Original article

Title: *Toll-like receptor 8 (TLR8)* polymorphisms are associated with non-progression of chronic hepatitis C in HIV/HCV coinfected patients

Running head: *TLR8* polymorphisms and CHC

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Transparency declarations: The authors do not have any commercial or other association that might pose a conflict of interest.

Character count of Title: 115

Character count of Running Head: 23

Word count of Abstract: 254

Word count of Keywords: 6

Word count of Text: 3241

Count of References: 41

Count of Tables: 4

Count of Figures: 1

Supplemental tables: 3

ABSTRACT

Objective: Variants within *toll-like receptor 8 (TLR8)* have been related to Hepatitis C virus (HCV) infection. We evaluate whether *TLR8* polymorphisms are associated with chronic hepatitis C (CHC)–related outcomes in human immunodeficiency virus (HIV)/HCV coinfected patients. Sex-dependent effects could be detected due to *TLR8* gene is located on chromosome X.

Design: Cross-sectional study.

Methods: 220 patients, with a minimum follow-up time of 10 years with HCV infection, were genotyped for *TLR8* polymorphisms (rs17256081, rs4830807, rs1013151, rs5741886, rs5744069, rs3764880) using GoldenGate® assay. The outcome variables were non-fibrosis (F0), mild-inflammation (A0/A1), non-steatosis [fatty hepatocytes (FH) <10%], and non-insulin resistance (IR). The analysis was performed under a dominant model of inheritance.

Results*:* Four polymorphisms were analyzed (rs1013151, rs5744069, rs17256081 and rs1013151). The latter was tag SNP of the haplotype block formed by rs4830807, rs1013151 and rs5741886. Two *TLR8* polymorphisms (rs1013151 and rs5744069) were significantly associated with non-fibrosis for male patients [(adjusted odds ratio (aOR)=4.49 (95%CI=1.08; 18.62) (p=0.039) and aOR=6.17 (95%CI=1.45; 26.20) (p=0.014); respectively], and patients infected with HCV genotype 1 (GT1) [aOR=5.79 (95%CI=1.44; 23.32) (p=0.013) and aOR=8.01 (95%CI=2.16; 35.65) (p=0.005); respectively]. In the latter group, rs3764880 polymorphism was also related to non-fibrosis [aOR=5.65 (95%CI=1.26; 25.36); p=0.024]. In addition, three *TLR8* polymorphisms (rs17256081, rs1013151 and rs5744069) were significantly associated with non-IR for male patients [aOR=2.38 (95%CI=1.11; 5.09) (p=0.026), 3.19 (95%CI=1.40; 7.27) (p=0.006), and 2.33 (95%CI=1.02; 5.31) (p=0.045); respectively].

Conclusions*: TLR8* polymorphisms seem to be related to non-progression of liver fibrosis and absence of IR in HIV/HCV coinfected patients, particularly in males and those patients infected with GT1.

Key words: AIDS; chronic hepatitis C; insulin resistance; fibrosis; SNPs, TLR8.

INTRODUCTION

Chronic hepatitis C (CHC) represents a leading comorbidity in human immunodeficiency virus (HIV) and hepatitis C virus (HCV) coinfected patients during the combination antiretroviral therapy (cART) era [1, 2]. Additionally, HIV infection modifies the natural history of CHC, with a faster progression of fibrosis in HIV/HCV coinfected patients [3]. Moreover, although the published data suggests that cART might be beneficial for HIV/HCV-coinfected patients [4], the interactions among HIV, HCV and cART are also associated with several metabolic disorders [5], including dyslipidemia, lipodystrophy, steatosis, insulin resistance, and type 2 diabetes mellitus [5, 6]. These metabolic disturbances have been directly related to fibrosis progression, irrespective of the HCV genotype [7, 8].

Toll-like receptors (TLRs) are specific sensors of microbial and endogenous danger signals, highly conserved across species, which play a fundamental role in pathogen recognition and activation of innate immunity, and may induce a strong response against HCV [9]. TLRs are also involved in the pathophysiology of a wide range of diseases, and the genetic variability observed in TLRs has been related to differences in susceptibility to infectious and inflammatory diseases [10].

TLR8, which seems to be critical for eliminating RNA viruses, is an intracellular receptor that may recognize double-stranded RNA (dsRNA) from viruses in endosomal compartments. When TLR8 is activated induces the production of both nuclear factor (NF)-κB-dependent cytokines and type I interferons (IFNs), such as IFN-β [11]. *TLR8* gene is located on chromosome X, which is known to house the largest number of immune-related genes of the whole genome [12]. *TLR8* single nucleotide polymorphisms (SNPs) have been related with an altered innate immune response, susceptibility to HCV infection [13, 14], and progression of HIV disease [15, 16]. HCV may also directly activate the innate immune system via TLR8, leading to chronic inflammation and hepatic fibrosis; and may impair TLR signaling in order to evade clearance and promote chronic infection [17]. In addition, there are sex differences in innate and adaptive immune response, such as differences in the pathogenesis of infectious disease, being females less prone to suffer them. These differences in immune response are attributed to X chromosome gene contributions, besides sex hormones and environmental factor effects [18].

The aim of this study was to analyze the association between *TLR8* polymorphisms and four CHC-related outcomes (liver fibrosis, liver activity grade, liver steatosis, and insulin resistance) in HCV/HIV coinfected patients.

METHODS

Study design

We carried out a cross-sectional study in HIV/HCV coinfected patients that underwent a liver biopsy at Hospital Gregorio Marañón (Madrid, Spain) between September 2000 and November 2008. All patients were of European ancestry.

Liver biopsies were performed on patients who were potential candidates for anti-HCV therapy and had not received previous interferon therapy. Selection criteria were: no clinical evidence of hepatic decompensation, detectable HCV RNA by polymerase chain reaction (PCR), negative hepatitis B surface antigen, CD4+ lymphocyte count higher than 200 cells/μL, stable antiretroviral therapy or no need for antiretroviral therapy. Patients with active opportunistic infections, active drug addiction, and other concomitant severe diseases were excluded. From our cohort of 361 HIV/HCV coinfected patients with liver biopsy data, 220 patients had data available of *TLR* genotypes.

The study was conducted in accordance with the Declaration of Helsinki and patients gave their written consent for the study. The Institutional Review Board and the Research Ethic Committee of the Instituto de Salud Carlos III (ISCIII) approved the study.

Clinical and laboratory data

Clinical and epidemiological data were obtained from medical records. Consumption of more than 50 g of alcohol per day for at least 12 months was considered as a high intake. Body mass index (BMI) was calculated as the weight in kilograms divided by the square of the height in meters. The degree of insulin resistance (IR) was estimated for each patient using the homeostatic model assessment (HOMA): fasting plasma glucose (mmol/l) times fasting serum insulin (mU/l) divided by 22.5 [19].

The duration of HCV infection for patients with a history of intravenous drug use (IDU) was estimated starting from the first year they shared needles and other injection paraphernalia, which are the most relevant risk practices for HCV transmission [20]. The duration of HCV infection was not calculated when the date of initiation of their HCV infection could be determined with certainty (n=19).

Liver biopsies were performed as we described previously [21]. Liver fibrosis and necroinflammatory activity were estimated according to Metavir score as follows: F0, non-fibrosis; F1, mild fibrosis; F2, significant fibrosis; F3, advanced fibrosis; and F4, definite cirrhosis. Activity grade was scored as follows: A0, non-activity; A1, mild activity; A2, moderate activity; A3, severe activity. Liver steatosis was evaluated according to the existence of hepatocytes containing visible macrovesicular fat droplets. We considered hepatic steatosis to be clinically significant when fatty hepatocytes (FH) exceeded 10% of the hepatic parenchyma.

Genotyping of *TLR8* polymorphisms

TLR8 polymorphisms were elected using the databases of HapMap Proyect (http://snp.cshl.org/cgi-perl/gbrowse/hapmap_B35/) and NCBI (dbSNP) (http://www.ncbi.nlm.nih.gov/entrez/), and next we selected the TLR8 polymorphisms with an allelic frequency greater than 20% in European people.

Genomic DNA was extracted from peripheral blood with Qiagen kit (QIAamp DNA Blood Midi/Maxi; Qiagen, Hilden, Germany). Six *TLR8* polymorphisms (rs17256081, rs4830807, rs1013151, rs5741886, rs5744069, rs3764880) were genotyped at the Spanish National Genotyping Center (CeGen; http://www.cegen.org/) using GoldenGate® assay with VeraCode® Technology (Illumina Inc. San Diego, CA, USA).

Outcome variables

HIV/HCV coinfected patients were classified into groups according to CHC-related outcomes, which have been developed after a minimum follow-up time of 10 years with HCV infection. Four outcome variables were analyzed: i) Non-fibrosis (F0): Values of Metavir score for fibrosis equal to 0; ii) mild-inflammation (A0/A1): Values of Metavir score for activity grade ≤1; iii) Non-steatosis (FH <10%): hepatic steatosis was not clinically significant when fatty hepatocytes did not exceed 10% of parenchyma hepatic; iv) Non-IR, defined as HOMA values <2.5.

Statistical analysis

For the description of the study population, p-values were estimated with Chi-square test for categorical variable. All SNPs were analyzed for Hardy-Weinberg equilibrium (HWE) by Chi-square test, considering equilibrium when p >0.001.

For the genetic association study, logistic regression analysis was used to compare the outcome variables according to *TLR* polymorphisms under a dominant genetic model, which was the model that best fit to our data. These analyses were adjusted by the most important clinical and epidemiological characteristics, which were selected by "Stepwise" algorithm (a p-value for entry and exit of 0.15 and 0.20, respectively). The covariables used were gender, age, alcohol intake, BMI, HOMA, nadir CD4+ T-cells, AIDS, undetectable HIV-RNA (<50 copies/ml), CD4+ T-cells, cART (protease inhibitor or non-nucleoside analogue), HCV-RNA ≥500,000 IU/ml, HCV genotype, and specific antiretroviral drugs (protease inhibitor, tenofovir, abacavir, thymidine analogues, etc.). Thus, each logistic regression analyses were always adjusted for the most significant covariates associated with each one of the outcome variables, avoiding the over-fitting of the regression.

These analyses were performed by using the Statistical Package for the Social Sciences (SPSS) 19.0 software (IBM Corp., Chicago, USA). In addition, pair-wise linkage disequilibrium (LD) analysis was computed by Haploview 4.2 software, and HWE was performed using the PLINK software. All p-values were two-tailed and statistical significance was defined as p <0.05.

***In silico* analysis**

We analyze the possible functional implication of each significant polymorphism, and we look for transcription factor binding sites generated or disrupted by them. The LASAGNA-Search 2.0 [22] web tool (http://biogrid-head.engr.uconn.edu/lasagna_search/) was used to search for transcription factor from TRANSFAC® (TRANScription FACtor database), a public eukaryotic transcription factor database, (http://www.biobase-international.com/product/transcription-factor-binding-sites), which provides experimentally-proven binding sites, consensus binding sequences and regulated genes.

RESULTS

Characteristics of the patients

The epidemiological and clinical characteristics of 220 HIV/HCV coinfected patients are shown in **Table 1**. The median age was 39.8 years, 73.6% were male and 27.3% had prior aids-defining conditions. At the time of liver biopsy procedure, 85.2% patients were on cART. The mean CD4+ count was 468 cells/mm^3, 74.18% had an HIV-RNA <50 copies/mL, and 75.2% had an HCV-RNA >500,000 UI/mL.

Characteristics of *TLR8* polymorphisms

Six *TLR8* polymorphisms were genotyped: one at exon one (rs3764880) which is a missense variant (Met1Val), and would ablate the putative start codon in one of the transcripts encoded by this gene; one located at intron one (rs17256081); and four at intron two (rs4830807, rs1013151, rs5741886, rs5744069) (**Supplemental Table 1**). All of them had a minimum allele frequency (MAF) >5%, and were in HWE (p >0.001). Due to *TLR8* gene is located in chromosome X, Hardy-Weinberg equilibrium was calculated excluding males X chromosomes.

The six *TLR8* polymorphisms showed a strong LD (**Figure 1**), particularly three SNPs (r^2=0.84) located at intron two (rs4830807, rs1013151, rs5741886). In order to avoid redundancy, we analyzed the association of only one SNP of this block (rs1013151) with the outcome variables. The missense rs3764880 polymorphism was in high LD (r^2=0.83) with this block, although not completely. In summary, four SNPs (rs3764880, rs17256081, rs1013151 and rs5744069) were used for the genetic association study.

Significant differences were found in the sex ratio between *TLR8* polymorphisms genotypes under a dominant model of inheritance (**Table 2**).

TLR8 polymorphisms and CHC-related outcomes

Table 3 shows the genotypic frequencies of *TLR8* polymorphisms under a dominant model of inheritance for each one of the outcome variables analyzed. For liver fibrosis, two SNPs (rs1013151, and rs5744069) showed significant differences. Thus, T allele for both rs1013151 and rs5744069 (p=0.001 and p=0.001; respectively) were more frequent in patients without fibrosis (F0). Rs3764880 was close to reach statistical significance (p=0.051). When data were stratified by gender and major HCV genotypes (GT1 and GT3), these differences remained significant and were more pronounced for males and patients infected with GT1. For IR, three SNPs (rs3764880, rs17256081, and rs1013151) had significant differences. Thus, G allele for rs3764880 (p=0.043), and T allele for both rs17256081 and rs1013151 polymorphisms were more frequent in patients without IR (HOMA <2.5) (p=0.015 and p=0.025; respectively). When data were stratified by gender and major HCV genotypes (GT1 and GT3), these differences remained for rs1013151 (p=0.020) in male patients and for rs17256081 (p=0.033) and rs1013151 (p= 0.040) in

patients infected with GT1. No significant differences were found when the liver activity grade and steatosis were analyzed (*data not shown*).

Table 4 shows the association analysis of *TLR8* polymorphisms and CHC-related outcomes. For liver fibrosis, the presence of T allele for both rs1013151 and rs5744069 polymorphisms showed higher odds for non-fibrosis (F0) [adjusted odds ratio (aOR)=4.42 (p=0.006) and aOR=4.76 (p=0.003); respectively]. When data were stratified by gender, both polymorphisms remained significant for male patients [aOR=4.49 (p=0.039) and aOR=6.17 (p=0.014); respectively]. When data were stratified by major HCV genotypes (GT1 and GT3), GT1 patients had significant values of aOR for rs3764880 [aOR=5.65 (p=0.024)], rs1013151 [aOR=5.79 (p=0.013)], and rs5744069 [aOR=8.01 (p=0.005)]. Similar tendency was observed when stratified by GT1 male patients for rs1013151 and rs57444069 [OR=5.37 (p=0.067) and aOR=5.71 (p=0.057); respectively] (**Supplemental Table 2**). Regarding IR, the presence of T allele for both rs17256081 and rs1013151 polymorphisms was related to increased odds of non-developing IR (HOMA <2.5) [aOR=2.23 (p=0.014) and aOR=2.06 (p=0.035); respectively]. When data were analyzed by gender, similar results were obtained for rs17256081 [aOR=2.38 (p=0.026)] and rs1013151 [aOR=3.19 (p=0.006)] in male patients. In this case, rs57444069 was also significantly associated with non-IR (HOMA <2.5) [aOR=2.33 (p=0.045)]. When data were stratified by major HCV genotypes (GT1 and GT3), no significant results were found. However, when stratified by both gender and HCV genotype (**Supplemental Table 2**), rs1013151 and rs57444069 polymorphisms remained significantly associated with non-IR in male patients infected with GT1 (HOMA <2.5) [OR=6.50 (p=0.005) and aOR=4.07 (p=0.029); respectively]. Finally, none of the *TLR8* polymorphisms were significantly associated with mild-inflammation or non-steatosis (*data not shown*). Neither relationship was found between TLR8 polymorphisms and CHC-related outcomes for GT3 patients (**Supplemental Table 3**).

DISCUSSION

The major findings of the present study were: (1) the minor alleles at rs3764880, rs1013151, and rs5744069 polymorphisms were significantly associated with non-fibrosis (F0), particularly in men and in patients infected with GT1; (2) the presence of minor alleles at rs17256081, rs1013151, and rs5744069 polymorphisms were associated with non-IR, particularly in male patients; and (3) a lack of association among *TLR8* polymorphisms and liver activity grade and steatosis. Polymorphisms within *TLR8* gene have been studied in numerous infectious and immune diseases, showing a key role in the innate immune response and thus, in the disease pathogenesis [10]. However, to our knowledge, this is the first study that reports the relationship between *TLR8* polymorphisms and the CHC-related outcomes in HIV/HCV coinfected patients.

The significant results detected in our study were all limited to males, which is understandable due to the location of *TLR8* gene at chromosome X. Genetic association studies on sex chromosomes are special due to several issues: association may be confounded by differences in sex ratio between genotypes, the phenomenon of X inactivation, and the under-representation of females in clinical studies. In our case, there are significant differences in the sex ratio between genotypes, for this reason sex was included as a covariate in the logistic regression analysis, as it was stated by Clayton [23]. The X inactivation -randomly transcriptional silencing of one X chromosome at early stage of female fetal development -, provides dosage compensation for X-linked genes between XX females and XY males [24]. Thus, the effect of A allele in males and A alleles in homozygous females are equivalent. The problem arises for heterozygous females, because they will be mosaics. Thus, this could be one of the causes for the absence of significant results for females. In addition, because of the p-value is dependent on the sample size, the limited number of females enrolled in our study (26.4%) will also inevitably affect the results. The general under-representation of women in clinical trials has been previously stated [24], which in most cases is related with the immunological advantage of women, that have a lower burden of bacterial, viral and parasitic infections [18]. The outcome and survival rates for illnesses are better in women than in men, where the X chromosome play a key role, although the mechanisms are not clear [25]. The X chromosome contains the largest number of immune-related genes of the whole human genome, moreover contains 10% of microRNAs, some of them with interesting roles in immunity such as Mir-448 which is induced by IFNβ and inhibits the Hepatitis C virus replication [12].

The *TLR8* rs3764880 is a non-synonymous polymorphism located at exon 1, which generates a change from A to G that modifies the start codon from ATG (methionine) to GTG (valine), resulting in a truncated TLR8 isoform with a shorter signal peptide (1038 residues for the *TLR8*-rs3764880G allele *vs.* 1041 residues for the *TLR8*-rs3764880A allele) [16]. This modification is a functional polymorphism that decrease NF-kappa B activation, as well as response to different TLR8 ligands. Immune cells carrying the rs3764880 G allele had augmented TNFα-responses, but decreased translation of truncated TLR8 isoform B and NF-κB production relative to those carrying the rs3764880

A allele [26, 27]. Interestingly, previous research indicated that the *TLR8* rs3764880 G allele is associated with 0.78 log_{10} copies/ml higher peak of plasma HIV-RNA [28], but slower natural course of HIV disease [16]. Besides, *TLR8* rs3764880 G allele protects against tissue damage in active tuberculosis [29]. In HCV infection, rs3764880 G allele might be responsible for the enhanced immune activation, which will explain why the natural course of HCV infection is enhanced in these patients.

For the remaining *TLR8* polymorphisms studied, we performed an *in silico* analysis to speculate on the biological implications of the TLR8 polymorphisms and pathogenesis: i) The *TLR8* rs1013151 polymorphisms is located at intron 2. Introns may harbor alternative transcription start sites [30], where transcription factors (TF) may regulate elongation or splicing rather than initiation. We found that they could generate possible targets for TF (TRANSFAC database [31]), which could probably affect *TLR8* expression. Regarding rs1013151 polymorphisms, the presence of the protector allele T may generate a putative target site for signal transducer and activator of transcription 5 (STAT5), which has been related to hepatoprotective genes against liver fibrosis [32]; while the presence of risk allele C at rs1013151 may generate a putative target site for heat shock factor (HSF)1, whose inactivation has been related to liver fibrosis prevention through suppressing collagen production in human hepatic stellate cells [33]. ii) The *TLR8* rs5744069 polymorphism is located at intron 2. The risk allele C generates a target site for the myocyte enhancer factor 2 (MEF2), a transcription factor that regulates multiple aspects of human stellate cells (HSC) activation during the development of liver fibrosis [34]; while the protective allele T does not generates any target site. iii) The *TLR8* rs17256081 polymorphism, located at intron 2, does not generate any target site for TF. However rs17256081 is also located at TLR8-antisense (TLR8-AS1) [35], which encodes for a single stranded RNA that is complementary to TLR8 messenger RNA (mRNA). Antisense-RNAs are able to inhibit translation by base pairing to its complementary mRNA. Therefore, variation within its sequence might affect the base pairing to TLR8 mRNA leading to differential gene expression.

At the molecular level, insulin resistance is promoted by inflammation and activation of the innate immune [36], and TLRs have been also implicated in the pathogenesis of IR [37]. Thus, TLR2 and TLR4 bind to components of the gram -positive and -negative bacteria respectively, activating the immune system and promoting the development of IR [37, 38]. In our study, *TLR8* polymorphisms (rs17256081, rs1013151, and rs5744069) were associated with decreased odds of developing IR, just like were associated with non-fibrosis. Just as we discussed earlier, the protective allele of *TLR8* polymorphisms would be associated with less inflammation, decreasing the risk of having IR.

Moreover, microbial products have recently emerged as potential drivers of this immune activation in HIV infection, increasing the risk of insulin resistance and liver fibrosis [39]. TLR2 is involved in intestinal permeability regulation, which is also related to bacterial translocation [40, 41]; and TLR2 expression is up-regulated following bacterial

activation of TLR8 [11]. Therefore, we could hypothesize that TLR8 is related to IR and liver fibrosis through regulation of TLR2. However, it is difficult to decipher the exact mechanism, because multiple aspects are influencing insulin resistance in HIV/HCV coinfected patients [6].

Several aspects have to be taken into account for the correct interpretation of the results. Firstly, this is a cross-sectional study with a limited number of patients, which could limit achieving statistically significant values. Secondly, metabolic disturbances are caused by several interacting genetic and environmental determinants, being complicated to find the true individual effects of each disease-associated factor. Thirdly, the patients selected for our study were patients who met a set of criteria for starting HCV treatment and it is possible that this may have introduced a selection bias. Moreover, since the study was carried out entirely in Caucasians, and the frequency of these alleles differs among different ethnicities, it would be interesting to perform an independent replication of this study for different ethnic groups. Further analyses are needed in HCV-monoinfected patients in order to evaluate only the effect of CHC, and in HIV-monoinfected patients for assessing the impact of HIV infection and cART in the development of metabolic disturbances. Moreover, further studies are needed in order to assess the possible functional implication of each polymorphism in vitro.

In conclusion, *TLR8* polymorphisms seem to be related to non-progression of liver fibrosis and absence of IR in HIV/HCV coinfected patients, particularly in male patients and infected with GT1. These results could be useful for therapeutic decision-making in clinical practice, because patients with protective *TLR8* alleles will display a better prognosis of the disease.

ACKNOWLEDGEMENTS

The authors thank the Spanish National Genotyping Center (CeGen) for providing the SNP genotyping services (http://www.cegen.org).

Authors contribution: AFR and SR performed all statistical analysis, interpretation of the data and wrote the manuscript. JB and SR participated in the study concept and design. JB, TAE, AC, CD and FT participated in patient selection, collection of samples and acquisition of data. AFR, MAJS, MGA, DPT, MGF and VB participated in sample preparation, DNA isolation and genotyping pre-procedure, and contributed with critical revision of the manuscript. SR supervised the study.

Sources of Funding: This work has been supported by grants from *Fondo de Investigación de Sanidad* (FIS) [PI08/0738, PI11/00245; PI08/0928, and PI11/01556], and *Fundación para la Investigación y la Prevención del Sida en España* (FIPSE) [361020/10]. Besides, this work has been (partially) funded by the RD12/0017/0024 and RD12/0017/0004 projects as part of the Plan Nacional R + D + I and cofinanced by ISCIII- Subdirección General de Evaluación y el Fondo Europeo de Desarrollo Regional (FEDER).

DPT, MGF, MAJS and MGA are supported by *Instituto de Salud Carlos III* [grant numbers CM12/00043, RD12/0017/0024, CD13/00013 and CD12/00442, respectively]. VB is supported by the *Fondo de Investigación Sanitaria* through the *Miguel Servet* program [grant number CP13/00098]. JB is an investigator from the *Programa de Intensificación de la Actividad Investigadora en el Sistema Nacional de Salud* (I3SNS) [INT10/009 and INT12/154].

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