Height Discordance in Monozygotic Females is not Attributable to Discordant Inactivation of X-linked Stature Determining Genes

S.C. Healey[1], K.M. Kirk[2], V.J. Hyland[1], C.F. Munns[3], A. K. Henders[2], J.A. Batch[3], A.C. Heath[4], N. G. Martin[2], and I.A. Glass[5]

[1]*Molecular Genetics Section, Pathology Department, Royal Brisbane Hospital*
[2]*Queensland Institute of Medical Research and Joint Genetics Program, University of Queensland, Brisbane, Australia*
[3]*Royal Children's Hospital, Brisbane, Australia*
[4]*Department of Psychiatry, Washington Unviersity School of Medicine, St Louis, Missouri, USA*
[5]*Queensland Clinical Genetics Service, Brisbane, Australia*

We tested the hypothesis that X-linked genes determining stature which are subject to skewed or non-random X-inactivation can account for discordance in height in monozygotic female twins. Height discordant female monozygotic adult twins (20 pairs) were identified from the Australian Twin Registry, employing the selection criteria of proven monozygosity and a measured height discordance of at least 5 cm. Differential X-inactivation was examined in genomic DNA extracted from peripheral lymphocytes by estimating differential methylation of alleles at the polymorphic CAG triplet repeat of the Androgen receptor gene (XAR). There were 17/20 MZ pairs heterozygous at this locus and informative for analysis. Of these, 10/17 both had random X-inactivation, 5/17 showed identical X-inactivation patterns of non random inactivation and 2/17 (12%) showed discordant X-inactivation. There was no relationship between inactivation patterns and self-report chorionicity. We conclude that non-random X-inactivation does not appear to be a major contributor to intra-pair height discordance in female MZ twins.

The utility of twins in the estimation of heritability of various traits is based on the assumption that MZ twins are genetically identical (Martin, Boomsma et al., 1997). There is growing evidence, however, that monozygotic (MZ) twins are rarely completely identical and that genetic discordance and differences in intrauterine environment may result in MZ twin discordance for various phenotypes (Machin, 1996). For example, the process of X-inactivation, a possible cause of MZ twinning in itself (Goodship, Carter et al., 1996), may relate to various types of discordance arising within the cells of the developing embryo (Hall, 1996a).

In somatic cells of normal females, one X-chromosome is inactivated in early embryogenesis to enable dosage compensation for most (but not all) X-linked genes, so that males and females have approximately equal amounts of gene products (Lyon, 1961). X-inactivation in early human development is believed to be random and females are accordingly mosaic for two populations of cells, expressing alleles from one or the other X-chromosome (Davidson et al., 1963). Non-random X-inactivation of otherwise structurally normal X-chromosomes has been shown to be the cause of discordant phenotypic expression of X-linked disorders, notably Duchenne muscular dystrophy, X-linked mental retardation, red-green color blindness and Hunter disease (Machin, 1996). What has not been investigated is whether discordant X-inactivation patterns in MZ co-twins contribute to intra-pair differences in quantitative phenotypes.

Stature is a classic example of a quantitative trait with a high heritability. Using parental-offspring measurement data, Galton was the first to estimate the heritability for height. His estimate of 0.83 is almost identical to that from contemporary Finnish twin data reported in this issue, confirming the predominant role of genetic variance for at least the last 150 years (Vogel & Motulsky, 1997). However, the well documented increase in height over the same period indicates that stature is also influenced by secular trends in environmental conditions to which the whole population is subjected (almost certainly improved childhood nutrition) (Vogel & Motulsky, 1997).

In this communication, we estimate the heritability of height in a large Australian twin sample and from it select height-discordant female MZ twins to investigate whether X-linked stature determining genes, subject to X-inactivation processes could account, at least in part, for intrapair discordance of this polygenic trait in female MZ twins.

Materials And Method

Subjects

Twins participating in this study were first surveyed via a self-report Health and Lifestyle Questionnaire which was

Address for Correspondence: Dr Nicholas G. Martin, Genetic Epidemiology Laboratory, Queensland Institute of Medical Research, Post Office, Royal Brisbane Hospital, Brisbane QLD 4029, Australia. E-mail: nickM@qimr.edu.au

Received 7 November, 2000, accepted 5 December 2000.

https://doi.org/10.1375/twin.4.1.19 Published online by Cambridge University Press

mailed in 1980–1982 to all twin pairs over 18 years of age who were registered with the Australian Twin Registry (5867 pairs). Responses were received from 3808 twin pairs and 576 singles, including self-report information on height from 3667 complete pairs. Self-report information on birthweight (Treloar et al., 2000) and chorionicity (Duffy, 1993) was also available. The item on chorionicity was phrased as follows: "How many placentas (afterbirths) were there at birth? (single / 2 joined / 2 separate / don't know)". Where possible, self-report birthweight and chorionicity information was checked against information provided by the twins' mothers.

Between 1993 and 1998, subsets of this cohort of twins were involved in one or more of a range of studies involving a clinical examination (Bellamy et al., 1999; Duffy et al., 1998; Heath et al., 1997). As part of three of these studies, participants' heights were recorded, resulting in standardised clinical measures of adult height for 2463 individuals from whom a blood sample was also collected and lymphocyte DNA extracted.

For the selection of twins for the X-inactivation study, the clinical height measurements of adult MZ female twins from the above sample were inspected. Those MZ twins with height differences of 5 cm (equivalent to ± 0.83 SD for adult height) or more were identified and their DNA was submitted for molecular analysis.

Zygosity Determination

In the overall sample of 3808 twin pairs, zygosity was determined by twins' responses to standard items about physical similarity and the degree to which others confused them with one another. This method has been shown to give at least 95% agreement with diagnosis based on extensive blood typing (Eaves et al., 1989; Martin & Martin, 1975).

Since the analysis of data from monozygotic twins only is crucial to the testing of our hypothesis regarding height and X-inactivation, the zygosity of those height-discordant twin pairs judged to be MZ by the above method was confirmed by molecular analysis of 9 standard markers (short tandem repeat loci D3S1358, vWA, FGA, D8S1179, D21S11, D18S51, D5S818, D13S317 and D7S820) plus a segment of the amelogenin gene indicating sex using a commercial kit (AmpFlSTR Profiler Plus Amplification Kit, ABI). These results were cross-checked with blood group results for the ABO, MNS and Rh systems (Australian Red Cross Blood Service) and/or phenotypic data (hair, skin and eye colour), giving an overall probability of monozygosity for those pairs of greater than 99.99%.

Statistical Methods

Preliminary statistical analyses were conducted using SAS 6.11 (SAS Institute, 1996), while Pearson correlations between the self-reported stature of twins and their co-twins calculated using PRELIS 2.1e (Jöreskog & Sörbom, 1993). However, while significant twin correlations establish the fact that there is familial aggregation for the measures of interest, they do not distinguish between the possible mechanisms by which this arises. Structural equation modelling is used to make this distinction, by considering which combination of additive genetic (A), shared environment (C) and unique environment (E) effects provides the most parsimonious explanation for the observed pattern of MZ and DZ twin correlations. Models were fitted in LISREL 8.1e (Jöreskog & Sörbom, 1993) using the maximum likelihood fit function which employs the covariance matrices estimated by PRELIS 2.1e. We began by specifying a complete decomposition for three sources of variance — additive genes, shared environment and unique environment. This full model was then simplified by successive dropping of non-significant parameters (i.e., by seeing whether dropping a parameter resulted in a significant increase in the goodness-of-fit chi-square).

Molecular Analysis of X-Inactivation Status

The human AR gene contains a polymorphic CAG repeat in the coding region of Exon 1. Since only one allele on the X chromosome is commonly active in female cells, a differential methylation assay based on restriction enzyme analysis using HpaII and HhaI allows identification of the inactive allele. Results of this assay can be confirmed by performing PCR, specific for positive methylation status. Thus a PCR product will only be obtained for the inactive X allele. Where non-random X inactivation is present, the more commonly inactivated allele will be preferentially amplified.

Genomic DNA was extracted from peripheral lymphocytes obtained from blood samples provided by the MZ pairs, using standard techniques (Miller et al., 1988). Screening these pairs through the X-linked AR locus by amplifying this region using specifically designed primers and the polymerase chain reaction (PCR) enabled detection of heterozygotes (Allen et al., 1992). Modifications were made to this protocol to improve the amplification efficiency of this reaction, with the addition of 10% DMSO to the PCR reaction buffer and increasing primer concentrations. The PCR products were resolved on a 5% denaturing polyacrylamide sequencing gel (PAGE), with MZ twins being loaded in adjacent lanes. DNA from individuals who were identified as being heterozygous at this locus was then digested in two separate reactions with HpaII (New England Biolabs) and its isoschizomer Hha I (New England Biolabs). These digested products were then reamplified for the AR locus (using the same primers). These second round amplified products were then run against the first round non-digested PCR products on a 5% denaturating PAGE gel with the same loading configuration of adjacent lanes for each MZ twin member. Determination of inactivation status at this locus was determined by visually inspecting band intensities between the resolved PCR fragments. Inactivated alleles preferentially amplify and produce a stronger band in the second round amplification compared to the amplification of the undigested DNA.

Results

Through the participation of many twin pairs in multiple studies, it was possible to compare self-reported stature in the questionnaire-based study conducted between 1980 and 1982 with clinical height measurements taken in subsequent studies (1993–1998). The correlation between self-report and clinically measured height was 0.90 ± 0.01 (s.e.) for both females (1660 subjects) and males (803 subjects).

https://doi.org/10.1375/twin.4.1.19 Published online by Cambridge University Press

Twin correlations obtained for the various zygosity groups for self-reported and clinically-measured stature are shown in Table 1. These correlations are substantially higher for male and female MZ twin pairs than for their DZ counterparts, indicating the presence of additive genetic effects. Structural equation modelling of the self-report data gives the results presented in Table 2. The full ACE model (incorporating additive genetic, shared environment and unique environment effects) fits the data quite well, as does the reduced AE model obtained when shared environmental effects are removed from the model ($\chi^2_1 = 2.46$, $p = 0.117$). Inspection of the fit of the CE model (which does not include additive genetic effects) demonstrates that shared and environmental influences alone cannot adequately explain the pattern of similarities in stature between twins, while a comparison of the fourth (E) model to the AE model confirms highly significant familial aggregation for stature. The heritability estimate for stature in this sample is given by the proportion of variance attributable to additive genetic influences (0.88), remarkably consistent with Galton's first estimate over 100 years ago.

Clinically measured height data were available for 488 monozygotic female twin pairs. Intrapair height differences were found have a small but statistically significant correlation with the self-reported difference in birthweight between the twins ($r = 0.16$; $p < 0.001$). However, there were no significant differences in means between the twin pairs reporting themselves to be monochorionic monozygous (MCMZ) and those reporting themselves to be dichorionic (DCMZ).

Table 1

Twin Pair Correlations (with standard error) for Self-report and Clinical Measures of Stature

	Self-report height measure		Clinical height measure	
	Number of pairs	Correlation ± Standard Error	Number of pairs	Correlation ± Standard Error
MZ female	1193	0.88 ± 0.01	526	0.92 ± 0.01
MZ male	546	0.90 ± 0.01	202	0.91 ± 0.02
DZ female	726	0.47 ± 0.03	277	0.47 ± 0.05
DZ male	330	0.42 ± 0.05	108	0.34 ± 0.09
DZ female-male	445	0.48 ± 0.04	159	0.40 ± 0.06
DZ male-female	427	0.46 ± 0.04	112	0.41 ± 0.09

Table 2

Model Fitting for Self-report Measure of Stature. Proportions of variance attributable to additive genetic, shared environment and unique environment effects are represented as A, C and E respectively. The chi-square goodness-of-fit (χ^2) and degrees of freedom (*df*) are also shown, along with the corresponding *p*-value.

Model	Proportions of Variance			Model fit		
	A	C	E	χ^2	*df*	*p*-value
ACE	0.83	0.05	0.12	16.51	15	0.35
AE	0.88	—	0.12	18.97	16	0.27
CE	—	0.66	0.34	989.51	16	< 0.001
E	—	—	1.00	1794.82	17	< 0.001

Molecular Analysis of X-Inactivation Status

We identified from the above sample 20 adult MZ female twin pairs in which clinically measured adult intrapair height differences of 5 cm or more were present. The mean age of these MZ twins was 49 years (32–77 years) at the time of measurement and DNA collection, and the range of the intrapair height differences was 5cm to 9cm with a mean height discrepancy of 6.25cm.

As an initial step, we determined that 17 of these 20 pairs were heterozygous for the AR triplet CAG repeat. Of those 17 pairs, 10 pairs showed approximately equal band intensities for digested and undigested DNA for both members of the MZ pair, indicating random X-inactivation in this tissue in both twins. Of the remainder, 5 pairs of MZ twins displayed identical patterns of non-random X-inactivation to be present between twin 1 and twin 2. Band intensities differed between digested and undigested DNA, but for each twin member the bands were identical, indicating concordant skewed X-inactivation to be present, which could not therefore explain intrapair height differences.

Two MZ twin pairs had discordant skewed X-inactivation present in lymphocytes. In one of these two pairs (intrapair height difference = 6 cm), both twin members showed preferential amplification of the same allele in the digested DNA-PCR, compared with the undigested DNA-PCR, but this amplification was markedly more pronounced in one twin member, indicating discordant non random X-inactivation to be present. In the second of these discordant MZ twin pairs (intrapair height difference = 5 cm), twin 1's digested DNA-PCR showed preferential amplification of the larger allele, while twin 2's digested DNA-PCR showed preferential amplification of the smaller allele, again indicating discordant non random X-inactivation to be present. As shown in Figure 4, these two pairs reported themselves to be monochorionic MZ. The two twin pairs reporting themselves to be dichorionic MZ have larger intrapair height differences, but random X-inactivation or X-inactivation skewed in the same direction.

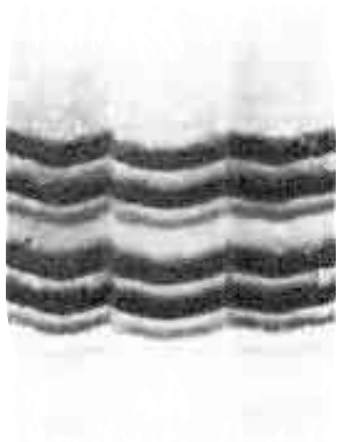

Figure 1

Random 50 50 concordant inactivation

https://doi.org/10.1375/twin.4.1.19 Published online by Cambridge University Press

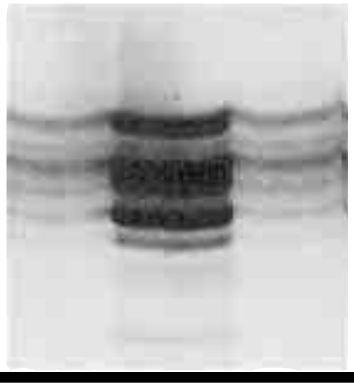

Figure 2

Skewed concordant inactivation

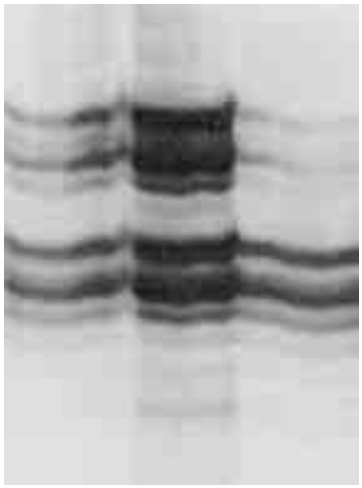

Figure 3A

Example of skewed discordant inactivation

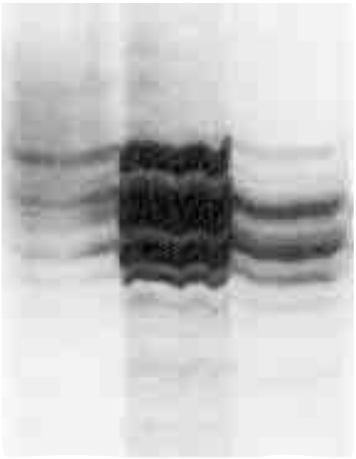

Figure 3B

Example of skewed discordant inactivation

Figures 1–3 *Lane one* Twin 1 digested with HpaII prior to PCR, *Lane 2* standard PCR, *Lane 3* Twin 2 digested with HpaII prior to PCR

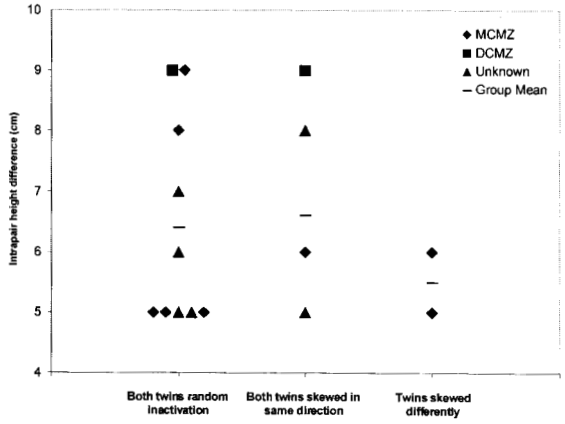

Figure 4

Intrapair height difference and X-inactivation pattern in monozygotic twins, by self-reported chorionicity.

Discussion

As expected from other studies, variance in stature in this sample can be attributed mostly to additive genetic influences and some unique environmental effects, with a heritability estimate of 0.83, and the possibility of a small shared environmental effect accounting for approximately 5% of variance. However, as observed elsewhere (Eaves et al., 1999, Phillips & Matheny, 1990) we found a significant amount of spousal correlation for height (μ = 0.20, based on 2322 husband-wife pairs). Once this is taken into account in this sample (Baker et al., 1996) it is found that environmental factors shared by twins probably have little or no effect on their final adult heights, as was found in Finnish twins (accompanying paper).

Although this analysis was conducted on self-report measures, these were found to correlate highly with clinical measures of height used in the remainder of our analysis (0.90 in both men and women). In fact, the correlations of twins' self report height with clinically measured height was only just higher than the correlation of MZ cotwins' self reported (or clinically measured) height, indicating that once measurement error is accounted for, the true heritability of stature may approach 100 percent.

In line with other studies, and even despite our additional selection of discordance for a trait (height), we found the majority of MZs (58%) were either concordant for random X-inactivation (10/17; 59%) or concordant for skewed inactivation (5/17; 29%) (Bamforth et al., 1996; Goodship et al., 1996; Trejo et al., 1994). Our proportion of height discordant MZs with intrapair discordant X-inactivation (2/17; 12%), does not significantly differ from the proportion (6%) of MZ pairs that demonstrate intrapair X-inactivation discordance in umbilical cord tissues (Bamforth et al., 1996) documented from a much larger dataset of X-inactivation status in 79 pairs of otherwise normal MZ twins (Fisher's Exact test, 2-sided; p = 0.12). In addition, no significant difference exists in intrapair discordance of X-inactivation between our MZ sample and that

https://doi.org/10.1375/twin.4.1.19 Published online by Cambridge University Press

of normal DZ twins as reported by Goodship et al (Fisher's Exact test, 2-sided; $p = 1.00$) (Goodship et al., 1996).

These results indicate, at least in our small sample of MZ twins, that intrapair height discordance at this level (range 5 to 9cm or 0.83SD to 1.80 SD for height) cannot easily be attributed to an excess of MZ twins having intrapair discordant X-inactivation. The two MZ pairs in our dataset manifesting intrapair discordance for X-inactivation status in lymphocytes did not possess the maximum height discordance observed among the 20 MZ twin pairs selected for this study (6 and 5cm respectively, versus the range of height discordance of 5 to 9cm) (Figure 4). Furthermore, it is interesting that both these pairs report themselves as monochorionic, which is inconsistent with the notion that they have seeded each other's bone marrow with identically X-inactivated stem cells. This either suggests that they have misreported their chorionicity, or that the bone marrow is screened in some way from circulating stem cells shared through vascular anastomoses. Ideally we would use DNA from another tissue, such as buccal cells, in which such vagaries did not have to be entertained. Similarly, it would be good to confirm self-report chorionicity with accurate hospital records, although this is a formidable task. In this regard, it would also be beneficial to validate and extend our observation that the maximum MZ intrapair difference in height was in the two pairs who report themselves as dichorionic.

Our sample is small and it is possible that our choice of a minimum of 5cm MZ intrapair height difference (close to one adult SD for height) for the purposes of assignment of an MZ pair as height discordant, was insufficiently stringent to adequately address this hypothesis. Clearly, a much larger investigation, concentrating on MZ female pairs with even greater height differences might be able to address this issue in a more focused manner, although it is possible that selecting for such extreme discordance may concentrate cases of gestational anomalies unrelated to X-inactivation. Although it is thought that many different genes contribute to variance in normal height, it is also possible that no important QTLs are located on the X chromosome, or that the causal polymorphisms were not heterozygous in our two X-inactivation discordant MZ pairs.

Alternatively, the possibility that X-inactivation patterns may vary in different tissues (Trejo et al., 1994) may mean that the tissue available to us to study this biological phenomenon (peripheral lymphocytes) does not accurately reflect the X-inactivation patterns of cells in the growth plate, the organ of linear advancement in humans (Monteiro et al., 1998). In addition, because the approximately 70% of MZ twins who are monochorionic have shared their circulation during gestation, true X-inactivation discordance in effector tissues may potentially be masked by use of peripheral lymphocytes for determining X-inactivation patterns (Hall, 1996b). Other causes of phenotypic variation in genotypically identical MZ twins are known and of these, differences in intrauterine environment (such as differential intrauterine nutrition or twin-twin transfusion syndrome; Martin et al., 1997) may have played an important role in promoting height differences between MZ twin pair members. Unfortunately, perinatal and placental data were not available to examine this possibility.

Acknowledgments

We thank Judith Hall for helpful comments on the manuscript and the twins for their cooperation.

References

Allen, R.C., Zoghbi, H.Y., Moseley, A.B., Rosenblatt, H.M., & Belmont, J.W. (1992). Methylation of HpaII and HpaI sites near the polymorphic CAG repeat in the human androgen-receptor gene correlates with X chromosome inactivation. *American Journal of Human Genetics, 51,* 1229–1239.

Baker, L.A., Treloar, S.A., Reynolds, C.A., Heath, A.C., & Martin, N.G. (1996). Genetics of educational attainment in Australian twins: sex differences and secular changes. *Behavior Genetics, 26,* 89–102.

Bamforth, F., Machin, G., & Innes, M. (1996). X-chromosome inactivation is mostly random in placental tissues of female monozygotic twins and triplets. *American Journal of Medical Genetics, 61,* 206–216.

Bellamy, N., Klestov, A., Muirden, K., Kuhnert, P., Do, K.A., O'Gorman, L., & Martin, N. (1999). Perceptual variation in categorizing individuals according to American College of Rheumatology classification criteria for hand, knee, and hip osteoarthritis (OA): observations based on an Australian Twin Registry study of OA. *Journal of Rheumatology, 26,* 2654–8.

Davidson, R.G., Nitowsky, H.M., & Childs, B. (1963). Demonstration of two populations of cells in the human female heterozygous for glucose-6-phosphate dehydrogenase variants. *Proceedings of the National Academy of Sciences of the United States of America, 50,* 481–485.

Duffy, D.L. (1993). Twin studies in medical research. *Lancet, 341,* 1418–1419.

Duffy, D.L., Mitchell, C.A., & Martin, N.G. (1998). Genetic and environmental risk factors for asthma: a cotwin-control study. *American Journal of Respiratory and Critical Care Medicine, 157,* 840–845.

Eaves, L.J., Eysenck, H.J., & Martin, N.G. (1989). *Genes, culture and personality: an empirical approach.* London: Academic Press.

Eaves, L.J., Heath, A.C., Martin, N.G., Neale, M.C., Meyer, J.M., Silberg, J.L., Corey, L.A., Truett, K., & Walters, M.S. Biological and cultural inheritance of stature and attitudes. In: C.R. Cloninger (Ed.), *Personality and psychopathology* (pp. 269–308). Washington: American Psychopathological Association.

Goodship, J., Carter, J., & Burn, J. (1996). X-inactivation patterns in monozygotic and dizygotic female twins. *American Journal of Medical Genetics, 61,* 205–208.

Hall, J. (1996a). Twinning: mechanisms and genetic implications. *Current Opinion in Genetics and Development, 6,* 343–347.

Hall, J. (1996b). Twins and twinning. *American Journal of Medical Genetics, 61,* 202–204.

Heath, A.C., Bucholz, K.K., Madden, P.A.F., Dinwiddie, S.H., Slutske, W.S., Bierut, L.J., Statham, D.J., Dunne, M.P., Whitfield, J.B., & Martin, N.G. (1997). Genetic and environmental contributions to alcohol dependence risk in a

national twin sample: consistency of findings in women and men. *Psychological Medicine, 27,* 1381–1396.

Jöreskog, K.G., & Sörbom, D. (1993). *New features in PRELIS 2 and LISREL 8.* Chicago: Scientific Software Inc.

Lyon, M. (1961). Gene action in the X-chromosome of the mouse (*Mus muscularis L.*). *Nature, 190,* 372–373.

Machin, G. (1996). Some causes of genotypic and phenotypic discordance in monozygotic twin pairs. *American Journal of Medical Genetics, 61,* 216–228.

Martin, N.G., & Martin, P.G. (1975). The inheritance of scholastic abilities in a sample of twins. I. Ascertainment of the sample and diagnosis of zygosity. *Annals of Human Genetics, 39,* 213–218.

Martin, N.G., Boomsma, D.I., & Machin, G.A. (1997). A twin-pronged attack on complex traits. *Nature Genetics, 17,* 387–392.

Miller, S.A., Dykes, D.D., & Polesky, H.F. (1988). A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Research, 16,* 1215.

Monteiro, J., Derom, C., Vlietinck, R., Kohn, N., Lesser, M., & Gregersen, P.K. (1998). Commitment to X inactivation precedes the twinning event in monochorionic MZ twins. *American Journal of Human Genetics, 63,* 339–346.

Phillips, K., & Matheny, A.P. (1990). Quantitative genetic analysis of longitudinal trends in height: preliminary results from the Louisville Twin Study. *Acta Geneticae Medicae et Gemellologiae, 39,* 143–163.

SAS Version 6.11 (1996). Cary, North Carolina: SAS Institute.

Trejo, V., Derom, C., Vlietinck, R., Ollier, W., Silman, A., Ebers, G., Derom, R., & Gregersen, P.K. (1994). X chromosome inactivation patterns correlate with fetal-placental anatomy in monozygotic twin pairs: implications for immune relatedness and concordance for autoimmunity. *Molecular Medicine, 1,* 62–70.

Treloar, S.A., Sadrzadeh, S., Do, K-A., Martin, N.G., & Lambalk, C.B. (2000). Birth weight and age at menopause in Australian female twin pairs: exploration of the fetal origin hypothesis. *Human Reproduction, 15,* 55–59.

Vogel, F. & Motulsky, A. (1997). *Human Genetics*. Berlin: Springer-Verlag.

https://doi.org/10.1375/twin.4.1.19 Published online by Cambridge University Press