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Interleukin-18 gene polymorphisms predict risk and outcome of Alzheimer's disease

Paola Bossù¹, Antonio Ciaramella¹, Maria Luisa Moro¹, Lorenza Bellincampi², Sergio Bernardini², Giorgio Federici²,¹, Alberto Trequattrini³, Fabio Macciardi⁴, Ilaria Spoletini¹, Fulvia Di Iulio¹, Carlo Caltagirone¹,⁵ and Gianfranco Spalletta¹,⁶.

¹IRCCS Santa Lucia Foundation, Via Ardeatina 306, 00179 Rome, Italy
²Dept. Internal Medicine and Dept. Laboratory Medicine, University “Tor Vergata”, Rome, Italy
³ASL Città di Castello, Perugia, Italy
⁴Policlinico MultiMedica and Department of Science and Biomedical Technologies, University of Milano, Italy
⁵Dept. of Neuroscience, University “Tor Vergata”, Rome, Italy
⁶Neuropsychology Outpatient Clinic, Tor Vergata Hospital, University “Tor Vergata”, Rome, Italy

Corresponding author:
Dr. Paola Bossù,
IRCCS Santa Lucia Foundation, Department of Clinical and Behavioural Neurology,
Experimental Neuro-psychobiology Lab
Via Ardeatina, 306, I-00179 Roma – Italy
E-mail: p.bossu@hsantalucia.it
Tel. +39 06 51501520; Fax. +39 06 51501552;

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ABSTRACT
Inflammation has been extensively implicated in Alzheimer's disease (AD) pathogenesis. Although there is evidence of a key role for cytokines in neuroinflammation processes, so far the pro-inflammatory cytokine IL-18 has not yet been associated to AD. This study was aimed to investigate the impact of two polymorphisms of human IL-18 gene promoter at positions -607 (C/A) and -137 (G/C) on both susceptibility to and progression of AD. The results revealed that the genotype distribution of -607 (C/A) polymorphism was different between AD patients and control subjects ($\chi^2=7.99$, df= 2, p=0.0184). In particular, carriers of CC genotype were at increased risk of developing AD (OR=2.33; 95% CI=1.29-4.22; p=0.0052). The observed genotypes were in Hardy-Weinberg equilibrium as for -607 polymorphism, whereas the -137 polymorphism appeared in Hardy-Weinberg disequilibrium only in patient group (p=0.0061). Finally, in a two year follow up study, the -137 CC genotype was strongly and specifically associated with a faster cognitive decline (F=4.024; df=4,192; p=0.0037 for time by IL-18 -137 G/C group interaction) with no interaction effect with the ApoE $\epsilon4$/non $\epsilon4$ allele presence. Since IL-18 cytokine promoter gene polymorphisms have been previously described to have functional consequences on IL-18 expression, it is possible that individuals with a prevalent IL-18 gene variant have a dysregulated immune response, suggesting that IL-18 mediated immune mechanisms may play a crucial role in AD.
INTRODUCTION
Alzheimer's Disease (AD), the most common form of dementia among elderly people, is a neurodegenerative disorder characterized by progressive formation of amyloid senile plaques, tangles and selective neuronal death in the brain, probably caused by the effect of the amyloid beta peptide (Aβ) accumulation.[1] AD aetiology has not yet been fully clarified, possibly because multifactorial causes consisting of an interaction between ageing, environmental factors and genetic predisposition have been implicated. In particular, CNS is susceptible to inflammatory diseases and AD has been largely shown to be linked to chronic inflammatory reactions within the brain.[2, 3] Indeed, an early and consistent activation of microglia cells, the mononuclear phagocytes that participate in mechanisms of innate and adaptive immunity within the brain, have been demonstrated in the cerebral areas of neurodegeneration in AD patients.[4, 5] Although activated microglia could have a beneficial role by participating in Aβ clearance because of their ability to phagocyte, they can also produce many toxic factors.[6] Several and growing evidence suggests that chronic microglia-mediated immune response during Aβ deposition can prime an inflammatory destructive cascade and contribute to Aβ plaque formation, thus participating in AD aetiopathogenesis.[7] Cytokines, such as Interleukin (IL)-1, IL-6 and Tumour Necrosis Factor alpha (TNF-α) have been clearly involved in this neuroinflammatory process and their production has been found mostly increased in AD patients and impaired in the late stage of the disease.[8-11] Moreover, several studies described an association between cytokine gene polymorphisms and AD,[8, 9, 12, 13] suggesting that cytokine variant genes can be also included among genetic risk factors for AD, although a functional consequence of such polymorphisms has not always been characterized. Taken all together, such observations converge in delineating a dysregulation or impairment of the immune response, which may not only reflect an epiphenomenon, but may causally be related to AD pathology.[14] Among the factors capable of modulating the immune response, the cytokine IL-18 has been shown to have potent immunoregulatory activity on both innate and adaptive response and its involvement in ageing and in neurodegenerative brain diseases has been also recently highlighted.[15, 16] IL-18, belonging to the IL-1 cytokine superfamily, is a pleiotropic cytokine, produced by a variety of cell types, including activated microglia and astrocytes.[17] Similarly to IL-1β, IL-18 is synthesized as a biologically inactive precursor molecule, which in order to became active needs to be cleaved by the intracellular cystein-protease caspase-1, a caspase which is up-regulated in brain tissues from both subjects at high risk of developing AD and AD patients.[18] Originally identified as IFN-γ inducing factor, IL-18 possesses strong pro-inflammatory activities, based on its capability to induce the synthesis of cell adhesion molecules, nitric oxide, chemokines and other pro-inflammatory cytokines such as IL-1β, TNF-α and IL-8.[15, 19] At molecular level, the regulation of IL-18 expression has been extensively studied; the sequences upstream of the human IL-18 cDNA with promoter activity have been characterised and the functional properties of some of their polymorphic variants described.[20] In the present study, the distribution of the human IL-18 gene promoter polymorphisms at positions -607 (C/A) and -137 (G/C) was analyzed in a group of AD patients and control subjects in order to determine its impact on the risk of developing the disease, by means of a case-control study. Afterwards, a longitudinal clinical evaluation in the subgroup of patients who completed a two-year follow-up period has been performed and the influence of the two IL-18 gene promoter polymorphisms on AD outcome evaluated.

MATERIALS AND METHODS
Study design and population
Patients selection criteria
Patients with a diagnosis of probable AD were considered appropriate for enrolment. All the 339 included subjects (66.7 % women, mean age at study 74.3 ± 7.5 SD years, education 6.4 ± 3.9 SD years) were unrelated Caucasian patients and were consecutively recruited in memory clinics located in Central Italy. These subjects were drug naïve and underwent the first clinical examination
for the diagnosis of AD. After the first diagnostic evaluation, patients were treated with acetylcholinesterase inhibitors (AChE-I), according with the guidelines for the treatment of AD of the Italian Neurological Society and the international guidelines.[21] The nature and purposes of this study were presented to them and explained to their responsible caregiver and/or legal guardian. Written informed consent was obtained from patients or patients’ representative and from caregivers prior to beginning detailed screening activities. Approval for the study had been obtained from the local ethics committee. Inclusion criteria were: 1) diagnostic evidence of late-onset (age of onset > 65 years) probable AD consistent with NINCDS-ADRDA criteria; 2) being healthy and able to walk independently or with a walker or cane; 3) vision and hearing sufficient for compliance with testing procedures; 4) laboratory values within normal limits, or considered to be clinically insignificant by the investigator. Exclusion criteria included: 1) lack of a “reliable” caregiver defined as: being able to report to clinic, to ensure compliance to treatment and clinic visits, to contact the patient at least twice weekly, one of which had to be a personal visit; 2) major medical illness, i.e. diabetes not stabilized, obstructive pulmonary disease or asthma, haematological/oncological disorders, B12 or folate deficiency as evidenced by blood concentrations below the lower normal limit, pernicious anaemia, clinically significant and unstable active gastrointestinal, renal, hepatic, endocrine or cardiovascular system disease, newly treated hypothyroidism, liver function tests (ALT or AST) greater than 3 times the upper normal limit, creatinine concentrations greater than 150 µmol/L; 3) comorbidity of primary psychiatric or neurological disorders (i.e., schizophrenia, major depression, stroke, Parkinson’s disease, seizure disorder, head injury with loss of consciousness within the past year); 4) known suspected history of alcoholism or drug abuse; 5) CT or MRI evidence of focal parenchymal abnormalities; 6) scan evidence of neoplasm. Of the total number of enrolled AD subjects, 3 subjects were not genotyped for -137 locus and other 3 subjects were not genotyped for -607 locus, due to technical reasons linked to a limited source of blood DNA.

Among the patient group, 108 subjects with mild to moderate AD were followed up in the two-year longitudinal study. We opted for a more stringent selection of the longitudinal subgroup of patients, who had a Mini Mental State Examination (MMSE)[22] score >10 at the baseline. In fact, patients with severe AD are already in the latest stage of the illness and their cognitive outcome is not measurable. Within this longitudinal patient group, 4 died during the follow-up period and 2 dropped-out due to non-compliance. Data from the 102 patients (68.6 % women, mean age at study 76.5 ± 5.8 SD years, education 4.7 ± 3.0 SD years) who completed all the evaluations during the 2-year period were analyzed. Compared to the total patient group, the longitudinal subgroup of AD patients did not statistically differ for distribution of gender ($\chi^2$=0.137; df= 1; p=0.712); whereas, as expected by the selection criteria of the longitudinal patients, who were in their mild to moderate phase of illness in comparison with the total group that included severe patients also, other sociodemographic characteristics, such as age (t=-2.799; df= 439; p=0.0054) and educational level (t=-3.910; df= 439; p=0.0001) were different.

Clinical evaluations
A clinical neurologist used the NINCDS-ADRDA[23] criteria for assessing diagnosis of AD. A clinical psychologist administered patients at day 0 (baseline) using the MMSE. The same psychologist administered, after 1 and 2 years of follow-up the MMSE, which is a commonly used neurocognitive test measuring, by means of 16 items, orientation, language, verbal memory, attention, visuospatial function and mental control with scores ranging from 30 (no impairment) to 0 (maximum impairment).

Control subjects
One hundred and thirty-nine subjects were included as controls (55.4 % women, mean age 69.4 ± 6.4 SD years, education 7.3 ± 3.6 SD years). The control subjects were neither related to one another nor to AD patients; in particular, we selected only those subjects that were in the same
patients' age range (66-96 years), had a MMSE score ≥24 and did not satisfy the NINCDS-ADRDA criteria for diagnosis of AD, as clinically assessed and confirmed by the memory tests of the Mental Deterioration Battery (MDB).[24] Indeed, the normal cognitive level of the Italian population could be so defined, according to Measso et al.[25] In our study, the subjects enrolled as controls were Caucasian, did not show any neurological signs or symptoms at a clinical neurological examination, and were recruited from the general population of the same geographic region of the patients. Furthermore, of the total number of 139 enrolled control subjects, only 131 subjects were genotyped for -137 locus due to technical reasons linked to a limited source of blood DNA.

**Genotyping**

Genomic DNA was purified from 200 µl of human whole blood using the MagNA Pure LC DNA Isolation Kit I (Roche Diagnostics, Mannheim, Germany) in an automated extractor from the kit's manufacturer (MagNA Pure LC). The polymorphisms at the position -607 and -137 in the promoter of IL-18 gene were genotyped by sequence-specific PCR (PCR-SSP) as previously described.[20] For the position -607C/A-specific PCR, a common reverse primer 5’-TAACCTCATTCCAGACTTTCC-3’ and two sequence-specific forward primers 5’-GTTGCAGAAAGTGTAAAAATTATTTAC-3’ and 5’-GTTGCAGAAAGTGTAAAAATTATTTA-3’ were used. A control forward primer 5’-CTTGTGCTATCCATTCCAGGA-3’ was used to amplify a 301-bp fragment covering the polymorphic site as an internal positive amplification control. All reactions were carried out in a Perkin-Elmer 9600 Thermocycler (Applied Biosystems, Foster City, CA). Samples were initially denatured at 94°C for 2 min, followed by seven cycles of 94°C for 20 s, 64°C for 40 s and 72°C for 40 s and 25 cycles 94°C for 20 s, 57°C for 40 s, 72°C for 40 s, and final step of elongation at 72°C for 5 min. For the -137 genotyping, a common reverse primer 5’-AGGAGGGCAAAATGCACTGG-3’ and two sequence specific forward primers 5’-CCCCACTTTTACGGAAGAAAG-3’ and 5’-CCCCAACTTTTACGGAAGAAAC-3’ were used. A control forward primer 5’-CCAATAGGACTGATTATTTCCGA-3’ was used to amplify a 446-bp fragment covering the polymorphic site to serve as an internal positive amplification control. After 2 min of denaturation at 94°C, PCR was performed by five cycles of 94°C for 20 s, 68 °C for 1 min and 25 cycles of 94°C for 20 s, 62°C for 40 s, 72°C for 40 s and 72°C for 5 min. All PCR products were visualized by 2% agarose gel electrophoresis stained by ethidium bromide. The ApoE genotyping was performed by Real-time PCR on a Light-Cycler Instrument (Roche Diagnostics GmbH, Mannheim, Germany) using the Light-Cycler ApoE Mutation Detection Kit, commercially available from Roche Diagnostics (Roche Diagnostics GmbH, Mannheim, Germany).

**Statistical analyses**

We analyzed our case-control data with Stata 9SE (StataCorp, 2005), looking for case-control association with single SNPs. Before performing inferential test, we controlled for deviation from Hardy-Weinberg equilibrium. Comparisons of categorical variables were made by using the χ² test. Differences in continuous variables among different genotypes were analyzed by factorial analyses of variance (ANOВAs). The MMSE scores during the two years of follow-up were analyzed using a repeated-measures ANOVA with the three IL-18 -137 (G/C) genotypes and ApoE ε4 vs. non ε4 genotypes as between-subjects factors and rating scale scores during time as a within-subjects factor with three levels. The same analysis was also used for IL-18 -607 (C/A) genotypes.

**RESULTS**

The distribution of ApoE variants in AD patients and control subjects is shown in Table 1. The distribution of IL-18 -607 and -137 genotypes in AD patients and control subjects is shown in Table 2. The analysis of IL-18 promoter polymorphisms in the total group of patients and controls revealed statistically significant differences in genotype distributions for the locus -607 (χ²=7.99; df= 2; p=0.0184): an higher frequency of -607 CC carriers has been observed in AD patients, contrarily to control subjects, who showed a more frequent distribution of AA genotypes at this locus. Interestingly, heterozygous subjects were equally distributed among the two groups.
Furthermore, a logistic regression analysis examining the relationship between the presence of the three -607 C/A genotypes and the risk of developing AD, revealed that CC homozygotes were at increased risk of developing AD (OR=2.33; 95% CI=1.29-4.22; p=0.0052). No significant association has been found between IL-18 polymorphism at position -137 and the susceptibility to AD. The observed genotypes were in Hardy-Weinberg equilibrium as for -607 C/A polymorphism, both in the total group of patients and controls, whereas, the -137 G/C polymorphism appeared in Hardy-Weinberg disequilibrium only as for the total patient group, contrarily from controls (estimated disequilibrium for cases D=-0.028, exact p=0.0061; estimated disequilibrium for controls D=-0.013, exact p=ns). At the same time, in the described population of control subjects and AD patients, the presence of Apolipoprotein E (ApoE) ε4 polymorphic variant, which is known to be a potent risk factor for the developing AD,[26] has been analyzed. As expected, the presence of ApoE ε4 allele in the total AD patient population was significantly higher than in control subjects (44% vs. 15.8%; χ²=33.94; df=1; p<0.0001). However, no significant association among ApoE variant alleles and the studied IL-18 -607 and -137 gene promoter polymorphisms was found in control subjects (χ²=1.634; df=2; p=0.442 and χ²=2.612; df=2; p=0.271, respectively) as well as in AD patients (χ²=3.646; df=2; p=0.162 and χ²=4.400; df=2; p=0.111, respectively).

Table 1. Frequency of genotypes of ApoE in control subjects and AD patients of the total and longitudinal subgroups

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>CNT (N=139)</th>
<th>AD TOT. (N=339)</th>
<th>AD LONG. (N=102)</th>
<th>χ²; df; p-value (AD LONG. vs. AD TOT.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ε2/ε2</td>
<td>0.7% (N=1)</td>
<td>0% (N=0)</td>
<td>0% (N=0)</td>
<td></td>
</tr>
<tr>
<td>ε2/ε3</td>
<td>9.4% (N=13)</td>
<td>4.4% (N=15)</td>
<td>6.9% (N=7)</td>
<td></td>
</tr>
<tr>
<td>ε2/ε4</td>
<td>1.4% (N=2)</td>
<td>2.1% (N=7)</td>
<td>1% (N=1)</td>
<td></td>
</tr>
<tr>
<td>ε3/ε3</td>
<td>74.1% (N=103)</td>
<td>51.6% (N=175)</td>
<td>46.1% (N=47)</td>
<td></td>
</tr>
<tr>
<td>ε3/ε4</td>
<td>14.4% (N=20)</td>
<td>34.8% (N=118)</td>
<td>38.2% (N=39)</td>
<td></td>
</tr>
<tr>
<td>ε4/ε4</td>
<td>0% (N=0)</td>
<td>7.1% (N=24)</td>
<td>7.8% (N=8)</td>
<td></td>
</tr>
<tr>
<td>ε4 vs. non ε4</td>
<td></td>
<td></td>
<td></td>
<td>χ²=0.306; df=1; p=0.5801</td>
</tr>
</tbody>
</table>

Table 2. Frequency of genotypes of IL-18 gene promoter polymorphisms in control subjects and AD patients of the total and longitudinal subgroups

<table>
<thead>
<tr>
<th>Locus</th>
<th>Genotypes</th>
<th>CNT (N=139)</th>
<th>AD TOT. (N=336)</th>
<th>AD LONG. (N=102)</th>
<th>χ²; df; p-value (AD LONG. vs. AD TOT.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-18 -607</td>
<td>CC</td>
<td>27.3% (N=38)</td>
<td>36.9% (N=124)</td>
<td>36.3% (N=37)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CA</td>
<td>51.1% (N=71)</td>
<td>50.6% (N=170)</td>
<td>58.8% (N=60)</td>
<td>χ²=5.227; df=2; p=0.073</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>21.6% (N=30)</td>
<td>12.5% (N=42)</td>
<td>4.9% (N=5)</td>
<td></td>
</tr>
</tbody>
</table>
Factorial ANOVAs did not detect significant differences in age at onset among AD patients carrying different IL-18 -607 (F=1.159; df=2,333; p=0.315) and -137 (F=0.443; df=2,333; p=0.642) polymorphisms.

In order to study the influence of IL-18 gene promoter polymorphisms on AD progression, among the total AD group, a subset of 102 patients with mild to moderate AD has been selected and followed-up yearly for two years. The distributions of ApoE variants, IL-18 -607 and -137 genotypes of the longitudinal AD patient group are reported in Tables 1 and 2 and they were not found significantly different with respect to the whole group of 339 AD patients. MMSE scores during 2 years of follow-up are shown in figure 1. A significant change in MMSE total score during time (F=42.590; df=2,192; p<0.0001) and a significant time by IL-18 -137 G/C group interaction (F=4.024; df=4,192; p=0.0037) was observed. No statistically significant time by IL-18 -137 G/C by ApoE ε4/non ε4 group interaction (F=1.111; df=4,192; p=0.353) was found. In particular, the C/C genotype at position -137 was specifically associated with faster cognitive decline regardless the presence of the ApoE ε4 allele. Additional statistical factorial ANOVA and χ² analysis showed no statistically significant differences between the groups of patients with IL-18 -137 G/C polymorphisms as for age (F=1.438; df=2,99; p=0.242), gender (χ²=4.127; df=2; p=0.127), years of education (F=1.141; df=2,99; p=0.324).

DISCUSSION

The present study demonstrates for the first time an association between cytokine IL-18 gene promoter polymorphisms and both the susceptibility and the clinical outcome of AD. Interestingly, this effect on risk and outcome of AD was not influenced by the presence of different ApoE ε allelic variants. The cytokine IL-18 is a pleiotropic factor involved in the amplification of inflammatory response, which plays a role in many illnesses with a relevant chronic inflammatory component. In addition, IL-18 has been recently suggested to be a key player also in neuroinflammation and neurodegeneration by exerting a double role, both beneficial and detrimental, in autoimmune, ischemic, traumatic and infectious disorders of CNS.[16] In AD brains a chronic inflammatory response has been largely demonstrated[2, 3, 11] and it is therefore conceivable that IL-18 could make a crucial contribution also in AD neurodegeneration. From a genetic point of view, AD results to be influenced by multiple susceptibility loci, whose combinations contribute to the development of this disorder.[27] Accordingly, we have recently shown that genomic polymorphisms in GSTs, a family of enzymes that appear to be critical for the protection against oxidative stress, a phenomenon strictly linked to inflammation, are associated with AD susceptibility[28] and clinical course.[29] These effects also were independent from the ApoE ε4 allelic variant possession. Similarly, several polymorphisms in pro-inflammatory or even anti-inflammatory cytokine genes have been described associated to the disease.[8, 9, 12, 13] Regarding polymorphisms of IL-18 gene, an important recent study analyzing the genetic variability of the whole IL-18 system (IL-18, the two IL-18R chains and IL-18 Binding Protein) in patients with cardiovascular diseases demonstrated that certain haplotype variations of the IL-18 gene consistently influence both circulating levels of IL-18 and clinical outcome of the disease.[30] Moreover, previous studies have analysed the association of -607(C/A) and -137(G/C) IL-18 gene promoter polymorphisms with immune driven pathologies such as Crohn’s disease, diabetes and rheumatoid arthritis. Although the results of these studies are conflicting, in particular regarding diabetes or rheumatoid arthritis,[31-35] it is possible that these polymorphisms contribute to the pathogenic mechanisms of some inflammatory diseases. Interestingly, one publication[20] based on a promoter transcription activity

| IL-18 -137 | GG | 49.6% (N=65) | 53.3% (N=179) | 49% (N=50) | χ²=0.780; df=2; p=0.677 |
|-----------|----------------|----------------|
| GG        | GC | 43.5% (N=57) | 43.1% (N=145) | 46.1% (N=47) |
| CC        | 6.9% (N=9) | 3.6% (N=12) | 4.9% (N=5) |
assay, reported that such two polymorphisms of IL-18 gene promoter have functional consequences, being of relevance for nuclear transcriptional factor binding. In fact, nucleotide substitution at position -607 (C→A) is suggested to impair a cAMP-responsive element-binding site. Furthermore, the C allele presence at position -137 is hypothesised to modify a H4TF-1-binding site.[20] The resulting -607A/-137C haplotype have functional consequence by lowering the activity of IL-18 gene promoter. On the contrary, patients homozygous for genotype -607 CC and -137 GG had increased IL-18 mRNA levels, in comparison to patients with other genotypes.[20] In further functional studies, an increased transcriptional activity of the wild-type genotype -137 GG of human IL-18 gene has been described[36] and monocytes from subjects with -137 GG genotype have been shown to produce more IL-18 than and monocytes from subjects with -137 GC genotype.[37] On the basis of such results and in the light of our data, it is conceivable to hypothesize that -607 CC genotype is associated with an higher expression of IL-18 gene and that sustained levels of the cytokine can increase the susceptibility to AD. Whereas, the CC genotype at position -137 could correspond to a lower activity of the gene and, the tendency to react with lower IL-18 production following AD onset could cause a worsening of the disease course. However, further studies are needed to characterize the modulation of IL-18 expression in AD. As for the -137 G/C polymorphism, a significant and consistent deviation from Hardy-Weinberg equilibrium has been identified in AD patients, suggesting the presence of selective forces, at least in our population. Such observation, although appears not to be supported by any significant association of this polymorphism with the increased risk of developing AD, is in line with the longitudinal data, which show that patients carrying the CC genotype at this position are fast decliners relatively to cognitive performance, thus indicating that homozygosity for such variant allele can be considered as a negative prognostic factor for the disease. Although the group of patients carrying -137 CC genotype is small (n=5), the data we obtained in the longitudinal study are statistically highly significant and further supported by other personal observations (Spalletta G and Bossù P). However, due to the small sample, the present longitudinal results should be considered as the basis for a new hypothesis and should be replicated in further expanded studies. Furthermore, it is somehow interesting to notice that the human IL-18 gene maps to 11q22.2-q22.3, a region near the tip of the long arm of chromosome 11, which has been previously reported in linkage studies on AD families.[38]

Our findings indicate a relevant association between IL-18 gene polymorphisms and AD, suggesting that this cytokine may play a role in the disease pathogenesis. So far no consistent evidence have been produced about an implication of IL-18 in AD neurodegeneration, and conflicting results were obtained about IL-18 production in AD patients. In fact, a recent study reported that serum levels of the cytokine did not significantly differ among MCI, severe AD and control subjects,[39] whereas other authors have described more elevated levels of plasma IL-18 in AD as well as in vascular dementia patients, compared to control subjects.[40] In conclusion, our study provides a new evidence supporting a possible role of the IL-18 cytokine in AD. Further studies deserve to be performed in this direction, as they might add a valuable light to the complex mechanisms involving immune dysregulation and leading to the pathogenic pathways of AD.

Competing interests: none

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Figure legends

Figure 1. Mini Mental State Examination (MMSE) scores of Alzheimer’s disease patients with different –137 G/C genotypes over 2 years of follow-up. Carriers of CC genotype show a strong decrease in their global cognitive level, in comparison to the non CC patients.