titative-trait analysis was explicitly addressed nearly a decade ago for sib pairs (Kruglyak and Lander 1995) and, more recently, for larger pedigrees (e.g., Sham et al. 2002), although several methods still in use today have not fully accounted for this issue, and users should be cognizant of this fact (Cordell 2004). Also, although nonparametric linkage (NPL) analysis has always been recognized to be conservative when the data is not fully informative (Kruglyak et al. 1996), this problem has long been resolved either by calculating LOD scores (Kong and Cox 1997) or by estimating significance empirically through simulation (e.g., Kruglyak and Daly 1998), an approach that is becoming increasingly practical even for whole-genome scans. Other methods are examined in detail by Cordell (2004), who comes to similar conclusions. Of course, it is well appreciated that all linkage methods (and all statistical tests, in general) have lower power when faced with less informative data, but this broadly recognized effect is distinct from the "bias" claimed by Schork and Greenwood.

Schork and Greenwood (2004) also make a problematic statement about parametric linkage analysis. They correctly note that the contribution to the LOD score of completely uninformative families is zero—exactly the same as when such families are simply excluded from analysis—but then inexplicably conclude that "uninformative families detract from a linkage signal in parametric settings as well" (Schork and Greenwood 2004, p. 312). Since the final statistic in parametric analysis is simply the sum of individual family LOD scores, uninformative families, obviously, have absolutely no effect on the overall results.

In conclusion, the "bias" in linkage analysis claimed by Schork and Greenwood does not affect most modern nonparametric (and parametric) linkage analysis methods. The handling of incomplete information remains an active area of research in some specialized linkage settings.

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Got Bias? The Authors Reply

To the Editor:

We are happy to see that our colleagues have taken seriously the issue we raised in our article (Schork and Greenwood 2004), and, in essence, we do not disagree with much of the factual content of their letters (Abecasis et al. 2004; Mukhopadhyay et al. 2004; Visscher and Wray 2004 [all in this issue]). However, we strongly disagree with aspects of their commentaries and will concentrate on four related issues in our response: (1) the use of the word "bias" to characterize the effects of the treatment of non–fully informative observations as though they were fully informative, in a nonparametric linkage analysis setting; (2) the prevalence and pervasiveness of the inappropriate treatment of non–completely informative observations, in nonparametric linkage analyses; (3) the use of both simulation studies and published "guidelines" for the interpretation of linkage test statistics in the face of inappropriate treatment of non–fully informative observations; and (4) the difference between, and need for refinements in, paramet-

Table 1

SOLAR Variance-Components Analysis of a Quantitative Trait in which Some Families Are Forced to Be Uninformative

	LOD Scores				
Family ID	Original Data[a]	Linked[b]	Data Removed[c]	Homozygous[c]	Heterozygous[c]
1334	−.2215	...	−.0949	−.0949	−.0949
1345	−.2390	...	−.1149	−.1149	−.1149
1346	2.0463	2.1224	1.7108	1.7108	1.7108
1349	.1005	−.1837	.2588	.2588	.2588
1350	−.1488	...	−.2615	−.2615	−.2615
1358	.6113	...	.3778	.3778	.3778
1362	−.0085	...	−.4964	−.4964	−.4964
1377	.8464	1.4597	.6918	.6918	.6918
1408	.7518	1.1983	1.1969	1.1969	1.1969
1418	−.4073	...	−.4245	−.4245	−.4245
1421	.7240	.9921	1.1242	1.1242	1.1242
1424	.3491	.1458	.3342	.3342	.3342
Total LOD	4.4043	5.7346	4.3023	4.3023	4.3023

[a] Per-family scores produced by SOLAR with the original data.

[b] Scores computed on the basis of a reanalysis of only the families showing linkage in the original data.

[c] Per-family scores computed from an analysis in which the families contributing negative evidence for linkage in the original analysis were made uninformative at the marker locus by removing their genotype data (column 4), by making them homozygous for the same allele (column 5), or by making them heterozygous for the same alleles (column 6).

ric and nonparametric linkage–based gene-discovery strategies.

First, our commentators generally take offense to the use of the word "bias" in our description of what happens in a nonparametric linkage analysis when uninformative or partially informative observations (e.g., affected sibling pairs with non–completely informative marker-genotype data) are treated on equal footing with those that are fully informative. We are in no way wedded to the term "bias" and do not actually care how one refers to the issue we raised in our paper, whether as a "conservative handling" of partially informative observations or as a "power loss" due to the treatment of partially informative observations as though they were fully informative. We do want to emphasize that, as shown by Cordell (2004), the treatment of non–completely informative observations as though they were informative does, on average, deflate the test statistic toward values more consistent with the null hypothesis—as we showed in our simple and contrived example involving affected sibling pairs and the coin-flip example—and thus suggests that this phenomenon induces a tendency or "bias" (in a general sense) toward test-statistic values closer to the null hypothesis.

Second, our commentators dwell on the elegant work of Kong and Cox (1997), which considers the issue we describe in the context of affected–sibling-pair analyses. Kong and Cox (1997) provide a test statistic that appropriately combines uninformative and informative observations into a test statistic based on marker information. However, not all statistics currently in use exploit the principles described by Kong and Cox (1997). For example, a very recent survey by Cordell (2004) suggests that, indeed, statistics do exist that inappropriately treat non–completely informative observations as though they were fully informative, although the degree to which this phenomenon affects various linkage test statistics is context dependent. Thus, for example, of the six statistics for quantitative-trait analysis that Cordell examined, only one—the statistic implemented in the Merlin "Regress" software module—did not show the effects of this phenomenon. In this context, it could be said that perhaps the message of Kong and Cox (1997) simply has not reached the broader genetics community in the way our commentators would like. It should also be noted that Cordell's study was not exhaustive, suggesting that more research investigating other statistics is needed.

Since the SOLAR analysis program (Almasy and Blangero 1998) was not considered by Cordell (2004), we explored its handling of uninformative families in a simple study meant to showcase the issue of concern in a practical example. We want to emphasize that we believe SOLAR provides an excellent suite of genetic analysis tools despite the issue we expose (which is a result of the potential complexity of its handling in the setting of variance-components models). We used a subset of the data investigating the genetic determinants of a molecular phenotype in genotyped three-generation CEPH pedigrees (Greenwood et al. 2004; data available on re-

quest). We analyzed 12 CEPH pedigrees together and then forced 5 of them to be completely uninformative by three different methods. We then compared the results, which are presented in table 1, as per-family and overall LOD scores. Families with blank data in the second column not only contributed negative evidence for linkage to the overall original linkage signal (column 1 of table 1) but were forced to be completely uninformative in subsequent analyses—a phenomenon which, if accounted for properly (i.e., by not considering the contribution of the uninformative families to the linkage signal), should increase evidence for linkage, via the LOD score.

From table 1, it can be seen that the LOD score actually decreases (from 4.4 to 4.3) when the families providing negative evidence for linkage are made completely uninformative, which suggests that informativeness is not accounted for in this analysis. In addition, family 1358 was uninformative in the original data set yet contributed substantial positive evidence for linkage, which, again, is consistent with the potential for the inclusion of uninformative families to increase the value of the linkage statistic because of stochastic effects, as discussed in our article (Schork and Greenwood 2004) and Cordell's (2004). (Indeed, the individual informative and uninformative family LODs are simply summed without weighting, to give the total LOD, thus allowing the uninformative families to contribute to the LOD score for the sample.) We also found that the variance-components statistic implemented in the Merlin software package provided exactly the same overall LOD scores for these families as SOLAR did in each context, suggesting that Merlin is computing statistics in the same way as SOLAR.

Third, although simulation-based tests could be of value in helping determine the impact of the use of statistics that inappropriately treat non–completely informative observations as though they were informative in actual linkage studies (i.e., by simulating the process of including non–completely informative families in data sets and then estimating *P* values for observed statistics from these simulations), such practices can be problematic for a number of reasons:

1. Resorting to simulation studies merely reinforces the need to accommodate inappropriate handling of non–completely informative observations in the construction of a test statistic.
2. One would have to simulate in accordance with the exact mechanism creating the lack of informativeness (partial missing genotype data, marker informativeness, etc.), although the use of permutation tests of allele-sharing information in certain settings may ease this problem (note that not all computer programs provide, by default, *P* values for statistics based on simulation studies)—in addition, this would have to be pursued on a locus-by-locus basis to accommodate the marker information (and/or lack thereof) at each locus.
3. Point estimates of relevant parameters (sibling risk, variance explained, etc.) would not be as reliable as those obtained in a comparable sample of informative observations (as described in our analogy to flipping a coin).
4. Analyses that require simulation studies would produce actual test statistics that are highly context dependent (e.g., a low LOD score on one chromosome may have a low *P* value as a result of the reductions in the test statistic that arise from the inclusion of non–completely informative families as though they were completely informative, whereas a high LOD score on a different chromosome may have a high *P* value for the same reason), which would undermine conventional "guidelines" for assessment of linkage evidence based on test-statistic values—for example, to convey the value of a LOD score as an indication of linkage strength (Lander and Kruglyak 1995)
5. Because of the nonmonotonic relationships between test-statistic values that require simulations to assess significance, total sample size (i.e., a sample that is not adjusted for informativeness), and *P* values (from the simulations), one would have to be conscious not only of test statistics conveying linkage with artificially low values through these simulation studies but also of test statistics with artificially high values for the same reason—especially for statistics, such as variance-components statistics, that show wide variation in values when constructed without appropriate weighting for marker informativeness (Cordell 2004).

It is thus arguably better to use statistics that are designed to account for marker informativeness. In this context, however, studies that have not used, for example, locus-by-locus simulation studies to investigate the effect of the inclusion of non–completely informative observations on test-statistic values obtained throughout the genome might benefit from such studies, since interpretation of the statistical significance of their results is in doubt (see, e.g., the otherwise comprehensive and excellent studies by Panhuysen et al. [2003] and Arya et al. [2004])—a practice entirely consistent with the advice given in our article (Schork and Greenwood 2004).

Fourth, the problem of the inappropriate handling of non–completely informative observations is unique to nonparametric, as opposed to parametric, linkage analysis, since many conventional nonparametric linkage test statistics make use of assigned or imputed allele-sharing

values in their construction from available marker information. Thus, the inappropriate treatment of allele-sharing values assigned to observations that do not have informative marker data creates problems. This simply is not the case in conventional parametric linkage analysis, where, for example, uninformative observations simply do not contribute to a linkage statistic (i.e., they do not contribute positively or negatively to the signal but contribute a value of 0.0 to the overall LOD score, as though they were simply removed from the analysis).

To combat the issue we exposed, we suggest the following actions, all of which are consistent with our commentators' considerations: (1) software documentation should inform the user about (appropriate) potential problems in interpreting test statistics implemented in that software at face value (e.g., on the basis of the guidelines published by Lander and Kruglyak [1995] that focus on actual test-statistic values, such as LOD scores or *t* statistics); (2) simulation-based *P* values should be provided by default for problematic test statistics; and (3) greater emphasis should be placed on the derivation and use of statistics that, like the statistic in Kong and Cox (1997), are based on sound statistical principles for the treatment of non–completely informative observations.

The problems plaguing the reconciliation of multiple nonparametric linkage analysis results—in, for example, the combination of evidence to guide a positional cloning effort—are both numerous and vexing. Consider a recent example in which a LOD score of 11.68 implicating a susceptibility locus for myocardial infarction was reported (Wang et al. 2004*a*; see also the correspondence of Newton-Cheh et al. [2004] and Wang et al. [2004*b*]). On the basis of conventional guidelines, this LOD score should have (and was reported to have) an associated nominal *P* value of ~.00000000001, making it one of the (if not the single) most significant linkages ever reported for a complex trait. However, after simulation studies, this LOD score was found to have a *P* value of .0001 (still impressive but much less so). Although it is unclear if the statistic used to produce the LOD score of 11.68 was plagued by the stochastic effects of treating of non–fully informative observations as though they were informative, our article (Schork and Greenwood 2004) (and Cordell's [2004]) suggests that some statistics could (and, in fact, do) treat them this way and hence could lead to interpretive difficulties and discrepancies of this type. It is in this context that we provided the conclusion in our article, which we restate here with minor parenthetical qualifications (in brackets): "…researchers who have actually conducted relevant linkage studies (without completely informative data) in the past and ignored, or were not aware of, [the allele-sharing information] problem [i.e., by, e.g., knowingly or unknowingly using an available, though problematic, statistic without adjustment via, e.g., extensive locus-by-locus simulation studies] should go back and revisit their analyses" (Schork and Greenwood 2004, p. 316).

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Germline *PHOX2B* Mutation in Hereditary Neuroblastoma

To the Editor:

We read with interest the study by Trochet and colleagues (2004), published in the April 2004 issue of *The American Journal of Human Genetics,* that described germline mutations of the paired-like homeobox 2B gene (*PHOX2B* [MIM 603851]) in neuroblastoma (MIM 256700). We have also considered *PHOX2B* as a candidate gene for predisposition to neuroblastoma, and we now report on a germline *PHOX2B* mutation in a pedigree with neuroblastoma. However, we also show that there is no evidence for mutation of this gene in eight other pedigrees with neuroblastoma screened to date. We think these data establish *PHOX2B* as the first bona fide gene that can predispose to neuroblastoma when mutated in the germline, and the findings further emphasize the complex genetics of this important pediatric malignancy.

We previously demonstrated linkage of hereditary neuroblastoma to 16p12-13 by use of a genomewide screening strategy (Maris et al. 2002). Positional cloning of a putative 16p12-13 hereditary neuroblastoma-predisposition gene (*HNB1*) is ongoing, but the critical genomic region for this gene remains large. We had previously considered and excluded other genes known to be mutated in Hirschsprung disease (MIM 142623) and/or in congenital central hypoventilation syndrome (CCHS [MIM 209880]) as candidates for *HNB1,* because these disorders can occur coincident with both sporadic and hereditary neuroblastoma (Maris et al. 2002). Because of the recent reports that the vast majority of patients with CCHS harbor *PHOX2B* mutations, including two patients also affected with neuroblastoma (Amiel et al. 2003; Weese-Mayer et al. 2003), we initiated a screen for germline mutations in this gene in our series of pedigrees with neuroblastoma.

Oligonucleotide primer pairs flanking the coding regions of exons 1, 2, and 3 of *PHOX2B* were designed by use of the program Primer 3.0; these primer pairs were used for PCR amplification and bidirectional sequencing of purified PCR products (primer sequences available on request). We screened germline DNA from the proband and an unaffected family member for each of the seven families that showed cosegregation of a 16p haplotype with disease, as well as for two pedigrees that consisted of cousins with neuroblastoma with no cosegregation of 16p marker haplotypes (see Maris et al. [2002] for details of pedigrees). We also sequenced 109 control DNA samples from the Coriell SNP500 Cancer Panel (Coriell Cell Repositories). All sequence aberrations were confirmed by repeat sequencing after cloning of purified PCR products (TOPO TA Cloning Kit [Invitrogen]), and DNA samples from the remaining available members of the pedigree were also screened for the variant. The Children's Hospital of Philadelphia institutional review board approved this work.

A heterozygous single-base deletion (676delG) was discovered in a complex pedigree with neuroblastoma (fig. 1) (see dbSNP Home Page). This family has seven members in three generations affected with neuroblastoma, and two of these individuals were also shown to have Hirschsprung disease. The proband was affected with neuroblastoma, Hirschsprung disease, and neurofibromatosis type 1 (MIM 162200). The putative nonsense mutation 676delG segregated with neuroblastoma through all three generations, and the frameshift was predicted to produce a slightly truncated protein that would no longer code for the second polyalanine tract. This family had previously been shown to cosegregate a 16p12-13 haplotype with neuroblastoma, and the proband was also shown to have an inactivating mutation in *NF1* (3775delT) that was not present in either of her parents (Maris et al. 2002). Tumor material was available only for patient 1-001, and the tumor exon 3 sequence remained heterozygous for the 676delG mutation. In addition, loss-of-heterozygosity studies using microsatellite markers (D4S2912, D4S1587, D4S405, D4S2971, and D4S428) that are closely linked to the *PHOX2B* locus showed no evidence for allelic deletion. The only other sequence variant discovered in the remaining eight pedigrees was a putative SNP (C552T) in pedigree 12 that is not predicted to affect the resultant protein sequence (S184S) (see dbSNP Home Page). This