

# Evidence of activation of the Toll-like receptor-4 proinflammatory pathway in patients with schizophrenia

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**Background:** Alterations in the innate immune/inflammatory system may underlie the pathophysiology of schizophrenia, but we do not understand the mechanisms involved. The main agents of innate immunity are the Toll-like receptors (TLRs), which detect molecular patterns associated with damage and pathogens. The TLR first reported was TLR4, and it is still the most studied one. **Methods:** We aimed to describe putative modifications to the TLR4 proinflammatory pathway using 2 different strategies in 2 cohorts of patients with schizophrenia and matched controls: 1) quantification of protein and mRNA expression in postmortem prefrontal cortex samples from 30 patients with schizophrenia and 30 controls, and 2) identification of single nucleotide polymorphisms associated with the risk of schizophrenia using whole blood samples from 214 patients with schizophrenia and 216 controls. **Results:** We found evidence of alterations in the expression of the initial elements of the TLR4 signalling pathway (TLR4, Myeloid differentiation primary response gene 88 [MyD88] and nuclear factor- $\kappa$  B [NF- $\kappa$ B]) in the PFC of patients with schizophrenia. These alterations seem to depend on the presence/absence of antipsychotic treatment at death. Moreover, a polymorphism within the *MyD88* gene was significantly associated with schizophrenia risk. **Limitations:** The use of 2 different approaches in 2 different cohorts, the lack of a complementary neuropsychiatric group, the possible confounding effects of antipsychotic treatment and suicide are the main limitations of our study. **Conclusion:** The evidence from this dual approach suggests there is an altered innate immune response in patients with chronic schizophrenia in which the TLR4 proinflammatory pathway could be affected. Improved understanding of the stimuli and mechanisms responsible for this response could lead to improved schizophrenia treatment and better control of the side effects of current antipsychotics.

## Introduction

There is increasing evidence of activation of the innate immune system in psychosis, although the precise role this activation plays in the etiology, degree and evolution of the structural/functional brain alterations remains unclear.<sup>1,2</sup>

Innate immunity is a nonspecific homeostatic response; however, if uncontrolled, it becomes harmful.<sup>3</sup> The response is initiated through the family of Toll-like receptors (TLRs), which are pattern recognition receptors that detect circulating pathogen-associated molecular patterns (PAMPs) that are present in pathogens but not in mammalian cells. These patterns trigger a complex proinflammatory cascade that can regulate central nervous system (CNS) homeostasis and even promote pathology.<sup>4</sup> Toll-like receptors are highly expressed in immune cells<sup>5</sup> and in different types of CNS cells.<sup>6</sup> This ubiquitous distribution suggests that TLRs play other roles in non-pathogen-associated

CNS diseases/injuries, presumably through recognizing a number of endogenous molecules released from damaged tissues (damage-associated molecular patterns [DAMPs]).<sup>7</sup>

The most studied member of the TLR family is TLR4, which mostly responds to lipopolysaccharide (LPS) from Gram-negative bacteria<sup>8</sup> through its coreceptor: myeloid differentiation protein-2.<sup>9</sup> Through recruiting some other adaptor proteins, such as the myeloid differentiation factor 88 (MyD88), TLR4 acquires specificity to intracellular signalling. After various consecutive steps in the transduction pathway (i.e., activation of specific kinases, such as interleukin [IL]1 receptor-associated kinase 1 [IRAK1]), the prototypic inflammatory nuclear factor- $\kappa$  B (NF- $\kappa$ B) is activated. This in turn triggers expression of the enzymes inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) and the activation of cytokines, such as IL-1 $\beta$  and IL-6. The activation of these proinflammatory mediators in the brain can produce an

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accumulation of oxidative and nitrosative molecules, which can attack membrane phospholipids thereby causing cell damage via lipid peroxidation.<sup>10</sup> There are endogenous counterbalancing mechanisms, such as activation of the anti-inflammatory nuclear receptor peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ )<sup>11,12</sup> (Appendix 1, Fig. S1, available at [jpn.ca](#)).

Recently, a peripheral imbalance of these pro-/anti-inflammatory pathways was reported in patients with first-episode psychosis (FEP) and in patients with chronic schizophrenia with an acutely exacerbated condition.<sup>13–15</sup> However, only a few studies report altered TLR expression or response to immune stimuli in patients with schizophrenia.<sup>16,17</sup>

A recent update reviews the randomized controlled trials of the efficacy of anti-inflammatory compounds in patients with schizophrenia.<sup>18</sup> However, the effects of long-term antipsychotic treatment on the TLR4 signalling pathway and the putative therapeutic use of compounds capable of modulating this pathway remains largely unexplored.

Interestingly, results from several genome-wide association studies (GWAS) in patients with schizophrenia have reported significant associations in chromosome regions that contain different genes related to the immune system and inflammation.<sup>19–22</sup> Although no genetic association studies include *TLR4* in patients with schizophrenia, an increased frequency of the *TLR4* rs11536891 polymorphism was recently reported in patients with early-onset bipolar disorder compared with controls.<sup>23</sup>

Several circumstances provide good reasons to study TLR4 in schizophrenia populations specifically. First, some studies indicate that TLR4 may play a significant role in neurodevelopment and plasticity.<sup>24</sup> Second, TLR4 could participate decisively in the priming alterations of the fetomaternal immune system after infection/stress.<sup>25</sup> Third, an altered microbiome and signs of intestinal inflammation, increased intestinal barrier permeability and bacterial translocation are present in patients with schizophrenia.<sup>26</sup> Finally, paliperidone regulates stress-induced activation of TLR4 in animal models.<sup>27</sup>

Taking into consideration all this background, in the present study we aimed to determine and report putative modifications of the TLR4 signalling pathway by using 2 different approaches: 1) quantification of protein and mRNA expression in postmortem prefrontal cortex (PFC) samples and 2) genetic association analysis to identify single nucleotide polymorphisms (SNPs) associated with schizophrenia.

## Methods

### *Postmortem human brain studies*

#### **Sample**

Human brain samples were obtained from autopsies (Basque Institute of Legal Medicine, Bilbao, Spain) in compliance with research policies and ethical committees for postmortem brain studies. After a retrospective search for antemortem medical information, we obtained postmortem PFC brain samples from 30 individuals who had a diagnosis of schizophrenia according to DSM-IV and from 30 controls matched for sex and age ( $\pm 4$  yr) in a paired design. The criteria for selecting controls

were absence of neuropsychiatric disorders and absence of drug abuse. We preferentially selected samples from controls with sudden and unexpected deaths in order to avoid possible confounding agonal influences. We screened blood from all sample donors to determine the presence of antipsychotics, other drugs and ethanol. According to the absence or presence of antipsychotic drugs in the toxicological screening, the schizophrenia samples (and their matched controls) were divided into 2 subgroups: antipsychotic-free ( $n = 17$ ) and antipsychotic-treated ( $n = 13$ ). Characteristics of the definitive schizophrenia-control pairs are shown in Appendix 1, Table S1. After completion of the assays, because the results of the 2 matched control subgroups did not differ for confounding factors (Appendix 1, Table S1), they were pooled for statistical analysis.

Specimens of PFC (Brodmann area [BA] 9) were dissected at autopsy (0.5–1 g tissue) following standard procedures.<sup>28</sup> They were immediately stored at  $-80^{\circ}\text{C}$  until assayed. Brain pH values were obtained at autopsy, and the RNA integrity number (RIN) was also assayed, as previously reported, as quality indexes.<sup>29</sup> The group averages for these parameters are shown in Appendix 1, Table S1.

#### **Preparation of nuclear and cytosolic extracts from tissue samples**

We used a modified procedure based on the method of Schreiber and colleagues<sup>30</sup> (see Appendix 1 for details).

#### **Western blot analysis**

To determine the expression levels of TLR4, MyD88, iNOS and COX-2, PFC samples were homogenized by sonication in phosphate-buffered saline (PBS) mixed with a protease inhibitor cocktail (Complete®, Roche Farma) (pH = 7), followed by centrifugation at 12 000g for 10 min at  $4^{\circ}\text{C}$ . In the case of NF- $\kappa$ B and PPAR $\gamma$ , nuclear extracts were analyzed, whereas for the inhibitory subunit of NF- $\kappa$ B, I $\kappa$ B $\alpha$ , cytosolic extracts were used. Detailed information on primary and secondary antibodies can be found in Appendix 1.

#### **Real-time polymerase chain reaction analysis**

We prepared total cytoplasmic RNA from the PFC samples using TRIzol (Invitrogen). Aliquots were converted to complementary DNA (cDNA) using random hexamer primers. Quantitative changes in messenger RNA (mRNA) levels were estimated using real-time polymerase chain reaction. We calculated relative mRNA concentrations from the take-off point of the reactions using the software provided; glyceraldehyde 3-phosphate dehydrogenase (GADPH) and tubulin levels were used to normalize the data. The sequences of the primers can be found in Appendix 1.

#### **Lipid peroxidation**

Lipid peroxidation was measured using the thiobarbituric acid test for malondialdehyde (MDA) following the method described by Das and Ratty<sup>31</sup> (Appendix 1).

#### **Nitrite levels**

As the stable metabolites of nitric oxide, NO $_2^-$  levels were measured using the Griess method (see Appendix 1 for details).

### Statistical analysis

Data were analyzed using the GraphPad and InvivoStat programs. We processed each pair of samples for a case and matched control in parallel to control for experimental variance. Semiquantitative values for mRNA and protein were tested for a Gaussian distribution and compared using a Student unpaired *t* test and 1-way analysis of variance (ANOVA), followed by the Fisher least significant difference (LSD) post hoc test. Correlations were analyzed to evaluate the influence of the postmortem delay, storage time and body mass index (BMI). When positive, we tested the results using analysis of covariance (ANCOVA) with the corresponding factor as a covariate. The results are expressed as means  $\pm$  standard errors of the mean. We considered results to be significant at  $p < 0.05$ .

### Genetic association study

#### Participants

A total of 216 patients with schizophrenia diagnosed using the Structured Clinical Interview for DSM-IV (SCID-DSM-IV)<sup>32</sup> were recruited at the Psychiatric Service of the Hospital Clínic, Barcelona, Spain. We excluded 2 patients from the study owing to technical genotyping problems, leaving 214 patients (139 men, 75 women, mean age  $30.6 \pm 11.3$  yr) for our analysis. We selected 216 healthy controls matched for age and sex (140 men, 76 women, mean age  $30.4 \pm 11.2$  yr) for the purpose of this study. A questionnaire was administered to each participant in an interview to elicit demographic information, smoking habits and personal medical history. Moreover, the

clinical information was checked against clinical records. Controls were excluded if they reported a history of cancer or mental disorder. All the participants were white and had previously participated in other genetic studies of schizophrenia risk.<sup>33</sup> We obtained written informed consent and blood samples from each participant, and the Ethics Committee of the Hospital Clínic approved the study protocol.

#### Sample preparation

We collected blood samples from participants in ethylenediaminetetraacetic acid (EDTA; BD Vacutainer EDTA tubes, Becton Dickinson), and genomic DNA was extracted with the MagNA Pure LC DNA Isolation Kit III and an LC MagNA Pure system (Roche Diagnostics GmbH). We measured DNA concentration and quality spectrophotometrically using a NanoDrop 2000 (Thermo Fisher Scientific).

#### SNP selection and genotyping

We selected SNPs located in 9 genes known to be involved in the regulation of the TLR4 pathway, in accordance with data published in PubMed, Ensembl and the Genetic Association Database. Our aim was to find SNPs with functionality based on the consequence of the nucleotide change (e.g., missense), the location within the gene (e.g., regulatory region) or previously described associations (with protein levels, inflammatory processes or schizophrenia risk and other mental disorders; Table 1). We excluded SNPs with frequencies lower than 5%. Some SNPs were rejected before genotyping owing to the assay rules. A total of 22 SNPs (Table 1) were genotyped

**Table 1: Twenty-two single nucleotide polymorphisms included in the genetic association study**

Gene	SNP	Chr. position	Gene position	Major/minor alleles	Minor allele frequency	References*
<i>LBP</i>	rs2232571	20:36974084	5' upstream	T/C	0.134	62
<i>CD14</i>	rs5744455	5:140013307	5' upstream	C/T	0.208	63
	rs5744441	5:140016847	5' upstream	C/T	0.213	64
<i>TLR4</i>	rs4986790	9:120475302	Nonsynonymous	A/G	0.056	61
	rs4986791	9:120475602	Nonsynonymous	C/T	0.053	61
<i>MyD88</i>	rs11536889	9:120478131	3'UTR	G/C	0.144	65
	rs4988453	3:38179254	5' upstream	C/A	0.042	66
	rs4988457	3:38182136	Noncoding exon	C/G	0.043	66
	rs7744	3:38184021	3'UTR	A/G	0.153	50
<i>IRAK4</i>	rs4251545	12:44180295	Nonsynonymous	G/A	0.110	67
<i>IRAK1</i>	rs3027898	X:153275890	3' downstream	A/C	0.199	68
<i>NFKB1</i>	rs28362491	4:103422155	5' upstream	ATTG /-	0.332	69
	rs72696119	4:103422504	5'UTR	C/G	0.329	70
	rs2272676	4:103423326	Splicing region	G/T	0.274	NR
	rs230529	4:103457418	Intron region	G/A	0.329	71
	rs4699030	4:103503824	Intron region	G/C	0.338	71
	rs148268461	4:103534740	Splicing region	- /A	0.053	NR
<i>IL6</i>	rs1800796	7:22766246	5' upstream	G/C	0.069	72
	rs1800795	7:22766645	5' upstream	G/C	0.350	58
<i>IL1B</i>	rs1143634	2:113590390	Synonymous	C/T	0.227	58
	rs1143627	2:113594387	5'UTR	T/C	0.378	73
	rs16944	2:113594867	5' upstream	G/A	0.375	58

NR = no reference; SNP = single nucleotide polymorphism; UTR = untranslated region.

\*Includes 1 relevant reference on which the polymorphism selection was based. In cases of NR, the polymorphism was selected based just on its gene position. These references can be found in Appendix 1, available at [jpn.ca](http://jpn.ca).

using the MassArray assay with the Sequenom genotyping system at the Santiago de Compostela node of the Spanish National Genotyping Centre (CeGen). For quality control, 10 samples were genotyped in duplicate for all the SNPs analyzed, with 100% concordance.

### Statistical analysis

Sample size and statistical power calculations were performed using Quanto 1.2 software. These calculations were based on allele frequencies in population genetics data from 1000 genomes (Ensembl). Given the sample size and assuming a 5% level of significance, we were able to detect odds ratios of 1.9–2.3 with more than 80% statistical power when we analyzed SNPs with allele frequencies of 0.05–0.4.

We calculated means and standard deviations for continuous variables. Univariate analysis ( $\chi^2$  test for categorical variables and Student *t* test for continuous variables) was used to identify variables associated with the risk of schizophrenia using SPSS statistics software version 20 (IBM). No differences in age or sex were detected. However, we observed significant differences in smoking habits (50.0% of patients v. 37.9% of controls,  $p = 0.024$ ). To account for this, the statistical analysis was adjusted accordingly.

After quality control of the genotyping, all the SNPs were included in the statistical analysis, as all of them showed consistent clustering and high genotyping rates (> 90% of samples), and none of them was out of Hardy–Weinberg equilibrium. To estimate the independent contribution of each SNP to schizophrenia risk, we assessed genotype frequencies by means of multivariate methods based on logistic regression analysis, with the analysis adjusted for variables significantly more common in patients with schizophrenia (smoking habits). For this, we used the SNPAssoc R package.<sup>34</sup> The haplotype analysis was also performed using the SNPAssoc R package. We performed SNP interaction analysis using the multifactor dimensionality reduction (MDR), as described elsewhere,<sup>35</sup> and the MDR 2.0 software, available from the open-source MDR project ([www.epistasis.org/software.html](http://www.epistasis.org/software.html)). Further information on the methods used in the SNP interaction analysis can be found in Appendix 1.

To account for the multiple testing issue, we applied a Bonferroni correction (0.05/number of SNPs analyzed) for the single SNP analysis (after Bonferroni correction, results were considered to be significant at  $p < 0.002$ ) and permutation for the interaction analysis (the  $p$  value of the best model was corrected for multiple testing by 1000 permutation cycles using the MDR Permutation Testing Module 1.0).

## Results

### *TLR4 pathway in postmortem human brain samples of patients with schizophrenic and matched controls*

TLR4 and MyD88 protein expression were higher in the post-mortem PFC samples of patients with schizophrenia than matched controls (Fig. 1A and B). In addition, TLR4 displayed a positive correlation with storage time. The subsequent ANCOVA, controlling for storage time, confirmed the ele-

vated TLR4 expression in patients with schizophrenia versus controls ( $p = 0.010$ ). MyD88 mRNA levels did not differ between the groups (Fig. 1C). NF- $\kappa$ B expression in nuclear extracts was also higher in patients with schizophrenia than controls (Fig. 1D), but there were no changes in the protein levels of I $\kappa$ B $\alpha$  in cytosolic extracts (Fig. 1E); IL-1 $\beta$  and IL6 mRNA levels were also the same for the control and schizophrenia groups (Fig. 1F and G).

Expression of the iNOS and COX-2 enzymes was unaltered in patients with schizophrenia compared with controls (Fig. 1H and I). Similarly, PFC samples from patients with schizophrenia did not present a significant change in the lipid peroxidation index MDA (Fig. 1J) or NO<sub>2</sub> levels (Fig. 1K) when compared with controls.

In order to study counterbalancing mechanisms, we evaluated PPAR $\gamma$  expression. Western blot studies showed an increase in nuclear PPAR $\gamma$  protein levels in the PFC of patients with schizophrenia compared with controls (Fig. 1L). TLR4 pathway results were controlled for suicide, which was the cause of death of a large number of patients with schizophrenia. No influence was observed except for PPAR $\gamma$  protein levels, where the difference between patients with schizophrenia and controls was attenuated ( $F_{1,57} = 3.09$ ,  $p = 0.08$ ).

### **Effect of antipsychotic treatment on TLR4 pathway**

In order to determine the possible effect of antipsychotic treatment on the TLR4 proinflammatory pathway, patients with schizophrenia were divided into 2 groups: antipsychotic-free and antipsychotic-treated at time of death. The treated patients presented higher levels of TLR4 and MyD88 protein expression than controls (Fig. 2A and B). No significant differences were found between antipsychotic-free patients and controls or antipsychotic-treated patients in either parameter, although, especially for TLR4, the confidence interval for antipsychotic-free patients extended to the median of the treated patients. At the MyD88 mRNA expression level, treated patients presented higher levels than both control and antipsychotic-free patients, with no major differences between the latter 2 groups (Fig. 2C). This suggests that this effect could be not only medication-associated, but also genetically mediated.

Moreover, antipsychotic-free patients showed higher NF- $\kappa$ B expression than the control group (Fig. 2D). The remaining parameters studied in blood did not differ between the antipsychotic-free and treated patients at the time of death, except for MDA levels, which appeared lower in antipsychotic-free patients than controls and higher in antipsychotic-free patients than in treated patients (Fig. 2J). Post hoc differences in MDA levels between controls and antipsychotic-free patients ( $p = 0.020$ ) were attenuated when we controlled for suicide ( $p = 0.06$ ).

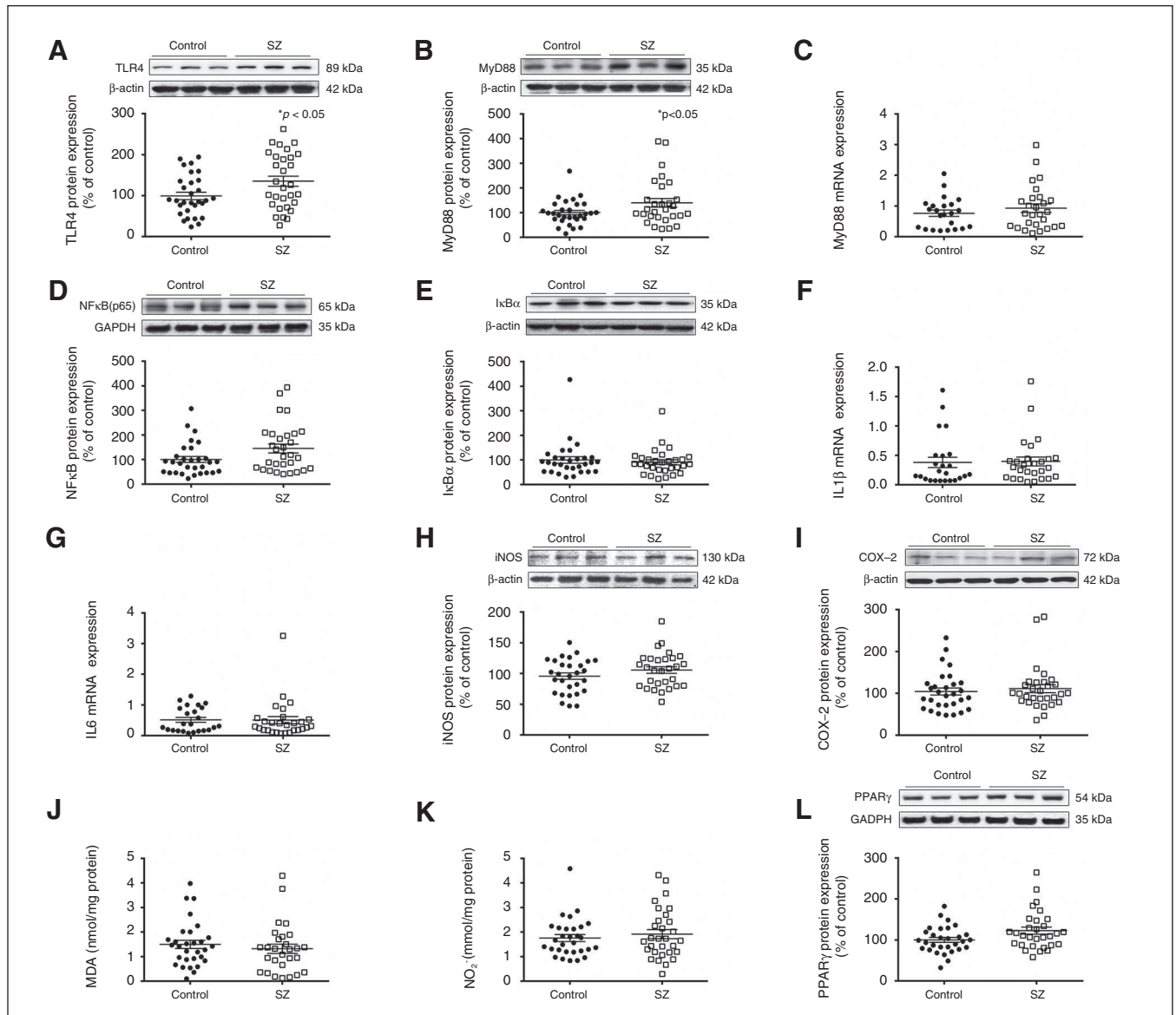
### *Genetic association study*

The genotype frequencies of 3 SNPs differed between controls and patients with schizophrenia (Table 2). While 4.2% of controls were homozygous for the minor frequency allele of *MyD88* rs7744, no cases were homozygous for the same allele ( $p = 0.001$ ). Regarding *IL6* rs1800795, about 20% more



controls than patients with schizophrenia were carriers of the minor frequency allele ( $p = 0.007$ ). Moreover, differences in genotype frequencies of *IL1B* rs1143634 were also found: while only 3.7% of controls were homozygous for the minor frequency allele, 7.5% of patients with schizophrenia were homozygous for the same allele ( $p = 0.037$ ). *MyD88* rs7744 was the only SNP that remained significantly associated with schizophrenia after Bonferroni correction. Haplotypes in *MyD88* (rs4988453–rs4988457–rs7744) and *IL6* (rs1800796–rs1800795) were nominally associated. The frequency of the

*MyD88* CCG haplotype, containing the minor frequency allele rs7744, was higher in controls than in patients with schizophrenia (18.5% v. 11.3%,  $p = 0.014$ ). In the same way, the *IL6* GC haplotype, containing the minor frequency allele rs1800795, was more common in controls than in patients with schizophrenia (18.5% v. 11.3%,  $p = 0.045$ ). In both cases, the haplotype associations were no stronger than those found with the single polymorphisms alone. In fact, rs7744 and rs1800795 were responsible for the *MyD88* and *IL6* haplotype associations, respectively.



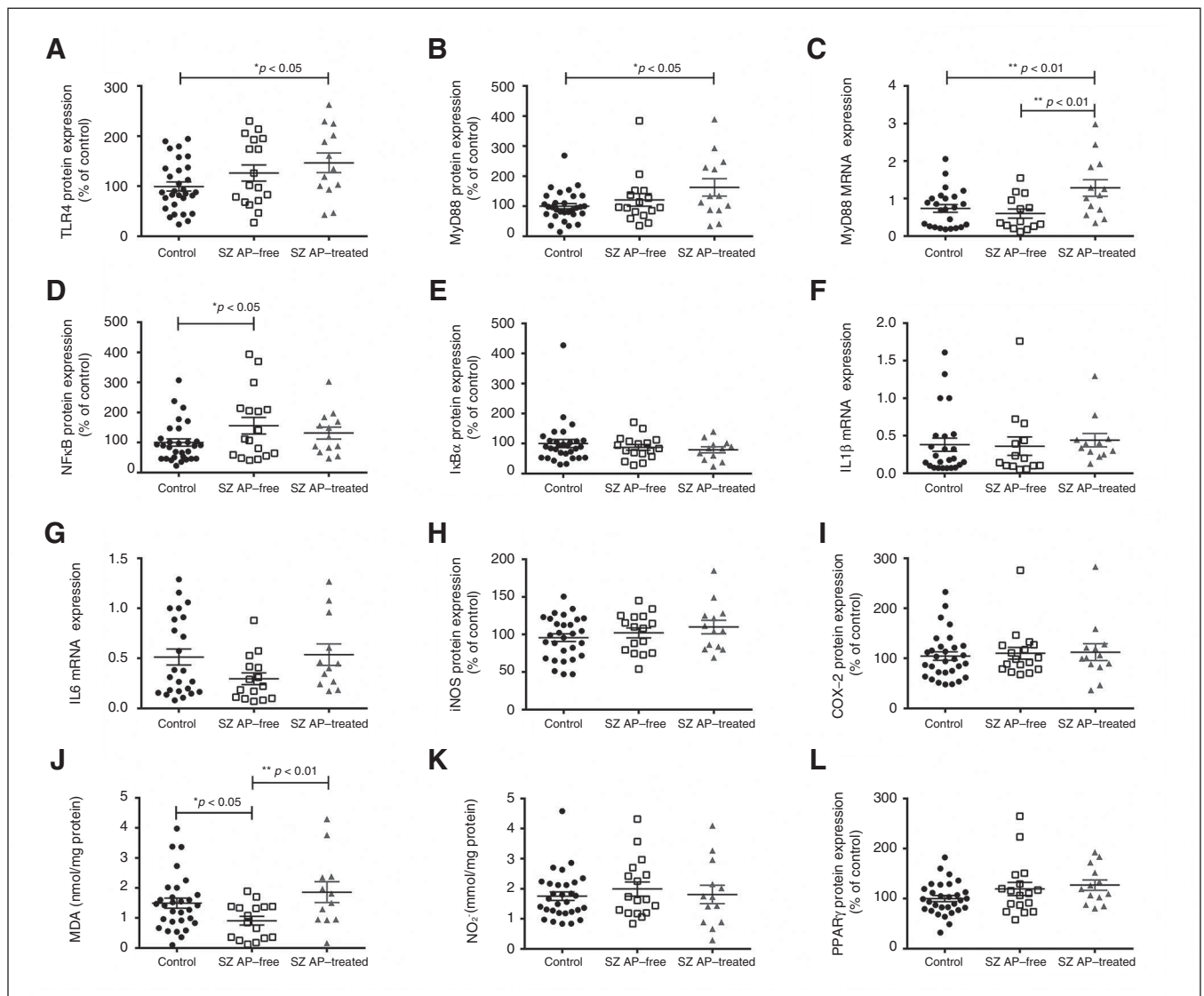
**Fig. 1:** Toll-like receptor-4 (TLR4) pathway in postmortem human brain samples from patients with schizophrenia (SZ) and matched controls. (A) TLR4 and (B) myeloid differentiation factor 88 (MyD88) protein levels. (C) Relative messenger RNA (mRNA) levels of MyD88. Protein levels of (D) nuclear factor  $\kappa$ B (p65) and (E) inhibitory protein  $\kappa$ B $\alpha$ . Messenger RNA levels of (F) interleukin (IL)1 $\beta$  and (G) IL6. Protein levels of (H) inducible nitric oxide synthase (iNOS), (I) cyclooxygenase-2 (COX-2), (J) malondialdehyde (MDA) and (K) nitrite levels (NO<sub>2</sub>). (L) Protein levels of peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) in the prefrontal cortex of patients with schizophrenia and matched controls. The densitometric data of the respective bands of interest are normalized with respect to  $\beta$ -actin/glyceraldehyde 3-phosphate dehydrogenase (lower band). \* $p < 0.05$  compared with controls; unpaired Student  $t$  test analysis. Data represent the means  $\pm$  standard errors of the mean.

The results of the exhaustive MDR analysis are given in Table 3. A 5-way model including the rs1800795 (*IL6*), rs7744 (*MyD88*), rs2272676 (*NFKB1*), rs1143627 (*IL1B*) and rs3027898 (*IRAK1*) variants performed best overall (training accuracy: 0.721; testing accuracy: 0.602) with a cross-validation consistency of 100/100 (permutation testing  $p = 0.010$ ). The stepwise addition of the 5 variants increased the accuracy from 0.561 to 0.602. We constructed a multilocus attribute with the 5 SNPs. Carriers of the predisposing attribute (69.9% of cases v. 30.1% of controls) were almost 7 times more likely to develop schizophrenia than those without the attribute (odds ratio 6.9, 95% confidence inter-

val 4.4–10.6,  $p < 0.001$ ). The predisposing genetic attribute we constructed correctly predicted 76.9% of cases (sensitivity), 67.3% of controls (specificity) and 72.1% of cases and controls (accuracy). Moreover, 69.9% of predicted cases were actual cases (precision).

## Discussion

In the present study, evidence of the involvement of an altered TLR4 pathway in patients with schizophrenia was found in both of the research approaches adopted. We considered it interesting to evaluate the TLR4 pathway at



**Fig. 2:** Effect of antipsychotic treatment on Toll-like receptor-4 (TLR4) pathway in postmortem human brain samples of patients with schizophrenia (SZ) and matched controls. (A) TLR4 and (B) myeloid differentiation factor 88 (MyD88) protein levels. (C) Relative messenger RNA (mRNA) levels of MyD88. Protein levels of (D) nuclear factor  $\kappa$ B (p65) and (E) inhibitory protein  $\kappa$ B $\alpha$ . Messenger RNA levels of (F) interleukin (IL)1 $\beta$  and (G) IL6. Protein levels of (H) inducible nitric oxide synthase (iNOS) and (I) cyclooxygenase-2 (COX-2), (J) malondialdehyde (MDA) and (K) nitrite levels (NO $_2$ ). Protein levels of (L) peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) in the prefrontal cortex of patients with schizophrenia free from or treated with antipsychotics and matched controls. \* $p < 0.05$ , \*\* $p < 0.01$ ; 1-way analysis of variance followed by the Fisher least significant difference post hoc test. Data represent the means  $\pm$  standard errors of the mean.

different stages, from genes to protein level, to strengthen the evidence of its role in the pathophysiology of schizophrenia.

In our search for polymorphisms, we tried to find possible etiological mechanisms, and through the postmortem study, we could check possible lifetime effects (including confounding factors, such as age, BMI or medication) on the state and response of the innate immune system in a chronic clinical manifestation of the disease.

First, we found evidence of higher expression of TLR4 and MyD88 in the postmortem PFC of patients with schizophrenia. TLR4 upregulation in this brain area has also been demonstrated in the PFC of alcoholic adolescents.<sup>36</sup> In accordance with our results, NF- $\kappa$ B upregulation had previously been found in the postmortem PFC of patients with schizophrenia and in patients with other psychiatric diseases, such as depression or autism.<sup>37–39</sup> Recently, meta-analysis of gene coexpression networks in the postmortem PFC of patients with schizophrenia and controls found quantitative and clustering alterations in the module including immunity-related genes, such as *TLR2*, *CD14*, *MYD88* and *NFKBIA*.<sup>40</sup>

Previous studies demonstrated a peripheral imbalance in the TLR4 signalling pathway in patients with schizophrenia. Enhanced TLR-dependent responses were observed, producing a massive release of IL6 or TNF- $\alpha$ .<sup>16</sup> In addition, other authors found increased TLR4 expression in the monocytes of patients with schizophrenia.<sup>17</sup> Interestingly, these monocytes are less reactive to viral infection.

However, the origin of TLR4 activation in individuals with psychotic diseases remains unknown. Some nonexcluding mechanisms have been proposed. First, given the key role played by LPS in TLR4 stimulation, increased intestinal barrier permeability and bacterial translocation have been reported in patients with schizophrenia, as in those with major depression. Patients present an altered microbiome and, in some cases, intestinal inflammation.<sup>26</sup> In addition, DAMPs are believed to be involved in the pathophysiology of psychotic disease,<sup>41</sup> but their precise role remains unclear. Second, there is growing preclinical evidence that preperinatal infections can induce maternal immune activation and inflammatory and oxidonitrosative stress that can lead to neurodevelopmental damage and behavioural abnormalities in the progeny.<sup>25,42–45</sup> In fact, activation of TLRs by infection has been reported to affect the fetomaternal immune response and cause behavioural abnormalities in descendants.<sup>44,45</sup>

Other possible sources of activation of the innate immune system have been recently suggested for major depressive disorder and could also be applicable to schizophrenia. These include psychosocial stressors, physical inactivity, obesity, smoking, poor dental care or overall hygiene and sleep deprivation.<sup>46</sup> All these putative mechanisms of activation could activate other TLRs; this is especially relevant considering that the degree and type of inflammatory response could vary depending on the specific combinations of TLRs activated at the microglial level.<sup>47</sup> This dynamic and heterogenic activation

**Table 2: Significant genotype associations**

SNP	Group; no. (%)		OR (95% CI)	<i>p</i> value*
	Schizophrenia	Control		
rs7744 ( <i>MyD88</i> )				
AA+AG	214 (100.0)	207 (95.8)	1.00	
GG	0 (0.0%)	9 (4.2)	0.00 (0.0–0.0)	0.001
rs1800795 ( <i>IL6</i> )				
GG	105 (49.1)	85 (39.4)	1.00	
CG+CC	109 (50.9)	131 (60.6)	0.56 (0.5–0.9)	0.007
rs1143634 ( <i>IL1B</i> )				
CC+CT	198 (92.5)	208 (96.3)	1.00	
TT	16 (7.5)	8 (3.7)	2.59 (1.0–6.5)	0.037

CI = confidence interval; OR = odds ratio; SNP = single nucleotide polymorphism.

\*Logistic regression analysis adjusted for smoking habit. Results were considered to be significant at  $p < 0.002$  after Bonferroni correction.

**Table 3: Results of the multifactor dimension reduction analysis showing the best model of all possible 1- to 5-loci interactions and their outcome parameters**

Model	Training accuracy*	Testing accuracy†	CVC‡	<i>p</i> value§
rs1800795	0.561	0.561	100/100	0.46
rs2272676–rs3027898	0.586	0.420	63/100	> 0.99
rs1800795–rs2272676–rs3027898	0.616	0.421	61/100	0.39
rs1800795–rs7744–rs2272676–rs3027898	0.661	0.406	44/100	0.99
rs1800795–rs7744–rs2272676–rs1143627–rs3027898¶	0.721	0.602	100/100	0.020

CVC = cross-validation consistency.

\*Accuracy in the 100-fold training data set.

†Accuracy in the 100-fold testing data set.

‡100-fold CVC.

§*p* value of the best model corrected by 1000 permutations.

¶Odds ratio 6.9 (95% confidence interval 4.4–10.6),  $p < 0.001$ , sensitivity 76.9%, specificity 67.3%, accuracy 72.1%, precision 69.9%.

further complicates the study and elucidation of the role of TLR4 in physiologic/pathophysiological CNS conditions.

Efforts should be made to determine whether symptomatic onset of the disease occurs in a vulnerable brain (immunologically primed months or years before symptoms manifest) or whether symptoms that will increase the deleterious effects of inflammation or infection develop in genetically prone individuals.

It is worth discussing the differential results concerning the balance of pro-/anti-inflammatory pathways between the postmortem brain samples evaluated here and those previously reported at the systemic level in patients with FEPS and chronic schizophrenia.<sup>13-15</sup> A significant increase in intracellular components of the NF- $\kappa$ B-dependent main proinflammatory pathway (iNOS, COX-2 expression, and NO<sub>2</sub> and MDA levels), along with a significant decrease in the anti-inflammatory receptor PPAR $\gamma$ , have been reported in patients with FEPS and chronic schizophrenia. However, in our PFC samples, these proinflammatory parameters remained unaltered, and the expression of PPAR $\gamma$  increased. This switch in the pro-/anti-inflammatory balance suggests that the degree and progression of the inflammatory process together with the nature of its autoregulatory mechanisms vary in the different stages of the illness or the different organic compartments studied. Although mRNA expression of IL-1 $\beta$  and IL-6 was not altered in the postmortem PFC samples from patients with schizophrenia, a recent meta-analysis reported increased peripheral IL-1 $\beta$  and IL6 levels in acute psychosis that normalizes with antipsychotic treatment.<sup>48</sup>

In this particular clinical sample, the increased expression of TLR4/MyD88 seems to be predominantly influenced by the pharmacological treatment at the moment of death, which thus constitutes an important confounding factor. Although the anti-inflammatory/antioxidant role of antipsychotics has previously been reported,<sup>49</sup> their direct upstream effects on TLR4/MyD88 expression levels remained unexplored in human samples. Recent data in stress-based animal models show a potential regulatory role of paliperidone on TLR4 activation in the PFC.<sup>28</sup>

One of our most striking results is the higher content of MDA found in the PFC of patients with schizophrenia treated with antipsychotics compared with both antipsychotic-free patients and controls. Our results agree with previous findings that report a neurotoxic profile for high doses or chronic use of antipsychotics.<sup>50,51</sup> Further studies are needed to extend these findings to other brain areas or degrees of psychotic disease.

The results we obtained in the genetic association approach also suggest that the TLR4 signalling pathway is involved in the etiology of schizophrenia. Our results could indicate that individuals homozygous for the *MyD88* rs7744 minor allele (GG) may have protection from schizophrenia. There are only a few studies of the presence or functional role of this polymorphism in human disorders. *MyD88* rs7744 has been associated with autoimmune or inflammatory processes,<sup>52-54</sup> but its biological function has not yet been identified. As it is located in the 3' untranslated region (UTR) region, *MyD88* rs7744 could influence the stability of mRNA, thereby compromising

normal protein levels. Moreover, it has been reported that an alternatively spliced form of *MyD88* acts as a negative regulator of TLR4, which leads to a reduced inflammatory response.<sup>55</sup> It may be that rs7744 was in linkage disequilibrium with another polymorphism that could influence this alternative splicing of the *MyD88* gene. Either possibility would explain the reduced schizophrenia risk observed in individuals homozygous for the *MyD88* rs7744 minor allele. Interestingly, *MYD88* is located on chromosome 3p21.3-p22, a region previously linked with schizophrenia.<sup>56</sup> More recently, several GWAS of schizophrenia have consistently reported positive signals in the 3p21 region, particularly in the *ITIH* family of genes,<sup>22,57,58</sup> which codify for molecules that also seem to play an important role in inflammation.<sup>59</sup>

Our genetic results show that other genes related to the TLR4 signalling pathway could also be involved in schizophrenia. Nominal associations were found for *IL6* rs1800795 and *IL1B* rs1143634. Interestingly, both polymorphisms have previously been associated with the disease.<sup>60-62</sup> Moreover, *IL6* rs1800795, located in the promoter region, has been associated with increased *IL-6* blood levels<sup>61</sup> and with reduced hippocampal volume in antipsychotic-naive patients with schizophrenia.<sup>63</sup>

We also propose a predictive genetic model for schizophrenia risk that includes the *MyD88*, *IL6* and *IL1B* genes, as well as another 2 key genes in the intracellular signalling of the TLR4 pathway: *NFKB1* and *IRAK*. The model identifies different genotype combinations of these 5 genes that allow the classification of patients with schizophrenia and healthy controls with high sensitivity and specificity.

Our genetic results suggest that polymorphisms in genes related to the TLR4 pathway play a role in the pathophysiology of schizophrenia, which would be in line with results from GWAS that report significant signals in regions that include different genes also related to the immune system and inflammation.<sup>19-22</sup>

### Limitations

An important limitation of the present study was that the 2 approaches were applied in 2 different cohorts. No immune marker other than genetic ones could be examined in the genetic cohort because no suitable samples were collected for such a purpose. Then, in order to detect a possible genetic effect, we did not consider it appropriate to increase the heterogeneity by considering the 2 cohorts together, particularly taking into account the small increase of sample size that would have been achieved. In the future, when more post-mortem samples have been collected, it will be interesting to assess the effect of the SNPs in that independent cohort.

At the moment, the significance of the higher expression of TLR4 and MyD88 in patients treated with antipsychotics compared with higher NF- $\kappa$ B in antipsychotic-free patients is difficult to interpret in a meaningful way. However, we must consider the fact that NF- $\kappa$ B is a master inflammatory molecule that could also be activated in mechanisms that are not directly related to the TLR4/MyD88 pathway.

In this vein, it is worth noting that the great majority of patients have experienced chronic treatment, even in the case of



the antipsychotic-free group. The presence of a statistically non-significant trend toward elevated TLR4/MyD88 expression in antipsychotic-free patients could be interpreted in the context of a previous treatment period. With this type of postmortem brain samples, it is almost impossible to find medication-naïve patients, but the toxicological screening that we performed guarantees no antipsychotic medication use in the month preceding death in antipsychotic-free patients.

Unfortunately, cytokine levels are studied only at the mRNA level, and there is a lack of concordance between the mRNA findings concerning cytokines and the genetic polymorphisms and haplotypes found. Further studies are required to check the functional consequences of these polymorphisms.

The predictive capacity of our genetic model for schizophrenia, which includes SNPs in 5 TLR4 pathway genes, would need to be validated in independent populations. It is important to highlight that *MyD88* rs7744 and *IL6* rs1800795 were the genetic variants that explained the highest percentage of the total entropy of the predictive model.

Finally, another limitation was the presence of several potentially confounding factors in some of the individuals included in the postmortem study, such as suicide as the cause of death.

## Conclusion

The evidence from this dual approach suggests the existence of an altered innate immune response in patients with chronic schizophrenia, in which the TLR4 pathway could be one of those affected. Further research is needed to determine the state of the innate immune system in the different grades of psychotic disease (paying special attention to the potential confounding factor of antipsychotic treatment), the etiologic relevance of the polymorphisms found, and whether the pharmacological modulation of TLR4-dependent inflammatory and oxidonitrosative stress is useful for the management of the symptomatology and chronic treatment of psychotic pathologies.

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