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Effect of the XbaI polymorphism of estrogen receptor alpha on postmenopausal gray matter

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Abstract

The frequent polymorphism XbaI (A351G) in the estrogen receptor alpha (ERalpha) gene has been associated with some postmenopausal pathologies' risk such as Alzheimer's disease (AD) or cognitive decline. In the present study, we explored whether the XbaI polymorphism leads to different gray matter volumes using voxel-based morphometry (VBM) on 20 magnetic resonance images of healthy postmenopausal women. Subjects carrying the less common XbaI/X allele were contrasted to non-carriers in groups well balanced by relevant confounding variables. The XbaI/X allele carriers displayed clusters ranging from 9 to 28% of tissue reductions in the cerebellar (cluster size, z, stereotactic coordinates: 16 mm^3; 3.17; 14, −94, −38) and cerebral cortex, in particular in the occipital lobe (272 mm^3; 3.76; −38,−68,−16), in the middle frontal gyrus (192 mm^3; 3.71; 38, 12, 38) and in the middle temporal gyrus, while the opposite comparison was negative. The XbaI/X allele in ERalpha gene is associated to smaller gray matter volumes of the cerebral and cerebellar cortex. This allele might increase the susceptibility for senile neurodegenerative conditions, being associated to smaller cerebral reserve.

Keywords: Estrogen receptor alpha; XbaI; Neuroimaging; MR; VBM; Morphometry

Estrogens' beneficial effects on the prevention of some pathologies, from osteoporosis to Alzheimer's disease (AD), in women during aging are generally accepted and attributed to the activation of estrogen receptors (ERs), among which estrogen receptor alpha (ERalpha) and ERbeta are the most widely investigated. The protective effect could be mediated by variants in the genes coding for the estrogen receptors. At this regard, a common restriction fragment length polymorphism XbaI (A351G) has been described in intron 1 of the ERalpha gene, that is in strong linkage disequilibrium with another known polymorphism, PvuII, in Caucasoid populations [2].

A protective role of the XbaI/X (351G) allele has been evidenced on physical aging in women after menopause. In particular, the XX genotype has been associated with higher bone mineral density or otherwise reduced fracture risk [12,24]. A trend for lower bone loss rates and greater benefit from estrogen replacement treatment (ET) was also observed [30]. Lower mammographic densities in postmenopausal women carrying the XbaI/X allele were observed *per se* [33], and in response to ET [34].

Much more controversial is the role of the XbaI polymorphism on the brain. The majority of the studies recorded an increased frequency of the less common XbaI/X allele in patients with AD [13,14,5,6,21] or cognitive impairment [27],

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and evidence exists suggesting that the increased risk might be specific for late onset AD [15]. Some state that the risk provided by the XbaI/X allele can raise up to 7.6 times the usual risk in the interaction with the ε4 APOE allele [5,6,21,27,15].

Nonetheless, these findings are not systematically replicated [19,22,18,7,32,35]. In one study they have even been clearly disconfirmed, with detection of higher frequency of the XbaI/x (351A) allele in subjects with AD [25]. Two other researches detected increased risk in allele XbaI/x carriers for familial early onset [23], or sporadic AD [29], although this was observed in the interaction with the APOE genotype in both studies.

Finally, an association of the XbaI polymorphism with a trend for different amygdaloid and hippocampal morphology has been recently reported [7], but further studies are needed to clarify the role of this polymorphism on the cerebral morphology and on cognitive decline.

In this study, we aimed to investigate the relationship between the XbaI polymorphism of ERalpha and gray matter morphology, in a sample of healthy women in their postmenopausal age, well balanced by important confounding factors. The a priori hypothesis was that the less frequent XbaI/X allele would be associated to a disadvantage, based on the evidence that the modulatory action of the XbaI/X allele is less effective in mediating estrogenic effects on the cerebral cortex [20]. This implies that the negative effects of menopausal reduction of estrogen levels would be amplified in XbaI/X carriers. To test this hypothesis, we quantitatively analyzed magnetic resonance (MR) images with a sensitive technique investigating the gray matter at the voxel level.

Subjects in this study were drawn from a wider group of 83 non-demented postmenopausal women aged 50 or over, recruited to investigate the effect of ET on cognition [10]. Subjects were healthy volunteers, whose cognition was checked, besides MMSE (Mini Mental State Examination), by administration of an extensive neuropsychological battery tapping learning, verbal and spatial long- and short-term memory, attention, executive and visuospatial functions, praxis and non-verbal intelligence (detailed neuropsychological findings are reported in Ghidoni et al. [10]. ET users were all taking estrogen hormone therapy not combined with progestin (i.e., Estraderm, 0.05/0.1 mg of estradiol USP per day transdermally). A subgroup of 37 women gave consent to undergo both MR imaging and blood drawing.

The study was approved by the local ethical committee (CEIOC, Brescia, Italy, Prot. No. 48/2001) and informed consent was undersigned by all participants.

Among the 37 subjects who underwent both blood drawing and MR, 22 were currently treated with ET or underwent ET in the past, 15 never received ERT. The proportion of treated and untreated women was not homogeneous among carriers and non-carriers of XbaI/X. Since ET was strongly associated with greater gray matter volumes in previous studies on this sample [10,4], a subgroup where XbaI/X carriers and non-carriers were balanced as to treatment was drawn. The minimum subgroup size was 5 subjects (5 non XbaI/X carriers + ERT), therefore other 15 subjects (5 non XbaI/X carriers + no ERT; 5 XbaI/X carriers + no ERT; 5 XbaI/X carriers + ERT) were chosen trying to reach the best match of confounding variables (age, education, APOE genotype, etc.) among groups.

DNA was extracted according to standard procedures. ERalpha XbaI polymorphism was analyzed by PCR amplification followed by restriction analysis (XbaI, Fermentas) as described in Kobayashi et al., 1996 [17]. Capital "X" stands for absence of the restriction site (indicating nucleotide G) and lower case letter "x" stands for the presence of the restriction site (nucleotide A).

APOE genotyping was carried out by PCR amplification and HhaI restriction enzyme digestion. The genotype was resolved on 4% Metaphor Gel (BioSpa, Italy) and visualized by ethidium bromide staining [11].

3D high resolution MR scans were collected at the Città di Brescia Hospital, Brescia, with a 1.0 Tesla Philips Gyroscan (TR = 20 ms, TE = 5.0 ms, flip angle = 30°, field of view = 220 mm, acquisition matrix = 256 × 256, slice thickness = 1.3 mm). Images were preprocessed with SPM2 (http://www.fil.ion.ucl.ac.uk/spm/software/spm2/) following an optimized voxel-based morphometry (VBM) protocol including customized template, customized prior probability maps, and main VBM steps (normalization, segmentation, modulation and smoothing) [8,9,26]. The customized template, rather than the SPM template, was used, in order to minimize the deformation of individual images to the common space. In fact, the customized template is a mean of the brains of the subjects under examination, while the SPM one is built on different subjects. Images of two subjects of the present study were not properly normalized in the first step of the template creation, where they should have been mapped onto the SPM template, and were therefore excluded from the creation of the customized template. The customized template in this study was therefore a mean of the brains of our subjects, with the exclusion of two of them, i.e. it was anyway a more appropriate template than other standard spaces. The two subjects have been successfully normalized onto this final customized template, and could properly enter each further stage of the statistical comparisons.

Smoothed gray matter images of XbaI/X allele carriers and non-carriers were contrasted in direct and opposite comparisons.

Voxel-by-voxel comparisons were carried out using an ANCOVA model, modelling the effects of groups and parametric nuisance covariates (cranial size and age) ("Single Subjects: conditions and covariates" procedure). The threshold for significance was set at 0.001 uncorrected. Percent changes were computed from the VBM values of brain volumes by subtracting, in each voxel, the mean value of non-carriers minus the mean value of the carriers, and dividing this difference by the mean value of non-carriers.

Non-parametric tests (Kruskall–Wallis, Mann–Whitney and Fisher's exact test) were used to compare sociodemographic features among groups. P threshold was set at 0.05 for statistics of sociodemographic characteristics.

All women scored normally in the overall evaluations and neuropsychological tests [10].

For the whole group of 37 women from which the balanced subgroup was drawn, genotype frequencies were in Hardy–Weinberg equilibrium ($p = 1.00$). The minor allele fre-

quency (MAF) was 0.43; the genotypic frequencies of XX, Xx, xx were 0.19 (7/37), 0.49 (18/37), 0.32 (12/37), respectively. The XbaI genotype and allele distributions were similar to those previously reported in an Italian population ($\chi^2 = 1.27$; $p = 0.53$ and $\chi^2 = 0.48$; $p = 0.49$, respectively) [25].

The distribution of ET treatment was not homogeneous in subjects carrying different genotypes (XbaI/X non-carriers: 5 ET vs. 7 no ET; XbaI/X carriers: 17 ET vs. 8 no ET). Although the proportion only approached statistical significance at Fisher's exact test ($p = 0.086$, computed among all genotypes), the strong effect of ET previously detected on this sample [4] made it necessary to balance by ERT, to allow detection of subtle genotype effects.

In the subgroup balanced by ET exposure, age and education level did not differ significantly among XbaI/X allele carriers and non-carriers. Of particular interest, no statistical differences were detected both in duration of treatment and in the time lag between menopause and ET (Table 1). The APOE/ε4 allele was homogeneously represented across the subgroups (Table 1), as well as APOE/ε3 (exact $p < 0.628$). None of the subjects in the selected group carried the APOE/ε2 allele.

The XbaI/X allele carriers displayed smaller gray matter volumes (Fig. 1) in a set of clusters scattered in the cortical associative regions, namely, the occipital cortex (mm^3; z; stereotactic coordinates: x, y, z: 272; 3.76; −38, −68, −16), the middle frontal (192; 3.71; 38, 12, 38) and middle temporal (32; 3.13; 70, −22, −8 and 8; 3.09; 60, −24, −22) gyri, and in the cerebellum (16; 3.17; 14, −94, −3 and 16; 3.12; 22, −74, −62). At the chosen threshold of $p < 0.001$, the difference in gray matter covered a total of 536 mm^3, and the significant clusters corresponded to regions with a tissue reduction ranging from 9 to 28% compared to controls. Opposite comparisons were negative.

Table 1
Sociodemographic features of the subjects selected in order to balance carriers and non-carriers of the XbaI/X allele of ERalpha for ET exposure and APOE genotype

	Non-carriers ($n = 10$)	Carriers ($n = 10$)	p
Age	57.5 (5.7)	56.8 (2.1)	0.290
Education	10.3 (3.3)	11.6 (5.8)	0.818
Age at menopause	47.2 (6.3)	50.8 (2.2)	0.253
Time on untreated menopause	7.8 (6.2)	5.0 (2.9)	0.494
ET treatment	5/10	5/10	1.00
ET duration	8.3 (5.5)	5.4 (2.6)	0.249
Lag ET	2.2 (3.2)	1.6 (3.6)	0.700
APOE ε4[a]	2	3	0.628

Numbers denote mean (S.D.) years of age, of education, of ET (estrogen replacement therapy) duration or (Lag ET) lag from beginning of menopause to beginning of ET.
p denotes significance on Mann–Whitney or Fisher's exact tests.
[a] Number of alleles.

Due to the small sample size, individual values of brain volumes were plotted for each peak of significance (Fig. 1). The dispersion of the values was reasonably limited, at least for the three most significant peaks.

In this study we investigated the influence of the XbaI polymorphism on gray matter morphology in healthy postmenopausal women well matched as to ET treatment and comparable as to the other relevant confounders (APOE genotype, ET duration, distance of ET from menopause onset, etc.), and XbaI/X carriers displayed smaller gray matter volumes. The extent of the difference covered a total of 536 mm^3 at the chosen threshold ($p < 0.001$ uncorrected), and the change ranged from 9 to 28%, quite notable considering that it was found in

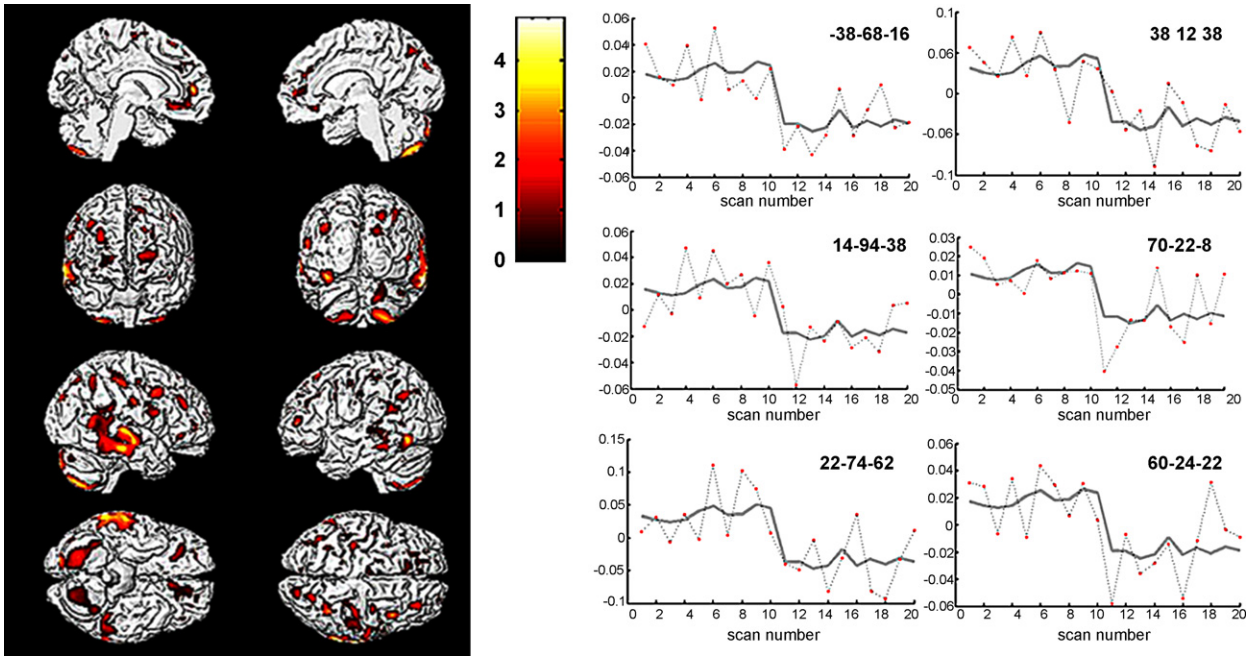

Fig. 1. Smaller gray matter structures in subjects carrying the XbaI/X allele of estrogen receptor alpha ($p = 0.05$ for illustrative purpose) and plots showing individual values of gray matter volume at the peaks of significance at $p = 0.001$.
Lighter voxels correspond to higher Z value. In each graph, subjects 1–10 are the non-carriers, subjects 11–20 are the carriers of the XbaI/X allele.

healthy subjects. The result is consistent with the a priori hypothesis that postmenopausal XbaI/X carriers would have displayed smaller gray matter volumes. Moreover, the regions displaying smaller gray matter volume lie within the pattern of cortical structures previously associated to greater volume after ET [4], independently of genotype, in a sample including these subjects, showing that the effect is found in regions sensitive to a trophic effect of estrogens in this age range.

Disagreement exists about the effect of the two alleles of the XbaI polymorphism on AD susceptibility, as attested by a recent meta-analysis [3]. Indeed, both the XbaI/X and the XbaI/x alleles have been associated with AD, mainly in its late onset form [13,14,5,4,22,16,7,32,23,29,16], or at least to cognitive decline [14,27], with and without interaction with the ε4 allele of the APOE.

Nonetheless, dementia and cognitive decline are complex conditions related to a great number of confounding factors independently affecting risk. We hypothesized that direct information about healthy gray matter morphology might be informative about the role that the XbaI polymorphism might have on age-related cognitive changes, and tried to match subjects for the most relevant confounding factors affecting cerebral health. To date, the only available study about human brain morphology associated with this polymorphism described a trend for smaller amygdaloid and hippocampal volumes in women carrying the most common XbaI/x allele [7]. Nonetheless, the trend for smaller volumes entirely vanished after stratification according to APOE genotype, indicating that neither the XbaI/x nor the XbaI/X alleles influenced medial temporal volumes by themselves.

Therefore, we investigated the whole gray matter in a sample of healthy postmenopausal women, paying attention to the matching of important confounding variables such as ET exposure. In the original group who underwent both blood drawing and MR scanning, the proportion of those who underwent ET was unbalanced, with XbaI/X carriers more often undergoing ET. This leads to two considerations. First, the disadvantage associated to the XbaI/X allele might lead women carrying it to undergo ET more often than non-carriers. This possibility can plausibly be drawn, as postmenopausal women with a less efficient receptor might experience greater sufferance from the menopausal estrogen dearth. Second, this proportion of treated subjects among XbaI/X carriers and non-carriers only approached statistical significance at exact test, but the effect of ET on the brain demonstrated particularly strong, at least on the sample described in the present study [10,4]. This disproportion could therefore mask the effect of the genotype under investigation, with a strong and opposite effect on the gray matter volumes. We ruled out this confounding effect by selecting subjects balancing by ET, but it can be hypothesized that little attention to this variable might be a source of uncontrolled variability possibly explaining part of the heterogeneity of findings from different studies. As to APOE, in our sample, the ε4 allele was homogeneously represented across the subgroups. Evidence exists that ε4 carriers of the APOE, well known risk factor for AD, have different cerebral morphology, be they affected by AD or normal young controls [20]. It is not clear to which extent this morphological difference mediates the risk for developing AD, nonetheless its primary effect on brain morphology is likely to hide, as well as to interact with, other genotype's effect.

We used a fine technique, detecting volume changes at the voxel level at direct *t*-test comparison in the different cerebral districts, and found the less common XbaI/X allele to be associated with significantly smaller gray matter volumes in regions other than the medial temporal, namely the associative neocortex, in the occipital, frontal and temporal lobes and in the cerebellum. This pattern of smaller cortical volume in the XbaI/X carriers is compatible with the majority of epidemiological studies, detecting increased risk for dementia in the XbaI/X carriers and with an increased risk for cognitive decline. Indeed, altered functional connectivity with frontal, temporal, occipital and cerebellar regions has been detected in patients with AD, the most frequent cause of cognitive decline [1]. Nonetheless, we suggest to interpret conservatively the findings, as conferring a generic higher vulnerability, as posterior regions are involved also in other kinds of dementia, such as Lewy Bodies disease. The plausible reduced response of the ERalpha to the neurotrophic effect of estrogens in the XbaI/X carriers might increase risk for different kinds of cognitive decline. Findings from studies in HeLa cells [22], indicated that the modulatory action of the XbaI/X allele tends to be less effective in mediating estrogenic effects on the cerebral cortex, and this may interact with a variety of pathological processes. On the other hand, the observed pattern of smaller volumes is restricted to neocortical regions, while ERalpha are more massively represented in the limbic structures [28]. This finding may be due to the fact that VBM is not the most appropriate technique to detect subtle tissue changes in structures as small as, for example, the amygdala. Nonetheless, ERalpha are present in the associative cortex of both rat and human brains [28,18], and are known to mediate the effect of estrogens of augmenting the cholinergic transmission [28]. Moreover, quite recent evidence suggests that ERalpha are importantly represented in the cerebral and cerebellar vasculature, an often underestimated target of estrogen effect throughout the whole brain [31]. This is consistent with the posterior clusters of smaller tissue located in the cerebellum, a structure with particularly dense vascularization.

Anyway, far from providing definitive answers to the debated question of the role of the XbaI polymorphism on cerebral ageing, this study suffers from important limitations, first of all the small sample size. In this study, a $p < 0.001$ was accepted due to the a priori hypothesis. Moreover, the opposite comparison is negative, and the difference is found in comparing normal women vs. normal women, a kind of comparison aimed to detect really subtle differences. Nonetheless, the findings must be replicated with a more powerful experiment, therefore in larger samples, which allow a more powerful test and can lead to results surviving correction for multiple comparisons. Therefore, caution in the interpretation of these data, which should considered as having only an explorative value, is recommended: their use for any clinical application is discouraged as improper. Replication on larger samples is required, provided a careful check of important confounders often disregarded such as ET and APOE genotype. Availability of a larger sample would also

allow to explore a number of interesting issues, like the effect of different haplotypes in the ERalpha gene or cumulative effect in genes belonging to the same pathway as estrogen receptor beta gene.

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