopped directly after you stopped taking oral contraceptives?	**1**	**No**
		2	**Yes**
C17.	Did your mother had problems with menopause?	**1**	**No**
		2	**Yes**
		3	**Don't know**
C18.	Were these problems	**1**	**Very severe**
		2	**Severe**
		3	**Moderately**
		4	**Light**

C19.	Have you had a hysterectomy?	**1**	**No**
		2	**Yes**
C19a.	How old were you when you had your hysterectomy?	**Age**	_____
C19b.	When did you have your hysterectomy? Was it	**1**	**Before menopause**
		2	**During menopause**
		3	**After menopause**
C19c.	Were both Ovaries removed?	**1**	**No**
		2	**Yes**
C20.	Have you ever been diagnosed with any of the following forms of cancer?	**1**	**Ovarian cancer**
		2	**Cervical cancer**
		3	**Breast cancer**
		4	**Other, specify**

_____

		No	**Yes**
C21.	Have you ever been diagnosed with any of the following?	**1**	**2**
C21.1.	Endometriosis	**1**	**2**
C21.2.	Fibroids in the uterus	**1**	**2**
C21.3.	Pre-eclampsia	**1**	**2**
C21.4.	Caesarean (for first pregnancy)	**1**	**2**

C.22	Have you <u>ever</u> suffered from any of the following health problems?	**No**	**Yes**
C.22.1.	High Blood Pressure	**1**	**2**
C.22.2.	Cardiovascular disease	**1**	**2**
C.22.3.	High Cholesterol	**1**	**2**
C.22.4.	Diabetes type 1	**1**	**2**
C.22.5.	Diabetes type 2	**1**	**2**
C.22.6.	Bone Fractures	**1**	**2**
C.22.7.	Osteoporosis	**1**	**2**
C.22.8.	Arthritis (osteo, rheumatoid)	**1**	**2**
C.22.9.	Hepatitis	**1**	**2**
C.22.10.	Angina	**1**	**2**
C.22.11.	Asthma or Bronchitis	**1**	**2**
C.22.12.	Eczema	**1**	**2**
C.22.13.	Allergy or Hay Fever	**1**	**2**
C.22.14.	PCO (polycystic ovary syndrome)	**1**	**2**
C.22.15.	Migraine	**1**	**2**

		No	Yes
C.22.16.	Thyroid	**1**	**2**
C.22.17.	Anorexia Nervosa	**1**	**2**
C.22.18.	Obesity	**1**	**2**
C.22.19.	Other, specify	**1**	**2**

D. FAMILY STRUCTURE

Mow I'd like to ask you some questions about your family

D1.	Is your mother still alive?	**1**	**No**
		2	**Yes**
D1a.	How old is your mother now?	**Age**	_____
D1b.	What is her date of birth?	**Date**	_____
D2.	Is your father still alive?	**1**	**No**
		2	**Yes**
D2a.	How old is your father now?	**Age**	_____
D2b.	What is his date of birth?	**Date**	_____
D3.	How many brothers do you have?	**Number**	_____
D4.	How many sisters do you have?	**Number**	_____
D5.	Is pedigree MODZT drawn? (see separate sheet)	**1**	**No**
		2	**Yes**
D6.	Is pedigree of father of the twins drawn? (see separate sheet)?	**1**	**No**
		2	**Yes**

D7.	We are interested to know if there is any history of female infertility in your family? Do any of your female relatives have problems with infertility?	**1** **2**	**No** **Yes**

D7a.	Which relatives are these?	**No**	**Yes**
	Sister	**1**	**2**
	Daughter	**1**	**2**
	Mother's sister	**1**	**2**
	Father's sister	**1**	**2**
	Other, specify _____	**1**	**2**

E. ANCESTRY

Now I'd like to ask you some questions about your parents and their parents, that is, your grandparents

E1.	In which country was your mother born?	**Record** _____
E1.a.	In which country was your mother's mother, that is, your maternal grandmother, born?	**Record** _____
E1.b.	In which country was your mother's father, that is, your maternal grandfather, born?	**Record** _____
E2.	In which country was your father born?	**Record** _____
E2.a.	In which country was your father's mother, that is, your paternal grandmother, born?	**Record** _____
E2.b.	In which country was your father's father, that is, your paternal grandfather, born?	**Record** _____

We are also interested in knowing the ancestry of your parents and grandparents. That is, the place or ethnic group where most of their ancestors came from.

E3.	What is the ancestry of your mother's mother, that is, your maternal grandmother?	**Record** _____
E3.a.	What is the ancestry of your mother's father, that is, your maternal grandfather?	**Record** _____
E4.	What is the ancestry of your father's mother, that is, your paternal grandmother?	**Record** _____
E4.a.	What is the ancestry of your father's father, that is, your paternal grandfather?	**Record** _____

END THE INTERVIEW BY THANKING THE RESPONDENT

DZ TWEELINGEN INTERVIEW
(Moeders van twee-eiige tweelingen)

NTRID _____

Link nummer _____

Geboorte datum _____

Naam interviewer _____

Datum interview _____

A1. Hoe oud bent u nu? _____

A2 Wat is uw geboorte gewicht? _____

A3 Bent u op dit moment zwanger? _____

A3.a Hoe vaak bent u zwanger geweest? _____

A4. Hoe veel kinderen heeft u gekregen? _____

A5. Hoeveel van uw zwangerschappen waren tweeling zwangerschappen _____

A5a. Hoe wist u dat het om een tweelingzwangerschap ging?

1 Echo
2 Miskraam
3 Geboorte
4 Anders

Specificeer

A5b. Indien meer dan 1 tweelingzwangerschap:
Hoe wist u dat het tweelingzwangerschappen waren?

1 Echo
2 Miskraam
3 Geboorte
4 Anders

Specificeer

A6. Hoeveel tweelingparen heeft u?

1 Een
2 Twee
3 Drie

A7. Heeft u tweeling meisjes, tweeling jongens of een tweeling jongen en een meisje?

Vraag dit voor ieder tweelingpaar apart. Indien er maar een tweelingpaar is codeer deze dan onder het eerste paar

A7.1 Eerste paar		**A7.2 Tweede paar**		**A7.3 Derde paar**	
MZF (identieke meisjes)	**1**	MZF (identieke meisjes)	**1**	MZF (identieke meisjes)	**1**
DZF (niet identieke meisjes)	**2**	DZF (niet identieke meisjes)	**2**	DZF (niet identieke meisjes)	**2**
MZM (identieke jongens)	**3**	MZM (identieke jongens)	**3**	MZM (identieke jongens)	**3**
DZM (niet identieke jongens)	**4**	DZM (niet identieke jongens)	**4**	DZM (niet identieke jongens)	**4**
DZO (jongen, meisje)	**5**	DZO (jongen, meisje)	**5**	DZO (jongen, meisje)	**5**

ATTENTIE!
VRAAG A8. TOTEN MET A16. MOETEN VOOR ALLE TWEELINGPAREN AFZONDERLIJK UITGEVRAAGD WORDEN

Hoeveel lijkt de tweeling op elkaar wat betreft?

A8. Gezicht

1 Precies
2 Enigszins
3 Helemaal niet

A9. Haar kleur

1 Precies
2 Enigszins
3 Helemaal niet

A10. Gelaatskleur

1 Precies
2 Enigszins
3 Helemaal niet

A11. Oog kleur

1 Precies
2 Enigszins
3 Helemaal niet

Als de tweeling OS (meisje/jongen) is ga verder met B.

Hoe vaak verwart u de tweeling met elkaar?

A12.

1 Altijd
2 Vaak
3 Soms
4 Nauwelijks
5 Nooit

A13. Hoe vaak verwaren familieleden (of leraren) de tweeling met elkaar?

1 Altijd
2 Vaak
3 Soms
4 Nauwelijks
5 Nooit
9 Weet niet

A14. Hoe vaak hebben vreemden moeite om ze uit elkaar te houden?

1 Altijd
2 Vaak
3 Soms
4 Nauwelijks
5 Nooit
9 Weet niet

A15. Lijkt de tweeling op elkaar als twee druppels water of lijken ze net zoveel op elkaar als gewone broers of zussen?

1 Als twee druppels
2 Als gewone broers/zussen

A16. Volgens uw mening is de tweeling

1 Absoluut identiek
2 Waarschijnlijk identiek
3 Absoluut niet identiek
4 Waarschijnlijk niet identiek

A16a Hoe bent u tot deze conclusie gekomen?

1 Bloed test
2 DNA test
3 Uiterlijk
4 Bij de geboorte vertelt dmv placenta
5 Bij de geboorte vertelt, onbekende reden
6 Het is later door de arts verteld
7 Karakter
8 Anders

Specificeer anders _____

A17. Is een van uw zwangerschappen (inclusief miskraam of doodgeboorte) tot stand gekomen d.m.v. een hormoonbehandeling die door een arts is voorgeschreven?

1 Nee
2 Ja

A18. Is een van uw zwangerschappen (inclusief miskraam of doodgeboorte) tot stand gekomen d.m.v. IVF?

1 Nee
2 Ja

A19. Is een van uw zwangerschappen (inclusief miskraam of doodgeboorte) tot stand gekomen d.m.v. een alternatieve of natuurlijke vruchtbaarheidsbehandeling?

1 Nee
2 Ja

Specificeer _____

B. GEBOORTE GESCHIEDENIS

Ik zou u nu wat vragen willen stellen over de geboortes en zwangerschappen van uw kinderen. We beginnen met uw eerste zwangerschap.

ZWANGERSCHAP	**EERSTE**	**TWEEDE**	**DERDE**
B1. Hoe oud was u aan het begin?			
B2. Was deze zwangerschap gepland?	NEE 1 JA 2	NEE 1 JA 2	NEE 1 JA 2
B3. Hoe lang heeft u geprobeerd om zwanger te worden?	_____ MAAND	_____ MAAND	_____ MAAND
B4. Was deze zwangerschap bijgestaan door IVF of een gelijkwaardige behandeling?	NEE 1 JA 2	NEE 1 JA 2	NEE 1 JA 2
B5. Was deze zwangerschap bijgestaan door een alternatieve of natuurlijke vruchtbaarheids behandeling?	NEE 1 JA 2	NEE 1 JA 2	NEE 1 JA 2
B6. Hoe is deze zwangerschap geëindigd? 1. Geboorte (vaginaal) 2. Geboorte (keizersnede) 3. Miskraam 4. Abortus 5. Dood geboorte			
B7. Hoeveel weken heeft de zwangerschap geduurd?			
B8. Heeft u medicatie gebruikt zoals hormonen om de zwangerschap te ondersteunen? **Indien ja, specificeer** _____ _____	NEE 1 JA 2	NEE 1 JA 2	NEE 1 JA 2
B9. Alleen leven geboren kinderen Wat is de voornaam van dit kind of deze kinderen? Bij een tweeling de voornaam van de OUDSTE bovenaan.			
B10. Geboorte datum	___/___/___ dd mm jjjj	___/___/___ dd mm jjjj	___/___/___ dd mm jjjj
B11. Sekse	Vrouw 2 Man 1	Vrouw 2 Man 1	Vrouw 2 Man 1

ZWANGERSCHAP	**VIERDE**	**VIJFDE**	**ZESDE**
B1. Hoe oud was u aan het begin?			
B2. Was deze zwangerschap gepland?	NEE 1 JA 2	NEE 1 JA 2	NEE 1 JA 2
B3. Hoe lang heeft u geprobeerd om zwanger te worden?	_____ MAAND	_____ MAAND	_____ MAAND
B4. Was deze zwangerschap bijgestaan door IVF of een gelijkwaardige behandeling?	NEE 1 JA 2	NEE 1 JA 2	NEE 1 JA 2
B5. Was deze zwangerschap bijgestaan door een alternatieve of natuurlijke vruchtbaarheids behandeling?	NEE 1 JA 2	NEE 1 JA 2	NEE 1 JA 2
B6. Hoe is deze zwangerschap geëindigd? 1. Geboorte (vaginaal) 2. Geboorte (keizersnede) 3. Miskraam 4. Abortus 5. Dood geboorte			
B7. Hoeveel weken heeft de zwangerschap geduurd?			
B8. Heeft u medicatie gebruikt zoals hormonen om de zwangerschap te ondersteunen? **Indien ja, specificeer** _____ _____	NEE 1 JA 2	NEE 1 JA 2	NEE 1 JA 2
B9. Alleen leven geboren kinderen Wat is de voornaam van dit kind of deze kinderen? Bij een tweeling de voornaam van de OUDSTE bovenaan.			
B10. Geboorte datum	___/___/___ dd mm jjjj	___/___/___ dd mm jjjj	___/___/___ dd mm jjjj
B11. Sekse	Vrouw 2 Man 1	Vrouw 2 Man 1	Vrouw 2 Man 1

C. GEZONDHEID EN MEDICATIE

Nu ga ik wat vragen stellen over uw gezondheid en uw medische voorgeschiedenis.

C1.	Hoe lang bent u?	**cm**	_____
C2.	Hoeveel weegt u op dit moment?	**Kg**	_____
C3.	Hoeveel heeft u maximaal gewogen sinds uw 18e, uw zwangerschappen niet meegerekend?	**Kg**	_____
C3a.	Hoeveel heeft u maximaal gewogen sinds uw 18e, inclusief uw zwangerschappen?	**Kg**	_____
C3b.	Hoeveel heeft u minimaal gewogen sinds uw 18e, exclusief periodes van ziekte?	**Kg**	_____
C4.	Hoe oud was u toen u voor het eerst menstrueerde?	**Leeftijd**	_____

Deze vragen gaan over de periode VOORDAT u kinderen heeft gekregen

C5.	Hoeveel dagen gemiddeld per natuurlijke cyclus verloor u bloed?	**Dagen**	_____
C6.	Was uw menstruatieperiode	**1**	**Zwaar**
		2	**Gemiddeld**
		3	**Licht**
		4	**Weet niet**
C7.	Was uw menstruatieperiode	**1**	**Erg pijnlijk**
		2	**Gemiddeld**
		3	**Geen probleem**
		4	**Weet niet**
C8.	Was uw menstruatieperiode	**1**	**Erg beperkend**
		2	**Gemiddeld**
		3	**Geen probleem**
		4	**Weet niet**
C9.	Hoeveel dagen zaten er gemiddeld tussen de start van een periode en de start van de daarop volgende?	**Dagen**	_____
C9a.	Te wisselt om te specificeren	**9**	

Roken

C10. Heeft u voorafgaand aan de tweelingzwangerschap gerookt?

1 Nee

2 Ja, maar zodra ik wist dat ik zwanger was ben ik gestopt. Dit was in ___ week van mijn zwangerschap. Voor mijn zwangerschap rookte ik gemiddeld ___ sigaretten/shag per dag en dit deed ik ___ jaren.

3 Ja, voordat ik zwanger werd. Ik ben____ weken ____ maanden _____ jaren voordat ik zwanger werd gestopt met roken en ik rookte gemiddeld ____ sigaretten/shag per dag en dit deed ik ___ jaren.

4 Ja, alleen tijdens de zwangerschap. Ik heb ___ weken gerookt en ik rookte gemiddeld ____ sigaretten/shag per dag.

5 Ja, voordat ik zwanger werd en gedurende de zwangerschap. Voordat ik zwanger werd rookte ik gemiddeld ____ sigaretten/shag per dag en dit deed ik ___ jaren. Tijdens mijn zwangerschap rookte ik gemiddeld ____ sigaretten/shag per dag.

Anticonceptie

C11.	Heeft u voorafgaand aan de tweelingzwangerschap de anticonceptiepil gebruikt?	**1**	**Nee**
		2	**Ja**
	Indien ja, welke?		
	Indien ja, hoeveel weken bent u met de pil gestopt voordat u zwanger werd van tweeling?	**Weken**	_____

Foliumzuur

C12.	Heeft u voorafgaand aan of tijdens de tweelingzwangerschap de foliumzuur gebruikt?	**1**	**Nee**
		2	**Ja**
	Ja, alleen voordat ik zwanger werd	**Weken**	_____
	Ja, alleen tijdens de zwangerschap	**Weken**	_____
	Ja, voor en tijdens de zwangerschap	**Weken**	_____
C13.	Gebruikt u momenteel anticonceptie?	**1**	**Nee**
		2	**Ja**

C13a. Welke methode?

1 **Combinatie pil**
2 **Progesteron alleen pil**
3 **Hormoon pleisters**
4 **Nuvaring**
5 **Prik pil**
6 **IUD (hormoon/koper)**
7 **Sterilisatie**
8 **Anders, specificeer**

C14.	Bent u in de menopauze?	**1** **2**	**Nee** **Ja**
C14a.	Hoe oud was u toen uw menstruatie stopte of onregelmatig werd?	**Leeftijd**	_____
C14b.	Heeft u tot aan de menopauze de pil geslikt?	**1** **2**	**Nee** **Ja**
C14c	Bent u nadat u stopte met de pil niet meer ongesteld geworden?	**1** **2**	**Nee** **Ja**
C15.	Heeft u problemen met de menopauze?	**1** **2**	**Nee** **Ja**
C15a.	Waren deze problemen?	**1** **2** **3** **4**	**Zeer ernstig** **Ernstig** **Gemiddeld** **Licht**
C16.	Is uw moeder in de menopauze?	**1** **2** **3**	**Nee** **Ja** **Weet niet**
C16a.	Hoe oud was uw moeder toen haar menstruatie stopte of onregelmatig werd?	**Leeftijd**	_____
C16b.	Heeft uw moeder tot aan haar menopauze de pil geslikt?	**1** **2** **3**	**Nee** **Ja** **Weet niet**
C16c.	Is uw moeder nadat ze stopte met de pil niet meer ongesteld geworden?	**1** **2**	**Nee** **Ja**
C17.	Heeft uw moeder problemen gehad met de menopauze?	**1** **2** **3**	**Nee** **Ja** **Weet niet**
C18.	Waren deze problemen	**1** **2** **3** **4**	**Zeer ernstig** **Ernstig** **Gemiddeld** **Licht**

C19.	Heeft u uw baarmoeder weg laten halen? (hysterectomie)	**1**	**Nee**
		2	**Ja**
C19a.	Hoe oud was u toen u uw baarmoeder weg liet halen?	**Leeftijd**	_____
C19b.	Wanneer had u de operatie? Was dit	**1**	**Voor de menopauze**
		2	**Tijdens menopauze**
		3	**Na de menopauze**
C19c.	Zijn beide eierstokken verwijderd?	**1**	**Nee**
		2	**Ja**
C20.	Is er bij ooit een van de volgende soorten kanker vastgesteld?	**1**	**Eierstok kanker**
		2	**Baarmoederhals kanker**
		3	**Borst kanker**
		4	**Anders, specificeer**

_____

		Nee	**Ja**
C21.	Heeft u ooit …… gehad?	**1**	**2**
C21.1.	Endometriose	**1**	**2**
C21.2.	Bindweefselvorming in de baarmoeder	**1**	**2**
C21.3.	Zwangerschapsvergiftiging	**1**	**2**
C21.4.	Keizersnede	**1**	**2**

C.22	Heeft u ooit aan een van de volgende gezondheidsproblemen geleden?	**Nee**	**Ja**
C.22.1.	Hoge bloeddruk	**1**	**2**
C.22.2.	Hart en vaatziekten	**1**	**2**
C.22.3.	Hoog cholesterol	**1**	**2**
C.22.4.	Diabetes type 1	**1**	**2**
C.22.5.	Diabetes type 2	**1**	**2**
C.22.6.	Bot breuk	**1**	**2**
C.22.7.	Osteoporose	**1**	**2**
C.22.8.	Artritis	**1**	**2**
C.22.9.	Hepatitis	**1**	**2**
C.22.10.	Angina	**1**	**2**
C.22.11.	Astma of Bronchitis	**1**	**2**
C.22.12.	Eczeem	**1**	**2**
C.22.13.	Allergie of Hooikoorts	**1**	**2**
C.22.14.	PCO (polycysteus ovarium syndroom)	**1**	**2**
C.22.15.	Migraine	**1**	**2**

		Nee	Ja
C.22.16.	Schildklierafwijking	**1**	**2**
C.22.17.	Ondergewicht (anorexia nervosa)	**1**	**2**
C.22.18.	Overgewicht (obesitas)	**1**	**2**
C.22.19.	Anders, specificeer	**1**	**2**

D. FAMILIE STRUCTUUR

Nu zou ik u wat vragen willen stellen over uw familie

D1.	Leeft uw moeder nog?	**1** **2**	**Nee** **Ja**
D1a.	Hoe oud is uw moeder nu?	**Leeftijd**	_____
D1b.	Wat is haar geboortedatum?	**Datum**	_____
D2.	Leeft uw vader nog?	**1** **2**	**Nee** **Ja**
D2a.	Hoe oud is uw vader nu?	**Leeftijd**	_____
D2b.	Wat is zijn geboortedatum?	**Datum**	_____
D3.	Hoeveel broers heeft u?	**Aantal**	_____
D4.	Hoe veel zusters heeft u?	**Aantal**	_____
D5.	Is de stamboom getekend? (los vel)	**1** **2**	**Nee** **Ja**
D6.	Is de stamboom van de vader van de tweeling getekend? (los vel)?	**1** **2**	**Nee** **Ja**

D7.	We willen graag weten of in uw familie vrouwelijke familieleden zijn die problemen hebben gehad met onvruchtbaarheid. Hebt u vrouwelijke familieleden met vruchtbaarheidsproblemen?	**1** **2**	**Nee** **Ja**

D7a.	Om welke familieleden gaat het?	**Nee**	**Ja**
	Zus	**1**	**2**
	Dochter	**1**	**2**
	Moeders zus	**1**	**2**
	Vaders zus	**1**	**2**
	Anders, specificeer _____	**1**	**2**

E. VOORGESLACHT

Tot slot zou ik u graag wat vragen willen stellen over waar uw ouders en grootouders vandaan komen.

E1. In welk land is uw moeder geboren?

Record _____

E1.a. In welk land is uw moeders moeder geboren?

Record _____

E1.b. In welk land is uw moeders vader geboren?

Record _____

E2. In welk land is uw vader geboren?

Record _____

E2.a. In welk land is uw vaders moeder geboren?

Record _____

E2.b. In welk land is uw vaders vader geboren?

Record _____

We willen ook graag nog iets weten over de afkomst van uw grootouders. Ik zal een voorbeeld geven., uw vader is geboren in Nederland, maar zijn vader is bijvoorbeeld Duits of Surinaams.

E3. Wat is de afkomst van uw moeders moeder?

Record _____

E3.a. Wat is de afkomst van uw moeders vader?

Record _____

E4. Wat is de afkomst van uw vaders moeder?

Record _____

E4.a. Wat is de afkomst van uw vaders vader?

Record _____

END THE INTERVIEW BY THANKING THE RESPONDENT

Telephonic interview: descriptive results

In this appendix I describe the collection and the descriptive results of the interview data collected for the study "Genetics of dizygotic twinning". The study appertains to the Dutch part of a large-scale sample collection in Dutch and Australian families in which two or more sisters have given birth to spontaneous dizygotic (DZ) twins. The goal of this project is to collect biological samples and interview data from 1000 sister-pairs who both have given birth to spontaneous dizygotic (DZ) twins. The parents of these sister-pairs, and sometimes their siblings, are also asked for biological samples, but were not interviewed.

In the Netherlands, all mothers of twins participating in this study are interviewed by phone. In the 20-30 minutes during interview we collected data on offspring zygosity, pre-pregnancy health, previous pregnancies, and health of the mother, familial twinning and ancestry.

For this project 1,175 mothers of twins were selected from the Netherlands Twin Register. For this thesis I recruited 368 families, these families consist of mothers of twins together with their sister and/or other additional female family members with twins and their parents (total number of participants N=1,393).

Only the mothers of twins were interviewed. In total 368 probands and 339 sisters, 37 mothers, 33 aunts, 27 nieces and 4 daughters of the probands took part. There were 9 mothers of twins who did not want to be interviewed. In addition, after the data collection for this thesis stopped, the data collection of an additional 206 mothers of twins (indicated as "pending" in chapter 2, figure 2) was still ongoing. At this point in time an additional 104 interviews (49 probands and 47 sisters, 4 mothers, 2 aunts and 2 nieces of the probands) have been completed from 49 newly recruited families. In this appendix I summarize the descriptive results of my own completed interviews (N=808) and the additional completed interviews (N=104) together (N=912).

Description of samples

Selection of probands

Selection of participants was done in two waves, the first wave started in December 2003 by approaching mothers of DZ twins, who in previous survey studies of the NTR indicated that they have a sister with twins. In these studies mothers of twins with the NTR were asked to report on other women with twins in their family. In total 517 mothers were selected by using previous NTR survey information. Of these mothers, 188 mothers and their families agreed to participate in the study "Genetics of dizygotic twinning" and gave informed consent for biological sample collection and the telephonic interview. However 272 approached mothers did not want to participate or were excluded (see page 22).

The second wave of data collection started in July 2005. Mothers were selected on the basis of their answers in a questionnaire which was send to all mothers registered in the NTR in April 2005 (chapter 5 and 6). In this questionnaire mothers were asked whether they had a sister, a mother or/and an aunt with multiples. We selected all probands who replied that she had a sister with twins and all probands who said her mother and the sister of her mother (aunt) had twins. In addition to the 517 mothers selected from previous survey studies, 658 mothers were selected by the questionnaire. Of these mothers, 231 mothers and their families participated, 278 did not or were excluded (see page 22).

As shown in chapter 2, figure 2, there were 206 eligible, additional, mothers ('pending'). Of these mothers there were 149 mothers who were recruited from the questionnaire study in a second step and there were 57 mothers who were recruited through other ongoing NTR projects. Of these later recruited mothers 49 agreed to participate.

In most families only one of the sisters with twins (the proband) was registered with the NTR, the sister of the proband was registered with the NTR after she agreed in participation. However, in 51 families both sisters were already registered with the NTR. In most cases we were not able to identify families with both sisters

registered before we actually spoke to the mothers and asked for pedigree information. This was because mothers register independently of each other and mostly use their husband's family name. Both sisters were therefore initially approached as probands. In those 51 families we decided to refer to the first approached mother as the proband and to the second approached mother as the sister.

Recruitment of families

After the selection of probands was made we approached approximately 50 probands per time based on postal code. The proband was send a letter and information brochure to invite her and her family for the study "Genetics of dizygotic twinning". This was followed by a phone call to obtain pedigree information and permission to approach her sister, her parents and/or other siblings. At this point we made a first selection. Although the probands were selected on the criterion that these mothers have one or more sisters with twins, in some cases information obtained from these mothers were not correct or incomplete. These mothers were themselves twins and had a twin sister instead of a sister with twins, or the mothers did not have a sister but only a brother with twins. In those cases, when no other family members with twins were present, the proband was excluded. Also, if the proband was sure the zygosity of her own twins or of the twins of her sister was monozygotic (MZ) the proband was excluded (N=152). Another reason for exclusion was if the proband did not have contact with her sister (N=29) or the proband or her sister was deceased (N=20). Finally, 11 probands were excluded because they or their sister did not speak Dutch or lived abroad. In addition, 275 approached probands did not want to participate, 180 probands had no interest or time for the project and of 95 probands the sister had no interest or time. Finally, of 63 probands the current address was unknown, and the invitation letters were returned to us.

All family members were initially informed about the study by the proband mother. After giving their permission to be contacted, they also received a letter and information brochure about the study. If one or both parents were unavailable or deceased, other siblings were asked for their participation. The additional recruitment of siblings is important because in this way it is still possible to examine the parental genotypes if one or both parents are deceased. This is important for future linkage and association studies.

When a family agreed to participate all members received a confirmation letter with an informed consent form. After informed consent forms were returned to us, we made an appointment for the telephone interview and the blood collection.

Results (descriptive interview data)

In this part I will describe the descriptive results from the 912 completed interviews. There were 417 probands, 386 sisters, 41 mothers, 35 aunts, 29 nieces and 4 daughters of the probands.

PART A

Part A consisted of 19 items regarding birth weight of the mother, the number of children the mother had and the mother's judgment of zygosity of the twins. The mothers were only asked to judge the zygosity of their twins if they were of the same sex. Information was obtained on similarity of the children and experiences of mistaking one twin for the other (eight items). The last three questions in part A asked about fertility treatments in any pregnancy. Age at time of the interview, at first pregnancy and at twin pregnancy was obtained by subtracting respectively the interview date, the birth date of first child and the birth date of the twins from the birth date of the mother.

The mean maternal age at time of the interview was 45.9 years old (SD 10.6). The oldest interviewed mother of twins was 83 years and the youngest mother was 23 years. There were 828 mothers with one twin pair

and 20 mothers with 2 twin pairs. There were 17 mothers with triplets and 1 mother with a twin pair and triplets. In addition, there were 46 mothers who had had a miscarriage (28) or stillbirth (18) of one of the twins or both twins. The zygosity of twin offspring of mothers who had had a miscarriage or stillborn twins was uncertain or unknown. Zygosity of the twin offspring of mothers with live-born twins are listed in table 1 for the first twin pair (N=866) and in table 2 for the second twin pair (N=20).

Table 1 Zygosity of the first live-born twin pair according to the mother.

Zygosity	N	%
Identical girls	83	9.6
Non-identical girls	216	24.9
Identical boys	55	6.4
Non-Identical boys	190	21.9
Opposite sex	322	37.2
Total	866	100.0

Table 2 Zygosity of the second live born twin pair according to the mother.

Zygosity	N	%
Identical girls	2	0.2
Non-identical girls	6	0.7
Identical boys	1	0.1
Non-identical boys	9	1.0
Opposite sex	2	0.2
Not applicable, no second twin	892	97.8
Total	912	100.0

In total 189 mothers used artificial reproductive treatments in any of their pregnancies. Of those, 126 mothers used only ovulation induction (OI) medication, 58 mothers used in vitro fertilization (IVF) and 5 mothers used alternative medication like, herbal tea.

Table 3 summarizes maternal characteristics with respect to birth weight of the interviewed mother.

Table 3 Birth weight (grams) of the mother of twins.

	N	Missing	Minimum	Maximum	Mean	SD
Birth weight of mothers of twins	703	209	1,250	6,000	3,286	632,8

Tables 4 and 5 show the reported number of pregnancies and twin pregnancies, including miscarriages, for the interviewed mothers. In total there were 2,529 pregnancies.

Table 4 Reported number of pregnancies (including miscarriage).

Number of pregnancies	N	%
First	153	16.8
Second	338	37.1
Third	212	23.2
Fourth	102	11.2
Fifth	53	5.8
Sixth or more	54	5.9
total	912	100.0

Table 5 Reported number of twin pregnancies (including miscarriage).

Number of twin pregnancies	N	%
First	870	95.4
Second	41	4.5
Third	1	0.1
Total	912	100.0

PART B

In part B we asked information on every separate pregnancy the mother had, including miscarriages in 11 items.

Of all interviewed mothers 213 have had one or more miscarriages (singleton or multiple) and 699 mothers never had a miscarriage. Table 6 shows in which pregnancy the miscarriage occurred (e.g. in 111 mothers the miscarriage was in the first pregnancy). The total number of miscarriages is more than 213 because some mothers had more than one miscarriage. The percent of miscarriages is 295/ 2,529 = 11.7 %.

Table 6 Information on in which pregnancy a miscarriage occurred.

Number of pregnancy	N	%	Cumulative %
First	111	37.6	37.6
Second	79	26.8	64.4
Third	53	17.9	82.3
Fourth	34	11.5	93.8
Fifth	14	4.7	98.5
Sixth or higher	4	1.4	100.0
Total	295	100.0	

In 315 mothers the pregnancy of the twins or triplet was their first pregnancy. In 324 mothers it was their second pregnancy and in 273 mothers the multiple pregnancy was their third or higher. Table 7 shows the number of weeks it took to become pregnant with the twins.

Table 7 Time to become pregnant with the twins.

Time to become pregnant	N	Minimum	Maximum	Mean	SD
Number of weeks	729	0	144	9.1	17.2
Missing	183				
Total	912				

The length of all pregnancies, including twin pregnancies, and specific for the live-born twin pregnancies are listed in table 8. The mode of delivery of the live born twins is listed in table 9.

Table 8 Length of each pregnancy of live born children.

Pregnancy	N	Minimum	Maximum	Mean	SD
First	783	24	43	38.3	3.1
Second	657	21	44	38.3	2.8
Third	358	29	43	38.7	2.2
Fourth	171	27	43	38.7	2.2
Fifth	88	20	43	38.8	2.6
Sixth	45	32	46	39.5	2.2
Twins	852	24	44	36.8	2.8

Table 9 Mode of delivery of the live born twins.

Mode of delivery	N	%	Cumulative %
Natural	677	78.2	78.2
Caesarian	175	20.2	98.4
Missing	14	1.6	100.0
Total	866		

PART C

Part C consists of 19 items regarding health and medical history. Data were collected on height, weight (before, during and after the twin pregnancy), menstrual cycle, menopause (of proband and her mother), smoking before the twin pregnancy, use of folic acid before and during the twin pregnancy, use of contraception and health problems.

Table 10 summarizes maternal characteristics of the mothers of twins. BMI at time of the interview was calculated as BMI=weight $_{(kg)}$ / height^{2} $_{(m)}$.

Table 10 Height (cm), weight (kg) and BMI (kg/m^2) of mothers of twins.

Maternal characteristics	N	Missing	Minimum	Maximum	Mean	SD
Body mass index	905	7	15.6	60.1	24.9	4.2
Height	910	2	148	189	169.8	4.2
Weight at time of the interview	906	6	44	180	71.9	12.7
Maximal weight (excluding pregnancy)	903	9	45	180	76.3	14.3
Maximal weight (including pregnancy)	780	132	50	180	82.8	13.5
Minimal weight (without illness)	881	31	37	95	59.6	7.8

Table 11 through 14 show data obtained regarding the menstrual cycle. Mothers were explicitly asked about their menstrual cycle before their first pregnancy.

Table 11 Age in years at menarche of mothers of twins.

Menarche	N	Minimum	Maximum	Mean	SD
Age at menarche	896	8	18	13.2	1.5
Missing	16				

Table 12 Length of period for natural cycles before first pregnancy.

Length of menstruation	N	Minimum	Maximum	Mean	SD
Average days menstruation	883	2	15	5.3	1.4
Missing	29				

Table 13 Days of menstrual cycle before first pregnancy.

Days of cycle	N	Minimum	Maximum	Mean	SD
Days of menstrual cycle	741	18	35	27.4	2.2
Irregular cycle	142				
Missing	29				

Table 14 shows the answers of mother regarding the type of menstrual period. The mothers were asked to indicate what description fit best. For example 284 mothers said that "my period is very heavy" is the best description for their type of period.

Table 14 Type of menstrual period before first pregnancy.

Type of menstrual period	%	N
Heavy	31.1	284
Normal	50.4	460
Light	17.7	161
Don't know	0.4	4
Missing	0.4	3
Total	100.0	912
Type of menstrual period	%	N
Very painful	29.6	270
Normal	29.9	273
No pain	39.8	363
Don't know	0.2	2
Missing	0.4	4
Total	100.0	912
Type of menstrual period	%	N
Very restrictive	22.0	201
Normal	22.3	203
Not at all	55.2	503
Don't know	0.1	1
Missing	0.4	4
Total	100.0	912

The mothers were also asked if they already reached menopause. If so, they were asked if they reached menopause naturally and whether they used oral contraceptives, if yes they were asked when they stopped using the pill they also stopped menstruating (see Table 15).

Table 15 Age (in years) at menopause of the interviewed mother.

Menopause	N	Minimum	Maximum	Mean	SD
No (age at time of interview)	543	23	52	39.7	5.0
Yes (age of natural menopause)	278	29	58	46.2	5.0
Hysterectomy before menopause	59				
Yes, pill use	25				
Yes, but age is missing	7				

The interviewed mother was also asked whether her own mother reached menopause and if so at what age. Many of interviewed mothers did not know their mothers age (don't know/unknown) because they never discussed this or because their mother died before she reached menopause (don't know/unknown). In a few cases the mother of the interviewed mother did not reach menopause yet (N=7). See table 16.

Table 16 Age of menopause of the mother of the interviewed mother.

Menopause mother of mother	N	Minimum	Maximum	Mean	SD
No	7				
Yes (age of natural menopause)	568	29	60	48.9	5.1
Yes, pill use	26				
Don't know/unknown	138				
Yes, but age is missing	173				

Mothers were asked if they smoked before their first twin pregnancy. Not all mothers were asked this question because questions about smoking were implemented in a later version of the interview, when some mothers (N=275) already completed the interview (see table 17).

Table 17 Cigarette smoking before the twin pregnancy.

Smoking	Number of cigarettes per day before the twin pregnancy					Missing number of cigarettes
	N	Mean	SD	Min	Max	
Never smoked	382					0
Yes, including first weeks of pregnancy	71	8.7	5.4	1	25	6
Yes, before pregnancy	84	10.5	7.8	1	40	8
Before and during pregnancy	96	13.7	6.9	1	40	4
Don't know	3					0
Total	637					18
Missing	1					
Not asked	275					

Mothers were also asked if they used oral contraceptives before the twin pregnancy and how many weeks before they got pregnant they stopped taking them. Table 18 gives an overview of the mothers that did or did not use oral contraceptives and how many weeks they stopped taking the pill before they became pregnant.

Table 18 Use of oral contraceptives before the twin pregnancy.

Oral contraceptives	Number of weeks stopped taking OC before the twin pregnancy				
	N	< 4 weeks	4-12 weeks	12-24 weeks	> 24 weeks
No	374				
Yes	388	68	117	61	142
Yes, but missing number of weeks	28				
Missing	122				
Total	912				

Mothers were also asked whether they used folic acid before the twin pregnancy and if so for how many weeks. The mother could answer that she used folic acid before, during or before and during the twin pregnancy. See table 19.

Table 19 Use of folic acid before the twin pregnancy.

Folic acid	Number of weeks used				
	N	< 4 weeks	4-12 weeks	12-24 weeks	> 24 weeks
No	459				
Before the pregnancy	6	1	1	2	2
Only during	126	4	46	34	42
Before and during	141	8	31	26	76
Yes, but missing number of weeks	56				
Missing	124				
Total	912				

Tables 20 to 23 summarize all questions that were asked about the mother's medical history. The mothers were asked whether they were ever diagnosed with these specific diseases.

Table 20 Ever diagnosed with cancer.

Diagnosed with	Yes	No	Missing
Ovary cancer	3	907	2
Cervix cancer	15	896	1
Breast cancer	16	895	1

Table 21 Ever diagnosed with pre-eclampsia.

Pre-eclampsia	N	%
No	827	90.7
Yes	85	9.3
Missing	0	0.0
Total	912	100.0

Table 22 Ever diagnosed with endometriosis/uterine fibroid ever.

Endometriosis	%	N
No	94.1	858
Yes	5.6	51
Missing	0.3	3
Total	100.0	912
Uterine Fibroid	%	N
No	91.5	834
Yes	8.3	76
Missing	0.2	2
Total	100.0	912

Mothers were asked if experienced a number of health problems ever in life. Not all mothers were asked all questions because some questions for example cardiovascular disease and polycystic ovary syndrome (PCOS) were implemented in a later version of the interview, when some mothers (N=100) already completed the interview. Questions about migraine, pituitary pathology and hepatitis were implemented when 34 mothers already completed the interview. (see table 23).

Table 23 Health problems experienced ever in life.

Health problems ever in life	Yes	No	Missing	Not asked*	Total
High blood pressure	281	630	1	0	912
Cardiovascular disease	48	748	16	100	912
High cholesterol	73	837	2	0	912
Diabetes	20	888	4	0	912
Bone fracture	228	682	2	0	912
Osteoporosis	43	868	1	0	912
Arthritis	106	804	2	0	912
Hepatitis	32	845	1	34	912
Angina	389	521	2	0	912
Asthma or bronchitis	167	740	5	0	912
Eczema	242	669	1	0	912
Allergies	261	546	5	100	912
PCOS	14	777	21	100	912
Migraine	251	615	12	34	912
Pituitary pathology	76	799	3	34	912
Underweight	17	776	19	100	912
Overweight	106	691	15	100	912

* question added later

PART D

Part D asked about family history of twinning and fertility. Table 24 summarizes the maternal and paternal age of the parents of the mothers of twins.

Table 24 Age of the mother and father of the mother of twins when she herself was born.

Age of parents	N	Minimum	Maximum	Mean	SD
Maternal age mothers mother	875	16	45	29.4	5.7
Missing maternal age	37				
Paternal age mothers father	846	15	54	32.1	6.4
Missing paternal age	66				

The total number of siblings, brothers and sisters are summarized in table 25.

Table 25 Number of brothers and sisters, and total number of siblings of interviewed mothers.

Siblings	0	1	2	3	4	5	6 >	Missing
Brothers	203	271	181	115	63	34	37	8
Sisters	12	279	239	173	88	58	62	1
Total sibs	1	83	193	161	111	98	257	8

Finally, part D asked about fertility problems in female biological relatives. Fertility problems indicated as "Other, specify" are usually cousins of the interviewed mothers. Table 26 indicates which female family members were reported to have fertility problems.

Table 26 Fertility problems of female family members of mothers of twins.

Family member	Yes	No	Not asked*	Total
Daughter	16	753	143	912
Sister	158	615	139	912
Aunt on mothers side	36	733	143	912
Aunt on fathers side	20	750	142	912
Other, specify	111	660	141	912

* question added later

PART E

Part E contained 2 items about the birth country of the parents of the interviewed mothers and 2 items about the ancestry of their grandparents. Table 27 shows the birth countries of mother's side of the interviewed mother and table 28 shows the birth countries of father's side. Table 29 shows the ancestry on mother's and father's sides of the interviewed mother.

Table 27 Birth country of the mother of the interviewed mother and her parents.

Country	Mother	Grandmother	Grandfather
Netherlands	875	859	878
Belgium	2	7	3
Surinam or Dutch Antilles	6	7	6
Turkey	2	2	2
Indonesia	12	8	6
Western Europe (other)	8	23	9
Eastern Europe	5	5	5
USA	2	0	2
Asia (other)	0	1	0
Total	912	912	912
Asked not answered	0	0	1

Table 28 Birth country of the father of the interviewed mother and his parents.

Country	Father	Grandmother	Grandfather
Netherlands	876	885	871
Belgium	4	2	4
Surinam or Dutch Antilles	8	8	8
Turkey	3	2	2
Indonesia	12	4	12
Western Europe (other)	6	8	12
Eastern Europe	2	2	2
USA	0	0	0
Asia (other)	1	0	0
Total	912	912	912
Asked not answered	0	1	1

Table 29 Ancestry of the grandparents of the mother of twins on mothers and fathers side.

	Mothers side		Fathers side	
Country	Grandmother	Grandfather	Grandmother	Grandfather
Netherlands	824	844	834	830
Belgium	11	7	10	11
Surinam or Dutch Antilles	4	4	6	4
Turkey	2	2	13	2
Indonesia	10	5	2	4
Western Europe (Other)	36	27	30	40
Eastern Europe	7	7	3	5
Asia (Other)	2	1	1	3
South Africa	0	0	1	0
USA	0	1	0	0
Russia	1	0	0	1
Total	897	899	900	900
Missing	15	13	12	12

Appendix II

Survey study

Questionnaire for mothers of multiples

This questionnaire is completed by: ☐1 biological mother of the multiple ☐2 adoption-/foster-/ mother of the multiple

Date of birth: ___-____-___ Fill in date: _____-____-____

Date of birth of the multiple: ___-____-___ They are: ☐1 identical ☐2 fraternal ☐3 triplets ☐4 unknown

Date of birth possible 2nd multiple ___-____-___

Are you a part of a multiple yourself?

☐1 no ☐2 yes MZ ☐3 yes DZ ☐4 yes triplet ☐5 yes, unknown zygosity

Is the father of the multiple a part of a multiple?

☐1 no ☐2 yes MZ ☐3 yes DZ ☐4 yes triplet ☐5 yes, unknown zygosity

1 Which of your own biological family members are also parents of a multiple? More answers are possible. If one of the beneath listed family members have multiples, please indicate if they are girls (FF), boys (MM) or a girl and boy (FM), by circling FF, MM or FM. If you don't know the sex of the multiple than circle? Please also indicate zygosity by circling fraternal, identical or? If you don't know. **TN**. If there are multiples in the family of the father of the twins or if you have other remarks, please specify this by remarks.

I don't have relatives with multiples	☐1							
My daughter(s) has/have multiples	☐2	FF	MM	FM	?	identical	fraternal	?
My son(s) has/have multiples	☐3	FF	MM	FM	?	identical	fraternal	?
My sister(s) has/have multiples	☐4	FF	MM	FM	?	identical	fraternal	?
My brothers has/have multiples	☐5	FF	MM	FM	?	identical	fraternal	?
My mother has multiples	☐6	FF	MM	FM	?	identical	fraternal	?
The sister(s) of my mother has/have multiples	☐7	FF	MM	FM	?	identical	fraternal	?
The brother(s) of my mother has/have multiples	☐8	FF	MM	FM	?	identical	fraternal	?
The parents of my mother has/have multiples	☐9	FF	MM	FM	?	identical	fraternal	?
The sister(s) of my father has/have multiples	☐10	FF	MM	FM	?	identical	fraternal	?
The brother(s) of my father has/have multiples	☐11	FF	MM	FM	?	identical	fraternal	?
The parents of my father has/have multiples	☐12	FF	MM	FM	?	identical	fraternal	?

Remarks:

2 How many brothers and sisters do you have?

Sister(s) with the same father and mother as I have	0☐	1☐	2☐	3☐	4☐	5☐	6☐	7☐	8☐ or more
Sister(s) with the same father, but not the same mother	0☐	1☐	2☐	3☐	4☐	5☐	6☐	7☐	8☐ or more
Sister(s) with the same mother, but not the same father	0☐	1☐	2☐	3☐	4☐	5☐	6☐	7☐	8☐ or more
Brother(s) with the same father and mother as I have	0☐	1☐	2☐	3☐	4☐	5☐	6☐	7☐	8☐ or more
Brother(s) with the same father, but not the same mother	0☐	1☐	2☐	3☐	4☐	5☐	6☐	7☐	8☐ or more
Brother(s) with the same mother, but not the same father	0☐	1☐	2☐	3☐	4☐	5☐	6☐	7☐	8☐ or more
Non biological brother(s) and sister(s)	0☐	1☐	2☐	3☐	4☐	5☐	6☐	7☐	8☐ or more

3 How old were you when you had your first menstrual period?

☐1 younger than 11 years
☐2 11 years
☐3 12 years
☐4 13 years
☐5 14 years
☐6 15 years or older

4 In which category fits your birth weight?

☐1 Less than 2000 gram
☐2 2000 - 2500 gram
☐3 2501 - 3000 gram
☐4 3001 - 3500 gram
☐5 3501 - 4000 gram
☐6 More than 4000 gram
☐7 Don't know

5 In which category fits the birth weight of the father of the twins?

☐1 Less than 2000 gram
☐2 2000 - 2500 gram
☐3 2501 - 3000 gram
☐4 3001 - 3500 gram
☐5 3501 - 4000 gram
☐6 More than 4000 gram
☐7 Don't know

6 Concerning the time of birth

☐1 I was born too early, ...weeks
☐2 I was born in time
☐3 I was born too late, ...weeks
☐4 Don't know

7 Concerning the time of birth of the father of the twins?

☐1 He was born too early, ...weeks
☐2 He was born in time
☐3 He was born too late, ...weeks
☐4 Don't know

8 How old were you when your first child was born? _____ **years old**

9	**How many pregnancies did you have?**	0☐	1☐	2☐	3☐	4☐	5☐	6☐	7☐	8☐ or more
10	**How many miscarriages did you have?**	0☐	1☐	2☐	3☐	4☐	5☐	6☐	7☐	8☐ or more
11	**How many twins did you have?**	0☐	1☐	2☐	3☐	4☐	5☐	6☐	7☐	8☐ or more
12	**How many triplets did you have?**	0☐	1☐	2☐	3☐	4☐	5☐	6☐	7☐	8☐ or more
13	**How many singletons did you have?**	0☐	1☐	2☐	3☐	4☐	5☐	6☐	7☐	8☐ or more
14	**How many pregnancies did you have (incl. miscarriage) before the twin pregnancy?**	0☐	1☐	2☐	3☐	4☐	5☐	6☐	7☐	8☐ or more

15 How did you get pregnant of your first twin pregnancy?

☐1 It was a natural pregnancy, and I became pregnant in
  ☐1a 0-2 months ☐1b 3-5 months
  ☐1c 6-12 months ☐1d more than 12 months
☐2 IVF (in vitro fertilization)
☐3 ICSI (intro cytoplasmatic sperm injection)
☐4 IUI (intra-uterine insemination)
☐5 ovulation induction
☐6 other, _____

16 How much did you weigh before your first twin pregnancy? _____kg
17 How much did you weigh at the end of that pregnancy? _____kg
18 How much do you currently weigh? _____kg
19 How tall are you? _____cm

20 Did you use oral contraceptives prior to your first twin pregnancy?

☐1 No
☐2 Yes → ☐2a I stopped taking OC less then 2 months prior to the pregnancy
  ☐2b I stopped taking OC between 2 – 6 months before the pregnancy
  ☐2c I stopped taking OC for longer than 6 months before the pregnancy
  ☐2d I don't know how long I stopped before I became pregnant

21 Did you used folic acid before/during your first twin pregnancy?

☐1 No
☐2 Yes, but only before the pregnancy
☐3 Yes, but only during the pregnancy
☐4 Yes, before and during the pregnancy
☐5 I don't know

22. Were you a smoker before/ during your first twin pregnancy?

☐1 no
☐2 Yes, but only before the pregnancy
☐3 Yes, but only during the pregnancy
☐4 Yes, before and during the pregnancy
☐5 I don't know

Many thanks for your cooperation!

Vragenlijst voor moeders van meerlingen

Dit formulier is ingevuld door: ☐1 biologische moeder van de meerling ☐2 adoptie-/pleeg-/stiefmoeder van de meerling

Geboortedatum invuller ___-____-___ Datum van invullen: _____-____-____

Geboortedatum van de meerling: ___-____-___ Ze zijn: ☐1 eeneiig ☐2 twee-eiig ☐3 drie-eiig ☐4 weet niet

Geboorte datum evt 2de meerling ___-____-___

Bent u zelf deel van een meerling?

☐1 nee ☐2 ja, eeneiig ☐3 ja, twee-eiig ☐4 ja, drieling ☐5 ja, weet zygositeit niet

Is de vader van de meerling deel van een meerling?

☐1 nee ☐2 ja, eeneiig ☐3 ja, twee-eiig ☐4 ja, drieling ☐5 ja, weet zygositeit niet

1 Welke van uw eigen biologische familieleden zijn ouders van een tweeling of meerling? U kunt meerdere hokjes aankruisen. Als een van uw familieleden een tweeling heeft, geef dan aan of het twee meisjes (MM), twee jongens (JJ) of een jongen en meisje (JM) zijn door MM, JJ, of JM te omcirkelen. Indien u het geslacht van de tweeling niet weet, kunt u dit aangeven door ? te omcirkelen. Geef ook aan of het om een ééneiige of twee-eiige tweeling gaat door het juiste antwoord te omcirkelen. **NB**. Als er veel meerlingen voorkomen in de familie van de vader van uw meerling kunt u dat bij opmerkingen noteren. Eventuele andere aanvullingen kunt u daar ook kwijt (bijvoorbeeld over drielingen of meerdere tweelingen).

Er komen géén andere meerlingen voor in mijn familie	☐1							
Mijn dochter(s) heeft/hebben een tweeling	☐2	MM	JJ	JM	?	ééneiig	twee-eiig	?
Mijn zoon(s) heeft/hebben een tweeling	☐3	MM	JJ	JM	?	ééneiig	twee-eiig	?
Mijn zuster(s) heeft/hebben een tweeling	☐4	MM	JJ	JM	?	ééneiig	twee-eiig	?
Mijn broer(s) heeft/hebben een tweeling	☐5	MM	JJ	JM	?	ééneiig	twee-eiig	?
Mijn moeder heeft een tweeling	☐6	MM	JJ	JM	?	ééneiig	twee-eiig	?
De zuster(s) van mijn moeder heeft/hebben een tweeling	☐7	MM	JJ	JM	?	ééneiig	twee-eiig	?
De broer(s) van mijn moeder heeft/hebben een tweeling	☐8	MM	JJ	JM	?	ééneiig	twee-eiig	?
De ouders van mijn moeder hebben een tweeling	☐9	MM	JJ	JM	?	ééneiig	twee-eiig	?
De zuster(s) van mijn vader heeft/hebben een tweeling	☐10	MM	JJ	JM	?	ééneiig	twee-eiig	?
De broer(s) van mijn vader heeft/hebben een tweeling	☐11	MM	JJ	JM	?	ééneiig	twee-eiig	?
De ouders van mijn vader hebben een tweeling	☐12	MM	JJ	JM	?	ééneiig	twee-eiig	?

Ruimte voor opmerkingen:

2 Hoeveel broers en zussen heeft u zelf? (wilt u op ieder regel een antwoord aankruisen?)

zus(sen) met dezelfde vader en moeder als ik	0☐	1☐	2☐	3☐	4☐	5☐	6☐	7☐	8☐ of meer
zus(sen) met dezelfde vader, maar niet dezelfde moeder	0☐	1☐	2☐	3☐	4☐	5☐	6☐	7☐	8☐ of meer
zus(sen) met dezelfde moeder, maar niet dezelfde vader	0☐	1☐	2☐	3☐	4☐	5☐	6☐	7☐	8☐ of meer
broer(s) met dezelfde vader en moeder als ik	0☐	1☐	2☐	3☐	4☐	5☐	6☐	7☐	8☐ of meer
broer(s) met dezelfde vader, maar niet dezelfde moeder	0☐	1☐	2☐	3☐	4☐	5☐	6☐	7☐	8☐ of meer
broer(s) met dezelfde moeder, maar niet dezelfde vader	0☐	1☐	2☐	3☐	4☐	5☐	6☐	7☐	8☐ of meer
niet biologisch verwante broer(s) / zus(sen)	0☐	1☐	2☐	3☐	4☐	5☐	6☐	7☐	8☐ of meer

3 Hoe oud was u toen u voor het eerst menstrueerde?

☐1 Jonger dan 11 jaar
☐2 11 jaar
☐3 12 jaar
☐4 13 jaar
☐5 14 jaar
☐6 15 jaar of ouder

4 In welke categorie valt uw geboortegewicht?

☐1 minder dan 2000 gram
☐2 2000 - 2500 gram
☐3 2501 - 3000 gram
☐4 3001 - 3500 gram
☐5 3501 - 4000 gram
☐6 meer dan 4000 gram
☐7 weet ik niet

5 En het geboortegewicht van de vader van uw meerling?

☐1 minder dan 2000 gram
☐2 2000 - 2500 gram
☐3 2501 - 3000 gram
☐4 3001 - 3500 gram
☐5 3501 - 4000 gram
☐6 meer dan 4000 gram
☐7 weet ik niet

6 Wat betreft het tijdstip van uw geboorte

☐1 ik ben te vroeg geboren, nl .....weken
☐2 ik ben op tijd geboren
☐3 ik ben te laat geboren, nl .....weken
☐4 weet ik niet

7 Het tijdstip van de geboorte van de vader

☐1 Hij is te vroeg geboren, nl .....weken
☐2 Hij is op tijd geboren
☐3 Hij is te laat geboren, nl .....weken
☐4 weet ik niet

8 Hoe oud was u toen u uw eerste kind/ kinderen kreeg? _____ **jaar**

9	**Hoe vaak bent u zwanger geweest?**	0☐	1☐	2☐	3☐	4☐	5☐	6☐	7☐	8☐ of meer
10	**Hoeveel miskramen heeft u gehad?**	0☐	1☐	2☐	3☐	4☐	5☐	6☐	7☐	8☐ of meer
11	**Hoeveel tweelingen heeft u gekregen?**	0☐	1☐	2☐	3☐	4☐	5☐	6☐	7☐	8☐ of meer
12	**Hoeveel drielingen heeft u gekregen?**	0☐	1☐	2☐	3☐	4☐	5☐	6☐	7☐	8☐ of meer
13	**Hoeveel eenlingen heeft u gekregen?**	0☐	1☐	2☐	3☐	4☐	5☐	6☐	7☐	8☐ of meer
14	**Hoe vaak was u zwanger (incl. miskramen) voordat u in verwachting raakte van uw eerst geboren meerling?**	0☐	1☐	2☐	3☐	4☐	5☐	6☐	7☐	8☐ of meer

15 Hoe is de zwangerschap van uw eerst geboren meerling tot stand gekomen?

☐1 Ik ben spontaan zwanger geworden na
  ☐1a 0-2 maanden ☐1b 3-5 maanden
  ☐1c 6-12 maanden ☐1d Meer dan 12 maanden
☐2 IVF (in vitro fertilisatie)
☐3 ICSI (intro cytoplasmatic sperma injectie)
☐4 IUI (intra-uterine inseminatie)
☐5 ovulatie inductie
☐6 anders, _____

16 Hoe zwaar was u voordat u in verwachting raakte van uw eerst geboren meerling? _____kg
17 Hoe zwaar was u aan het einde van deze zwangerschap? _____kg
18 Wat is uw huidige gewicht? _____kg
19 Hoe lang bent u? _____cm

20 Heeft u in de periode voorafgaande aan de zwangerschap van uw eerst geboren meerling de pil gebruikt?

☐1 Nee
☐2 Ja → ☐2a ik ben minder dan 2 maanden voor de zwangerschap gestopt
  ☐2b ik ben tussen de 2 en 6 maanden voor de zwangerschap gestopt
  ☐2c ik ben langer dan 6 maanden voor de zwangerschap gestopt
  ☐2d ik weet niet meer hoe lang voor de zwangerschap ik ben gestopt

21 Heeft u foliumzuur geslikt vóór en/of tijdens de zwangerschap van uw eerst geboren meerling?

☐1 nee
☐2 ja, alleen voordat ik zwanger werd
☐3 ja, alleen tijdens de zwangerschap
☐4 ja, voor en gedurende de zwangerschap
☐5 weet ik niet

22. Heeft u gerookt vóór en/of tijdens de zwangerschaps van uw eerst geboren meerling?

☐1 nee
☐2 ja, alleen voordat ik zwanger werd
☐3 ja, alleen tijdens de zwangerschap
☐4 ja, voor en gedurende de zwangerschap
☐5 weet ik niet

23 In Nederland worden registratiesystemen bijgehouden op het gebied van de volksgezondheid, zoals de Landelijke Vroedvrouwen Registratie. Geeft u het Nederlands Tweelingen Register toestemming om uw gegevens te vergelijken met de gegevens uit de registratiesystemen? Al uw gegevens worden te allen tijde strikt vertrouwelijk behandeld.

☐1 ik geef **wel** toestemming ☐2 ik geef **geen** toestemming

Hartelijk dank voor uw medewerking!

Appendix III

Invitation letter

Nederlands Tweelingen Register (NTR)

Datum	Uw brief van	Telefax	Bijlage(n)
datum postmerk		020-5988832	1

Ons kenmerk	Uw kenmerk	Telefoon	E-mail
NTR/DZGEN		020-5988731	c.hoekstra@psy.vu.nl

Postadres: Van der Boechorststraat 1, 1081 BT Amsterdam

vrije* Universiteit *amsterdam

Geachte mevrouw,

Het is u wellicht bekend dat tweelingen in sommige families vaker voorkomen dan in andere. In samenwerking met het Queensland Institute of Medical Research (een wetenschappelijk instituut in Australië), onderzoeken wij de overerving van het krijgen van tweelingen. Voor dit onderzoek vragen wij moeders van een tweeling die een zus hebben met ook een tweeling om bloed af te staan. Ook vragen wij de zus en de ouders van deze moeders hun bloed af te staan. Hieruit wordt erfelijk materiaal, hormoonwaardes en het cholesterol gehalte verkregen voor het onderzoek. Wij brengen u van de uitslag van uw cholesterol gehalte op de hoogte. Mochten wij in de andere bepalingen afwijkende waarden vinden dan zullen wij u hier over informeren. Deze eenmalige bloedafname kan door ons bij u thuis of op uw werk gebeuren. Voor dit onderzoek is het van belang om te weten of de tweelingen twee-eiig of eeneiig zijn. Mocht dat niet zeker zijn, dan kan dit door middel een DNA test gebaseerd op een monduitstrijkje bepaald worden. Daarnaast vragen we aan de moeders van een tweeling om mee te werken aan een telefonisch interview.

U hebt in het verleden in een vragenlijst opgegeven dat u een zus met een tweeling heeft. Onze vraag is dan ook of u en uw zus en uw ouders bereid zijn om aan dit onderzoek deel te nemen. Als uw ouders niet aan dit onderzoek mee kunnen werken is het wenselijk dat, zover dit mogelijk is, broers of zussen aan het onderzoek deelnemen. We willen u verzoeken uw zus en uw ouders van het onderzoek op de hoogte te stellen en te vragen of zij aan het onderzoek willen deelnemen.

In de loop van de volgende weken kunt u een van onze medewerkers aan de telefoon verwachten, dit om uw vragen naar aanleiding van deze brief te beantwoorden en eventueel het een en ander af te spreken. Uw deelname is uiteraard op vrijwillige basis en uw gegevens worden vertrouwelijk behandeld. U kunt op elk moment in het onderzoek zonder opgaaf van reden, besluiten om met het onderzoek te stoppen. Mocht u er prijs op stellen met een onafhankelijk arts over dit onderzoek te praten, dan kunt u kontact opnemen met Dr. C. B. Lambalk, Afdeling Voortplantingsgeneeskunde VUmc secretariaat 020-4440070.

De kennis die met dit onderzoek wordt verworven verschaft een beter begrip van de vrouwelijke vruchtbaarheid en onvruchtbaarheid. Uw deelname wordt dan ook zeer op prijs gesteld.

In de bijgevoegde folder vindt u meer informatie over de achtergrond van het onderzoek. Wij vertrouwen er op u voldoende te hebben geïnformeerd en hopen op uw medewerking.

Met vriendelijke groet,
Prof. D.I. Boomsma
Drs. C. Hoekstra

Appendix IV

Brochure

Meer informatie

Voor praktische informatie kunt u altijd contact opnemen met:
Mw. A.van.Bruggen 020 - 5989898

Verdere informatie over de wetenschappelijke achtergrond van de studie wordt verstrekt door:
Mw. Prof. Dr. D.I. Boomsma
Mw. Drs. C. Hoekstra

Of neem een kijkje op onze website:
http://www.tweelingenregister.org

Vrije Universiteit Amsterdam
Biologische Psychologie
Van der Boechorststraat 1
1081 BT Amsterdam
020-5988792

Versie 3.

INFORMATIE

Familieonderzoek naar
de erfelijkheid van de
twee-eiige tweelingzwangerschap

Achtergrond
Ieder jaar worden in Nederland meer tweelingen geboren. Veel mensen weten uit hun eigen omgeving dat het krijgen van een tweeling in sommige families meer voorkomt dan in andere. Uit onderzoek is gebleken dat dit vooral het geval is bij het krijgen van twee-eiige tweelingen.
Een van de belangrijkste factoren die het krijgen van een twee-eiige tweeling beïnvloeden is een meervoudige eisprong. De moeder produceert in plaats van één eicel, twee of meerdere eicellen binnen één menstruele cyclus. We denken dat dit een van de mechanismen is, waarlangs de erfelijke aanleg werkt.

Het doel van het onderzoek is om in de families waarin veel twee-eiige tweelingen voorkomen na te gaan welk gen verantwoordelijk is voor de meervoudige eisprong en uiteindelijk het krijgen van twee-eiige tweelingen. Het vinden van dit gen is belangrijk omdat we dan in staat zijn om meer inzicht te krijgen in de vruchtbaarheid en onvruchtbaarheid van vrouwen. Deze kennis zal misschien in de toekomst gebruikt kunnen worden bij de behandeling van onvruchtbaarheid. Daarnaast brengt een tweeling zwangerschap meer risico's met zich mee dan een eenling zwangerschap.

Methode
Al een aantal jaren doet het NTR in samenwerking met wetenschappers in Australië onderzoek naar de erfelijke aanleg voor het krijgen van tweelingen bij de mens.

Nu is vast komen te staan, dat het krijgen van twee-eiige tweelingen erfelijk is, zal een volgende stap in het onderzoek zijn om dit gen te vinden. Daartoe benaderen we moeders van twee-eiige tweelingen met een zus die ook een twee-eiige tweeling heeft.

Als u niet zeker weet of uw tweeling eeneiig of twee-eiig is kunnen wij dit testen. Hiervoor hoeft bij de tweeling geen bloed afgenomen te worden. De zygositeit (een of twee-eiig) kan bepaald worden door met een wattenstaafje wat mondslijmvlies uit de mond te halen.

Het is voor de genetische analyse van belang dat ook de ouders van de moeder en zus meedoen. Als de ouders niet aan het onderzoek kunnen deelnemen is het wenselijk dat, als dit mogelijk is, broers of zussen aan het onderzoek deelnemen, ook als ze zelf geen tweeling hebben.

Om te kunnen onderzoeken welke specifieke delen van het DNA (de drager van erfelijke informatie) van invloed zijn op het krijgen van een tweeling is een kleine hoeveelheid bloed nodig. Gekeken wordt of er een stukje DNA is, dat samen met het krijgen van tweelingen overerft. Daartoe willen we bij u en bij uw familieleden een aantal buisjes bloed afnemen. In het bloed wordt ook het cholesterol en de hoeveelheid geslachtshormonen bepaald. Van de hormonen wordt gedacht dat zij een rol spelen bij het krijgen van twee-eiige tweelingen. Ook vragen wij u een kleine hoeveelheid ochtend urine te bewaren, waarin stofwisselings-producten kunnen worden bepaald.

Omdat hormoonwaarden tijdens de menstruele cyclus sterk variëren wordt bij alle vrouwen geprobeerd op hetzelfde moment in de cyclus bloed af te nemen. Bij vrouwen die de pil gebruiken kan het bloed afgenomen worden op een willekeurige dag in de pil vrije week. Voor vrouwen in de menopauze kan de bloedafname op ieder tijdstip.

Wij brengen u van de uitslag van uw cholesterol gehalte op de hoogte. Mochten wij in de andere bepalingen afwijkende waarden vinden dan zullen wij u hier over informeren

Naast het afnemen van het bloed wordt bij de moeders van een tweeling een telefonisch interview afgenomen, waarin ook een stamboom van de familie gemaakt wordt.

Voor de voortgang van het onderzoek is de medewerking, van zoveel mogelijk moeders van twee-eiige tweelingen met hun zussen die ook een twee-eiige tweeling hebben en hun ouders essentieel! Dit geldt zowel voor de mensen die mogelijk al eerder meegedaan hebben als voor mensen die voor het eerst meedoen.

We hopen van harte dat u bereid zult zijn om aan dit onderzoek deel te nemen. Wij benadrukken dat alle informatie strikt vertrouwelijk wordt behandeld. Soms werken we samen met onderzoekers van andere universiteiten of bedrijven, waarbij uw data worden gebruikt en worden opgenomen in een gemeenschappelijke database. Uw identiteit blijft beschermd en wordt niet aan derden bekend gemaakt.

Verzekering voor eventuele schade die het gevolg is van het onderzoek is in overeenstemming met de wettelijke vereisten een verzekering afgesloten. Het contactadres van de verzekeringsmaatschappij is Onderlinge Waarborg maatschappij Centramed b.a., Postbus 90504, 2509 LM te 's Gravenhage. Gezien de uiterst geringe risico's van dit onderzoek verwachten wij geen problemen. Indien u meent schade te hebben opgelopen dan kunt u hierover contact opnemen met het secretariaat Biologische Psychologie of met het bureau medische zaken van het VU Medisch Centrum. Voor verdere informatie m.b.t. verzekering verwijzen wij u naar de bijlage.

Appendix V

Confirmation letter and informed consent

Nederlands Tweelingen Register (NTR)

Datum	Uw brief van	Telefax	Bijlage(n)
datum postmerk		020-5988832	1
Ons kenmerk	Uw kenmerk	Telefoon	E-mail
NTR/DZGEN		020-5988731	c.hoekstra@psy.vu.nl

Postadres: Van der Boechorststraat 1, 1081 BT Amsterdam

vrije* Universiteit *amsterdam

Geachte mevrouw/meneer,

U heeft zich bereid verklaard om mee te werken aan het onderzoek naar de overerving van de meerlingzwangerschap. Hiervoor onze hartelijke dank. Het doel van dit onderzoek is om genen te lokaliseren die belangrijke bijdragen leveren in het krijgen van meerlingen. De kennis die hiermee wordt verworven, verschaft een beter begrip van de vrouwelijke vruchtbaarheid. Daarvoor hebben wij moeders van twee-eiige tweelingen benaderd die een zus hebben met ook een twee-eiige tweeling. Bij deze moeders en bij de ouders van deze moeders worden een aantal buisjes bloed afgenomen. De buisjes bloed worden opgeslagen en gebruikt voor het doen van bepalingen.

Samen met deze brief ontvangt u, een verklaring en een retourenvelop. De verklaring dient door u ingevuld en ondertekend te worden.

Soms wonen meerdere personen die aan dit onderzoek deelnemen op een adres. Het is dan belangrijk dat u de verklaring, die aan u persoonlijk geadresseerd is, invult en opstuurt.

U wordt door een van onze medewerkers gebeld voor het maken van een afspraak voor de bloedafname. De bloedafname wordt door ons bij u thuis gedaan.
Het is voor het onderzoek van **groot belang**, dat bij de moeders van de tweelingen het bloed op **de derde dag van hun cyclus** geprikt wordt. Dit is belangrijk omdat de hormoonwaarden die wij willen bepalen fluctueren over de cyclus. Wij willen alle moeders op het zelfde moment in de cyclus prikken. Bij vrouwen die de anticonceptie pil gebruiken kan het bloed afgenomen worden op een willekeurige dag in de pil vrije week. Voor vrouwen in de menopauze of voor de ouders van de moeders met een tweeling is het moment van bloedafname niet van belang.

Wij vertrouwen erop dat wij u hiermee voldoende hebben geïnformeerd. Mocht u nog vragen hebben, dan kunt u ons altijd bellen.

Met vriendelijke groet,

Prof. dr. D.I. Boomsma
Drs. C. Hoekstra

Het onderzoek naar de overerving van de meerlingzwangerschap

Wilt u de onderstaande verklaring invullen en naar ons terug sturen?

ONDERGETEKENDE,

DE HEER/MEVROUW (NAAM) :…….

GEBOORTEDATUM :…….

ADRES :…….

POSTCODE :…….

WOONPLAATS :…….

VERKLAART:

1. In volledige vrijheid te willen meedoen aan het wetenschappelijke onderzoek naar de genetische oorzaak van meerlingzwangerschappen.
2. Begrepen te hebben dat hij/zij zich op ieder gewenst moment zonder consequenties uit het onderzoek kan terugtrekken.
3. Begrepen te hebben wat het onderzoek inhoudt en akkoord te gaan met de bloedafname.
4. Toestemming te hebben gegeven voor het bepalen van hormoonwaarden en cholesterol in het bloed. De cholesterol waarden worden tevens gebruikt voor onderzoek naar hart en vaatzieken.
5. Toestemming te geven om restanten DNA op te slaan voor eventuele aanvullende bepalingen in de toekomst.
6. Toestemming te geven dat hij/zij voor eventueel vervolgonderzoek door het NTR benaderd kan worden.

DATUM : …….
HANDTEKENING : …….

Ik, ondergetekende, bevestig hierbij dat deze studie zowel mondeling als schriftelijk aan de bovengenoemde deelnemer is uitgelegd.

Naam arts/onderzoeker: .....
Handtekening: ..... Datum: .....

Vrije Universiteit Amsterdam, afdeling Biologische Psychologie
Nederlands Tweelingen Register
Onderzoek naar de overerving van de meerlingzwangerschap
Van der Boechorststraat 1, 1081 BT Amsterdam, tel 020 - 5988731

List of publications

Papers

Hoekstra C, Meijer P, Kluft C, Heutink P, Smit G, de Geus E, Smit JH, van Bruggen A, Montgomery GW, Boomsma DI (2004) Genetics of dizygotic twinning. A feasibility study for a biobank. Twin Res, 7, 6, 556-563.

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Book Chapter

Montgomery GW and **Hoekstra C** (2008) Tweelingonderzoek: wat meerlingen vertellen over de mens. DI Boomsma (eds) pp 25-34.

. Dankwoord

Dit hoofdstuk is voor de mensen zonder wie dit proefschrift er niet zou zijn geweest. Dit zijn uiteraard in de eerste plaats alle tweelingmoeders die de tijd hebben genomen om de vragenlijst voor moeders van meerlingen in te vullen en terug te sturen. Daarnaast wil ik alle tweelingmoeders en hun familieleden bedanken die mee hebben gedaan naar de studie "de overerving van de meerlingzwangerschap". Ik wil deze moeders en hun familieleden bedanken voor de flexibiliteit en de inzet om dit project tot een succes te maken. Voor dit onderzoek hebben tweelingmoeders heel de familie bij elkaar getrommeld, zodat we iedereen tegelijkertijd konden prikken en niet van huis naar huis moesten rijden. Het kwam dus wel eens voor dat we met zeven personen om 7 uur 's ochtends aan de koffie zaten. We hebben mensen onderweg naar hun werk geprikt, in een wegrestaurant waar onze routes elkaar toevallig kruisten en we hebben zelf ook eens iemand in een kelder tussen de meelzakken geprikt!

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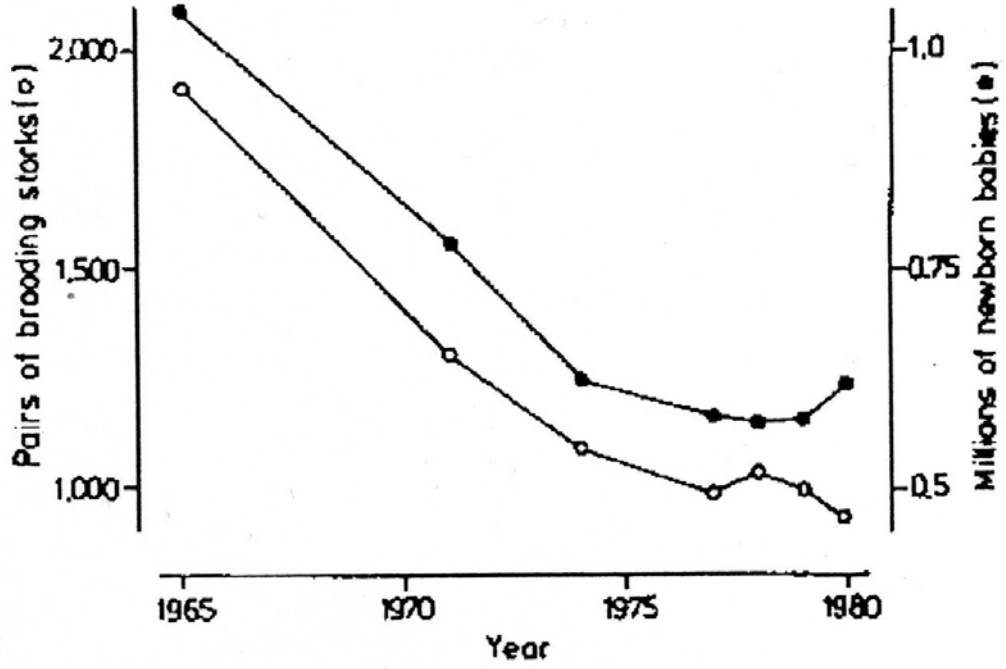
A new parameter for sex education

SIR—There is concern in West Germany over the falling birth rate. The accompanying graph[1,2] might suggest a solution that every child knows makes sense.



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