Positive Association of the Cathepsin D Ala224Val Gene Polymorphism With the Risk of Alzheimer's Disease in Ecuadorian Population

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Abstract: Background: Alzheimer' s disease (AD) is the most common cause of senile dementia. In Ecuador, the number of deaths caused by AD increases each year. Epidemiologically, the Ecuadorian population is composed of a mixture of several genetic backgrounds along with environmental factors, that make it unique and ideal for population studies. The main objective of this study was to determine the prevalence of Cystatin C (CST3), Cathepsin D (CTSD) and Manganese superoxide dismutase(MnSOD) amino acid-altering polymorphisms and their influence on the development of AD in the Ecuadorian population. Methods: This is a case-control study consisting of 56 patients with AD, from the Department of Neurology at Carlos Andrade Marín Hospital. The control group (n5 55) comprised healthy elderly adults. The inclusion period was from January to August of 2012. Peripheral blood was collected from both groups for DNA extraction, polymerase chain reaction and capillary sequencing.Results: There was a positive association between aCTSD polymorphism (Ala224Val) and the development of AD (odds ratio5 8.1, 95% confidence interval: 0.9–85.7; P , 0.025). However, the 3 other polymorphisms investigated did not show significant associations with AD. Conclusions: Variations in CTSD andMnSOD showed no association with the development of AD, whereas the presence of the Ala224Val polymorphism inCTSD had a positive association with the development of AD.

Key Indexing Terms: Alzheimer' s disease; Capillary sequencing; Genetic polymorphisms. [Am J Med Sci 2015;00(00):1 –6.]

Alzheimer' s disease (AD) is the most common cause senile dementia, involved in approximately 60% to 70% of cases of cognitive decline in the elderly.Worldwide, nearly 95.6 million people suffer from this disease, and it is expected that by 2030 this will increase to 65.7 million.[2] According to the Instituto Nacional de Estadística y Censo, 0.29% of all deaths in Ecuador in 2008 was caused by AD. In 2011, this increased to 0.4%. Therefore, it is imperative to determine the genetic risk factors involved in the development of this disease in Ecuador. The Ecuadorian population is composed of 60% mestizos, 30% native Amerindians, 8% Afro-descendants and the remaining 2% is composed of whites, Arabs and Mongols.[3] Some studies performed in the Ecuadorian population have shown that the incidence of certain polymorphisms is similar to some European and Asian countries and others are not. For instance, when comparing Cystic Fibrosis, the incidence in Latin America is 1/10,000, whereas in whites the incidence is 1/2,000. In Ecuador, only 150 cases have been reported.[4]

AD begins with subtle memory loss, which progresses to a more severe, irreversible and incapacitating form.[5] Other symptoms include confusion, poor judgment, language problems and hallucinations, among others.[6] In the brain, cerebral cortical atrophy is a product ofintraneural neurofibrillary tan-gles and large extracellular accumulations of amyloid b(Ab) in the form of senile plaques and cerebrovascular deposits. These Ab aggregates are thought to trigger an inflammatory response, neuronal cell death and gradual cognitive decline.[7,8]

Genetically, late-onset AD is considered a polygenic and multifactorial disorder.[5,6,9–11] This disorder displays no single or simple mode ofinheritance. However, early-onset familial AD presents dominant and fully penetrant forms that are caused by variations in 3 genes;amyloid precursor protein(APP), prese-nilin 1 (PSEN1) and presenilin 2 (PSEN2).[12,13] Early-onset AD is considered rare because it is only present in 5% of all cases.[11,14] In contrast, late-onset or sporadic events represent 95% of cases and seem to be governed by an array of common risk alleles across a number of different genes. All of these genes appear to be related to the production, aggregation and removal of Ab. Other than Ab accumulation, AD has also been associated with oxidative damage, mitochondrial dysfunction, neurodegeneration and dementia.[15]

Recent advances in molecular genetics suggest that many genes can work together to impact the predisposition of the disease and the age of onset. The Abcascade and mitochondrial stress hypothesis suggest that once Abstarts to accumulate, it promotes oxidation and compromises mitochondrial function.[13–15]

The gene for manganese superoxide dismutase (MnSOD), a tetrameric isoenzyme, is found on the long arm of chromosome 6 (6q25). In addition to MnSOD, there are 2 other superoxide dismutase isoenzymes (SODs), one found in the cytosol CuZnSOD and the other an extracellular SOD, both of which contain copper and zinc in the active center. SODs convert superoxide radicals (reactive oxygen species) into molecular oxygen and H_2O_2, which can then be neutralized by glutathione peroxidase, catalase and peroxiredoxin reductase. The biological activity of MnSOD takes place inside the mitochondrial matrix.[16] Some studies suggest that MnSOD is critical for neuronal survival following oxidative damage[1.7] There are 2 main functional polymorphisms in MnSOD, which may be responsible for the development of AD; a Ile58Thr variant located in exon 3, affecting the interface stability of the tetramericprotein and reducing the activity of the protein [18,19] and Ala9Val (GCT/GTT) in exon 2, a polymorphic substitution in the MnSOD gene, which induces a conformational change in the mitochondrial targeting sequence and turns the b-pleated sheet into an a-helix,

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making the transportation of this protein across the 2 mitochondrial membranes into the matrix, impossible.[20]

On the other hand, recent genetic evidence suggests the existence of a connection among AD, neurodegeneration and the lysosomal system, which is exceptionally well-developed in the brain. Therefore, it is not surprising that defects in the synthesis, sorting and transport of lysosomal enzymes produce disorders of mental dysfunction.[21] Cathepsin D (CSTD) is a major intracellular acidic protease of the endosomal-lysosomal pathway generated by the *CSTD* gene that is located on chromosome 11 (11q12) and ubiquitously expressed. *CSTD* is involved in the degradation, cell invasion, apoptosis, internalization and initial processing of APP at the cell surface where a possible interaction with the *APOE* genotype has been noted.[22] An exonic variant of *CTSD* resulting in an Ala224Val amino acid change has been associated with an increase of pro-CTSD secretion and was over-represented in an AD population.[21,23] Carriers of this polymorphism had a 3.1-fold increased risk for developing AD compared with non-carriers.[24,25]

Another genetic risk factor is Cystatin C (CST3), which is an endogenous proteinase inhibitor of the cathepsins B, H, L and S. Cystatin is synthetized in the brain by neurons, asctrocytes and choroid plexus cells. It has an inhibitory activity within the endosomal-lysosomal system and forms a part of a family of inhibitor proteins that are classified into 3 groups; cystatins type 1 and 2 and kininogens. Cystatin levels are increased in AD patients. The *CST3* gene is located on chromosome 20p11.2 and contains 3 exons. The Ala73Thr polymorphism maps to the penultimate position of the signal peptide.[26] The aim of this study was to determine whether there is an association between the development of AD and the Ile58Thr and Ala9Val polymorphisms in *MnSOD*, Ala73Thr in *CST3* or Ala224Val in *CTSD* in patients from the Ecuadorian population.

METHODS

Participants and Samples

All sampled individuals diagnosed with late-onset AD were examined by the Neurology Department at Carlos Andrade Marín Hospital in Quito, Ecuador. The clinical diagnosis was made according to the National Institute on Aging and Alzheimer's Association criteria.[27] Several psychometric tests were applied. (1) SM-IV is a diagnostic and statistical manual adopted by the American Psychiatric Association, which is correlated with the ICD-10 classification of mental and behavioral disorders by the World Health Organization. This test uses a multi-axial approach to diagnose and organize between different mental disorders. (2) The Mini-Mental State Examination was used for complaints of memory problems or to diagnose dementia and to assess its progression and severity.[28] (3) The Frontal Assessment Battery was used to screen for global executive dysfunction, which analyzes cognitive and behavioral function by using 6 subtests.[29] (4) The Clinical Dementia Rating (CDR) evaluates the severity of dementia stages. CDR is a 5-point scale in which CDR-0 means no cognitive impairment and the remaining 4 points correspond to various stages of dementia according to the Alzheimer's disease Research Center. Additionally, medical tests and MRIs were performed to discard hypothyroidism or hydrocephalia, respectively. Normal healthy control samples came from the Biomedical Research Institute of the Universidad de las Américas DNA biobank. The authors analyzed 55 control samples with an average age of 72.38 ± 9.89 years, ranging from 60 to 89 years and with a median age of 68 years. These subjects lacked neurological or psychiatric diseases. Included were 56 AD patients with an average age of 73.12 ± 10.38 years, ranging from 59 to 95 years and having a median age of 74 years. More information about patients and controls is detailed in Table 1. All patients were recruited from the Neurology Department at Carlos Andrade Marín Hospital. In both groups, additional parameters were collected, such as age, sex, clinical history and written informed consent. Exclusion criteria included the presence of psychiatric disorders, cerebrovascular diseases, or types of dementia such as Lewy body dementia, drug consumption that could affect cognition, head trauma, psychomotor retardation detected since infancy, acute concomitant disease, diabetes, chronic kidney disease, liver disease or a history of multiple heart attacks. For this study, only mestizos (European and Amerindian mixed ancestry) were included. The Universidad de las Américas Bioethics Committee approved this study.

Genotyping

DNA was extracted from peripheral blood samples using the PureLinkT Genomic DNA Kit (Invitrogen, Carlsbad, CA) followed by DNA quantification using NanoDrop2000 (ThermoScientific, Waltham, MA). *CST3*, *CTSD* and *MnSOD* genotypes were determined using polymerase chain reaction (PCR). PCR was performed in a final volume of 50 μL containing 4 μL of DNA template, 34 μL H_2O Milli-Q, 0.4 μM of forward and reverse primers, 1.5 mM $MgCl_2$, 5 μL 10× buffer (200 mM Tris–HCl pH 8.4, 500 mM KCl), 0.2 μM each of deoxynucleotide triphosphate and 2.5 U Taq Platinum DNA polymerase (Invitrogen). The authors used the following primers: for Ile58Thr, forward (F): 5′-TCCAGGGGAAGTACTGTTTG-3′ and reverse (R): 5′-GCAGACCTCTTTGATGGTTG-3′; for Ala9Val, F: 5′-GCTGTGCTTTCTCGTCTTCA-3′ and R: 5′-CAACGCCTCCTGGTACTTCT-3′; for Ala73Thr, F: 5′-TATCTAGCTCCAGCCTCTCG-3′ and R: 5′-TACATGTCGTTGCTGGCTTT-3′; and for Ala224Val, F: 5′-GTCCATGTAGTTCTTGAGCA-3′ and R: 5′-GGTGACCACTTCTTAGGACT-3′. The standard PCR protocol for all the polymorphisms consisted of an initial denaturation step of 10 minutes at 95°C followed by 35 cycles of 45 seconds at 95°C, 1 minute at 60.3°C, 45 seconds at 72°C and a final extension step of 3 minutes at 72°C. The only variation was in the annealing temperature, which was 60.3°C for Ile58Thr, 62°C for Ala9Val, 53°C for Ala73Thr and 60°C for Ala224Val. Amplicons were confirmed using electrophoresis in

TABLE 1. Participant demographics

					Cultural background, %		
	N	Median age, yr	Females, %	Males, %	PS	HS	B
Patients	56	73.12 ± 10.38	53.57	46.43	21	63.8	15.2
Controls	55	72.38 ± 9.89	43.64	56.36	13.9	60.2	25.9

B, bachelor or higher; HS, high school; PS, primary school.

a 2% agarose gel and observed using an ImageQuant 300 transilluminator (General Electric, Fairfield, SC). Finally, the fragments were purified with Agentcourt Cleanseq (Beckman Coulter, Miami, FL), sequenced using a BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Austin, TX), and analyzed by using Seq-Scape Software v2.6 (Applied Biosystems).

Statistical Analysis

Descriptive statistical analysis was used. Allelic and genotypic frequencies of each SNP were used to calculate genotype information, and the Hardy–Weinberg equilibrium was determined using online software (http://www.genes.org.uk/software/hardy-weinberg.shtml). All information obtained from the subjects was compiled into a database, and statistical analyses were carried out using SPSS v.17 (SPSS, Chicago, IL). Chi square (χ^2) analysis was performed to determine whether there were significant differences between the allele frequencies of cases and controls from the Ecuadorian population. To determine the risk of developing AD for each polymorphism, the odds ratio (OR) was calculated. The data were analyzed using a 2×2 contingency table.

RESULTS

The observed genotypes and the allele distributions in the study population are shown in Table 2. For the Ala73Val polymorphism, most of the analyzed individuals (85.14%) carried the normal G allele. Similarly, for the Ala224Val polymorphism, there was a higher percentage of the normal C allele in the population (77.03%). Meanwhile, for Ile58Thr, only the normal G allele was found. Because these alleles represented the whole analyzed population, no additional statistical analysis was performed. Finally, the data obtained for the *MnSOD* (Ala9Val) polymorphism revealed that the minor C allele was present in a higher percentage (58.56%) of cases than in the normal population.

The Hardy–Weinberg analysis is shown in Table 3 where the distribution of allelic and phenotypic frequencies is described. Regarding the T allele (wild type) of the Ala9Val polymorphism, we observed that the allele frequency was higher in the affected group (0.45) than in the control group (0.38). Using χ^2 analysis to compare cases and controls, we found that the 2 groups were in Hardy–Weinberg equilibrium, and that there were no significant differences between them. For Ala73Thr, the frequency of the G allele (wild type) was 0.8 for the affected group and 0.9 for the control group. Finally, the frequency of the C allele (wild type) in the *CTSD* gene was higher (0.9) for the control group than for the affected group (0.64).

In terms of risk (Table 4), the *MnSOD* (Ala9Val) and *CST3* (Ala73Thr) polymorphisms did not present a relevant OR value. For Ala9Val, the OR was 0.6 (95% CI: 0.2–1.6; $P = 0.42$) for both the T/C and C/C genotypes. For Ala73Thr, the OR for G/A was 2.3 (95% CI: 0.5–9.7; $P = 0.43$) and for A/A it was 2.3 (95% CI: 0.6–8.1; $P = 0.32$), which in both cases was nonsignificant. However, the heterozygous C/T allele of Ala224Val at *CTSD* had a highly significant OR of 8.0 (95% CI: 3.2–19.8; $P < 0.0001$). The rare homozygous genotype, T/T, had an OR of 9.0 (95% CI: 0.9–85.7; $P = 0.025$), and the group formed by the heterozygous and homozygous mutant genotypes (C/T + T/T) had an OR of 8.1 (95% CI: 3.4–19.5; $P < 0.0001$).

DISCUSSION

Globally, the prevalence of AD is increasing in older populations because of longer lifespans, which makes aging a major risk factor.[30] However, the molecular causes of AD are still poorly understood. The discovery of the Aβ peptide led to the formulation of the amyloid cascade hypothesis, in which mutations in *APP*, *PSEN1* and *PSEN2* were identified as the main pathogenic variants for the early-onset form of AD.[31] However, for the late-onset form, the causes remain unclear. For instance, *ApoE* has been proposed to be the highest risk factor found thus far, but in some populations, such as the Ecuadorian one, even though the ε2, ε3, ε4 polymorphisms are present, other polymorphisms represent a higher risk for AD, including the Pro198Leu polymorphism in *GPX-1*.[32]

To date, more than 20 non-*APOE*-related loci have been associated with disease risk in systematic meta-analyses.[13] Specifically, Ala9Val and Ile58Thr in *MnSOD* were potentially associated with Parkinson's disease, but no association was found between these variants and depressive disorders.[33] In this study, the Ile58Thr polymorphism was not related to the development of AD because none of the groups presented the rare homozygous genotype C/C. The Ala9Val polymorphism had a higher frequency of the rare C allele (0.62) in the control group than in the affected population (0.55). However, the Ala9Val polymorphism had no association with AD.

TABLE 2. Allele distribution of the population

Gen (Polimorphism)	Genotype	Individuals	Percent	Allele	Percent
CST3 (Ala73Val) G→A	G/G	90	81.08	G	85.14
	G/A	9	8.11	A	14.86
	A/A	12	10.81		
CSTD (Ala224Val) C→T	C/C	65	58.56	C	77.03
	C/T	41	36.94	T	22.97
	T/T	5	4.5		
MnSOD (Ile58Thr) T→C	T/T	111	100	T	100
	T/C	0	0	C	—
	C/C	0	0		
MnSOD (Ala9Val) T→C	T/T	21	18.92	T	41.44
	T/C	50	45.06	C	58.56
	C/C	40	36.02		

TABLE 3. Hardy-Weinberg analysis for the polymorphisms studied

Gene	Population	Genotype	Number of individuals	Percent	Genotypic frequency	Expected frequency	Allelic frequency	HWE *P* value
MnSOD Ala-9Val T→C	Affected	T/T	13	23.21	0.23	0.20	0.45	>0.05
		T/C	24	42.86	0.43	0.49		
		C/C	19	33.93	0.34	0.31	0.55	
		Total	56					
	Controls	T/T	8	14.55	0.15	0.15	0.38	>0.05
		T/C	26	47.27	0.47	0.47		
		C/C	21	38.18	0.38	0.38	0.62	
		Total	55					
CST3 Ala73Thr G→A	Affected	G/G	42	75.00	0.75	0.65	0.80	>0.05
		G/A	6	10.71	0.11	0.32		
		A/A	8	14.29	0.14	0.04	0.20	
		Total	56					
	Controls	G/G	48	87.27	0.87	0.81	0.90	>0.05
		G/A	3	5.45	0.05	0.18		
		A/A	4	7.27	0.07	0.01	0.10	
		Total	55					
CTSD Ala224Val C→T	Affected	C/C	20	35.71	0.36	0.41	0.64	>0.05
		C/T	32	57.14	0.57	0.46		
		T/T	4	7.14	0.07	0.13	0.36	
		Total	56					
	Controls	C/C	45	81.82	0.82	0.81	0.90	>0.05
		C/T	9	16.36	0.16	0.18		
		T/T	1	1.82	0.02	0.01	0.10	
		Total	55					

Concerning *CST3*, some studies have shown that the Ala73Val variant has been associated with an increased risk of susceptibility to AD in white populations. However, a study in the Italian population found no such association.[34] The results are similar to studies done in Japan, the Netherlands, Germany and Italy, where no association with the development of the disease was found.[35–37]

A report on vascular dementia performed in the German ($P = 0.009$), British ($P < 0.001$) and American ($P < 0.001$) populations determined that polymorphism in *CTSD* was related to the disease, unlike studies in the Korean population.[38] In 2006, it was suggested that Ala224Val polymorphism increases the relative risk of AD.[21] In the case-control study, the heterozygous C/T allele was more frequent in the affected individuals than in the controls. It was also determined that the relative risk of the heterozygous genotype C/T was highly significant. Also, the rare homozygous T/T genotype was significantly associated with disease status in the groups analyzed.

TABLE 4. Odds ratio and χ^2 analysis for the different genes studied with their respective polymorphisms

Gene	Genotype	Affected N (%)	Controls N (%)	OR	95% CI	*P*
MnSOD Ala9Val T→C	T/T	13 (23.2)	8 (14.5)	1	Reference	
	T/C	24 (42.9)	26 (47.3)	0.6	0.2–1.6	0.42[a]
	C/C	19 (33.9)	21 (38.2)	0.6	0.2–1.6	0.42[a]
	T/C + C/C	43 (76.8)	47 (85.5)	0.6	0.2–1.5	0.36[a]
CST3 Ala73Thr G→A	G/G	42 (75.0)	48 (87.3)	1	Reference	
	G/A	6 (10.7)	3 (5.5)	2.3	0.5–9.7	0.43[a]
	A/A	8 (14.3)	4 (7.3)	2.3	0.6–8.1	0.32[a]
	G/A + A/A	14 (25.0)	7 (12.8)	2.3	0.8–6.2	0.16[a]
CTSD Ala224Val C→T	C/C	20 (35.7)	45 (81.8)	1	Reference	
	C/T	32 (57.1)	9 (16.4)	8.0	3.2–19.8	0.000[b]
	T/T	4 (7.1)	1 (1.8)	9.0	0.9–85.7	0.025[b]
	C/T + T/T	36 (64.2)	10 (18.2)	8.1	3.4–19.5	0.000[b]

[a] non significative.
[b] significative.
CI, confidence interval, values calculated with 1 degree of liberty; OR, odds ratio.

The same analysis was performed for the heterozygous C/T and rare homozygous T/T genotypes, which were also significantly associated. This suggests that the presence of the T allele increases the risk of developing AD. Thus, this polymorphic variant may play an important biological role in the pathogenesis of late-onset AD. Further studies with a bigger sample size are required to verify these results.

CONCLUSIONS

Several polymorphisms in many different genes have been proposed as risk factors for late-onset AD. However, the incidence and association pattern vary between populations, possibly because of environmental factors or genetic interactions, making the identification of the main factors causing AD difficult. In conclusion, the presence of Ala224Val increases the risk of developing AD by 8.1 times in the Ecuadorian population, unlike in other populations, possibly because of environmental and genetic modifiers present in the mestizo population studied.

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REFERENCES

1. **Jorde LB, Carey JC, Bamshad MJ.** Medical Genetics, 4th ed. China: Elsevier; 2009.
2. **World Health Organization.** Dementia cases set to triple by 2050 but still largely ignored. Geneva 2012.
3. **Paz-y-Miño C, López-Cortés LE.** Genética Molecular y Citogenética humana: Fundamentos, aplicaciones e investigaciones en el Ecuador, 1st ed. Ecuador: Yachay EP; 2014.
4. **Paz-y-Miño C, Pérez JC, Burgos R, et al.** The DeltaF508 mutation in Ecuador, South America. Hum Mutat 1999;14:348–50.
5. **Loy CT, Schofield PR, Turner AM, et al.** Genetics of dementia. The Lancet 2014;383:828–40.
6. **Greenfield JP, Tsai GK, Gouras B, et al.** Endoplasmic reticulum and trans-Golgi network generate distinct populations of Alzheimer β-amyloid peptides. Proc Natl Acad Sci U S A 1999;96:742–47.
7. **Yanker BA, Duffy LK, Kirchner DA.** Neurotrophic and neurotoxic effects of amyloid-b protein: reversal by tachykinin neuropeptides. Science 1990;12:279–82.
8. **Segovia de Arana J.M, Mora T.** Enfermedades Neurodegenerativas. Serie Científica 2002.
9. **Liddell MB, Bayer A, Owen AJ.** No evidence that common allelic variation in the Amyloid Precursor Protein (APP) gene confers susceptibility to Alzheimer's disease. Hum Mol Genet 1995;4:853–8.
10. **Bird T.** Alzheimer's disease overview. Gene Rev 2014;1–19.
11. **Pagon RA, Adam MP, Bird TD, et al.** Alzheimer's disease overview. Gene Rev 2014.
12. **Turner PR, O'Connor K, Tate WP, et al.** Roles of amyloid precursor protein and its fragments in regulating neural activity, plasticity and memory. Prog Neurobiol 2003;70:1–32.
13. **Bertram L, Tanzi RE.** Thirty years of Alzheimer's disease genetics: the implications of systematic meta-analyses. Nat Rev Neurosci 2008;9:768–78.
14. **Guimerá A, Girones X, Cruz-Sánchez F.** Actualización sobre la patología de la enfermedad de Alzheimer. Rev Esp Patol 2002;1:21–48.
15. **Esposito L, Raber J, Kekonius L, et al.** Reduction in Mitochondrial Superoxide Dismutase Modulates Alzheimer's Disease-Like Pathology and Accelerates the Onset of Behavioral Changes in Human Amyloid Precursor Protein Transgenic Mice. Neurobiology of Disease 2006.
16. **Church SL, Grant JW, Meese EU, et al.** Sublocalization of the gene encoding manganese superoxide dismutase (MnSOD/SOD2) to 6q25 by fluorescence in situ hybridization and somatic cell hybrid mapping. Genomics 1992;823–5.
17. **Galecki P, Śmigielski J, Florkowski A, et al.** Analysis of two polymorphisms of the manganese superoxide dismutase gene (Ile-58Thr and Ala-9Val) in patients with recurrent depressive disorder. Psychiatry Res 2010;30:43–6.
18. **Borgstahl GE, Parge HE, Hickey MJ, et al.** Human mitochondrial manganese superoxide dismutase polymorphic variant Ile58Thr reduces activity by destabilizing the tetrameric interface. Biochemistry 1996;35:4287–97.
19. **Kowalski M, Bielecka-Kowalska A, Oszajca K, et al.** Manganese superoxide dismutase (MnSOD) gene (Ala-9Val, Ile58Thr) polymorphism in patients with age related macular degeneration (AMD). Med Sci Monit 2010;16:CR190–196.
20. **Shimoda-Matsubayashi S, Matsumine H, Kobayashi T, et al.** Structural dimorphism in the mitochondrial targeting sequence in the human manganese superoxide dismutase gene. A predictive evidence for conformational change to influence mitochondrial transport and a study of allelic association in Parkinson's disease. Biochem Biophys Res Commun 1996;226:561–5.
21. **Nixon RA, Cataldo AM, Mathews PM.** The endosomal-lysosomal system of neurons in Alzheimeŕs disease Pathogenesis: a Review. Neurochem Res 2000;25:1161–72.
22. **Steinfeld R, Reinhardt K, Schreiber K, et al.** Cathepsin D deficiency is associated with a human neurodegenerative disorder. Am J Hum Genet 2006;78:988–98.
23. **Mariani E, Seripa D, Ingegni T.** Interaction of CTSD and A2M polymorphisms in the risk for Alzheimer's disease. J Neurol Sci 2006;25:187–91.
24. **Touitou I, Capony F, Broullet JP, et al.** Missense polymorphism (C/T224) in the human cathepsin D pro-fragment determined by polymerase chain reaction single strand conformational polymorphism analysis and possible consequences in cancer cells. Eur J Cancer 1994;3:390–4.
25. **Papassotiropoulos A, Bagli M, Kurz A, et al.** A genetic variation of cathepsin D is a major risk factor for Alzheimer's disease. Ann Neurol 2000;47:399–403.
26. **Lin C, Wang S, Wu C, et al.** The association of a cystatin C gene polymorphism with late-onset Alzheimer's disease and vascular dementia. Chin J Physiol 2003;46:111–15.
27. **Mckhann GM, Knopman DS, Chertkow H, et al.** The diagnosis of dementia due to Alzheimeŕs disease: recommendations from the national Institute on Aging-Alzheimeŕs Association workgroups on diagnostic guidelines for Alzheimeŕs disease. Alzheimer Dement 2011;7:263–9.
28. **Alzheimer's society.** The Mini-Mental State Examination (MMSE) a guide for people with dementia and their carers. Information Sheet 2001.
29. **Appollonio I, Leone M, Isella V, et al.** The Frontal Assessment Battery (FAB): normative values in an Italian population simple. Neurol Sci 2005;26:108–16.
30. **Mucke L.** Alzheimer's disease. Nature 2009;15:895–7.
31. **Chistyakov DA, Savost'anov KV, Zotova EV, et al.** Polymorphisms at the SOD2 and SOD3 genes and their relation to diabetic Neuropathy in type 1 diabetes mellitus. BMC Med Genet 2000;1:2–4.
32. **Paz-y-Miño C, Carrera C, López-Cortés A, et al.** Genetic polymorphisms in apolipoprotein E and glutathione peroxidase 1 genes in the ecuadorian population affected with Alzheimer's disease. Am J Med Sci 2010;340:373–7.

33. **Wang V, Chen SY, Chuang TC, et al.** Val-9Ala and Ile+58Thr polymorphism of MnSOD in Parkinson's disease. Clin Biochem 2010;43: 979–82.

34. **Nacmias B, Bagnoli S, Tedde A, et al.** Angiotensin converting enzyme insertion/deletion polymorphism in sporadic and familial Alzheimer's disease and longevity. Arch Gerontol Geriatr 2007;45:201–6.

35. **Maruyama H, Izumi Y, Oda M, et al.** Lack of an association between cystatin C gene polymorphisms in Japanese patients with Alzheimer's disease. Neurology 2001;24:337–9.

36. **Dodel RC, Du Y, Depboylu C, et al.** A polymorphism in the cystatin C promoter region is not associated with an increased risk of AD. Neurology 2002;26:664.

37. **Roks G, Cruts M, Slooter AJ, et al.** The cystatin C polymorphism is not associated with early onset Alzheimer's disease. Neurology 2001; 24:366–7.

38. **Jeong BH, Lee KH, Lee YJ, et al.** Genetic polymorphism in exon 2 of cathepsin D is not associated with vascular dementia. Acta Neurol Scand 2011;123:419–23.