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The Problem of Determining Twin Zygosity for Epidemiological Studies

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The use of epidemiological studies of twins as a means of determining the existence of genetic factors in various conditions is well known. To be of value, the zygosity of the twin pairs must be identified with as great a degree of confidence as possible. A method of determining twin zygosity, using concordance in general likeness, hair colour, hair type, eye colour, ear form, tongue roll and phenylthiocarbamide (PTC) taste sensitivity, was developed for use in a survey of 244 pairs of like-sexed twins aged 5 to 15 years. The results obtained and the problems raised when applying this method are discussed. The method was simple to apply to a large group of twin pairs, and monozygotic pairs were identified with a degree of confidence greater than 95%.

Key words: Twin zygosity, General likeness, Hair colour, Hair type, Eye colour, Eye form, Tongue rolling, PTC taste sensitivity

The study of twins as a means of identifying genetic influences in various conditions has become a widely recognised approach in educational, medical and dental investigations. The existence of genetic factors may be confirmed by demonstrating that monozygotic (MZ) twin pairs show a significantly greater degree of similarity (concordance) for the condition under investigation than dizygotic (DZ) pairs. It is, therefore, a prerequisite of any such study that the diagnosis of zygosity should be as accurate as possible. Hogben in 1933 [15] and Kempthorne in 1978 [16] have pointed out, however, that the comparison of MZ and DZ twins cannot be used to partition quantitatively the relative roles of heredity and environment in the determination of any trait. Thus it would seem reasonable to omit from an investigation any pairs of twins for whom zygosity is in doubt rather than allow misdiagnosis to obscure genuine differences which might exist between the MZ and DZ twins in the sample. It is possible, however, that the differences may in fact be exaggerated by such omissions.

For a survey of 244 like-sexed twin pairs aged 5 to 15 years [7], a series of tests was devised which gave a certainty greater than 95% that a pair of twins diagnosed as MZ was in fact "identical."

Selection of Tests

The so-called "similarity" method of twin diagnosis was first described by Siemens in 1927 [20]. It relies on the theory that MZ twins, having an identical genetic constitution, must show strong resemblances in those features and traits that are known to be genetically determined, whereas DZ twins will show no greater similarity than might appear in any two sibs of the same family. Siemens listed certain traits, such as hair and eye colour, for which MZ twin pairs are almost always concordant whereas DZ pairs are more likely to be discordant. He considered body build and the form of the face and of the ear to be traits for which MZ twins usually show strong resemblances. He suggested that observations of this kind could be used as tests of zygosity in twins.

Further developments of this method have involved the intrapair comparison of fingerprints and blood and serum groupings. The use of these techniques in a study involving large numbers of twin pairs is impracticable. The necessary parental consent for fingerprinting would not be forthcoming in all cases because of the emotive issues associated with the procedure. To obtain the quantity of blood required for serological typing, venepuncture is necessary and this procedure, of no benefit to the subjects in the investigation, carries with it certain risks and is particularly difficult in the younger child. In both instances—fingerprinting and blood typing—the analysis of the results would present difficulties and it would not improve substantially upon other simpler, and more practical tests of zygosity.

Several studies have been made of certain individual traits considered to be genetically determined, and these provided valuable criteria in establishing zygosity.

The use of ear form in the diagnosis of zygosity was described by Dahlberg in 1926 [6]. He suggested that concordance in ear form could be used as a criterion of monozygosity in very young twins.

Sturtevant in 1940 [22] first put forward the theory that the ability to roll the tongue into a longitudinal tube is genetically determined. He described the "trick" as being due to the inheritance of a single dominant gene, "but with fairly frequent complications." Some doubt was also cast on the simple inheritance of this trait by Matlock [17], who described "monozygotic" twins discordant for tongue roll. In an investigation which included 92 pairs of MZ and 83 pairs of DZ twins, Vogel [24] came to the conclusion that "tongue curling is to a certain degree, but not exclusively, hereditary."

Individuals who are able to taste phenylthiocarbamide (PTC) and phenylthiourea compounds are known as "tasters," and those unable to detect them as "nontasters." In the early thirties, Fox [8,9] reported that 40% of the volunteers he had tested were unable to taste crystals of para-ethoxyurea or other related compounds. Blakeslee and Salmon [3], using PTC in solution as well as crystals, confirmed Fox's findings and concluded that inability to taste PTC is inherited as a Mendelian recessive characteristic. Snyder [24], working independently, attributed an inability to taste this compound to a single dominant gene that was neither sex-linked nor sex-influenced. His results, however, are similar to those of Blakeslee and Salmon, and imply that the trait is recessive in nature. Ardashnikov and his fellow workers [1] and Rife [19] reported pairs of identical twins discordant for PTC taste sensitivity. Unfortunately, it is not possible to assess the implications of their findings because in neither paper is the method of testing described.

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The concentration used for testing has been found to influence the distinction between "tasters" and "nontasters." Blakeslee and Salmon [3] noticed that the number of "tasters" in their sample increased with increasing concentration of the compound. In 1953 Harris [11] found that most people could readily be categorised by giving them a fairly strong solution to taste. Harris and Kalmus [12, 13] and Chung and others [5] used a concentration of 130 mg% phenylthiocarbamide as the basic stock solution for serial dilution tests and, because only a simple positive/negative response was required, this was the concentration chosen for the present study (test 6).

In 1961 Cederlöf and his co-workers [4] reported on an investigation in which mailed questionnaires were used to diagnose the zygosity of like-sexed twins. Six hundred pairs of like-sexed twins were asked whether, when growing up, they had been "as like as two peas" or had only a family likeness. Each twin was asked to complete the questionnaire without consulting the other. In 521 cases both members of a pair replied. Of these, a sample of 200 pairs was selected for a further investigation in which five independent systems were used to determine the blood group of each twin. In 72 cases both members of the pair had replied that they were "as like as two peas" when young; 71 of these pairs were found to be serologically concordant. In 108 pairs both twins claimed only family likeness; of these 99 were found to be serologically discordant. The remaining 20 pairs gave conflicting replies.

In considering the validity of this method, the WHO Meeting of Investigators on the Use of Twins in Epidemiological Studies in 1966 [26] came to the conclusion that where "both members of the twin pairs had answered this question consistently, 95% were correctly identified" as MZ or DZ "on the evidence of blood and serum grouping." One of the simplest methods for zygosity diagnosis, that of assessing general likeness, would thus appear to be very reliable. "General likeness" was, therefore, used as the major deciding factor in the determination of zygosity in the present study.

CLINICAL MATERIAL AND METHODS

As part of a dental epidemiology study, 244 pairs of like-sexed twins were examined during the period May 1966 to April 1968. Of these, 114 pairs were male and 130 female. The children, aged 5 to 15 years inclusive, attended schools in Dewsbury, Huddersfield, Leeds, and York in the County of Yorkshire.

Zygosity Tests

The following tests were used in the determination of twin zygosity. They are described in the order in which they were performed. Each member of a twin pair was assessed separately for the first six tests.

1. Hair colour. The colour of the hair was determined by comparison with a shade guide constructed from a hairdressers' dye shade card (Clynol Gelée). The guide consisted of 35 detachable clips each containing a short bundle of hair fibres dyed a specific colour. The basic colours were black, red, brown, and blonde. The last two were subdivided into various basic shades: ash, matt, gold, and hazelnut. Mahogany and chestnut brown were also included. The basic shades were subdivided further to give a range of intensity: dark, medium, light and super. Where the colour of an individual's hair varied over different parts of the head, the hair near the scalp in the region of the parting was compared with the shade guide. Where no absolute match could be found, the nearest shade was recorded. With some of the older girls it was necessary to enquire whether or not the hair contained any dye or colour rinse.

2. Hair type. The type of hair was recorded as straight, wavy or curly (Fig. 1). In female twins care was taken to discover whether the wave or curl was natural or not.

3. Eye colour. The colour of the iris was determined by comparison with a shade guide provided by an artificial eye manufacturer (W.H. Shakespeare & Sons, Ltd.). The basic colours on the guide were blue, blue-grey-hazel, blue-grey, hazel, grey, and brown. There was a choice of six shades for each of the first five basic colours and of three shades of brown. Where no absolute match could be found the nearest shade was recorded.

Fig. 1. Hair types; top–straight; middle–wavy; bottom – curly.

4. Ear form. The presence of a free lobe to the ear was recorded as positive; that of a fixed lobe as negative (Fig. 2).

5. Tongue roll. The ability to roll the tongue into a longitudinal tube without the aid of the teeth, but with the help of the lips where necessary, was recorded as positive; inability to do so as negative (Fig. 3).

6. PTC taste sensitivity. The ability to taste phenylthiourea in a concentration of 130 mg% was recorded as positive and inability to taste the chemical as negative.

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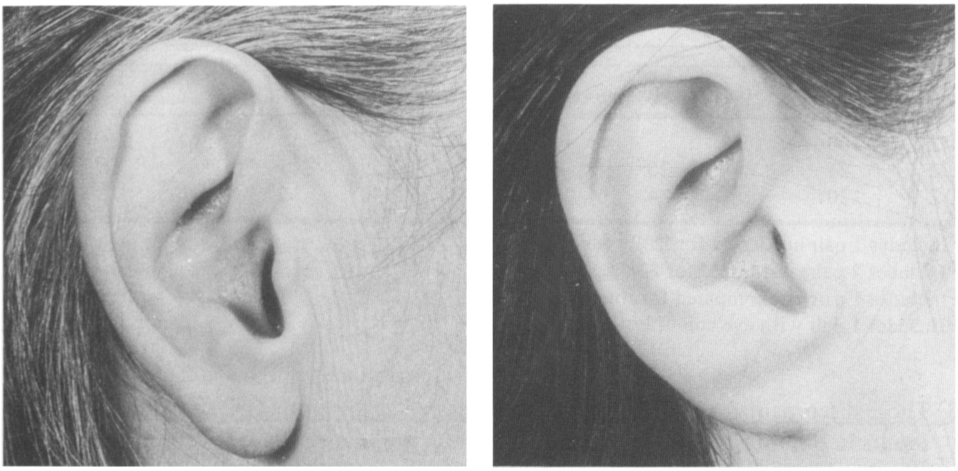

Fig. 2. Ear form – attachment of the ear lobe. Left, free lobe; right, fixed lobe.

Fig. 3. Tongue roll. Left, positive; right, negative.

7. General likeness. The final test was a visual comparison of the two members of each twin pair standing side by side. A general assessment of likeness in height, build, facial appearance and colouring (distribution of freckles, etc) was made. Those twins who were "as like as two peas" were recorded as "general likeness positive." Older pairs of twins who looked very much alike were asked if people had had difficulty in telling them apart when they were younger (eg, of primary school age). When the answer was "yes" from both twins, and when further questioning revealed that those experiencing the difficulty had been in almost daily contact with the pair, they were recorded, once again as "general likeness positive." All other pairs were recorded as "general likeness negative."

RESULTS

The criteria used in allocating a pair of twins to a particular zygosity group were as follows:

1) Twins concordant for general likeness and also in the remaining six tests were considered to be monozygotic (MZ);
2) Those concordant for general likeness but discordant in one or more of the other tests were classed as of uncertain zygosity (UZ);

TABLE 1. Distribution of the Twin Pairs by Zygosity and Number of Discordant Tests

		Number of discordant tests							
	N	0	1	2	3	4	5	6	7
MZ	100	100[a]							
UZ	24		20[c]	4					
DZ	120		4	25	42[b]	40[d]	7[a]	2	0

[a]Includes 1 pair not tested for PTC taste sensitivity.
[b]Includes 2 pairs not tested for PTC taste sensitivity.
[c]Includes 1 pair not compared for tongue roll.
[d]Includes 1 pair with eye colour not recorded.

3) Those discordant for general likeness were classed as dizygotic (DZ) regardless of their results in other tests. (In fact only 4 of the 120 pairs classed as DZ were concordant for all tests other than general likeness—see Table 1.)

Four pairs of twins were not tested for PTC taste sensitivity because one, or both, had shown extreme apprehension during the preceding examination. No decision was possible about the ability of one twin to roll his tongue because of severe tongue-tie and hence no comparison with the cotwin could be made. The eye colour of one member of a pair of twins discordant for general likeness was inadvertently omitted from the records.

Table 1 shows the numbers of twin pairs finally allocated to each of the zygosity groups. Of the 124 pairs of twins diagnosed as concordant for general likeness, 100 (44 male and 56 female) were finally classed as MZ and 24 (13 male and 11 female) as UZ. Of the 120 pairs who were discordant for general likeness (and, therefore, classed as DZ), 57 pairs were male and 63 pairs were female.

Results of Tests Other Than General Likeness

In these tests no significant differences were observed between the sexes within the MZ and DZ groups. Data for the sexes have therefore been combined. It was not possible to test the UZ group for differences between the sexes because of the small numbers involved.

The distribution of the twin pairs into Concordant and Discordant for each of the individual tests can be seen in Tables 2 to 7. For hair colour and eye colour a pair of twins was classified as:

1) *Absolutely concordant* in either test if both members of the pair were found to have the same "score" on the shade guide;

TABLE 2. Distribution of the Twin Pairs by Concordance for Hair Colour

	Absolutely concordant	Considered concordant	Discordant
MZ	68	32	
UZ	11	10	3[a]
DZ	23	33	64

[a]All 3 pairs discordant for this test only.

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TABLE 3. Distribution of the Twin Pairs by Concordance for Eye Colour

	Absolutely concordant	Considered concordant	Discordant
MZ	59	41	
UZ	7	12	5[a]
DZ[b]	25	35	59

[a]4 pairs discordant for this test only.
[b]1 pair eye colour not recorded.

TABLE 4. Distribution of the Twin Pairs by Concordance for Hair Type

	Concordant	Discordant
MZ	100	
UZ	23	1
DZ	83	37

TABLE 5. Distribution of the Twin Pairs by Concordance for Shape of Ear Lobe

	Concordant free lobes	Concordant fixed lobes	Discordant
MZ	67	33	
UZ	14	10	0
DZ	68	17	35

TABLE 6. Distribution of the Twin Pairs by Concordance for the Ability to Roll the Tongue

	Concordant positive	Concordant negative	Discordant
MZ	66	34	
UZ[b]	4	7	12[a]
DZ	58	20	42

[a]9 pairs discordant for this test only.
[b]1 pair not tested (one twin with tongue-tie).

2) *Considered concordant* if the difference in "score" was of shade rather than of basic colour. The decision as to which "scores" would be considered concordant was made before the study was undertaken;
3) *Discordant* if the difference was one of basic colour.

For all the other tests the distinction was simply between concordant and discordant.

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TABLE 7. Distribution of the Twin Pairs by Concordance for PTC Taste Sensitivity

	Concordant "tasters"	Concordant "nontasters"	Discordant
MZ[b]	67	32	
UZ	15	2	7[a]
DZ[c]	69	17	31

[a] 4 pairs discordant for this test only.
[b] 1 pair not tested.
[c] 3 pairs not tested.

DISCUSSION

The purpose of a series of tests to determine the zygosity of pairs of like-sexed twins is to enable the investigator to separate MZ from DZ pairs with as great a degree of certainty as possible. For any test to be of value, the characteristic on which it is based must be known to be genetically determined and the test itself should, as far as possible, be objective in nature. It should also be shown that the prevalence of the trait within the survey group does not differ from that in the population at large and that neither mechanism of "twinning" associates, positively or negatively, with the investigated trait.

There is considerable agreement that the tests used in the present study are based on genetically determined traits. Only two of them were purely objective, however–tongue roll and PTC taste sensitivity. A further two–hair colour and eye colour– were made as objective as possible by the use of shade guides: each twin was compared with the guide, rather than twin with twin in a direct comparison. (The use of some standard for eye colour comparison was recommended by Grieve and Morant [10].) In the remaining three tests subjective decisions were inevitable. In the case of hair form and ear form it was a matter of deciding into which category an individual should be placed rather than whether the pair of twins under investigation was concordant or discordant for that particular characteristic. The consideration of general likeness, which involved a direct comparison of one twin with the other was, perhaps, the most subjective of the tests.

Serological typing and fingerprint comparison may be considered more objective tests of zygosity than those used in the present study. However, for reasons already stated, it was thought impracticable and unnecessary to apply them to such a large sample of twins.

Whereas it is generally accepted that the characteristics used in the first four tests are genetically determined, doubt has been expressed about tongue rolling and about PTC taste sensitivity. Because of this reservation the results of these two tests were analysed in greater detail. It was also possible to compare the frequencies for both these tests with other studies. The frequency distributions for the twin pairs in this survey are shown in Tables 8 and 9.

An analysis of the frequencies of "rollers" and "nonrollers" within the zygosity groups revealed no significant difference between the groups. In a survey of 480 male and 529 female American Caucasoid students, Urbanowski and Wilson [23] found that 315 (65.6%) of the males and 379 (71.6%) of the females were able to roll their tongues. Of the MZ twins in the present study, 64 (72.7%) of the males and 68 (60.7%) of the females were tongue-rollers. Corresponding values for the DZ twins were 72 (63.2%) of the males and 86 (68.3%) of the females. Analysis of Urbanowski and Wilson's results reveals a probably significant difference between the sexes ($P = 0.041$, Fisher's exact test). There are no signifi-

TABLE 8. The Frequencies of Tongue "Rollers" and "Nonrollers" Within the Zygosity Groups

	"Rollers"	"Nonrollers"
MMMZ	64	24
FFMZ	68	44
MZ	132	68
MMDZ	72	42
FFDZ	86	40
DZ	158	82

TABLE 9. The Frequencies of PTC "Tasters" and "Nontasters" Within the Zygosity Groups

	"Tasters"	"Nontasters"
MZ	134	64
DZ	169	65

*TABLE 10. The Probability of a Pair of DZ Twins Being Concordant for Each Test Used in the Determination of Zygosity**

	Number examined	Number concordant	Probability of concordance
Hair colour	120	56	0.47
Hair type	120	83	0.69
Eye colour	119	60	0.50
Ear lobe	120	85	0.71
Tongue roll	120	78	0.65
PTC taste	117	86	0.74

*Based on the number found to be concordant in the present study.

cant differences between the sexes within the zygosity groups in the present study. When comparison of the results of the present study is made with those of Urbanowski and Wilson, no significant differences are observed except for MZ females where P = 0.024. These results show certain inconsistencies which are difficult to interpret.

Using the observed frequencies among the DZ twins, the expected distribution, due to chance, into concordant positive, concordant negative and discordant is 52.0, 14.0 and 54.0. Comparing these values with the observed distribution shows no significant difference. On the basis of this result it would appear that like-sexed DZ twins are no more likely to be concordant for tongue-roll than any two individuals taken at random. This tends to confirm the reservations expressed by Sturtevant [22], Matlock [17] and Vogel [26] about the genetic basis of this trait.

In the examination of the results for PTC taste sensitivity a comparison of the frequencies of "tasters" and "nontasters" within the zygosity groups revealed no significant differences.

Hartmann [16] found that 73% of the individuals he tested were able to taste PTC in the concentration used in this study. Harris [11] stated that approximately 70% of

European populations fall into the "taster" class. In the present study 68% of the MZ and 72% of the DZ twins were classified as "tasters," values not incompatible with the findings of both Hartmann and Harris.

On the basis of observed frequencies for the DZ twins in the present study the distribution into concordant "tasters", concordant "nontasters" and discordant expected by chance would be 61.0, 9.0 and 47.0, respectively. The difference between this and the observed distribution is significant ($\chi^2 = 13.488$ with 2 df, $0.01 > P > 0.001$).

It would appear unlikely that environment could play any part in determining whether an individual becomes a "taster" or a "nontaster," and hence the difference between the observed and expected distributions may be taken as a measure of the influence of a common genetic heritage on the members of a DZ twin pair.

The value of any particular test in aiding the diagnosis of zygosity depends upon the probability of DZ twins being concordant for that test: the smaller the probability, the greater the value of the test. Table 10 shows the probability of a pair of DZ twins being concordant for each individual test other than general likeness in the present study. (The calculations have been based on the proportion found to be concordant for each test.) Hair colour, with a probability of 0.47, contributes most, and PTC taste sensitivity (P = 0.74), least towards the diagnosis of zygosity.

The probability of DZ twins being concordant for all the tests is the product of the probabilities of concordance in each individual test, provided that the tests were examining independent characteristics. Table 11 shows the probability of DZ twins being concordant (Pc) for all six tests, excluding general likeness, for five out of six, four out of six, etc, assuming all six tests to be independent. For all six tests, Pc = 0.055. The observed distribution of concordance in the 116 pairs of twins diagnosed as DZ who completed all six tests is shown in Table 12. The expected distribution has been calculated from the probabilities in Table 11. A χ^2 analysis reveals no significant difference between the observed and expected distributions. This result implies that the characteristics used in the six zygosity tests are independent of one another. Had this not been the case, the observed frequency distribution would have revealed significantly more pairs of twins concordant in a greater number of tests than expected.

Because of the way in which pairs of twins were classified as UZ, it is not possible to make use of them in the above analysis. However, of the 23 pairs of UZ twins who completed all six tests, 19 pairs were concordant for five of the six tests and four pairs were concordant for four of them. This is obviously a greater degree of concordance than would be expected on the basis of Table 11 and could be interpreted as indicating that the UZ group is a mixture of MZ and DZ twin pairs.

In their examinations to determine the value of general likeness as a means of diagnosing zygosity, Cederlöf and his co-workers [4] found only one serologically discordant pair of twins out of the 72 claiming to have been "as like as two peas" when young. The tests used in their study gave "an estimated mean probability of about 96%" that twins found to be serologically concordant would be MZ, so that 3 of the remaining 71 pairs could also have been DZ. Thus, 4 of the 72 pairs (5.6%) may in fact have been DZ.

On the basis of this figure, combined with the other results obtained, the probability of DZ twins in the present study having been diagnosed as MZ would be 0.0031, if general likeness and the other six tests used were completely independent. However, the assessment of general likeness must have included such items as hair colour, hair form, etc. So the probability of diagnosing a pair of true DZ twins as MZ by the method described here is likely to be greater than 0.0031 but certainly less than 0.055 (the value for the tests other than general likeness).

*TABLE 11. The Probability of DZ Twins Being Concordant for all Combinations of the Tests Used in the Determination of Zygosity**

Test combination	Probability
All six tests	0.055
Any five of the six tests	0.214
Any four of the six tests	0.331
Any three of the six tests	0.261
Any two of the six tests	0.112
Only one of the six tests	0.025
None of the six tests	0.0022

*Based on the probabilities in Table 10.

*TABLE 12. A χ^2 Analysis of the Difference Between the Observed and the Expected Frequency of Concordance in the Tests Used in the Determination of Zygosity**

Number of concordant tests	Frequency observed	Frequency expected
6	4	6.4
5	25	24.8
4	40	38.4
3	39	30.3
2	6	13.0
1	2	2.9
0	0	0.3
Total	116	116.1

*Based on the results for the 116 pairs of DZ twins who completed all the tests, and on the probabilities in Table 11. $\chi^2 = 6.790$ with 3 df; $P > 0.05$. (Data combined to give cell sizes $\geqslant 5$).

In 1847 Bertillon [2] stated that the number of like-sexed twins in a population would be expected to equal the number of opposite-sexed pairs and that the excess of the observed over the expected was due to the presence of MZ twins. Weinberg [25] revived this theory and provided a rule by which it is possible to estimate the number of MZ twins in a population: the number is obtained by subtracting twice the number of male-female pairs from the total twin population. Applying this method to the figures for live births in England and Wales for the years 1951 to 1964 inclusive (Registrar General's Statistical Review, 1953-1964), the years during which the twins used in this survey were born, gives an average MZ:like-sexed DZ ratio of 100:131.14 ± 9.69 (SD). The observed ratio of 100:120 found in the present study is, therefore, 1.15 standard deviations below the national average, a difference which cannot be regarded as significant.

CONCLUSION

If we exclude the inconclusive work of Cederlöf [4] referred to earlier, the use of twins in the investigation of genetic traits has been limited to numerically small samples. There is

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no recognised method of diagnosing zygosity with a one hundred per cent certainty, even at birth. The seven tests which have been used in this study were simple to apply and gave an immediate diagnosis. Later statistical analysis has shown this diagnosis to have been of considerable accuracy.

It is suggested that these tests could provide a very useful method of zygosity diagnosis for large-scale twin surveys not only in medicine but in many other fields of research into the influence of heredity.

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