

Pharmacogenetics and Pharmagenomics, Trends in Normal and Pathologic al Aging Studies: Focus on p53

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Abstract: In spite of the fact that the aging organism is the result of complex life-long gene/environment interactions, making peculiar the susceptibility to diseases and the response to drugs, pharmacogenetics studies are largely neglected in the aged. Altered response to drugs, cardiovascular and metabolic alterations, cancer and dementia are among the age associated ailments. The latter two are the major contributors to illness burden for the aged. Aging, dementia and cancer share a critical set of altered cellular functions in the response to DNA damage, genotoxic stress, and other insults. Aging in higher animals may be influenced by the balance of cell survival versus death, a decision often governed by checkpoint proteins in dividing cells. The paper is mainly focused on one of such proteins, p53 which has been recently shown to be involved in aging and Alzheimer's Disease (AD). Within this reference frame we studied p53 in aged controls and demented patients finding that with aging there is an increase of mutant like conformation state of p53 in peripheral blood cells, which is more pronounced in AD patients. As a result of such conformational change, p53 partially loses its activity and may become unable to properly activate an apoptotic program when cells are exposed to a noxious stimulus. Moreover we found that the tertiary structure of p53 and the sensitivity to p53-dependent apoptosis are affected by low concentrations of soluble beta amyloid, the peptide that accumulates in AD brain but also present in peripheral tissues. It is possible that p53 conformers may occur in the presence of misfolded molecules such as, but not limited to, beta amyloid. In particular at neuronal level the altered function of cell cycle proteins may affect synaptic plasticity rather than cell duplication.

Key Words: pharmacogenetics, pharmacogenomics, aging, Alzheimer's disease, beta-amyloid, conformationally altered p53, biomarker.

PHARMACOGENETICS AND PHARMACOGENOMICS: OPPORTUNITIES IN AGING STUDIES

The terms pharmacogenomics and pharmacogenetics tend to be used interchangeably, and a precise consensus definition of either remains elusive. However, pharmacogenetics refers to the role of genetic variation affecting drug response or adverse reactions to drugs [1]. While, pharmacogenomics better fits the definition of the science dealing with the analysis of genome (DNA) and of its products (RNA and proteins), aimed to correlate genomic information to cell or tissue response in order to find new targets for therapy, to develop new drugs and to study the response to them (see Drug Information Association website at www.diahome.org).

The aging organism is the result of complex life-long gene/environment interactions, making peculiar the susceptibility to diseases and the response to drugs. Nowadays, altered response to drugs, cardiovascular and metabolic alterations, cancer and dementia are among the age associated

ailments. In this context, the shift from broad treatment strategies to more individually and genetically selected approaches would ensure that therapies would be both safer and more effective. In fact with an increased understanding about how genes and drugs interact, many patients could undergo a genetic test to predict their response and help ensure the medicine and dose is right at the first time. However, pharmacogenetic studies in aged patients are largely neglected, even if in elderly people pharmacokinetic and/or pharmacodynamic changes occur compared to younger people, increasing the variability of the response. Aging is characterized by a progressive loss of functional capacities of most if not all organs, a reduction in homeostatic mechanisms and a response to receptor stimulation. Also, loss of water content and an increase of fat content in the body are reported. These changes have to be taken into consideration when the prescription of particular (cardiovascular or neuropsychiatric) drugs in elderly patients increases the risk of adverse drug reactions [2]. Pharmacodynamic changes in the elderly can result in greater, or sometimes even lesser, drug sensitivity than that seen in a younger individual. A greater drug sensitivity is particularly noticed with those drugs which act on the central nervous system, such as benzodiazepines [3]; on the other hand, β -adrenergic agents are an

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example of a reduction in responsiveness in elderly individuals [4]. Even if there is a general trend of greater pharmacodynamic sensitivity in the elderly, however, this is not universal, and these age-related changes must be investigated agent-by-agent. Thus, inter-individual differences in drug response and adverse effects may be caused by variability in drug metabolism due to genetic polymorphisms, induction or inhibition of the metabolism by concomitant drug intake, environmental or physiological factors or pathological conditions. As an example, the metabolism of donepezil, a selective acetylcholinesterase inhibitor used in the treatment of Alzheimer's disease (AD), is dependent upon the genetic polymorphisms of the gene encoding CYP2D6, which has a large number of allelic variants, causing either absent, decreased or increased enzyme activity [5-6]. Varsaldi and coworkers [7] observed a large inter-individual variability in the concentration to dose ratio of donepezil among AD patients and noted that this variability partially correlated with the CYP2D6 genotype, thus suggesting that genetic polymorphism of CYP2D6 influences the metabolism and therapeutic outcome of donepezil.

Compared to pharmacogenetics, an abundant literature on pharmacogenomic studies is available even if not yet well organized. To date, pharmacogenomic studies are mainly focusing on many questions related to aging, concerning the research for aging genes, disease causing genes, longevity genes and are also pointing to identify susceptibility genes related to specific diseases, which allow health care providers to predict more accurately the risk of an individual of developing a specific disease [8-9]. A number of laboratories are making substantial and exciting progresses in the understanding of the genetics of aging and longevity; the principal aim of this studies are that these gene discoveries will lead to the identification of drug targets-drugs that would slow down the aging process and permit people to delay and perhaps escape age-associated diseases. Interestingly, by overviewing PubMed Citations from 1950 to 2008, the choice of model organism for aging is represented by *Homo sapiens*. In fact, while we found many data about developmental biology from worms, flies, and mice, there is a paucity of detailed information on the pathophysiology of aging, particularly in worms and flies; in contrast, there is a vast literature on these and all other aspects of human biology, including remarkable progress in human genetics [10].

Aging and susceptibility to diseases associated with aging are likely to be influenced by thousands of genes [11-12]. The vast amount of available literature generated in this field needs, however, a systematic revision and confirmation studies for the frequently contrasting results. Lacking genetic variations that predispose to diseases (so called "disease genes") as well as having variations that confer disease resistance (so called "longevity-enabling genes") are probably both important to confer a remarkable survival advantage [13-14]. Conversely, defective functions of genes associated with longevity may influence premature neuronal survival [8].

About aging genes, the debate regarding the existence of genetic mutations actually able to either cause or accelerate aging is still open. Some researchers have proposed a genet-

cally set biological clock which establishes when a species begins to age. Telomere length and/or telomerase function have been selected as time keeper candidates because of their potential role in regulating the number of cell divisions [15]. However, reduction in telomerase activity with older age might actually be a defensive measure against the increased potential for age-associated cancer. Another genetic defense against cancer is the p53 gene. Tyner and colleagues have noted an increase in p53 activity as a result of a deletion mutation in the first six exons of the p53 gene associated with cancer prevention but also accelerated aging [16]. In fact, by generating transgenic mice carrying this mutation they found that mutant p53 mice exhibit an early onset of phenotypes associated with aging (reduced longevity, osteoporosis, generalized organ atrophy and a diminished stress tolerance), thus suggesting that p53 has a role in regulating organismal aging [16].

Among susceptibility genes, there are now numerous examples of genetic variations that substantially predispose to age-related disease (for example Alzheimer's disease and apolipoprotein E ϵ 4). The apolipoprotein E (APOE) gene is the most prevalent risk factor for AD, especially in those subjects harboring the APOE-4 allele, whereas carriers of the APOE-2 allele might be protected against dementia [8, 17-18]. Because these variations are also associated with increased mortality risk, it is likely that centenarians do not have many of these predisposing variations [18-20]. Within this context, Schachter and colleagues found that the frequency of the apolipoprotein ϵ 4 allele decreases markedly with advancing age, whereas, one of its counterparts, the ϵ 2 allele, becomes more frequent with advanced age among Caucasians [18].

STRATEGIES FOR PHARMACOGENOMIC STUDIES

Cardiovascular and metabolic alterations, cancer and dementia represent complex age-associated diseases with multiple gene variations. Within the context of complex diseases, two different approaches are at the base of pharmacogenomic studies: one relies on a wide-genome screening and the other is driven by a specific hypothesis.

A genome-wide screening strategy underpins on the fact that, with the completion of the human genome project, single nucleotide polymorphisms (SNP) genotyping is being undertaken in a large number of pharmacogenomic studies to identify variants associated with responses to specific drugs. Throughout the human genome, several types of genetic polymorphisms can be found, but among these, SNPs are the most common polymorphisms and the attention of researchers has been focused on these in the last years. Genome-wide association studies have emerged as an increasingly effective tool for identifying genetic contributions to complex diseases and represent the next frontier to further understand the underlying etiologic, biological, and pathologic mechanisms associated with chronic complex disorders. There have already been success stories for diseases such as diabetes mellitus [21]. Another recent example of wide-genome screening comes from the Framingham Heart Study (FHS) [22]. AD provides another good example of a complex age-associated disease with multiple genetic etiologies, including

specific sub-types inherited as autosomal dominant traits, as well as common forms related to the inheritance of susceptibility genes. By performing genome-wide associations, an expectation is that the multiple contributing loci (as in AD) can be identified simultaneously, and then integrated into pathways contributing to a pathogenetic process for which drugs can be identified [23-24]. There is now experimental and clinical trial evidence to support this prediction [21, 25-27]. Roses and coworkers described the design of high throughput disease-association SNP studies in AD [28], by confirming, from this initial screen that, among others, APOE4 is genetically associated with AD. Another screening strategy based on the use of microarray technology was also adopted for a new molecular test for AD in blood plasma [29]. Despite the great impact and the variety of available information, the screening strategy in pharmacogenomic studies shows to date limitations yet. Currently, costs limit the widespread use of pharmacogenomics. Execution of pharmacogenomic studies is dependent on many pieces of information: access to accurate clinical and demographic data, DNA samples from well designed studies, SNPs, genotyping technologies, informatics technologies to handle large quantities of data, statistical methodologies for data analysis and interpretation. In this regard, other limitations in the progress of pharmacogenomics include tools used for collecting, archiving, organizing and interpreting the huge amount of data generated in a pharmacogenomics study so that data from different experiments can be compared.

Specific hypothesis driven studies represent another approach in pharmacogenomics. Differently from wide-genome screening, the strategy is focused on the knowledge of well-known genes involved in specific diseases. Taking as example AD, functional genomics studies in AD revealed that age of onset, brain atrophy, cognitive decline, apoptosis, immune function and amyloid deposition are associated with AD-related genes. The genetic defects identified in AD during the past 25 years are represented by mutations in the amyloid precursor protein (APP) gene, on chromosome 21, in the presenilin 1 (PS1) gene, on chromosome 14, and in the presenilin 2 (PS2) gene, on chromosome 1 [8, 30-31]. In addition, polymorphic variants of risk in more than 200 different genes can increase neuronal vulnerability to premature death [18], with APOE gene (19q13.2) as the most prevalent risk factor for AD. Thus, focusing the study on one of these genes is possible to understand its specific role on specific events typical of AD. On the other hand, instead, all together, these genetic factors could interact in still unknown genetic networks leading to a cascade of events characterized by abnormal protein processing and misfolding with subsequent accumulation of abnormal proteins, excitotoxic reactions, oxidative stress, mitochondrial injury, synaptic loss and deficiencies in neurotransmitter function [8, 30, 32-33].

Pertaining to the context of aging, another example of hypothesis-driven study is based on checkpoint control by specific proteins. Aging in higher animals may be influenced by the balance of cell survival versus death, a decision often governed by checkpoint proteins in dividing cells. One of such proteins is p53; in mammals, p53 loss increases tumorigenesis, while specific gain-of-function alleles reduce tumor incidence but accelerate aging, suggesting a trade-off between tumor surveillance and stem cell maintenance [34].

p53: AGING AND CRITICAL DECISION ABOUT CELL FATE

The transcriptional network of p53-responsive genes produces proteins able to interact with a large number of other signal transduction pathways in the cell. p53 protein can trigger the onset either of reversible or permanent growth arrest [35-37] or of apoptosis [38-39]. However, the mechanisms involved in the decision between these cellular responses are not well understood. Cell type, the presence of growth factors or oncogenes, the intensity of the stress signal, and the cellular level of p53 have been cited as important factors in determining a specific p53-induced response [40-42]. Posttranslational modifications of the p53 gene have also been reported to influence the response observed [43]. For example, p53 phosphorylation by different kinases in response to stress can select for arrest or apoptosis, suggesting the involvement of modifiers upstream of the p53 protein [43-44].

Once a cell has been damaged and the DNA damage response and p53 are activated, a complex signaling network is engaged to result in a long-term cell fate decision. Rodier and coworkers reported an intriguing scheme showing four options adopted in normal mammalian cells [34]. Activation of cell cycle checkpoints by p53 leads to transient cell growth arrest [45]; p53 physically localizes to sites of DNA damage to promote repair [46] and simultaneously stimulates the transcription of direct effectors of the cell growth arrest (e.g. the cyclin-dependent kinase inhibitor p21) as well as effectors required for efficient DNA repair of complex lesions that require longer processing (e.g. GADD45) [47]. At this point, several potential cellular outcomes can occur, most of which are heavily influenced by the cell type as well as the severity of the DNA lesions: transient cell cycle arrest (when DNA damage is not severe), defective repair (resulting in mutation, such as chromosomal aberrations), cell death (apoptosis) or permanent cell cycle arrest (cellular senescence) [34]. Thus, p53 protects the genome by promoting the repair of potentially carcinogenic lesions in the DNA, thereby preventing mutations. In addition, p53 eliminates or arrests the proliferation of damaged or mutant cells by the processes of apoptosis and cellular senescence [48-49].

Loss of function in p53 is usually associated to many common human cancers. Mutant p53 is almost always defective for sequence-specific DNA binding, and thus for transactivation of genes upregulated by the wild-type protein [50]. Interestingly, when p53 is mutated in non-dividing cells, such as neurons, a dysfunctions accumulation can occur; Yang and coworkers, in fact, demonstrated the existence of aberrant neurons in AD brain, by showing that neurodegeneration is correlated to neurons reentering a lethal cell cycle [51].

CONFORMATIONALLY ALTERED p53 IN AGED CONTROLS AND ALZHEIMER'S DISEASE PATIENTS

We have studied p53 in aged controls and demented patients finding that with aging there is an increase of conformationally altered p53, which is more pronounced in AD patients, to the point that it has been proposed as a putative biomarker in the early stages of the disease [52-53]. As a

result of such conformational change, p53 partially loses its activity and may become unable to properly activate an apoptotic program when cells are exposed to a noxious stimulus [52]. In this study we described and demonstrated an abnormal response of AD fibroblasts to an acute oxidative injury; in particular, fibroblasts from AD patients were found to be less vulnerable to the oxidative injury induced by H_2O_2 in comparison with fibroblasts from non-AD subjects. Fibroblasts from sporadic AD patients represent an important starting point in the research for novel biomarkers because of their various abnormalities in metabolic and biochemical processes, which reflect some of the events in AD brain [54-55]. Furthermore, on the basis of immunoprecipitation studies with conformation-specific p53 antibodies (PAb1620 and PAb240), which discriminated folded vs unfolded p53 tertiary structure, we found that in fibroblasts from AD patients a significant amount of total p53 assumes an unfolded tertiary structure; such alteration can compromise p53 response to an acute injury elicited by an excess of free radical production. Mutant p53 found in AD fibroblasts has been demonstrated to be independent from gene mutations on the basis of sequence analysis of the p53 gene, thus suggesting that one of the peripheral events associated to the disease is responsible for generating a conformationally altered p53 isoform [52]. In the attempt of investigating on the mechanism of such alteration, we assessed the contribution of APP metabolic products to the change in p53 conformational state. We found that the exposure to nanomolar concentrations of beta-amyloid ($A\beta$) 1-40 peptide induced the expression of an unfolded p53 protein isoform in fibroblasts derived from non-AD subjects [56]. These data suggest that the tertiary structure of p53 and the sensitivity to p53-dependent apoptosis are influenced by low concentrations of soluble $A\beta$. On this basis, we hypothesised that low amounts of soluble $A\beta$ induce early pathological changes at cellular level that may precede the amyloidogenic cascade. One of these changes is the induction of a novel conformational state of p53. If low amounts of $A\beta$ peptide, not resulting in cytotoxic effects, are responsible for p53 structure changes, it could be possible to consider the unfolded p53 both as an agent participating to the early pathogenesis and as a specific marker of the early stage of AD.

We then investigated the altered p53 isoform in more accessible cells, such as peripheral blood cells [53] to determine the frequency and the extent of this defect in AD patients and to explore the possibility to develop an assay to validate conformationally altered p53 as a putative peripheral marker of the disease. The identification of new biological markers, to date, can be greatly useful both to improve diagnostic accuracy and/or to monitor the efficacy of putative therapies, since the confirmatory diagnosis of AD is possible only *post mortem*, based on recognition and quantification of senile plaques and neurofibrillary tangles. Furthermore, we set up a rapid and easy flow-cytometric approach to identify the different expression of conformationally altered p53 between AD and non-AD subjects. Differently from other data in literature regarding the development of biomarkers for AD or in CSF [57-58] or in blood [59-60], the main advantage of our study was the development of a biological sample preparation procedure as well as of an analytical method that could permit a routine analysis. If this method should be

confirmed in a larger population and further validated, it could be useful in improving the knowledge regarding the clinical diagnosis of AD, because we could have the possibility to recognize AD and non-AD cases, by starting from small blood volumes thus using a less invasive technique [53]. Interestingly, we observed that the expression of conformationally altered p53, both in controls and AD, is an age dependent event, while it is independent from the length of illness and from the MMSE score; this linear correlation with age would suggest that its significance would be different within specific age interval segments. In order to determine the diagnostic performance of conformationally altered p53 as an AD marker, we worked out sensitivity and specificity within different age intervals and found that these values were more significant in subjects up to 70 years of age (sensitivity of 90% to discriminate AD patients from non demented aged individuals at a specificity value of 77%) compared with the corresponding values for individuals older than 70 years [53]. Although the age stratification is admittedly a post-hoc analysis it serves to indicate that the putative marker proposed appears to be more important in the younger patients, also considering that the positivity to p53 conformational mutant induced a calculated O.R. of 29.2 for AD. The fact that the most significant differences are observed in the youngest patients indicate that the measurement of conformational mutant p53 may be usefully applied to detect AD at the early stages, perhaps applied to those patients falling in the ill defined category of mild cognitive impairment (MCI). Whether this expression of conformationally altered p53 will be suitable as an adjunctive diagnostic tool to predict the conversion from MCI to AD is under investigation.

In addition, in order to better characterize the nature of this different expression between AD and control subjects, we also evaluated whether the expression of conformational mutant p53 showed a correlation with the APOE status. We recruited a population of 75 patients with sporadic AD, 69 healthy age-matched controls (CTR) (Table 1). Genomic DNA was extracted from peripheral leukocytes by proteinase K digestion and standard phenol/chlorophorm extraction procedure. The APOE gene polymorphisms (isoforms APOE ϵ 2, APOE ϵ 3, APOE ϵ 4) were determined by Hha I restriction endonuclease digestion of PCR products, according to Hixson and Vernier [61]. After having determined APOE gene polymorphisms, we then divided this population in four groups, control subjects and AD patients with and without the ϵ 4 allele, to establish if the presence of the ϵ 4 allele might further affect the expression of mutant-like p53. The expression of mutant conformational p53 was independent from distribution of ϵ 4 allele of APOE, thus highlighting that the presence of ϵ 3/ ϵ 4 and ϵ 4/ ϵ 4 genotypes was not linked to a higher expression of p53 conformational status (Fig. 1). AD patients without the ϵ 4 allele showed a higher expression of mutant-like p53 compared to respective controls (percent of PAb positive cells, mean $\pm$ SD; control subjects without APOE ϵ 4: 22.8 ± 12.2 ; AD without APOE ϵ 4: 39.3 ± 15.0 ; AD without APOE ϵ 4 versus respective control $P < 0.0001$). The comparison of the APOE ϵ 4 positive cases did not show a statistically significant difference between AD and the respective controls possibly because of the small size of the control sample (8 patients). On the other hand, complexively

the p53 may be a marker useful in the $\epsilon 4$ negative younger patients (< 70 years).

Table 1. Demographic and Clinical Variables of all the Subjects

	AD	CTR
N (M;F)	75 (20;55)	69 (34;35)
mean age $\pm$ SD	79 $\pm$ 9	78 $\pm$ 10
L.O.I. (month)	53 $\pm$ 26	
MMSE	14 $\pm$ 6	28 $\pm$ 1.6

N: number; M: male; F: female; L.O.I.: length of illness; MMSE: Mini-Mental State Examination. Data are expressed as mean $\pm$ standard deviation.

CONCLUSIONS/PERSPECTIVES

Aging in higher animals may be influenced by the balance of cell survival versus death, at least in part regulated by a fine timing of checkpoint proteins and preservation of DNA integrity and correct repair [62-63]. With our study we propose an example pointing to a peculiar aspect of pharmacogenetic/pharmacogenomics research in aging and age associated diseases, that is the possibility to define novel tar-

gets for therapeutic interventions aimed to checkpoint proteins. p53 has been recently shown to be involved in aging and AD. Recent evidence suggests that increased p53 activity can, at least under some circumstances, promote organismal aging [34]. We showed a link between AD pathology and conformationally altered p53 [52-54], by finding that with aging a higher increase of an unfolded state of p53 in AD patients compared to age-matched controls occurs. What can be the contribution of a conformational change of a protein to the aging process is under investigation. We could also address the issue whether a generalization of this phenomenon within the context of the "gain and loss of function" of protein conformers will be possible. It is worth to underline that the observation that aging and AD interfere with proteins controlling the duplication and cell cycle, such as p53, is interesting and may lead to the speculation that, in senescent neurons, derangements in proteins commonly dealing with cell cycle control and apoptosis could affect neuronal plasticity and functioning rather than cell duplication.

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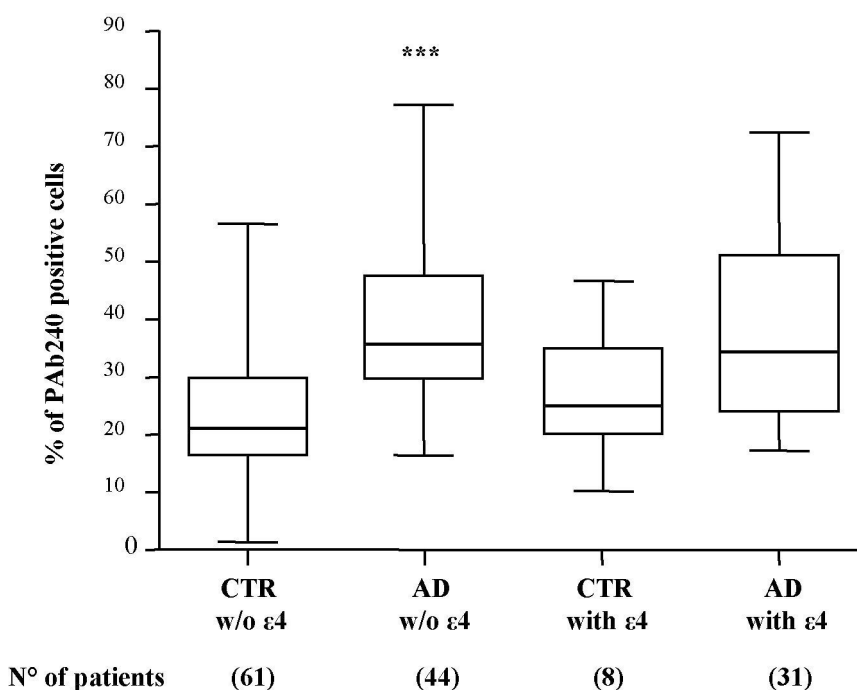


Fig. (1). Correlation between the APOE status and conformational mutant p53.

Box plot of the amount of conformational mutant p53 (%) expressed in peripheral blood cells of all the subjects classified on the basis of their APOE genotypes. The figure is based on a rather innovative application in flow cytometry on peripheral blood cells. Venous blood samples were collected from a group of 75 patients with sporadic AD and 69 healthy age-matched controls (CTR). Peripheral blood mononuclear cells were then analyzed for the expression of conformationally altered p53 with a flow cytometer. The APOE gene polymorphisms (isoforms APOE $\epsilon 2$, APOE $\epsilon 3$, APOE $\epsilon 4$) were determined by Hha I restriction endonuclease digestion of PCR products. Bonferroni multiple comparison test was used to compare values in different groups. Differences were considered significant when a p-value < 0.05 was attained. ***p < 0.001 vs control without $\epsilon 4$ allele. We previously published [53] the observation that the elevated conformationally altered p53 was specific for AD and not observed in other neurodegenerative diseases, such as Parkinson's disease, vascular dementia and supranuclear palsy.

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ABBREVIATIONS

A β	=	Beta-amyloid
AD	=	Alzheimer's disease
APO E	=	Apolipoprotein E
APP	=	Amyloid precursor protein
FHS	=	Framingham Heart Study
SNP	=	Single nucleotide polymorphisms
PS1	=	Presenilin 1
PS2	=	Presenilin 2

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