A twin-pronged attack on complex traits

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Before one starts the hunt for quantitative trait loci (QTLs) for a complex trait, it is necessary to show that the trait is genetically influenced. This evidence is most likely to come from the classical twin study—the demonstration that monozygotic twins are more similar for the trait than dizygotic twins. The strengths and weaknesses of twin studies are discussed, and it is suggested that, far from becoming irrelevant with advances in molecular biology, they can improve the efficiency of QTL detection and play an important role in unravelling developmental genetic mechanisms.

As human genetics turns from its recent successes in finding the monogenic disease genes to the much harder task of unravelling the etiology of the complex diseases, the time is ripe for a new look at an old design—the classical twin study, which compares the similarity of monozygotic (MZ) and dizygotic (DZ) twins. There is a growing realization that the maximum return on expensive molecular epidemiological investigations comes when they are conducted in the context of a well-designed family study (see Box 1).

Familial does not equal genetic

The starting point for the genetic dissection of complex traits is the demonstration that genes are an important determinant of whether an individual lies higher or lower on a scale of measurement or disease predisposition. The fact that a trait 'runs in families' is not sufficient evidence to assume that its etiology is genetic, for families may share predisposing environments as well as genes.

One solution to this problem has been to compare the similarity of adopted individuals with their foster parents in comparison to their biological parents from whom they were separated early in life. Heston's finding of five cases of schizophrenia among the offspring of 47 schizophrenic mothers given up for adoption[1] and Goodwin's similar finding of a high alcoholism rate in adopted offspring of alcoholics compared with adopted offspring of nonalcoholics[2] proved to be turning points in our appreciation of the role of genetic factors in the etiology of those diseases. For other traits, however, the adoption study is sometimes flawed by selective placement—the attempts by adoption agencies to match the foster home to the attributes of the biological parents. Modern contraceptive options have made adoptions increasingly rare and atypical, and so, valuable as its past contributions have been, this design has a limited future.

The perfect natural experiment

Fortunately, nature has provided a near-perfect design whose future seems as secure as that of humankind. MZ twins crop up with monotonous regularity in about 4 per 1,000 confinements, regardless of ethnicity or maternal age. DZ twins are also plentiful, although more so among black Africans (about 16/1,000 confinements) and Europeans (8/1,000) than in Japanese or Chinese (2/1,000; ref. 3). A woman of 37 is four times more likely to produce DZ twins than one of 18, so the tendencies to later childbearing and use of assisted reproduction techniques (which frequently produce multiple births) are likely to keep the DZ rate high.

From a strictly genetic viewpoint, there is no advantage in using DZ twins rather than ordinary sibling pairs. However, as virtually every trait of interest varies in expression with age (and, indeed, a trait may be influenced by different genes at different ages), DZ twins have the great virtue of removing this confounder from consideration, as well as matching MZ twins as closely as possible in the pre- and post-natal circumstances of gestation and rearing. From a practical viewpoint, twins have the additional advantage that they tend to be very cooperative research subjects, and around the world there now exist several large twin registries with longitudinal data on a wide range of biomedical variables. DZ twins are also less likely to have different fathers, a problem that besets other sibling studies.

Because MZ twins share all their genes (but see Box 2), whereas DZ twins share, on average, only one half of their segregating genes, comparison of MZ with DZ similarity allows one to estimate the relative importance of genes and environment. Behind this bald statement of the rationale of the classical twin study lie several important qualifications and assumptions—most of which, fortunately, are susceptible to empirical test[4]. The simplicity of the concept is also apt to mislead the novice into thinking that estimating the role of genes and environment is straightforward, whereas uncritical use of the twin method without due regard to sampling and design issues has too often led to dubious and even erroneous inferences[5].

Well-conducted twin studies are able to completely change the way we view problems and to re-orient entire research programs. For instance, autism had been attributed to a variety of causes, such as emotional coolness in the mother, until a landmark twin study found much higher concordance in MZ than DZ twins[6]. For decades, the focus of research on attention deficit hyperactivity disorder (ADHD) in children was on the possible role of dietary imbalance, food additives and the like. Several recent twin studies, however, have found heritabilities for ADHD around 0.8, suggesting a very major role for genetic factors[7]. It is harder to obtain good evidence for rare diseases, but by systematically asking all Canadian patients with multiple sclerosis whether they were a twin, researchers collected a large enough sample to show an MZ concordance of 26% *versus* a DZ concordance of 2%[8]. Recent successes in finding QTLs for multiple sclerosis[9] can in part be attributed to the impetus from these striking findings in twins.

Other cases are less clear-cut. Twin studies suggesting genetic influences on male and female homosexuality have depended on highly biased samples ascertained through the homosexual press, but they have nevertheless contributed to a radical re-

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Box 1 • Structural equation modelling

Until recently, the arcane mysteries of structural equation modelling in human quantitative genetics could perhaps be dismissed as irrelevant by molecular geneticists working with monogenic diseases that have a favorable signal-to-noise ratio. For complex traits, however, the signal is much weaker, and the most sensitive techniques of genetic epidemiology are needed to detect QTLs against the background of environmental and polygenic noise.

The term 'complex' usually implies that a trait is influenced by many factors—in which case, as the number of factors gets larger, it will have a distribution that is continuous and approximately normal, like height or cholesterol level. Maximum information about such traits resides in their continuous scale of measurement, and any attempt to divide the scale into categories will cause a loss of power for genetic analysis. For complex traits that can be scored only as dichotomous (for example, affected/unaffected, present/absent), nonparametric methods such as the use of affected relative pairs are likely to be the most robust for detection of linkage[36]. For many such dichotomous traits, however, there exist one or more continuously distributed liability scales (for instance, blood pressure for hypertension, bronchial hyperreactivity and serum IgE for asthma) or strong risk factors (for instance, moliness for melanoma) that may lend more power to detect relevant QTLs than the complex trait itself. For example, the diagnostic criteria of DSM-IV assume that attention deficit hyperactivity disorder (ADHD) is a discrete category, requiring that an affected child show a number of symptoms. A child with twelve ADHD symptoms scores the same as a child with eighteen symptoms (both are 'affected'). A child with five inattention and five hyperactivity symptoms (ten in total) does not qualify at all, whereas a child with six inattention and four hyperactivity symptoms (also ten in total) qualifies as ADHD affected[37]. It is clear that in this case, the affected sib-pair method would be less powerful than using the complete distribution of symptom counts.

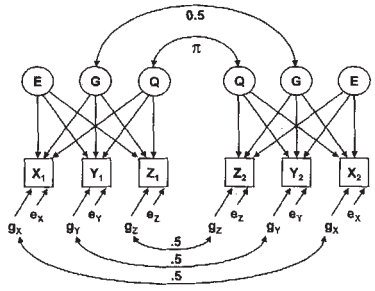


Fig. 1 Multivariate path model showing quantitative trait locus (Q), genetic background (G) and environmental factors (E) common to three phenotypes (X, Y and Z) plus genetic (g) and environmental (e) factors unique to each trait. Traits are measured in two siblings, or DZ twins.

If a QTL pleiotropically influences a number of continuous variables, causing them to be correlated, in most cases there will be a substantial increase in power to detect the QTL if a multivariate analytic method is used rather than analysing variables singly. One approach is to use structural equation modelling (SEM), which allows the co-variance among phenotypes to be adecomposed into a genetic part, which indicates the extent to which the same genes influence multiple phenotypes, and an environmental part, which indicates the extent to which phenotypic covariance is induced by the environment.

Applications of SEM to twin and family data usually model the genotype and the environment as latent, unobserved, variables whose presence is inferred, and effect size estimated from the inequalities in genetic relatedness of MZ and DZ twins and other relationships. However, measured information on the genotype or the environment is easily incorporated. Identity-by-descent (IBD) status in DZ twins, based on multi-point marker data, can be incorporated in a path diagram (Fig. 1). There are three measured phenotypes X, Y and Z (in boxes) that are observed in a series of DZ twin pairs or sibs. The correlation between sibs 1 and 2 for the common QTL that contributes to all three phenotypes is π (the proportion of genes at a marker locus identical by descent). Correlations between phenotypes and between sibs can also arise from background polygenic variation G (correlated 0.5 between sibs). Correlations between phenotypes, but not between sibs, can also arise from environmental influences unique to individuals (E). Each of the phenotypes can also be influenced by its own specific genetic (g_x,g_y,g_z) and environmental (e_x,e_y,e_z) factors. With the addition of MZ twins, the model could be further elaborated to include the effects of shared family environment and non-additive genetic influences.

This model is used to explore the gain in power to detect the QTL, as compared with univariate analysis. The total heritability (the sum of contributions from Q, G and gi) for all three phenotypes is 57%, but they are influenced to a different degree by the QTL. The variance due to the QTL is set at 28%, 14% and 7%, respectively. Analysing each variable separately, the number of sib pairs required to detect the QTL (with 80% power and a=0.001) is 1288, 5225 and 20938 pairs for X, Y and Z respectively.

Table 1 • Number of sib pairs required to detect a QTL (Q) influencing traits X,Y and Z

TRUE MODEL (% variance in X,Y,Z due to:)																ANALYTIC METHOD (sib pairs required to find QTL)	
Case	X					Y					Z						
	Q	G	g	E	e	Q	G	g	E	e	Q	G	g	E	e	multi-variate	mean
1	28	.	29	.	43	14	.	43	.	43	7	.	50	.	43	777	737
2	28	14	15	.	43	14	14	29	.	43	7	14	36	.	43	1,021	1,035
3	28	.	29	14	29	14	.	43	14	29	7	.	50	14	29	1,174	1,248
4	28	14	15	14	29	14	14	29	14	29	7	14	36	14	29	1,387	1,639
5	.	43	14	14	29	14	14	29	14	29	7	14	36	14	29	3,310	17,111
6	28	14	15	14	29	.	28	29	14	29	7	14	36	14	29	1,180	6,949
7	28	14	15	14	29	14	14	29	14	29	.	21	36	14	29	1,039	4,147

Numbers are shown for two different analytic strategies: multivariate and mean analysis.

To explore the power of multivariate analysis, four cases are considered: 1) the QTL is the only source of covariance between the three measures, 2) in addition to a common QTL there is also correlated genetic background (G), or 3) correlated environmental factors (E) or 4) both G and E. As well as a full multivariate genetic analysis, we consider the simpler strategy of a univariate analysis of the mean of the X,Y and Z phenotypic values. Table 1 shows the number of sib pairs needed to detect the common QTL using both methods. When the QTL is the only cause of correlation between measures (case 1), the gain in power (compared to the univariate case) is greatest. If the correlation between measures is due to other common factors in addition to the QTL (cases 2,3,4), the QTL influences become harder to detect but still compare favourably to the univariate situation. Depending on the multivariate model that is appropriate for the data, the greatest increase in power is achieved either by taking the average value of the three measures or by fitting the full multivariate model to the data. Suppose, however, that of the three correlated phenotypes, only two are influenced by the QTL (cases 5,6,7). In these cases, taking the mean value of the phenotypic measures gives drastically less power than fitting the full multivariate model. The numbers will vary according to the particular case, but the general point is that while one will occasionally be lucky analysing single variables or the mean of several correlated variables, the most powerful general approach for QTL linkage analysis in a set of correlated phenotypes will be explicit modelling of all the potential sources of covariation and specific variance. These simulations are for unselected samples. Although selection can greatly improve the power to detect a QTL, it is usually not easy in multivariate designs to decide which phenotype to select on. If, in addition to DZ twins, a sample of MZ twins measured for the same multiple phenotypes is available, it is possible to select sib pairs on their genetic scores on the (unobserved) common genetic factor that may harbour a QTL effect. By selecting on a high genotypic deviation, power is increased substantially[38]. Phenotypic data from the untyped MZ and DZ twins can be analysed simultaneously with phenotypic and genotypic data from the selected group of DZ twins and a large gain in power is achieved if the same QTL influences multiple phenotypes. With the new generation of software packages for structural equation modelling[35], these models are readily implemented.

thinking on the causes of homosexuality[10,11]. However, a recent large national twin study of sexual orientation failed to find any evidence for genetic influence on female homosexuality and only marginal evidence for a genetic contribution to male homosexuality[12]. More whimsically, twin studies have suggested a major role for genes in traits as diverse as television watching[13], social attitudes[14], divorce[15] and, at the sublime end of human existence, happiness itself[16]!

The use of twins to study the relative roles of 'nature and nurture' is most frequently attributed to Galton (1875), but in fact he had only the haziest idea that there were two sorts of twins, although the distinction between MZ and DZ twins had been proposed in France the previous year. The first use of the classical twin study, comparing similarities of MZ and DZ twins, was in 1924 by Siemens, who studied melanocytic naevi in twins[17]. For the next 50 years, a host of twin studies were performed on a wide variety of physical and mental traits.

MZ or DZ?

Many early twin studies were beset by doubts about the accuracy of zygosity diagnosis. However, with the discovery of blood groups in the 40's and 50's, and enzyme polymorphisms in the 60's, extremely low probabilities of misclassification could be achieved. With the increasing use of DZ twins for QTL linkage analysis, the need for accurate zygosity diagnosis is greater than ever, as the erroneous inclusion of MZ twins with DZ twins who are identical by descent for both parental alleles at a marker locus (IBD = 2) can generate artificial evidence for linkage, a fate that almost befell the first reported QTL linkage to a behavioural trait —dyslexia[18]. Fortunately, zygosity diagnosis is now easier and cheaper than ever; six to eight highly polymorphic DNA markers that can be run together in a single gel lane are sufficient to obtain an accurate zygosity diagnosis. However, because of the high postzygotic mutation rate of short tandem repeat polymorphisms, it is safest not to diagnose dizygosity until two differences between co-twins have been found.

How large do studies need to be?

A much greater problem with early twin studies was that generally they were far too small; experimenters used to the sample sizes required to compare means assumed that these were sufficient for the comparison of correlations between MZ and DZ groups. The field was revolutionionized in 1970 by publication of a now classical paper by Jinks and Fulker, which laid down a rigorous framework for a hypothesis-testing approach to the study of the genetic and environmental causes of individual differences[19]. In subsequent papers by Eaves and co-workers, these methods were extended to explore the utility of a range of twin, family and adoption designs, and to estimate the sample sizes required to discriminate between genetic and environmental hypotheses of causation[20,21].

The results of the power studies were quite alarming—to obtain a reasonably accurate estimate of the degree of genetic influence on a quantitative trait, at least 200 pairs need to be studied for a trait of high heritability, whereas ten or twenty times this number may need to be studied for traits of intermediate or low heritability—especially if other influences, such as family environment or genetic nonadditivity (dominance, epistasis), are also important[22]. For 'all or nothing' traits, such as disease status (affected *versus* unaffected), in which information from a continuous measure is unavailable, even larger samples of unselected twins need to be studied—the rarer the trait, the higher the number[23]. Thus, from earlier small-scale twin studies, alcoholism in females was previously thought not to be genetically influenced; only with a study of 2,000–3,000 twin pairs was it shown to be under the same degree of genetic influence in males and females, with a heritability of 0.7 (ref. 24). For rarer diseases, however, it is often more efficient to ascertain twins from among diseased individuals, particularly if there is a population-based disease register. Such small selected samples, in reality drawn from huge unselected samples, can contain dramatic messages about etiology, as in the cases of autism and multiple sclerosis mentioned above. Even so, the power may still be low, and it is vital to be on guard for ascertainment biases. It is important, too, to realize that the fact that traits have only moderate heritability does not mean they are unsuitable for molecular genetic analysis. For example, a Swedish twin study of breast cancer found a heritability of only about 0.4, but we now know that this averages some highly genetic, some moderately genetic and some very environmental etiologies[25].

These sobering power calculations reinforced the value of the large population-based twin registers that already existed in the Scandinavian countries[25,26], and led to the establishment of large volunteer twin registers in Australia, The Netherlands, Virginia and elsewhere. A modern windfall from the investment in big twin registries is the availability of large numbers of DZ twins that can be screened to ascertain extreme discordant sib pairs for QTL linkage analysis[28].

Advances in statistics—QTL linkage analysis

Hybridization of the Birmingham school's methods of biometrical genetics with the methods of path analysis, first developed by Sewall Wright and extended in the 70's by Morton, has led to a new flowering of human quantitative genetics. Many of the methodologic developments have occurred within the discipline of behaviour genetics, but they have equal application to non-behavioural phenotypes in the wider arena of genetic epidemiology[29,30].

Potential problems that may confound the simple dissection of genetic and environmental influence include variation in environmental sensitivity of different genotypes (genotype-environment interaction) and non-random placement of genotypes in the range of available environments, sometimes called the double-advantage effect (genotype-environmental co-variation; ref. 31). A special case of the latter is the effect that social interactions between relatives may have on the expression of certain traits (cooperation/competition or imitation/contrast effects; ref. 32). It is sometimes stated that such potential effects invalidate simple-minded attempts at variance decomposition. However, the empirical finding after twenty or so years of studies with adequate design and power to detect such effects is that few good examples of them have been documented, let alone replicated in humans.

A final caveat is that the results of variance decomposition in one population may not apply to a second population in which there are differences in exposure to the relevant environmental factors. For example, one might not expect the proportions of genetic and environmental variance in naevus density (moliness) in white Australians with tropical sun exposure to be the same as in people of similar genetic stock living in northern Europe or Canada.

The most fruitful developments in human quantitative genetics have been in the area of multivariate analysis, in which the concepts of biometrical genetics have been blended with maximum-likelihood factor analysis to explore the genetic basis of covariation between traits[33]. Initial applications made use of the structural equation modelling package *LISREL*[34], but Neale has now produced his own program, *Mx*, which has tailored structural modelling techniques to the needs of genetic epidemiologists in a powerful and versatile fashion (for example, missing values, ascertainment and, most recently, multivariate QTL linkage analysis[35]). The aim is to determine in what combinations

common genes pleiotropically influence a series of traits, and to what extent there are genetic effects specific to each trait (Box 1).

Getting the multivariate model right may greatly enhance the power for QTL detection[38], a point not lost on the new gene-hunting companies that have been competing to recruit structural modellers. One of the most striking results of such analysis has been the consistent finding that anxiety, depression and most of the other neurotic disorders appear to be influenced by just a single set of genes, and that their differentiation into distinct clinical entities appears to be due to environmental factors[39].

Frequently, however, the results of multivariate analysis can have a number of different interpretations, as the axes representing genetic and environmental factors underlying co-variation can be rotated arbitrarily. However, this problem, which has plagued factor analysis since the beginnings of the subject early in this century, can now be solved[40]. In the first published example of QTL linkage analysis, six QTLs were found for tomato weight and four QTLs for the correlated trait of solids concentration. The correlation between the traits is likely to be explained by the fact that three QTLs appeared to influence both traits[41]. In the behavioural domain, QTL linkage analysis was recently performed for four correlated tests for emotionality in mice; approximately six QTLs were found to be influencing each trait, of which three QTLs were common to all four traits and presumably central to the core dimension of emotionality[42].

As genome scans become cheaper, there is at last the prospect of untangling the complex web of pleiotropic gene effects on correlated traits. The results of such analyses will have immense practical implications both in food production and in medicine. In the human arena, where many confounding effects cannot be designed away as they can with experimental organisms, such complex analyses of pleiotropy are going to be most powerful within the structure of a twin-family design in which complicating factors and residual effects can be jointly estimated and allowed for, as shown in recent simulation studies[40,43].

Of course, once QTLs have been detected by linkage analysis, there remains the problem of identifying and cloning the culprits. Although one is painfully aware of the difficulty of doing this for traits in which critical recombinants cannot be unequivocally identified[44], one lives in hope that the age of positional cloning really is giving way to the age of positional candidacy[45], and that the era of high-density linkage disequilibrium mapping will soon be with us[46].

Problems—real and imaginary

As good as the twin design is, it is not without its problems and detractors. The most constant and potentially the most damaging criticism is that MZ twins share more similar post-natal environments than DZ twins, so their excess similarity cannot necessarily be attributed to their genetic identity. The objection has face validity, as it has been repeatedly shown that MZs do indeed experience more similar environments both as children and later in life. However, a series of ingenious studies, involving ethological observations and making use of mistaken zygosity diagnosis and differing degrees of separation, have all pointed to the conclusion that, for the most part, the more similar treatment of MZs is not the cause of their greater phenotypic similarity but, rather, a consequence of their genetic identity and the more similar responses this elicits from the environment[4].

The ideal way to altogether avoid the problem of more similar MZ environments is to use MZ twins separated at birth and reared apart in quite different surroundings. Unfortunately for genetic epidemiologists, MZ twins reared apart are very rare. Since 1979, however, Bouchard has accumulated a sample of more than 60 pairs and, for a wide range of behavioural and biomedical phenotypes, has found correlations close to those of MZ twins reared together[47]. Further confidence is lent to the generalizability of findings from twin studies when they are extended to include other relatives. In the 'Virginia 30,000' study, 20,000 relatives (parents, sibs, spouses and offspring) have been added to 10,000 twins in a study of various bio-behavioural phenotypes. Whereas the dissection of effects is much more detailed in such a powerful design, the point estimates of total genetic and environmental influence hardly differ from those of the twin data alone[48,49].

The validity of the twin method gets further empirical support from studies of biochemical measures whose causative genes are already known. A twin study recently found a heritability of 69% for plasma levels of histidine-rich glycoprotein, which is involved in coagulation and fibrinolysis. When the responsible polymorphism was identified, it turned out to explain that nearly the same amount of variance as was found in the twin data[50]. A similar story was found for the protease inhibitor polymorphism and serum levels of α_1-antitrypsin in twins[51]. The attraction of performing one's polymorphism association studies within the context of a twin study is that not only does one have in-built ethnically matched controls[27] but also one gets simultaneous estimates of how much genetic variance is accounted for, and is *not* accounted for, by the polymorphism.

Another challenge to the twin-study concerns the biological typicality of twins, and MZ twins in particular. It has been alleged that the circumstances of MZ twin gestation and presentation are so atypical, and fraught with danger, that many types of disease are more common in MZs both at birth and later in life[52]. Although this fear may be valid in certain conditions (for instance, cerebral palsy), it is otherwise greatly exaggerated. There is some evidence that twins are in fact disadvantaged in gestation and early rearing, but equally good evidence that most such disadvantages are 'washed out' by age five[53,54].

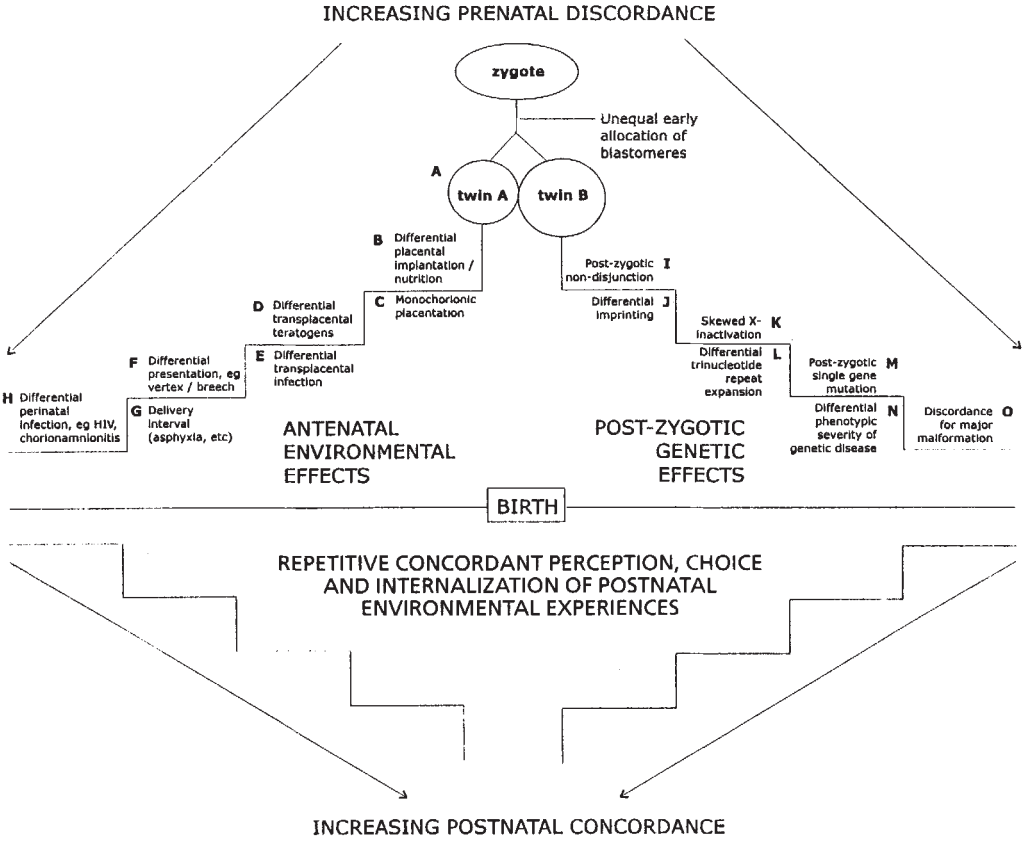
Nevertheless, the criticism reinforces the value of collecting good placentation data on twins. About 65% of MZ twins are mono-chorionic (MCMZ), and it is in these that the major gestational anomalies occur. If MCMZ twins can be shown to have quite different correlations from the rarer class of di-chorionic MZ twins (DCMZ), the assumptions of the twin method may indeed be called into question. The effect could go in either direction, with MCMZ twins more, or less, similar than DCMZ pairs. The former might occur if some consequence of shared blood supply (viral infection, for instance) make MCMZ twins more similar, whereas the latter would be expected if the anastomoses in the shared vasculature—or other anomalies due to sharing a crowded chorion—produce greater differences between MCMZ than less cramped DCMZ pairs. There are few twin resources with good placentation data and adult phenotypes for comparison, so such effects remain the field of speculation. However, investment in such a resource is likely to yield rich rewards in our understanding of developmental etiology.

What makes MZ twins different?

Another, less queried but more important assumption of the classical twin study is that MZ twins are in fact genetically identical[55]. It is assumed that the extent to which MZ twins differ from each other must be due to environmental differences, by which it is tacitly assumed that these are the exogenous environmental factors that are the focus of epidemiologists. The singular lack of success in identifying such exogenous environmental factors to account for MZ discordance for behavioural and all but a few disease phenotypes[56–61] might lead the sceptic to ask whether MZ discordance might be due to endogenous accidents of development and differentiation, from perturbations in the gradients of developmental fields to somatic mutation, somatic recombina-

Box 2 · How identical are MZ twins?

There are many reasons why MZ twins might be less than fully identical[61]. A wide range of ante-natal genetic and environmental influences can cause phenotypic and genotypic divergence. Reconvergence may occur after birth because the twins do not passively undergo differing experiences; on the contrary, it now seems likely that they actively seek, select and perceive similar environments because of genetic similarities in brain physiology.



A, Evidence from X-inactivation studies in MZ female pairs heterozygous for Duchenne muscular dystrophy shows larger 'clones' of dystrophin positivity and negativity in muscle biopsies of the affected than non-affected twin, implying origin of tissues from a smaller number of 'founder' cells. Affected twins always have lower birth weights. If critical numbers of embryonic cells are required at specific times and places to respond to molecular signals for initiation and completion of key steps in differentiation and growth (including paracrine signalling), such cell numbers may not be present in the twin with a smaller number of founder cells.

MZ and DZ twins do not always share similar intrauterine environments. B, There is only one optimal placental implantation site, and it is unlikely that both twins, whether di-chorionic DC or monochorionic $(MC)_7$ will be able to benefit from this site. In DC twins, both MZ and DZ, one placenta may be differentially affected by disease, with sub-optimal nutritional function for that fetus. **C**, MCMZ twins frequently have severe complications from inter-fetal vascular connections in their truly single (not fused) placentas. These include twin–twin transfusion, twin reversed arterial perfusion, unequal sharing of the parenchyma and organ infarction after fetal death of one twin. **D,E**, Equal doses of transplacentally transmitted agents are not necessarily delivered to both twins, particularly if the twins are DC. **F**, It is common for twin pairs to be delivered from different presentations. Breech presentations are associated with risks of brain trauma, causing discordance. **G**, If the delivery of the second twin is significantly delayed, there are risks of asphyxia and brain damage to that twin. (This effect may be confounded by different presentations.) In rare cases, delivery of the second twin is delayed for days or even weeks. There are no published data on the post-natal development of such twins, who would all be DC, and some, therefore, MZ. **H**, Ascending infection (chorionamnionitis) affects the gestation sac lower in the uterus, and this twin is usually born first. Perinatally acquired HIV infection more commonly affects the first-born twin.

MZ twins may be genetically discordant. I, MZ twin pairs may have different chromosome constitutions because of post-zygotic non-disjunction. This can even result in discordant external genital sex (for example, in a 46,XY and 45,X pair). This type of non-disjunction could be termed 'confined twin mosaicism', and is further complicated by the potential in MC twins for transfer of hematogenous stem cells *via* interfetal vascular anastomoses, giving 'pseudo-mosaic' results from lymphocytes when fixed somatic cells (for instance, fibroblasts) may give pure cell-line results from each twin. **J**, Several MZ twin pairs have been found who are discordant for Beckwith-Wiedemann syndrome, in which the gene is imprinted. Differential methylation in multiple sites could have an overall effect on the phenotypes of MZ twin pairs. **K**, Skewed X-chromosome inactivation has been found in some female MZ twins who are discordant for the phenotypic expression of X-linked diseases. The twins may show 'reciprocal skewing', or one may have skewed and the other random X inactivation. The latter pattern may be caused by the sequestration in the twinning process of a small group of blastomeres that are, by chance, non-randomly X-inactivated (A). It has been suggested that apparently singleton females with skewed X inactivation may be 'MZ twin survivors'. **L**, Differential trinucleotide repeat expansion has been reported in MZ twin pairs. This raises the possibility that DNA methods of zygosity diagnosis will never permit the establishment of monozygosity because genetic differences could be found in all twin pairs if carried out exhaustively. There is a need to establish confidence limits within which MZ status is determined on the basis of genetic differences that are not large enough to imply dizygosity. Ultimately, perhaps only MC twins could be accepted as MZ, and this would be unfortunate because of the atypical phenotypic features caused by monochorionicity (C). **M**, No convincing cases have yet been reported of MZ twins discordant for single-gene diseases on the basis of post-zygotic mutation. However, MZ twins could be discordant for single-gene disease through reversion from a trisomic zygote, with uniparental disomy in one twin but not the other. **N,O**, MZ twin pairs seldom show equivalent severity of expression of genetic diseases and malformations. Possible explanations include A, above.

tion, differences in tissue-specific methylation patterns and the timing of such events[62,63]. Any of these mechanisms, beginning with perhaps some slight inequality during development, might ultimately lead to a difference in disease phenotype[64] (Box 2).

There are already well-documented examples of such phenomena for X-linked diseases for which differences in X-inactivation patterns between female MZ co-twins heterozygous for a disease allele can result in striking discordance for X-linked diseases such as Duchenne muscular dystrophy[65,66]. Might not similar inequalities in timing or degree of methylation of autosomal genes in specific tissues cause discordance for other diseases? Such speculations might be nothing more than the recondite hobby of gemellologists if there were not a nagging feeling that such phenomena might hold the key to much that we do not understand in disease etiology. The challenge is now for twin researchers, in concert with molecular and developmental biologists, to find new techniques to exploit this superb natural experiment.

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