

The timing of monozygotic twinning: a criticism of the common model

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Summary

In the dominant model, monozygotic (MZ) twinning is universally accepted as a post-fertilization event resulting from splitting of the embryo along its first 2 weeks of development. The stage at which splitting occurs determines chorionicity and amnionicity. A short history on how the model was built is presented, stressing the role played by some embryologists, in particular George Corner, in its completion and final success. Strikingly, for more than 60 years no deep criticisms have been raised against the model, which, in virtue of its rational and plausible character, enjoys the status of undisputed truth. At close examination, the embryological support of the model shows some important weak points, particularly when dealing with late splitting. In the author's view, the model not only has contributed to 'suspend' our knowledge on the timing of MZ twinning, but seems indefensible and claims to be substituted. That factor could imply relevant consequences for embryology and bioethics. As an alternative to the model, a new theory to explain the timing of MZ twinning is proposed. It is based on two premises. First, MZ twinning would be a fertilization event. In that case, due to an alteration of the zygote–blastomere transition, the first zygotic division, instead of producing two blastomeres, generates twin zygotes. Second, monochorionicity and monoamnionicity would not depend on embryo splitting, but on fusion of membranes. Some support for this theory can be found in recent embryological advances and also in some explanations of old.

Keywords: MZ twinning, Timing of MZ twinning

Introduction

This article analyzes the history and validity of the current explanation on the genesis and timing of human monozygotic (MZ) twinning (referred to in the following as the 'model'). At close examination, the model is less than satisfactory in explaining some important aspects of the timing of MZ twinning. This article is written to invite a re-examination of the question, and also to offer an alternative explanation.

The model

The model, proposed more than a half century ago, enjoys today an unreserved support by embryologists, reproduction scientists and bioethicists. It rests on two basic postulates:

1. MZ twinning is a post-fertilization event resulting from the splitting of an early embryo.
2. The timing of twinning establishes the structure of membranes; in other words, the developmental stage at which splitting takes place determines chorionicity and amnionicity.

In scientific and popular publications alike, the model has crystallized in formalized patterns of verbal and graphic presentation. It follows one of two ways for measuring embryologic time: either the successive stages of early development (2-cell stage, cleavage, morula, early and late blastocyst, bilaminar embryonic disc, primitive streak); or, more usually,

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the sequence of post-fertilization days. Accordingly, it is said that splitting from day 1 to 3 (i.e. before compaction of the morula) results in the production of dichorionic/diamniotic (DC/DA) twins; splitting between days 5 and 8 (by division of the inner cell mass at the blastocyst stage) results in the production of monochorionic/diamniotic (MC/DA) twins; splitting between days 9 and 12 (by partition of the bilaminar embryonic disc at the late blastocyst stage) results in the production of monochorionic/monoamniotic (MC/MA) twins; and splitting around day 14 or later (when the primitive streak is formed) results in the appearance of conjoined twins (see Note 1). The standard graphic representation of the model consists in a dual combination of embryological and obstetrical diagrams. Ordinarily, such diagrams show besides the developmental stages at which the embryo splits and the ways the embryonic membranes are affected, a representation of an open uterus depicting twins, placentas, and chorionic and amniotic sacs.

An extensive bibliographical search has shown that the model not only has met with general approval (see Note 2), but it is put forth in a factual, assertive style, conveying the certitude that it is not a hypothetical explanation, but a sound description of hard facts (see Note 3). Such pretension is, as I will try to demonstrate, an educated but deceptive guess. The apparent soundness of the model is the result of a curious epistemic evolution that changed an initial and tentative explanation into an accepted and undisputed truth.

The matter is relevant in two ways: first, the timing of MZ twinning is a biological issue; therefore, in the age of evidence-based medicine, we must be interested in knowing in what measure the information we possess on the matter is reliable. Second, the issue has the utmost bioethical relevance, as debates on the beginning of human life and the ethics of *in vitro* fertilization (IVF) or embryo experimentation have been persistently focused on the timing of MZ twinning.

The building of the model: a short history

The history of the embryology of human twinning is long and complex. Here, attention will be centred on the birth and evolution of the model (see Note 4; Sobotta, 1901; Schwalbe, 1906; Patterson, 1913).

Although there are some remote precedents, it seems reasonable to choose as the starting point of the model an article published by Corner in 1922, in which he described three pairs of MC twins in the pig. Corner did not hide his discontent over the inadequacy

of the then current explanations on the origin of identical twins (see Note 5; Corner, 1922). However, after acknowledging the scarcity of facts and the abundance of conjectures, Corner dared at the end of his article to offer a new hypothesis. '...we may permit ourselves to indulge in a brief speculation regarding the morphogenesis of human monochorionic twins... [H]uman single-ovum twins may be of two types. One... arising by duplication of the inner cell mass before formation of the amniotic cavity (pig type) would typically give rise to two embryos in a single chorion with two independent amniotic cavities... The second kind of twinning, occurring by duplication of the actual embryonic areas after formation of the amniotic cavity (armadillo type), would produce two embryos within a single amnion.' (see Note 6; Corner 1922) Corner did not consider the timing of MZ twinning of the DC DA type influenced by the then prevailing opinion that DC twins were always dizygotic (DZ), and MZ twins always MC.

The data were not new, but its interpretation supposed a fundamental innovation. Corner introduced for the first time the idea that differences in the structure of the fetal membranes in MC twins were dependent on the moment of partition of the embryo. Henceforth, time became a decisive factor for the understanding of twinning.

Before Corner, the timing of the twinning was considered irrelevant, because, as things were then understood, the exclusive condition for MC twinning was the presence of two embryonic areas within a single blastodermic vesicle. To justify amnionicity, a 'topological' reasoning was then adduced: if the two embryonic areas within the blastocyst were sufficiently apart, each could develop separately its own amnion, with the result of MC DA twins; if they were near together, a common amnion would result, and, therefore, MC MA twins (see Note 7; Schultze, 1897; Bumm, 1902; Wilder, 1904; Newman, 1917). In Corner's proposal, the old 'geometrical' view ought to be abandoned, so that timing could get the leading role in the explanation of MC twinning. But, regrettably, the immediate impact of the 1922 article by Corner was minimal (see Note 8; Streeter, 1924; Grosser, 1927; Hughes, 1927; Klein, 1927; Potter, 1927).

The next important step in the building of the model came in the 1920s, when the statement 'zygosity equals chorionicity' was demonstrated false. Siemens, applying his method of polysymptomatic (dermatologic) similarity diagnosis to the study of twins, established firmly that many DC twins were MZ, and not DZ (see Note 9; Siemens, 1925). Siemens' findings, soon confirmed by others, forced some important amendments to Corner's model. That was the task of von Verschuer. In 1932, he expanded

the model to include DC DA twins among the MZ. Verschuier attributed the origin of the newly characterized type to a partition of the embryo during early cleavage stages, before the beginning of the differentiation into embryoblast and trophoblast (Verschuier, 1932). The timing of twinning began now with the appearance of the two first blastomeres.

In 1947, the model moved forward. Until then, the dating of the twinning event was referred to embryological stages of imprecise chronology (splitting of blastomeres, formation of two inner cell masses within the blastocyst, splitting of the germ disc, formation of two primitive streaks). Coulton, Hertig and Long, based on Hertig's observation that, in the human embryo, the amnion forms between the 7th and the 13th day after fertilization (Hertig, 1945), offered an estimation of the time of splitting for MA twinning. They reasoned that if the germ disc had split before the formation of the amnion [on day 7], presumably two amnions would then have formed; alternatively, in the case of MA twins, the split ought to occur after that day, but before day 13^{1/2}, when the primitive streak initiates its development (Coulton *et al.*, 1947). In this way, the first, although incomplete, daily calendar was established for MA twinning. The age of the embryo, measured as post-fertilization days, became hereafter a meaningful element for the description and understanding of MZ twinning. Hertig's article had a wide repercussion in the literature (see Note 10; Conybeare, 1954; Hanes, 1954).

In 1955, Corner completed his model (Corner, 1955). In the Baer Lecture of the Chicago Gynecological Society he delivered the previous year, Corner updated his ideas on the embryological theory of human twinning, considered the support it received from recent findings, and presented some illustrative cases he chose mostly from the Carnegie collection (see Note 11). Corner emphasized the theoretical character of the model: 'Embryologists and obstetricians have built up on paper the morphological theory of single-ovum twinning, tracing the various ways in which one egg cell might ultimately develop into two embryos. All this information is in the textbooks; it has chiefly been worked out by deduction from the arrangement of the placentas and fetal membranes as seen at birth or in fetal life. Embryonic specimens early enough to provide direct corroboration of the theory are rare' (see Note 12; Corner, 1955).

To provide the necessary background of early human embryology for the subsequent analysis of the three critical stages of twinning of the finished model he presented, Corner offered a series of five microphotographs of the stages of development corresponding to the times at which twinning may occur: the 2-cell stage and the morula (about days 2 to 4 after ovulation); the blastocyst (about day 5); the

implanted embryo, pre-villous stage (about day 11) and a later embryo (about day 17) (see Note 13).

Then he analyzed each of the three critical stages. First, he discussed the twinning by separation of the early blastomeres, which he thought highly probable, but which can never be absolutely proven by inspection of the products of conception. His conclusion was that 'unless the age arrives of 'test-tube babies' and of the experimental embryology...this type of human twinning must remain a plausible conjecture' (see Note 14; Corner, 1955). Second, the twinning at the blastocyst stage (days 4 to 7 after ovulation). Corner rationalized that 'if during this period some accident of development causes division of the inner cell mass, or starts the growth of two inner cell masses, then we have the beginning of twin embryos that will be enclosed in a single chorion'. He illustrates this possibility with Assheton's and Streeter's non-human specimens, and describes the findings from four own specimens (see Note 15; Corner, 1955). He concluded that 'obstetricians are familiar with this arrangement as seen at term, when careful examination of the afterbirth reveals the doubled amnion and permits the retrospective deduction that the two infants must have come from two inner cell masses in one blastocyst' (see Note 16; Corner, 1955). Third, the latest variety of twinning, the duplication of the embryonic rudiment of the germ disc, at about 15 days. This event can occur 'if two embryonic nodes develop instead of one. Speaking in the technical language of general embryology, this process is one of double gastrulation...When such twinning occurs in man...two embryos will appear on the floor of the amniotic cavity, and will have a common yolk sac. This arrangement will persist until birth, when the obstetrician will find two infants in a single chorioamniotic chamber'. Corner could not find a satisfactory illustration of this kind of twinning with normal embryos in an early stage (only damaged specimens and some cases of conjoined twins).

The triumph of the model

In a few years, the model evolved from a morphological theory into a precise record of facts. The reception of the model by the scientific community was almost immediate and approving. Published at the end of 1955, the next year Corner's article was included in some bibliographical reviews (see Note 17; Anonymous, 1956; Author, 1956; Ebert, 1956; Greenhill, 1956); in 1957 it was repeatedly cited in books, journal articles or short communications (see Note 18; Beck & Rosenthal, 1957; Craig, 1957; Librach & Terrin, 1957; Morton, 1957; Osborne &

de George, 1957). In successive years the number of citations grew steadily both in journals and books (see Note 19; Stern, 1960; Benirschke & Driscoll, 1967; Strong & Corney, 1967; Bulmer, 1970; Boyd & Hamilton, 1970; O'Rahilly, 1973). Fifteen years after its publication, the model became the standard wisdom (see Note 20; Corner, 1955; Benirschke & Driscoll, 1967; Strong & Corney, 1967; Dollander, 1970; Leroy, 1985; Nance, 1990). Remarkably, despite the lack of direct embryological evidence, the model was accepted as proven. Contrasting views were few and, as time passed, weaker (see Note 21). For decades, no other alternative theory disputed its supremacy.

Various factors have contributed to the rapid diffusion and acceptance of the model. Undoubtedly, the indisputable scientific prestige of Corner was a determining factor for its success (see Note 22; Hertig *et al.*, 1956; Corner 1981). Corner's ideas were supported by his outstanding academic achievements in the fields of endocrinology, human reproduction, medical education and history, and his 15 years as Director of the Department of Embryology of the Carnegie Institution (see Note 23; Hartman, 1956; Lord Zuckerman, 1983; Ramsey, 1994).

Another factor responsible for the triumph of the model was its internal, straightforward, logic: the model is quite reasonable. It connects mutually two variables: on one side, the morphology of the fetal membranes with its gradations of double/single (DC/DA, MC/DA, MC/MA); and, on the other, the 'three critical stages' (see Note 24; Corner, 1955), i.e. the successive stages of embryonic development at which twinning was thought to occur.

Finally, a third factor aiding acceptance of the model has been the convincing force of its graphic representation. Corner's original diagram and its numerous adaptations, as well as new versions produced by others over the years, made it readily comprehensible, and above all, gave a semblance of 'reality' to the model (see Note 25).

Surprisingly, Corner did not hold his theory in great esteem (see Note 26; Corner, 1955; Ebert, 1956; Corner, 1958; Corner, 1981). It would be interesting to know the reasons behind such an attitude. Probably, he realized that the embryological data he used could not confirm the core of his theory, because in none of the specimens was he able to observe directly the central event of MZ twinning: the splitting of the embryo. The specimens he examined belonged to post-splitting stages, not to the original moment of twinning: his 'observed embryology' was compatible with his theory, but unable to prove it.

Despite that fact, Corner convinced himself that his model corresponded to what really happened. Some meaningful modifications in his 1955 article attest to his change of mind. It suffices to compare

his 1922 and 1955 articles: *The Morphological Theory of Monochorionic Twins* was the title chosen by the young Assistant Professor of Anatomy of the Johns Hopkins University, a title revealing the tentative character of his proposal; while *The Observed Embryology of Human Single-Ovum Twins* suggests a matter-of-fact assertion by the prestigious Director of the Department of Embryology of the Carnegie Institution. Another example of the change of tone is the caption for Fig. 6 in his first article, which reads 'Hypothetical Diagrams of Human Monochorionic Twins'. Thirty-three years later, the same figure bears this other legend: 'Diagrams illustrating two types of single-ovum twinning in man'. The transition of theoretical proposal to factual description appears unmistakably clear.

Corner was not alone in 'seeing the light'. Benirschke, the leading authority on human placenta and twinning for the last half of the 20th century, made a similar transition. In 1965, he acknowledged the lack of data to establish with a certain degree of certitude the timing of twinning (see Note 27; Benirschke, 1965). Then, without any good reason in 1973, ignorance appears to be overcome and the old doubts cleared up (see Note 28; Benirschke & Kim, 1973).

Now for more than a half century, the model dwells in the realm of facts. Now, its adherents are not only biologists and physicians: with the expansion of bioethics, its main supporters are philosophers, theologians, jurists, bioethicists and politicians. The model has played a decisive role in the disputes on the legal and ethical status of the human embryo.

The weaknesses of the model

In fact, the rationality and persuasive force of the model have sufficed to quench the few criticisms raised against it, so that it has remained practically unchanged and, what is worse, unchallenged. But, if closely examined, one can find some weak points in its apparent solidity. The difficulties to explain the genesis of twinning increase as development of the embryo goes forward: for example, the suggested mechanism of formation of conjoined twins is complex and elusive in comparison with the simple mechanism proposed for the genesis of DC DA twins. The following are some weaknesses are found in all three critical stages.

Part (a)

Let us begin with the simplest case. It is said that DC DA twins result from the first cleavage division. Thenceforth the two cells become reciprocally independent; each of them initiates its separate

individual existence, and each one develops as a whole embryo. Despite inhabiting both the same pellucida, both make their own independent and autonomous passage from cleavage to hatching. Afterwards, they implant separately in the endometrium and separately develop their sets of membranes (see Note 29).

This short story is practically all the model indicates regarding the origin of DC DA twins. It conveys the comfortable idea that this type of twinning is due to a casual separation of the blastomeres, a random or fortuitous event. Some causes have been suggested for the partition of the embryo at this critical stage, as, for example, subtle genetic differences which translate into a repulsive force that leads to the splitting of the zygote (Hall, 1996), alterations of the calcium levels in the maternal environment or inside the embryo (Steinman & Valderrama, 2001), or a supposed absence or scarcity of binding molecules between embryonic cells (Bamforth *et al.*, 2003).

Also some objections have been raised against the plausibility of twinning at this critical stage. The co-existence of two embryos within the same zona has been considered unlikely by those convinced that early embryos tend to fuse together in the closed and progressively overcrowded space of the pellucida. They think, therefore, that twinning is by necessity a post-hatching phenomenon (see Note 30; Allen, 1969; Leroy, 1985). As pre-compaction morulas tend to fuse, others suppose that the independence of MZ twins within the zona is only possible after the differentiation of the trophoctoderm, so that twinning must be a post-compaction event (Tarkowski & Wojewodzka, 1982). To make more credible the separation of early blastomeres as a mechanism of twinning, a few authors have dispensed with the pellucida, at least in their graphic representations, as if twinning were easier in 'naked' zygotes (see Note 31; Salerno, 1959; Derom & Derom, 2005).

One last weakness of the model on the origin of DC DA twins consists in its inability of finding its own confirmation in IVF. Despite the countless human embryos produced and examined *in vitro*, the problem remains unsolved: the splitting and growth of twins within the pellucida has been never observed or live-recorded (see Note 32; Verpoest *et al.*, 2009; Knopman *et al.*, 2010).

Part (b)

The second critical stage (days 4 to 8 after fertilization) is, according to the model, the time when MC DA twins are generated. It is customary to attribute this type of twinning to the splitting of the ICM (see Note 33; Roberts & Fisher 2011; Roode *et al.*, 2012) or, rarely, to a supposed spontaneous development of two original ICMs (see Note 34; Sills *et al.*,

2000). Blastocysts with two separate ICMs have been observed in some non-human species (see Note 35; Assheton, 1893; Corner, 1955); in more recent times, they have been occasionally found in the course of human IVF (Meintjes *et al.*, 2001). To date and despite the use of time-lapse cinematography techniques, no-one has observed directly the instant at which an ICM splits into two, neither the simultaneous formation of two separate ICMs (see Note 36; Mio & Maeda, 2008). Occasionally the visualization of the hatching of two blastocysts, instead of one, from the same pellucida has been reported (Van Langendonck *et al.*, 2000).

The increase of MZ twinning linked to the practice of IVF has provoked a great interest in identifying the etiologic factors, in particular those responsible for the more frequent MC DA placentation. Many potential causes have been suggested for the fission of the ICM in two: a fissure in a hard and rigid pellucida can provoke an atypical hatching, with the result of a split ICM within an trophoctoderm (see Note 37), or of two separate complete blastocysts (see Note 38; Leroy, 1985; Edwards *et al.*, 1986; Behr & Milki, 2003); a disruption of intercellular communication inside the ICM (see Note 39; Aston *et al.*, 2008); a confluence into a continuous dissecting plane of dispersed groups of apoptotic cells in the ICM (see Note 40; Corner, 1955; Ménézo & Sakkas, 2002); and, finally, the ectopic adhesion of a piece of the ICM to the inner surface of the abembryonic trophoctoderm when the blastocyst collapses, with the result that, at re-expansion, the torn apart portion of the ICM can give origin to a twin (see Note 41; Payne *et al.*, 2007).

All these suggestions are a display of the ingenuity of their authors to find an answer to the riddle. Only suggestions that point to the possibility that an altered zona could bisect the blastocyst and the ICM have received a limited, but questionable support, in the literature.

Supposedly, *in vitro* embryo culture could afford some opportunities to observe the alleged process of twinning, but until now graphic evidence has been elusive or unconvincing. Certainly, twin blastocysts inside the pellucida are not subjects easy to detect, and, because of their thickness, more difficult still to photomicrograph (see Note 42). Moreover, photographs provide an image frozen in time: the use of continuous time-lapse video could help to observe what happens from beginning to end, that is, the whole course of the event (see Note 43). For now, one can only conclude that twinning took place before hatching when the ICMs appear situated at opposite poles of the twin blastocysts (see Note 44; Behr & Milki, 2003). Recently, there has been convincing documentation of the presence inside the pellucida of two independent blastocysts prior to hatching (See Note 45; Shibuya & Kyono, 2012).

Part (c)

The difficulties in explaining the mechanism of MZ twinning grow as embryo development progresses and its structure becomes more complex. According to the model, MC MA twins result from the splitting of the embryonic disc on days 8 to 12. By this time, implantation has begun, the amnion and the exocoelomic cavity have formed, and the ICM is deeply changed by hypoblast delamination and epiblast epithelialization. Such evolved embryonic structure represents an important challenge to the model.

The model affirms that with the splitting of the germ disc (now, the floor of the amniotic cavity) into two portions, two embryonic anlagen result that develop to MC MA twins. Although much attention has been devoted to the obstetrical consequences of this type of placentation, practically nothing new has been added to the understanding of its morphogenesis. Hertig suggested that MC MA twinning was possible on the condition that the cells of the germ disc were sufficiently undifferentiated to form two equally potential halves, a circumstance only possible before the appearance of embryonic axial arrangement (see Note 46; Coulton *et al.*, 1947).

The vague explanations offered to justify the low frequency of MC MA twinning (weakening of the twinning impetus, resistance of the embryo to splitting) have not contributed to the clarification of its genesis. There are no acceptable descriptions on how the disc can split into two halves, nor on how the resulting parts distance one from the other. This issue is important, as the type of separation (complete or incomplete) decides if the twins become separate or conjoined. Many questions (such as which forces are behind the splitting of the disc and the separation of its halves; how the amniotic epithelium can insinuate between those halves; and, if the splitting follows the longitudinal axis of the disc, how each half rebuilds its complementary missing parts, left and right) remain unanswered.

Part (d)

The deficiencies of the model culminate when the splitting theory tries to elucidate the twinning of the trilaminar embryo at 14 days or later. The majority of followers of the model limits their commentaries to state that the late splitting of the embryonic shield gives origin either to MC MA twins, or, if incomplete separation results, to conjoined twins. Other authors put forward the alternative hypothesis of a double gastrulation (see Note 47; Arey, 1922; Corner, 1955; McLaren, 1982). Only very few authors mention the hypothesis of embryonic fusion, but apparently

no serious critical evaluation on the plausibility of Spencer theory has been published (Spencer, 2000).

It must be asked: how and when a second primitive streak can be inserted in a germ disc? Until 40 years ago, drawing two parallel streaks on the same embryonic disc could be a simple mental experiment because, on the wake of Spemann–Mangold organizer’s transplant experimentation, the disc was considered then a clean slate, apt to receive new determinations at any moment, at any place or in any direction. Now, however, we know that, at least in the mouse, the embryonic shield is a highly organized structure, its cellular population show gradients of specific gene activation and signalling activity, and that these gradients flow in the proximal–distal direction as well in a centripetal direction from the ring of extraembryonic tissues to the centre of the disc (Tam & Gad, 2004). As regards the theory of double gastrulation it must be considered very unlikely (see Note 48; McGeady *et al.*, 2006).

When the complex organization of the embryonic disc at the molecular and cellular levels is taken into account, the splitting theory of the model does not seem a reasonable explanation for the origin of both late MC MA and conjoined twins. Notwithstanding, some authors insist on the capacity of the embryonic axes to divide into two parallel ones capable of inducing two new primitive streaks (see Note 49; Kaufman, 2004), or on the unlikely possibility that ‘if the streak splits along its length prior to completion of formation, conjoined twins result’ (see Note 50; Downs, 2008).

Conclusion

From this critical review it can be concluded that the current model of the timing of MZ twinning is not based on facts but only on apparently reasonable conjectures. When the model is analyzed in detail, it reveals itself as fragile and untenable. Consequently, from the scientific and bioethical perspectives, the model ought to be presented not as a reliable record of observed facts, but as a hypothetical construct. It lacks the required strength to adjudicate on biological or bioethical issues related to the early embryo. Its acceptance by many is grounded on an uncritical acceptance of these conjectures and the passage of time (see Note 51; Boklage, 2005).

Although this critical assessment goes against the grain, it has, however, some support in recent bibliography (see Note 52; Matias *et al.*, 2011). The model is inadequate and unable to explain some instances of twinning associated with assisted reproduction practices (Klein *et al.*, 2005). To overcome

the present situation it seems necessary to 'disenchant' the model and invite the proposal of new assessable theories on the mechanism and timing of human twinning.

Addendum

After so much criticism on the model, the author is uncertain about the convenience of adding here a short note on a theory of himself on the origin of MZ twinning. After due consideration, he feels obliged to present it for discussion and critical evaluation. A very similar theory on the origin of MZ twins based on a process of long fertilization that gives origin through an intermediate stage to two zygotes has been recently presented by López-Moratalla and Cerezo (see Note 53; López-Moratalla & Cerezo, 2011). My theory is based in two premises:

1. All MZ twinning is the result of the first zygotic division. In other words, in the case of MZ twinning, the first cleavage division of the fertilized egg, instead of giving origin to two blastomeres, generates twin zygotes.
2. The structure of the fetal membranes does not depend on the splitting of an embryo, but on different modes of fusion of the membranes of the twin embryos within the pellucida (or, in the case of conjoined twins, of the two embryonic bodies).

With respect to the first premise: this theory puts forward that there is a unique timing for human twinning: the division of the zygote (see Note 54). That proposal presupposes that the zygote can adopt two basically different types of cleavage: (1) the common type, that gives origin to two blastomeres; and (2) the twinning type, that generates twin zygotes. The twin zygotes (if there is no further MZ twinning to triplets or quadruplets) initiate their own embryonic development when each of them, shifting immediately to the standard developmental mode, cleaves to form its first two blastomeres.

As the cleavage of the zygote to form the first two blastomeres can be legitimately considered as the last event of the process of fertilization (see Note 55; Hertwig, 1906; Jones Jr & Schrader, 1987; Silver, 1987; Lejeune, 1992; Bompiani, 2006), it could be easily understood that twinning belongs to the fertilization process and, therefore, is not a post-fertilization event. An immediate consequence of this theory is a radical change in the chronology of MZ twinning: it shifts from the 14 days customarily assigned by the model based on embryo splitting, to around 24 hours after sperm penetration, when the process of transition from

zygote to blastomeres takes place. It could be the result of certain molecular and cellular decisions taken in that transition. Therefore the clarification of their nature and mechanisms acquires the utmost importance.

With respect to the second premise: in normal circumstances, the embryos, twinned or not, develop for the first 5 days within the pellucida. In the case of twinning, the eventual fusion of their membranes can in principle follow one of two basic paths.

In the first one, no fusion is produced between the two closely apposed trophoctoderms. Both twins follow their own independent course through the stages of cleavage, morula and blastocyst. They remain tightly packed together within the zona until the moment of hatching, when two independent blastocysts emerge and expand, to implant and develop as DC DA twins. This is the same and well known history of the DC DA twins of the model, with only one difference: the starting point of DC DA twins would not be, as the model proposes, the splitting of blastomeres during the first four days after fertilization, but the production of two independent zygotes at the fertilization process.

The second path concerns monochorionicity. This type of placentation in its two forms of amnionicity (DA or MA) requires, according to this the new proposed theory, the fusion of the trophoctoderms of the two twins, an event that in all probability happens within the zona or during hatching. There are divergent opinions on the ability of the trophoctoderm of two embryos for mutual adhesion and fusion. Some maintain that fusion of embryos before trophoblast differentiation results in the formation of aggregate chimeras, never in the production of twins (see Note 56; Boklage, 2010). Others think that there are distinct possibilities of trophoctoderm fusion that depend upon the developmental stage (early or late) of the blastocyst. Redline has suggested that there could be a short period, immediately preceding the blastocyst stage, when trophoblast fusion is possible and compatible with the independent growth of the twins (see Note 57; Redline, 2003). Another circumstance that would favour fusion of trophoblasts is the phenomenon of repeated cycles of collapse and expansion of blastocysts. Its mechanism and meaning have not been clarified until now, but it can be suspected that the collapse of the blastocyst, with the emptying of the blastocoelic cavity and the draining of the fluid into the perivitelline space, cannot be done without some disruption of the trophoctoderm (see Note 58; Niimura, 2003). It can be speculated that welding the edges of such discontinuities could facilitate the fusion of blastocysts and bring about monochorionicity. Mio and Maeda have suggested the possible relationship between cycles of collapse-expansion and MC twinning (Mio & Maeda, 2008).

Lastly, the type of amnionicity could be determined, as Redline observes, by the distance separating the two ICMs within the fused twin blastocyst: 'If the inner cells were sufficiently far apart, dizygotic twins with separate amnions could develop' (see Note 59; Redline, 2003). The concept that proximity-remoteness of ICMs within the blastocyst can decide amnionicity is not new; it has a long history. Although controversial, it was widely accepted at the beginning of the twentieth century (see Note 60; Bumm, 1902).

For the mechanism of origin of conjoined twins, the fusion theory of Spencer appears to offer a more reasoned and acceptable explanation than the simplistic theory of very late and incomplete splitting.

A few years ago, Blickstein devised two main requirements for future new theories on MZ twinning (see Note 61; Blickstein, 2006). The first was the capability of give some reason of the consistent increase of MZ twinning with every method of assisted conception. The new theory that considers twinning as a fertilization event is in the best position to explain that IVF and its technical variants can affect directly the process of fertilization and be responsible for the observed increased incidence of twins.

The second provision required by Blickstein is that any new theory on MZ twinning must be able to explain why embryologists do not observe any physical splitting of the embryo in *in vitro* fertilization. If, according to the present theory, there is no embryo splitting, such a condition could be excused. Certainly, the visualization of two separate embryos within the confined space a single pellucida is technically a very difficult task, due to cellular overcrowding and the limited room for blastocyst expansion. Perhaps, continuous time-lapse video micrographic analysis and confocal microscopy could help to overcome this difficulty. In any case, we need a more complete knowledge of the molecular markers of the zygote-blastomere transition. Only then, the riddle of MZ twinning could start to be solved.

Notes

¹ The modular, repetitive, character of the preceding description of the model tries intently to imitate the style used by practically all authors. That does not mean that all of them adopt exactly the same timing schedule. Although the general trend in recent years is to follow a regular sequence of 4-day periods (1–4, 5–8, 9–12, and 13–16 or simply after 12), there are countless combinations of the time measured in days assigned to the different types of MZ twinning.

² Are very few the authors, and every time less in number, who remind their readers that the model rests on a supposition.

³ Frequently some complementary information is added (on the per cent distribution, the prognostic significance, and the pathophysiology, of the different types of placentation), with the result that the model's rationality and acceptability are enhanced. In fact, the credit enjoyed by the model as common knowledge dispenses with the use of bibliographic references for support.

⁴ The history of the ideas, old and new, on the mode of origin of twins is fascinating. The subject of twins and twinning has been the object of many anecdotal stories, in academic as well as in popular publications. But, to my knowledge, there is no up-to-date systematic and detailed account of evolving ideas on the timing of MZ twinning written from a scientific perspective. Some interesting but partial contributions can be found in 19th and early 20th century bibliography, for example: Sobotta (1901); Schwalbe (1906) particularly the chapter on the history and bibliography on Teratology pp. 5–21; and Patterson (1913) especially the section on theories of polyembryony on pp. 625–43.

⁵ 'Almost the whole mass of discussion now current in the literature of embryology and genetics is pure hypothesis constructed by reasoning backward from the observed anatomy of late stages, chiefly from the disposition of the foetal membranes. Even the term 'single-ovum twins' is an assumption when applied to mammals', Corner (1922) on p. 389. The article is now easily accessible at: <http://archive.org/stream/johnshopkinsmedi33john#page/n471/mode/2up>

⁶ *Ibid*, on p. 391.

⁷ Newman (1917) on p. 13. This 'geometrical' explanation, suggested originally by Kölliker and Schultze (Schultze (1897) p. 176; and completed by Bumm (1902) on p. 282, was further elaborated by Wilder (1904) p. 391.

⁸ Corner's 1922 article was cited approvingly by Grosser (1927) p. 64; Potter (1927); and Streeter (1924) on p. 85. Klein (1927), on the contrary, did not perceive its innovative character. The article was referred to, but not discussed, by others (Hughes, 1927).

⁹ 'Contrary to the current opinion, my observations show that some one-ovum twins have two separate placentas and also apparently two separate chorions': Siemens (1925), p. 645.

¹⁰ It was due in great part to the interest among obstetricians to publish cases of monoamniotic twins, considered an extraordinary rarity during the 1950s. For example, in 1954, Coulter's article was cited in two successive articles, with almost identical titles, published in the same issue of a journal: Conybeare (1954) and Hanes (1954).

- ¹¹ Corner had been then for almost 15 years the Director of the Department of Embryology of the Carnegie Institution of Washington, probably the most prestigious institution in the world devoted to embryological research.
- ¹² Corner (1955), on p. 934.
- ¹³ The credit for the figures, previously published in the *Carnegie Institution's Contributions to Embryology* (no bibliographic reference is given), is attributed to Hertig, Rock & Heuser.
- ¹⁴ Corner (1955), on p. 936.
- ¹⁵ *Ibid*, on pp. 937–42.
- ¹⁶ *Ibid*, on pp. 940–1.
- ¹⁷ Anonymous, 1956. For example, in a Review of Current Literature (1956). *J. Obstet. Gynaecol. Br Emp.* **63**, 459–79); in the Greenhill (1956). *Year Book of Obstetrics and Gynecology*. Chicago: Year Book Publishers, p. 258; in the annual report of the Carnegie Institution (Ebert, J.D. (1956). *Department of Embryology. Carnegie Institution of Washington. Year Book, 1955–1956*. Washington DC: Carnegie Institution of Washington: pp. 269–92, on p. 290.
- ¹⁸ See, among others: Beck & Rosenthal (1957), p. 546; Craig (1957); Librach, S. & Terrin (1957); Osborne & de George (1957); and Morton (1957).
- ¹⁹ The model of Corner (his ideas and his diagram) is cited in the most important reference works on human twinning, embryology or genetics along the 1960s and 1970s. For example, Stern, K. (1960), pp. 538–9; Benirschke & Driscoll (1967), p. 168; Strong & Corney (1967), p. 17; Boyd & Hamilton (1970), p. 314; Bulmer (1970), p. 27; O'Rahilly (1973), p. 38, and many others. The model became a 'classic'.
- ²⁰ 'George W. Corner (1955) published a remarkable paper in which he discussed the embryological mechanisms which might be responsible for the occurrence of these three varieties of monozygotic twins. Ever since, obstetricians and authors writing on twinning and placentation (Benirschke & Driscoll, 1967; Strong & Corney, 1967) have been following his views without exercising the same caution in interpreting the available data' (Leroy, 1985, p. 395) The enthusiasm for the model induced some authors to use too forceful a language: Dollander, for example, speaking on the model, says that there is no other possible explanation for twinning; and that the different types of MZ twins are the necessary result of duplication at the model's indicated stages (Dollander, 1970, p. 328) Not without a touch of humour, Nance could say that 'the party line holds that MZ twinning may occur at any time up until about the 10–14 days of embryonic life' (Nance, 1990, p. 647).
- ²¹ Some of them will be referred to in the next section on the weaknesses of the model.
- ²² A convincing proof is the tribute of admiration that Hertig, Rock and Adams included in his 1956 recapitulative article on their epoch-making studies on early human development: 'The authors are ever mindful of Dr Corner's contributions to this joint study: his scholarly interest, his friendly encouragement, his expert embryologic interpretation of the specimens and his unique editorial help in the publication of these stages of human reproduction' (Hertig *et al.*, 1956, p. 436). The admiration was reciprocal. On the Hertig and Rock's contribution, Corner wrote that it 'was an achievement worthy of the Nobel Prize' (Corner, 1981, p. 290).
- ²³ Leaving aside the numerous tributes to Corner as a historian of medicine, some eulogies on his scientific contributions have been published during his life (Hartman, 1956); and after his death (Lord Zuckerman, 1983; Ramsey, 1994).
- ²⁴ The expression 'three critical stages', introduced by Corner: 'There are three critical stages at which twinning might occur.' Corner (1955), p. 934, has been widely used in the literature as an introductory locution to the timing of twinning.
- ²⁵ I intend to do a critical analysis of the different 'families' of graphic representation of the timing of twinning.
- ²⁶ In his short autobiography (Corner, 1958), he does not make mention of it. In his large one (Corner, 1981), he makes a cursory allusion to 'some studies of early embryonic abnormalities and a thorough review of single-ovum twinning and other multiple births...based largely on specimens in the Carnegie collection of embryos and fetuses' (Ebert, 1956, p. 294). However, Corner listed his article on the observed embryology (Corner, 1955) at the end of his most representative scientific work. This situation was not the case with his 1922 article, which he never mentioned again.
- ²⁷ He wrote: 'As Hertig and Corner have stressed many times, we need much more descriptive data to construct a definitive table of human embryonic development. This deficiency is particularly severely felt in the latter portion of embryogenesis (when does the twinning stop being effective?)'. In the same article, the legend of the diagram on the timing of twinning reads: 'This diagram is presented in an attempt to draw attention to the deficiency of our knowledge concerning the timing of the origin of monozygous twins' (Benirschke, 1965, pp. 61–62).
- ²⁸ 'If twinning occurs before the setting aside of the cells that eventually make the chorion – i.e., once the inner cell mass is distinct from the blastocyst wall (about 2 or 3 days) – two chorions develop (DCDA). Thereafter, twinning cannot split the chorionic

- cavity, and MCDA placentation develops. Once the amnion has formed, MCMA placentas occur.' In this same publication, Benirschke, as a mark of his new certitude, deletes from his diagram the question marks and the captions for intermediate stages (amniotic plica, bipartite yolk sac) (Benirschke & Kim, 1973, p. 1278).
- ²⁹ The origin of DC DA MZ twins is not restricted, according to the model, to the separation of the two first blastomeres: it includes the splitting, in two numerically equilibrated groups, of the blastomeres of the pre-compaction morula. For simplicity, it will be mentioned only the separation of the first two blastomeres.
- ³⁰ For example, Allen said: '...separation of early blastomeres... is not thought to be a factor in human twinning since the unyielding zona pellucida would make a mechanical separation of blastomeres unlikely' (Allen, 1969, p. 34). And Leroy affirms emphatically: 'Nobody has hitherto described two distinctly separated morulae or blastocysts within the same zona. When kept together blastomeres always stick to each other and organize between themselves the formation of a single embryo. This is even true when blastomeres of two different species such as rat and mouse are experimentally combined *in vitro* to produce a single chimeric blastocyst' (Leroy, 1985, p. 396).
- ³¹ Drawings of two naked blastomeres were frequent in the 1950s, following the graphic representation introduced by Salerno (1959), p. 206. It persists until today: see, for example, Fig. 24.1 taken from O'Rahilly & Müller (1998), p. 158 and Fig. 5.11, on p. 48.
- ³² 'We have never observed an embryo splitting in half before the blastocystic stage on its own initiative over 15 years of laboratory experience. The absence of a description of such embryos in the literature points to the conclusion that investigators in other IVF laboratories have also failed to observe such a phenomenon' (Knopman *et al.*, 2010). Curiously the fact of no having been able to see splitted morulas within the zona induced some authors to almost negate its existence (Verpoest *et al.*, 2009, p. 2948).
- ³³ In the human embryo, putative hypoblast is segregated by the 7th day of development (Roode *et al.*, 2012). Therefore, the agent to which ICM splitting is attributed must act upon two different embryonic tissues (epiblast and hypoblast). In any case, once the ICM is split by whatever mechanism it may be, a further problem remains: how the resulting halves of the ICM distance one from the other. In a blastocyst with two ICMs, these may appear nearby one another or separate, even situated at diametrically opposite poles. From what we know at present on the dynamics of trophoblast stem cells growth, these are situated at the pole in contact with the ICM (Roberts & Fisher, 2011). It is then unlikely that the migrating trophoblast could contribute to the separation of both ICMs.
- ³⁴ Sills *et al.* (2000), p. 219.
- ³⁵ For example, beautifully illustrated by Assheton (1893) and also by Corner (1955) Fig. 6, p. 938.
- ³⁶ Mio & Maeda (2008), p. 660.e4.
- ³⁷ In this case, MC DA twins would result.
- ³⁸ Behr & Milki (2003). In this case, the two separate blastocysts can develop a DC DA gestation. Such possibility was suggested for the first time in human reproduction by Leroy, but he thought that atypical hatching was not likely to occur *in vivo* because in many species there is no hatching; instead, the zona dissolves under the activity of embryonic and uterine proteinases, Leroy (1985) and popularized by Edwards *et al.* (1986).
- ³⁹ Aston *et al.* (2008), p. 379.
- ⁴⁰ Ménézo & Sakkas (2002). It is interesting to cite in this context a forgotten suggestion of Corner: that selective cellular death can act as a dissecting knife dividing the embryo into two. Corner wrote in 1955: 'Sometimes, in the segmentation stage, perhaps when the first two blastomeres give rise to four, or at the sixteen- or thirty-two-cells stage, those cells which are to form the inner cell mass are differentiated from the others. Some accident of growth, for example, faulty division of a cell or two at the middle of the little group of embryo-forming cells, might split the tiny germ into two, before it is anatomically distinguishable as an inner cell mass. Thus two contiguous or only partially separated cell masses might arise... It is easy to imagine bizarre consequences, for instance, formation of two amniotic cavities with only one yolk sac.' (Corner, 1955, pp. 944–5).
- ⁴¹ Payne *et al.* (2007). Payne's hypothesis does not take in account the difference in adhesiveness to ICM between polar and mural trophoblast.
- ⁴² In the case of blastocysts with two ICMs at different depth, ordinary photomicrographs are unsatisfactory, because they lack the required depth of focus. Probably, the use confocal reconstruction of images could help in this aspect. Some technical resources are incompatible with a careful handling of the human embryo *in vitro*.
- ⁴³ An isolated photographic image does not capture action in time nor direction of movement. It can be interpreted arbitrarily. The photograph of an athlete at the instant of throwing the javelin could be interpreted as if he was catching a javelin coming from the air. Similarly, a static image of the hatching of twin blastocysts cannot help by itself to decide if both blastocysts were already present inside the

pellucida before hatching or if only one resided previously there and the rigid pellucida cleaved it in the process of hatching.

- ⁴⁴ This is the case, for example, of Fig. 2 in Behr & Milki (2003) cited in Note 15, on p. 1503.
- ⁴⁵ Shibuya & Kyono (2012), p. 256.
- ⁴⁶ Coulton *et al.* (1947), p. 120.
- ⁴⁷ The hypothesis of double gastrulation was introduced by Arey: 'There is considerable evidence to support the belief that the twinning impulse occurs relatively late, at about the moment of gastrulation, which in mammals corresponds to the time of the formation of a primitive streak; the process, then, is simply one of double gastrulation' (Arey, 1922, p. 247; Corner, 1955, p. 942). The idea that 'the single cell mass forms two primitive streaks, giving rise to two separate individuals was untiringly publicized by Ann McLaren (McLaren, 1982, p. 19), but she never outlined by word or sketch how that could happen.
- ⁴⁸ 'No credible description of embryonic discs with more than one primitive streak has been reported in mammals' (McGeady *et al.*, 2006, p. 39).
- ⁴⁹ For example, Kaufman (2004), pp. 511–2.
- ⁵⁰ Downs (2008), pp. 46–68.
- ⁵¹ Paraphrasing some words of Boklage, it could be said that 'Every source I have seen either directly states *the model* assumption as a given or includes a reference to provide authority for a statement of *the model* as a given. The included reference may include an earlier reference, which may include another and so on, but, regardless of the chain's length, the other end of the chain of references is always a statement, without physical evidence, of the...common knowledge' (Boklage, 2005, p. 258) (*italics of the present author*).
- ⁵² A few and dissenting voices can be heard amidst the clamor of approval of the model. Blickstein, for example, has emphasized the hypothetical nature of the model and the speculative character of the different mechanisms proposed (Matias *et al.*, 2011, p. 462).
- ⁵³ According to these authors, MZ twins are the result of a peculiar fertilization process. In essence, the first mitosis of the fertilized egg takes place before its polarization by calcium ions, giving origin to two totipotent cells (zygote phenotype). Such peculiar twinning fertilization process 'would include an intermediate state in which a non-polarized cell would be formed with the new biological identity (a new inherited genome), but still without the phenotype proper to a zygote. The division of this cell and the simultaneous polarization of the two resulting cells would generate two zygotes.' Such theory takes account of the observations on the role of calcium in the fertilization pro-

cess (López-Moratalla & Cerezo, 2011, pp. 197–200).

- ⁵⁴ As it is understood in this article, the zygote is a short-lived cell, in which lifespan coincides with the duration of the fertilization process.
- ⁵⁵ That fertilization is not an instant but a process is now accepted by practically all. There is disagreement, however, on the event with which fertilization concludes. One of the opinions – which I support – maintains that fertilization ends with the completion of the first cleavage mitosis (Jones & Schrader, 1987, p. 191; Silver, 1987, p. 38; Lejeune, 1992, p. 197; Bompiani, 2006, p. 102). The idea is not new: in old time biology, fertilization includes primarily the stimulation to development: 'Fecundation is stimulation to development. Eggs, until then incapable to divide are incited, after the penetration of the sperm, to divide and so produce a new animal' (Hertwig, 1906, p. 487).
- ⁵⁶ 'There is no reason to imagine that monochorionic twins could arise...by fusion of separate trophoblasts to form a single shared precursor to the chorion and a single shared precursor of the placenta...In the production of many thousands of experimental embryonic chimeras, no fusion between two differentiated trophoblasts was ever reported' (Boklage, 2010, pp. 114–5).
- ⁵⁷ Although Redline is dealing with the formation of dizygotic MC twins, his suggestion is applicable to MZ MC twinning: 'One could speculate that there is a short period when two late-stage, preblastocyst embryos containing inner cells that are committed but not yet differentiated to form an embryo and outer cells that are committed but not yet differentiated to form trophoblast might partially fuse to form a single monochorionic placenta' (Redline, 2003, p. 114).
- ⁵⁸ Niimura has observed, in contracted blastocysts, 'wide spaces' between trophoblast cells and the disappearance of the ridges that mark, on scanning electron microscopy, the presence of intercellular junctions (Niimura, 2003, p. 416).
- ⁵⁹ Redline, *ibid* (Redline, 2003, p. 114).
- ⁶⁰ A known textbook of obstetrics taught: 'If the ICMs lie sufficiently apart, then each of them shall build up its own amnion. Only in very rare instances, when the ICMs are situated closely nearby, a common amnion enclosing them is formed' (Bumm, 1902, p. 282). Bumm presents an illustrative graphical representation of the two situations.
- ⁶¹ Blickstein (2006), p. 236.

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