Letters to the Editor

Am. J. Hum. Genet. 69:1146–1148, 2001

The Importance of Genealogy in Determining Genetic Associations with Complex Traits

To the Editor:

Most common diseases, such as asthma, type 2 diabetes, bipolar disorder, and cardiovascular disease, are known to have genetic components, but the susceptibility genes have been notoriously difficult to localize and to identify. These complex diseases likely have a large number of genetic and nongenetic risk factors that together have varying effects on phenotype. Many investigators have recommended founder populations for complex-trait mapping, with the expectation that fewer susceptibility alleles will be segregating in these restricted gene pools (Lander and Schork 1994; Wright et al. 1999; Shifman and Darvasi 2001). Some or all individuals in these populations are inbred, but often the exact relationships between all members are either unknown or not taken into account. It is tempting to use such populations for their presumed homogeneity, even in the absence of accurate pedigree information. The failure to take full pedigree information into account can either reduce the power to detect linkage (Dyer et al., in press) or inflate LOD scores (Miano et al. 2000). The failure to account for relatedness among individuals will also affect association studies. In particular, many statistical tests of association are not strictly valid, owing to the lack of true independence between individuals. Nonetheless, such populations have been used widely in association studies (e.g., de Silva et al. 1999; Laprise et al. 2000; Ospina-Duque et al. 2000; Summerhill et al. 2000; Bitti et al. 2001; Hegele et al. 2001), and some authors have even recommended the inclusion of founder populations in case-control studies, owing to their decreased heterogeneity (Shifman and Darvasi 2001). However, the impact that ignoring pedigree relationships has on tests of association has not been evaluated.

The Hutterites are an extreme example of a large, complex pedigree with multiple inbreeding loops. We are in the unique position of having complete genealogical information on this 12,903-person, 13-generation pedigree (Abney et al. 2000). Additionally, we have extensive phenotype characterization and a dense microsatellite map (of 568 short-tandem-repeat–polymorphism markers) of ~750 members of this population, who are descendants of just 64 Hutterite founders (Ober et al. 2000). Thus, we were able to assess the effect that ignoring pedigree information has on statistical tests of association.

We performed two separate genomewide scans of association on each of three quantitative phenotypes: serum immunoglobulin E (IgE), serum LDL, and body-mass index (BMI). These phenotypes were chosen to represent quantitative traits associated with diverse complex diseases (asthma, cardiovascular disease, and diabetes, respectively). All phenotypes were adjusted for age and sex and were transformed so that the residuals were approximately normally distributed (Abney et al. 2001). The heritabilities of IgE and BMI were completely accounted for by additive genetic variance, with heritabilities of .63 and .54, respectively; the heritability of LDL had a strong dominance component in addition to additive genetic variance, with a broad heritability of .96 (discussed in detail by Abney et al. [2001]). To estimate the effect that each allele at each locus has on the trait values, we used a statistical test of association, developed specifically for use in large, inbred pedigrees (Ober et al. 2001 [in this issue]). Pedigree structure is taken into account by the use of variance components to model the polygenic background (Abney et al. 2000, 2001). When pedigree structure is ignored, the method is equivalent to a linear regression of the trait on age, sex, and genotype, with a Bonferroni correction applied to the P value for the t test for significance of genotype. In the first scan, we included the variance components and therefore took into account the relatedness between individuals. In the second scan, we did not include any pedigree information (additive and dominance variances were 0). In both scans, the observed P values were adjusted for multiple comparisons, by use of a Bonferroni correction.

The two methods yielded dramatically different results. In general, the significance of association with a given marker was considerably inflated when pedigree structure was not included, although in some cases the reverse was true (see fig. 1). Only two loci were among the five most significant results, by both methods, for the three traits (the same marker at 7p21 was associated with IgE, by both methods, and the same marker at 8q12

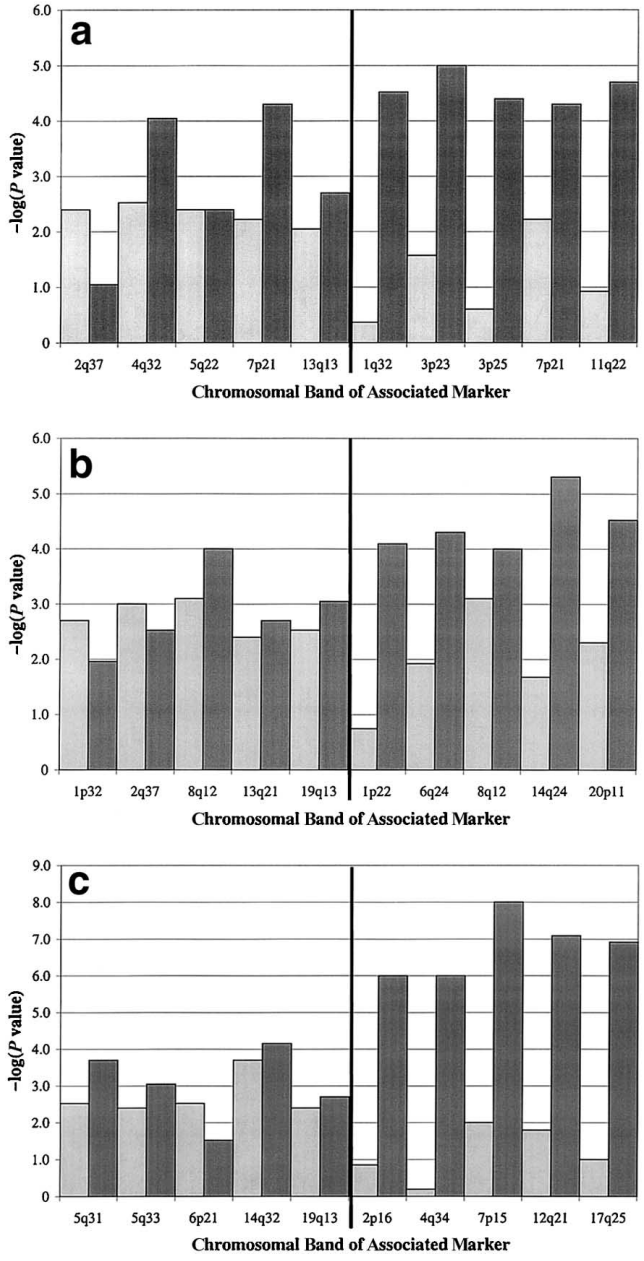

Figure 1 Results of two genome scans—one including pedigree structure (*lighter bars*) and one not including pedigree structure (*darker bars*)—for IgE (*a*), LDL (*b*), and BMI (*c*). The five most significant loci when structure is included (*left sides*) and when structure is not included (*right sides*) are shown.

was associated with LDL, by both methods). In addition, many more loci showed evidence of association when the pedigree structure was not included (see fig. 2). In fact, 10%–22% of all markers appeared to have a strong association ($P < .01$) with the phenotype, when pedigree structure was not included. Results for 58 single-nucleotide polymorphisms showed similar trends (data not shown).

Although the Hutterites are an extreme example of a complex pedigree, there are several other populations known to have similar structures (Badner et al. 1990; Slutsky et al. 1997; Hsueh et al. 2000). Moreover, individuals from a variety of smaller, island populations who either have been or are currently being studied may be more related to each other than can be discerned from the recently collected pedigree data (de Silva et al. 1999; Mathias et al. 2000; Bitti et al. 2001). In fact, even presumably outbred populations may contain hidden consanguinity (Broman and Weber 1999), and cryptic relatedness may be a problem in association studies of rare disorders (Bacanu et al. 2000). This problem may be avoided by the use of statistical tools designed to detect misspecified or cryptic relationships (McPeek and Sun 2000; Sun et al., in press).

We cannot prove that the inclusion of the pedigree structure in the method results in true associations, until the alleles contributing to these quantitative traits are found; however, we believe that the number of associations found when structure is ignored is unrealistic. Presumably, a profound failure of the assumption of independence between individuals, in method 2, results in a dramatically increased number of type 1 errors. Overall, our data suggest that failing to take into account extended-familial relationships can result in a large number of false-positive results, and some "true" associations may be missed. In addition, the level of significance could be overestimated by several orders of magnitude. In an association study in which it is not possible to take into account all familial relationships, as we have done with the Hutterites, another option is to use genomic controls (Devlin and Roeder 1999). Otherwise, naïve approaches to genetic-association analysis could result

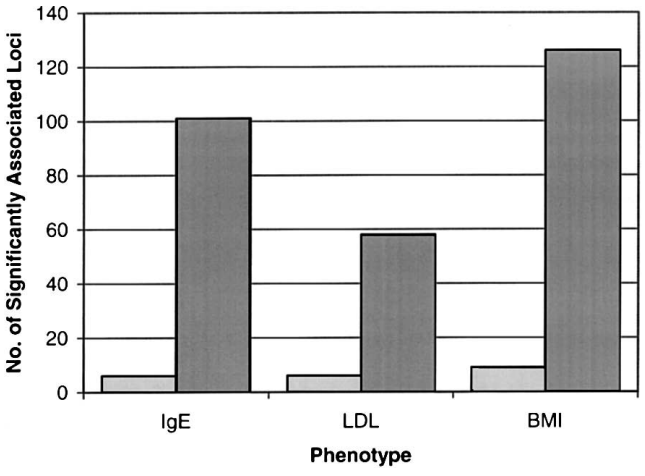

Figure 2 Number of significantly associated ($P < .01$) loci when pedigree structure is included (*lighter bars*) and when pedigree structure is not included (*darker bars*).

in an enormous amount of time and of money spent in following up artifactual associations.

Acknowledgments

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Reporting of Linkage Results

To the Editor:

In a recent report in the *Journal,* Province (2001, p. 661) correctly points out that a bias occurs if Fisher's method of combining *P* values is used naively to pool the results of "many of the very popular nonparametric (i.e., model free) linkage methods (in particular, variance components, affected sib pair, and extremely discordant sib-pair linkage)." Indeed, Morton (2000, p. 9) has stated that "methods that force lods to be non-negative values create a serious bias that should be avoided as far as possible." The earliest variance-component linkage method is that formulated by Haseman and Elston (1972), and the same methods as were described in that paper to estimate the proportion of alleles that a sib pair share identical by descent can be used, in the so-called mean test, to test for linkage on the basis of data on either affected sib pairs or extremely discordant sib pairs. This has long been implemented in the computer program SIBPAL. This program automatically computes one-tailed *P* values that are uniformly distributed between 0 and 1, under the null hypothesis of no linkage, and so do not suffer the bias that occurs whenever LOD scores truncated at 0 are reported. It has been argued elsewhere (Elston1998; Nyholt 2000) that it would be preferable to summarize the results of linkage analysis in terms of *P* values rather than in terms of LOD scores, and the problem discussed by Province is a further reason for doing so. Although the bias can be obviated in the manner described by Province, a more accurate pooling of linkage results would be obtained if every study in which there is evidence against linkage is assigned the relevant *P* value between $\frac{1}{2}$ and 1, rather than assigning to all such studies an average value. Pooling of *P* values in this manner was done by Wilson and Elston (1995) in a meta-analysis of linkage results for alcoholism. All that would be required for this method of pooling to become standard, if we are willing to make the asymptotic assumption to convert LOD scores to *P* values, is for software that currently truncates LOD scores at 0 to calculate and quote negative LOD scores, corresponding to negative variance components and/or recombination fractions $> \frac{1}{2}$, when this is appropriate.

Consider, for example, the sib-pair methods based on maximizing a LOD score over allele-sharing probabilities z_i, where z_i is the probability that a sib pair shares i alleles identical by descent at a genomic location. Because $z_2 = 1 - z_0 - z_1$, the parameter space $0 \leq z_0, z_1, z_2 \leq 1$ can be represented by the triangle depicted in figure 1, in which the smaller hatched triangle represents the possible parameter values for affected sib pairs when there is simple monogenic inheritance (Holmans 1993). When a complex disease is being studied, linkage can be missed if the maximization for affected sib pairs is restricted to this smaller hatched triangular area (Dizier et al. 2000); on the other hand, maximizing the LOD score over the whole parameter space depicted in figure 1 can lead to a positive LOD score when there is evidence against linkage. The dashed line in figure 1 divides the parameter space into two parts, corresponding to the proportion of alleles shared by a pair of sibs being either $> \frac{1}{2}(z_2 > z_0)$ or $< \frac{1}{2}(z_2 < z_0)$. Maximization should be over the entire parameter space, but the resulting maximum LOD score should be made negative if the result is in the direction opposite to linkage (see fig. 1). In this particular case, because >1 df is involved, the conversion of a maximum LOD score to a *P* value is not simple, even if based on asymptotic considerations. In other situations (e.g., see Kong and Cox 1997; Whittemore and Tu 1998), when only 1 df is involved, the conversion is simple if we are prepared to make asymptotic assumptions. In any cases of doubt, a permutation test (sampling from the entire permutation distribution, if necessary, for computational feasibility) can be performed. As pointed out elsewhere (Elston et al. 1996) ~10,000 sib pairs are required if, in an affected-sib-pair study, a quoted *P* value of 10^{-4} is to be within 20% of the true value when we rely on as-

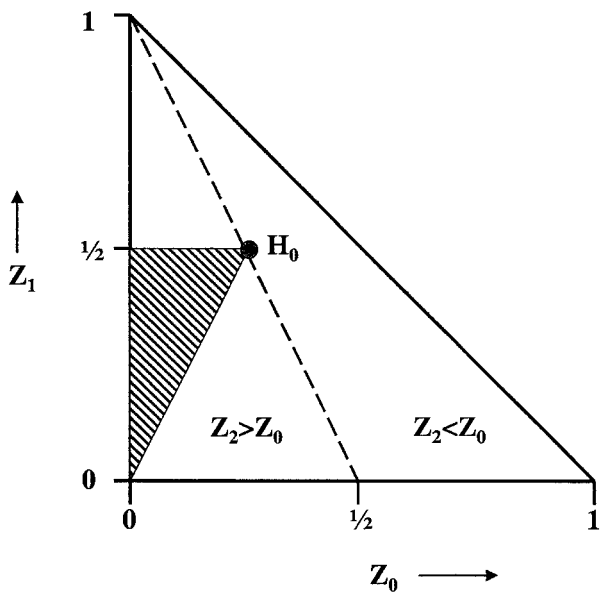

Figure 1 Parameter space for sib-pair allele sharing. H_0 is the null hypothesis (no linkage) point $z_0 = \frac{1}{4}, z_1 = \frac{1}{2}, z_2 = \frac{1}{4}$. When concordant sib pairs are studied, points to the left of the dashed line are in the direction of linkage, and points to the right are in the direction opposite to linkage; when discordant sib pairs are studied, the converse is the case. The smaller hatched triangle represents the parameter space for concordant sib pairs when there is simple monogenic inheritance.

ymptotic assumptions to calculate it. In any case, it would seem preferable for analysts to quote one-sided *P* values rather than to have to resort to the average bias-controlling procedure suggested by Province—although this should not be taken to imply that either *P* values or LOD scores are completely satisfactory summary statistics.

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Examinations of Methylenetetrahydrofolate Reductase C677T and A1298C Mutations—and In Utero Viability

To the Editor:

The recently published study by Isotalo et al. (2000) analyzed the methylenetetrahydrofolate reductase (MTHFR) C677T and A1298C mutations in neonatal and fetal groups, to determine whether particular MTHFR genotype combinations are associated with decreased in utero viability. Isotalo et al. (2000) observed all possible genotype combinations in the fetal group, but combined 677CT/1298CC and 677TT/1298CC genotypes were not observed in the neonatal group. Therefore, they hypothesized that decreased viability exists among fetuses carrying the 677CT/1298CC and 677TT/1298CC genotypes, with a possible selection disadvantage in fetuses with an increased number of mutant MTHFR alleles. They also did not observe the 677CT/1298CC and 677TT/1298CC genotypes in a population consisting of healthy adult controls.

We have tested for the MTHFR C677T and A1298C mutations in a Hispanic population of Mexican descent, to determine risk for spina bifida (SB) (Volcik et al. 2000). Although we observed all possible MTHFR 677/1298 ge-

Table 1

Combined MTHFR C677T/A1298C Genotype or Allele Frequencies in a Hispanic Population of Mexican Descent, Composed of Patients with SB, Their Parents, and Controls

Genotype or Allele	Observed Frequency in			
	Patients (n = 302)	Mothers (n = 281)	Fathers (n = 143)	Controls (n = 82)
MTHFR C677T/A1298C genotype:				
CT/CC	.003	.000	.007	.012
TT/AC	.020	.028	.021	.024
TT/CC	.000	.004	.000	.000
MTHFR allele:				
677C	.493	.477	.549	.527
677T	.507	.523	.451	.473
1298A	.854	.856	.815	.811
1298C	.146	.144	.185	.189

Table 2

Combined MTHFR C677T/A1298C Genotype or Allele Frequencies in a U.S. Population of European Descent, Composed of Patients with SB, Their Parents, and Controls

Genotype or Allele	Observed Frequency in			
	Patients (n = 160)	Mothers (n = 149)	Fathers (n = 107)	Controls (n = 66)
MTHFR C677T/A1298C genotype:				
CT/CC	.000	.020	.019	.015
TT/AC	.038	.007	.019	.000
TT/CC	.000	.000	.000	.000
MTHFR allele:				
677C	.578	.601	.551	.682
677T	.422	.409	.449	.318
1298A	.694	.721	.734	.712
1298C	.306	.279	.266	.288

notype combinations in this Hispanic population, the 677TT/1298CC combination was observed only once, in the mother of an affected individual (table 1). We have analyzed the MTHFR C677T and A1298C mutations in a U.S. population of European descent, composed of patients with SB, their parents, and controls, and have observed the 677CT/1298CC genotype combination (table 2). In addition, we have observed the MTHFR C677T and A1298C mutations in a Canadian population of European descent, composed of patients with SB and their parents (table 3). We observed only a single individual, a patient with SB, with the 677TT/1298AC genotype. However, because of the small size of our sample, we expected that only one or two individuals in each of the groups would have the 677TT/1298AC genotype. It is therefore difficult to reach conclusions, on the basis of the absence of this genotype in the small Canadian population that we studied. The presence of these genotypes in healthy parents and controls militates against the hypothesis, proposed by Isotalo et al. (2000), that the absence of the 677CT/1298CC genotype suggests that "additional MTHFR mutations in *cis* are potentially deleterious or lethal" (Isotalo et al. 2000, p. 989). Perhaps it is the combination of two mutant alleles at both sites (677TT/1298CC), reaching a threshold of four, rather than three, mutations that creates a disadvantage. Other groups have also identified individuals with the 677T and 1298C alleles in the *cis* configuration and individuals with the mutations in the *trans* configuration (Weisberg et al. 1998; Friedman et al. 1999).

Table 3

Combined MTHFR C677T/A1298C Genotype or Allele Frequencies in a Canadian Population of European Descent, Composed of Patients with SB and Their Parents

Genotype or Allele	Observed Frequency in		
	Patients (n = 46)	Mothers (n = 45)	Fathers (n = 30)
MTHFR C677T/A1298C genotype:			
CT/CC	.022	.067	.033
TT/AC	.022	.000	.000
TT/CC	.000	.000	.000
MTHFR allele:			
677C	.641	.722	.683
677T	.359	.278	.317
1298A	.696	.589	.667
1298C	.304	.411	.333

An additional concern is that Isotalo et al. (2000) fail to indicate the ethnicity of the population that they studied. Therefore, we have provided data from three ethnic groups (Hispanics of Mexican descent, U.S. individuals of European descent, and Canadians of European descent), to compare genotype and allele frequencies. If the population studied by Isotalo et al. (2000) was Canadian, it is notable that they did not observe the 677CT/1298CC genotype in their neonatal group, whereas we observed this genotype in 2%–6% of the Canadian population, of patients with SB and their parents, that we studied. Our data support the conclusion of Isotalo et al. (2000) concerning decreased viability among fetuses with the 677TT/1298CC genotype, because we did not observe this genotype in the U.S. and Canadian populations that we studied. Because we observed, in three different populations, the 677CT/1298CC genotype at frequencies nearing those expected, we conclude that this genotype does not result in a significant selective disadvantage.

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Reply to Volcik et al.

To the Editor:

Volcik et al. (2001 [in this issue]) provide supporting evidence that methylenetetrahydrofolate reductase (MTHFR) 677 and 1298 alleles have crossed over, as we have demonstrated in fetal tissue and in antenatal subjects (Isotalo et al. 2000). Their observations also support the findings of Hanson et al. (2001), who demonstrated, in a study of adults, a genotype frequency of 0.2% for both MTHFR 677CT/1298CC and MTHFR 677TT/1298AC. The only MTHFR crossover combination that we observed in an antenatal control group was 677TT/1298AC (Isotalo et. al 2000). We hypothesized that the allelic combinations 677TT/1298CC and 677CT/1298CC are potentially deleterious or lethal in utero; however, the existence of 677CT/1298CC in both adults and children with spina bifida (SB) casts some doubt on our hypothesis.

A major consideration not addressed by Volcik et al. (2001 [in this issue]), concerning their study group, is that their study focused on children with SB and on their parents. Children with SB have survived a fetal defect—one that is known to cause stillbirth and miscarriage. Without medical intervention, postgestational mortality in infants with SB is high. Therefore, the MTHFR genotype combinations and frequencies observed in the nonviable group that we studied may overlap with those in groups with SB.

Additionally, development of SB, as well as of other neural-tube defects, can be reduced by folate sufficiency and therefore is affected by diet and, possibly, by other defects within the folate-delivery and metabolic pathways. Folate status has been shown to affect the contribution of the MTHFR 677 genotype to SB (Christensen et al. 1999). Nutritional-status differences between the study groups may influence the survival of specific genotypes. The contribution that the mother makes to SB or to fetal demise may be, in part, genetic—as was possibly the case in the U.S. 677TT/1298AC representation—and most certainly is in part due to maternal folate sufficiency. The distribution of 677TT/1298AC combined genotypes was well represented in all children with SB and in Hispanic parents but not in U.S. parents. Notably, the only groups with a strong representation of 677CT/1298CC, relative to the 677TT/1298AC genotype, were the U.S. and Canadian mothers of children with SB. This is in contrast to the lack of 677CT/1298CC in our Canadian antenatal control group. Of 148 control subjects, Volcik et al. found 2 individuals with the 677CT/1298CC genotype.

Volcik et al.'s Canadian study group consisted of affected families with SB and did not contain controls; therefore, we find it difficult, in relation to our control group, to draw conclusions concerning their findings of the 677CT/1298CC combinations. Our Canadian group was predominantly of European (Celtic) descent, although some Canadians of other derivations were included. It is interesting to note that Weisberg et al. (1998) also studied a Canadian population of children with SB and their mothers and did not identify any individuals with the 677CT/1298CC or 677TT/1298CC genotypes.

We still hypothesize that combined common polymorphisms of MTHFR play a role in fetal demise and in the development of neural-tube defects. Maternal MTHFR is in the position to affect the quantity and form of folate delivered to the fetus, whereas fetal MTHFR may subsequently affect the utilization and distribution of the supplied folate. The most important determinant for the development of neural-tube defects, however, is likely the initial folate sufficiency of the mother. It is unfortunate that Volcik et al. did not examine patterns between the parental—in particular, the maternal—MTHFR genotypes and the genotypes of the children with SB that they studied. Perhaps there are specific maternal/fetal combinations of MTHFR that have a relationship with either fetal demise or the development of SB.

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