Letters to the Editor

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Failure to Detect Linkage of Preeclampsia to the Region of the *NOS3* Locus on Chromosome 7q

To the Editor:

Preeclampsia is an important inheritable pregnancy-related hypertension syndrome. Since it develops as a result of widespread endothelial dysfunction, the *NOS3* gene responsible for endothelium-derived nitric oxide (NO) production via its gene product, eNOS, has been suggested as an obvious candidate gene for preeclampsia (Morris et al. 1996). We were intrigued, therefore, by the report, by Arngrímsson et al. (1997), of linkage in the region of the *NOS3* gene and have attempted to repeat their findings in our own collection of preeclampsia families.

Evidence for linkage was sought by use of two, separately ascertained, affected sister-pairs (ASPs) collections, from Amsterdam and Cambridge (United Kingdom), that contained 104 sibships. In the Cambridge Centre, a total of 21 extended pedigrees were also identified, some on the basis of the ASPs suitable for conventional parametric linkage studies. All affected individuals were Caucasian and met the Redman and Jefferies criteria of preeclampsia: they were proteinuric (>300 mg/24 h) and hypertensive, with a blood pressure of >140/90 occurring after 20-wk gestation and with a ⩾25-mmHg rise in diastolic blood pressure (Redman and Jefferies 1988). These features all resolved by 3 mo postpartum, and none of the subjects had concurrent diabetes, renal disease, or essential hypertension. Some sibships (<5%) contained subjects showing features of pregnancy-induced hypertension (PIH) alone (i.e., they were not proteinuric). These members were not included in subsequent sib-pair analysis, and none of the larger pedigrees used contained subjects with PIH. For the purposes of linkage analysis, all males were assigned to the affection status of "unknown."

Subjects were genotyped for the CA-repeat marker within intron 13 of the *NOS3* gene (referred to here as "*NOS3i13*"). The Cambridge samples were also genotyped for the two flanking markers D7S483 and D7S505. Genotyping was performed by use of PCR amplification of the microsatellite markers by means of primer pairs, in which the forward primer had been 5′ end–labeled with a fluorescent amidite. CEPH primer sequences were used for the flanking markers, and primer sequences published elsewhere were used for *NOS3i13* (Nedaud et al. 1994). The PCR products were multiplexed and were run on an ABI 377, and allele sizes were determined by use of version 2 of GENOTYPER (Applied Biosystems).

The number of alleles shared identical by descent (IBD) was calculated for the ASPs by use of the maximum-likelihood method, as implemented in SPLINK (Holmans 1993; Holmans and Clayton 1995). Parental genotype data were used where available but were incomplete for the Cambridge ASPs and were not available for the Amsterdam ones. Comparison of allele frequencies and marker heterozygosity from the SPLINK output for the two groups showed no significant differences between the two collections. The allele sharing for the *NOS3i13* marker is shown in table 1, for both the Amsterdam and Cambridge ASPs. There is no evidence of excess allele sharing in either group. In fact, the Cambridge group shows a deficit of allele sharing, attributable to chance alone. To confirm this, allele sharing for the two flanking markers for *NOS3,* D7S483 and D7S505, was investigated. These two flanking markers are approximately equidistant from *NOSi13* and span a 4-cM region over 7q36. Again, there was no evidence of a significant positive deviation from a 1:2:1 distribution in the Cambridge group, either for D7S483 or for D7S505.

Parametric analysis of the preeclampsia pedigrees was performed by use of four of the models, employed elsewhere (Arngrímsson et al. 1997), as follows (q is the frequency of disease gene, and f is the penetrance): an autosomal model (AD) of high arbitrary penetrance ($q = .02$, $f = 0.9$), a partial-dominance model (AD/LP; $q = .10$, $f_{Aa} = 0.21$, $f_{AA} = 1.0$), a fully recessive model (AR; $q = .2$), and an affecteds-only model (AO; $q = .02$, $f_{Aa} = f_{AA} = 0.001$). The power of the preeclampsia pedigree collection to detect linkage was estimated by means of simulation of each model, by use of SLINK (Weeks et al. 1991; Ott 1989). The results of 500 replications of each model generated by SLINK, if a hypothetical marker with eight equally frequent alleles

Table 1

Allele Sharing for *NOS3i13* and Flanking Markers

ASP Collection and Marker	No. of Alleles Shared IBD			*P*
	0	1	2	
Amsterdam ($n = 46$):				
NOS3i13	11.1	20.2	14.6	.250
Cambridge ($n = 58$):				
D7S483	11.3	32.0	14.7	.388
NOS3i13	18.0	28.0	12.0	.584
D7S505	15.1	30.0	13.8	.584
Combined ($n = 104$):				
NOS3i13	28.9	47.8	26.3	.547

(PIC = .86) is assumed, are summarized in tables 2 and 3. These results show that such a marker linked tightly to preeclampsia provides an average LOD score sufficient to establish linkage in three of the four models. The power of the simulated marker to detect linkage in the AD/LP model is substantially less than that of the other three models, as has been noted elsewhere (Harrison et al. 1997).

The models were tested by use of all three markers for the 4-cM interval, on 7q, encompassing the *NOS3* locus. Table 4 shows the results of two-point linkage analysis, using allele frequencies provided by SPLINK; the findings were not altered significantly by the assignment of equal allele frequencies throughout. For all the models, linkage to *NOS3i13* could be excluded with LOD < -2. As expected from the SLINK modeling, the low-penetrance AD/LP model provided smaller LOD scores than did the other three models, and linkage to the flanking markers could not be excluded in our preeclampsia pedigrees. However, for neither of the markers under the AD/LP model did the LOD scores approach the required threshold of $3 + \log(4)$ (Kidd and Ott 1984).

These results, therefore, have failed to confirm linkage of preeclampsia to a chromosome 7q 4-cM region containing the *NOS3* gene, as reported by Arngrímsson et al. (1997). This failure occured despite use of a similar combination of ASPs and conventional two-point linkage in pedigrees. Our study used two independently ascertained collections of ASPs, containing a total of 104 ASPs that appeared to be powered adequately. We have estimated the λ_s for preeclampsia at ~10, assuming a local prevalence of preeclampsia (using our strict definition) of some 2%. Since our total ASP collection contains 70 fully informative pairs (estimated by SPLINK) for the *NOSi13* marker, an expected maximized LOD score of 7.6 is given (Risch 1990). Even if λ_s has been overestimated, a figure of 5 would still result in a LOD score substantially >3 (actually 5.7).

Our inability to replicate the earlier linkage report could reflect population differences, although both studies are based on white northern Europeans. It is possible that significant differences in our definition of the preeclampsia phenotype may be important, although the differences in our method of ASP analysis seem unlikely. We have focused in our study only on ASPs with a definite diagnosis of preeclampsia, excluding ones in which the diagnosis either is uncertain or is consistent only with isolated PIH. Nevertheless, Arngrímsson et al. (1997) report that the significance of the excess allele sharing was not substantially altered by removal of the milder phenotypes from the sib-pair analysis. Our method of sib-pair analysis relies on a maximum-likelihood method (SPLINK) to calculate allele sharing that is IBD. The SPLINK program is able to utilize parental genotype data, although this was not available for most of our ASPs. The previous linkage study on 7q also used a likelihood ratio–based method (SIBPAIR), as well as direct testing of increased identity by state (IBS) sharing (APM). A recent comparison of the various methods available to detect linkage in nuclear pedigrees, including sib pairs, showed the superiority of IBD-based programs versus IBS-based programs (Davis and Weeks 1997). In this comparison, SPLINK actually showed comparable power to detect linkage with the SIBPAIR program, except when additional genotypes were available from unaffected members, which was not the case in this study. Our choice of SPLINK, therefore, is unlikely to have increased the possibility of a false-negative result.

The previous positive sib-pair analysis reported by Arngrímsson's group was also supported by two-point linkage results from their pedigree collection (Arngrímsson et al. 1997). They investigated a number of different models, reported elsewhere, from the literature. The LOD score, maximized over the five models, was 4.03, by use of the D7S505 flanking marker rather than the *NOS3i13* marker. This suggests that the preeclampsia locus may be some distance away from the *NOS3* gene itself, although the *NOS3i13* marker and the preeclampsia locus must be in linkage disequilibrium, on the basis of their transmission/disequilibrium-test results. Adopting a similar parametric analysis in our own preeclampsia pedigrees, we have failed to demonstrate a LOD score high enough to confirm linkage in any of the models. In fact, the LOD scores generated actually have enabled us

Table 2

Results of Computer Simulations with SLINK, for 500 Replicates

Model	Average LOD Score at θ =				
	.0	.1	.2	.3	.4
AD	6.05	4.89	3.37	1.92	.75
AD/LP	1.70	1.49	.99	.53	.18
AR	4.89	3.81	2.48	1.26	.39
AO	3.88	3.12	2.05	1.08	.37

to exclude linkage in all of them, except the AD/LP model. Using the AD/LP model, we did find a small, nonsignificant LOD score, using a flanking marker, but it was by use of D7S483 and not D7S505—that is, at the opposite end of the 7q interval.

A key role for endothelium-derived NO in pregnancy is well supported. An eNOS inhibitor, for example, infused chronically into pregnant animals, produces a preeclampsia-like state, with hypertension, proteinuria, thrombocytopenia, and growth retardation (Molnar and Hertelendy 1992). More-recent findings also provide direct evidence for the role of NO production in the fall in peripheral vascular resistance (and blood pressure) seen in normal human pregnancy (Knock and Poston 1996). It is possible that NO forms part of an adaptation pathway, to accommodate the cardiovascular changes of pregnancy and to prevent the development of maternal hypertension and the clinical syndrome of preeclampsia. However, although these data provide a tantalizing circumstantial argument for *NOS3* being a candidate gene for preeclampsia, they do not prove that a *primary* abnormality in eNOS underlies the pathophysiology of preeclampsia. It is equally plausible that the observed changes in NO production during preeclampsia are secondary to free-radical damage of the vascular endothelium.

In summary, then, we have been unable to replicate the previous report of linkage of preeclampsia to the region of the *NOS3* gene. Although abnormalities in NO production have been observed in preeclampsia, we believe that the case for the *NOS3* gene or its product, eNOS, having a *primary* role in the pathophysiology of preeclampsia remains unproved.

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Table 3

Results of Two-Point Linkage Analysis

	% of Replicates with LOD-Score Threshold >		
Model	1	2	3
AD	100.0	99.4	97.6
AD/LP	78.4	41.4	10.8
AR	100.0	98.4	89.2
AO	98.8	89.6	72.4

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Table 4

Two-Point Linkage Results for the Four Models Tested

Model and Marker	LOD Score at θ =						
	.00	.01	.05	.10	.20	.30	.40
AD:							
D7S505	−7.22	−5.52	−3.01	−1.59	−.34	.08	.14
NOS3i13	−9.76	−8.40	−5.41	−3.44	−1.40	−.46	−.07
D7483	−2.37	−2.05	−1.27	−.80	−.41	−.26	−.15
AD/LP:							
D7S505	−1.56	−1.40	−.90	−.49	−.07	.07	.07
NOS3i13	−2.08	−1.86	−1.20	−.67	−.12	.07	.08
D7483	.34	.39	.51	.53	.39	.17	.02
AR:							
D7S505	$-\infty$	−7.02	−3.63	−2.08	−.74	−.23	−.04
NOS3i13	$-\infty$	−7.69	−3.98	−2.16	−.58	−.04	.07
D7483	$-\infty$	−3.80	−1.46	−.50	.11	.14	.03
AO:							
D7S505	−3.20	−2.51	−1.03	−.22	.32	.34	.20
NOS3i13	−6.03	−5.05	−2.92	−1.63	−.47	−.06	.04
D7483	−1.91	−1.59	−.86	−.43	−.13	−.09	−.08

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