Possible Association between *SNAP-25* Single Nucleotide Polymorphisms and Alterations of Categorical Fluency and Functional MRI Parameters in Alzheimer's Disease

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Handling Associate Editor: Marco Bozzali

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INTRODUCTION

The major cause of cognitive decline in the elderly is Alzheimer's disease (AD), a neurodegenerative disorder characterized by intracellular neurofibrillary tangles and extracellular amyloid β-peptide (Aβ) plaques. The pathogenesis of AD is complex and involves genetic factors that are still mostly unidentified and whose role is ill defined. Thus, the presence of the ε4 allele of apolipoprotein E gene (*APOE4*), the only known confirmed genetic risk factor for sporadic AD is neither necessary nor sufficient for disease manifestation [1].

A consistent finding in AD is synaptic loss, and synaptic abnormalities are suggested to be the pathologic finding more closely correlated with this disease [2] and to play a primary role in the characteristic cognitive deficits seen in AD. This could be mediated by the loss of synapses itself or by an altered exocytosis of neurotransmitters, proteins stored in synaptic vesicles that are released into the synaptic cleft [3]. Several SNARE proteins are involved in exocytosis [4]; synaptosomal-associated protein of 25 kDa (SNAP-25), in particular, is a vesicular SNARE protein playing an important role in the release of neurotransmitters. Thus, SNAP-25 interacts with voltage-gated calcium channels [5] inhibiting their function and reducing responsiveness to depolarization [6, 7]. Changes in SNAP-25 levels are present in schizophrenia, with levels of the protein being decreased in the hippocampus and the frontal lobe Broadman's area (BA) 10, and increased in prefrontal lobe BA 9 and in the cingulate cortex [8] as well as in cerebrospinal fluid (CSF) [9]. A *SNAP-25* promoter variant was also found to result in an augmented protein expression in the brain of patients with early-onset bipolar disorders [10]. Higher levels of this protein have also been involved in attention deficit hyperactivity disorder [11], a condition characterized by hyperactive behavior and impaired attentive ability resulting in social dysfunction [12], and neuroticism [13]. In this case, the observed association is between attention deficit hyperactivity disorder and two single nucleotide polymorphism (SNPs) localized between intron 3 and the 3′ untranslated region of *SNAP-25* gene (rs3746544 and rs1051312) [14, 15], whereas a third SNP (rs363043) correlates with hyperactive behavior in autistic children [16]. Notably, the *SNAP-25* gene lies in an area linked to intelligence (20p12–p11.2) [17], and a family-based genetic association test performed in children and adults showed that particular *SNAP-25* SNPs (rs363043, rs353016, rs363039, rs363050) are associated with variation in Intelligence Quotient (IQ) phenotypes. These SNPs are localized within intron 1 in a region spanning about 13.8 kb, and are known to affect transcription factor binding sites [18].

In this study, we verified a possible involvement of *SNAP-25* by comparing AD patients with age- and gender-matched healthy controls. In particular, the frequency of distribution of five *SNAP-25* gene polymorphisms (rs363043, rs363039, rs363050, rs3746544, rs1051312) was correlated with the degree of cognitive impairment evaluated with an extensive neuropsychological assessment. We considered a category fluency task as an indirect measure of long-term memory status (for a review, see [19]) allowing us to test verbal competences that rely on the structure of semantic network. Semantic fluency requires integrity of semantic concepts, and dysfunction occurs early in AD and causes significant disability with AD progression. Recently, evidences from neuropsychological [20, 21] and functional magnetic resonance imaging (fMRI) studies [22–24] showed that language deficits, especially those interesting verbal fluency functioning, are precursors of AD clinical condition.

Moreover, we replicated our findings in subjects with amnestic mild cognitive impairment (aMCI) and possible associations between *SNAP-25* SNPs and disease status, degree of cognitive impairment and fMRI parameters were analyzed.

MATERIALS AND METHODS

Ethical committee approvals

Written consent was obtained from all the participants in the study or from their care givers. The consent was in accordance with the declaration of Helsinki; the ethical committees of the Don C. Gnocchi Foundation and of the Fondazione Ca' Granda, IRCCS

Table 1
Baseline characteristic of patients with a diagnosis of Alzheimer's disease (AD) or amnestic mild cognitive impairment (aMCI); healthy controls (HC1 and HC2) are also included

	AD ($n = 607$)	HC1 ($n = 615$)	aMCI ($n = 148$)	HC2 ($n = 310$)
Parameters				
Age (y) [Mean ± SD]	76.7 ± 8.2	72.0 ± 6.9	76.8 ± 7.8	69.9 ± 6.3
Range (y)	53–96	44–104	59–96	41–86
Male %	32.3	31.7	46.0	44.7
MMSE score [mean ± SD]	18.2 ± 6.5	27.1 ± 3.1	25.2 ± 1.3	27.2 ± 2.8
Formal education (y) [mean ± SD]	8.0 ± 4.1	8.1 ± 3.8	7.7 ± 3.4	7.9 ± 3.9
APOE4+%	47.0[1]	19.0[1].	36.5[2]	22.2[2]

APOE4+: positivity for epslion4 allele SD: standard deviation. [1]AD versus HC1: $p < 0.001$ OR: 3.78 IC(95%): 2.09–4.93; [2]aMCI versus HC_2: $p < 0.001$ OR: 2.00 IC(95%): 1.30–3.08.

Ospedale Maggiore Policlinico in Milano approved the study.

Patients and controls

A total of 1680 Italians of Caucasian origin were enrolled; the study was designed as a case control comparing 607 AD patients and 615 healthy controls (HC1). A replication study was conducted enrolling 148 patients with aMCI and a second group of 310 HC (HC2); both HC1 and HC2 were age-and gender-matched with the patients (Table 1). Patients were consecutively recruited by the Neurology Departments of the Don C. Gnocchi Foundation and of the Fondazione Ca' Granda, IRCCS Ospedale Maggiore Policlinico in Milano.

AD patients had a clinical diagnosis of probable AD in mild to moderate stage of disease according to the NINCDS-ADRDA Work Group criteria [25] and DSM IV-R [26]. Reversible causes of dementia were excluded after medical and neurological evaluation, laboratory analysis, CT scan or MRI, and other investigations when necessary (e.g., electroencephalography, single-photon emission computerized tomography scan, CSF examination, etc.); all AD cases were sporadic. Outpatients diagnosed with aMCI according to Petersen criteria [27] were consecutively recruited as well from those attending the Memory Disorders Outpatients Service of the Don Gnocchi Foundation. Only aMCI individuals considered at high risk to develop AD were enrolled in the study. To be eligible, aMCI subjects had to meet the following operational criteria: memory complaint, confirmed by an informant; abnormal memory function, documented by extensive neuropsychological evaluation; normal general cognitive function, as determined by both Clinical Dementia Rating (CDR) [28] scale (CDR with at least a 0.5 in the memory domain); no impairment in functional activities of daily living as determined by a clinical interview with the patient and informant; no significant cerebral vascular disease (Hachinski score less than or equal to 4) [29]; no major psychiatric illnesses with particular attention to exclude subjects with history of depression (Hamilton Depression Rating Scale score less than or equal to 12) [30, 31]. Patients are followed with annual brain MRI and routine laboratory tests, and re-evaluated approximately every 6 months with neurological examination and a battery of neuropsychological tests and scales.

Two groups of HC: HC1 and HC2 of unrelated Italians that were age- and gender-matched with AD and aMCI patients respectively were recruited as well. These individuals were selected according to the SENIEUR protocol for immuno-gerontological studies [32, 33]; their cognitive status was assessed by Mini-Mental State Examination (MMSE) and mean raw data scores were reported in Table 1.

Neuropsychological evaluation and psychometric assessment

A randomly selected subgroup of 209 AD (72 males, 137 females) and 54 aMCI (24 males, 30 females) individuals underwent extensive neuropsychological evaluation that included MMSE [34], language functions tests (phonological and categorical fluency [35] and Token tests [36]), short-term memory tests (Corsi, Digit Span Forward and Backward tests [37]), long-term memory tests (Rey's Figure Delayed Recall [37]; Paired-Associate Learning test, and Story Recall test [35]) and frontal-executive functions (Raven Coloured Progressive Matrices [38]), visuo-spatial abilities (Rey's Figure Copy [37]). Categorical fluency impairment was also evaluated with fMRI verbal fluency task (the paced overt version of verbal fluency paradigm described by Basho and colleagues [39], see data analysis section). All the evaluation values were adjusted for age and educational level (conversion formulae are

reported in the appropriate references) and only the corrected scores were used for correlation analysis.

SNPs typing

Genomic DNA was isolated from peripheral blood mononuclear cells by phenol-chloroform extraction. SNPs were typed using the Taqman SNP Genotyping Assays (Applied Biosystems by Life Technologies, Foster City, CA, USA) on an ABI PRISM 7000 Sequence Detection System. For rs363039, rs363043, rs363050 and rs3746544, respectively, the C_327976_10, C_2488346_10, C_329097_10, and C_27494002_10. Human Pre-Designed Assays (Applied Biosystems by Life Technologies) were used. The restriction enzyme polymorphism rs1051312 was genotyped by DdeI digestion as previously described [14].

APOE4

Customer-designed taqman probes for the 112 and 158 codons were used. Primers and probes for the 112 codon are: 112 Forward primer: 5′-GGG CGC GGA CAT GGA G-3′, 112 Reverse primer: 3′–TCC TCG GTG CTC TGG CC-5′, 112 Arg Probe : 5′-CGT GCG CGG CCG-3′-FAM, 112 Cys Probe: 5′-ACG TGT GCG GCC GCC TG-3′-VIC. Primers and probes for the 158 codon are: 158 Forward primer: 5′-TCC GCG ATG CCG ATG-3′, 158 Reverse primer: 3′-GCT CGG CGC CCT CG-5′, 158 Arg probe: 5′-CCT GCA GAA GCG CCT GGC A-3′-FAM, 158 Cys probe: 5′-CCT GCA GAA GGG CCT GGG AGT-3′-VIC.

fMRI protocol and data analysis

MRI scans were obtained using a 1.5 Tesla scanner (Magnetom Avanto, Siemens, Erlangen, Germany). Functional images were acquired with single-shot gradient echo EPI sequence (TR/TE = 3000/50 ms, voxel size = $3.9 \times 3.9 \times 3$ mm^3, 38 axial slices, 120 volumes) using blood oxygenation level dependent (BOLD) contrast. A morphological three-dimensional T1-weighted MPRAGE sequence (TR/TE = 1900/3.37 ms, voxel size = $1 \times 1 \times 1$ mm^3, number of axial slices = 176) was also acquired to be used as anatomical scan for fMRI analysis.

Thirty-eight subjects (28 AD patients and 10 HC) were selected to perform the paced overt version of verbal fluency paradigm (ABAB block design) [40]. Eighteen of these patients carried the rs363050 (AA or AG) and rs363043 (CT or TT) genotype (group 1); the other 10 carried the rs363050 (GG) and rs363043 (CC) genotype (group 0). For each individual 6 semantic categories were randomly presented during the fMRI acquisition. Overt responses were obtained via an MRI-compatible patient response and sound system (VisuaStim Digital, Resonance Technology Inc.) The use of ePrime software (e-Prime 2.0 Psychology Software Tool, http://www.pstnet.com) ensured exact timing of prompts.

Statistical analysis

Chi-square analysis was used to exclude any deviation of SNP genotype distribution from Hardy -Weinberg equilibrium (HWE) and to compare case-control differences of SNPs distributions after gender stratification.

Haplotype analyses were performed using the SHEsis software freely available at http://202.120.7.14 /analysis/myAnalysis.php [40, 41]. The Kolmogorov-Smirnov (K-S) test was applied to verify normal distribution of numerical variable scores. Cognitive scores, which resulted normally distributed, were shown as mean and standard deviation (SD) and analysis of variance ANOVA was performed in relationship with SNPs distribution. For those variables, which were not normally distributed, Kruskall-Wallis test was applied. For genotype analyses p_c values were corrected for 2 degree of freedom (degree of freedom of the genotype distribution of the three different genotypes); p values of allelic comparison have only 1 degree of freedom because they analyze bialleic polymorphisms, therefore they did not need to be corrected for degree of freedom.

A multivariate logistic forward stepwise regression model corrected by gender and *APOE4* positivity was used. This model had categorical fluency scores <25 or >25 as response variables in AD and in aMCI and genotype (rs363050 (AA/AG versus GG) and rs363043 (CT/TT versus CC)) as explanatory covariates, *Post hoc* power analysis were performed for all logistic regression and reported if the actual power was lower than 90%.

Haplotype association analysis was performed using plink [42] by logistic regression; haplotype probabilities of individual subjects were incorporated as covariates in the regression model, which estimate the Odds ratios and p values associated with having a score of categorical fluency impairment ≤ 25, adjusting for gender and *APOE4* positivity.

Statistical analysis on fMRI data was performed using SPM8 (SPM8, http://www.fil.ion.ucl.

ac.uk/spm). Preprocessing of functional images involved realignment, co-registration to the anatomical image, spatial normalization to the Montreal Neurological Institute (MNI) space, and spatial smoothing with a 8 mm full-with at half-maximum (FWHM) Gaussian kernel. Single subjects statistical analysis was then performed with general linear model (GLM) approach [43] to detect the activation areas during the task (t-contrast: categorical fluency A versus control condition B). The contrast images obtained at the single-subject level were used to compute the second level analyses. An ANOVA full factorial design was employed with a 3 level factor to model the groups (AD gr0, AD gr1, and HC), and with task performance, intracranial volume (ICV) (obtained by adding up white matter volume + grey matter volume + CSF volume) and MMSE scores as nuisance covariates, to adjust for potential confounds. We used a t-Student's contrast to assess the main effect of categorical fluency versus control condition in HC and AD (both gr0 and gr1) and to describe the difference among the genotypes (HC versus AD gr0; HC versus AD gr1; AD gr0 versus AD gr1). The maps resulting from the second level analyses were thresholded with two approaches: first, the activation clusters that survived after correction for multiple comparisons (Family wise correction, pFWE-corr <0.05) and an extent threshold of voxel size of 10 adjacent voxels were considered; then, for exploratory purposes, group differences were also described considering an uncorrected $p < 0.001$ threshold with 50 or more contiguous voxels.

The contrast maps (one for each subject) entered in a second level analysis (ANOVA). ANOVA was performed to describe the difference among the three groups (HC, group 0, Group 1). Only activation clusters that survived after correction for multiple comparisons (Family wise correction, $p_{FWE\text{-}corr} < 0.05$) and an extent threshold of voxel size of 10 adjacent voxels were considered in the results section. Task performance entered the fMRI second level statistical analyses as a covariate due to the different performance obtained from AD (99%) and HC (78%).

RESULTS

SNAP-25 polymorphisms distribution

The five *SNAP-25* gene SNPs were in Hardy Weinberg equilibrium in patients and controls, as shown by molecular genotyping. Genotype and allelic distribution comparisons revealed the presence of a significant association between rs363050 (AA) and AD ($p_c = 0.002$, OR:1.47) (Table 2a). Allelic distribution analyses confirmed that the rs363050 (A) allele is more frequently present in AD patients compared to HC1 ($p = 0.01$, OR:1.24) and showed that the rs363043 (T) allele is statistically more frequent as well in AD than in HC1 ($p = 0.01$, OR:1.29). After stratification for gender, the rs363050 (AA) genotype and (A) allele resulted to be statistically more frequent in AD female patients compared to HC1 of the same gender ($p_c = 0.001$, OR:1.64 and $p = 0.0003$, OR:1.46 respectively). Similarly, the rs363043 (T) allele was statistically more frequent in female AD patients than in controls ($p = 0.02$, OR:1.29). No significant differences were seen in males, possibly because of the lower analyzed numbers (Table 2a).

The rs363050 (A) allele and the rs363043 (T) allele were also significantly associated with aMCI when these patients were compared with HC2 ($p = 0.01$, OR:1.42 and $p = 0.04$, OR:1.35) (Table 2b). Because the replication cohorts of aMCI and controls were relatively small (148 versus 310), the *post hoc* power of the OR estimate (H_0 OR = 1) was calculated. Considering the size of the cohorts, an OR point estimate of 1.39 and frequencies of 34.9 and 28% respectively, the actual power was 0.814.

The ε4 allele of *APOE4* is the only known confirmed genetic risk factor for sporadic AD. All the individuals were thus genotyped for *APOE4* polymorphism and, as expected, results confirmed the presence of a positive association between *APOE4* and both AD ($p < 0.001$, OR: 3.78 versus HC1) and aMCI ($p < 0.001$, OR: 2.00 versus HC2) (Table 1). *SNAP-25* genotypes were equally distributed in *APOE4* + and *APOE4*- individuals, indicating that *SNAP-25* SNPs correlate with AD and aMCI independently of *APOE4* (data not shown). Haplotype analysis of *SNAP-25* SNPs and *APOE* evidenced a linkage disequilibrium between rs363050 and rs363043 and between rs3746544 and rs1051312 (Fig. 1), with the rs363050/rs363043 A-T haplotype being statistically more frequent in AD compared to HC ($p = 0.002$, OR: 1.45) and in aMCI compared to HC2 ($p = 0.03$, OR: 1.35).

Finally no association was detected between the *SNAP-25* SNPs (rs363039 (G/A), rs3746544 (T/G), and rs1051312 (T/C)) and either AD or aMCI (Supplementary Table 1a, b).

SNAP-25 polymorphisms and neuropsychological evaluation

Because the results above suggest an association between *SNAP-25* genotypes and both AD and aMCI

Table 2a

Genotype and allele distribution of the rs363050 and rs363043 *SNAP-25* SNPs in Alzheimer's disease (AD) patients and in age- and gender-matched healthy controls (HC1). Only results obtained in females are shown in details, as no significant differences were observed in male patients. OR: Odds ratio; 95%CI: Interval of confidence. p_c: *p* value corrected for two degree of freedom for genotype distribution

SNAP-25		AD		HC1		*p* value	OR; 95% CI
		Females ($n = 411$) %	Males + Females ($n = 607$) %	Females ($n = 420$) %	Males + Females ($n = 615$) %		
rs363050							
Genotypes	A/A	46[2]	44[1]	34[2]	35[1]	[1]$p_c = 0.002$	1.47; 1.16–1.86
						[2]$p_c = 0.001$	1.64; 1.23–2.20
	A/G	44	42[3]	50	50[3]	[3]$p_c = 0.008$	0.72; 0.57–0.90
	G/G	10	14	15	15		
						[1]pc = 0.003	
						[2]pc = 0.04	
Alleles	A	68[5]	65[4]	59[5]	60[4]		[4]1.24; 1.05–1.47
							[5]1.46; 1.18–1.79
	G	32	35	41	40		[4]0.80; 0.68–0.95
							[5]0.69; 0.56–0.84
						[4]$p = 0.01$	
						[5]$p = 0.0003$	
rs363043							
Genotypes	C/C	44	44	51	49		
	C/T	44	43	41	41		
	T/T	12	13[6]	8	9[6]	[6]$p_c = 0.08$	1.49; 1.02–2.18
						[6]$p_c = 0.04$	
Alleles	C	66	65	71	70		[7]0.80; 0.67–0.95
							[8]0.78; 0.63–0.96
	T	34[8]	35[7]	29[8]	30[7]		[7]1.29; 1.05–1.45
							[8]1.29; 1.04–1.59
						[7]$p = 0.01$; [8]$p = 0.02$	

[1]p_c: AD versus HC1; [2]p_c: female AD patients versus female HC1; [3]p_c: AD versus HC1; [4]p AD versus HC1; [5]p: female AD versus female HC1; [6]p_c: AD versus HC1;[7]p: AD versus HC1; [8]p: female AD versus female HC1.

Table 2b

Genotype and allele distribution of the rs363050 and rs363043 *SNAP-25* SNPs in amnestic mild cognitive impairment (aMCI) patients and in age- and gender-matched healthy controls (HC2). OR: Odds ratio; 95% CI: Interval of confidence; p_c: *p* value corrected for two degree of freedom for genotype distribution

SNAP-25		aMCI	HC2	*p* value	OR; 95% CI
		Males + Females ($n = 148$) %	Males + Females ($n = 310$) %		
rs363050					
Genotypes	A/A	39	30		
	A/G	50	51		
	G/G	11[1]	19[1]	[1]$p_c = 0.04$	0.52; 0.28–0.94
				[1]pc = 0.04	
Alleles	A	64[2]	60[2]		[2]1.42; 1.07–1.90
	G	36	40		[2]0.70; 0.52–0.94
				[2]$p = 0.01$	
rs363043					
Genotypes	C/C	43	49		
	C/T	45	41		
	T/T	12	9		
				[3]$p_c = 0.09$	
Alleles	C	65	70		[4]0,73; 0,54–0,99
	T	35[4]	30[4]		[4]1,35; 1,01–1,82
				[4]$p = 0.04$	

[1]p_c: aMCI versus HC2; [2]p: aMCI versus HC2; [3]p_c: aMCI versus HC2; [4]p: aMCI versus HC2.

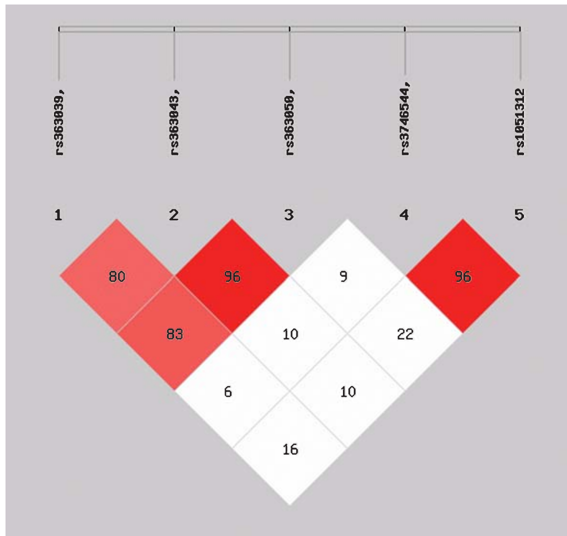

Fig. 1. LD pattern of (r^2) for the five selected SNPs within the *SNAP-25* gene on chromosome 20 p12-p11.2.SNPs. (1) rs363039, (2) rs363043, (3) rs363050, (4) rs3746544, (5) rs1051312.

independently of *APOE4*, we verified possible correlations between such genotypes and AD-associated clinical parameters in a randomly selected subset of 209 AD and 54 aMCI patients. ANOVA analysis regarding categorical fluency scores showed that the rs363050 and rs363043 genotypes resulted as pathological ($\leq$25) in AD (df:2, F = 4.65, p = 0.01 and df:2, F = 3.85, p = 0.03, respectively) (Table 3). Further supporting the importance of these SNPs in modulation of categorical fluency, data indicated that the cognitive skewing between pathological and normal value score increased when AD were stratified according to the presence/absence of the rs363050 (A) and rs363043 (T) alleles. Therefore lower categorical fluency scores were detected in patients carrying the rs363050 (A) allele in either homozygote rs363050 (AA) or heterozygote (AG) compared to those carrying rs363050 (GG) (df:1, F = 9.52, p = 0.002 and df:1, F = 7.54, p = 0.01). Similarly, reduced categorical fluency scores were seen in rs363043 (TT) homozygote AD patients compared to those carrying the rs363043 (CC) genotype (df:1, F = 5.36, p = 0.02). Notably, the skewing of categorical fluency scores was lower in patients carrying the rs363043 (CT) heterozygous pattern compared to those carrying the rs363043 (CC) genotype (df:1, F = 3.75, p = 0.05) (Table 3). Finally, no correlations were detected between *SNAP-25* genotypes and categorical fluency scores in aMCI, in whom such scores were above the 25 points cut-off.

The relative contribution of rs363050 (AA/AG) versus rs363050 (GG) and of rs363043 (TT/CT) versus rs363043 (CC) in the model was evaluated next in a multivariate binary logistic regression model taking in account *APOE4* positivity and gender as covariates. Categorical fluency scores $\leq$25 or >25 were adopted as the response variable; *SNAP-25* genotypes, $APOE4^{pos}/APOE4^{neg}$ and gender as covariates. Stepwise binary logistic regression analysis evidenced that both rs363050 (AA/AG) and rs363043 (TT/CT) are statistically correlated to categorical fluency impairment when they are inserted singularly in regression analysis and adjusted for *APOE4* and gender (rs363050 (AA/AG) p = 0.005; OR: 3.93) (Table 4; Model 1); (rs363043 (TT/CT) p = 0.04 OR: 1.82) (Table 4; Model 2). When both these variables and their interaction were evaluated in the same model, rs363050 (AA/AG) alone remained associated to categorical fluency impairment (p = 0.01 OR: 3.167). These results suggest that, even if both rs363050 (A) and rs363043 (T) alleles correlate with categorical flu-

Table 3

Categorical Fluency and *SNAP-25* polymorphisms in AD and aMCI patients. SD, standard deviation, df, degree of freedom, *p*, *p* value corrected for degree of freedom

		AD						aMCI					
SNAP-25		Mean	n	SD	f	df	*p*	Mean	n	SD	f	df	*p*
rs363050													
Genotypes	A/A	24.2[1]	110	8.3	[1]9.5	1	0.002	33.3	20	8.7			
	A/G	24.3[2]	74	8.9	[2]7.5	1	0.01	33.3	25	8.9			
	G/G	29.7[1,2]	25	6.8				33.6	9	6.1			
	Total	24.9	209	8.5	4.65	2	0.01	33.4	54	8.3			n.s
rs363043													
Genotypes	C/C	26.7[3]	82	9.0	[3]5.4	1	0.02	34.7	27	7.9			
	C/T	24.1[4]	97	8.4	[4]7.5	1	0.05	31.6	24	8.5			
	T/T	25.5[3,4]	30	6.1				36.0	3	8.5			
	Total	24.9[3,4]	209	8.5	3.85	2	0.03	33.4	54	8.3			n.s.

[1]AA versus GG; [2]AG versus GG; [3]CC versus TT; [4]CT versus TT.

Table 4
Categorical Fluency and *SNAP-25* polymorphisms in AD patients. Results of multivariate stepwise logistic regression analysis. Responsible variable: Categorical Fluency Score categorized as ≤25 (pathological) or >25 (normal). OR, odds ratio; 95% CI, interval of confidence

		wald	*p* value	OR	95%CI
Model 1		2.16	0.141	0.804	0.60–1.07
Selected Variables	rs363050 (AA/AG)	7.79	0.005	3.93	1.50–10.32
Unselected Variables	$APOE4^{pos}$		0.58		
	Gender		0.45		
Model 2		2.82	0.093	0.74	0.52–1.05
Selected Variables	rs363043 (TT/CT)	4.33	0.04	1.82	1.04–3.18
Unselected Variables	$APOE4^{pos}$		0.72		
	Gender		0.60		

Model 1: AD patients (n = 209); covariates: $APOE4^{pos}$ (e4/e4 e4/e3 e2/e4), Gender (female versus male), *SNAP-25* rs363050 (AA/AG versus GG). Model 2: AD patients (n = 209); covariates: $APOE4^{pos}$ (e4/e4 e4/e3 e2/e4), Gender (female versus male), *SNAP-25* rs363043(TT/CT versus CC).

Table 5
Logistic regression analysis by plink software adjusting for gender and *APOE4* positivity. Categorical Fluency and *SNAP-25* haplotype rs363050 /rs363043 polymorphisms in AD patients. Responsible variable: Categorical Fluency Score categorized as ≤25 (pathological) or >25 (normal); covariates: $APOE4^{pos}$ (e4/e4 e4/e3 e2/e4), Gender (female versus male) OR: odds ratio

SNP1	SNP2	haplotype	wald	*p* value	OR
rs363050	rs363043	AT	4.84	0.0278	1.25
		GC	1.95	0.163	0.87
		AC	0.562	0.454	0.923

ency impairment, the involvement of rs363050 (A) is stronger than the one of rs363043 (T). Therefore, when analyzed together, one masks the other.

Finally, logistic regression analysis was performed using the Plink software to evaluate haplotype distribution in relationship with categorical fluency impairment (≤25 or >25) and adjusting for gender and *APOE4* positivity. Results confirmed that the rs363050/rs363043 A-T haplotype is significantly associated with lower categorical fluency scores (p = 0.03 OR: 1.25) (Table 5).

SNAP-25 and functional MRI

To verify possible correlations between *SNAP-25* genotypes and imaging patterns, a group of AD patients and HC1 underwent fMRI evaluation. Ten HC1, 18 AD patients in whom the *SNAP-25* SNPs being more frequent in AD were present (group 1), and 10 AD patients in whom the *SNAP-25* SNPs being more frequent in AD were NOT present (group 0) were analyzed. After discarding 4 patients (3 out of group 1 and 1 to out of group 0) for excessive motion artifacts, significant differences (ANOVA $p < 0.001$) emerged. Bonferroni-corrected ANOVA results indicated that task performance was significantly better in HC1 (mean task performance 99.24%±1.07%) compared to AD patients (mean group 0 : 76.37%±12.91%, $p = 0.002$; mean group 1 : 80.14%±4.37%, $p < 0.001$); no significant differences were found between the two AD groups. Demographical, neuropsychological and behavioral task-fMRI characteristics were shown in Table 6.

Imaging results for category-driven word generation showed an activation in the bilateral (left > right) frontal cortex (inferior and middle frontal gyri), the left premotor cortex, the bilateral cingulate gyrus, the medial temporal lobe cortex, and the basal ganglia in HC1. The overall pattern of brain activation seen in AD was similar to that seen in HC1, even if a significantly reduced recruitment was detected in the frontal cortex (Fig. 2A, B). Raising the statistical thresh-

Table 6
Demographical, neuropsychological and behavioral task-fMRI characteristics

	AD gr0 (N = 9)	AD gr1 (N = 15)	HC (N = 10)	Group comparison
Age (y) [Mean ± SD]	75.56 ± 5.85	74.67 ± 5.39	70.80 ± 3.85	
Range (y)	69–83	61–81	64–77	
Male %	44.4	33.3	40.0	
MMSE score [mean ± SD]	19.37 ± 3.24[1]	20.03 ± 2.44[2]	28.31 ± 1.8[1,2]	$p < 0.001$
Performance on fMRI task				
Accuracy	77.37 ± 12.918[1]	80.14 ± 4.378[2]	99.24 ± 1.078[1,2]	$p < 0.001$

Comparisons of MMSE and fMRI task variables between controls (HC) and patients with Alzheimer's disease (AD) were all significant at $p < 0.001$; *Post Hoc* Test [1]HC versus AD gr0: $p < 0.05$ [2]HC versus AD gr1: $p < 0.05$. AD gr1 = patients carried the rs363050 (AA or AG) and rs363043 (CT or TT) genotype; AD gr0 = patients carried the rs363050 (GG) and rs363043 (CC) genotype.

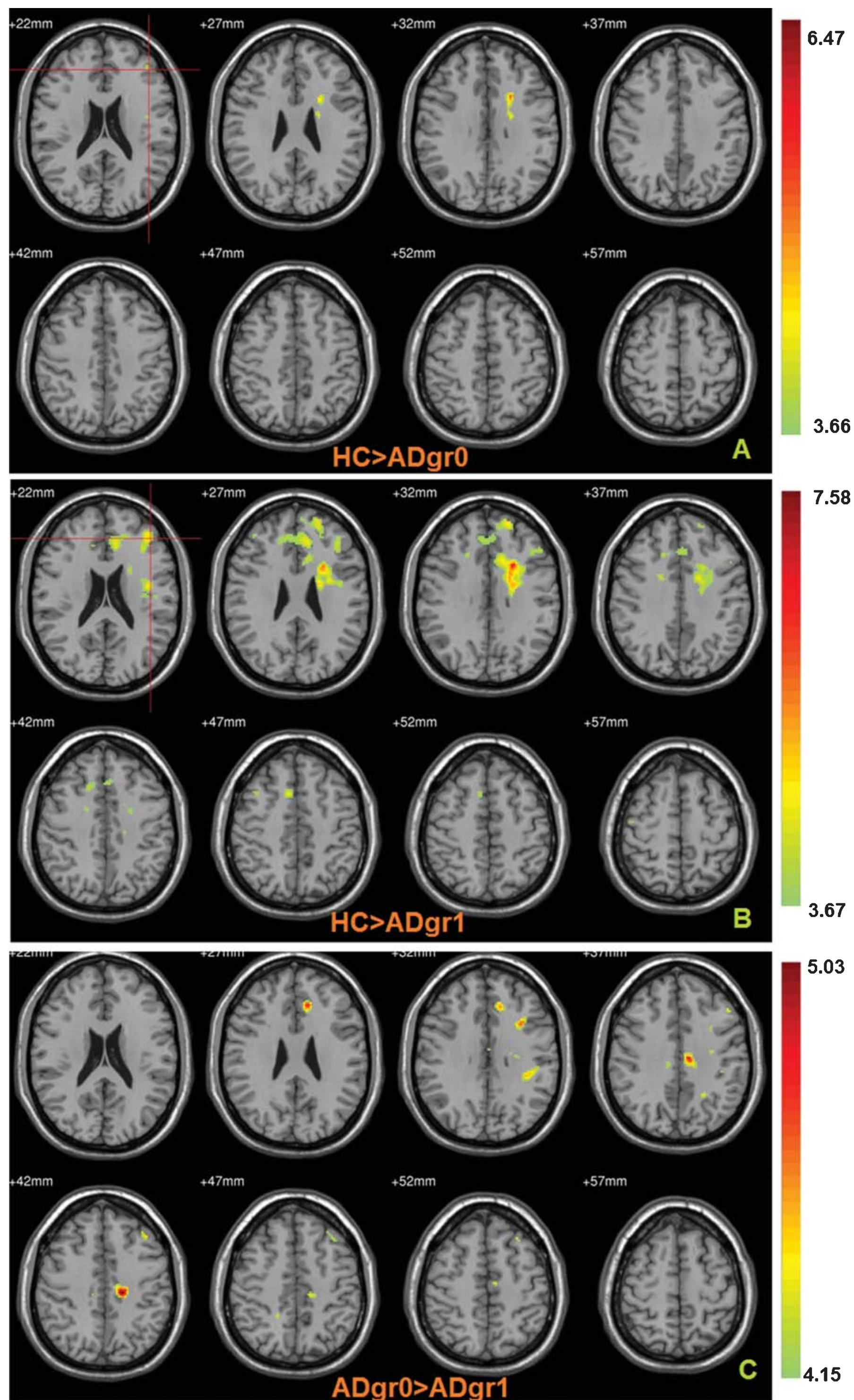

Fig. 2. Main BOLD effect due to the paced overt categorical verbal fluency task: comparison among the three groups: healthy controls (HC) (10 subjects), group 0 (10 AD patients carrying the rs363050 (GG) and rs363043 (CC) genotypes), and group 1 (18 AD patients carrying the rs363050 (AA or AG) and rs363043 (CT or TT) genotypes) (ANOVA). Brain areas which are significantly more activated in: HC1 versus AD group 0 (A); HC1 versus AD group 1 (B); and AD group 0 versus group 1 (panel C) are shown. See text for statistical thresholds and further details. The right side on the images corresponds to the left side of the brain.

old to an uncorrected level for statistical purposes ($p_{unc} < 0.001$), we noticed in AD patient compared to HC a significant hypoactivation also in temporal cortex (temporal pole and the fusiform gyrus). Nevertheless, direct comparison between the two AD groups (ANOVA) showed significant differences ($p_{FWE\text{-}corr} < 0.05$ at cluster level). Thus, brain activity was significantly reduced in group 1 patients in the cingulate cortex and in the frontal (middle and superior gyri) and the temporo- parietal (angular gyrus) area (Fig. 2C); in the opposite comparison (group 1 > group 0) increased activation was not detected in any of the brain regions involved in the task.

DISCUSSION

SNAP-25 is a vesicular SNARE protein that plays an important role in the release of neurotransmitters via its interaction with voltage-gated calcium channels. *SNAP-25* gene polymorphisms are suggested to associate with both variations in IQ phenotypes and a number of neurologic conditions, including the age-related decline of cognitive function [44]. Because anatomical and functional synapsis alterations are present in AD we evaluated the possible involvement of *SNAP-25* polymorphisms in this disease. *SNAP-25* is a highly polymorphic gene as it includes 225 SNPs. We focused on those that had previously been shown to correlate with human diseases and variations in intelligence and that localize within intron 1, in a region spanning about 13.8 kb known to affect transcription factor binding sites [18].

Results herein suggest that *SNAP-25* gene polymorphism associate with AD and aMCI in Italian patients. Thus, the frequency of carriers of the *SNAP-25* rs363050 (A)(AA/AG) and the rs363043 (T)(TT/CT) alleles was significantly increased in AD and in aMCI compared with gender- and age-matched healthy controls. Notably, a declining degree of prevalence of these alleles was present when AD (higher prevalence) were compared to aMCI (intermediate prevalence) and HC (lower prevalence); this observation is possibly due to the fact that not all aMCI will evolve into AD. It will be interesting to evaluate how many aMCI carrying the above-mentioned *SNAP-25* genotypes will indeed develop AD.

As a result of the *SNAP-25* allelic distribution, the rs363050/rs363043 A-T *SNAP-25* haplotype was statistically more frequent in both AD and aMCI compared to HC. These two SNPs are in linkage disequilibrium, therefore their contribution may be due either to the fact that the rs363050 (A) genotype drags the rs363043 (T) genotype, or to the possibility that other genotypes within the haplotype are associated to AD development.

Having observed a possible association between particular *SNAP-25* SNPs, AD, and aMCI, we next verified the presence of correlations between such SNPs and clinical parameters. Results showed that the *SNAP-25* haplotypes more frequently seen in AD, are associated with altered scores at the categorical fluency test. In particular, pathological mean scores in this test (≤ 25) were associated with the rs363050 (AA/AG) and rs363043 (CT/TT) alleles in AD, with the strongest association being seen with rs363050 (AA/AG). These alterations were present in AD alone, as in aMCI, scores were higher than the pathological cut off of 25. Results were analyzed next taking into account gender and *APOE4* positivity by performing stratified analysis of different *SNAP-25* polymorphisms in relationship with gender and with *APOE4* positivity. Results indicated that the *SNAP-25* SNPs-associated categorical fluency impairment is independent of both *APOE4* and gender. Results of further multivariate logistic stepwise regression showed that, although both the rs363050 (A) and rs363043 (T) allele are associated with categorical fluency impairment, the role played by rs363050 (A) is stronger than that of rs363043 (T).

Verbal fluency, and in particular Category fluency, is altered in AD [20, 21], and a category fluency task is incorporated in the CERAD (Consortium to Establish a Registry for Alzheimer's Disease) protocol for the diagnosis and the clinical staging of AD [45, 46]. Category fluency relies on the structure of the semantic network, on the availability of sound lexical-semantic representations, and on the access to semantic knowledge, three facets of cognition that are affected in AD [20, 21]. It seems thus biologically relevant that data herein suggest that impairments of these key components of language functioning are associated with the *SNAP-25* SNPs that prevail in AD patients.

Possible anatomical relationships between *SNAP-25* SNPs and categorical fluency impairment were finally analyzed by fMRI in a subset of AD patients. We adopted the verbal fluency paradigm described by Basho and colleagues [39] to test language function. This fMRI task was chosen because it allows an appropriate response monitoring and a tight control over and reduced individual variability of task performance, making it suitable for the application in patients with cognitive deficits. Results showed that brain activation and brain area recruitment (prefrontal regions, parieto-temporal area, and cingulate cortex) are signif-

icantly diminished in patients carrying the *SNAP-25* SNPs that are more frequent in AD and correlate with pathological categorical fluency scores. This activation pattern could reflects pathologic alterations within critical nodes of the neural networks subserving working memory and attentional–executive functions [47, 48]. Recent studies have shown a relationship between reduced neural metabolism and poor performances in semantic memory tasks [24, 49, 50]. Our results are in line with these works, and especially with those focusing on the left temporoparietal and left prefrontal cortex [48, 49]. The most impaired areas we found in AD were the frontal cortex and the anterior cingulated. It is proven that damage in these areas could reasonably influence the efficiency of attentive processing in task accomplishment. Finally, though at lower levels of significance, AD patients presented a hypoactivation also in temporal pole cortex, an area that is commonly considered as integral part of the semantic network [51], and fusiform gyrus. Interestingly, the impairment in the fusiform gyrus, an area that mediates between lateral semantic memory and medial episodic memory encoding networks, is coherent with recent evidence [52] showing that impairment in frontal and temporal areas involved in language network characterize the clinical evolution of AD.

Brain activation impairments were significantly more frequent in AD patients carrying the rs363050 (AA/AG) and/or rs363043 (CT/TT) genotypes, and, on the other hand, an augmented brain activity was seen in bilateral parietal and frontal brain area and in the cingulate cortex of AD patients carrying the rs363050 (GG) and/or rs363043 (CC) compared to those carrying the rs363050 (AA/AG) and/or rs363043 (CT/TT) genotypes. The higher activation of group 0 could be attributable to some sort of residual compensatory mechanisms in frontal areas that accounts for a semantic deficit [53]. Interestingly, the left inferior parietal lobe and angular gyrus are considered to be crucial areas in the processing hierarchy underlying concept retrieval and conceptual integration. A damage of the left angular gyrus is responsible for a variety of cognitive impairments, such as, among others, anomia, sentence comprehension impairment and dementia [54]. Notably, our AD patients did not show differences in activations of bilateral anterior temporal lobe (ATL) and TP. This lack of activation in ATL and TP can be read as a index of impairment of these areas that reflects a connective degeneration in temporal cortices in AD. Coherent with present results are recent findings on AD population [52] that show a decrease in neuro plasticity of temporal lobes with the progression of the disease.

Categorical verbal fluency tests assess medial temporal lobe function [22] and are positively correlated with neurobiological hippocampal and parietal lobe neurochemical abnormalities in AD [55]. The *SNAP-25* gene is highly expressed in the hippocampus [56], a brain structure that plays a crucial role in semantic fluency performance [57]. Animal studies showed that the hippocampal SNAP-25 protein is involved in memory consolidation and long-term memory formation in rats [58, 59]; additional results indicate that changes of hippocampal *SNAP-25* expression contribute to age-related decline of cognitive function [44]. It is therefore tempting to speculate that the SNPs described herein could influence the neuronal density and connectivity of the hippocampus, modulating synaptic plasticity and neurogenesis in the left hippocampus. Both the SNPs described herein, rs363050 (A/G) and rs363043 (C/T), localize within intron 1 in a region spanning about 13.8 kb which is known to affect transcription factor binding sites [18]. We analyzed the functional effect, of rs363050 and 363043 SNPs on transcriptional activity using luciferase a reporter gene assay. Our preliminary results showed that the rs 363050 (A) allele associates with a significantly higher *SNAP-25* expression compared to the rs363050 (G) allele (Braida et al., unpublished results). This could be due to the impairment of binding of factors involved in the modulation of the *SNAP-25* gene expression level or to the binding of other factors, different from the ones that recognize the sequence of the parental allele, acting as repressor. Both reduced and excessive *SNAP-25* activity has been implicated in various disease states that involve cognitive dysfunctions such as attention deficit hyperactivity disorder, schizophrenia, and AD [14, 60–63]. An excess of *SNAP-25* activity during adulthood was shown to be sufficient to mediate significant deficits in the memory formation process. Expression of *SNAP-25* in the adult dorsal hippocampus was also demonstrated to result in the dysregulation of memory consolidation machinery in this brain region [64]; finally, overexpression of *SNAP-25* in cultured hippocampal neurons was associated with impaired synaptic transmission [65]. Altogether, these results suggest that an increased *SNAP-25* level does impair synaptic maturation and/or neurotransmission. Moreover, as SNAP-25 is an age-related protein which is present in two isoforms, SNAP-25a which prevails in younger individuals, and SNAP-25b which takes over is seen in adults, contradictory results could be due to the fact that these isoforms may be differently regulated by the same gene polymorphisms. We have not yet obtained definite results on the possible correlation between *SNAP-25*

polymoprphisms and splicing and miRNA expression; these analyses are undergoing in our laboratory

Further analysis and independent validation in additional cohorts will be required to assess the relevance of these variants in AD and to establish the possible predictive value of *SNAP-25* polymorphisms in the evolution of this disease.

ACKNOWLEDGMENTS

Funding for this study was provided by 2011 Ricerca Corrente [Italian Ministry of Health and 5 × 1000, 2008] and Monzino Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Authors' disclosures available online (http://www.j-alz.com/disclosures/view.php?id=2312).

SUPPLEMENTARY MATERIAL

The supplementary table is available in the electronic version of this article.

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