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Editors

Twin and Higher-order Pregnancies

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Biology

Contents

Biology and Genetics of Dizygotic and Monozygotic Twinning

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Trailer

This chapter summarizes what is known and what remains unknown about the human twinning process. The interest of this chapter is a description of the processes underlying twinning, with a specific focus on the biological and genetic aspects. While the mechanisms and contributory factors to dizygotic twinning are becoming well established, much remains unknown about the etiology of monozygotic twinning. Here we provide an overview of the incidence of twinning across the globe and present what is known about the influences of twinning based on the findings of historical, epidemiological, and more recent molecular studies.

Definitions

1. Zygosity – The number of zygotes that become fertilized leading to a multiple birth, or the genetic makeup of the pregnancy.
2. Dizygotic twins – Non-identical or fraternal twins that are the result of two independent ova that are fertilized by two separate spermatozoa.
3. Monozygotic twins – Identical twins that arise from a single fertilized ovum.

Learning Objectives

- Define and differentiate between the biological mechanisms that give rise to monozygotic and dizygotic twins.
- Compare and contrast the composition of fetal membranes of monozygotic and dizygotic twins.
- Describe the genetic and non-genetic factors that are associated with spontaneous dizygotic twinning events.
- Explain the strengths and weaknesses of zygosity assessment methods.
- Describe the differences in rates of monozygotic and dizygotic twins.
- Introduce the first genome-wide association study of dizygotic twinning.

3.1 Introduction

Twins and higher-order multiples have piqued the interest of humankind for many centuries. The remarkable similar resemblance often ascribed to twins has been observed in many literary texts and philosophical works. Twins with similar outward appearance but with noticeable personality differences have been well characterized throughout history. Observations of twins are noted as far back in time as the Biblical accounts of Jacob and Esau, by philosophers Augustine of Hippo and Aristotle, and by poets like Shakespeare (e.g., *The Comedy of Errors* and *Twelfth Night*).

From a scientific perspective, it was in the nineteenth century that the Scottish obstetrician J Matthews Duncan recognized and documented that two types of twins existed, now commonly referred to as identical and fraternal (non-identical) twins [2]. Sir Francis Galton was the first the recognize the value of studying twins to elucidate the genetic contribution to variation in human traits [3]; however, he was not aware of the distinction between monozygotic (MZ or identical) and dizygotic (DZ or fraternal) twins. Even in the early twentieth century, the existence of two types of twins was debated with the famous statistician Sir Ronald Fisher (who was the second of twins himself) proving mathematically that it was highly unlikely that there was more than one type of twin [4]. Nevertheless, the idea put forth by Galton was to compare trait concordance in twins in an attempt at disentangling the genetic (nature) and environmental (nurture) influences. Galton's proposal laid the foundation for modern era twin studies aimed at discerning the genetic contribution of complex traits and etiology of disease.

The theoretical basis of quantitative genetics proved to be fundamental to the creation and application of the classical twin design, which builds on the – by now firmly established – differential genetic relatedness of MZ and DZ twin pairs. Realizing the enormous potential of quantitative genetic theory to studies that apply the classical twin design for

studying human traits, large twin registries were established in the 1950s [5], although studies of twins had already been done in Russia [6–8] and elsewhere. The first scientific studies of twins in medicine were by Poll [9] and Siemens [10] who investigated the different levels of similarity between MZ and DZ twins for mole counts. Findings from their work suggested that MZ twin pairs were nearly genetically identical, whereas DZ twin pairs shared on average 50% of their genetic variation; this was reflected in the twice as large resemblance for mole counts in MZ than in DZ twin pairs. By now, twin registries are increasingly popular, and have proven strengths in longitudinal data collection and inclusion of biological samples to evaluate the genetic variation in susceptibility to disease.

Remarkably, despite the rapid gain in knowledge about the importance of genetic variation due to advancements in genotyping technology combined with the development of powerful linkage and genetic association studies, there is not a comprehensive understanding of the twinning process. For example, there are no estimates for the heritability of twinning. A number of factors influencing the DZ twinning process are well described, and the first genetic factors for DZ twinning have been characterized [11]. However, the heritability of DZ twinning remains unknown, and the knowledge regarding the etiology of MZ twinning is even more limited.

This chapter summarizes the current body of knowledge surrounding the epidemiological, biological, and genetic aspects of human twinning. Included are explanations of the biological mechanisms of twinning, descriptions of the underlying genetic contributions to the twinning process, and details on the frequency of twinning within populations.

3.2 Zygosity, Chorionicity, Placentation of Twinning

Zygosity refers to the genetic makeup of the pregnancy, or the number of zygotes that become fertilized leading to a multiple birth. Twins, in rarer cases triplets, quadruplets (four), quintuplets (five), arising from a single fertilized ovum are termed monozygotic (MZ). The first identical quintuplets known to have survived their infancy were the Canadian Dionne quintuplets, all five of which survived to adulthood (Fig. 3.1).

Fig. 3.1 The Dionne quintuplets. *The Dionne quintuplets, born May 28, 1934, outside of Callander, Ontario, Canada. Despite being born 2 months premature, all five quintuplets survived to adulthood.* (Source: ▶ https://commons.wikimedia.org/wiki/File:Dionne_quintuplets.jpg)

Alternatively, twins or multiples originating from two or more ova that are fertilized by separate spermatozoa are called dizygotic (DZ) [12], or trizygotic in the case of triplets. Nowadays, higher-order births of non-identical twins often are the result of assisted reproductive technologies (ART).

Multiple gestation pregnancies are inherently high risk to both the mother and the developing fetuses. Certain twin pregnancies, specifically those possessing a single chorion (monochorionic), exhibit an even higher risk for numerous pre- and peri-natal complications. Therefore, information about the status of the developing fetal membranes is helpful for monitoring and improving the outcome of a multiple gestation pregnancy.

In early stage human development, the membranes of the placenta begin to form around day 4. Examination of placental membranes by ultrasound imaging serves as a non-invasive method for determining zygosity/twin status [13–16]. According to the traditional models of twinning (Fig. 3.2), DZ twins have distinct placentas and fetal membranes and are therefore dichorionic diamniotic, although fused membranes are possible. Approximately two-thirds of all MZ twins share one placenta with monochorionic diamniotic membranes, while roughly one-third have completely dis-

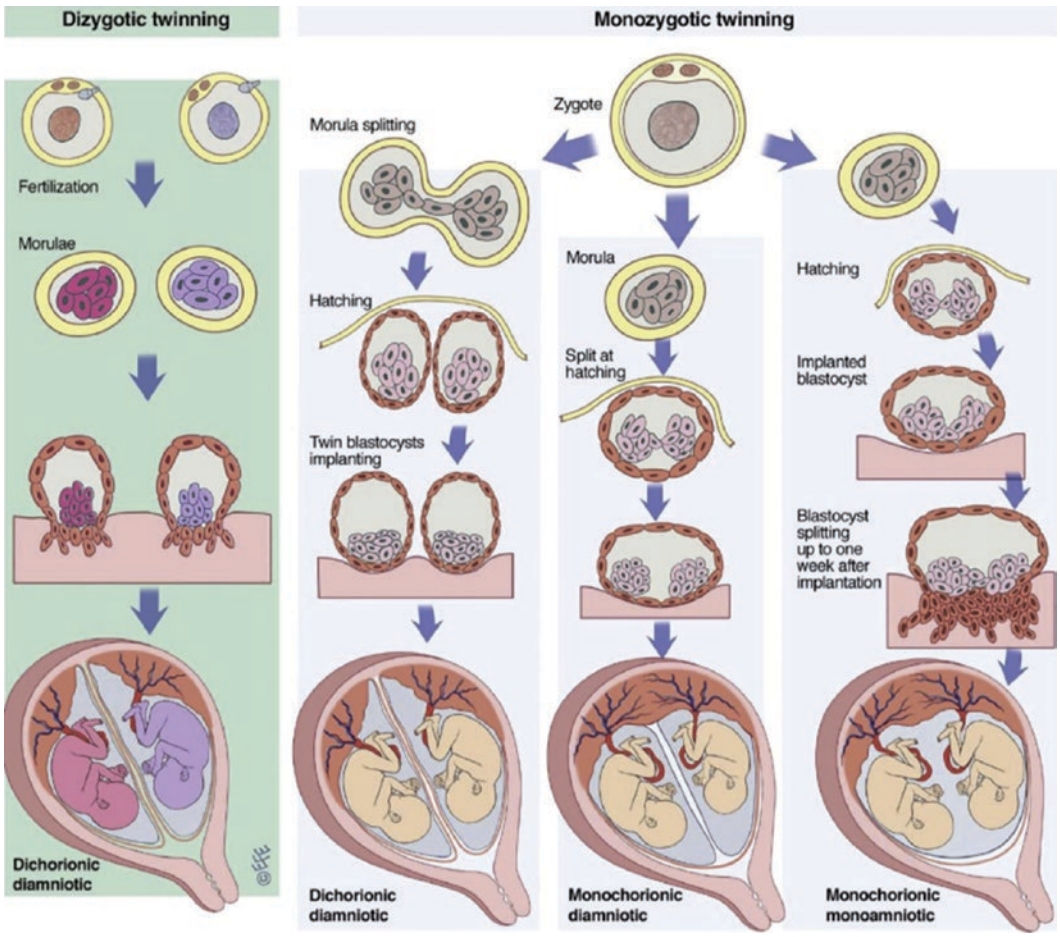

Fig. 3.2 The traditional model of twinning. *The formation of the two main types of twins according to the traditional model of twinning. DZ twins are the result of two distinct fertilization events and are dichorionic and diamniotic. MZ twins result from the postzygotic splitting of a single embryo early in gestation with varying numbers of fetal membranes depending on the timing of embryo splitting.* (This figure was obtained from McNamara et al. [21])

tinct placentas and membranes (dichorionic diamniotic). Only about 1% of MZ twins have one set of membranes and one placenta, making them monochorionic monoamniotic. The latter case poses highest risk for pre- and perinatal morbidity and mortality.

The placenta and membranes of a multiple gestation pregnancy represent a first means of identifying some MZ twins due to the presence of a single chorion. Although MZ twins will be of the same sex, not all same-sex twins are MZ. Therefore, for all same-sex twin pairs, DNA typing is the most useful and reliable method for determining zygosity [17]. Challenging the assumption that a single chorion indicates monozygosity, it is also possible for DZ twins to possess monochorionic diamniotic placentas. Thus, the dogma of a single chorion being synonymous with monozygosity is no longer proper due to chimeric DZ twins [18], a phenomenon in which one individual is composed of cells from two or more zygotes.

In normal embryogenesis, the chorionic membrane begins to form at about day 3. It is believed that if zygote separation takes place early, typically between day 1 and day 3, the result is MZ twins with separate placentas and membranes (dichorionic diamniotic). Alternatively, monochorionic diamniotic MZ twins result after the chorion has formed (day 3), but before the amnion has formed (typically between day 6 and day 8). Therefore, postzygotic splitting resulting in monochorionic diamniotic MZ twins typically occurs between day 3 and day 8. MZ twins with monochorionic monoamniotic membranes likely arise by splitting between day 8 and day 13. Conjoined twins are thought to arise after the beginning of the formation of the primitive streak, likely after day 14. However, the timing and mechanism(s) are not clear and empirical evidence is rare.

3.3 Zygosity Determination

3.3.1 Physical Appearance

For research purposes, twin zygosity is often times and most easily determined from questionnaires regarding similarity of physical characteristics. For instance, twins that are equal on most physical features and that are often times confused for one another are typically judged to be MZ. Alternatively, twins that differ on two or more physical characteristics and/or are not often mistaken for one another are often classified as DZ. As a whole, zygosity assessment from survey responses of physical traits corresponds rather well with zygosity determination through DNA typing [19, 20]. Apart from DNA testing, other basic rules that are routinely employed for zygosity determination are if the twins are opposite sex – DZ, of discordant blood groups – DZ, or possess a single, non-fused, placenta – MZ (please note the presence of two placentas does not imply DZ).

3.3.2 Placentation

Placental examination of a twin birth is common to establish the type of chorion and infer zygosity. DZ twins, except for chimeric twins, have two placentas with two chorions and two amnions. Thus, most DZ twins have dichorionic diamniotic placentation, although the placentas of DZ twins can sometimes appear as fused; yet they are functionally independent and with no inter-placental communication. Placentation in MZ twins is thought to vary depending on the timing of postzygotic splitting following a single fertilization event. Dichorionic diamniotic MZ twins (~33%) are formed if the split occurs early, on days 1–3, up to the morula stage. Monochorionic diamniotic MZ twins (~66%) result if the split happens between days 3 and 8, during which blastocyst hatching starts. Monochorionic monoamniotic MZ twins (~1%) occur if the split occurs between days 8 and 13. If no split has occurred by day 13, conjoined twins form [12, 21]. Examples of varying numbers of fetal membranes are also observed in triplets and higher-order multiples as shown in Fig. 3.3 (images adapted from Lamb et al. [22]).

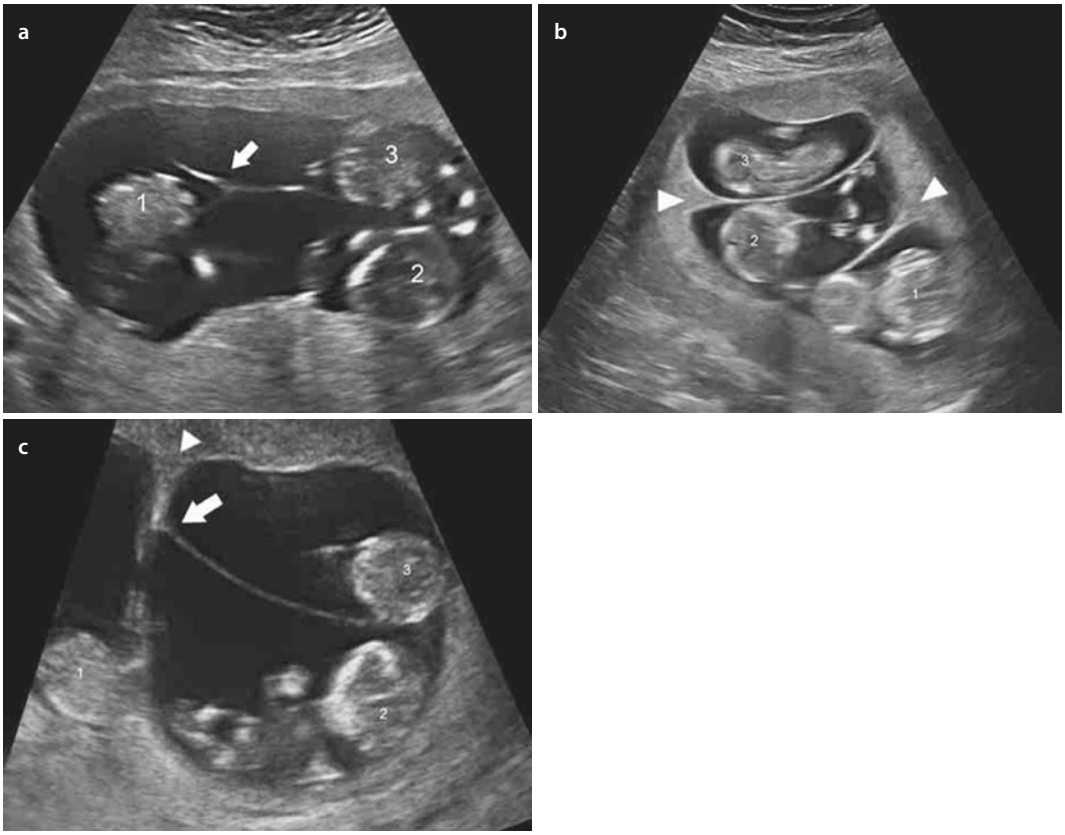

Fig. 3.3 Ultrasound images of triplets with varying numbers of fetal membranes. **a** *Monochorionic and therefore monozygotic triplets at 12 weeks of gestational age. The arrow indicates the meeting point of three amniotic membranes. Numbers indicate the three fetuses.* **b** *Trichorionic triplets at 12 weeks gestational age. The arrowheads indicate the separation between each fetus. These three fetuses do not share their placentas. This set of triplets can be trizygotic, dizygotic (one identical pair), or monozygotic. Numbers indicate the three fetuses.* **c**. *Dichorionic, triamniotic triplets at 13 weeks gestational age. The arrowhead indicates the separation of the chorionic membranes, which proves that this fetus does not share a placenta with fetuses 2 or 3. The arrow indicates the amniotic membarnes of fetuses 2 and 3, which are a monzygotic pair. At this point in time, it is unsure if fetus 1 shares zygosity with fetuses 2 and 3. Numbers indicate the three fetuses.*(This figure was adapted from Lamb et al. [22] with written permission from Cambridge University Press)

3.3.3 DNA Typing

The most robust and reliable way of determining zygosity is by DNA typing. The advent of cost-effective DNA genotyping has allowed for the opportunity to accurately determine zygosity through quantitative measures of allele sharing between twins when biological samples are available [23, 24]. Genetically, MZ twins will share (close to) 100% of their alleles and on average, DZ twins will share 50% of their alleles: similar to the allele sharing pattern of siblings. The recommended minimum number of single nucleotide polymorphisms (SNPs) needed to assess zygosity is around 50 [24]; however, utilization of around 20,000–30,000 SNPs bolsters confidence in zygosity determination. Procedurally, after genotyping and quality control, an optimal number of SNPs are selected and allele sharing in all pairs is determined. Sharing is reported as a proportion of markers for which a pair shares zero alleles (Z_0), one allele (Z_1), and two alleles (Z_2). From the proportions, total allele sharing (represented by $\hat{\pi}$) is calculated with the following formula: $\hat{\pi} = Z_2 + 0.5 * Z_1$. MZ pairs are identified by finding pairs with a $\hat{\pi} > 0.90$, allowing for some measurement error.

DZ pairs are defined as pairs with a $\hat{r} \neq$ and Z_1 between ~0.30 and ~0.70 [23].

3.4 Etiology of Twinning

3.4.1 Genetic Causes of MZ Twins

There have been several reports of families in which MZ twinning occurs more frequently than expected [25–30], although there is no compelling evidence to support an underlying genetic contribution to MZ twinning. Instances of familial MZ twinning from both maternal and paternal lineages have been documented, yet it has also been suggested that, independent of the sex of the parent transmitting the gene, a single gene is responsible for MZ twinning [29, 31]. There is additional evidence to suggest that there is no paternal effect on familial MZ twinning [32].

More recently, a gene thought to likely play a role in MZ twinning is *PITX2*. The *PITX2* gene was found as a candidate of monozygotic twinning in a molecular screening of an "experimental twinning" model in chickens [33]. *PITX2* encodes a protein that acts as a transcription factor, regulating the expression of genes involved with the formation of the embryonic axis.

3.4.2 Generation of MZ Twins

The universally accepted model of MZ twinning, often times referred to as the "fission model," rests on the hypothesis of postzygotic splitting of the conceptus within the first 2 weeks of development [21]. As exemplified by the model, the number of fetuses, chorions, and amnions are the result of the timing of embryo division. An alternative model of MZ twinning, sometimes called the "fusion model," challenges the traditional postzygotic splitting conjecture. The fusion model has been suggested due to criticisms of the traditional model lacking scientific evidence and the lack of specification of cleavage initiating factors [34]. The proposed alternate theory is based on two premises: (1) – MZ twinning occurs during the first cleavage division, resulting in twin zygotes and (2) – the structure of the fetal membranes is dependent on the various modes of fusion of the fetal membranes within the zona pellucida. Despite the two theories, the embryological processes that govern MZ twinning are still largely unknown and up for debate [35].

3.4.3 Genetic Causes of DZ Twins

DZ twinning is a complex trait that is likely under the influence of multiple genes. In the last decade, astonishing progress in characterizing the genes responsible for DZ twinning has been made. However, a comprehensive understanding of the genetic factors underlying the human tendency to conceive DZ twins is still lacking. Bearing this in mind, there are a number of genes with known roles in ovulation, female fertility, and DZ twinning in humans [36]. For example, mutations resulting in amino acid changes of the follicle-stimulating hormone receptor (FSHR) protein have been shown to influence DZ twinning [37]. Additionally, a variant within the promoter region of the *FSHR* gene was found to segregate with DZ twinning in a large family [38]; however, other studies have not replicated the involvement of this gene [39]. Several other candidate gene studies have provided evidence suggesting the involvement of other genes in the DZ twinning process, namely, serpin family A member 1 (*SERPINA1*) commonly referred to as alpha-1-antitrypsin [40, 41], peroxisome proliferator activated receptor gamma (*PPARG*) [42], and the fragile X "premutation" (*FRAXA*) [43, 44], although results were not replicated in future studies.

Linkage studies in family-based study designs have not provided evidence of enhanced genetic sharing among affected family members over chromosomal regions harboring the previously described candidate genes [38, 45]. However, linkage studies have indicated chromosomal regions that may possess novel candidate genes for DZ twinning [38, 45, 46]. For example, a study of 525 Australian and Dutch families of DZ twinning demonstrated the presence of new candidate DZ

twinning genes on chromosomes 6, 12, and 20 [38]. The results reaffirmed the notion that DZ twinning is a complex phenotype influenced by numerous genes. Mutations in growth differentiation factor-9 (*GDF9*) appear to influence DZ twinning in humans, albeit such mutations appear to be rare. Screening in large numbers of families with a rich history of DZ twinning revealed a two-base deletion in *GDF9* in heterozygous form resulting in a loss-of-function mutation in three families [47, 48]. It was also discovered that overall genetic variation in *GDF9* is more prevalent in mothers of DZ twins compared to controls [48]. Findings from non-human studies (e.g., sheep) of DZ twinning have implicated bone morphogenetic protein 15 (*BMP15*) and bone morphogenetic protein receptor 1B (*BMPR1B*) in the DZ twinning process; however, similar effects have not been found when studying *BMP15* [49] or *BMPR1B* [50] in humans. Both *GDF9* and *BMP15* are expressed in the oocyte and are essential for follicular development and have been implicated in premature ovarian failure [51]. *BMPR1B* is expressed in multiple cell types of the ovary and is the cognate receptor for *BMP15*. Surprisingly, while heterozygous mutations (one copy present) in *GDF9* and *BMP15* increase twinning rates, homozygous mutations (two copies present) result in female infertility.

More recently, genome-wide association studies (GWAS) have made it possible to scan the entire human genome for SNPs that are associated with a trait of interest in humans (e.g., twinning). In 2016, the first meta-analysis of GWAS in European-ancestry populations (Netherlands, Australian, Minnesota [United States of America]) identified the first common genetic variants associated with spontaneous DZ twinning [11]. Three statistically significant SNPs were found: in the upstream region of the follicle-stimulating hormone beta subunit (*FSHB*) gene, within an intron of the mothers against decapentaplegic homolog 3 (*SMAD3*) gene, and in an intergenic region on chromosome 1. The two SNPs near *FSHB* and within *SMAD3* were replicated in an independent Icelandic cohort. The former encodes the beta subunit of FSH, while the gene product of the latter is transcription factor involved in gonadal responsiveness to FSH. Sixty-three candidate genes of DZ twinning [52] were also tested, but only *FSHB* was associated with DZ twinning in gene-based tests. Interestingly, the replicated SNPs associated with twinning were also found to be associated with higher serum FSH levels, and with multiple aspects of female fertility, including earlier age at menarche, earlier age at first child, higher lifetime parity, earlier age at menopause, and later age at last child. Polygenic risk scores for DZ twinning were found to be significantly associated with DZ twinning in the independent Icelandic cohort, with a higher likelihood of having children, higher lifetime number of children, and an earlier age at first child. Together these findings corroborate the link between fertility and DZ twinning.

3.4.4 Generation of DZ Twins

Mechanisms leading to dizygotic twins operate on the selection of developing follicles within the ovary when instead of one ovum being released mid-cycle, two follicles mature, and both oocytes are released for fertilization. The subsequent fertilization of two eggs by two sperm during a pregnancy result in DZ twins. The processes of ovarian folliculogenesis and dominant follicle selection are governed by both circulating and intra-ovarian concentrations of FSH. Spontaneous DZ twinning tends to run in families and is associated with elevated concentrations of FSH in the mother [53]. FSH amounts seem to vary with geography, season, ethnic origin, and increasing parity, and are increased in tall, heavy, and older mothers with a peak at around 37 years of age [54]. Following this logic, it has been proposed that age-dependent twinning may also be due to natural selection favoring double ovulation events in response to declining fertility with increasing age [55]. It has also been documented that mothers of DZ twins have an increased number of FSH pulses during the early follicular phase, without a concurrent luteinizing hormone (LH) pulse [56].

It is well known that improved nutrition is a contributing factor for increases in multiple ovulation (i.e., twinning frequency) in other

species [57, 58], yet this has not been demonstrated in humans. In fact, human twinning rates do not appear to reflect average nutritional status as established from longitudinal studies of countries experiencing extended periods of starvation, such as the Dutch hunger winter [1]. In general, above a specific, yet undetermined, threshold, nutrition seems to be of minimal importance for twinning and reproduction in general.

3.5 Incidence of Twins

In the early twentieth century Wilhelm Weinberg postulated a method, referred to as the "Weinberg differential rule," for approximating population estimates of the number of MZ versus DZ twins [59]. Weinberg's proposal assumed that all MZ twins and half of DZ twins would be of the same sex, with the other half of DZ twins being of opposite sex. Therefore, he suggested multiplying the number of opposite-sex twins by two, which served as an estimate for the total number of DZ twins. The excess of same-sex twins would then be the number of MZ twins. Thought of in a different way, subtracting the total number of opposite-sex twins from the number of same-sex twins would yield an estimate of the number of MZ twins. Weinberg's calculation has been widely adopted because it serves as a simple method for estimating twinning frequency in populations; however, it rests on the assumption that the frequency of same-sex twins is the same as that of opposite-sex twin pairs, which may not always be the case [60, 61].

The rate of twinning includes stillbirths (≥28 weeks) and live births and is defined as the number of twin maternities per 1000 maternities. Differences in twinning rates between geographical regions have been studied extensively. In the 1970s, Bulmer studied twinning frequencies in three distinct geographical regions: Europe/North Africa, Sub-Saharan Africa, and Asia [62]. Bulmer found that the highest rate of twinning occurred in Sub-Saharan Africa (~23 per 1000 maternities), while the lowest rate occurred in Asia (~5–6 per 1000 maternities). Twinning rates exhibit considerable temporal and spatial variation (see Fig. 3.4). Provided that

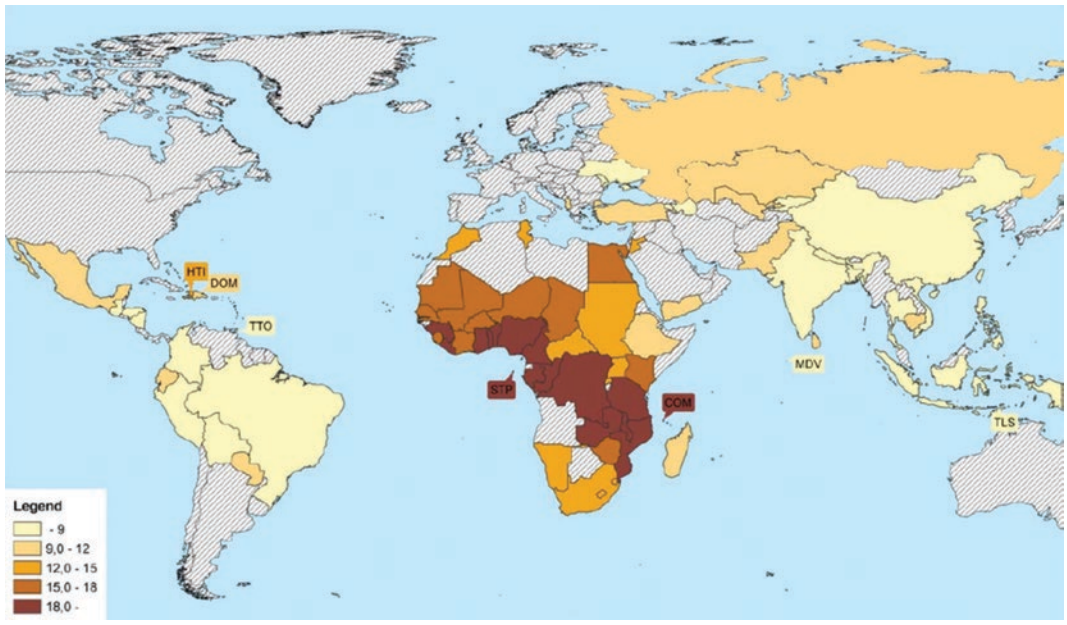

Fig. 3.4 Rates of twinning worldwide. *Heatmap showing the number of twins per 1000 births in 77 countries. Huge variation in twinning rates can be observed across the different regions of the developing world.* (The figure was adapted from Smits and Monden [76] by Veronika Odintsova to include 2011 Russian twinning rates.)

Table 3.1 Multiple births in Russian Federation in 2017

		Total number			Proportion in all deliveries			Birth rate per 1000		
	All deliveries, including born outside clinic	Multiple (all)	Twins	Triplets and higher order	Multiple (all)	Twins	Triplets and higher order	Multiple (all)	Twins	Triplets and higher order
Russian Federation	1,649,782	20,239	19,938	301	1.23	1.21	0.02	12.27	12.09	0.18

Table 3.1 was created and provided by Veronika Odintsova based on Russian national statistics [63]

the MZ twinning rate is known to be fairly constant around the world, the variation in overall twinning rate is generally attributed to the variation in DZ twinning rates.

Global twinning rates have predominantly been reported from Western countries with less being known about twinning rates in Eastern countries. In 2017, data received from open resources of national statistics of the Ministry of Health of the Russian Federation report a multiple birth rate of 12.27 births per 1000 deliveries (alive or stillbirth) as seen in Table 3.1 [63]. The overall twinning rate during this time was reported to be 12.09 per 1000 and the rate of higher-order multiples occurred at a rate of 0.18 per 1000.

3.5.1 Incidence of MZ Twins

Worldwide, and across all races, MZ twin birth rates occur at a constant rate of approximately 4 in every 1000 pregnancies [64]. The remarkable consistency in MZ twinning rates among all populations suggests that identical twinning is an occurrence that is not influenced by genetics. Unlike DZ twinning, the incidence of MZ twins is independent of maternal age, height, weight, or parity [65]. Although MZ twinning appears to be a sporadic event, instances of familial MZ twinning of varying modes of inheritance have been reported, with one report of autosomal dominant inheritance with variable penetrance [66]. The introduction of assisted reproductive technologies (ART) greatly enhances rates of DZ twinning and to some extent MZ twinning [67]. The increase in MZ twinning rates due to ART has been attributed to mechanical forces affecting the zona pellucida, or to the effects of incubation media and late implantation in in vitro fertilization procedures [68–70].

3.5.2 Incidence of DZ Twins

DZ twinning is common, yet large regional differences in DZ twinning rates exist around the world. The rate of DZ twins range from approximately 6 per 1000 maternities in Asia, to 10–20 per 1000 in Europe and the United States, to as high as 40 per 1000 in certain regions of Africa [12]. DZ twinning rates also vary substantially over time. In the United States, the observed incidence of twin births increased by a factor of 1.9 between 1971 and 2009 [71]. A considerable portion of the increase is attributable to fertility treatments, with an estimated 36% of all twins born in the United States in 2011 resulting from ART [71, 72]. DZ twinning rates peaked in the mid-2000s, following the number of ART pregnancies. In developed countries, with technological advancements and careful monitoring, DZ twinning rates due to ART have dropped substantially. In contrast to MZ twinning, spontaneous (i.e., no ART) DZ twinning is also very dependent on many maternal factors including age, nationality, parity, height, weight, and family history [36, 54].

3.5.3 Triplets and Higher-Order Multiples

The pattern of variation in triplet rates across the world is remarkably similar to that observed for twin births. That is, rates of triplet births are highest in African countries, intermediate in European populations, and lowest in Asian countries. More generally, the rate of triplets is in accordance with Hellin's law [73], which states that there is on average one twin maternity per N singleton maternities and that there is one X-tuplet maternity per $N^{(X-1)}$ [74, 75]. Thus, if the number of twin maternities is one in N singleton maternities, then the number of triplets is one in $N^{(3-1)}$. It follows that quadruplets (see ◘ Fig. 3.5) would occur at a rate of $N^{(4-1)}$. According to this logic, if there is one twin in every 80.05 births, this predicts that triplets occur at a rate of about 1 in ever 6408 births, which is only 4% higher than the incidence actually observed [76].

3.6 Factors Affecting Twinning

Many of the risk factors for DZ twinning are rather well established and include assisted reproductive technologies (ART), higher maternal age, parity, body composition, and smoking [12, 36, 77]. For MZ twinning, there is little to no agreement regarding the involved risk factors and/or causes [78]. Given that the two types of twinning are biologically distinct phenomena, it is not surprising that many of the factors involved in DZ twinning are not,

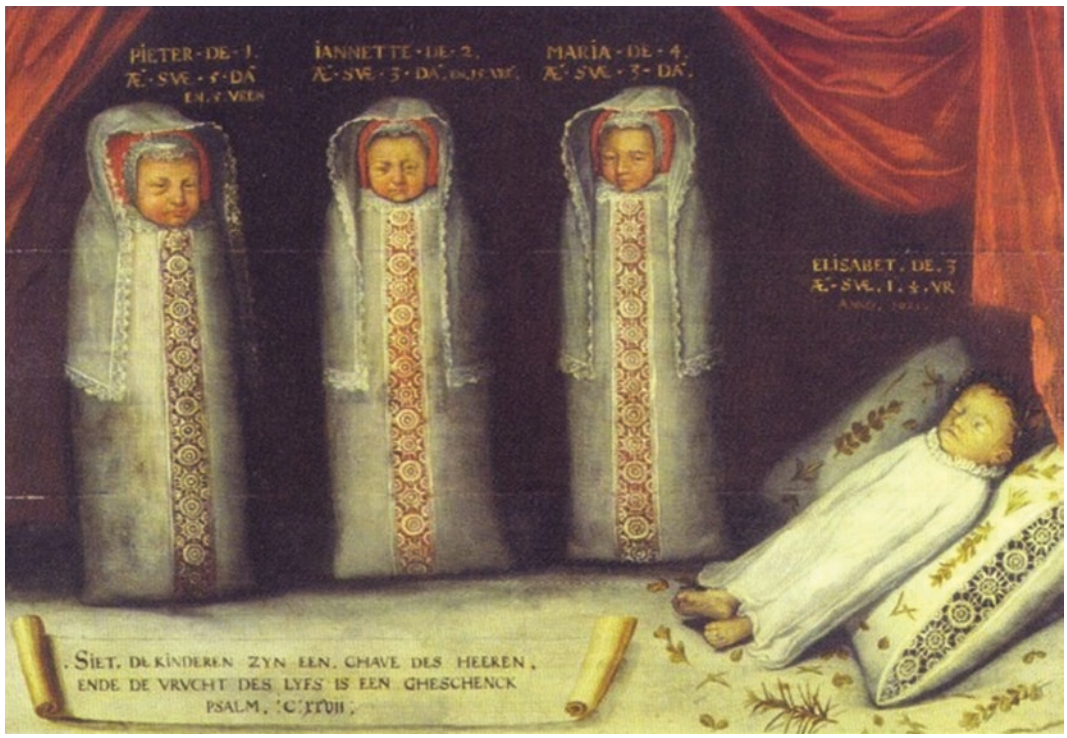

Fig. 3.5 Painting of Dutch quadruplets. *"Vierling Costerus" – Painting of quadruplets born on June 9, 1621, in Dordrecht, the Netherlands. Remarkably, the birth of the first (Pieter) and the last (Maria) was separated by 53 hours indicating an extremely difficult birthing process. Prior to the quad birth of one boy and three girls as pictured here, the parents mention the birth of twins 7 years before. Sadly, due to the high infant mortality rate in the seventeenth century, about 40–50% of children did not reach the age of 18, and the chance of survival was even smaller for multiple births. In the case of the quadruplets pictured here, one died an hour and a half after birth (Elisabet), and two others deceased within the first year.* (This figure was obtained by written permission from the Dordrecht Museum. The Noordbrabants Museum in 's-Hertogenbosch received this painting as a gift in 1925 and presented it on loan to the Dordrechts Museum in 1986. Painter and client are unknown.)

or to a lesser extent, found in MZ twinning. Below we describe established risk factors involved in multiple pregnancy and multiple birth.

3.6.1 Assisted Reproductive Technologies (ART)

Assisted reproductive technologies, especially in vitro fertilization (IVF) and ovulation induction (OI), are well-established risk factors for DZ and to some extent MZ twinning [78]. Ovulation inducing agents such as clomiphene citrate, human pituitary gonadotropins, and human menopausal gonadotropins are known to increase ovulation rate and hence the probability of multiple pregnancy [79]. In case of IVF, increased multiple pregnancy and multiple birth are due to the transfer of multiple embryos [12, 80]. Although less well understood, there is also a slight increase in MZ twinning after IVF and other ART, with estimates ranging from a two- to 12-fold increase in MZ twinning rate after ART procedures [78] and a two- to fivefold increase in MZ twinning following IVF [81, 82]. Notably, OI agents are often used in concert with IVF; thus, the increased chance of multiple pregnancy following IVF cannot be merely attributed to multiple embryo transfer [36].

3.6.2 Other Risk Factors

Higher maternal age is another well-established risk factor for DZ twinning [36, 62]; however, conflicting reports have been made for MZ twinning [62, 83, 84]. Paradoxically, while fertility decreases with age, the spontaneous twinning rate increases. Both polyovulation and embryonic survival rate increase with maternal age, suggesting that increased ovulation rate and decreased spontaneous abortion of potentially unhealthy offspring could act together as an insurance system to produce one last round of reproduction, with twinning being merely a by-product [55, 85]. Additionally, increased parity (the number or maternities prior to twin pregnancy) is associated with a higher risk of DZ, but not MZ twinning [12, 86], independent of maternal age [36, 87]. Moreover, maternal body composition has been reported rather consistently in relation to DZ twinning, with both obesity and tall stature increasing the risk of DZ twinning [53, 84, 88]. Furthermore, an unexpected relation between maternal smoking and higher probability of DZ twinning is sometimes observed although the mechanism behind this observation remains unclear [77, 89, 90].

3.7 Endocrinology of DZ Twinning

Mothers of spontaneous DZ twins have a predisposition to multiple ovulation events due to interference with selection of a single dominant follicle. Multiple follicle growth and subsequent multiple ovulation events have been observed in mothers of hereditary dizygotic twins [91, 92]. Follicular recruitment, selection, and dominance is controlled by a complex regulatory network within the hypothalamic-pituitary-ovarian axis (Fig. 3.6). The two main pituitary-derived hormones essential for reproductive function are FSH and LH and are secreted in response to the pulsatile secretion of gonadotropin-releasing hormone. FSH is the main hormone controlling follicular growth and its secretion is controlled by the main secretory products of the large dominant follicle(s), namely, estradiol and inhibin. Circulating concentrations of FSH, other intra-ovarian factors (e.g., GDF9 and BMP15), and their cognate receptors physiologically regulate ovarian folliculogenesis and ovulation quota. Transcriptional regulators of *FSH*, such as SMAD3, also regulate gonadal responsiveness to FSH. Ongoing development of a single follicle takes place when a certain threshold level of plasma FSH is marginally exceeded [93, 94]. When FSH levels are much higher than the threshold level, or exceed the threshold for an extended duration, multiple follicle growth can result [95]. In accordance with the endocrine model of dizygotic twinning [96], high levels of pituitary gonadotropins (i.e., FSH) are responsible for increased multiple ovulation in mothers of DZ twins.

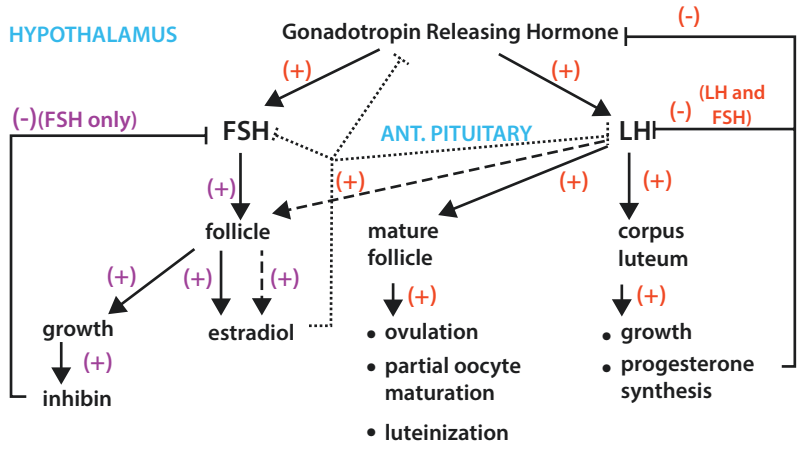

Fig. 3.6 Hormonal feedback and endocrine regulation of female reproductive physiology. + *signs denote positive feedback, whereas blunted arrows indicate negative feedback.* (This figure was obtained by written permission from the lecture materials of Dr. Kathleen Eyster (University of South Dakota, United States))

A number of studies, but not all [91], have shown increased levels of plasma gonadotropins in mothers of DZ twins [56, 97–99].

3.8 Non-human Twinning

Studies of non-human species have revealed several genes that contribute to DZ twinning. For example, in sheep, which are typically uniparous, certain breeds have higher incidences of multiple births [100]. Several genes, namely, *GDF9*, *BMP15*, and *BMPR1B*, have been confirmed to influence twinning rates in sheep by increasing follicle development and oocyte maturation [50, 101–103]. Major genes that increase ovulation rate and litter size in sheep and humans have been shown to have implications in other species. Evidence exists for genetic effects on twinning in cattle [104], the marmoset monkey [105], and on hormone-induced ovulation rate in mice [106].

Genes specific to MZ twinning remain elusive. Animal studies (originally in rabbit and roe deer) have suggested that MZ twinning results from disturbances to developmental thresholds and that delayed fertilization/implantation play a role [62]. These hypotheses have been further tested in nine-banded armadillos (*Dasypus novemcinctus*), which bear obligate MZ quadruplets each and every time they breed [107–110].

3.9 Sex Ratio

Sex ratio is defined as the ratio of males to total births. For DZ twins and singletons the ratio is 0.514, meaning a slight excess of males [62, 111]. For spontaneous MZ twins, triplets, and quadruplets, the sex ratio is lower (0.496) due to a slight excess of females [61, 111]. The value is even lower for monoamniotic twins, including conjoined twins, with a sex ratio of 0.2 [111]. There does not seem to be an excess of males in aborted twins. Dichorionic MZ twins exhibit the smallest increase in females, whereas monochorionic diamniotic MZ twins show the largest increase; thus, the rise may be due to later twinning events. Monochorionic monoamniotic twins and conjoined twins show an even greater increase in the number of females.

3.10 Discussion and Conclusion

Twinning is a common and multifactorial phenomenon, and elements of the twinning process remain poorly uncharacterized. Improved understanding of the underlying biological and genetic aspects of MZ and DZ twins and of the twinning process as a whole has been enhanced by the development of molecular and cytogenetic techniques. The influences on

DZ twinning are well studied, including contributions of numerous maternal (age, height, weight, parity, family history), environmental (ART), and associated genetic factors (most notably common variants near *FSHB* and within *SMAD3*). However, despite the robust associations with DZ twinning, the ability to predict DZ twinning events remains imprecise due to the myriad of genetic and non-genetic contributions. Likewise, comprehensive models of MZ twinning lack compelling evidence and are challenged by instances of atypical twinning. MZ twinning is likely influenced by some equivocal combination of non-genetic and genetic factors that likely result in delayed fertilization, embryo development, implantation, or some form of mechanical disruption of the early embryo. Further elucidation of the mechanisms by which twinning processes occur will have significant merit for predicting, managing, and improving the outcomes of multiple gestation pregnancies.

3.10.1 Review Questions

1. What are the etiological similarities and differences of monozygotic and dizygotic twins?

2. What is the role played by assisted reproductive technologies in the incidence of twinning over time?

3.10.2 Multiple-Choice Questions

1. What is the most conclusive method for determining zygosity status of twins?
   (a) Examination of placentation
   (b) Sex status (same or opposite sex)
   (c) Evaluation of survey responses
   (d) DNA testing
   (e) Assessment of outward physical appearance

Answer: (d). Although the other methods are convenient and relatively robust approaches for determining zygosity status of twins, only DNA testing provides a quantitative and accurate assessment of allele sharing for all twins, including same-sex twin pairs.

2. What is another term for dizygotic twins?
   (a) Identical twins
   (b) Fraternal twins
   (c) Typical twins
   (d) Atypical twins

Answer: (b). Dizygotic, or non-identical twins, develop from separate ova and are therefore genetically distinct. Thus, because their genetic relatedness is the same as for other sibling pairs, dizygotic twins are commonly referred to as fraternal twins.

3. The rarest form of monozygotic twins (with the exception of conjoined twins) exhibit which of the following fetal membrane states?
   (a) Dichorionic diamniotic
   (b) Dichorionic monoamniotic
   (c) Monochorionic diamniotic
   (d) Monochorionic monoamniotic

Answer: (d). Monochorionic monoamniotic monozygotic twins represent about 1% of all twin pregnancies. Approximately 66% of monozygotic twins are monochorionic diamniotic, whereas 33% are dichorionic diamniotic.

4. Genome-wide association studies have identified common genetic variants associated with spontaneous dizygotic twinning and female fertility in which genes?
   (a) *FSHB* and *SMAD3*
   (b) *GDF9* and *BMP15*
   (c) *BMP4* and *WFIKKN1*
   (d) *FSHR* and *BMPR1B*

Answer: (a). Single nucleotide polymorphisms near *FSHB* and within *SMAD3* are significantly associated with a higher rate of spontaneous dizygotic twinning and several other aspects of female fertility (e.g., earlier age at menarche, earlier age at first child, and higher lifetime parity).

5. In order of highest to lowest incidence, which of the following captures the large regional differences that are observed for dizygotic twinning?
   (a) Asia, Africa, Europe
   (b) Africa, Europe, Asia
   (c) Europe, Asia, Africa
   (d) Europe, Africa, Asia

Answer: (b). Whereas monozygotic twinning rates are relatively constant worldwide (~3 per 1000 births), large regional differences exist in dizygotic twinning rates with the highest incidence in African populations (~40 per 1000 births), followed by European populations (~10–20 per 1000 births), and the lowest incidence in Asian populations (~6 per 1000 births).

6. Which of the following is *not* a known non-genetic risk factor for spontaneous dizygotic twinning?
   (a) Parity
   (b) Maternal age
   (c) Nutritional status
   (d) Smoking status
   (e) Body mass index
   (f) Height

Answer: (c). Spontaneous dizygotic twinning is associated with parity, as well as increased maternal age, increased body mass index, increased height, and smoking status prior to pregnancy. Nutritional status is not known to be a direct contributor to dizygotic twinning as longitudinal studies in countries that experienced periods of starvation demonstrated consistent rates of twinning (e.g., Dutch hunger winter [1]).

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