Mol Neurobiol (2018) 55:2340–2349
DOI 10.1007/s12035-017-0489-3

Association of the *CX3CR1*-V249I Variant with Neurofibrillary Pathology Progression in Late-Onset Alzheimer's Disease

Alan López-López[1] · **Ellen Gelpi**[2] · **Diana Maria Lopategui**[1,3] · **Jose M. Vidal-Taboada**[1,4]

Received: 8 January 2017 / Accepted: 14 March 2017 / Published online: 25 March 2017

Abstract Neuroinflammation and microglial dysfunction have a prominent role in the pathogenesis of late-onset Alzheimer's disease (LOAD). *CX3CR1* is a microglia-specific gene involved in microglia-neuron crosstalk and neuroinflammation. Numerous evidence show the involvement of *CX3CR1* in AD. The aim of this study was to investigate if some functional genetic variants of this gene could influence on LOAD's outcome, in a neuropathologically confirmed Spanish cohort. We designed an open, pragmatic, case-control retrospective study including a total of 475 subjects (205 pathologically confirmed AD cases and 270 controls). We analyzed the association of the two CX3CR1 functional variants (V249I, rs3732379; and T280M, rs3732378) with neurofibrillary pathology progression rate according to Braak's staging system, age at onset (AAO), survival time, and risk of suffering LOAD. We found that individuals heterozygous for *CX3CR1*-V249I presented a lower neurofibrillary pathology stage at death (OR = 0.42, 95%CI [0.23, 0.74], p = 0.003, adj-p = 0.013) than the other genotypes. Eighty percent of the subjects homozygous for 249I had higher neurofibrillary pathology progression (Braak's stage VI). Moreover, homozygosis for 280M and 249I could be associated with a higher AAO in the subgroups of AD with Lewy bodies and without Lewy bodies. These *CX3CR1* genetic variants could represent new modifying factors of pathology progression and age at onset in LOAD. These results provide further evidence of the involvement of CX3CR1 pathway and microglia/macrophages in the pathogenesis of LOAD.

Keywords Alzheimer disease · CX3CR1 · Fractalkine receptor (OMIM 601470) · Modifying gene · Progression · Age at onset

Electronic supplementary material The online version of this article (doi:10.1007/s12035-017-0489-3) contains supplementary material, which is available to authorized users.

✉ Jose M. Vidal-Taboada
josevidal@ub.edu; josevidalt@gmail.com

[1] Biochemistry and Molecular Biology Unit, Department of Biomedical Sciences, Faculty of Medicine–IDIBAPS, University of Barcelona, Barcelona, Spain

[2] Neurological Tissue Bank of the Biobank, Hospital Clinic, IDIBAPS, Barcelona, Spain

[3] Miami Clinical and Translational Science Institute, University of Miami, Miami, Florida, USA

[4] Institut de Neurociencies, University of Barcelona, Barcelona, Spain

Introduction

Alzheimer's disease (AD) is the most common type of dementia in our clinical setting. It mostly affects the elderly, and its prevalence reaches up to 45% in the population older than 85. Characterized by a progressive and serious decline in cognitive, memory, and language functions, it leads to severe disability and dependence with an enormous personal, familial, and social impact [1].

Although the ultimate cause of AD remains unknown for most cases, the pathogenesis of the disorder has been widely studied: an abnormal excision of amyloid precursor protein (APP) leads to the parenchymal and vascular deposition of insoluble fibrils of beta-peptide and to the formation of neuritic plaques and amyloid angiopathy. This phenomenon is accompanied by an abnormal activity of several neuronal kinases, which in turn lead to oxidative stress, mitochondrial dysfunction, activation and recruitment of microglia and astrocytes, and in some cases, to hyperphosphorylation, abnormal configuration, and accumulation of Tau protein. This last event destabilizes neuronal microtubules and generates

neurofibrillary tangles, the second main histological hallmark of AD [2]. About 15–20% of patients with AD dementia also present cortical and subcortical Lewy bodies (LB) [3]. It has been suggested that subjects with AD and LB comprise a distinct subgroup referred to as LBV/AD (Lewy bodies variant/Alzheimer's disease) [4].

Misfolded and aggregated proteins interact with microglia and astrocytes, triggering the innate immune response. This is part of the neuroinflammatory process in AD, which contributes to disease progression and severity and is associated also with neurotoxicity and synaptic dysfunction [5–7]. In fact, microglia has been pointed to have a major role in the clearance of oligomeric beta-amyloid, and different microglia phenotypes, such as microglial priming, may be associated to different disease outcomes [8–11].

For many years, only a few genes were associated with susceptibility to AD: *APP*, *PSEN1*, and *PSEN2* with genetically determined early-onset familial AD; and *APOE* for the most common late-onset AD (LOAD). Since the introduction of genome wide association studies (GWAS) in 2009, the knowledge of a much more complex genetic background has emerged. More than 20 loci have been identified which increase the susceptibility/risk to LOAD with small effect, including *BIN1*, *CR1*, *CLU*, *CD33*, *EPHA1*, *MS4A4/MS4A6*, *ABCA7*, *CD2AP*, *HLA-DRB5/DRB1*, *PTK2B*, *SLC24A4-RIN3*, *INPP5D*, *MEF2C*, *NME8*, *ZCWPW1*, *CELF1*, *FERMT2*, *CASS4*, *TREM2*, *PLD3*, *TBK1*, *CTNNA3 PICALM*, *SORL1*, *GAB2*, and *MTHFR* [12–16]. Interestingly, some patterns emerge when analyzing the functional relations of these genes, and three main pathways in the pathogenesis of AD have been established: the lipid-processing pathway (*APOE*, *CLU*, and *ABCA7*), the immune system (*CLU*, *CR1*, *ABCA7*, *CD33*, *TREM2*, *IL1A*, *IL1B*, *2*, and *EPHA1*), and the synaptic-cell-functioning pathway (*PICALM*, *BIN1*, *CD33*, and *CD2AP*) [17–19]. Also, single nucleotide polymorphisms (SNPs) in the SNCA genes have been reported to contribute to LB pathology in LBV/AD patients, possibly via interaction with SNPs in the LRRK2 gene [20].

Recently, two rare mutations (in TREM2 and CD33) support the view that microglia has a fundamental role in AD. The SNP rs75932628 of TREM 2 is associated with increased risk of AD [21, 22]. TREM2 encodes for the triggering receptor highly expressed by microglia which mediates phagocytic activity and debris clearance [14]. Similarly, CD33-rs3865444 carriers have been shown to have increased β amyloid deposition [23], caused by a defective microglial β-amyloid uptake [24].

The *CX3CR1* gene (chemokine (C-X3-C motif) receptor 1, also known as fractalkine receptor, OMIM: 601470) in the CNS is a microglia-specific gene that mediates its neurotrophic functions, and it is involved in the microglia-neuron crosstalk [25]. Its ligand, fractalkine (CX3CL1), appears to be neuroprotective in some settings, whereas it contributes to neuronal damage in others. AD, and many progressive neuroinflammatory disorders associated with increased microglial activation, shows disruption of the CX3CL1/CX3CR1 communication system [26]. The impairment of CX3CL1/CX3CR1 signaling has a direct influence on the processes of neuroinflammation, neurotoxicity, and synaptic plasticity in the pathogenesis of AD and other neurodegenerative diseases [7, 8, 26]. Fractalkine has been suggested as an endogenous neuronal modulator that may limit microglial activity in AD [27, 28] by reducing the inflammatory reaction through fractalkine/NRF2/heme oxygenase 1 pathway [29]. Two of its genetic variants (V249I and T280M) have been described to affect CX3CR1 protein activity [30, 31]. Recently, our group has found an association of the CX3CR1 V249I and T280M variants with the survival and disease progression in amyotrophic lateral sclerosis (ALS) [32]. These variants also have been associated with several inflammatory and degenerative human conditions, including multiple sclerosis [33], Crohn's disease [34], AIDS [35], and age-related macular degeneration [36].

Despite numerous evidence showing involvement of *CX3CR1* in AD, and in several central nervous system conditions, no human genetic studies have been performed to investigate its influence on the pathogenesis of Alzheimer's disease. We hypothesized that *CX3CR1* genetic variants may influence AD outcome. Here, we analyzed the association between these two *CX3CR1* variants, and the disease risk, age at onset, and pathologic progression rate in a Spanish late onset AD (LOAD) cohort, including a subgroup of LOAD patients with LB (LBV/AD).

Subjects and Methods

Study Population

A total of 955 subjects were recruited for the study. 205 brain donors with pathologically proven late onset AD (LOAD) were recruited from the Neurological Tissue Bank of the Biobank-Hospital Clinic-IDIBAPS, Barcelona, Spain. Their DNA was obtained from fresh frozen cerebellar cortex. 750 healthy controls were obtained from the Spanish National DNA Bank (BNADN), and their DNA sample obtained from peripheral blood samples (270 controls were randomly selected by age and gender to match the cases).

The diagnosis of LOAD was defined as an age of onset or diagnosis ≥65 years according to clinical charts and required a high or intermediate score of AD neuropathological change according to the current National Institute on Aging, Alzheimer's Association guidelines, characterized by the presence of moderate or severe neuritic plaque density and neocortical or limbic neurofibrillary pathology (tangles and threads) in the context of dementia [37]. The presence of

concomitant Lewy body (LB) pathology was assessed in brainstem nuclei, limbic system, and cortical areas by hematoxylin-eosin stain and anti-alpha-synuclein immunohistochemistry in selected regions. LB pathology was subclassified as brainstem, limbic, neocortical, or amygdala only [38]. Information regarding age, gender, and clinical diagnosis was collected for all samples with available information. Age at onset (AAO), survival time, and rate of progression were recorded for AD cases (see demographic data at Table 1). Braak's staging system of neurofibrillary pathology (transentorhinal stages I and II, limbic stages III and IV, and neocortical stages V and VI) was used for the assessment of progression [38].

DNA Purification and Genotyping

DNA was isolated from postmortem fresh frozen cerebellar tissue using a variant of the silica affinity in guanidine tiocianate method developed in-house by our group. Briefly, 25–50 mg of brain tissue were thawed in 100 μl of lysis buffer (EDTA 20 mM, guanidine tiocianate 6M, K_3PO_4 20 mM, 1% triton X-100, and DTT 6.5 mM, pH = 6.8) and were broken down using an automatic pestle. The sample was further processed by squeezing the tissue homogenate five times through a 20 G syringe after adding 600 μl lysis buffer. After incubation for at least 3 min at room temperature, centrifugation (1 min at >13,000×*g*) and removal of the pellet, the supernatant was incubated in a silica-based affinity chromatographic column (Econospin, Epoch Life Sciences) for 1 min at room temperature. The DNA retained in the silica column was washed twice with 300 μl wash solution (25%ethanol, 25% isopropanol, 10 mM Tris-Cl, and 100 mM NAcl, pH = 8.0) and was centrifuged (1 min at 13000×*g*). DNA was finally eluted in 50 μl TE-reduced buffer (10 mM Tris-Cl and 0.1 mM EDTA, pH = 8.0), was prewarmed at 65 °C, and was centrifuged (1 min at 13000×*g*). This elution step was repeated once.

CX3CR1 V249I (rs3732379) and T280M (rs3732378) alleles were genotyped using the KASPar® SNP Genotyping system (LGC genomics, UK) according to the standard provider procedures. *CX3CR1* gene T280M and V249I variants were identified after amplification with V249I primers set (249_V:CTT CTG GAC ACC CTA CAA CG; 249_I:CCT CTT CTG GAC ACC CTA CAA CA; 249rev:GAG CTT AAG YGT CTC CAG GAA AAT CAT) and T280M primer set (280_T:GGC CCT CAG TGT GAC TGA GAC; 280_M:GGC CCT CAG TGT GAC TGA GAT; 280_rev:GAG AGG ATT CAG GCA ACA ATG GCT A). Fluorescence was measured at 25 °C (final point) in a 7300 real time PCR System (Applied Bioscience). Genotype calling was carried out using 7300 system SDS software v1.4 (Applied Biosytems, USA) and Klustercaller software (LGC genomics, UK).

Statistical Design, Analyses, and Statistical Power

We designed an open, pragmatic, case-control retrospective study with a total of 475 subjects divided into two cohorts: the control cohort of 270 individuals without neurodegenerative diseases (randomly selected by age and gender according to cases), and the case cohort of 205 patients with pathologically proven LOAD. The case cohort was further divided in 68 LOAD patients with LB (LBV/AD subgroup) and 137 LOAD patients without LB pathology (LB−/AD subgroup). Statistical data analyses were performed using the linear generalized models implemented in the SNPassoc R software package [39]. For progression analysis, ordinal logistic regression was carried out using SPSS software.

Sample population was tested for Hardy-Weinberg equilibrium using Fisher's exact test. The analyses of allelic frequencies proved that cases and controls' genotypes were in Hardy-Weinberg equilibrium (V249I $p = 0.912$, T280M $p = 0.243$).

Table 1 Demographic data of the cohorts: late-onset AD (LOAD) or LOAD without LB (LB−/AD) or LOAD with LB (LBV/AD) patients

		Controls	LOAD	LB−/AD	LBV/AD
Subjects	All	270	205	137	68
Gender	Men[b]	94 (34.8%)	70 (34.63%)	48	22
	Women[b]	176 (65.2%)	134 (65.37%)	89	46
Age	All[a]	64.97 ± 14.14	83.67 ± 6.12	83.43 ± 6.39	84.15 ± 5.54
	Men[a]	63.5 ± 11.39	82.30 ± 5.74	82.52 ± 6.20	81.82 ± 4.68
	Women[a]	65.75 ± 15.38	84.42 ± 6.21	83.92 ± 6.47	85.40 ± 5.60
Age at onset	All[a]		75.25 ± 5.87	75.57 ± 5.87	74.55 ± 5.87
	95% CI		74.379–76.126	74.54–76.63	72.96–76.13
Braak's stage Progression	IV		12(5.85%)	9 (6.6%)	3 (4.4%)
	V		83(40.49%)	52 (38.0%)	31 (40.5%)
	VI		110(53.66%)	76 (55.4%)	34 (53.7%)

Statistics format: [a] Mean ± SD, [b] *n* (%)

In the LOAD cohort, the effect of the two *CX3CR1* genetic variants was analyzed for association with AD risk, survival time, progression, and AAO. Analyses were performed for five different inheritance models: dominant, co-dominant, additive, recessive, and over-dominant. Sex, age, and/or AAO were introduced as adjusting variables. *P* values were modified by a factor of 4.3 to correct for multiple test comparison with Bonferroni method [39, 40]. Using this criterion, the uncorrected level for statistical significance was established at $p < 0.0116$. The uncorrected and corrected *p* values (adjusted or adj-*p*) are shown.

This study has a prior statistical power of 80% (alpha = 0.05) for detecting a difference in age of onset between groups of patients (mean population age of onset = 75.25 ± 5.87 years; 280M minor allele frequency = 0.145) greater than 2.3 years. Concerning the risk to suffer AD, this study has a power of 80% (alpha = 0.05) for detecting an OR > 1.49 between cases and controls for the 249I (minor allele frequency of 0.28).

Results

CX3CR1 Variants and AD Susceptibility

In the analysis of *CX3CR1* variants V249I and T280M with the disease risk in the whole LOAD group, or LBV/AD or the LB−/AD subgroups, we did not find any statistical significant association of neither variant with AD risk under different inheritance models (Tables 2 and 3). But nevertheless in the LBV/AD subgroup, we observed a statistical association tendency of V249I variant with LOAD risk ($p = 0.074$, Table 2).

CX3CR1 Variants and Neurofibrillary Pathology Progression

We found a statistically significant association of the V249I variant with neurofibrillary pathology stages, when assuming a co-dominant, over-dominant, and recessive inheritance pattern (Tables 2 and 4). Patients with CX3CR1 249V/I genotypes were over-represented in Braak stage IV subgroup (limbic pathology stage) and were under-represented in the higher neocortical neurofibrillary Braak stage VI subgroup (Table 4, Fig. 1). The protective effect of the 249V/I genotype for neurofibrillary pathology progression was higher (OR = 0.416, 95%CI [0.232, 0.737], $p = 0.003$, adj-$p = 0.013$) under an over-dominant genetic model. Instead, under a recessive model, patients with the CX3CR1 249I/I genotypes were more frequent (80%) in the highest neocortical pathology subgroups (Braak stage VI that includes primary cortical fields) than in the lower neocortical pathology group (20%; Braak stage V, that includes cortical association areas but preserves primary cortical fields) while no 249^{I} homozygous individuals were observed in the lower limbic stages (Table 4, Fig. 1). The risk effect of the CX3CR1 249I/I genotype on the progression of neurofibrillary pathology was OR = 3.967 (95%CI [1.271, 12.391], $p = 0.018$) under a recessive model.

Table 2 Summary of *P* values for genetic analyses of the CX3CR1 V249I variant with the different phenotypes analyzed in LOAD or LB−/AD or LBV/AD groups using different genetic models

	Co-dominant	Dominant	Recessive	Over-dominant	Additive
Disease risk					
LOAD	0.118	0.549	0.091	0.117	0.793
LB−/AD	0.189	0.465	0.168	0.138	0.997
LBV/AD	0.147	0.838	0.074	0.217	0.528
Progression					
LOAD	*0.003*	0.175	*0.008*	*0.003*	0.901
LB−/AD	*0.001*	0.218	*0.003*	*0.004*	0.961
LBV/AD	0.677	0.899	0.449	0.515	0.794
Age of onset					
LOAD	0.084	0.896	0.563	0.831	0.725
LB−/AD	0.822	0.692	0.548	0.962	0.575
LBV/AD	0.121	0.460	0.038	0.618	0.138
Survival					
LOAD	0.851	0.779	0.718	0.609	0.977
LB−/AD	0.371	0.323	0.193	0.809	0.188
LBV/AD	0.140	0.642	0.046	0.509	0.224

Numbers represent non corrected *p* values adjusted by sex (sex and age in disease risk assessment). Multiple test correction cut-off is set to $p < 0.0116$. Statistically significant results are highlighted in italics

Table 3 Summary of *p* values of the genetic analyses of the CX3CR1 T280M variant with the different phenotypes analyzed in LOAD or LB−/AD or LBV/AD groups using different inheritance models

	Co-dominant	Dominant	Recessive	Over-dominant	Additive
Disease risk					
LOAD	0.986	0.871	0.995	0.867	0.884
LB−/AD	0.905	0.743	0.819	0.688	0.812
LBV/AD	0.864	0.988	0.598	0.867	0.864
Progression					
LOAD	0.430	0.315	0.292	0.510	0.229
LB−/AD	0.300	0.357	0.151	0.642	0.215
LBV/AD	0.819	0.565	0.925	0.525	0.645
Age of onset					
LOAD	0.171	0.838	0.062	0.675	0.467
LB−/AD	0.038	0.546	*0.010*	0.785	0.193
LBV/AD	0.830	0.722	0.575	0.847	0.634
Survival					
LOAD	0.545	0.441	0.036	0.611	0.343
LB−/AD	0.239	0.102	0.393	0.171	0.089
LBV/AD	0.551	–	–	–	–

Numbers represent non corrected *p* values adjusted by sex (sex and age in disease risk assessment). Multiple test correction cut-off is set to $p < 0.0116$. Statistically significant results are highlighted in italics

Analysis of the pathology progression in the LOAD LB- and LBV/AD subgroups showed the same effect of the V249I variant only in those patients that did not develop LB during AD pathology (Table 2 and Online Resource 1). Despite the smaller sample size of the LOAD LB- subgroup compared with the whole LOAD group, the heterozygous genotype 249V/I showed a larger protective effect for neurofibrillary pathology progression (OR = 0.352, 95%CI [0.173, 0.715], $p = 0.004$, adj-$p = 0.017$) under an over-dominant model (Online Resource 1). The homozygous genotype 249I/I also showed a larger risk effect for disease progression under a recessive model (OR = 10.57 (95%CI [1.315, 84.94], $p = 0.027$; Online Resource 1).

No association has been detected between the variant T280M and neurofibrillary pathology stage for LOAD group or the LBV/AD and LB−/AD subgroups (Table 3).

CX3CR1 Variants and Age at Onset

Our analyses indicated that LB−/AD patients homozygous for the 280^{M} allele showed a later AAO (84.0 years ±3.61) than individuals carrying one (74.85 ± 0.82) or both wild-type alleles (75.12 ± 0.53). Assuming a recessive inheritance model, the mean difference of 8.71 years (95%CI [2.06 to 15.37] years was statistically significant ($p = 0.0099$, adj-$p = 0.43$) (Tables 3 and 5, Fig. 1). No statistically significant association was found for T280M variant with the AAO for LOAD (all) group or LBV/AD subgroup (Table 3).

In the LBV/AD subgroup, patients with 249I/I genotype showed an earlier AAO (69.17 ± 1.76, $n = 6$) compared to genotypes 249V/I (74.76 ± 1.11, $n = 21$) and 249V/V (74.74 ± 1.15, $n = 27$). The AAO difference (69.17 ± 1.76 vs 74.75 ± 0.80, $n = 48$) was −5.04 years (95%CI [−0.28,

Table 4 CX3CR1 V249I marker analysis for Braak's neurofibrillary pathology progression in LOAD patients assuming different genetic models

Genetic model	Genotype	Braak's neurofibrillary stage (*n*)			Effect for neurofibrillary progression		
		IV	V	VI	OR (95%CI)	*p* value	Model *p* value
Co-dominant	*V*/V	5 (4.6%)	41 (37.6%)	63 (57.8%)	0.0		
	V/I	7 (10%)	35 (50%)	28 (40%)	0.478 (0.264, 0.862)	*0.014*	*0.003*
	I/I	0 (0%)	4 (20%)	16 (80%)	2.974 (0.930, 9.516)	0.066	
Over-dominant	V/V + I/I	5 (3.9%)	45 (34.9%)	79 (61.2%)	0.0		
	V/I	7 (10%)	35 (50%)	28 (40%)	0.416 (0.232, 0.737)	*0.003*	*0.003*
Recessive	V/V + V/I	12 (6.7%)	76 (42.5%)	91 (50.8%)	0.0		
	I/I	0 (0%)	4 (20%)	16 (80%)	3.967 (1.271, 12.39)	*0.018*	*0.008*

Genotype correspondence: *V* $rs3732379^{C}$ allele, *I* $rs3732379^{T}$ allele

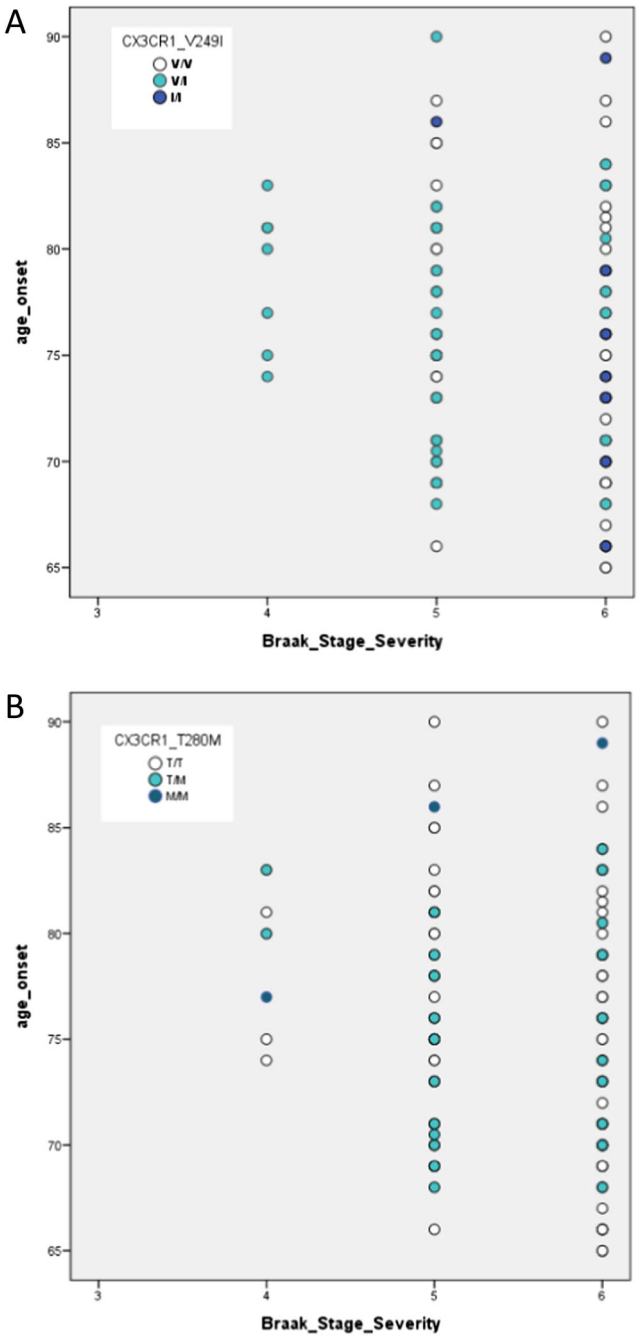

Fig. 1 Scatterplot for age at onset and Braak's neurofibrillary stage severity of the different *CX3CR1*-V249I (**a**) and T280M (**b**) genotypes, according to a recessive genetic model

−9.8], $p = 0.038$) for the homozygous patients, being statistically nominal significant under a recessive genetic model. No statistical association was found for V249I variant with the AAO for LOAD group or LB−/AD subgroup (Table 2).

CX3CR1 Variants and Survival

Analyses of the two *CX3CR1* variants using different genetic models did not show any statistically significant difference regarding survival among LOAD patients or the LB-subgroup (Tables 2 and 3). In the LBV/AD subgroup, under a recessive genetic model, the patients with genotype 249I/I showed a longer survival (163.2 months ± 20.3, $n = 5$) compared to genotypes 249V/I (110.9 months ± 14.1, $n = 21$) and 249V/V (113.3 ± 10.7, $n = 27$). Patients homozygous for the 249^{I} allele had a survival of 55.48 months longer (95%CI [097, 110], $p = 0.046$). Although there was a nominal statistically significance, the observed difference did not pass the multiple correction test (adj-$p = 0.207$).

Discussion

In our pathologically confirmed LOAD series, we have detected a novel association between the V249I variant of the microglial-specific gene *CX3CR1* and the progression of neurofibrillary pathology in LOAD; while the variants T280M and V249I seem to be associated with the age at onset. These results suggest that the CX3CR1 gene could act as a disease-modifying gene in the pathogenesis of AD.

The most significant observation of our study concerns the neurofibrillary pathology progression. We have observed that the *CX3CR1* V249I allele was related to the pathology stage in our Spanish cohort of LOAD patients. Most homozygous patients for 249^{I} allele showed a worse AD neurodegenerative progression at death with high Braak's neurofibrillary stages (stages VI and V). This result is in accordance with the fact that the same *CX3CR1* 249I allele was associated with a worse disease progression in a Spanish ALS cohort [32].

Heterozygous patients carrying the two alleles of the 249I variant (249V/I genotype) showed a lower Braak neurofibrillary stage than homozygous patients (249V/V genotype, Fig. 1). This indicates that heterozygous patients had less involvement of neocortical anatomical areas by neurofibrillary pathology; hence, a less severe neurodegenerative process, which could possibly indicate a heterozygous advantage: patients harboring two different alleles of the CX3CR1 V249I variant could have better means and resources in different/specific brain areas, reducing or delaying topographical disease progression. This may be related to the functions of CX3CR1 as a specific microglial gene in the CNS, with a fundamental role in the neuron-microglial crosstalk [26] and on microglial activation [36, 41]. Having two alleles (V and I) at position 249 could influence the versatility of microglia to change from a predominantly cytotoxic phenotype to one mostly neuroprotective, or vice versa; either at the initial neuronal damage or during the progressive chronic lesions.

The CX3CR1 249^{I} variant has been associated with reduced number of fractalkine (CX3CL1) binding sites and reduced binding affinity on peripheral blood mononuclear

Table 5 CX3CR1-T280M variant for age at onset (AAO) for LOAD, LBV/AD, and LB−/AD, under a recessive model of inheritance

Group	Genotype	*n*	AAO (median ± SEM)	Difference (95% CI)	*p* value	Adj-*p*
LOAD	G/G-A/G	170	75.04 ± 0.44			
	A/A	4	80.50 ± 34.33	5.55 (2–0.27, 11.37)	0.062	0.271
LB−/AD	G/G -A/G	115	75.28 ± 0.54			
	A/A	3	84.00 ± 3.61	8.71 (2.06, 15.37)	*0.010*	*0.043*
LBV/AD	G/G-A/G	55	74.55 ± 0.79			
	A/A	1	70.00 ± 0.0	−3.37 (−15.16, 8.42)	0.575	1.00

Adjusted by sex. Genotype correspondence: T = rs3732378^{G} allele, M = rs3732378^{A} allele. Statistically significant results are highlighted in italics

cells, resulting in a loss of function [42]. It has been suggested that CX3CL1/CX3CR1 may act in a neuroprotective pathway and as first-line of defense response to neuronal injury and neuroinflammation [26]. An altered modulation of CX3CL1/CX3CR1 signaling would promote neuroinflammation, neurotoxicity, and changes in synaptic plasticity in AD pathogenesis [7, 26, 29]. Thus, the decreased CX3CR1 activity of the 249^{I} allele would reflect an impaired microglial function that may contribute to AD pathogenesis, in part, by enhancing inflammatory activity of microglia and also neuronal degeneration leading to disease progression.

Concerning to the association of the CX3CR1 variants with the age at onset (AAO), the development of Lewy bodies (LB) during AD pathology (LBV/AD subgroup) modifies the effect of the variants under a recessive genetic model. In our Spanish LBV/AD subgroup, the V249I/I genotype could be associated with an earlier AAO (−5.04 years). In contrast, in the LOAD LB-subgroup, homozygosis for 280^{M} allele seems to be associated with a later AAO. It has been described that LBV/AD patients show both overlaps and differences with LB−/AD patients, and it has been suggested that LBV/AD subjects may comprise a distinct subset. At the present moment, however, the nosological position of LBV/AD within the broad spectrum of AD and LBD remains disputable [20, 43–46]. Our results indicate that genetic differences exist between late onset LBV/AD and LB−/AD subgroups, but further research is needed to address this issue.

Besides the influence of the presence of LB in AD pathology, another factor that could explain the opposed effects of *CX3CR1* variants (the earlier AAO for 249^{I} in LBV/AD and the later AAO for 280^{M} in LB−/AD) is the counter-acting effects of these two SNP's. Naissner et al. postulated that increased fractalkine concentration would outweigh the moderately reduced binding site density in subjects carrying the 249^{I} variant [47]. Similarly to those findings, McDermott et al. suggested that any harmful effects of 249^{I} allele could be reversed by the protective effect of 280^{M} allele [30]. Therefore, 249^{I} and 280^{T} variants might have counter-acting effects. Something which makes evolutionary sense as the allele 280M occurs mostly in the presence of 249^{I} allele [48].

In our Spanish cohort, 280M/M and 249I/I genotypes seem to modify the AAO, with an unexpectedly high mean difference of 8.7 and −5.4 years, respectively. If this is confirmed by further studies in different cohorts, *CX3CR1* variants could be the second strongest genetic factor described up to date which modify disease onset in LOAD. The strongest factor modulating AAO in AD is the APOE ε4 allele, first described in 1993 (0 copies: 84.3 years; 1 copy: 75.5; 2 copies: 68.4) [49] and widely reproduced [50, 51]. Other genes have been proposed as strong modifiers of disease onset in LOAD, such as *SORL1*(rs1784933AA genotype causes an AAO of 2.5 years earlier in homozygosis) [52] and *HMGCR* (in women delayed about 3.6 years the AAO) [53].

None of the GWAS studies that analyzed AAO in LOAD have found any association with the *CX3CR1* gene or loci [12, 54–57]. However, the magnitude of the effect we observed is unexpectedly high. This might be related to the sample heterogeneity because GWAS studies are using only clinically diagnosed cases and not neuropathologically confirmed cases. Another factor to take into account is the fact that the frequency of the variants are low (14.2% for 280M and 28% for 249I); and even lower for the genotype in homozygosis (2.5% of the cases for 280M and 10.1% for 249I); as such, in our Spanish series, only three patients were homozygous for 280^{M} allele and six for 249^{I} allele. This low frequency could also explain why the effect of *CX3CR1* 280M variant on AAO remained undetected in previous GWAS. Therefore, while rare variants may remain undetected in GWAS, targeted hypothesis studies have more statistical power to succeed [58].

Achieving more accurate diagnoses is essential to make progress in this field of research, and Brain Banks constitute a highly valuable source of neuropathologically confirmed neurodegenerative diseases. They have the potential to contribute in clarifying the existent unknowns concerning AD's causes and mechanisms [59]. The homogeneity and appropriate diagnostic classification of patients is always an important point to take into account when studying genetics of neurodegenerative diseases. In this respect, postmortem neuropathological diagnostic confirmation is an added value to our study.

It has been previously reported that the origin of the samples regarding clinical vs. pathologically confirmed AD has effect on the results [60]. Strengths of our design are the homogeneity of clinical-pathological diagnoses, and the fact that all samples were analyzed at the same Brain Bank, which minimizes observer variation and sources of bias. Even though our study has enough power to detect the differences observed in disease progression and age on onset, the possibility of a "statistically" spurious association cannot be disregarded completely, and we encourage the replication of our findings in larger cohorts and different populations.

Our finding supports the well-known intervention of neuroinflammation in the pathogenesis of LOAD. These *CX3CR1* associated variants, added to the previously described in *TREM2* and *CD33* [24, 61], provide further evidence of the fundamental role of microglia/macrophages in the outcome of LOAD. Interestingly, *TREM2* and *CX3CL1* (*CX3CR1* ligand) are over-expressed in AD hippocampal samples, thus, suggesting that dysfunction is those pathways may be an important hallmark of pathological neuroinflammatory processes in AD [62]. In this regard, it would be interesting to further investigate the possible interactions of these three microglia-associated genes (*CX3CR1*, *TREM2*, and *CD33*) in LOAD cases.

The reported results could be relevant in the clinical setting, considering CX3CR1variants as genetic markers and prognostic factors for AD progression, and point to CX3CR1 as an interesting target for AD therapy development. Also, CX3CR1 could be proposed as a potential pharmacogenetic marker useful for the development of new targeted AD treatments and personalized therapies, in a similar way that have been applied in the development of therapies for cancer (imatinib metasilate, transtuzumab, and others) or cystic fibrosis (Ivakaftor).

As conclusions, the variants V249I (rs3732379) and T280M (rs3732378) of the *CX3CR1* microglial gene could represent new modifying factors of age at onset and pathology progression in a neuropathologically confirmed Spanish LOAD population. Homozygous patients for the 249^{I} allele have a higher neurofibrillary Braak stage at death, i.e., a more advanced AD-related neurodegenerative process than heterozygous patients. Homozygous patients for the 280^{M} allele could have a delayed onset of the illness. Replication of this study with a larger sample size will be required to confirm the observed associations in other populations.

Acknowledgements The authors are indebted to the patients and their relatives for their cooperation. Authors acknowledge the Spanish National DNA Bank (Salamanca, Spain) and Neurological Tissue Bank of the Biobank-Hospital Clinic-IDIBAPS (Barcelona, Spain) for the data and sample procurement.

Compliance with Ethical Standards The protocol of this study was approved by the IRB from the Hospital Clinic of Barcelona, the Spanish National DNA Bank, and the IDIBAPS Biobank Ethics and Scientific Committees. All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration. All patients and/or close relatives, in case of brain donors, gave their informed written consent for the use of brain tissue for research purposes. This article does not contain any studies with animals performed by any of the authors.

Funding This study was funded by Ministerio de Economia y Competitividad (grant IPT-2012-0614-010000) and by Generalitat de Catalunya (grant 2014SGR1115). ALL held a fellowship from the Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS).

Conflict of Interest The authors declare that they have no conflict of interest.

References

1. Querfurth HW, LaFerla FM (2010) Alzheimer's disease. N Engl J Med 362:329–344. doi:10.1056/NEJMra0909142
2. Glass CK, Saijo K, Winner B et al (2010) Mechanisms underlying inflammation in neurodegeneration. Cell 140:918–934. doi:10.1016/j.cell.2010.02.016
3. Parkkinen L, Soininen H, Alafuzoff I (2003) Regional distribution of alpha-synuclein pathology in unimpaired aging and Alzheimer disease. J Neuropathol Exp Neurol 62:363–367
4. Hansen LA (1997) The Lewy body variant of Alzheimer disease. J Neural Transm Suppl 51:83–93
5. Rao JS, Kellom M, Kim H-W, Rapoport SI (2012) Neuroinflammation and synaptic loss. Neurochem Res 37:903–910. doi:10.1007/s11064-012-0708-2
6. Fan Z, Okello AA, Brooks DJ, Edison P (2015) Longitudinal influence of microglial activation and amyloid on neuronal function in Alzheimer's disease. Brain J Neurol 138:3685–3698. doi:10.1093/brain/awv288
7. Chen P, Zhao W, Guo Y et al (2016) CX3CL1/CX3CR1 in Alzheimer's disease: A target for Neuroprotection. Biomed Res Int 2016:8090918. doi:10.1155/2016/8090918
8. Simard AR, Soulet D, Gowing G et al (2006) Bone marrow-derived microglia play a critical role in restricting senile plaque formation in Alzheimer's disease. Neuron 49:489–502. doi:10.1016/j.neuron.2006.01.022
9. Perry VH, Teeling J (2013) Microglia and macrophages of the central nervous system: The contribution of microglia priming and systemic inflammation to chronic neurodegeneration. Semin Immunopathol 35:601–612. doi:10.1007/s00281-013-0382-8
10. Heneka MT, Carson MJ, El Khoury J et al (2015) Neuroinflammation in Alzheimer's disease. Lancet Neurol 14:388–405. doi:10.1016/S1474-4422(15)70016-5
11. Meyer-Luehmann M, Prinz M (2015) Myeloid cells in Alzheimer's disease: Culprits, victims or innocent bystanders? Trends Neurosci 38:659–668. doi:10.1016/j.tins.2015.08.011
12. Naj AC, Jun G, Beecham GW et al (2011) Common variants at MS4A4/MS4A6E, CD2AP, CD33 and EPHA1 are associated with late-onset Alzheimer's disease. Nat Genet 43:436–441. doi:10.1038/ng.801

13. Chouraki V, Seshadri S (2014) Genetics of Alzheimer's disease. Adv Genet 87:245–294. doi:10.1016/B978-0-12-800149-3.00005-6
14. Karch CM, Goate AM (2015) Alzheimer's disease risk genes and mechanisms of disease pathogenesis. Biol Psychiatry 77:43–51. doi:10.1016/j.biopsych.2014.05.006
15. Wang Z, Lei H, Zheng M et al (2016) Meta-analysis of the association between Alzheimer disease and variants in GAB2, PICALM, and SORL1. Mol Neurobiol 53:6501–6510. doi:10.1007/s12035-015-9546-y
16. Rai V (2016) Methylenetetrahydrofolate Reductase (MTHFR) C677T polymorphism and Alzheimer disease risk: A meta-analysis. Mol Neurobiol:1–14. doi:10.1007/s12035-016-9722-8
17. Morgan K (2011) The three new pathways leading to Alzheimer's disease. Neuropathol Appl Neurobiol 37:353–357. doi:10.1111/j.1365-2990.2011.01181.x
18. Schellenberg GD, Montine TJ (2012) The genetics and neuropathology of Alzheimer's disease. Acta Neuropathol (Berl) 124:305–323. doi:10.1007/s00401-012-0996-2
19. Dong X, Zhang L, Meng Q, Gao Q (2016) Association between interleukin-1A, interleukin-1B, and bridging integrator 1 polymorphisms and Alzheimer's disease: A standard and cumulative meta-analysis. Mol Neurobiol. doi:10.1007/s12035-015-9683-3
20. Linnertz C, Lutz MW, Ervin JF et al (2014) The genetic contributions of SNCA and LRRK2 genes to Lewy body pathology in Alzheimer's disease. Hum Mol Genet 23:4814–4821. doi:10.1093/hmg/ddu196
21. Guerreiro R, Wojtas A, Bras J et al (2013) TREM2 variants in Alzheimer's disease. N Engl J Med 368:117–127. doi:10.1056/NEJMoa1211851
22. Lu Y, Liu W, Wang X (2015) TREM2 variants and risk of Alzheimer's disease: A meta-analysis. Neurol Sci Off J Ital Neurol Soc Ital Soc Clin Neurophysiol. doi:10.1007/s10072-015-2274-2
23. Bradshaw EM, Chibnik LB, Keenan BT et al (2013) CD33 Alzheimer's disease locus: Altered monocyte function and amyloid biology. Nat Neurosci 16:848–850. doi:10.1038/nn.3435
24. Griciuc A, Serrano-Pozo A, Parrado AR et al (2013) Alzheimer's disease risk gene CD33 inhibits microglial uptake of amyloid beta. Neuron 78:631–643. doi:10.1016/j.neuron.2013.04.014
25. Wolf Y, Yona S, Kim K-W, Jung S (2013) Microglia, seen from the CX3CR1 angle. Front Cell Neurosci 7:26. doi:10.3389/fncel.2013.00026
26. Sheridan GK, Murphy KJ (2013) Neuron-glia crosstalk in health and disease: Fractalkine and CX3CR1 take centre stage. Open Biol 3:130181. doi:10.1098/rsob.130181
27. Cardona AE, Pioro EP, Sasse ME et al (2006) Control of microglial neurotoxicity by the fractalkine receptor. Nat Neurosci 9:917–924. doi:10.1038/nn1715
28. Lee S, Varvel NH, Konerth ME et al (2010) CX3CR1 deficiency alters microglial activation and reduces beta-amyloid deposition in two Alzheimer's disease mouse models. Am J Pathol 177:2549–2562. doi:10.2353/ajpath.2010.100265
29. Lastres-Becker I, Innamorato NG, Jaworski T et al (2014) Fractalkine activates NRF2/NFE2L2 and heme oxygenase 1 to restrain tauopathy-induced microgliosis. Brain J Neurol 137:78–91. doi:10.1093/brain/awt323
30. McDermott DH, Fong AM, Yang Q et al (2003) Chemokine receptor mutant CX3CR1-M280 has impaired adhesive function and correlates with protection from cardiovascular disease in humans. J Clin Invest 111:1241–1250. doi:10.1172/JCI16790
31. Daoudi M, Lavergne E, Garin A et al (2004) Enhanced adhesive capacities of the naturally occurring Ile249–Met280 variant of the chemokine receptor CX3CR1. J Biol Chem 279:19649–19657. doi:10.1074/jbc.M313457200
32. Lopez-Lopez A, Gamez J, Syriani E et al (2014) CX3CR1 is a modifying gene of survival and progression in amyotrophic lateral sclerosis. PLoS One 9:e96528. doi:10.1371/journal.pone.0096528
33. Arli B, Irkec C, Menevse S et al (2013) Fractalkine gene receptor polymorphism in patients with multiple sclerosis. Int J Neurosci 123:31–37. doi:10.3109/00207454.2012.723079
34. Brand S, Hofbauer K, Dambacher J et al (2006) Increased expression of the chemokine Fractalkine in Crohn's disease and association of the Fractalkine receptor T280M polymorphism with a Fibrostenosing disease phenotype. Am J Gastroenterol 101:99–106
35. Faure S, Meyer L, Costagliola D et al (2000) Rapid progression to AIDS in HIV+ individuals with a structural variant of the chemokine receptor CX3CR1. Science 287:2274–2277. doi:10.1126/science.287.5461.2274
36. Zhang R, Wang L-Y, Wang Y-F et al (2015) Associations between the T280M and V249I SNPs in CX3CR1 and the risk of age-related macular degeneration. Invest Ophthalmol Vis Sci 56:5590–5598. doi:10.1167/iovs.15-16830
37. Montine TJ, Phelps CH, Beach TG et al (2012) National Institute on Aging-Alzheimer's association guidelines for the neuropathologic assessment of Alzheimer's disease: A practical approach. Acta Neuropathol (Berl) 123:1–11. doi:10.1007/s00401-011-0910-3
38. Braak H, Alafuzoff I, Arzberger T et al (2006) Staging of Alzheimer disease-associated neurofibrillary pathology using paraffin sections and immunocytochemistry. Acta Neuropathol (Berl) 112:389–404. doi:10.1007/s00401-006-0127-z
39. González JR, Armengol L, Solé X et al (2007) SNPassoc: An R package to perform whole genome association studies. Bioinforma Oxf Engl 23:644–645. doi:10.1093/bioinformatics/btm025
40. Vidal-Taboada JM, Lopez-Lopez A, Salvado M et al (2015) UNC13A confers risk for sporadic ALS and influences survival in a Spanish cohort. J Neurol 262:2285–2292. doi:10.1007/s00415-015-7843-z
41. Maphis N, Xu G, Kokiko-Cochran ON et al (2015) Reactive microglia drive tau pathology and contribute to the spreading of pathological tau in the brain. Brain J Neurol 138:1738–1755. doi:10.1093/brain/awv081
42. Moatti D, Faure S, Fumeron F et al (2001) Polymorphism in the fractalkine receptor CX3CR1 as a genetic risk factor for coronary artery disease. Blood 97:1925–1928. doi:10.1182/blood.V97.7.1925
43. Colom-Cadena M, Gelpi E, Charif S et al (2013) Confluence of α-synuclein, tau, and β-amyloid pathologies in dementia with Lewy bodies. J Neuropathol Exp Neurol 72:1203–1212. doi:10.1097/NEN.0000000000000018
44. Compta Y, Parkkinen L, O'Sullivan SS et al (2011) Lewy- and Alzheimer-type pathologies in Parkinson's disease dementia: Which is more important? Brain J Neurol 134:1493–1505. doi:10.1093/brain/awr031
45. Sierra M, Gelpi E, Martí MJ, Compta Y (2016) Lewy- and alzheimer-type pathologies in midbrain and cerebellum across the Lewy body disorders spectrum. Neuropathol Appl Neurobiol. doi:10.1111/nan.12308
46. Singh N, Rai H, Sinha N et al (2012) Association of V249I and T280M polymorphisms in the chemokine receptor CX3CR1 gene with early onset of coronary artery disease among north Indians. Genet Test Mol Biomark 16:756–760. doi:10.1089/gtmb.2011.0256
47. Niessner A, Marculescu R, Haschemi A et al (2005) Opposite effects of CX3CR1 receptor polymorphisms V2491 and T280M on the development of acute coronary syndrome. A possible implication of fractalkine in inflammatory activation Thromb Haemost doi. doi:10.1160/TH04-11-0735

48. Murphy PM (1994) The molecular biology of leukocyte chemoattractant receptors. Annu Rev Immunol 12:593–633. doi:10.1146/annurev.iy.12.040194.003113
49. Corder EH, Saunders AM, Strittmatter WJ et al (1993) Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. Science 261:921–923
50. Blacker D, Haines JL, Rodes L et al (1997) ApoE-4 and age at onset of Alzheimer's disease: The NIMH genetics initiative. Neurology 48:139–147
51. Sando SB, Melquist S, Cannon A et al (2008) APOE ε4 lowers age at onset and is a high risk factor for Alzheimer's disease; a case control study from central Norway. BMC Neurol 8:9. doi:10.1186/1471-2377-8-9
52. Lee JH, Cheng R, Schupf N et al (2007) The association between genetic variants in SORL1 and Alzheimer disease in an urban, multiethnic, community-based cohort. Arch Neurol 64:501–506. doi:10.1001/archneur.64.4.501
53. Leduc V, De Beaumont L, Théroux L et al (2015) HMGCR is a genetic modifier for risk, age of onset and MCI conversion to Alzheimer's disease in a three cohorts study. Mol Psychiatry 20:867–873. doi:10.1038/mp.2014.81
54. Naj AC, Jun G, Reitz C et al (2014) Effects of multiple genetic loci on age at onset in late-onset Alzheimer disease: A genome-wide association study. JAMA Neurol 71:1394–1404. doi:10.1001/jamaneurol.2014.1491
55. Li Y-J, Scott WK, Hedges DJ et al (2002) Age at onset in two common neurodegenerative diseases is genetically controlled. Am J Hum Genet 70:985–993. doi:10.1086/339815
56. Lee JH, Barral S, Cheng R et al (2008) Age-at-onset linkage analysis in Caribbean Hispanics with familial late-onset Alzheimer's disease. Neurogenetics 9:51–60. doi:10.1007/s10048-007-0103-3
57. Zhao W, Marchani EE, Cheung CYK et al (2013) Genome scan in familial late-onset Alzheimer's disease: A locus on chromosome 6 contributes to age at onset. Am J Med Genet Part B Neuropsychiatr Genet Off Publ Int Soc Psychiatr Genet. doi:10.1002/ajmg.b.32133
58. Manolio TA (2010) Genomewide association studies and assessment of the risk of disease. N Engl J Med 363:166–176. doi:10.1056/NEJMra0905980
59. Balasa M, Gelpi E, Antonell A et al (2011) Clinical features and APOE genotype of pathologically proven early-onset Alzheimer disease. Neurology 76:1720–1725. doi:10.1212/WNL.0b013e31821a44dd
60. Crean S, Ward A, Mercaldi CJ et al (2011) Apolipoprotein E ε4 prevalence in Alzheimer's disease patients varies across global populations: A systematic literature review and meta-analysis. Dement Geriatr Cogn Disord 31:20–30. doi:10.1159/000321984
61. Jin SC, Benitez BA, Karch CM et al (2014) Coding variants in TREM2 increase risk for Alzheimer's disease. Hum Mol Genet 23:5838–5846. doi:10.1093/hmg/ddu277
62. Strobel S, Grünblatt E, Riederer P et al (1996) (2015) changes in the expression of genes related to neuroinflammation over the course of sporadic Alzheimer's disease progression: CX3CL1, TREM2, and PPARγ. J Neural Transm Vienna Austria 122:1069–1076. doi:10.1007/s00702-015-1369-5