The familial nature of migraine is high, with about 50% of those affected having an affected first-degree relative. Familial clustering suggests that genetic factors are involved in the disorder, but the mode of inheritance is controversial. Studies by Mochi et al. suggest there may be a common genetic background for the two main subtypes of migraine, migraine with and without aura, and that there may be a major gene contributing to the disease. A review of migraine twin, spouse, and family aggregation studies strongly suggested that these subtypes of migraine are genetically determined, with the mode of inheritance most likely multifactorial. However, autosomal dominant inheritance could not be excluded in either subtype of migraine. At present, the number of genes involved in the disorder is unknown and not identified, although a gene for a rare subtype of migraine, familial hemiplegic migraine, has been mapped to chromosome 19.

Nitric oxide (NO) is a messenger molecule that has different biological functions in different tissues. It plays an important role in the regulation of basal or stimulated vasodilation. Nitric oxide is involved in the central processing of pain stimuli and plays an important role in the regulation of basal or stimulated vasodilation. Nitric oxide synthase, which controls the synthesis of nitric oxide, could possibly be a cause, or candidate gene, in migraine etiology. In this study, we detected a polymorphism for endothelial nitric oxide synthase by polymerase chain reaction and tested this for association and linkage to migraine. Results from the study did not show an association of the nitric oxide synthase microsatellite when tested in 91 affected and 85 unaffected individuals. Using the FASTLINK program for parametric linkage analysis, the polymorphism did not show significant linkage to migraine when tested in four migraine pedigrees composed of 116 individuals, 52 affected. Total LOD scores excluded linkage up to 8.5 cM between the nitric oxide synthase polymorphism and migraine. Results using the nonparametric affected pedigree member form of analysis also did not support a role for this gene in migraine etiology.

Methods. Blood samples were collected from 91 migraine-affected and 85 unaffected individuals for the association studies and also from the members of 4 large multigenerational families for linkage studies. Prior to commencement, all research was approved by Griffith University's Ethics Committee for Experiments on Humans. All individuals donating blood samples gave informed consent, and all were of Caucasian origin. Volunteers for the association studies and migraine pedigree index cases were recruited from subjects involved in the Nambour Skin Cancer Trial and also from individuals responding to national media release requests. For the association studies, individuals were classified as migraine-affected if they indicated by questionnaire that they had a family history of the disorder and that they themselves displayed symptoms consistent with migraine diagnosis according to International Headache Society (IHS) criteria. Similarly, control individuals indicated by questionnaire that they had no personal symptoms and no family history of the disorder. For the linkage studies, all family pedigree members were personally interviewed by a clinical neurologist to establish diagnosis following the criteria specified by IHS, with affected individuals classified as having migraine with aura (MA) or migraine without aura (MO).

DNA was extracted with use of a standard salting-out procedure, and genotypes for the NOS3 polymorphism were determined by PCR with fluorescently labeled primers. The NOS3 (CA)n repeat was within intron 1 and the PCR primer pair was designed to be in intron 1.

**Article abstract**—Migraine shows strong familial aggregation. However, the number of genes involved in the disorder is unknown and not identified. Nitric oxide is involved in the central processing of pain stimuli and plays an important role in the regulation of basal or stimulated vasodilation. Nitric oxide synthase, which controls the synthesis of nitric oxide, could possibly be a cause, or candidate gene, in migraine etiology. In this study, we detected a polymorphism for endothelial nitric oxide synthase by polymerase chain reaction and tested this for association and linkage to migraine. Results from the study did not show an association of the nitric oxide synthase microsatellite when tested in 91 affected and 85 unaffected individuals. Using the FASTLINK program for parametric linkage analysis, the polymorphism did not show significant linkage to migraine when tested in four migraine pedigrees composed of 116 individuals, 52 affected. Total LOD scores excluded linkage up to 8.5 cM between the nitric oxide synthase polymorphism and migraine. Results using the nonparametric affected pedigree member form of analysis also did not support a role for this gene in migraine etiology.
Nonparametric analysis supported the parametric form of analysis detected any overall significant difference between the tested populations. Since association studies only test single points and those regions of the genome in linkage disequilibrium with the tested marker, we also performed linkage studies, which test not only the marker but areas linked to the marker, to determine the involvement of eNOS in migraine.

**Discussion.** From use of association studies and parametric and nonparametric forms of linkage analysis, our results do not support a role for endothelial nitric oxide synthase (eNOS) in migraine etiology. Association results showed no significant difference between migraine and nonmigraine subjects. One specific allele (164 bp) showed a small significant difference, most likely caused by low numbers in sample size, although further investigation with a larger sample may be more definitive. The NOS3 marker detected 20 alleles in the association study, reducing the accuracy of normalized chi-square analysis. To overcome this problem, we used a Monte Carlo test, applying the data set over 10,000 simulations to test for significant differences. Neither form of analysis detected any overall significant difference between the tested populations. Since association studies only test single points and those regions of the genome in linkage disequilibrium with the tested marker, we also performed linkage studies, which test not only the marker but areas linked to the marker, to determine the involvement of eNOS in migraine.

**Results.** The NOS3 polymorphism detected alleles ranging in size from 134 to 190 bp. In both association and linkage studies, most individuals were typed as heterozygotes. Twenty alleles were detected for the association study and 15 alleles from the four families used in linkage studies. Chi-square analysis showed no significant difference between the migraine-affected and -unaffected populations for the polymorphism, \( \chi^2 = 24.78; p = 0.168 \) (table 1). This was confirmed by Monte Carlo analysis with 10,000 simulations, \( \chi^2 = 24.78 \) and \( p = 0.155 \). One allele (164 bp), however, did show a small significant difference between the two populations (\( \chi^2 = 10.56; p = 0.001 \)), but the numbers of this allele in both populations were very small.

Individuals from four migraine pedigrees (MF1, MF7, MF14, and MF15) were genotyped for the polymorphism as shown in the figure. Four migraine pedigrees comprised of 116 individuals, 52 of whom were affected, were used in the linkage studies. The power of these pedigrees was calculated with use of the SIMLINK program, which estimates the power of a proposed linkage study for a complex genetic trait. These calculations showed that, using only three of these migraine pedigrees (MF1, MF7, and MF14) and a four-allele system, there is 85% power to detect significant linkage (LOD score \( \geq 3 \)) to a distance of 15 cM. Therefore, using a more informative marker such as the 20-allele NOS3 marker and using four pedigrees, we estimate that we have at least 90% power to detect linkage out to 20 cM.

Linkage results were analyzed with use of the FASTLINK program with two-point LOD scores calculated for the four pedigrees. LOD score results for all the tested families did not show probable linkage, with the total LOD scores for all four families, as presented in table 2, showing significant nonlinkage up to at least \( \theta = 0.05 \). In fact, linkage could be excluded up to 8.5 cM, since the families gave a total LOD score of \(-2.04\) at \( \theta = 0.085 \). Linkage results were also analyzed with use of the APM method with results presented in table 3. The significance of the APM statistics was calculated by the simulation of 1,000 replicates assuming an absence of linkage. As indicated in table 3, the \( p \) values obtained for all four families both individually and in total were not significant, with all \( p \) values >0.2.
Figure. Pedigrees of migraine families 1, 7, 14, and 15 (MF1, MF7, MF14, and MF15). All individuals have been diagnosed as having migraine with aura (MA), migraine without aura (MO), or as unaffected (blank), following IHS guidelines, and diagnoses are given directly under each symbol. Genotypes for the NOS3 microsatellite marker are indicated below migraine status.
Table 2 LOD scores* from migraine—NOS3 linkage analysis

<table>
<thead>
<tr>
<th>Pedigree no.</th>
<th>0.0</th>
<th>0.01</th>
<th>0.05</th>
<th>0.1</th>
<th>0.2</th>
<th>0.3</th>
<th>0.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MF1</td>
<td>−1.04</td>
<td>−0.78</td>
<td>−0.22</td>
<td>0.13</td>
<td>0.39</td>
<td>0.36</td>
<td>0.19</td>
</tr>
<tr>
<td>MF7</td>
<td>−0.53</td>
<td>−0.51</td>
<td>−0.42</td>
<td>−0.32</td>
<td>−0.16</td>
<td>−0.06</td>
<td>−0.01</td>
</tr>
<tr>
<td>MF14</td>
<td>−2.17</td>
<td>−2.03</td>
<td>−1.62</td>
<td>−1.23</td>
<td>−0.71</td>
<td>−0.36</td>
<td>−0.13</td>
</tr>
<tr>
<td>MF15</td>
<td>−0.65</td>
<td>−0.61</td>
<td>−0.49</td>
<td>−0.37</td>
<td>−0.20</td>
<td>−0.10</td>
<td>−0.04</td>
</tr>
<tr>
<td>Total</td>
<td>−4.39</td>
<td>−3.94</td>
<td>−2.74</td>
<td>−1.79</td>
<td>−0.68</td>
<td>−0.15</td>
<td>0.01</td>
</tr>
</tbody>
</table>

* LOD scores from four migraine families using complete pedigree data were computed using FASTLINK and calculated at recombination fractions (θ) varying from 0 to 0.40, θ_m = θ_p.

Table 3. NOS3-migraine APM analysis

<table>
<thead>
<tr>
<th>Marker</th>
<th>Family</th>
<th>t Statistic</th>
<th>p Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOS3</td>
<td>1</td>
<td>−0.31175</td>
<td>p value</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.61112</td>
<td>p value</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>−0.40588</td>
<td>p value</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>−0.08942</td>
<td>p value</td>
</tr>
<tr>
<td></td>
<td>All 4</td>
<td>−0.11883</td>
<td>p value</td>
</tr>
</tbody>
</table>

* p Values were simulated after 1,000 iterations with the intermediate, \( r(p) = 1/\sqrt{p} \), weighting function.

Hence, linkage data obtained from the NOS3 polymorphism did not indicate probable linkage of eNOS to migraine and significantly excluded close linkage of NOS3 to migraine in one large migraine pedigree. Although eNOS does not appear to be involved in migraine etiology, other genes involved in vasodilation and the central processing of pain may still play a role in this complex disorder.

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