

Research Article

A Novel Study and Meta-Analysis of the Genetic Variation of the Serotonin Transporter Promoter in the Italian Population Do Not Support a Large Effect on Alzheimer's Disease Risk

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Alzheimer's disease (AD) is a neurodegenerative disorder whose clinical onset is mainly characterized by memory loss. During AD progression, behavioral and psychological symptoms of dementia (BPSD) frequently occur. In this paper we evaluated the association between AD and the short/long (S/L) functional polymorphism of the promoter region of the 5-hydroxytryptamine (5-HT) transporter gene (*SLC6A4*). The S-allele shows a 2-fold reduced transcriptional rate, causing an imbalance in 5-HT intracellular availability that might in turn trigger behavioral and cognitive alterations. We also genotyped the *SLC6A4* promoter functional variant *rs25531* (A → G). By comparing the genotypic and allelic frequencies in an Italian population of 235 AD and 207 controls, we found an association between 5-HTTLPR and AD (odds ratio for the L-allele versus the S-allele: 0.74, associated *P* value = .03), while no difference was found for the *rs25531*. A meta-analysis of studies in Italy assessing 5-HTTLPR and AD risk gave an estimation of odds ratio for the L-allele versus the S-allele of 0.85 (associated *P* value = .08). Overall, our findings are not supportive of a large genetic effect of the explored polymorphisms on AD risk.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative process whose prevalence increases with age. The number of AD patients is expected to raise considerably in the next future [1]. More than 90% of AD cases are sporadic, and only a little percentage has a clear genetic cause [2–4]. The main clinical feature of AD at onset is memory loss, accompanied by behavioral and psychological symptoms of dementia (BPSD) encompassing agitation, aggression, sexual disinhibition, delusions, hallucinations, and sleeping or eating disorders that are an outstanding managing problem for the caregiver [5, 6].

Despite the fact that the most important neurochemical deficiency in AD is related to acetylcholine loss, a possible role for serotonin (5-HT) in AD was suggested by post-mortem assays showing reduced level of serotonin in AD brains [7]. 5-HT role in cognitive processes and memory has been recently suggested, both in animal models and in studies on human subjects [8, 9]. Consequently, 5-HT imbalance might contribute to AD pathological signs. The 5-HT transporter gene (*SLC6A4*, 17q11.1-q12) codes for a neuronal transmembrane protein that is devoted to 5-HT reuptake at presynaptic level, a key regulatory event for serotonergic transmission [10]. The promoter region of the *SLC6A4* gene bears a functional polymorphism,

named serotonin-transported linked-polymorphic region (5-*HTTLPR*), consisting of a 43-bp insertion or deletion (ind/del) leading to a hypofunctional short (S) or to a normal long (L) variant [11, 12]. This polymorphism has been investigated in association with AD risk (Table 1) [13–19], and a meta-analysis of the available data shows no significant effect [20]. A different *SLC6A4* promoter polymorphism, *rs25531* (A → G), is able to modulate 5-*HTTLPR* transcriptional efficiency, as the presence of the *rs25531* G-variant in an L-allele carrier reduces the normal transcriptional rate to a level comparable to the S-allele [21]. The genome-wide association studies performed so far did not report significant effect for these two genetic variants in relation to AD susceptibility [22]. In the Italian population, the 5-*HTTLPR* has been investigated as risk factor for AD with conflicting results, while to our knowledge no data are available for *rs25531*. To contribute in this field, we have made an association study in a population from Northern Italy.

2. Materials and Methods

2.1. Patients Recruitment. We recruited 235 independent AD subjects from two clinical centers: “Luigi Sacco” Hospital (Milan, Italy) and Ospedale Maggiore Policlinico (Milan, Italy). Probable AD was diagnosed according to the National Institute of Neurological and Communicative Disorders and Stroke criteria (NINCDS-ADRDA). A Hachinski Ischemic Score >4 was an exclusion criteria [23, 24]. Patients underwent physical and neurological examination, screening laboratory tests, cognitive evaluation, brain Magnetic Resonance Imaging (MRI), Computed Tomography (CT), or Positron Emission Computed Tomography (PET) when required. Dementia severity was assessed by the Minimal State Examination (MMSE) [25]. Controls ($n = 207$) were from the same clinical centers as above; they were mainly elderly outpatients coming to clinical attention for non-neurological illness or spouses of the cases. The absence of cognitive impairment in controls was measured by MMSE, at baseline and at least after one-year follow-up. All subjects (or their relatives) gave an informed consent to the participation in the study (approved by the local ethical committees) that followed the principles of the Declaration of Helsinki.

2.2. Blood Samples Collection, 5-*HTTLPR*, *rs25531*, and Apolipoprotein E Genotyping. About 5 mL of blood were collected by venipuncture, frozen at -20°C , and extracted to collect genomic DNA (gDNA) using a commercial kit according to the manufacturer's instructions (Promega, Madison, USA).

To assess 5-*HTTLPR* genotype, 50 ng of gDNA were amplified by polymerase chain reaction (PCR) with the following primers: forward: 5'-ggcgttgccgctctgaatgc, reverse-5'-gaggactgagctggacaacca (size of the amplified bands: L-allele 529 bp; S-allele 486 bp). The *rs25531* genotype was assessed by allele-specific PCR using as primers: forward A-allele specific: 5'-accctcgccgcatccccctgcaccaca-3'; forward G-allele specific: 5'-accctcgccgcatccccctgcaccacg-3'; common reverse: 5'-tggagtccgctggattctggtgccacct-3'. Finally, apolipoprotein E (*APOE*) genotype was assessed as previously published [26]. To avoid false genotyping, samples

were assessed at least twice and only unambiguous results were considered.

2.3. Meta-Analysis, Statistical Analysis, and Power Calculation. The meta-analysis was performed based on association studies data shown in the public available database <http://www.alzforum.org/> [20]. Calculations were done using the MetaEasy software v1.0.4 (<http://www.jstatsoft.org/v30/i07/paper>). Genotypic or allelic frequencies were compared using hypothesis-free χ^2 test by a free available online resource [27]. The odds ratios (ORs) were calculated by 2×2 contingency table at 95% confidence interval (CI) by GraphPad Prism 5.0. Power analysis (performed by G*Power 3.03). With the sample size of our novel study (a total of 417 subjects), we had 85% power to detect an increase of the S-allele of 7%, corresponding to a small-to-medium effect size of $w = 0.14$ (where w stands for the effect size conventional index for chi-square test [28]), and a power of 57% to detect a small effect size of $w = 0.10$ (an increase of S-allele of 5%). As for the meta-analysis performed in the Italian population, the total number of subjects was 1178, and in this case we had a power of 77% to detect an increase of the S-allele of 5%. The statistical significance limit was set at $P = .05$.

3. Results

3.1. 5-*HTTLPR*, *rs25531* Genotyping, and *APOE-ε4* Stratification. AD subjects ($n = 235$) and controls (CNTR, $n = 207$) were screened to assess 5-*HTTLPR* and *rs25531* genotype. Their demographic data are summarized in Table 2. AD cases and CNTR were people of Italian ancestry (self-reported, at least two generations before the patient were born and resident in Italy), balanced for age and sex proportion.

Genetic results are shown in Table 3. Of the available 235 AD and 207 CNTR, 220 AD (93.6%) and 197 CNTR (95.2%) were considered for subsequent analysis, with a genotyping efficacy of 99.5% and an accuracy of 94.8%. We checked at first Hardy-Weinberg equilibrium and found no deviation in cases and controls separately for 5-*HTTLPR*, while for *rs25531* controls had a significant difference from the expected genotypic frequencies ($\chi^2=8.29$, $P = .003$), probably due to a slight overrepresentation of the rare G/G homozygous genotype in this group. The distribution of genotypic frequencies of 5-*HTTLPR* and *rs25531* did not differ between AD and CNTR. However, by assuming that the presence of at least one S-allele was sufficient to modulate AD risk, we calculated the odds ratio OR (95% confidence interval (CI)) for the 5-*HTTLPR* L/L versus (S/L+S/S) genotype that was 0.62[0.41–0.94], with associated P -value of $P = .02$. For *rs25531*, the comparison considering (G/A+G/G) genotypes versus A/A genotype gave an OR (95% CI) of 0.83 (0.5–1.5), with associated P value of $P = .59$. As for allelic frequencies, the 5-*HTTLPR* S-allele was significantly more frequent in AD than CNTR (47.5% versus 40.1%). We have also verified whether the 5-*HTTLPR* allelic distribution was independent of the presence of the *APOE-ε4* allele (Table 4). The *APOE-ε4* allele by itself was a strong risk factor for AD (OR (95% CI) for carriers versus noncarriers: 5.4 (3.2–8.8), $P < .0001$). When we divided the 5-*HTTLPR*

TABLE 1: Literature overview of the 5-HTTLPR polymorphism in association with AD in the Caucasian population.

Reference	Population	No. of cases	No. of controls	Main result
[13]	UK	196	271	No association
[14]	Germany	84	118	No association
[15]	Germany	50	199	Association of S-allele with AD (S-allele frequency in AD and controls: 51% and 41%, resp.)
[16]	Italy	208	116	No association
[17]	Italy	105	114	No association
[18]	Austria	127	479	No association
[19]	Italy	164	54	Association of S-allele with AD (S-allele frequency in AD and controls: 47% and 34%, resp.)
This study (no overlapping with the above-cited populations)	Italy	220	197	Association of S-allele with AD (S-allele frequency in AD and controls: 47.5% and 40.1%, resp.)

AD: sporadic Alzheimer's disease.

TABLE 2: Demographics of the AD sample.

Diagnosis	No. of subjects (male : female)	Age at sampling (years \pm SD)	Age at onset (years \pm SD)	Disease duration (years \pm SD)	MMSE score at sampling (mean \pm SD)
AD	235 (74 : 161)	78.6 \pm 9.8	77.2 \pm 8.0	4.1 \pm 1.8	18.7 \pm 5.8
CNTR	207 (69 : 138)	77.0 \pm 9.3	NA	NA	28.2 \pm 2.6**

AD: sporadic Alzheimer's disease;

CNTR: controls;

MMSE: Minimental State Examination;

SD: standard deviation;

NA: not applicable;

** $P < .001$, Student's t -test versus AD.

cases and CNTR according to *APOE- $\epsilon 4$* status, we did not find a difference in the genotypic or allelic distributions. No variation was found by comparing 5-HTTLPR genotypic or allelic frequencies between AD *APOE- $\epsilon 4$* carriers and noncarriers.

We also performed a multivariate logistic regression considering variables: age, sex, *rs25531*, 5-HTTLPR, and *APOE- $\epsilon 4$* status. The contribution to AD of 5-HTTLPR was no longer significant (OR: 0.61, 95% CI: 0.38–1.05, $P = .10$). As for *rs25531*, no risk modulation was found ($P = .96$). We confirmed the strong influence of *APOE- $\epsilon 4$* allele (OR: 6.4, 95% CI: 3.5–11.8, $P < .00001$).

Finally, we have assessed whether 5-HTTLPR or *rs25531* influenced other clinical parameters as age at onset. We found no association between the 5-HTTLPR or *rs25531* genotype and dementia onset (data not shown).

3.2. Meta-Analysis for 5-HTTLPR Studies. Taking advantage from the public available database <http://www.alzforum.org/>, we have performed a meta-analysis of the Italian studies focused on 5-HTTLPR and risk of AD, including our own data. We found a marginal effect, with an odds ratio (OR) and 95% confidence interval (95% CI) of the L-allele versus the S-allele of 0.85 (0.70–1.03) (Figure 1). We compared

the Italian meta-analysis with a second meta-analysis based on thirteen studies (regardless of ethnicity) and a third including eight Caucasian studies only. In the general meta-analysis the OR (95% CI) was 0.97 (0.87–1.07), while for the Caucasian meta-analysis the OR (95% CI) was 0.90 (0.79–1.02) (Figure 1).

4. Discussion

5-HT imbalance might be the biochemical basis of the etiology of behavioral disturbances that are frequent features in late-onset AD [29, 30]. However, the genetic variability linked to the promoter region of the *SLC6A4* gene has been considered as predisposing factor for the development of AD dementia, too (Table 1). The increased frequency we have detected in 5-HTTLPR S-allele corresponds to a small-to-medium effect size (odds ratio (OR) of 1.6), a magnitude far below the *APOE- $\epsilon 4$* allele, but that seems independent of the presence of this strong risk factor as suggested by our stratification analysis. However, this observation should suffer from reduced sample size in the groups analyzed, even though both in AD *APOE- $\epsilon 4$* carriers (+) and AD noncarriers (–) the 5-HTTLPR S-allele frequency had a positive trend in comparison to controls. As for the significance of our

TABLE 3: 5-HTTLPR and rs25531 genotypic and allelic frequencies.

	Genotype count (%)			Allele count (%)		OR (95% CI) and P value for allelic distribution (L-allele versus S-allele)
	S/S	S/L	L/L	S	L	
<i>5-HTTLPR</i>						
AD (220)	51 (23.2)	107 (48.6)	62 (28.2)	209 (47.5)	231 (52.5)	
CNTR (197)	37 (18.8)	84 (42.6)	76 (38.6)*	158 (40.1)	236 (59.9)	0.74 (0.56–0.97) and 0.03
	Genotype count (%)			Allele count (%)		OR (95% CI) and P value for allelic distribution (G-allele versus A-allele)
	G/G	G/A	A/A	G	A	
<i>rs25531</i>						
AD (220)	0 (0.0)	31 (13.9)	189 (86.1)	31 (7.0)	409 (93.0)	
CNTR (197)	5 (2.5)	26 (13.2)	166 (84.3)#	36 (9.1)	358 (90.9)	0.75 (0.45–1.24) and 0.29

AD: sporadic Alzheimer's disease;

CNTR: controls;

* = 0.07; # = 0.06*, P-value calculated from χ^2 test for AD versus CNTR as for genotypic distribution;OR (95% CI): odds ratio and 95% confidence interval calculated from 2×2 contingency table.TABLE 4: Stratification of 5-HTTLPR genotype according to APOE- $\epsilon 4$ status.

	Genotype count (%)			Allele count (%)		P value
	S/S	S/L	L/L	S	L	
<i>APOE-$\epsilon 4$ (-)</i>						
AD (117)	25 (21.6)	57 (48.6)	35 (29.8)	107 (45.7)	127 (54.3)	.35 ^a
CNTR (171)	31 (18.1)	75 (43.8)	65 (38.1) ^a	137 (40.0)	205 (60.0) ^b	.17 ^b
	Genotype count (%)			Allele count (%)		P value
	S/S	S/L	L/L	S	L	
<i>APOE-$\epsilon 4$ (+)</i>						
AD (103)	27 (26.3)	50 (48.5)	26 (25.2)	104 (50.5)	102 (49.5)	.57 ^c
CNTR (26)	5 (19.2)	12 (46.1)	9 (34.7) ^c	22 (42.3)	30 (57.7) ^d	.29 ^d

AD: sporadic Alzheimer's disease;

CNTR: controls;

P value: P calculated from χ^2 test for AD versus CNTR;^{a,c} genotypic distribution;^{b,d} allelic distribution.

findings, the association found with unadjusted analysis was no longer significant after correction by logistic regression performed considering other variables, such as age, sex, and *APOE- $\epsilon 4$* status, even if the OR coming from the multivariate regression was similar to the uncorrected analysis, suggesting that the potential confounders age, sex, and *APOE- $\epsilon 4$* do not strongly influence the association result. A haplotypic study assessing 5-HTTLPR and rs25531 together might have been of interest, but our sample was too small to perform this analysis.

To our knowledge, none of the genome-wide association studies (GWASs) so far reported in the literature found an association signal for the *SLC6A4* promoter region and AD, while the case-control studies available to date addressing the same association are conflicting, even if they are mostly negative (Table 1). In the Caucasian population two studies linked 5-HTTLPR and AD, while others did not reproduce this association (including two studies in the Italian population, the largest enrolling $n = 324$ subjects) [16, 17]. To this respect, our population ($n = 417$ subjects with successful genotyping) is a bigger independent sample,

and the indication of an increased frequency of the 5-HTTLPR S-allele in AD deserves further replication in Italy, also considering the recent data by Lorenzi et al. reporting an increased frequency of 5-HTTLPR S-allele in sporadic demented subjects (AD and frontotemporal lobar dementia (FTLD)) from the same country [19]. Our data are also in agreement with the positive association between 5-HTTLPR and AD from Hu et al. ($n = 249$ subjects, AD group $n = 50$) [15], but represent a more accurate replication due to the increased sample size, in particular for the AD group (AD group genotyped in our study $n = 220$). Due to the Italian population structure and the age of included patients, we do not envisage a possible bias in our analysis due to population stratification, even if the Italian ancestry was self-reported.

The meta-analysis performed by comparing studies with mixed ethnicity, Caucasian ancestry and Italian ethnicity have pointed out a marked heterogeneity of results as demonstrated by the Cochran's Q-test for heterogeneity (significant in all the meta-analyses that we made). It is worth to notice that a trend for an increased risk of

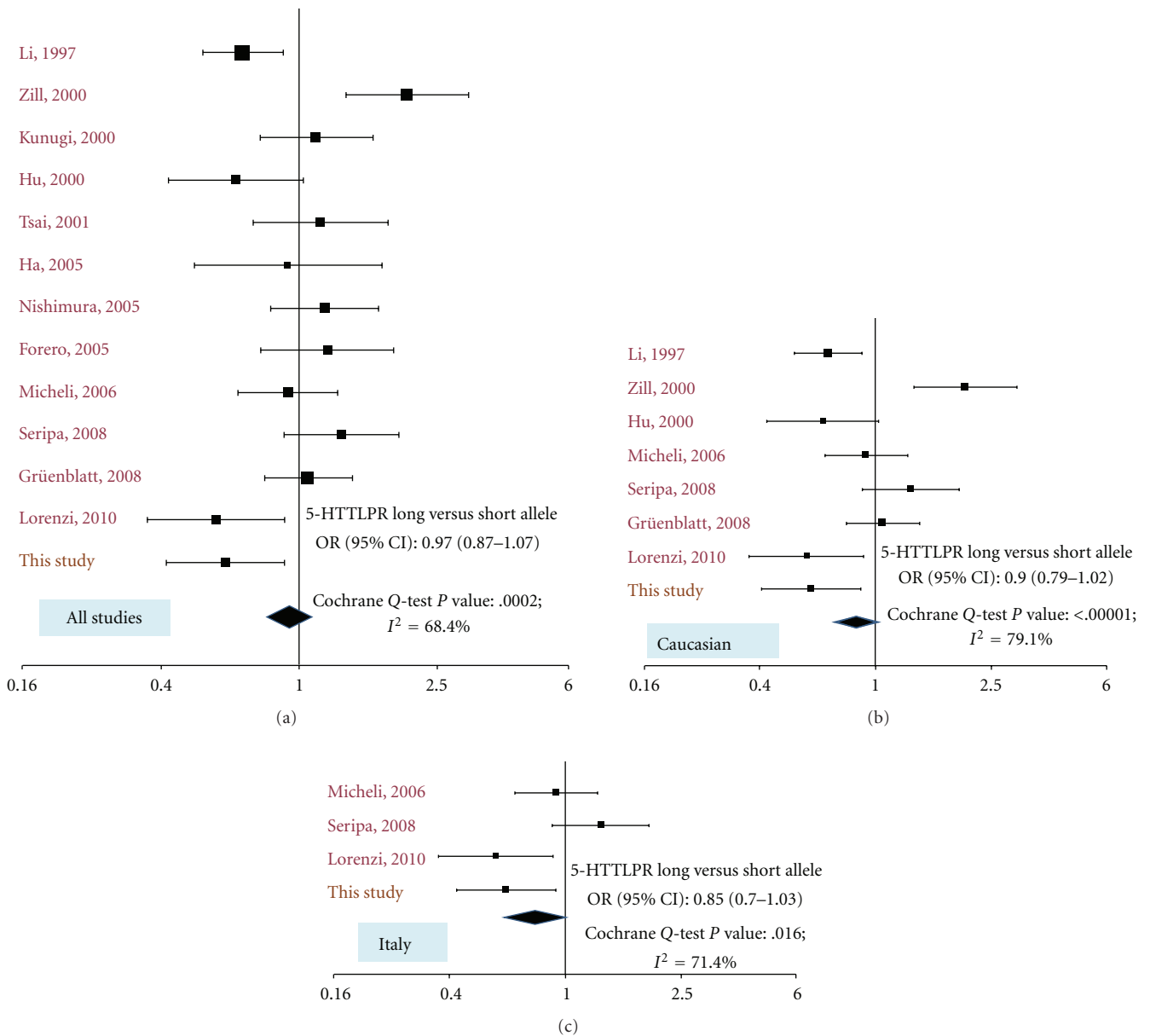


FIGURE 1: Meta-analysis of 5-HTTLPR. We have included our own data in three datasets: (a) all nonoverlapping studies regardless of ethnicity available online (<http://www.alzforum.org/> [20]); (b) Caucasian studies only; (c) Italian studies only. The odds ratio (OR) and 95% confidence interval (CI) are calculated for the long (L) versus short (S) allele. The diamond is the output of meta-analysis calculation, with its OR (95% (CI)).

the S-allele in comparison to the L-allele came to light, with the Italian population having the highest risk among the group considered, although not significant. We can speculate that 5-HTTLPR might be an AD risk factor with a selective ethnicity effect.

Overall, we acknowledge that the result of our novel association study is most likely inflated by type-I error, while the meta-analysis results do not provide nominally significant evidence for association. However, before a robust conclusion can be drawn, larger studies would be needed to definitely assess the role of this marker in AD pathogenesis.

The analysis on *rs25531* had first of all the important limitation of a deviation from Hardy-Weinberg equilibrium (HWE) in controls. They revealed that *rs25531* frequency in our population considered as a whole was quite limited (G-allele frequency of 8.0% and homozygous G/G genotype frequency of 1.2%), so it might explain a small percentage of AD genetic risk. Moreover, due to the frequency of this SNP and the departure from HWE in controls, our negative data should be confirmed in larger datasets. As for a possible linkage disequilibrium (LD) between *rs25531* and 5-HTTLPR we have already addressed this point with negative results [31].

Conflict of Interests

The authors declare no conflict of interests regarding the present manuscript.

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