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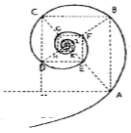

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Genetic Study of Biomarkers Involved in the Process of Aging Neurodegeneration

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Sommario

La proteina Sinaptosomiale di 25 KDa (SNAP-25) è una proteina SNARE coinvolta nel processo di esocitosi dei neurotrasmettitori dalle vescicole sinaptiche, processo che risulta alterato nella malattia di Alzheimer (AD). E' stato evidenziato che, variazioni dei livelli di SNAP-25, contribuiscono al declino di funzioni cognitive legate all'invecchiamento e che polimorfismi a singolo nucleotide (SNPs) del gene SNAP-25 correlano con patologie neuropsichiatriche, giocando un ruolo importante nella determinazione del fenotipo del QI. Per verificare il possibile coinvolgimento di SNAP-25 nell'AD sono stati analizzati cinque polimorfismi genici in pazienti affetti da AD (N=607), replicando lo studio in soggetti con decadimento cognitivo lieve di tipo amnesico (aMCI)(N=154) e in due gruppi di controlli sani, comparati per età e sesso (HC1: N=615 e HC2: N=310). I risultati hanno mostrato che gli alleli intronici rs363050(A) e rs363043(T), così come l'aplotipo rs363050/rs363043 A-T sono statisticamente più frequenti sia in AD che in aMCI. Ulteriori analisi hanno indicato che questi alleli ed aplotipo sono associati con punteggi patologici di fluenza categorica in pazienti AD. Infine, i genotipi di SNAP-25 correlano con una significativa ridotta attività cerebrale nella corteccia cingolata e nelle aree frontale (circonvoluzione media e superiore) e temporo- parietale (circonvoluzione angolare), come misurato dalle analisi di fMRI. In conclusione, dato che i polimorfismi di SNAP-25 sono associati con AD e correlano con alterazioni di fluenza categorica e con una ridotta e localizzata attività cerebrale, potrebbero essere utilizzati come markers per la diagnosi di AD e di deficit cognitivi. E' stata studiata, in seconda battuta, la possibile correlazione tra APOE4, gli SNPs di SNAP-25 e l'outcome della terapia a stimolazione multidimensionale (MST). Cinquantotto individui con diagnosi clinica di probabile o possibile AD, e fase di progressione da media a moderata, sono stati sottoposti a trattamento MST per 10 settimane. I soggetti sono stati sottoposti a test cognitivi, comportamentali e funzionali tramite le scale; Mini Mental Scale Evaluation (MMSE), Functional Living Skill Assessment (FLSA) e Neuropsychiatric Inventory Scale (NPI), sia prima del trattamento che alla fine della terapia. I genotipi analizzati per APOE e gli SNPs di SNAP-25 sono stati correlati con i punteggi ΔMMSE, ΔNPI and ΔFLSA tramite analisi di regressione logistica multinomiale. I risultati mostrano punteggi di MMSE più alti dopo la riabilitazione in soggetti APOE4 negativi comparati con i pazienti APOE4 positivi; mentre gli alleli di SNAP-25 rs363050(G) e rs363039(A) correlano con un significativo miglioramento comportamentale dopo trattamento MST. Le analisi aplotipiche degli SNPs di SNAP-25 hanno evidenziano la presenza di un associazione significativa tra l'aplotipo di SNAP-25 e bassi punteggi di ΔNPI. In particolare l'aplotipo rs363050(G)-rs363039(A)-rs363043(C): (GAC) è stato associato a miglioramento comportamentale secondo la scala NPI. In conclusione i polimorfismi in geni con attività di modulazione della plasticità neuronale potrebbero predire l'outcome di una riabilitazione multistrutturata per l'AD.

Abstract

Synaptosomal-associated protein of 25kDa (SNAP-25) is an age-regulated vesicular SNARE protein involved in the exocytosis of neurotransmitters from synapses, a process that is altered in Alzheimer's disease (AD). Changes in SNAP-25 levels are suggested to contribute to age-related decline of cognitive function, and single nucleotide polymorphisms (SNPs) in the SNAP-25 gene are present in neuropsychiatric conditions and play a role in determining IQ phenotypes. To verify a possible role of SNAP-25 in AD we analyzed five gene polymorphisms in patients with AD (N=607), replicating the study in subjects with amnestic mild cognitive impairment (aMCI)(N=154) and in two groups of age-matched healthy controls (HC1: N=615 and HC2: N=310). Results showed that the intronic rs363050(A) and rs363043(T) alleles, as well as the rs363050/rs363043 A-T haplotype are significantly more frequent in both AD and aMCI. Further analyses indicated that these alleles and haplotype are associated with pathological scores of categorical fluency in AD alone. Finally, SNAP-25 genotypes correlated with a significantly decreased brain activity in the cingulate cortex and in the frontal (middle and superior gyri) and the temporo- parietal (angular gyrus) area, as measured by fMRI. SNAP-25 polymorphisms are associated with AD and correlate with alterations in categorical fluency and a reduced localized brain activity. In conclusion SNAP-25 polymorphisms could be suggested as surrogate markers for the diagnosis of AD and of cognitive deficit; these SNPs might also have a possible predictive role in the natural history of AD. Moreover we investigated a possible correlations between APOE4 and SNAP-25 polymorphisms and the outcome of a multidimensional cognitive, behavioral and functional stimulation (MST). Fifty-eight individuals with mild-to-moderate AD underwent MST for 10 weeks. Mini Mental Scale Evaluation (MMSE), Functional Living Skills Assessment (FLSA) and Neuropsychiatric Inventory scale (NPI) were performed at baseline and after therapy. Molecular genotyping of ApoE4 and SNAP-25 SNPs were correlated with ΔMMSE, ΔNPI and ΔFLSA scores by multinomial logistic regression analysis. Results shown higher overall MMSE scores after rehabilitation in ApoE4 negative compared to ApoE4 positive patients, whereas the SNAP-25 rs363050(G) and rs363039(A) alleles correlated specifically with significant improvements in behavioural parameters after MST. Haplotype analysis of rs363050, rs363039 and rs363043 SNAP-25 SNPs showed the presence of a significant association between SNAP-25 haplotypes and lower ΔNPI. In particular the rs363050(G)-rs363039(A)-rs363043(C): (GAC) haplotype was statistically associated with a better outcome of treatment as measured by the NPI scale. In conclusion polymorphisms in genes known to modulate neural plasticity may predict the outcome of a multistructured rehabilitation protocol in AD.

INDICE

LIST OF ABBREVIATIONS

Alzheimer's Disease- AD

Mild Cognitive Impairment- MCI

Early-Onset Familial AD- EOFAD

Late-Onset AD- LOAD

Apolipoprotein E- APOE

Synaptosomal-Associated Protein of 25 kDa- SNAP-25

Presenilin 1- PSEN1

Presenilin 2- PSEN2

Amyloid Precursor Protein- APP

Functional Magnetic Resonance Imaging- fMRI

Cognitive Stimulation- CS

Multidimensional Stimulation Therapy- MST

Mini-Mental State Examination- MMSE

Functional Living Skills Assessment- FLSA

Neuropsychiatric Inventory scale- NPI

Chapter I

1. INTRODUCTION

1.1 Alzheimer's Disease

1.1.1 History

Alzheimer's Disease (AD) was described for the first time in 1907 by Aloysius Alzheimer a German neurologist and psychiatrist. He described the behavioral manifestations of “senile dementia” in a 51-year-old female named Auguste D. Moreover he was the first to recognize the significance of the senile plaques and neurofibrillary tangles found in her brain after her death at age 55 years [1,2]. Dr. Alzheimer’s lecture about the first case of the fatal progressive dementia did not receive special attention, although as a psychiatrist he was a pioneer at this time by associating pathological changes with dementia symptoms. Alzheimer’s colleague, Kraepelin, finally gave the disease its official name in 1910. Since then accumulating evidence and knowledge have been accumulated in this research field. Amyloid fibrils from tissue were visualized for the first time in 1959 by electron microscopy. X-Ray diffraction studies of isolated fibrils revealed the cross β structure as a common motif in 1968 [3]. In 1970, thanks to the amino acid analysis and protein sequencing tools was revealed that each amyloidosis is linked to specific protein [4]. In 1984, Aβ was identified as the major component of plaques from AD patients [5]. The Tau protein, already know as essential protein for microtubule assembly [6], was identified as the neurofibrillary tangle forming protein in 1985. The corresponding MAPT gene was cloned in 1988 [7], one year after the amyloid- β protein precursor (βAPP) gene containing the Aβ sequence was cloned from chromosome 21 [8]. Few years later, based on the strong

association between APOE and Aβ in the brain [9], APOE was suggested as Aβ-binding protein that induces a pathological β sheet conformational change in Aβ [10].

1.1.2 Introduction to the pathology

Alzheimer's disease is a chronic and progressive neurodegenerative disorder, and it is the most common age-related dementing illness. Actually is estimated that AD affect 35.6 million individuals worldwide and the number of patients increases every year and recent projections predict 65 millions of AD worldwide by 2030 [11]. AD is the most common type of dementia, accounting for 60-80% of all the cases [12] and affecting people aged 65 with an incidence of 25-50% [13].

Dementia is characterized by the loss of or decline in memory and other cognitive abilities. It is caused by various diseases and conditions that result in damaged brain cells. To make a diagnosis of dementia, physicians commonly refer to the criteria reported in the Diagnostic and Statistical Manual of Mental Disorder, Fourth Edition (DSM-IV) [14].

To be classified as dementia, the following DSM-IV criteria are required: symptoms must include decline in memory and decline in at least one of the following cognitive abilities:

- Ability to generate coherent speech;
- Ability to understand spoken or written language;
- Ability to identify or recognize objects, assuming intact sensory function;
- Ability to execute motor activities, that include motor and sensory abilities and comprehension of the required task;
- Ability to think abstractly, make sound judgments and plan and carry out complex tasks.

The impairment in cognitive abilities must be severe enough to interfere with daily life.

Moreover it is important for a physician to evaluate and determine the right cause of memory loss or other dementia-like symptoms. In fact some symptoms can be reversed if are caused by depression, delirium, drugs interaction, thyroid problems, alcohol abuse or vitamin deficiencies that are treatable condition. Otherwise, if symptoms of dementia are not caused by treatable condition, further assessments must be conducted by a physician to identify the form of dementia that is causing the symptoms. Different type of dementia are associated with distinct symptom patterns and characteristic microscopic brain abnormalities. Evidence from long-term observational and autopsy studies indicates that many people with dementia have brain abnormalities associated with more than one type of dementia. Despite the great scientific progress and the important discovery made in the last 100 years, the cause or causes of AD remain unknown. As yet, neither a satisfying therapy nor a preventative cure is available. Moreover AD can be precisely diagnosed post-mortem with analysis of neurological condition through the Braak staging, that could precisely classify the progress of the disease [15].

1.1.3 Symptoms of Alzheimer's disease

AD can affect people in different ways, but one of the most common signs of Alzheimer's is memory loss, especially forgetting recently learned information. This may be due to the disruption of brain cell function that usually begins in regions involved in the formation of new memories. As suggested by the Alzheimer's Association (*www.Alz.org*) there are 10 warning signs and symptoms. Every individual may experience one or more of these signs in different degrees: memory loss that disrupts daily life; challenges in planning or solving problems; difficulty completing familiar tasks at home, at work, or at leisure; confusion with time or place; trouble

understanding visual images and spatial relationships; new problems with words in speaking or writing; misplacing things and losing the ability to retrace steps; decreased or poor judgment; withdrawal from work or social activities; changes in mood and personality.

The pathological progression from mild to moderate and severe AD can take place at different rates. As the disease progresses the individual's cognitive and functional abilities decline. In advanced stage of AD, patients need help with the basic activities of the daily living (ADLs) as bathing, dressing and eating. In the final stage of the disease, people with AD lose their ability to communicate, to recognize loved ones and become bed-bound and reliant on a full-time caregiver. In the final stage of AD the inability to move becomes so dangerous that could expose the patient to infections including pneumonia. The AD-related pneumonia result fatal for the patients. While families generally prefer to keep the person with AD at home as long as possible, most of them eventually move into a nursing home where professional caregiver are available for better care [16].

1.1.4 Diagnosis of Alzheimer's disease

The National Institute on Aging (NIA) and the Alzheimer's Association recommended new diagnostic criteria and guidelines for AD in 2011. The new criteria and guidelines update, refine and broaden the guidelines published in 1984 by the Alzheimer's Association and the National Institute of Neurological Disorders and Stroke (NINDS). The work for the new criteria and guidelines began in 2009 when more than 40 researchers and clinicians in the AD field began an in-depth review of the 1984's criteria to decide how they might be improved by incorporating scientific advances.

The new criteria and guidelines differ from the original one, based chiefly on a doctor's clinical judgment about the cause of a patient's symptoms, taking into account reports from the patient, family members and friend,

results of cognitive testing and general neurological assessment, in two main aspects:

- Identification of three stages of AD;
- Inclusion of biomarker tests such as levels of certain proteins in fluids (e.g. levels of tau and amyloid-β in the cerebrospinal fluid and blood).

The three stages of AD identified are: preclinical AD; mild cognitive impairment (MCI) due to AD; dementia due to AD.

The first stage, preclinical AD, occurring before symptoms such as memory loss develop and before the affection of one's abilities to carry out everyday activities. Individuals in this stage have measurable change in the brain, cerebrospinal fluid and/or blood biomarkers that indicate the earliest signs of disease. When these very early changes in the brain are identified, an individual diagnosed using the new criteria would be said to have preclinical AD or MCI due to AD. The second stage, MCI due to AD, indicate individuals having mild, but measurable, changes in thinking abilities that are noticeable to the person affected, to the family members and friends, but that don't affect the individual's ability to perform the daily activities.

The third stage, dementia due to AD, is characterized by memory, thinking and behavioural symptoms that impair ability and function in daily life, caused by AD related processes.

1.1.5 Etiology of Alzheimer's disease

The causes of AD, a condition characterized by progressive neurodegeneration and gradual cognitive impairment, are still unclear. Experts believe that Alzheimer's develops as a complex result of multiple factors rather than any one overriding cause. Both age and genetics have been identified as risk factors. An estimated 1% of AD cases develop the disease as result of a genetic mutation [17], involving the gene for the amyloid precursor protein (*APP*) and the genes for the Presenilin 1 and 2 (*PSEN1* and *PSEN2*). Inheriting any of these genetic mutations guarantees that an individual will develop AD, in fact disease symptoms tend to develop before the age of 65 years. In this scenario the disease is considered early-onset familial AD (EOFAD) [18]. The other AD cases, like common chronic disease, appear to be sporadic and to have a later age at onset of more than 65 years (LOAD) [19].

Experts believe that AD, except for the sporadic cases, develops as a result of multiple factor rather than a single cause including: aging, injury, low educational levels, hyperlipidemia, homocysteinemia, diabetes mellitus and obesity [20-23].

1.1.5.1 **Age**

Aging is the most important known non-genetic risk factor for AD. Most people with AD are diagnosed at 65 years or older [24].

People younger than 65 years can also develop the disease, although this is much rarer. While age is the greatest risk factor, AD is not a normal part of aging and advanced age alone is not sufficient to cause the disease.

1.1.5.2 **Genetics**

Combination of environmental risk factors with genetic background, as *APOE4*, may further increase the risk for the LOAD and age-related decline [25]. As described before AD cases can be divided into LOAD and EOFAD subtypes. The EOFAD have an autosomal dominant inheritance linked to 3 genes: *APP, PSEN1* and *PSEN2*, whereas the LOAD form has been consistently associated with only one gene, *APOE*.

In EOFAD subjects, mutation affect APP processing, leading to an altered production of different Aβ peptides and, thus, their relative ratios. In Down's syndrome, patients carry an extra copy of chromosome 21, on which *APP* gene resides, it was noticed a develop of early-onset dementia with pathological hallmarks of AD in subject's brain [26], consistent with the idea that over-expression of APP could cause early-onset AD. In strong support of this idea, *APP* locus duplication leads to early-onset AD [27]. Moreover genetic variation in the promoter sequence, which might cause an increased *APP* gene expression, may be a risk factor for late-onset, with the levels of APP expression correlating inversely with age of disease onset [28].

While EOFAD is caused by rare and high penetrant mutation in three genes, the genetics of LOAD is more complex. Aging is the major non-genetic risk factor for LOAD. Instead, *APOE* gene, on chromosome 19, in particular the allele -ε4, has been identified as the major genetic risk factor. *APOE4*, genetically linked to sporadic AD, has a gene-dose effect on increasing the risk and lowering the age of onset of the disease [29]. Indeed, subject that inherit one copy of *APOE4* have an increased risk to develop AD, and subject that inherit two copy show an higher risk. Genome-wide association studies, performed on late-onset AD subjects from different population around the world, identified *APOE4* as the top gene with extremely high confidence in LOAD [30]. The estimated risk of developing AD in individuals with two copies of *APOE4* allele (~2% of the

population) is the ~60% at 85 years, and for those with one copy of the APOE4 allele (~25% of the population) is the ~30% by the same age. Compared to *APOE4* carriers, the risk for AD in subjects with two copies of *APOE3* allele is ~10% by the age of 85 years. Therefore, *APOE* gene should be considered as major gene, with semi dominant inheritance, for LOAD [31]. Unlike inheriting a known genetic mutation that causes AD, inheriting the ε4 form of the *APOE* gene does not guarantee that an individual will develop AD. Moreover, genome-wide association studies also identified other genes that modulate the risk of late-onset of AD, including *CLU, CR1, PICALM, BIN1, SORL1, GAB2, ABCA7, MS4A4/MS4A6E, CD2AP, CD33, EPHA1* and *HLA-DRB1/5* [30]. However, the relative contribution of these genes to AD is modest as compared to *APOE4*.

1.1.5.3 **Epigenetic**

Epigenetic is a "stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence" [32], these changes refers to modification in gene expression influenced by DNA methylation and/or chromatin structure, RNA editing and RNA interference [33]. Dysregulation in epigenetic mechanisms could lead to cognitive disorders that affect learning and memory as seen in AD [34]. Studies on post-mortem brain samples, as well as transgenic animal models, have shown that AD and aging are associated with epigenetic dysregulation, including abnormal DNA methylation and histone modifications. Despite this, it is not clear whether the epigenetic changes observed in AD represent a cause or a consequence of the disease, twin study support the hypothesis that epigenetic mechanism modulate AD risk [35].

1.1.5.4 **Education**

Researchers found that education plays a crucial role in the prevalence of AD or other dementia [36].

People with fewer years of education are at a higher risk than those with more years of education [37]. The exact cause for this relationship is unknown, but it is theorized that a higher education level leads to the formation of more synaptic connections in the brain. This creates a “cognitive reserve” in the brain [38], enabling subjects to better compensate for changes in the brain that could result in symptoms of AD by using alternate routes of neuron-to-neuron communication to complete a cognitive task [39].

1.1.6 Treatment of AD

1.1.6.1 **Pharmacologic treatment**

The aim of drugs administration is to slow or stop an illness or treat its symptoms. Unfortunately none of the treatments available today for AD slows or stops neurons malfunction and death in the brain. Until now, few drugs have been approved by the US Food and Drug Administration that temporarily improve symptoms of AD by increasing the amount neurotransmitters in the brain. The effectiveness of these drugs varies from person to person. Three of this drugs are acetylcholine esterase inhibitors (Donepezil, Rivastigmine, Galantamine), which are licensed for the treatment of people with mild-to-moderate AD, and Memantine, an NMDA receptor antagonist, which is licensed for the treatment of people with moderate-to-severe AD. The existing drugs do not specifically target the underlying pathology of AD. Instead, current treatments target cholinergic or glutamatergic function. Cholinergic function is compromised in AD [40] following early loss of basal forebrain cholinergic neurons. Treatments mainly focus on inhibition of acetylcholine esterase or modulation of muscarinic and nicotinic acetylcholine receptors.

Treatment with acetylcholinesterase inhibitors results in a moderate improvement in cognition (1.5-2 points on the MMSE over 6-12 months),

short-term (3-6 months) improvement in global outcome and some additional short-term stabilization of overall cognitive function [41, 42]. There is also more limited evidence of modest improvements in mood (particularly apathy) and social interaction [43].

1.1.6.2 **Non-pharmacologic treatment**

The non-pharmacological treatment are approaches that use physical therapy and reminiscence therapy. Even if, as pharmacologic treatment, non-pharmacologic therapies have not been shown to alter the course of AD, these therapies are often used with the goal of maintaining cognitive function, helping brain to compensate for impairment, improving quality of life or reducing behavioral symptoms such depression, apathy, wandering, sleep disturbances, agitation, and aggression. A wide range of non-pharmacologic therapies have been proposed and studied, but of all that's categories, reviewed in the Cochrane Database only cognitive stimulation suggested a beneficial effect. Moreover, of the high-quality studies reviewed, cognitive training, cognitive stimulation and ADLs training appeared most successful in reaching the aims of the intervention [44].

The most successful non-pharmacologic interventions, founded by a meta-analysis which combines results from many studies, seems to be a multicomponent intervention, which have the potential to reduce the frequency and severity of behavioral and psychological symptoms of dementia. This treatment is also tailored to the needs of the patient and the caregiver and delivered at home with periodic follow-up [45]. Based on this findings, a multidimensional stimulation approach [46] was developed using Cognitive Stimulation (CS), recreational/occupational and psychomotor/physical therapy to set up a Multidimensional Stimulation Therapy (MST) [47-49]. This MST was demonstrated to improve cognitive and behavioral disturbances. Moreover the MST treatment increase the

activation of temporal areas in the right insular cortex and in the thalamus, as determined by fMRI [50].

1.2 Genes and Biomarker research

1.2.1 Apolipoprotein E

The *APOE* gene is located on chromosome 19q13.2 and encodes a 299 amino acids long glycoprotein of 34.1 kDa [51]. It is synthesized in various tissues including liver, brain, skin and macrophages [52]. APOE plays multiple roles in the regulation of lipid and lipoprotein levels in the blood. APOE serves as ligand for members of very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL) receptor family, high-density lipoproteins (HDL) and is involved in the removal of lipoproteins from the circulation for the excretion in the liver. APOE and APOE isoforms functions may extend beyond lipid metabolism to include maintenance of normal brain function [53]. The protein contains two major structural domains, including a compact stable globular amino-terminal domain (amino acid residues 20-166) and a less-stable carboxy-terminal domain (amino acid residues 225-299). These domains are connected by a hinge region (amino acid residues 166-224) [54]. The LDL receptor-binding region is between residues 136-150 of the protein, where multiple basic amino acids are present [55]. The major lipid-binding region is contained in the carboxy-terminal domain [56]. The amino acid residues 245-266 are critical for binding to VLDL particles, whereas binding to HDL occurs even without the carboxyl-terminal domain [57].

Several individual Single Nucleotide Polymorphisms (SNPs) have been identified in the human *APOE* gene. The protein structure depends on the genetic polymorphisms, in particular two SNPs, rs7412 (C/T) and rs429358 (C/T), are responsible for the three major alleles: epsilon-2 (ε2),epsilon-3

(ε3) and epsilon-4 (ε4). Thus, because of the two copies of each gene present in human cells, there are six *APOE* genotypes: ε2/ε2, ε2/ε3, ε2/ε4, ε3/ε3, ε3/ε4, and ε4/ε4. They are responsible for three homozygous (ε2/ε2, ε3/ε3, and ε4/ε4) and three heterozygous (ε2/ε3, ε2/ε4, and ε3/ε4) genotypes [58]. The three APOE protein isoforms differ from each other by two cysteine/arginine interchanges at position 112 and 158. *APOE2, APOE3*, and *APOE4* contain cysteine/cysteine, cysteine/arginine, and arginine/arginine at these two positions, respectively [59].

APOE plays a role in the transport and metabolism of triglyceride-cholesterol. Genotyping could be used to improve the diagnosis of triglyceride cholesterol variants, and *APOE* polymorphisms were also associated with altered odds of having AD and other diseases. Determination of APOE level is of potential interest when studying different forms of brain damage and as a marker of ongoing regenerative processes in the brain.

APOE isoform-specific effects on APOE/Aβ complex levels may mediate the increase in soluble Aβ levels that correlate with *APOE4*. Allelic variations in *APOE* were consistently associated with plasma concentrations of total cholesterol, LDL cholesterol, and APOB (the major protein of LDL, VLDL, and chylomicrons). *APOE2* was studied in disorders associated with elevated cholesterol levels or lipid derangements such as type III HLP, coronary heart disease, stroke, peripheral artery disease, and diabetes mellitus [60]. But the ε2 allele is also associated with a lower risk of AD-related neurodegeneration [61], and with increased longevity in general [62]. *APOE4* is a major genetic risk factor for neurodegenerative diseases such as AD and PD [63-65].

1.2.1.1 **APOE and Alzheimer's Disease**

Understanding structural differences in APOE isoforms helped establish the molecular mechanism responsible for the associated pathology. Defects in APOE could result in alterations in its structure and function [66]. The critical effect of APOE in regulating plasma lipid and lipoprotein levels has been extensively and carefully studied. In particular, focusing on the APOE role in transporting cholesterol in and out of the central nervous system (CNS). The distribution of *APOE*'s major alleles, ε2, ε3, and ε4, in AD patients is 3.9%, 59.4%, and 36.7%, respectively [65]. Based on the strong association between APOE and Aβ in the brain [9], APOE was suggested as an Aβ-binding protein that induces a pathological β sheet conformational change in Aβ [10]. *APOE4* suggests probably increases the risk of AD by initiating and accelerating Aβ accumulation, aggregation, and deposition in the brain. Genome-wide association studies have shown that the *APOE4* allele is associated with AD [67,68] and was detected in homogeneous and heterogeneous populations in North America, Europe, and Asia.

It is estimated that the ε4 allele is the principal genetic factor in up to 50% of all cases of AD [69]. Moreover, risk for AD was increased two- to threefold in individuals who carried the heterozygous ε4 allele and about 12-fold in those who carried the homozygous ε4 allele [69]. Instead, there was evidence suggesting that *APOE2* allele may be protective against AD or associated with a marked reduction in AD risk [61]. AD risk in *APOE* ε2/ε2 or ε2/ε3 carriers was lower than in those carrying ε3/ε3 [70]. The APOE's role in AD is well established. Therefore, further studies are needed to understand the possible association between *APOE* and the rate of disease progression. *APOE* was classified as a risk factor for AD, but the molecular events that precede dementia remain elusive. *APOE4* was suggested to be associated with damage of the vascular system in the brain, leading to AD pathogenesis.

1.2.2 Synaptosomal-Associated Protein of 25 kDa

The Synaptosomal-Associated Protein of 25 kDa (*SNAP-25*) gene is located on chromosome 20p12-p11.2. It codes for a 206 amino acid presynaptic plasma membrane protein that is fundamental component of the SNARE (soluble N-ethylmaleimide-sensitive factor activating protein receptor) complex responsible for vesicle fusion and the regulation of synaptic vesicle exocytosis that are critical points in neurotransmission. It is a membrane bound protein anchored to the membranes of neurons via palmitoyl side chains located in the central region of the molecule, and together with syntaxin and the synaptic vesicle protein VAMP/synaptobrevin constitutes the initial SNARE docking complex for regulated exocytosis [71]. Besides its well characterized role in regulating exocytosis, there is increasing evidence that SNAP-25 interacts with a variety of other proteins involved in diverse functions; in particular it modulates various voltage gated ion channels [72] inhibiting their function and reducing responsiveness to depolarization [73,74].

Several studies demonstrate an association between *SNAP-25* gene polymorphism as well as altered expression of the protein with personality disorders, schizophrenia, and attention deficit and hyperactivity disorder; these studies reported that the *SNAP-25* gene might influence development of these disorders. 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 [75] as well as in cerebrospinal fluid (CSF) [76]. 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 [77]. Higher levels of this protein have also been involved in attention deficit hyperactivity disorder [78], a condition characterized by hyperactive behavior and impaired attentive ability resulting in social dysfunction [79], and neuroticism [80]. 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) [81,82], whereas a third SNP (rs363043) correlates with hyperactive behavior in autistic children [83]. Notably, the *SNAP-25* gene lies in an area linked to intelligence (20p12–p11.2) [84], and a family-based genetic association was reported between variation in intelligence quotient (IQ) phenotypes and two intronic variants on the *SNAP-25* gene. This study shows 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 [85]. *SNAP-25* gene polymorphisms are so suggested to associate with both variations in IQ phenotypes and a number of neurologic conditions, including the age related decline of cognitive function [86].

More recent data demonstrated that SNAP-25 expression negatively correlates with MMSE scores, as higher CSF concentrations of SNAP-25 were seen in patients suffering from more severe cognitive decline [87]. Notably: 1) an excess of SNAP-25 activity during adulthood was shown to be enough to mediate significant deficits in the memory formation process. 2) Expression of SNAP-25 in the adult dorsal hippocampus was demonstrated to result in the dysregulation of memory consolidation machinery in this brain region [88] and 3) over expression of SNAP-25 in cultured hippocampal neurons associates with impaired synaptic transmission [89]. Altogether, these results suggest that increased SNAP-25 levels do impair synaptic maturation and/or neurotransmission.

1.3 AIM

The principal aim of this study is to verify a possible involvement of *SNAP-25* in Alzheimer's disease 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 risk of AD development in elderly people and then replicated in MCI population.

AIM 2: Correlation of *SNAP-25* SNPs with cognitive decline: we correlated *SNAP-25* polymorphisms 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 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.

AIM 3: Correlation of *SNAP-25* SNPs and fMRI: Recently, evidences from neuropsychological [90,91] and functional magnetic resonance imaging (fMRI) studies [92-94] showed that language deficits, especially those interesting verbal fluency functioning, are precursors of AD clinical condition. Possible associations between *SNAP-25* SNPs and fMRI parameters were analyzed in AD patients.

AIM 4: Correlation of *SNAP-25* SNPs and *APOE* with the rehabilitative outcome: As an effective pharmacological treatment for AD is still missing, the potential usefulness of non-pharmacological interventions in both preventing and slowing cognitive decline in AD [95] is actively investigated. Because genetic patterns have convincingly been shown to modulate the clinical severity of AD, we investigated whether any correlations could be detected between *APOE4* positive/negative (*APOE4+/-*) and *SNAP-25* polymorphisms, and the outcome of rehabilitative treatment, in particular the MST therapy, in patients with mild-to-moderate AD.

CHAPTER II

2. MATERIAL AND METHODS

2.1 Subjects

A total of 1680 Italians of Caucasian origin were enrolled; the study was designed as a case control comparing 607 patients, with diagnosis of probable or possible AD and 615 healthy controls (HC1). A replication study was conducted enrolling 148 patients with amnestic mild cognitive impairment (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 [96] and DSM IV-R [97]. 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 [98] 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) [99] 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) [100]; 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) [101,102].

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 [103,104]; their cognitive status was assessed by Mini-Mental State Examination (MMSE) and mean raw data scores were reported in Table 1. All the individuals enrolled in the study, and their relatives when appropriate, provided written informed consent according to a protocol approved by a local ethics committee of the Don C. Gnocchi Foundation before admission to the study.

2.2 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 [105], language functions tests (phonological and categorical fluency [106] and Token tests [107]), short-term memory tests (Corsi, Digit Span Forward and Backward tests [108]), long term memory tests (Rey's Figure Delayed Recall [108]; Paired-Associate Learning test, and Story Recall test [106]) and frontal-executive functions (Raven Coloured Progressive Matrices [109]), visuo-spatial abilities (Rey's Figure Copy [108]). Categorical fluency impairment was also evaluated with fMRI verbal fluency task (the paced overt version

of verbal fluency paradigm described by Basho and colleagues [110], 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.

2.3 Rehabilitation treatment

2.3.1 Subjects

A subgroup of fifty-eight patients (32 females /26 males, mean age 75.8 years; SD=5.4 years) was enrolled, who underwent MST at the Neurology Departments of the Don Gnocchi Foundation. All patients had a clinical diagnosis of probable or possible AD with associated mild cerebrovascular disease in mild to moderate stage of progression according to the NINCDS-ADRDA Work Group criteria [96]. Every patient was examined twice: at baseline and at the end of the 10 weeks treatment.

2.3.2 Rehabilitative methods

The MST program involves three different actors: persons with Alzheimer (PWA), the dyade PWA-caregiver and the caregiver him/herself.

The MST program [50,111], involved 3 levels of treatment:

Level 1: The PWA performed 30 rehabilitation sessions (2.5 hours a day, 3 days a week, for a total of 10 weeks) in a room with materials necessary to carry out recreational–occupational activities of daily life. MST was administered by a psychologist and a rehabilitation therapist, both specialized in cognitive rehabilitation. Strong and tight interaction between participants and therapists was a crucial feature of the program. The treatment involved 4 steps: (*a*) Reality Orientation activities and cognitive exercises (about 45-minute); (*b*) physical activity (about 30-minute); (*c*)

occupational activities of daily living (about 30-minute); (*d*) recreational activities (about 45- minute).

Level 2: All caregivers of PWA had a single support interview with a psychologist at the beginning and at the end of the training. In these moments, family caregivers could freely express their psychological sufferance and their practical difficulties. Caregivers also followed a standardized short group educational program with a rehabilitation therapist designed to focusing on several points: AD clinical picture, pathogenetic mechanism, pharmacological therapy and recent advances in research, coping with behavioral problems, as well as legal and social aspects. The second level was offered (*a*) to collect data about past preferences and personality of the PWA in order to integrate this information into the rehabilitation program; (*b*) to offer psychological support to the caregiver; and (*c*) to promote the detection of practical coping solutions. Moreover, during psychoeducational meetings, caregivers were trained by the therapist in order to continue the treatment at home.

Level 3: All PWA subjects performed further stimulation at home: aerobic physical activity and specific but simple cognitive activities every day. This level was introduced to improve in the amount and intensity of the MST treatment and to favor a positive PWA–caregiver interaction at home.

2.3.3 Cognitive, Functional and Behavioral evaluation

All the 58 patients treated with rehabilitative therapy underwent cognitive, functional and behavioral evaluation status by an experienced neuropsychologist blinded to the treatment status who used psychometric tests and scales. MMSE was used as an index of change in cognitive functions, and, for the purpose of this work, MMSE raw scores were adjusted for age and educational level (conversion formulae are reported in [105]); only corrected scores were counted for correlation analysis. The Functional Living Skills Assessment - FLSA- [112] was used as a measure of functional-performance; FLSA assesses performance in tasks of everyday life through direct observation of role playing. Finally, behavioral disturbances were assessed with the Neuropsychiatric Inventory scale - NPI - [113].

2.4 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 [81].

2.5 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.

2.6 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 = 3.9×3.9×3mm3, 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 = 1×1×1mm3, 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) [114]. 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.

2.7 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 [114,115].

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 biallelic 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 [116] 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 8mm full-with at half-maximum (FWHM) Gaussian kernel. Single subjects statistical analysis was then performed with general linear model (GLM) approach [117] 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, pFWE-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%).

The Kolmogorov-Smirnov (K-S) test was applied also to verify normal distribution of numerical variable scores resulted by the group of 58 patients whom underwent rehabilitative MST therapy. All numerical scores, resulted normally distributed, and they were shown as mean and standard deviation (SD) and linear regression analysis was performed between

mean delta score of MMSE, NPI, FLSA evaluations after rehabilitative treatment and age, age of disease onset, disease duration, level of education and mean baseline score of each scale. ANOVA calculation was performed to analyse gender association with MMSE, NPI and FLSA delta scores. A multivariate logistic forward stepwise regression model was computed using *APOE4+* vs. *APOE4-* as response variables and difference between scores evaluated after rehabilitative treatment and at baseline (∆) of MMSE, NPI and FLSA scales as explanatory variables. Moreover, this model was corrected by MMSE at baseline (MMSEb), NPIb and FLSAb values, age of patients at the baseline, their level of education and gender Similarly a multinomial, multivariate logistic regression analysis was performed evaluating each *SNAP-25* polymorphic genotype as responsible variable and ∆MMSE, ∆NPI and ∆FLSA as explanatory variables, together with MMSEb, NPIb and FLSAb values, age of patients at the baseline, their level of education, gender and *APOE4+* genotype which were considered as covariates. Haplotype association analysis was performed using plink software [116] by logistic regression analysis; haplotype probabilities of individual subjects were incorporated as covariates in the regression model, which estimate the Beta value associated with ∆MMSE, ∆NPI and ∆FLSA.

CHAPTER III

3. RESULTS

3.1 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 (pc = 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 (pc = 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, indicating that the number of the study population is sufficient for a case-control study. 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 evidenced a linkage disequilibrium between rs363050 and rs363043 and between rs3746544 and rs1051312 (Figure 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.

3.2 SNAP-25 polymorphisms and neuropsychological evaluation

Because the results above suggest an association between *SNAP-25* genotypes and both AD and aMCI 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 (≤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 ≤25 or >25 were adopted as the response variable; *SNAP-25* genotypes, *APOE4+/APOE4-* 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 fluency 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).

3.3 SNAP-25 and functional MRI

A group of AD patients and HC1 underwent fMRI evaluation to verify the possible correlations between *SNAP-25* genotypes and imaging patterns. We analyzed 10 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). 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. As expected, the 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 (Figure 2A, B). Raising the statistical threshold 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 ($p_{FWE-corr} <0.05$ 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 (Figure 2C); in the opposite comparison (group 1 > group 0) increased activation was not detected in any of the brain regions involved in the task.

3.4 Rehabilitation results

Neuropsychological evaluations were performed at baseline and at the end of the 10 weeks treatment. Differences between the results obtained at 10 weeks and at baseline are reported as Delta scores (ΔMMSE, ΔNPI, ΔFLSA). Improvement are represented by ΔMMSE and ΔFLSA>0, whereas a behavioural improvement is described by ΔNPI <0. Results of correlation analyses between ΔMMSE, ΔNPI and ΔFLSA values and demographic factors, calculated by linear regression analysis considering the baseline value of each scale as covariate, are presented in Table 7.

A significant association between ΔMMSE values and gender (female *vs.* male) (negative Beta value of association to females: p_c=0.02 Beta=-0.33) was found together with a borderline significant positive correlation between MMSE values and age at baseline (p_c=0.05; Beta= +0.25).

Results also showed that NPI outcomes were negatively associated with the level of education (p_c=0.02; Beta:-0.31). In contrast, no correlations were found between cognitive and behavioral outcomes after treatment, and disease duration, age at baseline and age at onset.

3.4.1 APOE genotypes

A total of 27 out of the 58 patients carried the *APOE4* allele, (*APOE4+*): 4 were homozygous *E4+/E4+* and 23 were heterozygous *E4+/E3+.* Among *APOE4-* subjects: 4 carried *E2+/E3+* genotype and 27 were *E3+/E3+.*

Binomial logistic regression analysis was performed to calculate possible association between *APOE* polymorphisms and ΔMMSE, ΔNPI and ΔFLSA scores. *APOE4+* and *APOE4-* allelic association with Δvalues for each scale was calculated after correction for baseline values, age, level of education and gender, which were considered as covariates. Results indicated the presence of a statistical association between *APOE* polymorphism and ΔMMSE scores after treatment (p_c=0.041).Therefore, a weak positive effect of the *APOE4-* allele (OR: 1.4; 95%CI:1.0-1.9) on the outcome of MST was observed (ΔMMSE= 2.0±1.9) whereas the *APOE4+* allele was correlated with lower MMSE scores after therapy (ΔMMSE= 0.5±2.5). Graphical results are shown in Figure 3. No associations were observed between *APOE* polymorphisms and either ΔNPI or ΔFLSA results.

3.4.2 SNAP-25 genotypes

Frequency of distribution of the three analyzed single nucleotide polymorphisms (SNPs) of the *SNAP-25* gene (rs363043(C/T), rs363039(G/A), rs363050(A/G)), was evaluated next by multinomial logistic regression analysis in relationship with ΔMMSE, ΔNPI and ΔFLSA scores. *SNAP-25* genotypes categories for each SNP were considered as the dependent variable, and possible associations with the Δ values were calculated after correction for the baseline value, age, level of education, gender and *APOE* genotype (*E4-* vs. *E4+*), which were considered as covariates.

A statistically significant association was observed between ΔNPI values and the rs363050 and rs363039 *SNAP-25* genotypes (Figure 4 panel B,

Figure 5 panel E). In particular, subjects carrying the rs363050(G) as well as the rs363039(A) alleles were characterized by lower ΔNPI scores, which correspond to an improvement in behavioural functions after rehabilitative treatment. Notably, a significantly better outcome after treatment was observed in individuals carrying the minor rs363050 homozygote (GG)(ΔNPI = -2.4 ± 3.1) compared to those with the major homozygote (AA) genotype (ΔNPI = -0.1 ± 3.0)(p_c=0.015: OR: 6.6, 95%CI: 1.4-30.6), and in those with the heterozygote (AG)(ΔNPI = -2.7 ± 2.9) compared to those with the homozygote (AA)(ΔNPI = -0.7 ± 0.9) genotype (p_c=0.007, OR: 1.83, 95%CI:1.2-2.8)(Figure 4 panel B). A similar significantly better outcome was observed in subjects carrying the rs363039 minor homozygote (AA)(ΔNPI = -2.9 ± 3.1) compared to those with the major homozygote (GG) genotype (ΔNPI = -0.5 ± 3.2)(p_c= 0.019, OR: 10.8 95%CI: 1.5-78.2) and in (AA) homozygous (ΔNPI = -2.7 ± 2.2) compared to (AG) heterozygous individuals (ΔNPI = -1.5 ± 2.9) (p_c=0.025; OR: 9.6, 95%CI: 1.3-69.8)(Figure 5 panel E).

3.4.3 Haplotype analysis

The detection of a linkage disequilibrium between rs363050, rs363039 and rs363043 SNPs in intron 1 (figure 1) suggested us to further analyze possible associations between this haplotype and MST outcomes. Results showed the presence of a significant association between *SNAP-25* haplotypes and lower ΔNPI. In particular the rs363050(G)-rs363039(A)-rs363043(C)-(GAC) haplotype was statistically associated with a better outcome of treatment as measured by the NPI scale (p=0.03, Beta value= -1.27)(Table 8).

CHAPTER IV

4. 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 [86]. 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 [85]. 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 [90,91], 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 [118,119]. 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 [90,91]. 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 [110] 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 significantly 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 [120,121]. Recent studies have shown a relationship between reduced neural metabolism and poor performances in semantic memory tasks [94,122,123]. Our results are in line with these works, and especially with those focusing on the left temporo-parietal and left prefrontal cortex [121,122]. 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 [124]. 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 [125] 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 [126]. 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 [127]. 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 [125] that show a decrease in neuroplasticity of temporal lobes with the progression of the disease. Categorical verbal fluency tests assess medial temporal lobe function [92] and are positively correlated with neurobiological hippocampal and parietal lobe neurochemical abnormalities in AD [128]. The *SNAP-25* gene is highly expressed in the hippocampus [129], a brain structure that plays a crucial role in semantic fluency performance [130]. Animal studies showed that the hippocampal SNAP-25 protein is involved in memory consolidation and long-term memory formation in rats [131,132]; additional results indicate that changes of hippocampal *SNAP-25* expression contribute to age-related decline of cognitive function [86]. 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 could affect transcription factor binding sites

[85]. 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 rs363050 (A) allele associates with a significantly higher *SNAP-25* expression compared to the rs363050 (G) allele [133]. 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 [81,134-137]. An excess of SNAP-25 activity during adulthood was shown to be sufficient to mediate significant deficits in the memory formation process.

As previously described, AD is a highly prevalent neurodegenerative disorder with a complex and unclear pathogenesis; pharmacological therapies for AD are not yet available, and the efficacy of physical and occupational treatments is greatly variable amongst patients. As widely demonstrated, genetic factors play a role in the pathogenesis of AD. Thus, besides the presence of the *E4* allele of *APOE* gene*,* the only known confirmed genetic risk factor for sporadic AD, SNPs in various genes, including *interleukin-10*, *PIN-1* [138,139] and *SNAP-25*, have been shown to correlate with susceptibility to disease and to the rate of clinical progression. A further step of our research was to explore whether the outcome of multidimensional stimulation, including CS, in AD could be associated as well with the genetic background of the patient, focusing on *APOE4* and *SNAP-25* polymorphisms. Data herein suggest that this is indeed the case as significant associations were detected between such polymorphisms and the effect of rehabilitation.

Higher MMSE scores after rehabilitation were detected in *APOE4-* compared to *APOE4+* patients, though at a low level of significance and *SNAP-25* alleles and genotypes correlated with a significantly better outcome as measured by the NPI scale. These data are in agreement with recently published evidences indicating a poorer efficacy of Cognitive Stimulation on visuospatial memory performance measured using the Corsi test. [140] in *APOE4+* patients. The *APOE4* status has been reported to influence cognitive functions [141], possibly because *APOE* gene produces a protein that plays an important role in neuronal plasticity and repair [142-144]. Thus, the APOE lipoprotein stimulates synapse development [145], clearance of lipid debris [146], and promotion of granule cell mossy fiber sprouting [147]. The effectiveness of these processes is reduced when the *E4+* allele directs *APO*E production. Data in the animal model reinforce the idea that *APOE* alleles are involved in AD. Thus, a more efficient degradation of *APOE4* in astrocytes in transgenic animals results in lower levels of APOE in the brain and CSF, suggesting that the reduced levels of total APOE seen in *APOE4* carriers may directly contribute to disease progression. *APOE4+* is also less efficient than *APOE4-* in transporting brain cholesterol [65]. Other data show that *APOE* polymorphisms modify neuroinflammatory responses, and influence both outcome from acute brain injury and the risk of developing neurodegenerative disease. Traumatic brain injury (TBI) accelerates neurodegenerative pathology in double-transgenic mice expressing the human *APOE* alleles and mutated amyloid precursor protein: this pathology is exacerbated in the presence of the *APOE4* allele and the administration of an APOE-mimetic peptide markedly reduces the development of neurodegenerative pathology in these animals [142]. Very recently an increase of amyloid accumulation and allele frequency of *APOE4* was seen in the mild TBI patients with cognitive impairment [148,149], and a meta-analysis concluded that the *APOE4* allele may be associated with a poor prognosis in severe TBI patients [150].

These evidences suggest an *APOE4* cognitive phenotype associated with more inefficient mechanisms of neuronal repair and plasticity. It is therefore possible that people with AD carrying *APOE4* are less able to engage cognitive reserve mechanisms that could benefit from CS, with the result that carriers and non-carriers benefit differently from experiences that enhance cognition. A low level of significance was revealed in our evaluation of *APOE4* involvement in MST outcome. This may be considered as a point of criticism, but it is nevertheless important because we cannot exclude *APOE4* as covariate when involvement of other genetic marker as *SNAP*-25 has been evaluated in MST outcome.

Polymorphisms in the *SNAP-25* gene were instead mostly correlated with results at MST when behavioural improvement was measured. Thus, the *SNAP-25* rs363050(G) and rs363039(A) alleles, as well as the *SNAP-25* rs363050(G)-rs363039(A)-rs363043(C) (GAC) haplotype, which are in linkage disequilibrium, were statistically associated with lower NPI scores after treatment, i.e. with a more relevant effect of this treatment of behavioural parameters. As we have suggested in the first part of the research, a role for *SNAP-25* polymorphism in the risk of developing AD in individuals with a diagnosis of mild cognitive impairment (MCI), in particular, we have shown that the complementary rs363050(A) allele is significantly more frequent in patients with AD and MCI and is associated with pathological scores of categorical fluency and functional MRI parameters in individuals with a diagnosis of full-blown AD [151].

Previous studies suggested that synaptic loss correlates with the clinical manifestations of AD, while there is no relation between the number of accumulated parenchymal amyloid plaques and synaptic pathology [152,153]. More recent data demonstrated that *SNAP-25* expression negatively correlates with MMSE scores, as higher CSF concentrations of SNAP-25 were seen in patients suffering from more severe cognitive decline [87]. Notably: 1) an excess of SNAP-25 activity during adulthood

was shown to be enough to mediate significant deficits in the memory formation process. 2) Expression of *SNAP-25* in the adult dorsal hippocampus was demonstrated to result in the dysregulation of memory consolidation machinery in this brain region [88] and 3) over expression of SNAP-25 in cultured hippocampal neurons associates with impaired synaptic transmission [89]. Altogether, these results suggest that increased SNAP-25 levels do impair synaptic maturation and/or neurotransmission. These data thus suggest that, in AD patients, the lower success of rehabilitation seen in the presence of the rs363050(A) allele, or of a *SNAP-25* pattern in linkage disequilibrium with this allele, is the result of the higher SNAP-25 levels present in these patients.

It has been assumed that AD progression and functional restoration depend on the capacity for neural plasticity within residual neural tissues; such plasticity was recently suggested to be at least in part influenced by polymorphisms in several genes known to orchestrate this process [154]. Both APOE and SNAP-25 proteins play important roles in neuronal plasticity and repair; data herein suggest that SNPs in these genes are correlated with the outcome of non-pharmacological treatment in AD.

The promise of genomic medicine is to realize and to deliver a personalized approach to pharmacological and non-pharmacological treatment of diseases that is targeted to individual genetic pattern. Analysis of the genetic background of persons with AD could become useful in guiding the clinicians to define which rehabilitative intervention might better fit each patient, for example defining a more intensive or different kind of therapy in those subjects with a lower opportunity of success. The data presented here could, possibly in the near future, help in the optimization of the clinical management of individuals with neurological conditions.

CONCLUSION

Results herein suggest that *SNAP-25* gene polymorphisms (rs363050(A) and the rs363043(T) alleles as well as rs363050/rs363043 A-T haplotype) may be considered genetic risk factor for the development of AD and aMCI in Italian elderly people. The *SNAP-25* rs363050(AA/AG) and rs363043(TT/CT) genotypes are associated with altered scores at the categorical fluency test. Correlations between *SNAP-25* rs363050 (AA/AG) and/or rs363043 (CT/TT) genotypes and imaging patterns shown a significantly decreased brain activity in the cingulate cortex and in the frontal (middle and superior gyri) and the temporo- parietal (angular gyrus) area, as measured by fMRI.

Association between *APOE4* and *SNAP-25* with rehabilitation outcome was revealed. Higher MMSE scores after MST were detected in *APOE4*- compared to *APOE4*+ patients, as well as *SNAP-25* rs363050(G)(GG) allele and genotypes correlated with a significantly better outcome as measured by the NPI scale.

These data thus suggest a lower success of rehabilitation in AD patients carrying the rs363050(A) and *APOE4*+ alleles, maybe due to the lower neuroplasticity associated to these genotypes.

The data presented provide important indication of possible biomarkers for the prognosis and course of AD, and could provide a useful indication for the definition of rehabilitative interventions targeted to the patient.

The goal of genomic medicine is to realize and to deliver a personalized approach to pharmacological and non-pharmacological treatment of diseases that is targeted to individual genetic pattern.

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FIGURES AND TABLES

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

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

	AD (N=607)	HC1 (N= 615)	aMCI (N=148)	HC2 (N= 310)
Parameters				
Age (years) [Mean ±SD]	76.7**±8.2**	72.0**±6.9**	76.8**±7.8**	69.9**±6.3**
Range (years)	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 (years)[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]

Table 2a: Genotype and allele distribution of the rs363050 and rs363043 *SNAP-25* SNPs in Alzheimer's disease (AD) patients and in age- and sex-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.

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

SNAP-25		AD Females (N=411) %	AD Males+ Females (N=607) %	HC1 Females (N=420) %	HC1 Males+ Females (N=615) %	p value	OR; 95%CI
rs363050							
Genotypes	A/A	46[2]	44[1]	34[2]	35[1]	[1]p_c= 0.002 [2]p_c= 0.001	1.47; 1.16-1.86 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		
Alleles	A	68[5]	65[4]	59[5]	60[4]	[1]pc=0.003 [2]pc=0.04	[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 [6]p_c=0.04	1.49; 1.02 - 2.18
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	

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 sex-matched healthy controls (HC2).OR: Odds ratio; 95%CI: Interval of confidence;. pc: p value corrected for two degree of freedom for genotype distribution. [1]pc: aMCI vs. HC2; [2]p: aMCI vs. HC2;[3]pc aMCI vs. HC2; [4]p: aMCI vs. HC2;

SNAP-25		aMCI Males+ Females (N=148) %	HC2 Males+ Females (N=310) %	p value	OR; 95%CI
rs363050					
	A/A	39	30		
Genotypes	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					
	C/C	43	49		
Genotypes	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;	

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

		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**

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.

Model 1: AD patients (N=209); covariates: *APOE4*+ (ε4/ε4 ε4/ε3 ε2/ε4), Gender (female vs. male), *SNAP-25* rs363050 (AA/AG vs. GG)
Model 2: AD patients (N=209); covariates: *APOE4*+ (ε4/ε4 ε4/ε3 ε2/ε4), Gender (female vs. male), *SNAP-25* rs363043 (TT/CT vs. CC)

		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+		**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 +		0.72		
	Gender		0.60		

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+* (ε4/ε4 ε4/ε3 ε2/ε4), Gender (female vs. male) OR: Odds ratio;

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

Table 6: Demographical, neuropsychological and behavioral task-fMRI characteristics.

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 vs AD gr0:p<0.05; [2]HC vs 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

	AD gr0 (N=9)	**AD gr1 (N=15)**	**HC (N=10)**	**Group Comparison**
Age (years)[Mean ±SD]	75.56±5.85	74.67±5.39	70.80±3.85	
Range (years)	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

Table 7: Linear regression analysis between mean delta score of MMSE, NPI and FLSA evaluations after rehabilitative treatment and demographic data of AD patients. SD: standard deviation ; p_c: p value corrected for baseline values; *: Statistically significant p value. MMSE: Mini Mental Scale Evaluation; NPI: Neuropsychiatric Inventory scale; FLSA: Functional Living Skills Assessment; MMSEb : MMSE value at baseline, NPIb : NPI value at baseline; FLSAb: FLSA value at baseline.

Demographic data	N =58	ΔMMSE		ΔNPI		ΔFLSA	
		p_c	Beta	p_c	Beta	p_c	Beta
*Gender female / male (N)	32/26	**0.02***	**-0.33**	0.60	+0.07	0.59	-0.07
*Age (mean±SD)	75,8±5.4	**0.05***	**+0.25**	0.37	-0.11	0.89	+0.02
Age at onset (mean±SD)	73,5±5,6	0.06	+0.25	0.56	-0.09	0.39	-0.12
Disease duration (mean ±SD)	2.2±2.7	0.62	+0.81	0.21	-0.22	0.06	+0.30
*Level of education (mean±SD)	8,0 ±3,5	0.76	-0.04	**0.02***	**-0.31**	0.77	+0.04
*MMSEb (mean±SD)	20.8±3.6	**0.05***	**-0.26**		-		-
*NPIb (mean±SD)	9.8±7.1		-	**0.02***	**-0.30**		-
*FLSAb (mean±SD)	99.1±16.2		-		-	**0.02***	**-0.30**

Table 8: Haplotype association between *SNAP-25* rs363050, rs363039, rs363043 and ΔNPI values. *: Statistically significant p value.

SNP1	SNP2	SNP3	haplotype	Beta	p value
			AAT	0.64	0.69
			AGT	0.78	0.24
			GAC*	**-1.27**	**0.03***
rs363050	rs363039	rs363043	AAC	0.45	0.88
			GGC	-3.98	0.08
			AGC	1.27	0.08

Figure 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.

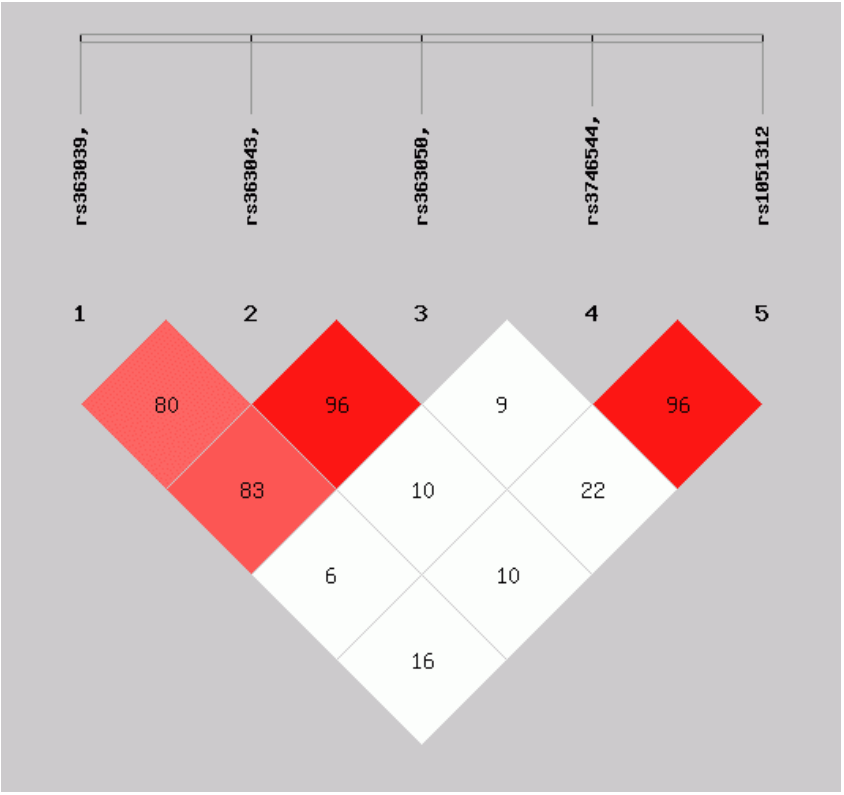


Figure 2: Main BOLD effect due to the paced overt categorical verbal fluency task: comparison among the three groups: healthy controls (HC)(10 subjects), group 0 (10 AD patients carrying the rs363050(GG) and rs363043 (CC) genotypes), and group 1 (18 AD patients carrying the rs363050 (AA or AG) and rs363043 (CT or TT) genotypes)(ANOVA). Brain areas which are significantly more activated in: HC1 *vs.* AD group 0 (panel A); HC1 *vs.* AD group 1 (panel B); and AD group 0 *vs.* 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.

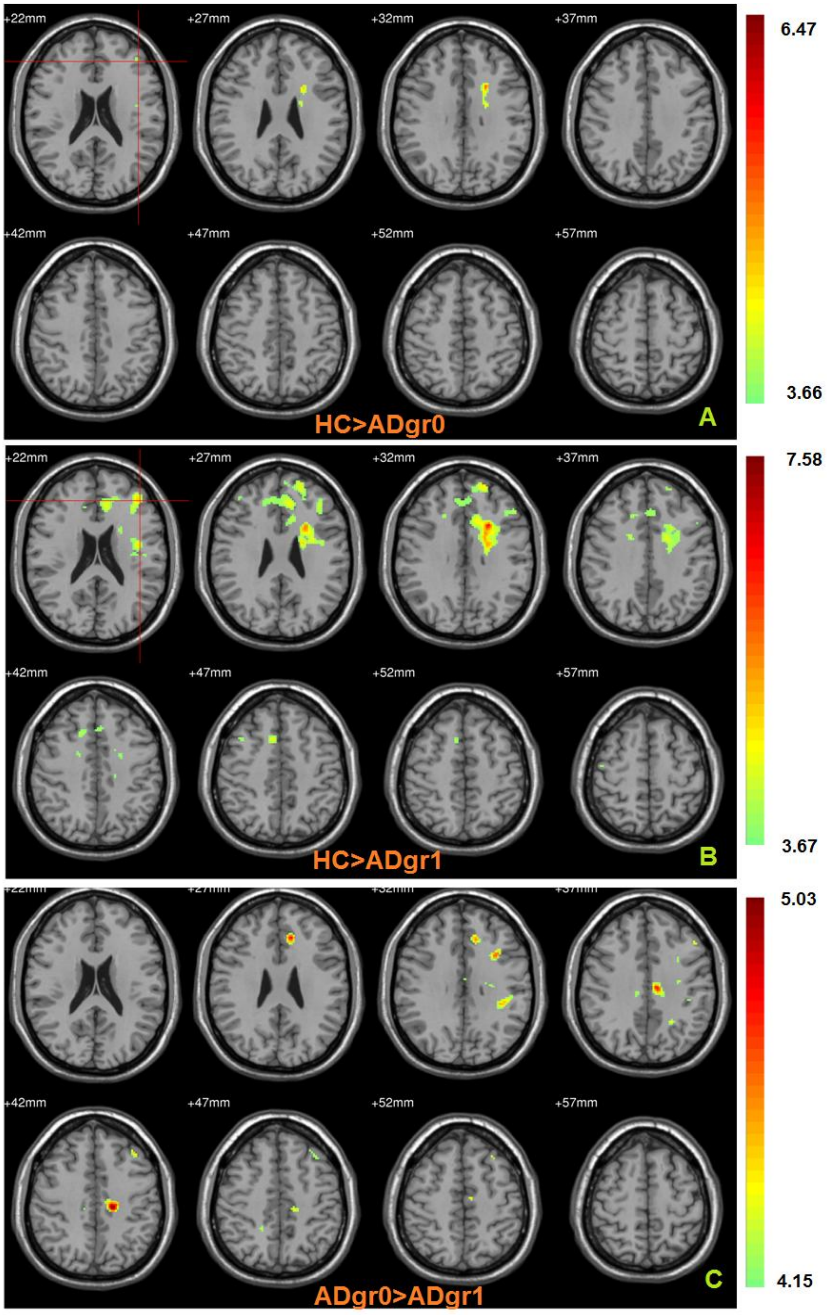

+22mm
+27mm
+32mm
+37mm
+42mm
+47mm
+52mm
+57mm
HC>ADgr0
A
6.47
3.66
HC>ADgr1
B
7.58
3.67
ADgr0>ADgr1
C
5.03
4.15

Figure 3: Binomial logistic regression analysis of *APOE* association to ΔMMSE (panel A), ΔNPI (panel B) and ΔFLSA (Panel C). *APOE4+* vs. *APOE4-* was considered as dependent variable, baseline values MMSEb, NPIb and FLSAb, age at baseline, level of education and gender were computed as covariates.

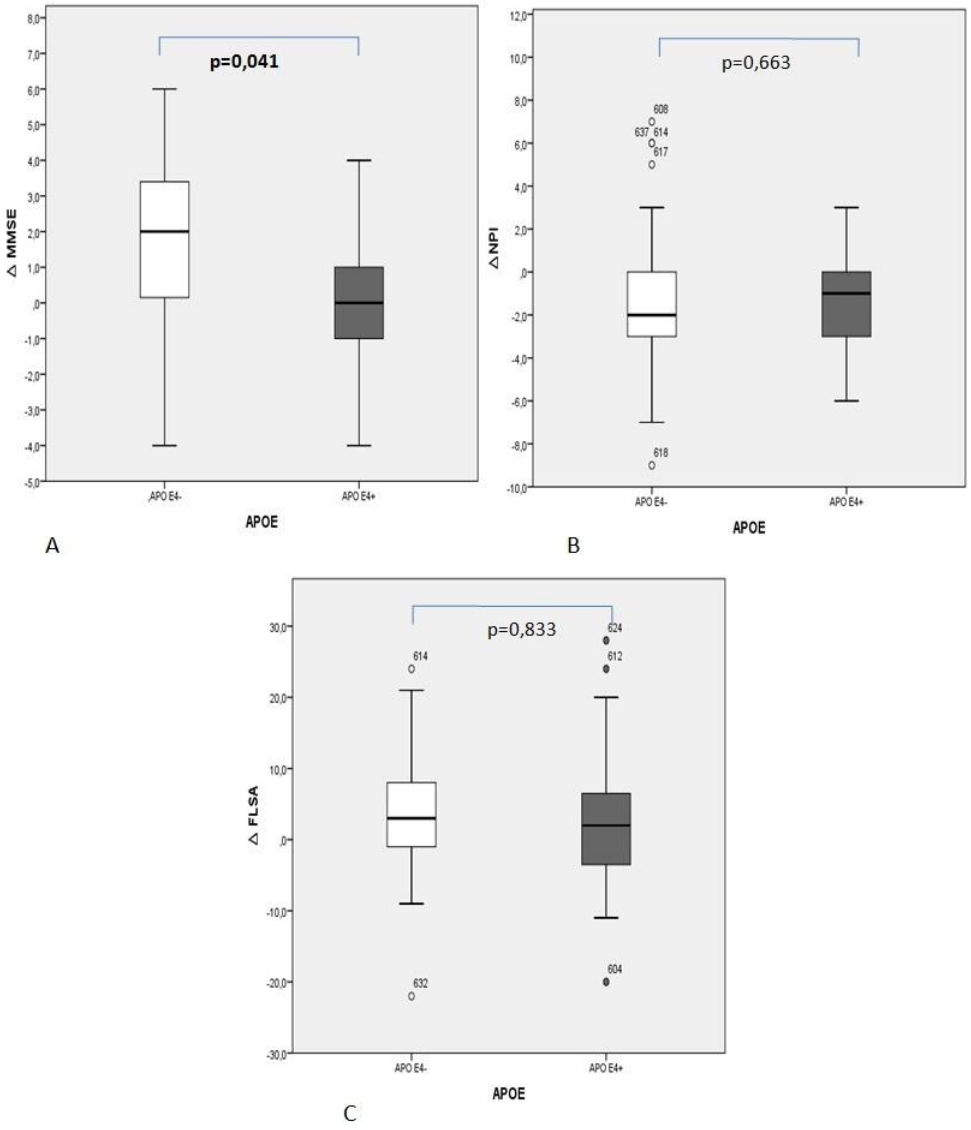


Figure 4: Box plot data were reported in figure 4 showing statistical association obtained after multinomial regression logistic analysis between delta scores variation in relationship with *SNAP-25* rs363050 genotypes and ΔMMSE score, (panel A) ΔNPI scores (panel B), ΔFLSA scores (panel C).

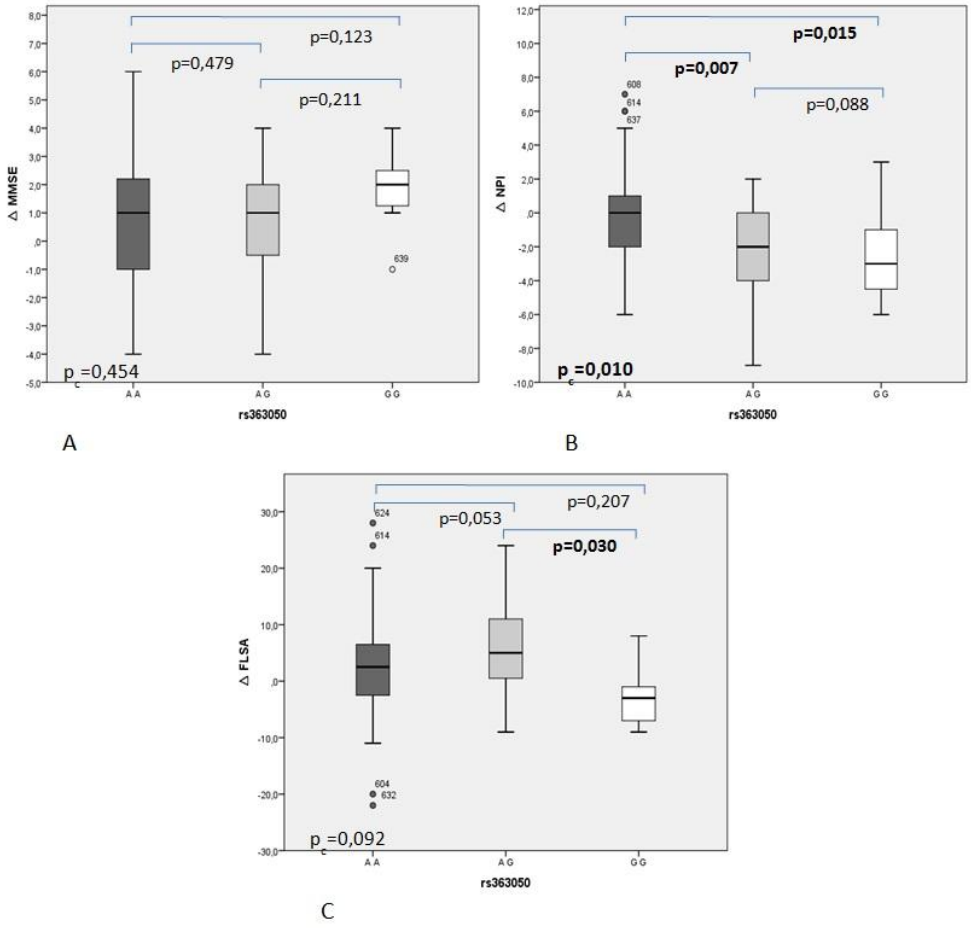
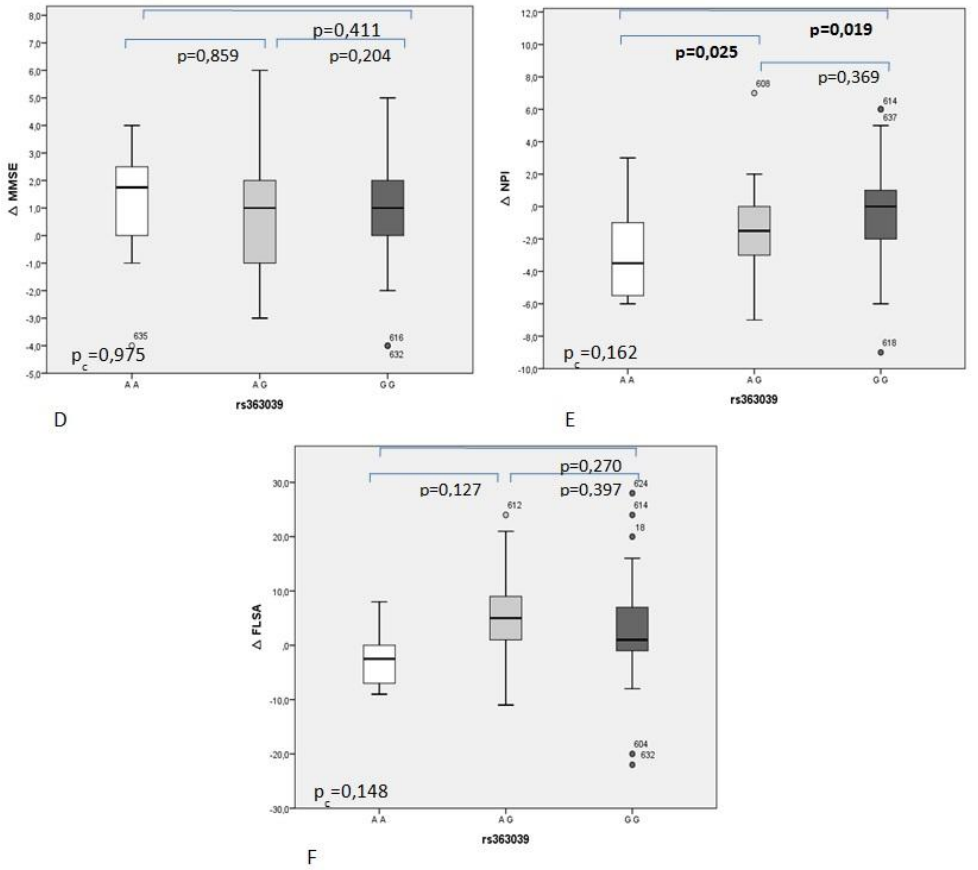


Figure 5: Box plot data were reported in figure 5 showing statistical association obtained after multinomial regression logistic analysis between delta scores variation in relationship with *SNAP-25* rs363039 genotypes and ΔMMSE score (panel D), ΔNPI scores (panel E), ΔFLSA scores (panel F).



SCIENTIFIC PRODUCTION RELATIVE TO THE PRESENT WORK:

1. **Possible association between SNAP-25 single nucleotide polymorphisms and alterations of categorical fluency and functional MRI parameters in Alzheimer's disease.** Guerini F.R., Agliardi C., Sironi M., Arosio B., Calabrese E., Zanzottera M., Bolognesi E., Ricci C., Costa A.S., Galimberti D., Griffanti L., Bianchi A., Savazzi F., Mari D., Scarpini E., Baglio F., Nemni R., Clerici M., *J Alzheimers Dis*, 2014;42(3):1015-28.

2. **Vitamin D receptor gene polymorphisms are associated with obesity and inflammosome activity.**
   Al-Daghri N.M., Guerini F.R., Al-Attas O.S., Alokail M.S., Alkharfy K.M., Draz H.M., Agliardi C., Costa A.S., Saulle I., Mohammed A.K., Biasin M., Clerici M., *PLoS One*. 2014;14;9(7):e102141.

3. **Synaptosomal Protein of 25 Kda (Snap-25) Polymorphisms Associate with Glycemic Parameters in Type 2 Diabetes Patients.** Al-Daghri N.M., Costa A.S., Alokail M.S., Zanzoterra M., Alenad A., Mohammed A.K., Clerici M., and Guerini F.R., *Journal of Diabetes Research.* 2015*. Article In Press.*

4. **APOE and SNAP-25 polymorphisms predict the outcome of MST rehabilitation in Alzheimer's disease.**
   Guerini F.R., Farina E., Costa A.S., Baglio F., Saibene F., Margaritella N., Calabrese E., Zanzottera M., Bolognesi E., Nemni R., Clerici M., *Neurorehabil Neural Repair.* 2015. *Article In Press.*