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Short communication

Assessing the association between common functional Nuclear Factor Kappa-b gene polymorphisms (NFKB1, NFKBIZ, NFKBIA) and Alzheimer's disease

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ABSTRACT

The Nuclear Factor Kappa-b (NF-Kb) pathway has been implicated in the pathogenesis of Alzheimer's disease (AD). We determined whether common variants in the NF-Kb genes were associated with the risk of developing late-onset AD (LOAD). A total of 639 Spanish LOAD and 500 controls were genotyped for the *NFKB1* rs28362491/rs7667496, *NFKBIA* rs696, *NFKBIZ* rs3217713 and *APOE-Ɛ2/3/4* polymorphisms. Rs7667496 C was increased in the patients ($p<0.001$) with the CC genotype showing a significant risk (CC vs T+, OR= 1.58, 95 % CI=1.25–2.01). The CC genotype was significantly associated with LOAD after correction by APOE-4+ genotypes, age and sex ($p=0.0003$, OR=1.88, 95 %CI=1.28–2.78). The *NFKB1* rs28362491 I - rs7667496 C haplotype was significantly increased in the patients ($p=0.02$). *NFKBIA* and *NFKBIZ* variants were not associated with the risk of LOAD in our population. In conclusion, *NFKB1* variants were associated with the risk of LOAD in our population. This finding encourages further studies to determine the involvement of the NF-kB components in LOAD.

1. Introduction

The NF-κB pathway has been implicated in the pathogenesis of neurological disorders such as Parkinson's (PD) and Alzheimer's (AD) through mechanisms that involve neuronal survival, regulation of synaptic plasticity and memory, or microglial activity and inflammation [1–5]. The pathway was dysregulated in peripheral mononuclear cells from AD-patients [6]. The p50 precursor, that is encoded by the *NFKB1* gene, was elevated in AD-brains [7]. Nfkb1$^{-/-}$ mice treated with anti-inflammatory drugs showed a reduced brain neuroinflammation and senescent cell burden, with significant improvements in cognitive function [8].

The presence of brain beta-amyloid plaques and neurofibrillary tangles is a pathological hallmark of Alzheimer's disease, and these structures driven pro-inflammatory stimuli with glial cells activation that could contribute to disease progression [9,10]. Amyloid-β aggregates activate peripheral monocytes in mild cognitive impairment [11]. Several studies showed that NF-κB levels were increased in the cerebral cortex of patients with AD patients, and this was correlated with the amount of the beta-amyloid precursor protein cleaving enzyme [12–14]. Moreover, p65 would induce the expression of this enzyme contributing to the formation of amyloid-fibrils and plaques [13,14].

The risk of developing LOAD is strongly associated with the presence of the Apolipoprotein-E (APOE) Ɛ4 isoform. APOE might regulate AD progression through the NF-κB pathway [15]. Apoe-Ɛ4 mice had increased expression of proinflammatory NF-κB-regulated genes [16]. After traumatic brain injury the Apoe-Ɛ4 mice showed increased activity of this pathway compared to mice without this ApoE variant [17]. Studies with ApoE knock-out showed that the expression of NF-κB and ApoE genes is mutually regulated. Apoe knock-out mice showed activation of the NF-κB pathway with increased inflammation and oxidative stress [18]. Several studies showed that the expression of APOE was

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affected by the treatment with NF-κB inhibitors [19].

Several variants in the NF-kB genes have been associated with cancer, cardiovascular, neurological, and immune-mediated diseases [20–24]. The best characterised was rs28362491, a 4-nucleotides indel in the promoter of the p50/*NFKB1* gene that has been recognised as a quantitative trait loci (QTL) for *NFKB1* expression in several cell and tissues (see https://gtexportal.org/home/snp/rs28362491). Variants in the *NFKBIA* and *NFKBIZ* genes have been associated with different transcript expression between the genotypes and the risk for immune mediated diseases [24].

In this work, we hypothesised that gene variants in the genes that encode NF-κB components might be involved in the susceptibility of developing LOAD.

2. Patients and methods

2.1. Study subjects

This study was approved by the Ethical Committee for Medical Research of Asturias, and all the participants gave their informed consent. All the patients (N=639 and controls (N=500) were of Spanish European ancestry and from the region of Asturias (Northern Spain, total population approx. 1 million). All the patients were diagnosed with probably Alzheimer's disease by Neurologists from the Hospital Universitario Central Asturias (HUCA) and **according to the criteria established by the "National Institute of Neurological and Communicative Disorders and Stroke–Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA;** https://www.ninds.nih.gov **/health-information/disorders/alzheimers-disease).** They had an onset age ≥65 years and were thus characterised as LOAD.

Controls were a total of 500 individuals aged ≥60 from the general population of Asturias without a diagnostic of cognitive impairment. **None of the patients and controls were related.**

2.2. NF-κB and APOE variants genotyping

The DNA of all the participants was obtained from blood leukocytes. We studied the *NFKB1* rs28362491, *NFKB1* rs7667496, *NFKBIA* rs696 and *NFKBIZ* rs3217713 variants. These polymorphisms were chosen due to their reported functional effects on gene expression or their association with diseases [20–24].

The *NFKB1* rs28362491 and *NFKBIZ* rs3217713 were genotyped by capillary electrophoresis in an ABI3130 sequencer (Fisher Scientific). The forward primers were 5-en labelleled with the 5-FAM fluorochrome and the alleles were thus visualised as peaks of different size (suppl figure). *NFKBIA* rs696 was genotyped with real-time PCR with TaqMan probes (assay id. C_145669_30; ThermoFisher Sci,). Briefly, a total of 25 nG of each genomic DNA was amplified in 96 well-plates and at final PCR volume of 20 μL. The TaqMan conditions were as provided by the manufacturer (https:// thermofisher.com/order/genome-database/details/genotyping/ C_145669_30).

The *NFKB1* rs7667496 was genotyped by digestion of PCR fragments with the restriction enzymes *MspI*, as previously reported [20]. Briefly, each genomic DNA was amplified in 96 well-plates in a final vol of 40 μL with primers Forward 5′-GAACACACTTTTCCTTACCCCCAGTC and reverse 5′-GTGACTGCATTTATC TTGGCCATACC (annealing at 64ºC). Each PCR was digested with the restriction enzyme *MspI* at 37ºC for 5 hours, followed by electrophoresis in 3 % agarose gels to visualize the allele sizes (allele T=603 bp; allele C=339+264 bp).

The APOE-Ɛ2/3/4 genotypes (SNPs rs429358 and rs7412) were determined by digestion of PCR fragments with the restriction enzyme *HhaI* as reported [25,26].

2.3. Statistical analysis

All the values were collected in an Excel file (available as reasonable request to the corresponding author). The statistical analyses were performed in R (www.r-project.org). Odds Ratio (OR) values and their 95 % confidence intervals (95 % CI) were determined by logistic regression (generalized linear model). A $p < 0.05$ was taken as the cutoff for significance. Haplotype frequencies were calculated online with CubeX program (http://apps.biocompute.org.uk/cubex/). **Testing for Hardy-Weinberg Equilibrium of the observed genotype frequencies was performed online (**https://wpcalc.com/en/equilibrium-hardy-weinberg/**).**

3. Results

The main values in our LOAD patients and controls are summarized in Table 1. As expected, the presence of *APOE-ε4* was significantly increased in the patients (multiple logistic regression with age and sex, $p=2\times10^{-16}$, OR=6.05, 95 %CI=4.37–8.48) (**suppl.** Table 1). **The observed genotype frequencies for the studied polymorphisms did not deviate from the Hardy-Weinberg equilibrium in both, patients and controls, and were close to the reported among European populations (suppl,** Table 2**).** For the *NFKB1* polymorphisms, there were no differences between male and female controls, and we pooled them as total population controls.

We found a significantly lower frequency of the rs7667496 MAF (T allele) in the patients ($p<0.001$). The two T-genotypes (TT and CT) were significantly reduced in the patients (Table 2). Thus, the C allele was a significant risk factor for LOAD in our study (OR=1.40, 95 % CI=1.18–1.68). We run a logistic regression using the number of C (risk) alleles and found the best value for the recessive model (CC vs CT+TT, $p<0.001$, OR=1.58, 95 %CI=1.25–2.01). The difference was significant after applying the Bonferroni's correction for multiple testing ($p<0.01$). Moreover, at a $p<0.01$ and the frequencies found for the risk genotypes the post hoc statistical power was >90 %. **This association was independent of sex, age and the presence of *APOE-ε4* (multiple logistic regression, CC vs TT+CT: p=0.001, OR=1.57, 95 %CI=1.20–2.05).**

The *NFKB1* rs28362491 insertion allele was more common in the patients compared to total controls, with a non-significantly higher frequency in male after applying the Bonferroni's correction (0.70 vs 0.64; p=0.03).

The two *NFKB1* polymorphisms were in linkage disequilibrium in our controls and patients (suppl table 3**). Haplotype rs28362491 I - rs7667496C was significantly increased in total patients vs controls (I-C vs other: p=0.003).**

The *NFKBIA* rs696 and *NFKBIZ* rs3217713 allele and genotype frequencies did not differ between the groups patients groups (Tables 1 and 2). There were no differences between the sex and APOE-ε4 groups for the studied polymorphisms in patients and controls (Table S4).

4. Discussion

The main finding of our study was a significant association between common variants in the *NFKB1* gene and the risk of developing LOAD. **The NF-kB pathway polymorphisms have been associated with other neurodegenerative diseases, such as Multiple sclerosis and Parkinson's disease** [23,27]**. However, to our knowledge there are no reports about their role in the risk of LOAD.**

Our findings were in agreement with studies that reported an effect of the NF-Kb pathway in the pathogenesis of Alzheimer's disease [1–8]. The rs7667496 and rs28362491 polymorphisms have been reported as quantitative trait loci (QTLs) for *NFKB1* transcript expression (Gtex portal; https://gtexportal.org/home/). At least one study showed that the common haplotype rs28362491 Ins – rs72696119C was *in vitro* associated with significant higher expression of *NFKB1* compared to the other haplotypes [28]. These polymorphisms were in linkage disequilibrium with rs7667496 (rs28362491-rs72696119-rs7667496) with two common European haplotypes: Ins-C-C and Del-G-T. Thus, the rs28362491 Ins – rs72696119C haplotype increased among LOAD

Table 1
Frequencies of the studied gene variants in patients and population controls.

	Alzheimer's MALE N=214	Alzheimer's FEMALE N=407	TOTAL Alzheimer's N=621	Controls MALE N=250	Controls FEMALE N=250	TOTAL Controls N=500
Age Range years	61–91	61–91	61–91	61–81	61–85	61–85
NFKB1 rs7667496						
TT	13 (6 %)	41 (10 %)	54 (9 %)	27 (11 %)	35 (14 %)	62 (12 %)
CT	94 (44 %)	162 (40 %)	256 (41 %)	128 (46 %)	116 (46 %)	244 (49 %)
CC	107 (50 %)	204 (50 %)	311 (50 %)	95 (38 %)	99 (40 %)	194 (39 %)
T	0.28	0.30	0.29	0.36	0.37	0.37
NFKB1 rs28362491						
II	105 (49 %)	179 (44 %)	284 (46 %)	100 (40 %)	105 (42 %)	205 (41 %)
ID	90 (42 %)	168 (41 %)	258 (41 %)	121 (48 %)	113 (45 %)	234 (47 %)
DD	19 (9 %)	60 (15 %)	79 (13 %)	29 (12 %)	32 (13 %)	61 (12 %)
D	0.30	0.36	0.33	0.36	0.35	0.36
NFKBIA rs696						
TT	39 (18 %)	74 (18 %)	111 113 (18 %)	38 (15 %)	41 (16 %)	79 (16 %)
CT	97 (45 %)	178 (44 %)	275 (44 %)	118 (47 %)	113 (45 %)	231 (46 %)
CC	78 (37 %)	155 (38 %)	233 (38 %)	94 (38 %)	96 (38 %)	190 (38 %)
T	0.41	0.40	0.40	0.39	0.39	0.39
NFKBIZ rs3217713						
II	125 (58 %)	261 (64 %)	-386(62 %)	152 (61 %)	151 (60 %)	303 (61 %)
ID	75 (35 %)	135 (32 %)	-210(34 %)	86 (34 %)	89 (36 %)	175 (35 %)
DD	14 (6 %)	11 (3 %)	25 (4 %)	12 (5 %)	10 (4 %)	22 (6 %)
D	0.24	0.19	0.21	0.22	0.22	0.22

Table 2
Univariate and multivariate (age and sex corrected) multiple logistic regression for the minor allele frequency genotypes.

	UNIVARIATE		AGE-SEX MULTIVARIATE	
	p-value	OR (95 % CI)	p-value	OR (95 %CI)
rs7667496 CT	0.003	0.65 (0.51–0.84)	0.01	0.70 (0.54–0.92)
rs7667496 TT	0.001	0.54 (0.36–0.82)	0.005	0.52 (0.34–0.82)
rs28362491 ID	0.08	0.80 (0.62–1.02)	0.25	0.85 (0.65–1.12)
rs28362491 DD	0.73	0.93 (0.64–1.37)	0.60	0.89 (0.59–1.36)
rs696 CT	0.82	0.97 (0.75–1.26)	0.43	0.89 (0.68–1.18)
rs696 TT	0.38	1.17 (0.83–1.65)	0.64	1.09 (0.75–1.59)
rs3217713 ID	0.60	0.94 (0.73–1.20)	0.51	0.91 (0.69–1.20)
rs3217713 DD	0.48	0.81 (0.45–1.46)	0.88	0.95 (0.51–1.80)

patients in our study could be associated with higher expression of *NFKB1*.

The presence of rs28362491 insertion was associated with lower basal *NFKB1* gene expression in neutrophils, monocytes and natural-killer cells, and lower basal expression in T and B cells [29]. However, this effect was reversed in monocytes stimulated with LPS or IFN-gamma where the presence of deletion was associated with lower expression. While the Del is located in a transcription factor site in the *NFKB1* promoter, the heterogeneous gene expression between the alleles might be also explained in part by the differences in promoter methylation. Thus, in neutrophils the DD-genotype was significantly more methylated compared to II genotype, and this could in part explain the increased expression among rs28362491 Ins [30]. Interestingly, the Del was associated with reduced antibody levels against several pathogens, increasing the risk of infection with influenza, legionella and papillomavirus. In the - same study the Del variant was associated with a higher risk of diseases associated with dysregulation of inflammatory responses and chronic inflammation, but was protective against allergic diseases which are associated with acute inflammatory responses [29].

Based on the reported studies, there are several mechanisms by which the functional *NFKB1* variants could modify the risk of developing LOAD. Among others, amyloid beta is a potent activator of the NF-kB pathway in primary neurons [31]. *NFKB1* expression was shared by LOAD and Down Syndrome (DS) caused by an extra copy of Chromosome 21, where the APP gene maps [32]. Many DS individuals develop Alzheimer's disease when they age, and this is due in part to the extra copy of the *APP* gene. *NFKB1* was thus proposed as important to both disorders in the context of direct association with APP processing, Tau post translational modification and network connectivity. **A recent study combining human brains and animal models showed the association of NF-κB and astrocytes in AD at a cell-specific transcriptomic level** [33]**.** In view of the implication of *NFKB1* in the pathogenesis of Alzheimer's disease, it is tempting to speculate that under pro-AD stimuli individuals with the rs28362491 Ins - rs7667496C haplotype might be at increased risk of developing LOAD through enhanced pro-inflammatory response.

Our study has several limitations. It was based on a limited number of - LOAD patients and from a single population. The reported associations require replication in different cohorts. The role of the NF-KB pathway on neurodegeneration makes the association of *NFKB1* variants with LOAD plausible, but functional studies to determine the different effect of these variants on these patients are necessary.

In conclusion, a common *NFKB1* haplotype was associated with LOAD in our population. If confirmed by other studies, the genotyping of this variants could help to identify individuals at an increased risk of developing this disease. Our results also encourage studies to determine usefulness of the pharmacological targeting of the NF-kB pathway in Alzheimer's disease [34].

Ethics and consent

This study was approved by the clinical research ethics committee of Hospital Universitario Central Asturias (HUCA). All the participants gave written or verbal consent. Data were handled in observance of Spanish legislation on data protection. The study complies with the principles of the Declaration of Helsinki ("Recommendations guiding doctors in biomedical research involving human subjects").

CRediT authorship contribution statement

Daniel Vazquez-Coto: Writing – review & editing, Visualization, Validation, Software, Methodology, Investigation, Formal analysis. **Sergio Perez-Oliveira:** Writing – review & editing, Validation,

Software, Methodology, Investigation, Formal analysis. **Manuel Menéndez-González:** Writing – review & editing, Resources, Investigation, Data curation, Conceptualization. **Eliecer Coto:** Writing – review & editing, Writing – original draft, Validation, Software, Methodology, Investigation, Formal analysis, Conceptualization. **Victoria Alvarez:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

None of the authors have competing interests related to this work.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbr.2024.115264.

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