Association between Parkinson’s disease and polymorphisms in the nNOS and iNOS genes in a community-based case–control study

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Excess of nitric oxide (NO) has been shown to exert neurotoxic impacts in the brain. Moreover, inhibition of two NO-synthesizing enzymes, neuronal NOS (nNOS) and inducible NOS (iNOS), displays neuroprotective effects in the MPTP model of Parkinson’s disease (PD). These data suggest a possible involvement of NOS as factors controlling the resistance of the nigral dopaminergic neurons to environmental insults. Therefore, we investigated whether polymorphisms present in these genes could contribute to the risk of developing PD. We carried out a community-based case–control study among subjects enrolled in the Mutualité Sociale Agricole, the French health insurance organization for workers connected to agriculture. Two-hundred and nine PD patients and 488 controls of European (mostly French) ancestry and matched for age, sex and region of residency were included in this study. Associations were observed with polymorphisms present in exon 22 of iNOS (OR for AA carriers = 0.50, 95% CI = 0.29–0.86, \( P = 0.01 \)) and in exon 29 of nNOS (OR for carriers of the T allele = 1.53, 95% CI = 1.08–2.16, \( P = 0.02 \)); no association was observed with a polymorphism in exon 18 of nNOS (OR for carriers of the T allele = 1.20, 95% CI = 0.85–1.69, \( P = 0.30 \)). Moreover, a significant interaction of the nNOS polymorphisms with current and ever cigarette smoking was found (nNOS 18, \( P = 0.05 \); nNOS 29, \( P = 0.04 \)). All together, these data favour an involvement of these two genes as new modifier genes in PD.

INTRODUCTION

Parkinson’s disease (PD) is the most common neurodegenerative disorder after Alzheimer’s disease. In the last 10 years, the identification of mutations in three different genes (α-synuclein, Parkin and Ubiquitin C-terminal Hydrolase) and the localization of at least five other loci have underscored the importance of genetic factors in the development of PD (1–9). Moreover, the observation that dopaminergic neurotoxin MPTP effects are similar to those observed in PD has led investigators to undertake association studies for polymorphisms in candidate genes involved in the dopamine pathway, mitochondrial function, lipoprotein metabolism or xenobiotic detoxification (10).

Nitric oxide (NO) is a biological messenger molecule with diverse physiologic roles. However, NO is also a free radical and can combine with superoxide anions to form peroxynitrite. Therefore, NO contributes to oxidative stress and induces numerous changes in the cells, including (1) lipid peroxidation, (2) functional alterations in proteins (e.g. nitrosylation), (3) DNA damage and (4) mitochondrial energy dysfunction (11). The formation of NO is catalysed from arginin and oxygen by the nitric oxide synthase (NOS). Three isofoms of NOS have been identified: neuronal NOS (nNOS); endothelial NOS (eNOS); and the inducible NOS (iNOS). Of the three isofoms, nNOS and iNOS are particularly relevant with respect to their potential implications in neurodegeneration and glial response occurring in PD. Indeed, nNOS is the main isoform constitutively expressed in neurons and glia. Moreover, iNOS can be induced in brain glial cells and invading macrophages in responses to a variety of injuries (12,13).

There are several lines of evidence in humans as well as in animal models favouring an implication of NO and NOS in the aetiology of PD. In particular, the inhibition of nNOS or iNOS, either using pharmacological substances or gene-deficient mice, prevents the destruction of dopaminergic neurons in MPTP models of PD (14–19).

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†The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.
We therefore explored the relation between polymorphisms in the nNOS and iNOS genes and PD in a community-based case–control study. Moreover, we investigated if exposure to pesticides or cigarette smoking modified the relation between these polymorphisms and PD. MPTP is an analog of the pesticide Paraquat, and exposure to pesticides is associated with PD (20). In addition, MPTP modulates nNOS and iNOS expression (18,21). Conversely, cigarette smoking is inversely associated with PD and may affect NO production (22). Our data tend to implicate both nNOS and iNOS genes in the development of PD.

RESULTS

Two-hundred and ninety-two subjects requested free health coverage for PD to the Mutualité Sociale Agricole (MSA). Of these, 184 were directly examined by the neurologist: 153 were found to have PD, 29 had another cause of parkinsonism, and two did not fulfill the criteria for parkinsonism. Of the remaining 108 patients, the neurologist who had requested free health coverage provided clinical information for 86 patients: he confirmed the PD diagnosis for 72 and diagnosed another cause of parkinsonism for 14. No detailed clinical information was obtained for 22 patients. However, based on the clinical examination by the MSA physician, all fulfilled the criteria for parkinsonism, and none had used neuroleptics prior to PD onset; they were therefore retained for our analyses. The 247 (153+72+22) patients were matched to 676 controls (194 quadruplets, 41 triplets, 12 pairs); 89% of the PD patients and 84% of the matched controls accepted blood sampling for DNA extraction. In addition, five patients and seven controls with at least one parent born outside Europe were excluded. The final sample includes 209 patients and 488 matched controls (108 quadruplets, 63 triplets, 38 pairs). Their general characteristics are shown in Table 1.

The results concerning the three biallelic polymorphisms in the two candidate genes are presented in Table 2. The control population was in Hardy–Weinberg equilibrium for all three polymorphisms (iNOS 22, P = 0.45; nNOS 18, P = 0.47; nNOS 29, P = 0.56).

AA homozygotes for the iNOS 22 polymorphism were more frequent among controls than among patients (OR = 0.56, 95% CI = 0.32–1.01; P = 0.05), whereas there was no significant difference in the frequency of heterozygotes between the two groups. Assuming a recessive effect of the A allele, the OR for AA carriers was 0.50 (95% CI = 0.29–0.86; P = 0.01) compared with the two other genotypes. This inverse association was stronger in men (OR = 0.40, 95% CI = 0.17–0.92; P = 0.03), than in women (OR = 0.61, 95% CI = 0.30–1.23; P = 0.16); however, the interaction between sex and iNOS 22 was not significant (P = 0.49). Similar results were obtained when considering only cases and matched controls without family history of PD (OR for AA carriers = 0.48, 95% CI = 0.27–0.86; P = 0.01) or with both parents born in France (OR for AA carriers = 0.53, 95% CI = 0.30–0.93; P = 0.03), and when cases with young onset PD (≤45 years, n = 4 and 10 matched controls) were excluded from the analyses (OR for AA carriers = 0.47, 95% CI = 0.27–0.82; P = 0.01). This association was not modified by age at onset of PD. (Data not shown.)

There were no statistically significant differences in the genotypic distributions of the nNOS 18 polymorphism between patients and controls (Table 2).

Conversely, genotypic distributions of the nNOS 29 polymorphism were different between patients and controls (P = 0.01). Both heterozygotes and TT homozygotes were at increased risk of PD. The OR increased with the number of T alleles (P for trend = 0.004). The OR for carriers of at least one T allele was 1.53 (95% CI = 1.08–2.16; P = 0.02). According to the Akaike Information Criterion of the codominant (484.281) and dominant (486.868) models, the codominant model was the best fitting model. Again, we noted that the association between the polymorphism and PD was stronger in men (OR = 1.84, 95% CI = 1.15–2.94; P = 0.01), than in women (OR = 1.21, 95% CI = 0.72–2.04; P = 0.47), although the interaction between sex and nNOS 29 was not statistically significant (P = 0.23). Similar results were obtained for cases and matched controls without family history of PD (OR for T carriers = 1.56, 95% CI = 1.08–2.26; P = 0.02), or with both parents born in France (OR for T carriers = 1.58, 95% CI = 1.10–2.26; P = 0.01), and when cases with young-onset PD were excluded from the analyses (OR for T carriers = 1.52, 95% CI = 1.07–2.15; P = 0.02). This association was not modified by age at onset of PD (data not shown).

Haplotypes were constructed for the two nNOS polymorphisms (Table 3). Among patients, there was an excess of haplotypes including the T allele for nNOS 29; this excess appeared to be independent of the nNOS 18 allele (case–control comparison of nNOS 18–nNOS 29 haplotypes C-T and T-T versus all others: OR = 1.39, 95% CI = 1.07–1.79, P = 0.009).

We explored the hypothesis of an interaction between the nNOS 29 and iNOS 22 polymorphisms (Table 4). Our data suggest that the protective effect of iNOS 22 was weaker for nNOS 29 TT or CT carriers than for CC homozygotes, or, in other words, that the effect of nNOS 29 was stronger among AA iNOS 22 carriers than among GA or GG carriers (observed OR for joint effects = 0.97 versus an expected OR of 0.29 × 1.41 = 0.41). However, the interaction test was not significant (P = 0.16). In a multivariate model that included both polymorphisms as main effects but no interactions, the association between PD and both nNOS 29 (OR for T carriers = 1.53, 95% CI = 1.08–2.17; P = 0.02) and iNOS 22 (OR for AA carriers = 0.48, 95% CI = 0.28–0.83; P = 0.01) remained significant.

Since smoking was inversely associated with PD in this population (Table 1), we tested the interaction between the two polymorphisms in the nNOS gene and cigarette smoking. An interaction was observed, as shown in Figure 1. For both nNOS polymorphisms, the inverse association between PD and smoking was lost among carriers of the polymorphic T allele, while it was present among homozygotes for the C allele (with a stronger effect of current smoking than ex-smoking). The interaction between the polymorphisms and current smoking was statistically significant (nNOS 18, P = 0.04; nNOS 29, P = 0.04), while the interaction between the polymorphisms and ex-smoking was not statistically significant (nNOS 18, P = 0.22; nNOS 29, P = 0.17). If ex- and current smoking were grouped together (ever smoking), the interaction between ever smoking and the polymorphism was not statistically significant (nNOS 18, P = 0.22; nNOS 29, P = 0.17).
smoking and the nNOS 18 (P = 0.05) and nNOS 29 (P = 0.04) polymorphisms remained significant.

Finally, because exposure to pesticides is positively associated with PD, we investigated the joint effect of exposure to pesticides and the polymorphisms, and found no significant interactions between exposure to pesticides and none of the polymorphisms (data not shown).

DISCUSSION

We have genotyped three polymorphisms in two NOS genes (nNOS and iNOS) in a large community-based case–control study of PD, composed essentially of sporadic patients. We found a significant over-representation of carriers of at least one nNOS 29 T allele and an under-representation of AA iNOS 22 homozygotes in PD cases compared with controls, this last polymorphism being newly reported. No association was observed between PD and the nNOS 18 polymorphism. Moreover, nNOS 18 and nNOS 29 may modulate the relation between cigarette smoking and PD.

These results suggest that nNOS and iNOS may play a role in the susceptibility to sporadic PD. This is in agreement with several studies in animals or humans. In animals, nNOS inhibitors are protective against MPTP, and mutant mice that lack the nNOS gene are more resistant to MPTP than wild-type mice (14–17,23). Similar experiments with iNOS show that this enzyme also plays a role in the sensitivity of dopaminergic neurons (18,19,24).

There is also evidence of NO overproduction as well as of an implication of nNOS and iNOS in PD patients. Increased nitrite concentrations have been found in the CSF of treated and untreated PD patients compared with controls (25), and nNOS was overexpressed in neutrophils of PD patients compared with controls (26). An increased NO signal was found using spectroscopy in the substantia nigra of PD patients compared with controls (27), and considerable amounts of iNOS were expressed in the substantia nigra of post-mortem PD samples, but not in age-matched control samples (28). Markers of NO formation (e.g. nitrotyrosine immunoreactivity) have been observed in Lewy bodies in PD (29) and MPTP-induced parkinsonism patients. Finally, bromocriptine, which is used as a treatment of PD, is a strong inhibitor of rat nNOS and, to a lesser extent, of murine iNOS (30).

We did not find a statistically significant interaction between the nNOS 29 and iNOS 22 polymorphisms. However, the protective effect of iNOS 22 was weaker for nNOS 29 TT or CT carriers than for CC homozygotes, and our study may have had insufficient power to detect an interaction of small size. When the effect of one of the polymorphisms was adjusted for the effect of the other, each of them remained associated with PD. These findings suggest that the two polymorphisms have independent effects.

The two polymorphisms in the nNOS gene appeared to modulate the protective effect of cigarette smoking. It has been reported, in post-partum placenta, that cigarette smoking was associated with decreased eNOS expression only among carriers of the rare allele of an eNOS polymorphism (31).
Table 2. The association between Parkinson’s disease and nNOS and iNOS polymorphisms

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Controls (%)</th>
<th>Cases (%)</th>
<th>OR (95% CI)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>nNOS 22</td>
<td>n = 482</td>
<td>n = 207</td>
<td>1.00 (reference)</td>
<td>—</td>
</tr>
<tr>
<td>GG</td>
<td>36</td>
<td>34</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>GA</td>
<td>47</td>
<td>57</td>
<td>1.21 (0.85–1.73)</td>
<td>0.28</td>
</tr>
<tr>
<td>AA</td>
<td>17</td>
<td>9</td>
<td>0.56 (0.32–1.01)</td>
<td>0.05</td>
</tr>
<tr>
<td>AA versus</td>
<td></td>
<td></td>
<td>0.50 (0.29–0.86)</td>
<td>0.01</td>
</tr>
<tr>
<td>GG+GA</td>
<td></td>
<td></td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>General test</td>
<td></td>
<td></td>
<td>0.02</td>
<td>—</td>
</tr>
<tr>
<td>Trend test</td>
<td></td>
<td></td>
<td>0.27</td>
<td>—</td>
</tr>
<tr>
<td>nNOS 18</td>
<td>n = 488</td>
<td>n = 209</td>
<td>1.00 (reference)</td>
<td>—</td>
</tr>
<tr>
<td>CC</td>
<td>46</td>
<td>42</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>CT</td>
<td>43</td>
<td>44</td>
<td>1.15 (0.80–1.64)</td>
<td>0.46</td>
</tr>
<tr>
<td>TT</td>
<td>11</td>
<td>14</td>
<td>1.45 (0.85–2.48)</td>
<td>0.17</td>
</tr>
<tr>
<td>CT + TT versus CC</td>
<td></td>
<td></td>
<td>1.20 (0.85–1.69)</td>
<td>0.30</td>
</tr>
<tr>
<td>General test</td>
<td></td>
<td></td>
<td>0.21</td>
<td>—</td>
</tr>
<tr>
<td>Trend test</td>
<td></td>
<td></td>
<td>0.18</td>
<td>—</td>
</tr>
<tr>
<td>nNOS 29</td>
<td>n = 488</td>
<td>n = 209</td>
<td>1.00 (reference)</td>
<td>—</td>
</tr>
<tr>
<td>CC</td>
<td>54</td>
<td>45</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>CT</td>
<td>38</td>
<td>43</td>
<td>1.41 (0.99–2.03)</td>
<td>0.06</td>
</tr>
<tr>
<td>TT</td>
<td>8</td>
<td>12</td>
<td>2.33 (1.27–4.26)</td>
<td>0.01</td>
</tr>
<tr>
<td>CT + TT versus CC</td>
<td></td>
<td></td>
<td>1.53 (1.08–2.16)</td>
<td>0.02</td>
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<tr>
<td>General test</td>
<td></td>
<td></td>
<td>0.01</td>
<td>—</td>
</tr>
<tr>
<td>Trend test</td>
<td></td>
<td></td>
<td>0.004</td>
<td>—</td>
</tr>
</tbody>
</table>

Linkage disequilibrium coefficients between the nNOS 18 and nNOS 29 polymorphisms in the nNOS gene are 0.46 in controls and 0.48 in patients. *Odds ratios (95% CI) and P-values were calculated using conditional logistic regression.

Nicotine injection or exposure to cigarette smoke leads to an increase in NO and/or NO metabolites in the brains of rats and rabbits, which may be mediated through the action of the NO (32,33). In this study, we observed, both for nNOS 29 and nNOS 18, that the inverse relation between smoking and PD was lost among carriers of the polymorphic T allele. This observation is in agreement with the hypothesis that an effect of smoking on NO production by nNOS is modulated by nNOS polymorphisms, but this hypothesis needs to be confirmed.

MPTP has been shown to increase nNOS and iNOS expression (18,19,21). However, we did not find evidence in favour of an interaction between exposure to pesticides and the polymorphisms that were tested in the present study. Therefore, if the effect of pesticides was mediated by an increase in NO production (as observed for MPTP), the increase is likely to be independent of these polymorphisms.

Since the functionality of these polymorphisms is unknown, it remains to determine whether the associations of the iNOS 22 and nNOS 29 polymorphisms with PD are the initiating event responsible for susceptibility to PD. Another possibility is that they are in linkage disequilibrium with another functional polymorphism or another gene nearby. iNOS 22 is an exonic polymorphism, but does not change the amino acids sequence. Another repeat polymorphism located in the promoter of the iNOS gene has been associated with dementia with Lewy bodies (34), a disease also characterized by the presence of Lewy bodies in the brain. The fact that both nNOS and iNOS genes are implicated in NO metabolism and were found to be associated with PD in this study argues against the implication of another gene. However, the Tau gene, for which a positive association with PD has been observed, is located at less than 15 cM of the iNOS gene on chromosome 17q (35–37). The strongest evidence of linkage from a genomic screen performed in late-onset PD was located at 7 cM from iNOS gene (38). We therefore investigated the linkage disequilibrium between Tau and iNOS 22 and adjusted our analyses of the relation between PD and iNOS 22 for the Tau genotype. Tau and iNOS 22 were not in linkage disequilibrium in cases (D = 0.05) and controls (D = 0.006), and the OR remained unchanged after adjustment (data not shown). The association between PD and iNOS is thus independent from the Tau gene.

nNOS 29 is located in an exon of the 5′ untranslated region (UTR). This region is often associated with the regulation of transcripts and mRNA stability. This polymorphism or another one located in the regulatory regions of gene expression may mediate this effect. This issue is difficult to solve, since the regulation of nNOS expression is very complex, with more than 12 transcripts (obtained by alternative splicing or promoter usage) and possible suppression of expression by an antisens RNA transcript. An association between PD and another repeat polymorphism in the nNOS gene promoter has been recently reported. Allele size distribution was significantly different between 64 Chinese patients and controls (39).

Association studies are at risk of population stratification bias if there are differences in ethnic background between patients and controls, and variations of allelic frequencies according to ethnic background. In this study, only subjects whose two parents were born in Europe were included. The majority of the participants had both parents born in France, and analyses restricted to this subgroup yielded results similar to the overall findings. In addition, for the nNOS 29 and nNOS 18 polymorphisms we found frequencies similar to those reported in a Caucasian population from the USA (40). Therefore, population stratification is unlikely to have biased our findings.

In conclusion, our findings suggest that nNOS and iNOS genes are susceptibility genes in PD, and that nNOS polymorphisms modify the relation between cigarette smoking and PD. Further studies should be undertaken in order to confirm this association in other populations, and to decipher the functionality of the polymorphisms that were studied.

Table 3. Estimated frequencies of nNOS haplotypes in Parkinson’s disease patients and controls

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Controls (n = 976)</th>
<th>Cases (n = 418)</th>
<th>ORa (95% CI)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-C</td>
<td>60</td>
<td>54</td>
<td>0.47 (0.37–0.60)</td>
<td>0.02</td>
</tr>
<tr>
<td>C-T</td>
<td>7</td>
<td>10</td>
<td>1.41 (0.97–2.02)</td>
<td>0.10</td>
</tr>
<tr>
<td>T-C</td>
<td>13</td>
<td>12</td>
<td>0.96 (0.69–1.34)</td>
<td>0.84</td>
</tr>
<tr>
<td>T-T</td>
<td>20</td>
<td>24</td>
<td>1.28 (0.94–1.73)</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Overall likelihood ratio test, P = 0.08. *Odds ratios and P-values are given for the case-control comparison of each haplotype versus all others.
Table 4. Individual and joint effects of the nNOS 29 and iNOS 22 polymorphisms on the risk of Parkinson's disease

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>nNOS 29</th>
<th>iNOS 22</th>
<th>Controls (%)</th>
<th>Cases (%)</th>
<th>OR (95% CI)*</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>GG+GA</td>
<td>45</td>
<td>43</td>
<td>1.00 (reference)</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>AA</td>
<td>9</td>
<td>3</td>
<td>0.29 (0.11–0.76)</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>CT + TT</td>
<td>GG+GA</td>
<td>38</td>
<td>48</td>
<td>1.41 (0.98–2.04)</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>CT + TT</td>
<td>AA</td>
<td>8</td>
<td>6</td>
<td>0.97 (0.49–1.93)</td>
<td>0.93</td>
<td></td>
</tr>
</tbody>
</table>

*ORs (95% CI) and P-values were calculated using conditional logistic regression.

MATERIALS AND METHODS

Participants

This study was conducted among subjects enrolled in a French social security organization, the MSA, which is responsible for the reimbursement of health-related expenses to workers connected to agriculture (i.e. farmers and farm workers, but also workers in silos, agricultural cooperatives and seed shops, as well as tertiary sector professionals). The study protocol has been described in detail elsewhere (41).

Briefly, individuals aged 18–75 years old who submitted an application for benefit from complete health care coverage for PD during an 18 months period were invited to participate. As part of the study protocol, they were examined by a neurologist with experience in movement disorders. Whenever it was impossible to directly examine the patient, the patient’s treating neurologist was contacted and asked to fill a clinical questionnaire. Parkinsonism was defined as the presence of two or more of the four cardinal signs: rest tremor, bradykinesia, rigidity, and impaired postural reflexes (42,43).

PD was defined as the presence of parkinsonism after exclusion of the following criteria: prominent or early signs of more extensive nervous system involvement not explained otherwise; supranuclear gaze palsy; repeated strokes with step-wise progression of parkinsonism; neuroleptic treatment before onset of the disease; unresponsiveness to levodopa (42,43). Patients who already benefitted from or requested free health coverage for dementia or who were bedridden were not included.

Controls were recruited among all MSA affiliates who made requests to be reimbursed for health expenses (e.g. rehabilitation, glasses, dental care, pediatric care, hydrotherapy, nursing care, respiratory function testing, holter monitoring). A maximum number of three controls were matched by age (± 2 years), sex and region of residency at the time of the study to each case. Participants were not included as controls if they already benefitted from free health coverage for PD or dementia, were treated for PD, were found to fulfil the criteria for parkinsonism, or were bedridden.

Information about demographic data, comorbidities, family history of PD, and the country of birth of the two parents were obtained. A face-to-face administered questionnaire elicited detailed information on cigarette smoking, including the average number of cigarettes smoked per day, as well as the starting and stopping age. In addition, if the number of cigarettes smoked per day varied during their lifetime, the participants were asked to divide their smoking history into a maximum of three time periods. Based on this information, participants were classified as current, ex- or never cigarette smokers, according to their smoking status at onset of PD (in patients), or index age (in controls; defined as the age at onset of PD in the matched case). The cumulative number of pack-years smoked until PD onset (in patients) or index age (in controls) was computed as the number of packs smoked per day (cigarettes smoked per day divided by 20) times the number of years smoked; our computation took into account variations in the number of cigarettes smoked per day according to the time periods defined above.

Exposure to pesticides was assessed using a two-phase case-by-case exposure assessment procedure that involved screening for professional exposure using a self-administered questionnaire and, for individuals professionally exposed according to this screening, a detailed interview at home with an occupational health physician from the MSA (41,44,45). Using a specific questionnaire, the physician completed the occupational history, and confirmed whether or not that the participant had personally sprayed or prepared pesticides in a professional setting; 20% of those who had declared to be professional users of pesticides in the self-administered questionnaire were not professional users according to the interview with the physician (11% were users for gardening exclusively, and 9% were never users). Finally, the questionnaires were reviewed by a panel of experts blinded to the case or control status, and we were able to classify the participants in three groups with respect to pesticides exposure: never users, users for gardening exclusively and professional users. We carried out a small validation study by interviewing over the phone subjects randomly chosen among those who had been classified as never users (n = 15) or users for gardening exclusively (n = 15); the self-administered questionnaire and the phone interview were always consistent and none of these 30 subjects had ever used pesticides in a professional setting.

The ethics committee of Hôpital du Kremlin-Bicêtre (Paris) approved the research protocol, and all subjects signed an informed consent.

Genotyping

Participants were blood sampled and genomic DNA was extracted from peripheral blood leukocytes using a standard technique.

For the NOS2 gene (NOS2A), we studied a G/A substitution in exon 22 (iNOS 22) that does not lead to
an amino acid change (PubMed, accession NT_024889), but creates a *B*gl restriction enzyme site. Primers were 5'-CTGCTGGCTTCCTGGTCCTTTCC-3' and 5'-CTCGGTTGTGTTGTTGACC-3'. DNA was initially denatured for 10 min at 95°C, then a touch-down program: 12 cycles at 95°C for 1 min, 72–66°C for 30 s, 72°C for 30 s and 23 cycles at 95°C for 1 min, 66°C for 30 s, 72°C for 30 s and, finally, 10 min at 72°C. The restriction fragments were visualized after electrophoresis on a 3% agarose gel: three fragments of 79, 24 and 23 bp defined the presence of the G allele, and two fragments of 103 and 23 bp the presence of the A allele.

We also genotyped two T/C polymorphisms of the nNOS gene, identified by Grasemann et al. in exon 18 (nNOS 18) and in exon 29 (nNOS 29) (40). Primers and PCR condition used
for exon 18 have been previously described. The polymorphism in exon 18 abolished a DraIII restriction site. The C allele was defined by the presence of two fragments of 124 and 55 bp, and the T allele by the presence of a fragment of 179 bp. We designed the following set of primers for the polymorphism in exon 29: 5′-TTGAGTCTTCCTGCTGCGATGT-3′ and 5′-GCTTTGCGCTAGTCTCTGCA-3′. PCR conditions were identical to those used for nNOS 18. The mutation created an NlaIII restriction site. The C allele was defined by three fragments of 102, 69 and 16 bp, and the T allele by the presence of four fragments of 94, 69, 16 and 8 bp.

Statistical methods

To minimize the risk of population stratification bias, only cases and controls with both parents born in Europe were included. Consistent with the matched design, matched analyses were performed, and the odds ratio (OR) was used to estimate the relative risk. For each studied variable, we calculated an OR, a 95% confidence interval (CI), and a P-value, using conditional logistic regression for matched sets with a variable number of controls. Conditional logistic regression models were also used to investigate the independent effect of the variables of interest, to adjust for potential confounding variables, and to test for multiplicative interactions.

To compare genotypic distributions in cases and controls, ORs were calculated separately for heterozygotes and homozygotes for the polymorphic allele, and a general test of association was performed by including dummy variables in the models and considering homozygotes for the wild allele as the reference group. In addition, we computed a P-value for trend by including an ordinal variable corresponding to the number of polymorphic alleles (codominant model). Analyses assuming a dominant model (nNOS 29 and nNOS 18) or a recessive model (nNOS 22) were also carried out. To compare the dominant and the codominant models for the nNOS 29 polymorphism, we used the Akaike Information Criterion, because the two models are not nested.

Haplotype frequencies for the two nNOS polymorphisms were estimated from genotype data using the E-M algorithm under the assumption of Hardy–Weinberg equilibrium. An overall likelihood ratio statistic was computed to compare the overall distribution of nNOS haplotypes between cases and controls, and tests for differences in individual haplotypes frequencies were performed using permutations (n = 10,000) (46). Linkage disequilibrium coefficients (D′) between the two nNOS polymorphisms were computed using standard formulas (47).

The SAS software release 6.11 was used for statistical analysis (SAS institute, Cary, NC, USA).

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