



UNIVERSIDAD DE JAÉN
FACULTAD DE CIENCIAS
EXPERIMENTALES
DEPARTAMENTO DE BIOLOGÍA
EXPERIMENTAL

TESIS DOCTORAL

**GENES DE LA INMUNIDAD INNATA Y
RESISTENCIA A LA INFECCIÓN POR VIH-1
EN USUARIOS DE DROGAS POR VÍA
PARENTERAL**

PRESENTADA POR:
MARÍA DEL ROCÍO HERRERO DEL RÍO

DIRIGIDA POR:
DR. ANTONIO JOSÉ CARUZ ARCOS
DR. LUIS MIGUEL REAL NAVARRETE

JAÉN, 4 DE FEBRERO DE 2016

ISBN 978-84-9159-014-9



Universidad de Jaén,

Departamento de Biología Experimental

Genes de la inmunidad innata y resistencia a la infección por VIH-1 en usuarios de drogas por vía parenteral

M^a del Rocío Herrero del Río

Jaén, 25 de febrero de 2016

Dr. Antonio José Caruz Arcos

Director

Dr. Luis Miguel Real Navarrete

Co-director



UNIVERSIDAD DE JAÉN

Área de Genética

Departamento de Biología Experimental

Facultad de Ciencias Experimentales

D. Antonio José Caruz Arcos, Profesor Titular del Área de Genética del Departamento de Biología Experimental de la Universidad de Jaén.

D. Luis Miguel Real Navarrete, Investigador Principal del Laboratorio de Investigación en la Unidad Clínica de Enfermedades Infecciosas y Microbiología del Hospital Universitario de Nuestra Señora de Valme.

CERTIFICAN:

Que el trabajo presentado para optar al Grado de Doctor en Biología Molecular y Celular titulado “Genes de la inmunidad innata y resistencia a la infección por VIH-1 en usuarios de drogas por vía parenteral” presentado por Dña. M^a del Rocío Herrero del Río y llevado a cabo en la Unidad de Inmunogenética del Departamento de Biología Experimental de la Universidad de Jaén, se ha realizado bajo nuestra dirección y que reúne los requisitos para proceder a la lectura y la defensa del mismo.

Para que así conste, firman la presente:

Fdo.: Antonio José Caruz Arcos

Fdo.: Luis Miguel Real Navarrete

En Jaén, a 24 de febrero de 2016

En Sevilla, a 24 de febrero de 2016

*Cuando emprendas tu viaje a Itaca
pide que el camino sea largo,
lleno de aventuras, lleno de experiencias.
No temas a los lestrigones ni a los cíclopes
ni al colérico Poseidón,
seres tales jamás hallarás en tu camino,
si tu pesar es elevado, si selecta
es la emoción que toca tu espíritu y tu cuerpo.
Ni a los lestrigones ni a los cíclopes
ni al salvaje Poseidón encontrarás,
si no los llevas dentro de tu alma,
si no los yergue tu alma ante ti.*

*Pide que el camino sea largo.
Que muchas sean las mañanas de verano
en que llegues -¡con qué placer y alegría!-
a puertos nunca vistos antes.
Detente en los emporios de Fenicia
y hazte con hermosas mercancías,
nácar y coral, ámbar y ébano
y toda suerte de perfumes sensuales,
cuantos más abundantes perfumes sensuales puedas.
Ve a muchas ciudades egipcias
a aprender, a aprender de sus sabios.*

*Ten siempre a Itaca en tu mente.
Llegar allí es tu destino.
Mas no apresures nunca el viaje.
Mejor que dure muchos años
y atracar, viejo ya, en la isla,
enriquecido de cuanto ganaste en el camino
sin aguantar a que Itaca te enriquezca.*

*Itaca te brindó tan hermoso viaje.
Sin ella no habrías emprendido el camino.
Pero no tiene ya nada que darte.*

*Aunque la halles pobre, Itaca no te ha engañado.
Así, sabio como te has vuelto, con tanta experiencia,
Entenderás ya qué significan las Itacas.*

Itaca - C.P. Cavafis

Tras resumir en datos cinco años de mi vida, me pongo a escribir los agradecimientos y no sé qué decir. ¡No será, porque no tengo a quién agradecer!... Pero si es verdad, que es más complicado resumir el tiempo en sensaciones que en datos; supongo que es porque ya se lleva un camino recorrido cuando se empieza la tesis, por lo que es imposible determinar en el terreno sentimental, el instante de inicio.

Tengo que agradecerle a Antonio Caruz, mi padre en la ciencia, la oportunidad que me ha dado de poder conocer este mundo tan apasionante como es la investigación; y a Luis Miguel Real, la ayuda que me has brindado a pesar de la distancia.

A mi familia. Sin vosotros, esto no habría sido posible. Jesús y M^a Luisa, mis padres, gracias por haberme inculcado los principios que tengo, la curiosidad por todo y por decirme siempre que los límites nos los ponemos nosotros mismos. A mi hermano, Jesús M^a, por ser el referente que siempre he tenido y por todas las veces que me has ayudado cuando te lo he pedido y cuando no. Los tres siempre me habéis apoyado en cada decisión que he tomado, en cada nuevo proyecto que he emprendido y de todas las formas posibles. Mi sacrificio ha sido el vuestro y sin vosotros, nunca habría llegado hasta aquí. Os lo agradezco y os lo agradeceré siempre.

Tengo que agradecer a todos los alumnos de grado y máster que han pasado durante estos años por el Grupo de Inmunogenética, cada uno de vosotros me habéis enseñado algo. Y en especial, agradecer a Emilio Valdivia el apoyo que me ha brindado durante estos dos últimos años; haces que los 9400 km que nos separan en línea recta y las 6 ó 7 horas de diferencia horaria, parezcan inexistentes. Gracias por ser la magnífica persona que eres.

Un agradecimiento a todo el Departamento de Biología Experimental. A las administrativas, en especial a Pilar Ogallar, hacéis un trabajo imprescindible y brillante que a veces no sabemos apreciar. A Juani, desde que llegaste el laboratorio es mucho más alegre. A Eva Siles y a Esther Martínez, del Área de Bioquímica y Biología Molecular. También al grupo de Paco Navarro, hemos compartido muchas cosas y tiempo juntos. Y por supuesto, al grupo de cardiovascular o como a mí me gusta llamarlos “las amables gentes de celular” (Estefanía, Fernando, Ángel, Dani, José y Feli), echaré de menos meterme con vosotros y que vosotros os metáis conmigo.

Azahara Salazar Fernández, pollito, creo que nos conocimos en un punto de inflexión en nuestras vidas. Sin tí, París no hubiese sido lo mismo. Prometo intentar poner en mi vida un poco más de: “Tú, sin mente como los pollos. Tú la lías, luego te sientas y dejas que las cosas se solucionen por si solas”. Este donde este, sabes que tienes una amiga para lo que sea.

Pero estos agradecimientos estarían incompletos si no menciono a toda la gente que durante mi vida he conocido y que en cierto sentido, me han ido modelando también como persona. A todos los enfermos que atendí y compartieron parte de sus vidas conmigo, me disteis lo mejor que una persona puede dar a otra, las experiencias vividas. A todas aquellas personas que creyeron en mí y me apoyaron. Y también, a aquellas personas que me pusieron zancadillas por el camino, porque, pese a ser amargas, gracias a esas caídas he crecido como persona y me han ayudado a ser quien soy.

A todos, gracias.

Índice/Table of Contents:

1. AGRADECIMIENTOS Y FINANCIACIÓN/ ACKNOWLEDGEMENTS AND FUNDING.....	13
2. ABREVIATURAS/ABBREVIATIONS.....	17
3. RESUMEN/ABSTRACT.....	25
4. INTRODUCCIÓN/INTRODUCTION	31
4.1.- LA SUSCEPTIBILIDAD A LA INFECCIÓN POR VIH-1 COMO RASGO MULTIFACTORIAL.....	33
4.2.- FACTORES GENÉTICOS, RESISTENCIA INNATA AL VIH-1 Y PROGRESIÓN DE LA ENFERMEDAD. .	34
4.3.- EVASIÓN DEL SISTEMA INMUNE Y POTENCIACIÓN DE LA INFECCIÓN.	42
4.4.- GENES DE LA INMUNIDAD INNATA Y RESISTENCIA A LA INFECCIÓN POR VIH-1: <i>CR2</i> , <i>CD209</i> E <i>IFNL4</i>	44
4.4.1.- <i>CR2/CD21</i>	44
4.4.2.- <i>DC-SIGN (CD209)</i>	44
4.4.3.- <i>IFNL4</i>	46
5. HIPÓTESIS Y OBJETIVOS/HYPOTHESIS AND OBJECTIVES.....	49
6. PACIENTES, MATERIALES Y MÉTODOS/PATIENTS, MATERIALS AND METHODS.....	55
6.1.- POBLACIONES DE ESTUDIO.	57
6.1.1.- <i>Población del estudio del complemento y resistencia innata al VIH-1</i>	58
6.1.2.- <i>Población del estudio de CD209 y resistencia al VIH-1</i>	58
6.1.3.- <i>Población de estudio de IFNL4 y su influencia en la infección por VIH-1</i>	58
6.2.- ASPECTOS ÉTICOS.....	59
6.3.- GENOTIPACIÓN.....	60
6.3.1.- <i>Genotipación mediante Veracode Golden Gate de Illumina</i>	60
6.3.2.- <i>Genotipación mediante PCR a tiempo real</i>	60
6.4.- SECUENCIACIÓN.....	69
6.4.1.- <i>Preparación de los pool de pacientes para la secuenciación</i>	70
6.5.- EXTRACCIÓN DE ARNM.....	70
6.6.- RETROTRANSCRIPCIÓN.	71
6.7.- CUANTIFICACIÓN DE LA EXPRESIÓN DE <i>CR2</i> , <i>C4BPA</i> Y <i>CD209</i>	72
6.7.1.- <i>Cuantificación de la expresión de CR2 y de sus isoformas</i>	72
6.7.2.- <i>Cuantificación de la expresión de C4BPA</i>	76
6.7.3.- <i>Cuantificación de la expresión de CD209</i>	77
6.8.- PURIFICACIÓN DE PLÁSMIDOS.	78
6.8.1.- <i>Transformación bacteriana por choque térmico</i>	78
6.8.2.- <i>Cultivo en medio líquido de bacterias transformadas</i>	79
6.8.3.- <i>Purificación de plásmidos</i> :.....	80
6.9.- CREACIÓN DE LÍNEAS CELULARES ESTABLES.	80
6.9.1.- <i>Amplificación del fragmento CR2_S y CR2_L, purificación y clonación en el vector lentiviral</i>	81
6.9.2.- <i>Generación de los vectores virales VSV-CR2_S y VSV-CR2_L</i>	86
6.9.3.- <i>Transducción de células HeLa p4 CCR5-CXCR4 con VSV-CR2_S y VSV-CR2_L</i>	93
6.10.- PRODUCCIÓN DE PSEUDOVIRUS.....	97

6.10.1.- Transfección con fosfato cálcico y recogida de pseudovirus.	97
6.10.2.- Titulación de los pseudovirus producidos.	97
6.11.- EXPERIMENTOS FUNCIONALES CD209.	99
6.11.1.- Amplificación del fragmento de la 5'UTR del promotor de CD209, purificación y clonación en el vector.....	99
6.11.2.- Medición de la actividad de los distintos promotores de CD209.....	103
6.12.- BIOESTADÍSTICA Y BIOINFORMÁTICA.....	105
7. RESULTADOS/RESULTS.....	107
ARTÍCULO 1/RESEARCH PAPER 1: ASSOCIATION OF COMPLEMENT RECEPTOR 2 POLYMORPHISMS WITH INNATE RESISTANCE TO HIV-1 INFECTION.	109
ARTÍCULO 2/RESEARCH PAPER 2: COMMON HAPLOTYPES IN CD209 PROMOTER AND SUSCEPTIBILITY TO HIV-1 INFECTION INTRAVENOUS DRUG USERS. (SUBMITTED)	141
ARTÍCULO 3/RESEARCH PAPER 3: IFNL4 RS368234815 POLYMORPHISM IS ASSOCIATED WITH INNATE RESISTANCE TO HIV-1 INFECTION.	163
RESULTADOS COMPLEMENTARIOS/COMPLEMENTARY RESULTS.....	169
Creación de las líneas estables: HELA p4 CCR5-CXCR4-CR2_S y HELA p4 CCR5- CXCR4-CR2_L.	171
Producción de virus y pseudovirus.....	176
8. DISCUSIÓN/DISCUSSION	179
9. CONCLUSIONES/CONCLUSIONS	187
10. REFERENCIAS BIBLIOGRÁFICAS/BIBLIOGRAPHY	193
11. ANEXOS/ANNEXES.....	203
12. OTROS TRABAJOS PUBLICADOS/OTHER PUBLISHED PAPERS	213
13. CURRICULUM VITAE	249

1. Agradecimientos y financiación/ Acknowledgements and funding

Agradecimientos y financiación/Acknowledgements and funding

Es de vital importancia, agradecer a todas las personas que han integrado las cohortes que en este trabajo se detallan; sin ellas, habría sido imposible llevar acabo los estudios que se describen en estas páginas.

También, agradecer a todos los hospitales y grupos participantes, sin cuya colaboración, la realización de este trabajo no habría sido posible: a Juan Antonio Pineda y a su equipo de la Unidad de Enfermedades Infecciosas y Microbiología Clínica del Hospital Nuestra Señora de Valme (Sevilla); a Antonio Rivero y su grupo del Hospital Reina Sofía (Córdoba), en colaboración con el Instituto Maimónides de Investigación Biomédica de Cordoba (IMIBIC); a Joan Fibla y a su grupo del Departamento de Ciencias Médicas Básicas de la Universidad de Lleida, en colaboración con el Hospital Arnau de Vilanova (Lleida); a Vicente Soriano y su grupo del Departamento de Enfermedades Infecciosas del Hospital Carlos III de Madrid; a Mario Clerici y su equipo de la Fundación Don Carlo Gnocchi de Milán (Italia), en colaboración con el Hospital S.M. Annunziata de Florencia (Italia); a los Dres. Antonio Carrero y María del Monte Trujillo del banco de sangre del Hospital de Jaén; y a Ángel Luis Corbí y su equipo del Centro de Investigaciones Biológicas del CSIC.

Este trabajo, ha sido financiado por diferentes proyectos de investigación, concedidos a los investigadores principales de los grupos participantes, siendo las entidades financiadoras: Ministerio de Sanidad, Servicios Sociales e Igualdad [PI021476 (Joan Fibla), PI051778 (Juan Antonio Pineda) y PI10/01232 y EC11-2086 (Antonio Caruz)], Instituto de Salud Carlos III-RETIC [RD06/006 y RD12/0017 (Juan Antonio Pineda)], Fundació Marató TV3 [020730 (Joan Fibla) y 020732 (Antonio Caruz)], Consejería de Salud de la Junta de Andalucía [PI-0335/2009 (Antonio Caruz), PI-0118-2013 (Luis Miguel Real), PI-0481-2012 (Jesús María Gómez), AC-0095-2013 (Juan Antonio Pineda)], la Consejería de Economía, Innovación, Ciencia y Empleo de la Junta de Andalucía [2014/00210/001 (Juan Antonio Pineda)], la Fundación Progreso y Salud de la Consejería de Salud de la Junta de Andalucía [AI-0021 (Juan Antonio Pineda)] y la Universidad de Jaén [UJA2013/10/03, 2014/00026/001 y 2015/00064/001 (Antonio Caruz)].

Por último, las estancias internacionales realizadas bajo la supervisión de: el Dr. Pierre Charneau en la Unidad de Virología

Agradecimientos y financiación/Acknowledgements and funding

Molecular y Vacunas del Instituto Pasteur de París, fue financiada por la Escuela de Doctorado de la Universidad de Jaén y su programa de Actividades y Ayudas al Doctorado EDUJA 2014; y el Dr. Fernando Arenzana Seis-Dedos, en la Unidad de Patogénesis Viral del Instituto Pasteur de Paris, actualmente Director General y Co-director Científico del Instituto Pasteur de Shanghai, que fue financiada por la European Molecular Biology Organization (EMBO) y su programa EMBO Fellowship Programme.

2. Abreviaturas/Abbreviations

APOBEC: Apolipoprotein B mRNA editing Enzyme Catalytic polypeptide like.

ATP: Adenosín Trifosfato.

BHQ: Black Hole Quencher.

BSA: Bovine Serum Albumin.

BST-2: Bone Marrow Stromal Cell Antigen 2.

C4BPA: Complement Component 4 Binding Protein, Alpha.

C´ADE: Complement-mediated Antibody-Dependent Enhancement.

CCR1: C-C chemokine Receptor type 1.

CCR2: C-C chemokine Receptor type 2.

CCR5: C-C chemokine Receptor type 5.

CD3: Cluster of Differentiation 3.

CD4: T-Cell Surface Glycorotein CD4.

CD8: T-Cell Surface Glycorotein CD8.

CD14: Cluster of Differentiation 14.

CD19: Cluster of Differentiation 19.

CD23 (Fc-epsilon-RII): Fc Fragment of IgE low affinity II receptor.

CD59: CD59 Molecule, Complement Regulatory Protein.

CFH: Complement Factor H.

CMV: Citomegalovirus.

CR1 (*CD35*): Complement Receptor 1.

CR2 (*CD21*): Complement Receptor 2.

CR2_L: Complement Receptor 2 Long Isoforms.

CR2_S: Complement Receptor 2 Short Isoforms.

Abreviaturas/Abbreviations

CRs: Complement Receptors.

CUL 5: Cullin 5.

CXCR4: Chemokine (C-X-C motif) Receptor 4.

DAF (*CD55*): Decay Accelerating Factor for complement.

DCs: Dendritic Cells.

DC-SIGN (*CD209*): Dendritic Cell-Specific Intercellular adhesion molecule-2-Grabbing Non-integrin.

DMEN: Dulbecco's Modified Eagle's Medium.

DMSO: Dimetilsulfóxido.

dNTPs: Deoxynucleotide.

DTT: Dithiothreitol.

EDTA: Ácido etildiaminotetraacético.

EGFP: Enhanced Green Fluorescence Protein.

FACS: Fluorescence Activated Cell Sorting.

FDCs: Follicular Dendritic Cells.

ENIS (HESN): Expuestos No Infectados.

FcRs: Fc Receptors.

FcγR: Receptors for the Fc región of IgG.

GWAS: Genome-Wide Association Studies.

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

HLA: Human Leukocyte Antigen.

HRM: High Resolution Melt.

ICAM-1 (*CD54*): Intercellular Adhesion Molecule 1.

ICAM-2 (*CD102*): Intercellular Adhesion Molecule 2.

ICAM-3 (*CD50*): Intercellular Adhesion Molecule 3.

IL28B: Interleukin 28 B.

IFNAR1: Interferon (Alpha, Beta and Omega) Receptor 1.

IFNL4: IFN-Lambda-4.

IFNs: Interferones.

IFN- γ R: Interferon-gamma Receptor.

IL: Interleucina.

JAK: Just Another Kinase.

LB: Luria-Bertanu medium.

LTR: Long Terminal Repeat.

MAPK: Mitogen-Activated Protein Kinases.

MBL: Manosa Binding Lectin.

MCP (*CD46*): Membrane Cofactor Protein.

MIP-1: Macrophage Inflammatory protein 1.

MOI: Multiplicity of Infection.

MX2: MX Dynamin-Like FTPase 2.

NCBI: National Center for Biotechnology Information.

NF- κ B: Nuclear Factor kappa-light-chain-enhancer of Activated B Cells.

NIH: National Institute of Health.

NK: Natural Killer.

ONUSIDA: Programa Conjunto de las Naciones Unidas sobre el VIH/SIDA.

PBMCs: Peripheral Blood Mononuclear Cells.

PBS: Phosphate Buffered Saline.

Pi3K: Phosphoinositide 3-kinase.

Abreviaturas/Abbreviations

PCR: Polymerase Chain Reaction.

RANTES: Regulated on Activation Normal T cell Expressed and Secreted.

RCA: Regulators of Complement Activation.

RNasas: Ribonucleasas.

RPMI: Roswell Park Memorial Institute medium.

SDF-1: Stromal Cell-Derived Factor-1.

SEIMC: Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica.

SIDA: Síndrome de Inmunodeficiencia Adquirida.

SLAM: Signaling Lymphocytic Activation Molecule.

SNP: Single Nucleotide Polymorphism.

STAT: Signal Transducers and Activators of Transcription.

TNFR2: Soluble Tumor Necrosis Factor Receptor 2.

TAR: Terapia Antirretroviral.

Th: Células T helper.

TIM: T-cell Immunoglobulin and Mucin.

TLRs: Toll-Like-Receptors.

TNF-alpha: Tumor Necrosis Factor Alpha.

TRIM: Tripartite Motif Proteins.

TRIM5: Tripartite Motif Containing 5.

U5R: Unique 5' Region.

UTR: Untranslated Region.

VDR: Vitamin D (1,25-Dihydroxyvitamin D3) Receptor.

VHB: Virus de la Hepatitis B.

Abreviaturas/Abbreviations

VHC (HCV): Virus de la Hepatitis C.

VIH (HIV): Virus de la Inmunodeficiencia Humana.

VSV: Vesicular Stomatitis Virus.

3. Resumen/Abstract

Introducción: Los patógenos pueden evadir los mecanismos de la inmunidad innata y usarlos para mejorar su infectividad. Algunos usuarios de drogas por vía parenteral y con comportamientos de alto riesgo, se han mantenido seronegativos para VIH-1 (ENIS). La principal causa genética identificada (*CCR5*), explica una proporción menor de los individuos fenotípicamente resistentes a la infección por VIH-1. El principal objetivo de este trabajo, ha sido analizar 21 genes de la inmunidad innata y su asociación con la infección del VIH-1. **Pacientes, materiales y métodos:** Contamos con una población española de 451 pacientes (250 VIH-1+/VHC+ y 201 ENIS VIH-1-/VHC+) de usuarios de drogas por vía parenteral, una cohorte italiana de 142 individuos de parejas serodiscordantes (62 VIH-1+ y 80 ENIS VIH-1-) y 200 donantes sanos españoles. Se genotiparon un total de 148 SNPs (65.149 genotipaciones), mediante el sistema GoldenGate de Illumina y sondas Taqman. La cuantificación de los ARNm mediante PCR a tiempo real. Los análisis estadísticos se han realizado con los programas PLINK V1.7, Haploview V4.1 y SPSS V14.0; y para el diseño de cebadores y clonaciones de plásmidos, se utilizaron el Bioedit V7.2.5, el Beacon Design V5.01 y el SerialCloner V2.5. **Resultados:** En este estudio, hemos identificado una asociación entre un menor riesgo de infección por VIH-1 por vía parenteral y dos polimorfismos ubicados en los genes del complemento: rs2842704 (*C4BPA*) y el rs1567190 (*CR2*). Este último, podría influir también en el riesgo de infección por vía sexual. Además, hemos identificado un haplotipo en el promotor de *CD209* (formado por los SNPs rs4804803, rs735239 y rs735240) que está asociado con protección a la infección por VIH-1 por vía parenteral. La caracterización fenotípica de estas asociaciones revela en ambos casos que, los genotipos protectores están correlacionados con menor nivel de expresión del ARN, o del promotor. Por último, la variante rs368234815 de *IFNL4*, asociada previamente con protección a la infección de VHC, también confiere protección a la infección por VIH-1 por vía parenteral. **Conclusión:** La protección contra la infección por VIH-1 tiene un origen multifactorial, quedando asociadas a este rasgo las variaciones genéticas ubicadas o ligadas a *CR2*, *C4BPA*, *CD209* e *IFNL4*. Por lo tanto, estas variaciones podrían usarse para evaluar el riesgo individual de infección al VIH-1. Además, nuestros datos sugieren que estas variaciones podrían ser usadas como nuevos marcadores farmacogenéticos para vacunas de nueva generación contra el VIH-1.

Introduction: Pathogens can evade the innate immunity mechanisms and even use them to enhance the infectivity. Some intravenous drug users with high-risk behaviour remain HIV-1 seronegative (HENS). The main identified host genetic cause (*CCR5*) explains a minor proportion of individuals phenotypically resistant to HIV-1 infection. The main objective of this work was to analyse 21 innate immunity genes and its association with HIV-1 infection. **Patients, materials and methods:** A Spanish sample of 451 intravenous drugs users (250 VIH-1+/VHC+ and 201 HESN HIV-1-/VHC+), an Italian cohort of 142 partners of serodiscordant couples (62 VIH-1+ y 80 HESN HIV-1-) and 300 healthy donors from Spain were the study populations. 148 SNPs were performed (65.149 genotyping) by GoldenGate system (Illumina) and Taqman. mRNA quantification was performed by PCR real time. Promoter activity was tested by reporter plasmid construction and transfection in cell culture. PLINK V1.7, Haploview V4.1, SPSS v14.0 were used for statistical analysis. Bioedit V7.2.5, Beacon design V5.01 and SerialCloner V1.5 were used for oligonucleotide design and the plasmids cloning. **Results:** In this study, we have identified an association between lower HIV-1 infection risk by parenteral route and two polymorphisms located in complement genes: rs2842704 (*C4BPA*) and rs1567190 (*CR2*). The last one, could influence the risk of infection by sexual route too. Furthermore, we have identified a haplotype within the *CD209* promoter (including the SNPs rs4804803, rs735239 and rs735240) that is associated with protection from HIV-1 infection by parenteral route. The phenotypic characterization of these associations revealed that in both cases the protective genotypes were correlated with either a lower mRNA expression levels or a lower promoter activity. Finally, the rs368234815 polymorphism located in *IFNL4*, previously associated with protection from HCV infection, also confers protection to HIV-1 infection by parenteral route. **Conclusions:** The protection against HIV-1 infection is a multifactorial trait. Genetic variation linked or within *CR2*, *C4BPA*, *CD209* and *IFNL4* loci are associated with this trait. Therefore, these variations could be used for evaluating the individual HIV-infection risk. In addition, our results suggest that these variations could have a value as new pharmacogenetics markers in the next generation HIV-1 vaccine programs.

4. Introducción/Introduction

4.1.- La susceptibilidad a la infección por VIH-1 como rasgo multifactorial.

A diferencia de las enfermedades genéticas sindrómicas, las enfermedades comunes o complejas suelen tener una etiología multifactorial, donde interviene el ambiente que rodea al individuo y su constitución genética (Donaldson et al. 2015).

La identificación de los factores ambientales que condicionan las enfermedades han sido el objeto de la epidemiología clásica. Sin embargo, el estudio de los condicionantes genéticos de las enfermedades y su evolución clínica es el reto actual de la genómica humana. Este conocimiento, permitirá la clasificación real de las patologías humanas y el diseño de nuevas y revolucionarias estrategias terapéuticas (Lander et al. 2001). Debido a la complejidad del genoma humano y a la multitud de genes que pueden estar involucrados en cada patología común, este reto es considerado como el más complicado de las ciencias biológicas.

La búsqueda de estos factores genéticos, se ha centrado en aquella proporción del genoma que es diferente de unos individuos a otros, y que, por tanto, es la base de la variabilidad fenotípica de los seres humanos. La mayoría de esas diferencias son polimorfismos de una sola base o SNPs. La metodología empleada para estos estudios es similar a la empleada en la epidemiología clásica, identificando las implicaciones de estos polimorfismos en las enfermedades según su presencia diferencial en grupos de casos y controles (Donaldson et al. 2015).

Las enfermedades infecciosas también son consideradas como enfermedades complejas, en donde el factor ambiental está totalmente identificado (el patógeno), y en donde la susceptibilidad a la infección, así como la evolución del paciente, pueden estar condicionados por la estructura genética del individuo (Donaldson et al. 2015).

En estas páginas, nos ocuparemos de una de las enfermedades infecciosas que se han convertido en una epidemia de ámbito mundial, la infección por VIH-1. En el último informe de ONUSIDA, se estima que un total de 36,9 millones de personas estaban infectadas por VIH en 2014 de las cuales, 17,1 millones, no lo sabían. En junio de 2015, 15,8 millones de personas estaban en tratamiento (<http://www.unaids.org/>).

El TAR ha cronificado esta infección a aquellas personas que tienen acceso al tratamiento y una buena adherencia al mismo (Essomba et al. 2015). Si bien es cierto, este tratamiento genera cuantiosos gastos económicos a los gobiernos actuales. En el caso de España, el costo medio de 48 semanas de tratamiento con TAR es de aproximadamente 8.380€ (Javier et al. 2015).

Los expuestos no infectados o ENIS son individuos que tras reiteradas exposiciones al VIH-1 permanecen seronegativos. Se han realizado estudios con grupos de ENIS (utilizando tanto la estrategia de genes candidatos como la de GWAS) (Joubert et al. 2010; Petrovski et al. 2011; Lingappa et al. 2011; Luo et al. 2012; Lane et al. 2013). Estos individuos con un fenotipo tan extremo, como es la resistencia completa o la baja susceptibilidad a la infección por VIH-1, son fundamentales para identificar factores genéticos relacionados con resistencia natural a la infección. Este conocimiento permitirá evaluar el riesgo de infección de cada individuo y será clave para el desarrollo de nuevas estrategias terapéuticas más eficaces contra este virus.

Hasta el momento se han descrito factores genéticos que influyen en la resistencia innata al VIH-1, así como otros que intervienen en la progresión de la enfermedad (Zhao et al. 2012).

4.2.- Factores genéticos, resistencia innata al VIH-1 y progresión de la enfermedad.

El único caso conocido de mutación que provoca una resistencia prácticamente total a la infección por VIH-1 es la mutación $\Delta 32$ en el receptor de quimiocina CCR5. Se trata de una delección de 32 pares de bases en el gen *CCR5*. Si se encuentra en homocigosis, se produce un correceptor truncado no funcional que genera resistencia genética a esta infección por cepas R5 dependientes. Este alelo está presente de forma natural en el 1% de la población caucásica. Este descubrimiento ha permitido el desarrollo de inhibidores específicos de CCR5, como el Maraviroc, comercializado por Pfizer (Hütter et al. 2015). *M303* es una mutación rara, ubicada en este mismo correceptor viral, que confiere resistencia al VIH-1 en individuos que sean homocigotos para ella o heterocigotos para $\Delta 32$ (Lane et al. 2013).

La existencia de individuos expuestos al VIH-1 no infectados y que no son portadores de la mutación *CCR5-Δ32*, indica que existen otros factores genéticos relacionados con este fenotipo. Hasta el momento, no se han identificado alelos de resistencia de alta penetrancia que pudieran explicar este fenotipo; sin embargo, se han descrito alelos que pueden influir no solo en la baja susceptibilidad a la infección, sino también en la progresión de la enfermedad. La mayoría de estos alelos se encuentran en genes que de una u otra forma están implicados en el proceso infeccioso (Tabla 1).

Los estudios recogidos en la tabla 1, son algunos ejemplos que sugieren que el grado de resistencia que presentan ciertos individuos no homocigotos para la mutación *Δ32*, puede ser un rasgo multigénico. Es por ello, que la identificación de todos los alelos implicados en ese rasgo, podrá servir en un futuro para poder estimar el riesgo de cada individuo expuesto. Por otra parte, este conocimiento permitiría establecer diferentes estrategias de protección y/o terapia que podrían ser aplicadas a diferentes individuos según su constitución genética.

Tabla 1: Polimorfismos genéticos asociados a la infección por VIH-1

Grupo	Gen	SNP	Fenotipo asociado
Receptores de quimiocinas	<i>CCR5</i>	$\Delta 32$ (rs333)	Delección de 32 pb asociada a resistencia al VIH en homocigosis (Lane et al. 2013).
		<i>M303</i>	Resistencia al VIH para homocigotos o heterocigotos de $\Delta 32$ (Lane et al. 2013).
	<i>CCR2</i>	V64I	El alelo A está relacionado con progresión lenta a SIDA (Kaur et al. 2013).
Ligandos de receptores de quimiocinas	<i>RANTES</i>	<i>n1.1</i>	Progresión rápida a SIDA en portadores del alelo C (An et al. 2002).
	<i>MIP-1beta</i>		Se han descrito diferencias de expresión de <i>MIP-1beta</i> entre ENIS y controles sanos (Zapata et al. 2015).
	<i>SDF-1</i>	rs1801157	El alelo A se relaciona con resistencia al VIH-1 en ENIS (Garcia-moruja et al. 2009). El mismo alelo está asociado a menor supervivencia en pacientes con SIDA (Kaur et al. 2013).
	<i>MCP-1</i>		Menor riesgo de infección por VIH-1 (Kaur et al. 2013)
Citoquinas	<i>IL-2</i>		Delección en homocigosis se relaciona con progresión de la enfermedad (Lama & Planelles 2007).
	<i>IL-4</i>		Progresión a SIDA (Lama & Planelles 2007).
	<i>IL-8</i>		Niveles de IL-8 en plasma es un marcador de progresión de la enfermedad (Lama & Planelles 2007).

Tabla 1: Polimorfismos genéticos asociados a la infección por VIH-1 (Continuación 1)

Grupo	Gen	SNP	Fenotipo asociado
HLA tipo I	<i>HLA-B</i>	<i>HLA-Bw4</i>	Progresión lenta de la enfermedad (Martin & Carrington 2013).
		<i>HLA-B*51</i>	
		<i>HLA-B*58:1</i>	
		<i>HLA-B*13</i>	
		<i>HLA-B*81:01</i>	
		<i>HLA-B*35-Px</i> <i>HLA-B*48:02</i>	Progresión rápida de la enfermedad (Martin & Carrington 2013).
		<i>HLA-B*57</i>	Progresión lenta de la enfermedad (Kaur et al. 2013).
		<i>HLA-B*27</i>	Protección de la infección por VIH-1 (Kaur et al. 2013).
	<i>HLA-C</i>	rs9264942	Control de carga viral (Martin & Carrington 2013; Kaur et al. 2013). Disminución de carga viral en afroamericanos (Zhao et al. 2012).
HLA tipo II	<i>HLA-DR</i>	<i>HLA-DRB1*13:03</i>	Reducción de la carga viral (Martin & Carrington 2013)
		<i>HLA-DRB1*15:03</i>	Susceptibilidad al VIH-1 (Martin & Carrington 2013).
Receptores	TIM	Haplotipos	Mayor respuesta de los Th1: Proliferación de las células T CD4+, aumento de la respuesta de los linfocitos T CD8+ y aumento de la replicación del VIH-1 (Kaur et al. 2013).
	Fc gamma IIa		Influyen en la transmisión vertical del virus en una cohorte de Kenia (Lama & Planelles 2007).

Tabla 1: Polimorfismos genéticos asociados a la infección por VIH-1 (Continuación 2)

Grupo	Gen	SNP	Fenotipo asociado
Receptores	CD209 (DC-SIGN)	rs4804803 (-336)	El alelo G se ha asociado con susceptibilidad a la tuberculosis y protección frente a la infección por VIH-1 en hindúes (Selvaraj et al. 2009). Este mismo alelo, se asoció con protección a la infección por VIH-1 por vía sexual en parejas serodiscordantes de Burkina Faso en modelo aditivo (Kagone et al. 2014).
			El alelo A se asocia a protección a la infección por VIH-1 por vía parenteral en europeos-americanos (Selvaraj et al. 2009).
		Haplotipo	Este haplotipo genera que el receptor tenga menos de 5 unidades de repetición en su cuello y esto se ha asociado con menor riesgo de infección por vía sexual en parejas serodiscordantes de China (Zhang et al. 2008).
	TLR-3	rs3775291 (Leu412Phe)	El alelo 412Phe confiere protección a la infección por VIH-1 en modelo dominante independientemente de la vía de transmisión (Sironi et al. 2012)
	TLR-8		Progresión más lenta de la enfermedad y reducción de la replicación viral (Mackelprang et al. 2014). Elevación del TNF-alpha y disminución de IL-10 y la prostaglandina E2 (Mackelprang et al. 2014).

Tabla 1: Polimorfismos genéticos asociados a la infección por VIH-1 (Continuación 3)

Grupo	Gen	SNP	Fenotipo asociado
Receptores	<i>VDR</i>	Haplotipo	El haplotipo GATGL (rs11568820, rs4516035, rs10735810, rs15444410, rs17878969) de <i>VDR</i> , se asocia en modelo dominante con protección al VIH-1 por vía parenteral (de la Torre et al. 2008). Niveles elevados de <i>VDR</i> y Vitamina D están asociados a resistencia al VIH-1 por vía sexual (Aguilar-Jiménez et al. 2013).
Factores de restricción	<i>TRIM5</i>	Haplotipo que genera la variante TRIM5-alpha	Protección frente al VIH-1 en trabajadoras sexuales de Pumwani de Naerobi (Kenia) (Price et al. 2011).
	<i>BST-2</i>		Inhibe la liberación de partículas virales. Polimorfismos en este gen se han asociado a la progresión de la infección (Laplana et al. 2013).
	<i>MX2</i>		El alelo G está asociado en modelo recesivo a protección por VIH-1 independientemente de la vía de entrada, siendo asociado con un aumento de la expresión de <i>MX2</i> en respuesta a IFN-alpha (Sironi et al. 2014).
	<i>APOBEC3</i>	<i>APOBEC3G-H186R</i> (rs8177832)	Se asocia en homocigosis a mayor carga viral y progresión más rápida a SIDA (Kaur et al. 2013).

Tabla 1: Polimorfismos genéticos asociados a la infección por VIH-1 (Continuación 4)

Grupo	Gen	SNP	Fenotipo asociado
Factores de restricción	<i>CUL5</i>		*No es un factor de restricción. Participa en la ubiquitinación de APOBEC3G.
		rs11212495	Retraso en la progresión a SIDA en afroamericanos (Zhao et al. 2012). Marcador incluido en un haplotipo que se asocia en estadounidenses a la velocidad de destrucción de células T en la infección por VIH-1 (An et al. 2007).
Interferones y sus receptores	<i>IFNAR1</i>		Susceptibilidad al VIH-1 y progresión a SIDA (Diop et al. 2006).
	<i>IFN-gamma</i>	rs2430561	El alelo A en homocigosis, se ha relacionado con susceptibilidad a neuropatía periférica en pacientes con VIH-1 (Thames et al. 2015).
	<i>STNFR2</i>		Altos niveles de este receptor de IFN-gamma en líquido cefalorraquídeo podría repercutir en la neuropatogénesis y el daño neurocognitivo del VIH (Thames et al. 2015).
	IFN-lambda	rs12979860 (<i>IFNL3</i>)	Respuesta al tratamiento del VHC con interferón pegilado (Real et al. 2014). No está relacionado con la infección o la progresión del VIH-1 y tampoco con la curación del VHB (Martin et al. 2010).
		rs368234815 (<i>IFNL4</i>)	Mismo valor predictivo en la respuesta al tratamiento que el rs12979860; ambos están en alto desequilibrio de ligamiento (Real et al. 2014).

Tabla 1: Polimorfismos genéticos asociados a la infección por VIH-1 (Continuación 5)			
Grupo	Gen	SNP	Fenotipo asociado
Sistema del complemento	MBL		Polimorfismos que disminuyen los niveles séricos de las MBL se han vinculado con mayores tasas de infección por VIH-1 en adultos y niños (Ballegaard et al. 2014).
			Niveles séricos elevados de MBL se han asociado en población finlandesa con mayor riesgo a contraer VIH (Ballegaard et al. 2014).
			Implicado en enfermedades autoinmunes y en el caso del VIH es usado como instrumento para potenciar la infección (Frank et al. 2014).

Tabla 1 | Polimorfismos genéticos asociados a la infección por VIH-1. Tabla resumen en la que se recogen los polimorfismos genéticos más destacados de los últimos 15 años y su papel en la infección por VIH-1.

4.3.- Evasión del sistema inmune y potenciación de la infección.

Los virus han ido evolucionando con el tiempo y han desarrollado diferentes estrategias de evasión del sistema inmune del hospedador (Assil et al. 2015); un ejemplo de esto es la capacidad de Vif para degradar a APOBEC3G o APOBEC3F, eliminando el efecto antiviral de estas enzimas (An et al. 2007).

Estudios recientes han demostrado que virus como el VIH, VHC y el Virus de la Hepatitis A, son capaces de empaquetar su ácido nucleico en endosomas. Crean una estructura en la que la cápsida y el material genético en su interior, quedan rodeados por una bicapa lipídica. Esta estructura mantiene su infectividad y es capaz de evitar la neutralización por anticuerpos, ya que carecen de glicoproteínas virales de superficie (Assil et al. 2015).

Se ha descrito como diferentes familias de virus utilizan RCA para camuflar su superficie viral; esto, es un claro ejemplo de convergencia adaptativa (Tortorella et al. 2000). En algunos casos, codifican análogos de proteínas reguladoras del complemento, como los poxvirus y los herpesvirus (Tortorella et al. 2000); y en otros, las incorporan durante el proceso de salida de la célula hospedadora, como los flavivirus (Chung et al. 2006) y retrovirus (Stoiber et al. 1997). Esto provoca que la superficie viral pase inadvertida, disminuyendo la deposición de fragmentos activos de C3 y del complejo de ataque de membrana en la superficie. Además, se ha observado en patógenos como: *B. burgdorferi* (Kenedy et al. 2009), *N. gonorrhoeae* (Ram et al. 1998), *S. albicans* (Oliver et al. 2008), *S. pneumonia* (Ogunniyi et al. 2009) y *S. pyrogenes* (Haapasalo et al. 2008).

En el caso del VIH-1, incorpora en el proceso de gemación a DAF, CD59, MCP, etc. También, es capaz de interaccionar con el Factor H mediante gp120 y gp41; el Factor H presente en el plasma, es secuestrado por estas interacciones produciendo la opsonización completa del virus y facilitando de este modo, la interacción del virus con los receptores del complemento presentes en las células. Esto, le aporta una ventaja al VIH-1 para la infección de las células permisivas (Bánki et al. 2005).

Lejos de ejercer su función de ayuda en la eliminación de inmunocomplejos en el caso del VIH-1, los eritrocitos que expresan CR1

intervienen, junto con el Factor I, en el procesamiento proteolítico de C3b en iC3b y C3d, aumentando la opsonización del virus. Esto produce una mayor interacción de los virus opsonizados con las células dendríticas y las células B, a través de receptores del complemento presentes en su superficie, como CR2 (Bánki et al. 2005), creando un gran reservorio viral en los tejidos linfoides (Frank et al. 2014). Además, en el caso de las células dendríticas, el VIH-1 interacciona mediante gp120 con receptores de ICAM-1, como DC-SIGN, mejorando la transmisión en *cis* del VIH en células permisivas, como los macrófagos que co-expresan DC-SIGN, CD4 y los correceptores virales (Zhang et al. 2008; Celerino da Silva et al. 2011). Este receptor también se expresa en células B y en plaquetas, pudiendo mejorar la infección en *trans* de los linfocitos T (Zhang et al. 2008).

Los anticuerpos es otro mecanismo que puede utilizar el VIH-1 para mejorar la infección. La formación de anticuerpos neutralizantes suele ocurrir tras años de infección persistente. Durante ese tiempo, se encuentran anticuerpos no neutralizantes y su existencia puede ser debida a: las diferentes conformaciones que el heterodímero gp120/gp41 puede presentar en los viriones que presentan a la inmunidad humoral, así como, la variabilidad que presentan gp120 y gp41 debido a la alta tasa de mutación que se produce por errores en la retrotranscripción, haciendo muy difícil la generación de anticuerpos neutralizantes. Esto hace que solo en porcentaje de esas estructuras, puedan presentar epítomos que sean susceptibles a la neutralización, dejando el resto, expuestos a la unión por anticuerpos no neutralizantes y a la opsonización (Stieh et al. 2015) y uniéndose directamente a los FcγR (Bánki et al. 2005; Willey et al. 2011).

El proceso de potenciación de la infección vía CRs de virus opsonizados con fragmentos de complemento y anticuerpos, se denomina C'ADE. Este fenómeno no sólo ocurre en el VIH-1, sino que también se da en otras familias virales como los flavivirus (Dengue) (Boonnak et al. 2008) o filovirus (Virus de Marburgo y del Ébola) (Nakayama et al. 2011). En el caso del VIH-1, existen posibles explicaciones de este fenómeno. En primer lugar, puede que además de la interacción del complejo gp120/gp41 con el receptor y correceptor viral, las interacciones que se producen entre los fragmentos opsonizados del complemento y los anticuerpos con los CRs y FcR respectivamente, haga que aumente el número de interacciones físicas entre el virus y la

célula diana, ayudando a que se produzca la infección. En segundo lugar, que se produzcan eventos de señalización que mejoren la infección por rutas alternativas de entrada, mejorando la replicación viral y suprimiendo la respuesta antiviral celular (Willey et al. 2011).

4.4.- Genes de la inmunidad innata y resistencia a la infección por VIH-1: *CR2*, *CD209* e *IFNL4*.

Este trabajo se centra en el estudio de rutas claves de los procesos inmunológicos y en las primeras etapas de la infección por VIH-1. *CR2* y DC-SIGN (*CD209*), son receptores esenciales en la activación de la inmunidad adaptativa, mientras que *IFNL4* pertenece a la familia de los IFNs, moléculas de gran capacidad antiviral.

4.4.1.- *CR2/CD21*.

CR2 es un glicoproteína que se expresa en muchas células del sistema inmune, como por ejemplo en linfocitos B, células dendríticas foliculares y algunos linfocitos T. Es el receptor del virus de Epstein-Bar y tiene como ligandos naturales C3d, C3dg e iC3b (Cruickshank et al. 2012).

Este receptor es clave en la interacción de la inmunidad innata y adaptativa, así como en la activación de los linfocitos B. Una vez que el VIH se encuentra opsonizado con fragmentos de C3d, C3dg e iC3b, se une a *CR2*. En el caso de los linfocitos B, se une también a los receptores de células B activando al linfocito y amplificando la señal. Y en el caso de las FDCs, el virus opsonizado se une a *CR2* y a los receptores Fc. Todo esto conlleva a la propagación de la infección (Xu et al. 2009).

Existen polimorfismos en *CR2* que se han asociado a susceptibilidad al lupus eritematoso sistémico (Douglas et al. 2010; Cruickshank et al. 2012). Sin embargo, se desconoce la repercusión que puedan tener variantes genéticas ubicadas en *CR2* en la infección por VIH-1.

4.4.2.- DC-SIGN (*CD209*).

CD209 codifica a DC-SIGN. Se trata de una lectina de tipo C, que está presente en forma de receptor transmembrana en las células presentadoras de antígenos, como las DCs y los macrófagos. Se une a ligando endógenos (ICAM-2, ICAM-2, MAC-1) y exógenos (Vannberg et al. 2008). Está implicada en la captura de bacterias, hongos, parásitos y

ciertos virus como el del Ébola, VHC, el Virus del Dengue, el CMV y el VIH. Este último, se une a DC-SIGN mediante la interacción con gp120 (Celerino da Silva et al. 2011).

DC-SIGN juega un papel fundamental en la captura del VIH-1 a nivel de las mucosas. Posteriormente, migra a los órganos linfoides secundarios que albergan grandes cantidades de células T aumentando de esta forma, la propagación de la infección (Zhang et al. 2008). Es por esto precisamente que, *CD209* es un blanco de los estudios de asociación genética (Selvaraj et al. 2009).

Polimorfismos ubicados en el promotor de este gen, se han asociado a una mayor susceptibilidad a infecciones como: tuberculosis (Yi et al. 2015), Virus de Chikungunya, así como un mayor riesgo de desarrollar sus síntomas clínicos (Chaaithanya et al. 2016), mayor agravamiento de la enfermedad hepática en personas infectadas por VHC (Ryan et al. 2010), mayor hospitalización por agravamiento de síntomas en infectados por el Virus del Dengue (Alagarasu et al. 2013), etc.

Sin embargo, existe cierta controversia con polimorfismos ubicados en *CD209* y su papel en la infección por VIH-1. Hay variantes que generan un acortamiento del cuello del receptor y que se han asociado a menor susceptibilidad a la infección en población China (Zhang et al. 2008). En un estudio realizado por Liu H y colaboradores, detectan variantes que afectan a la longitud del cuello del receptor en población estadounidense y que podrían reducir el riesgo de infección por VIH-1 (Liu et al. 2004). Sin embargo, en otros estudios realizados sobre población hindú, variaciones que determinan la longitud de cuello del receptor, no se asocian con susceptibilidad a la infección por VIH-1 (Rathore et al. 2008; Chaudhary et al. 2008). Estas diferencias encontradas podrían deberse a las diferencias existentes entre las poblaciones estudiadas, ya que las variables del estudio de Zhang y colaboradores son raras en individuos de África, América, Europa y Oceanía (Zhang et al. 2008).

Existen variaciones en la zona del promotor de *CD209*, como el alelo C de la variante -336, que se asocian con una mayor susceptibilidad a la infección por VIH-1 por vía parenteral, que los portadores del alelo T; sin embargo, por vía sexual no se encuentra asociación (Martin et al. 2004). Por otro lado, el alelo C en homocigosis, se asocia a protección frente a la infección por VIH-1 en hindúes (Selvaraj et al. 2009) y en el caso de

parejas serodiscordantes de Burkina Faso, se asocia a protección por vía sexual (Kagone et al. 2014) (Tabla 1).

Todos estos estudios ponen de manifiesto la necesidad de realizar nuevos análisis, encaminados a esclarecer el posible efecto de las variaciones de *CD209* en la resistencia a la infección por VIH-1 en nuestra población.

4.4.3.- IFNL4.

Cuando una célula es infectada, detecta diferentes componentes virales en su interior, tales como proteínas y ácidos nucleicos virales. Esto hace que se activen una serie de mecanismos de señalización a través de los TLR internos, que inducen la secreción de IFNs y citoquinas, desencadenando la respuesta inmune.

Existen tres tipos de interferones: IFN tipo I, formados por 12 subtipos; el IFN tipo II o IFN-gamma y el IFN tipo III o IFN-lambda. Cuando el IFN tipo I y tipo II se unen a sus receptores IFNAR e IFN-γR respectivamente, activan la ruta JAK-STAT (pudiendo activar otras rutas como: Pi3K, MAPK, o NF-κB) (Lin & Young 2014). Como consecuencia de esto, se produce la transcripción de múltiples genes asociados con la proliferación, diferenciación, activación funcional, supervivencia o apoptosis celular (Duarte & Frank 2000). Estos interferones, intervienen en la replicación viral mediante la inducción de enzimas que bloquean la traducción o degradan el ARN viral. También, pueden interferir en la liberación de partículas virales a través de la inducción de proteínas TRIM. Inducen la apoptosis de células infectadas, la maduración de DCs y la diferenciación de linfocitos T. También, activan y reclutan a las NK, células B y T. Además, el IFN tipo II induce una mayor expresión de los IFN tipo I, lo que estimula a las células endoteliales a producir quimioquinas que atraigan a macrófagos, neutrófilos y células T. También, promueve la producción de IL-12 por las células presentadoras de antígenos, lo que impulsa la diferenciación de las células T (Lin & Young 2014).

En el caso de los IFN tipo III se unen a IL-10R2 e IFN-λR1. Estos receptores se expresan en células epiteliales, células B y T, macrófagos y DCs. El IFN tipo III activa la vía de señalización JAK-STAT, induciendo actividad antiviral en estas células y la apoptosis; además, facilita la detección y la destrucción de células infectadas (Xu et al. 2015). Se ha

demostrado que el IFN tipo III, inhibe la replicación del VHB en hepatocitos y del VIH-1 en macrófagos y células T (Lin & Young 2014).

El papel de los IFN-lambda en la infección por VIH-1 es contradictorio. En un estudio llevado a cabo por Serra C y colaboradores, mostraron como en PBMCs tratados con IFNL2 e IFN tipo 1, se producía un aumento en la unión y replicación del VIH-1. Al parecer, esto es debido a que los interferones aumentan la expresión de CD4, CXCR4 y CCR5 (Serra et al. 2008). Hou W y colaboradores, muestran como el IFNL1 e IFNL2 inhiben la infección y replicación en macrófagos, al parecer debido a que el IFNL induce la producción de quimiocinas que bloquean la entrada del VIH-1 (MIP-1alpha y MIP-1beta) y a que además, induce la producción de IFN tipo I (Hou et al. 2009).

El papel de IFNL3 e IFNL4 en la infección por VIH-1 es desconocido. Numerosos estudios han evidenciado la existencia de SNPs que influyen en la respuesta al tratamiento de la infección por VHC con interferón pegilado. Es el caso del rs12979860, ligado a *IFNL3* (*IL28B*) (Tabla 1). Si bien es cierto, otro SNP ubicado aguas arriba, el rs368234815 (*IFNL4*), tiene el mismo valor predictivo en la respuesta al tratamiento en europeos pero no en africanos. Ambos marcadores, que en realidad se encuentran dentro de *IFNL4*, están en alto desequilibrio de ligamiento (Real et al. 2014). Es por todo esto, por lo que se intenta esclarecer que función tiene *IFNL4* en la infección por VIH-1 en este trabajo.

5. Hipótesis y objetivos/Hypothesis and objectives

Hipótesis global: Existen usuarios de drogas por vía parenteral que, pese a tener conductas de riesgo permanecen seronegativos al VIH-1 (ENIS). Todos los casos de resistencia al VIH-1 no pueden explicarse a través de la mutación de *CCR5*, por lo que los polimorfismos ubicados en los genes de la inmunidad innata podrían explicar el fenotipo extremo de ENIS.

Objetivo global: Identificar factores genéticos asociados a resistencia innata al VIH-1, utilizando una estrategia de genes candidatos.

Hipótesis 1: Mutaciones que reduzcan la capacidad funcional de las proteínas reguladoras de la activación del complemento podrían proteger de la infección por VIH-1, aumentando la virólisis y la citotoxicidad a través de la activación efectiva de las diferentes rutas del complemento.

Objetivo 1.1: Identificar y caracterizar nuevos polimorfismos genéticos de los genes RCA que podrían asociarse con resistencia a la infección por VIH-1 en drogadictos por vía parenteral.

Objetivo 1.2: Determinar, si los polimorfismos encontrados en los genes RCA que confieren resistencia por vía parenteral, también la confieren por vía sexual.

Objetivo 1.3: Desarrollar las herramientas necesarias para caracterizar funcionalmente a *CR2* mediante experimentos de infección: crear líneas celulares estables que expresen las dos variantes de *CR2* y optimizar el proceso de creación de pseudovirus.

Hipótesis 2: Los haplotipos de *CD209* podrían influir en el riesgo de infección por VIH-1. Esto se ha observado en otros virus como el del Dengue o en infecciones bacterianas como la tuberculosis.

Objetivo 2.1: Determinar la asociación genética de los SNPs y los haplotipos ubicados en el promotor de *CD209* en la susceptibilidad a la infección por VIH-1.

Objetivo 2.2: Determinar en un modelo *in vitro*, el efecto funcional de los haplotipos de *CD209* así como, realizar experimentos de infección en células mononucleares de sangre periférica de donantes sanos.

Hipótesis y objetivos/Hypothesis and objectives

Hipótesis 3: Un polimorfismo en *IFNL4* se ha asociado con curación espontánea y una mejor respuesta al tratamiento contra VHC. Esta variante podría tener un efecto protector frente la infección por VIH-1, ya que también está asociada a progresores lentos.

Objetivo 3.1: Determinar el papel del rs368234815 (inserción/delección) del gen *IFNL4* en la susceptibilidad a la infección por VIH-1.

Global hypothesis: Some intravenous drug users have remained HIV-1 seronegative (HESN), even with high-risk behaviour. The main identified host genetic cause (*CCR5*) cannot explain all cases of resistance to HIV-1 infection. Polymorphisms in innate immunity genes could explain this extreme phenotype.

Global objective: To identify genetic factors related to innate resistance to HIV-1 using a candidate genes association strategy.

Hypothesis 1: Polymorphisms that reduce the functional capabilities of regulators of complement activation (RCA) could induce a protective background against the HIV-1 infection, increasing the virolysis and cytotoxicity with a more effective activation of complement.

Objective 1.1: To identify and characterize new genetic polymorphisms located in RCA genes that could be associated with resistance to HIV-1 infection on intravenous drug users.

Objective 1.2: To confirm whether polymorphism within RCA genes previously identified can confer resistance to HIV-1 infection in individuals at risk of infection through sexual route.

Objective 1.3: To develop necessary tools to characterize *CR2* functionally through infection experiments: to create stable cell lines which express the two *CR2* isoforms and optimize pseudo-viruses creation process.

Hypothesis 2: The haplotypes of *CD209* may influence the risk of HIV-1 infection as has been observed in other viral (Dengue) or bacterial diseases (tuberculosis).

Objective 2.1: To test the genetic association of SNPs and haplotypes located in the *CD209* promoter with HIV-1 infection susceptibility.

Objective 2.2: To study the functional effect of *CD209* haplotypes using an in vitro model as well as expression experiments in PBMC from healthy donors.

Hipótesis y objetivos/Hypothesis and objectives

Hypothesis 3: One *IFNL4* polymorphisms is associated with spontaneous clearance and better response to HCV treatment. This variant could have a protective effect to HIV-1 infection since it is associates to long-term non-progressors too.

Objective 3.1: To determine the influence of rs368234815 INDEL in the susceptibility to HIV-1 infection.

6. Pacientes, materiales y métodos/Patients, materials and methods

6.1.- Poblaciones de estudio.

Para la realización de esta tesis se utilizaron 3 grupos de individuos.

El primer grupo está formado por individuos varones españoles de origen caucásico, usuario de drogas por vía parenteral que han compartido jeringuillas durante más de tres meses. En esta población de 451 individuos, contamos con 250 sujetos coinfectados con los virus VIH-1 y VHC y con 201 ENIS para VIH-1 pero, con serología positiva para VHC (Figura 1).

Estas muestras provienen de los hospitales: Arnau de Vilanova (Lleida), Nuestra Señora de Valme (Sevilla) y Reina Sofía (Córdoba) (Figura 1). El ADN se extrajo de una muestra de sangre periférica, que se tomó a cada individuo, mediante el kit de extracción de ADN de NucleoSpin® Blood QuitckPure (Macherey-Nagel, Düren, Germany) o Magna Pure System (Sigma-Aldrich, ST. Louis, MO, EE.UU.). El ADN genómico de todas las muestras se procesó en las instalaciones de microbiología de los respectivos hospitales, donde cuentan con el material de trabajo necesario para el procesamiento adecuado de las muestras.

La segunda población está formada por hombres y mujeres de nacionalidad italiana y de origen caucásico, que han mantenido más de 4 relaciones sexuales sin protección y al menos 3 de ellas, dentro de los 4 meses anteriores al ser inscritos en esta cohorte, teniendo un promedio de 30 contactos sexuales no protegidos al año (rango, 18 a >100). Forman parte de una cohorte de pareja heterosexuales serodiscordantes bien caracterizada y que se ha seguido desde 1997 (Biasin et al. 2010).

La procedencia de estas muestras de ADN fue el Hospital S.M. Annunziata, Florencia, Italia (Figura 1). El ADN de cada paciente se extrajo de una muestra de sangre periférica, en las instalaciones de dicho hospital, siguiendo el procedimiento de fenol-cloroformo.

La tercera y última población, está integrada por donantes sanos procedentes del banco de sangre del Hospital Ciudad de Jaén (Figura 1) a los que se les extrajo ADN de células mononucleares de sangre periférica aisladas, siguiendo el procedimiento de centrifugación en gradiente de ficoll. La extracción de ADN se realizó mediante el kit de extracción de ADN NucleoSpin® Blood QuitckPure (Macherey-Nagel, Düren, Germany) o Magna Pure System (Sigma-Aldrich, ST. Louis, MO, EE.UU.).

Pacientes, materiales y métodos/Patients, materials and methods

6.1.1.- Población del estudio del complemento y resistencia innata al VIH-1.

Para este estudio se utilizaron 250 muestras de individuos coinfectados con los virus VIH-1 y VHC, y 201 muestras de ENIS para VIH-1, pero con serología positiva para VHC de población española.

De la cohorte italiana se utilizaron un total de 142 muestras de las cuales, 62 fueron individuos infectados con el virus VIH-1 y 80 ENIS para VIH-1.

Y como controles, se utilizaron individuos del grupo de donantes sanos (Figura 1).

6.1.2.- Población del estudio de CD209 y resistencia al VIH-1.

Un total de 295 muestras de la población española se utilizaron para realizar este estudio, de las cuales 165 eran coinfectados con los virus del VIH-1 y VHC, y 130 ENIS para VIH-1 pero con serología positiva para VHC.

De las 165 muestras VIH-1(+)-VHC(+), 140 pertenecían a pacientes que se trataron con interferón pegilado- α -2a o con interferón pegilado- α -2b a dosis de 180 μ g/semana y 1,5 μ g/kg/semana respectivamente. También recibieron tratamiento con ribavirina a una dosis que se encontraba entre los 800 y 1200 mg/día.

Como controles se utilizaron 142 individuos del grupo de donantes sanos (Figura1).

6.1.3.- Población de estudio de IFNL4 y su influencia en la infección por VIH-1.

Para este estudio solo se utilizaron individuos de la población española. Un total de 401 de los cuales, 213 eran individuos coinfectados con los virus del VIH-1 y VHC y 188 muestras de ENIS para VIH-1, pero con serología positiva para VHC.

Al igual que en los anteriores estudios, los controles fueron individuos del grupo de donantes sanos de Jaén. (Figura 1).

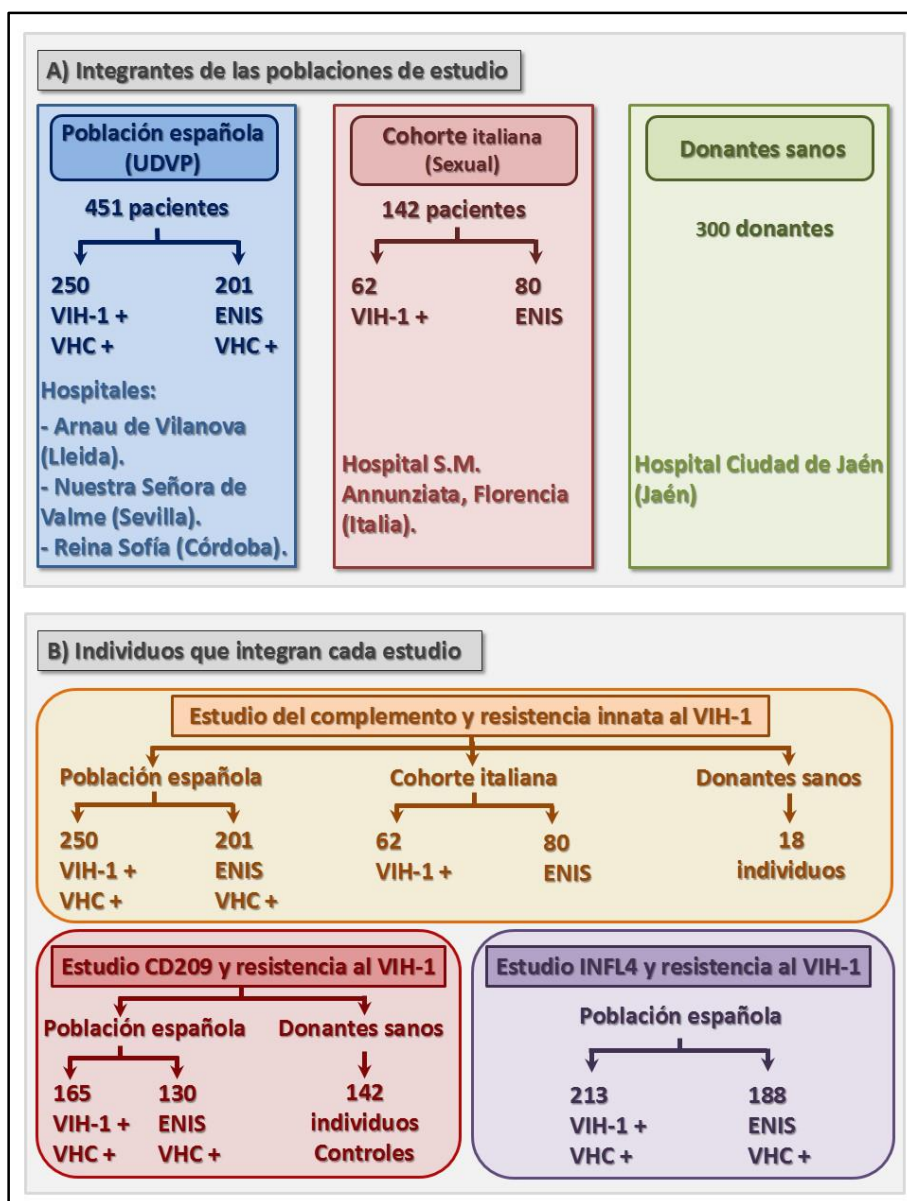


Figura 1 | Poblaciones y composición de los integrantes de cada estudio. A) Individuos que integran los tres grupos que se utilizan en este trabajo. B) Individuos que componen las poblaciones de cada estudio.

6.2.- Aspectos éticos.

Todos los estudios fueron diseñados y realizados de acuerdo con la declaración de Helsinki; fueron aprobados por los comités de bioética de los hospitales participantes y de la Universidad de Jaén (Anexo 1).

Pacientes, materiales y métodos/Patients, materials and methods

Todas las personas participantes en los estudios firmaron un consentimiento informado (Anexo 2).

6.3.- Genotipación.

6.3.1.- Genotipación mediante Veracode Golden Gate de Illumina.

Con este sistema se genotiparon 143 SNPs para realizar un estudio de genes candidatos. Estos SNPs, se encuentran distribuidos en 19 genes reguladores de la activación del complemento (criterios de inclusión en el apartado 6.12.).

Estas genotipaciones se llevaron a cabo mediante la técnica Veracode Golden Gate (Illumina, San Diego, CA, EE.UU.), en el Departamento de Genómica Estructural de Neocodex SL. (Sevilla, España). Los marcadores seleccionados se pueden ver en el Artículo 1: “Association of complement receptor 2 polymorphisms with innate resistance to HIV-1 infection” ubicado en el apartado 7. Más concretamente en la tabla 1 del mismo: “Table 1: Chromosomal location and genomic characteristics of selected markers”.

6.3.2.- Genotipación mediante PCR a tiempo real.

Todas las genotipaciones se realizaron mediante PCR a tiempo real en el equipo MX3005 (Stratagene, La Jolla, CA, EE.UU.) o Eco Real-Time PCR System (Illumina, San Diego, CA, EE.UU.).

6.3.2.1.- Genotipación del SNP rs1567190 de CR2.

Material:

-Sonda taqman rs1567190_CR2 (40X) de Applied Biosystem (Thermo Fisher Scientific, Carlsbad, CA, EE.UU.). Esta sonda va marcada para el alelo T con FAM y el alelo C con HEX, teniendo como quencher BHQ 1 y BHQ 2 respectivamente.

- Tampón de PCR Quantimix Easy Probes (Biotools B&M Labs, Madrid, España).

- DMSO.

- H₂O destilada autoclavada.

- ADN genómico humano (10 ng/μl).

Método:

Mezcla de reacción de PCR de genotipación del rs1567190 de CR2	
Material	Volumen por muestra (μl)
H ₂ O	4
Quantimix Easy Probes	6,3
DMSO	0,6
Sonda rs1567190_CR2	0,1
ADN	1
Volumen final de reacción	12 μl/pocillo

Condiciones de la PCR de genotipación del rs1567190 de CR2			
Etapas de la PCR		Tª (°C)	Tiempo (hh:mm:ss)
Activación de la Taq polimerasa (1 ciclo)		95	00:05:00
Amplificación (45 ciclos)	Desnaturalización	95	00:00:20
	Hibridación y extensión	64	00:01:00 (medición de fluorescencia)

Pacientes, materiales y métodos/Patients, materials and methods

6.3.2.2.- Genotipación del SNP rs2842704 de C4BPA.

Material:

- Sonda Taqman rs2842704_C4BPA (40X) de Applied Biosystem (Thermo Fisher Scientific, Carlsbad, CA, EE.UU.). Esta sonda va marcada con FAM para el alelo G y con HEX para el alelo A, teniendo como quencher BHQ 1 y BHQ 2 respectivamente.
- Kapa Probe Fast universal Q-PCR Kit Master Mix (2X) Universal (KapaBiosystem, Wilmington, MA, EE.UU.).
- H₂O destilada autoclavada.
- ADN genómico humano (10 ng/μl).

Método:

Mezcla de reacción de PCR de genotipación del rs2842704 de C4BPA			
Material		Volumen por muestra (μl)	
H ₂ O		4,6	
Kapa Probe Fast Universal Q-PCR		6,3	
Sonda rs2842704_C4BPA		0,1	
ADN		1	
Volumen final de reacción		12 μl/pocillo	

Condiciones de la PCR de genotipación del rs2842704 de C4BPA			
Etapas de la PCR		Tª (°C)	Tiempo (hh:mm:ss)
Activación de la Taq polimerasa (1 ciclo)		95	00:05:00
Amplificación (45 ciclos)	Desnaturalización	95	00:00:20
	Hibridación y extensión	63	00:01:00 (medición de fluorescencia)

6.3.2.3.- Genotipación del SNP rs2287886 de CD209.

Material:

- Sonda Taqman rs2287886_CD209 (40X) de Applied Biosystem (Thermo Fisher Scientific, Carlsbad, CA, EE.UU.). Esta sonda va marcada para el alelo A con VIC y el alelo G con FAM, teniendo como quencher BHQ2 y BHQ 1 respectivamente.
- Tampón de PCR Quantimix Easy Probes (Biotools B&M Labs, Madrid, España).
- H₂O destilada autoclavada.
- ADN genómico humano (10 ng/μl).

Método:

Mezcla de reacción de PCR de genotipación del rs2287886 de CD209	
Material	Volumen por muestra (μl)
H ₂ O	4
Quantimix Easy Probes	6,3
Sonda rs2287886_CD209	0,1
ADN	1
Volumen final de reacción	12 μl/pocillo

Condiciones de la PCR de genotipación del rs2287886 de CD209			
Etapas de la PCR		Tª (°C)	Tiempo (hh:mm:ss)
Activación de la Taq polimerasa (1 ciclo)		95	00:05:00
Amplificación (45 ciclos)	Desnaturalización	95	00:00:20
	Hibridación y extensión	63	00:01:00 (medición de fluorescencia)

Pacientes, materiales y métodos/Patients, materials and methods

6.3.2.4.- Genotipación del SNP rs4804803 de CD209.

Material:

- Sonda Taqman rs4804803_CD209 (40X) de Applied Biosystem (Thermo Fisher Scientific, Carlsbad, CA, EE.UU.). Esta sonda va marcada para el alelo A con VIC y el alelo G con FAM. Teniendo como quencher BHQ 2 y BHQ 1 respectivamente.
- Tampón de PCR Quantimix Easy Probes (Biotools B&M Labs, Madrid, España).
- H₂O destilada autoclavada.
- ADN genómico humano (10 ng/μl).

Método:

Mezcla de reacción de PCR de genotipación del rs4804803 de CD209			
Material		Volumen por muestra (μl)	
H ₂ O		4	
Quantimix Easy Probes		6,3	
Sonda rs4804803_CD209		0,1	
ADN		1	
Volumen final de reacción		12 μl/pocillo	

Condiciones de la PCR de genotipación del rs4804803 de CD209			
Etapas de la PCR		Tª (°C)	Tiempo (hh:mm:ss)
Activación de la Taq polimerasa (1 ciclo)		95	00:05:00
Amplificación (45 ciclos)	Desnaturalización	95	00:00:20
	Hibridación y extensión	60	00:01:00 (medición de fluorescencia)

6.3.2.5.- Genotipación del SNP rs735239 de CD209.

Material:

- Sonda Taqman rs735239_CD209 (40X) de Applied Biosystem (Thermo Fisher Scientific, Carlsbad, CA, EE.UU.). Esta sonda va marcada con VIC para el alelo A y con FAM para el alelo G, teniendo como Quencher BHQ 2 y BHQ 1 respectivamente.
- Tampón de PCR Quantimix Easy Probes (Biotools B&M Labs, Madrid, España).
- H₂O destilada autoclavada.
- ADN genómico humano (10 ng/μl).

Método:

Mezcla de reacción de PCR de genotipación del rs735239 de CD209	
Material	Volumen por muestra (μl)
H ₂ O	4
Quantimix Easy Probes	6,3
Sonda rs735239_CD209	0,1
ADN	1
Volumen final de reacción	12 μl/pocillo

Condiciones de la PCR de genotipación del rs735239 de CD209			
Etapas de la PCR		Tª (°C)	Tiempo (hh:mm:ss)
Activación de la Taq polimerasa (1 ciclo)		95	00:05:00
Amplificación (45 ciclos)	Desnaturalización	95	00:00:20
	Hibridación y extensión	63	00:01:00 (medición de fluorescencia)

Pacientes, materiales y métodos/Patients, materials and methods

6.3.2.6.- Genotipación del SNP rs735240 de CD209.

Material:

- Sonda Taqman rs735240_CD209 (40X) de Applied Biosystem (Thermo Fisher Scientific, Carlsbad, CA, EE.UU.). Esta sonda va marcada para el alelo A con VIC y para el alelo G con FAM, teniendo como quencher BHQ 2 y BHQ 1 respectivamente.
- Tampón de PCR Quantimix Easy Probes (Biotools B&M Labs, Madrid, España).
- H₂O destilada autoclavada.
- ADN genómico humano (10 ng/μl).

Método:

Mezcla de reacción de PCR de genotipación del rs735240 de CD209			
Material		Volumen por muestra (μl)	
H ₂ O		4	
Quantimix Easy Probes		6,3	
Sonda rs735240_CD209		0,1	
ADN		1	
Volumen final de reacción		12 μl/pocillo	

Condiciones de la PCR de genotipación del rs735240 de CD209			
Etapas de la PCR		Tª (°C)	Tiempo (hh:mm:ss)
Activación de la Taq polimerasa (1 ciclo)		95	00:05:00
Amplificación (45 ciclos)	Desnaturalización	95	00:00:20
	Hibridación y extensión	60	00:01:00 (medición de fluorescencia)

6.3.2.7.- Genotipación del SNP rs333 de CCR5 ($\Delta 32$).

Esta genotipación de llevo a cabo en el termociclador Eco Real.Time PCR System (Illumina, San Diego, CA, EE.UU.).

Material:

- Cebadores:

Cebador_sentido: 5'-TTC ATT ACA CCT GCA GCT CTC-3'.

Cebador_antisentido: 5'-GAC CAG CCC CAA GAT GAC TAT C-3'.

- Kit SsoFast Evagreen Supermix (BIO-RAD, Hercules, CA, EE.UU.) que incluye la enzima polimerasa (Sso7d-fusion polymerase), los dNTPs, $MgCl_2$ el tampón de reacción y Eva Green.

- H_2O destilada autoclavada.

- ADN genómico humano (10 ng/ μ l).

Método:

Mezcla de reacción de PCR de genotipación del rs333 de CCR5	
Material	Volumen por muestra (μ l)
H_2O	8,5
SsoFast EvaGreen Supermix	10
Cebador_sentido (500 μ M)	0,5
Cebador_antisentido (500 μ M)	0,5
ADN	0,5
Volumen final de reacción	12 μ l/pocillo

Pacientes, materiales y métodos/Patients, materials and methods

Condiciones de la PCR de genotipación del rs333 de <i>CCR5</i>			
Etapas de la PCR		Tª (°C)	Tiempo (hh:mm:ss)
Activación de la Taq polimerasa (1 ciclo)		95	00:02:00
Amplificación (45 ciclos)	Desnaturalización	95	00:00:10
	Hibridación y extensión	56	00:01:00 (medición de fluorescencia)
		95	00:01:00
Curva de disociación	Medida de fluorescencia con cada incremento de 0,1 °C para la curva de disociación o HRM	55	00:00:30 hasta
		95	00:00:30

6.3.2.8.- Genotipación del SNP rs368234815 de IFNL4.

Material:

- Sonda Taqman rs368234815_*IFNL4* (Isogen Life Science, Veldzigt, Países Bajos) marcada con HEX para la inserción TT: 5'-ATCGCAAGAAGGCC-3'. El quencher en este caso fue BHQ 2.
- Sonda Taqman rs368234815_*IFNL4* (Isogen Life Science, Veldzigt, Países Bajos) marcada con FAM para la inserción ΔG: 5'-ATCGCAG_CGGCCC-3'. Siendo el quencher BHQ 1.
- Cebadores:

Cebador_sentido: 5'-GCC TGC TGC AGA AGC AGA GAT-3'.

Cebador_antisentido: 5'-GCT CCA GCG AGC GGT AGT G-3'.
- Tampón de PCR Quantimix easy Probes (Biotools B&M Labs, Madrid, España).
- H₂O destilada autoclavada.
- ADN genómico humano (10 ng/μl).

Método:

Mezcla de reacción de PCR de genotipación del rs368234815 de <i>IFNL4</i>	
Material	Volumen por muestra (μl)
H ₂ O	4
Quantimix Easy Probes	6,3
Cebador_sentido (200 μmol/μl)	0,2
Cebador_antisentido (200 μmol/μl)	0,2
Sonda HEX_ <i>IFNL4</i> (100 μmol/μl)	0,2
Sonda FAM_ <i>IFNL4</i> (100 μmol/μl)	0,1
ADN	1
Volumen final de reacción	12 μl/pocillo

Condiciones de la PCR de genotipación del rs368234815 de <i>IFNL4</i>			
Etapas de la PCR		Tª (°C)	Tiempo (hh:mm:ss)
Activación de la Taq polimerasa (1 ciclo)		95	00:05:00
Amplificación (45 ciclos)	Desnaturalización	95	00:00:20
	Hibridación y extensión	60	00:01:00 (medición de fluorescencia)

6.4.- Secuenciación.

Para secuenciar el fragmento de 515.844 pb del cromosoma 1 (desde la posición 207.224.455 pb hasta las 207.740.29 pb, NCBI GRCh37/hg19) que comprende los genes reguladores del complemento *C4BPA*, *CD55*, *CR2* y *CR1*, se utilizó la tecnología HiSeq2000 (Illumina, San Diego, CA, EE.UU.).

Los datos obtenidos se analizaron mediante un programa informático de computación en la nube llamado DNAnexus (www.dnanexus.com, Mountain View, CA, EE.UU.).

Pacientes, materiales y métodos/Patients, materials and methods

6.4.1.- Preparación de los pool de pacientes para la secuenciación.

Se secuenciaron un total de 8 pools (mezcla) de pacientes: tres de ellos formados por ADN de un total de 33 ENIS, con genotipo homocigoto CC para el polimorfismo rs1567190 de *CR2* asociado a protección frente a la infección por VIH-1; los otros 5, formados por ADN de un total de 46 individuos infectados con VIH-1, con genotipo homocigoto TT para el polimorfismo rs1567190 de *CR2* asociado a riesgo frente a la infección por este virus. La media de individuos que formaban cada pool era de 9 pacientes.

Para la realización de los pools, se seleccionaron a los pacientes a los que se iba a secuenciar de los que previamente habían sido genotipados, mediante la tecnología Veracode GoldenGate (illumina, San Diego, CA, EE.UU.) Posteriormente, se mezclaron sus ADN en un tubo de 1,5 ml, se añadió el 10% del volumen total de la mezcla de ADN de acetato sódico 3 M a pH 5.3 y dos volúmenes del total de la mezcla de ADN de etanol al 100%.

Se incubaron durante toda la noche a -20 °C. Pasado este tiempo, se centrifugaron a 12000 rpm durante 25 minutos para que precipitase el ADN. Se quitó el sobrenadante y se les incorporó etanol al 70% para eliminar las sales del acetato sódico. Se volvieron a centrifugar a 12000 rpm durante 25 minutos, para volver a quitar el sobrenadante y dejar secar el ADN. Posteriormente, se resuspendieron los ADN secos en un tampón de elución Tris con pH 8.5. Luego, se cuantificaron las concentraciones de los diferentes pools mediante espectrofotometría, obteniendo una media de 210 ng/μl aprox. en cada pool.

Los pools se enviaron a la sede de Otogenetics Corporation (Otogenetics Corporation, Atlanta, EE.UU.) para su secuenciación.

6.5.- Extracción de ARNm.

Para llevar a cabo la extracción de ARNm total de PBMCs, se utilizó el reactivo TriPure Isolation Reagent (Sigma-Aldrich, ST. Louis, MO, EE.UU.) siguiendo el protocolo que proporciona el fabricante.

6.6.- Retrotranscripción.

Para realizar la retrotranscripción del ARNm extraído (mediante el método mencionado en el apartado anterior, 6.5.) a ADNc se siguió el siguiente procedimiento:

Material:

- Kit SuperScript™ III Reverse Transcriptase (200U/μl) (Thermo Fisher Scientific, Carlsbad, CA, EE.UU.) que contiene la enzima retrotranscriptasa SuperScript™ III RT (200U/μl), el tampón de reacción 5X First-Strand Buffer y DTT 0.1 M.
- Recombinant RNase Inhibitor (40U/μl) (Thermo Fisher Scientific, Carlsbad, CA, EE.UU.).
- Hexámeros aleatorios, Random Hexamer Primers (Thermo Fisher Scientific, Carlsbad, CA, EE.UU.).
- dNTPs 10 mM (Sigma-Aldrich, ST. Louis, MO, EE.UU.).
- H₂O destilada autoclavada.
- ARN (10 pg - 5 μg).

Método:

Mezcla 1 – Preparar en hielo	
Material	Volumen por muestra (μl)
H ₂ O	Completar hasta los 13 μl
dNTPs	1
Random Hexamer Primers	1
ARNm	-
Volumen final de reacción	13 μl
Se incubó a 65 °C durante 5 min y se puso en hielo al menos durante 1 min	

Pacientes, materiales y métodos/Patients, materials and methods

Mezcla 2 – Preparar en hielo	
Material	Volumen por muestra (μl)
5X First Strand Buffer	4
DTT	1
RNaseOUT	1
SuperScript™ III Reverse Transcriptase	1
Volumen a añadir a la mezcla 1	7 μl
Volumen final de reacción	20 μl
Se incubó a temperatura ambiente 5 min, luego a 50 °C durante 45-60 min y a 70 °C durante 15 min.	

6.7.- Cuantificación de la expresión de *CR2*, *C4BPA* y *CD209*.

Todas las cuantificaciones se realizaron mediante PCR a tiempo real en el equipo MX3005 (Stratagene, La Jolla, CA, EE.UU.).

Los ADNc que se utilizaron en todas las cuantificaciones fueron obtenidos siguiendo el protocolo detallado en el apartado anterior 6.6.

6.7.1.- Cuantificación de la expresión de *CR2* y de sus isoformas.

6.7.1.1.- Cuantificación de la expresión global de *CR2*.

Material:

- Sonda Taqman Hs00153398_m1 de cuantificación de *CR2* (20X) (Thermo Fisher Scientific, Carlsbad, CA, EE.UU.) marcada con FAM y con BHQ 1 como quencher.
- Sonda Taqman Hs01047410_g1 de cuantificación de *CD19* (20X) (Thermo Fisher Scientific, Carlsbad, CA, EE.UU.) marcada con VIC y con BHQ 2 como quencher. *CD19* fue el normalizador interno.
- Tampón de PCR Quantimix Easy Probes (Biotools B&M Labs, Madrid, España).
- H₂O destilada autoclavada.
- ADNc.

Método:

Mezcla de reacción de PCR de cuantificación global de <i>CR2</i>		
Material	Volumen por muestra (µl)	
H ₂ O	2,7	
Quantimix Easy Probes	6,3	
Sonda Hs00153398_m1 de <i>CR2</i>	0,5	
Sonda Hs01047410_g1 de <i>CD19</i>	0,1	
ADNc	2	
Volumen final de reacción	12 µl/pocillo	

Condiciones de la PCR de cuantificación global de <i>CR2</i>		
Etapas de la PCR	Tª (°C)	Tiempo (hh:mm:ss)
Activación de la Taq polimerasa (1 ciclo)	95	00:05:00
Amplificación (45 ciclos)	Desnaturalización 95	00:00:25
	Hibridación y extensión 60	00:01:00 (medición de fluorescencia)

6.7.1.2.- Cuantificación de la expresión de la isoforma corta de *CR2*.

Material:

- Sonda Taqman de cuantificación de *CR2_S* (Isogen Life Science, Veldzigt, Países Bajos) marcada con Texas-RED y con BHQ 2 como quencher: 5'-CATGTTTCTTTTCACAAAC-3'.

- Sonda Taqman Hs01047410_g1 de cuantificación de *CD19* (20X) marcada con VIC (Thermo Fisher Scientific, Carlsbad CA, EE.UU.) con BHQ 2 como quencher. *CD19* fue el normalizador interno.

- Cebadores:

Cebador_sentido de *CR2_S*: 5'-CACCTGGGATCCTGAAATACCA-3'.

Pacientes, materiales y métodos/Patients, materials and methods

Cebador_antisentido de *CR2_S*: 5'-TCTTGAAGACTCTGTCTCACAT GCT-3'.

- Tampón de PCR Quantimix Easy Probes (Biotools B&M Labs, Madrid, España).

- H₂O destilada autoclavada.

- ADNc.

Método:

Mezcla de reacción de PCR de cuantificación de <i>CR2_S</i>	
Material	Volumen por muestra (μl)
H ₂ O	2,6
Quantimix Easy Probes	6,3
Cebador_sentido	0,5
Cebador_antisentido	0,5
Sonda Taqman de <i>CR2_S</i>	1
Sonda Hs01047410_g1 de <i>CD19</i>	0,1
ADNc	1
Volumen final de reacción	12 μl/pocillo

Condiciones de la PCR de cuantificación de <i>CR2_S</i>			
Etapas de la PCR		Tª (°C)	Tiempo (hh:mm:ss)
Activación de la Taq polimerasa (1 ciclo)		95	00:05:00
Amplificación (45 ciclos)	Desnaturalización	95	00:00:25
	Hibridación y extensión	60	00:01:00 (medición de fluorescencia)

6.7.1.3.- Cuantificación de la expresión de la isoforma larga de CR2.

Material:

- Sonda Taqman de cuantificación de *CR2_L* (Isogen Life Science, Veldzigt, Países Bajos) marcada con Texas-RED y con BHQ 2 como quencher: 5'-CTGGCAGCCTTTTT-3'.

- Sonda Taqman Hs01047410_g1 de cuantificación de *CD19* (20X) marcada con VIC (Thermo Fisher Scientific, Carlsbad CA, EE.UU.) con BHQ 2 como quencher. *CD19* fue el normalizador interno.

- Cebadores:

Cebador_sentido de *CR2_L*: 5'-TGATAACACCTGGGATCCTGAAAT A-3'.

Cebador_antisentido de *CR2_L*: 5'-AGAAGACCGTATTTCCACC TGTATG-3'.

- Tampón de PCR Quantimix Easy Probes (Biotools B&M Labs, Madrid, España).

- H₂O destilada autoclavada.

- ADNc.

Método:

Mezcla de reacción de PCR de cuantificación de <i>CR2_L</i>	
Material	Volumen por muestra (µl)
H ₂ O	2,6
Quantimix Easy Probes	6,3
Cebador_sentido	0,5
Cebador_antisentido	0,5
Sonda Taqman de <i>CR2_L</i>	1
Sonda Hs01047410_g1 de <i>CD19</i>	0,1
ADNc	1
Volumen final de reacción	12 µl/pocillo

Pacientes, materiales y métodos/Patients, materials and methods

Condiciones de la PCR de cuantificación de <i>CR2_L</i>			
Etapas de la PCR		Tª (°C)	Tiempo (hh:mm:ss)
Activación de la Taq polimerasa (1 ciclo)		95	00:05:00
Amplificación (45 ciclos)	Desnaturalización	95	00:00:25
	Hibridación y extensión	60	00:01:00 (medición de fluorescencia)

6.7.2.- Cuantificación de la expresión de *C4BPA*.

Material:

- Sonda Taqman Hs00426339_m1 de cuantificación de *C4BPA* (20X) (Thermo Fisher Scientific, Carlsbad, CA, EE.UU.) marcada con FAM y con BHQ 1 como quencher.
- Sonda Taqman Hs00169122_g1 de cuantificación de *CD14* (20X) marcada con VIC (Thermo Fisher Scientific, Carlsbad, CA, EE.UU.) con BHQ 2 como quencher. *CD14* fue el normalizador interno.
- Tampón de PCR Quantimix Easy Probes (Biotools B&M Labs, Madrid, España).
- H₂O destilada autoclavada.
- ADNc.

Método:

Mezcla de reacción de PCR de cuantificación de <i>C4BPA</i>	
Material	Volumen por muestra (μl)
H ₂ O	3,3
Quantimix Easy Probes	6,3
Sonda Hs00426339_m1 de <i>C4BPA</i>	1,2
Sonda Hs00169122_g1de <i>CD14</i>	0,2
ADNc	1
Volumen final de reacción	12 μl/pocillo

Condiciones de la PCR de cuantificación de <i>C4BPA</i>			
Etapas de la PCR		Tª (°C)	Tiempo (hh:mm:ss)
Activación de la Taq polimerasa (1 ciclo)		95	00:05:00
Amplificación (45 ciclos)	Desnaturalización	95	00:00:25
	Hibridación y extensión	60	00:01:00 (medición de fluorescencia)

6.7.3.- Cuantificación de la expresión de *CD209*.

Material:

- Sonda Taqman Hs01588349_m1 de cuantificación de *CD209* (20X) marcada con FAM y con BHQ 1 como quencher: 5'-CTGGCAGCCTTTT-3'.
- Sonda Taman 4310881E de cuantificación de *β-actina* (20X) marcada con VIC (Thermo Fisher Scientific, Carlsbad, CA, EE.UU.) y con BHQ 2 como quencher. *β-actina* fue el normalizador interno.
- Tampón de PCR Quantimix Easy Probes (Biotools B&M Labs, Madrid, España).
- H₂O destilada autoclavada.
- ADNc.

Pacientes, materiales y métodos/Patients, materials and methods

Método:

Mezcla de reacción de PCR de cuantificación de <i>CD209</i>	
Material	Volumen por muestra (μl)
H ₂ O	3
Quantimix Easy Probes	6,25
Sonda Taqman de <i>CD209</i>	1
Sonda 4310881E de <i>β-actina</i>	0,1
ADNc	2
Volumen final de reacción	12,5 μl/pocillo

Condiciones de la PCR de cuantificación de <i>CD209</i>			
Etapas de la PCR		Tª (°C)	Tiempo (hh:mm:ss)
Activación de la Taq polimerasa (1 ciclo)		95	00:10:00
Amplificación (45 ciclos)	Desnaturalización	95	00:00:15
	Hibridación y extensión	60	00:01:00 (medición de fluorescencia)

6.8.- Purificación de plásmidos.

6.8.1.- Transformación bacteriana por choque térmico.

Material:

- Plásmidos de interés reconstituidos.
- Bacterias competentes, StrataClone SoloPack (Agilent Technologies, Santa Clara, CA, EE.UU.).
- Medio de cultivo LB líquido autoclavado.
- Placas de Petri con medio LB agar con el antibiótico de resistencia que aporta el plásmido. Estos antibióticos fueron en unos casos ampicilina (Affymetrix, Santa Clara, CA, EE.UU.), a una

Pacientes, materiales y métodos/Patients, materials and methods

concentración de 0,1 mg/ml y en otros, kanamicina (Affymetrix, Santa Clara, CA, EE.UU.) a una concentración de 0,03 mg/ml.

- Hielo, baño maría a 42 °C, estufa en agitación a 37 °C, mechero Bunsen y asa de siembra.

Método:

Con las bacterias competentes en hielo, se agregó 1 µl de plásmido (añadiendo siempre una cantidad que oscila entre 1 y 10 ng) y se dejó el tubo en hielo durante 15 minutos. Transcurrido este tiempo, se metió en un baño a 42 °C durante 40 segundos. Posteriormente, se puso el tubo de 1,5 ml en hielo durante 2 minutos. Luego, se añadió 1 ml de medio LB líquido, previamente calentado a 37 °C, y se puso en la estufa con agitación a 37 °C durante 45-60 minutos. Se sacó de la estufa y se centrifugo el tubo durante 1 minuto. Se tiraron los 2/3 del sobrenadante y el otro 1/3 se utilizó para resuspender el botón de células. Posteriormente, se sembró la suspensión de bacterias en placas de Petri y se incubó en la estufa a 37 °C toda la noche.

6.8.2.- Cultivo en medio líquido de bacterias transformadas.

Material:

- *E. coli* transformadas crecidas en la placa de Petri.
- Medio de cultivo LB líquido autoclavado con el antibiótico de resistencia que aporta el plásmido. Estos antibióticos fueron en unos casos ampicilina (Affymetrix, Santa Clara, CA, EE.UU.), a una concentración de 0,1 mg/ml y en otros, kanamicina (Affymetrix, Santa Clara, CA, EE.UU.) a una concentración de 0,03 mg/ml.
- Estufa en agitación a 37 °C, matraces autoclavados, mechero Bunsen/campana de flujo.
- Cloranfenicol (Affymetrix, Santa Clara, CA, EE.UU.) a una concentración de uso de 0,17 mg/ml (optativo).

Método:

Dependiendo del proceso de purificación que se fuera a realizar después, se puso un volumen u otro de cultivo.

Pacientes, materiales y métodos/Patients, materials and methods

Se añadió LB líquido autoclavado a un matraz estéril. Se picó una colonia de bacterias transformadas con la punta de una pipeta y se desechó en el matraz. Posteriormente, se agregó el antibiótico de resistencia en un volumen adecuado para que quedase a la concentración final de trabajo y se incubó el cultivo en una incubadora con agitación a 37 °C toda la noche.

A la mañana siguiente, se recogieron los cultivos por centrifugación.

*En algunos cultivos bacterianos que partieron de una transformación cuyo plásmido era de bajo número de copias, se procedió a un tratamiento con cloranfenicol a una concentración de 0,17 mg/ml.

6.8.3.- Purificación de plásmidos:

Para realizar la purificación de los plásmidos se utilizó el Kit NucleoBond® Xtra Maxi (Macherey-Nagel, Düren, Alemania) siguiendo el protocolo proporcionado por el fabricante.

6.9.- Creación de líneas celulares estables.

Esta fase se llevó a cabo en la Unidad de Virología Molecular y Vacunas del Instituto Pasteur de París, bajo la dirección de PhD. Pierre Charneau (Director de la unidad) y la supervisión de Philippe Souque y Catherine Blanc.

Para crear las líneas celulares estables que expresaran CR2_S y CR2_L se partió de dos plásmidos, cuyos tamaños eran 8453 pb y 8630 pb respectivamente. Estos plásmidos contenían las dos isoformas del gen: la isoforma corta, de 3109 pb y la isoforma larga, de 3279 pb (Anexo 3). Ambas isoformas fueron introducidas en el vector lentiviral pFLAP-CMV-EGFP-WPRE al que se le extrajo el gen de la *EGFP* (Anexo 4).

Para ello, se diseñaron unos cebadores específicos que amplificaran ambas isoformas y que además, le diesen un sitio de corte compatible con el sitio de corte del vector lentiviral. Posteriormente, se ligaron correctamente las isoformas del vector generándose dos vectores lentivirales: pFLAP-CMV-CR2_S-WPRE y pFLAP-CMV-CR2_L-WPRE (Anexo 5).

Pacientes, materiales y métodos/Patients, materials and methods

Para producir las dos líneas celulares estables: HeLa p4 CCR5-CXCR4-CR2_S y HeLa p4 CCR5-CXCR4-CR2_L, se transfectaron con fosfato cálcico las células 293T con los vectores lentivirales creados. Esto generó los vectores víricos con los que posteriormente, se realizaron diferentes transducciones sobre las células HeLa p4 CCR5-CXCR4 para conseguir que expresasen unos niveles fisiológicos de los receptores CR2_S y CR2_L.

6.9.1.- Amplificación del fragmento CR2 S y CR2 L, purificación y clonación en el vector lentiviral.

6.9.1.1.- Diseño de oligonucleótidos de amplificación de CR2_S y CR2_L.

Se eligieron las enzimas de restricción BamH I y Xho I para el vector lentiviral. Ambas enzimas cortaban a las isoformas CR2_S y CR2_L, según el análisis realizado con el programa SerialCloner V2.5 (Franck Perez, Serial Basics, Instituto Curie, París, Francia). Se buscaron entonces dos enzimas compatibles en la ligación con BamH I y Xho I, siendo Bgl II y Sal I respectivamente. Ninguna de las dos cortaba en las isoformas.

Se diseñaron 3 oligonucleótidos: 2 cebadores sentido con diana para Bgl II y 1 cebador antisentido con diana para Sal I. los oligonucleótidos se diseñaron con el programa SerialCloner V2.5 (Franck Perez, Serial Basics, Instituto Curie, París, Francia).

Posteriormente, se cuantificaron los plásmidos pCDNA31_CR2_SHORT y pCDNA31_CR2_LONG mediante espectrofotometría (Eppendorf Biophotometer Spectrophotometer UV/VIS (Eppendorf AG, Hamburgo, Alemania)). Tras esto, se procedió a realizar un PCR en gradiente de temperatura en el termociclador Mastercycler Pro (Eppendorf AG, Hamburgo, Alemania).

Material:

- Cebadores, todos ellos de la misma casa comercial (Eurofins Scientific, Luxemburgo, Luxemburgo).

Cebador sentido 1-Bgl II_CR2: 5'-AGATCTGCCACCATGGGCGCCGC GGGCCTGCTCG-3'.

Cebador sentido 2-Bgl II_CR2: 5'-AGATCTGCCACCATGGGCGCCG CGGGCCTGCTCGGGGTTTCTTGG-3'.

Pacientes, materiales y métodos/Patients, materials and methods

Cebador antisentido – Sal I_CR2: 5'-GTCGACTCAGCTGGCTGGG TTGTATGG-3'.

- La enzima ADN polimerasa, Pfu turbo DNA polymerase (2,5U/μl) (Agilent Technologies, Santa Clara, CA, EE.UU.).

- Tampón de reacción, 10X cloned Pfu reaction buffer (Agilent Technologies, Santa Clara, CA, EE.UU.).

- dNTPs 25 mM (Sigma-Aldrich, ST. Louis, MO, EE.UU.)

- H₂O destilada autoclavada.

- Plásmidos pCDNA31_CR2_SHORT y pCDNA31_CR2_LONG (GenScript EE.UU. Inc., NJ, EE.UU.) (20 ng/μl).

Método:

Mezcla de reacción de PCR para amplificación de CR2_S/CR2_L	
Material	Volumen por muestra (μl)
H ₂ O	40,6
Pfu reaction buffer	5
dNTPs	0,4
Cebador sentido 1/2	1
Cebador antisentido	1
ADN polimerasa	1
Plásmido pCDNA31_CR2_S/CR2_L	1
Volumen final de reacción	50 μl/pocillo

Condiciones de la PCR para amplificación de <i>CR2_S/CR2_L</i>		
Etapas de la PCR	Tª (°C)	Tiempo (hh:mm:ss)
Activación de la Taq polimerasa (1 ciclo)	94	00:02:00
Amplificación (30 ciclos)	Desnaturalización	00:00:15
	Hibridación (Gradiente de temperatura)	55
		57
		60
		62
	Extensión	00:03:00
Extensión final	72	00:01:00
Verificación de la amplificación: Se cargaron las muestras en un gel de agarosa al 1% con bromuro de etidio (0,7 mg/ml) (Eurobio AbCys, París, Francia) a una concentración aproximada de 0,014 mg/ml y se corrió durante 1 hora y 30 minutos a 100V.		

6.9.1.2.- Purificación de bandas en geles de garosa.

Las purificaciones de todos los fragmentos de PCR amplificados (insertos o vectores) se realizaron con el mismo procedimiento.

Se corrieron las muestras de interés en un gel de agarosa al 1% con bromuro de etido (0,7 mg/ml) (Eurobio AbCys, París, Francia) a una concentración aproximada de 0,014 mg/ml. En este caso, se cargaron en el gel 45 µl de los dos productos de la PCR de amplificación y se corrió durante 2 horas y 30 minutos a 50 V.

Se cortaron las bandas y se realizó la purificación de las mismas mediante el Kit NucleoSpin® Gel and PCR Clean up (Macherey-Nagel, Düren, Alemania) siguiéndose las instrucciones detalladas por el fabricante.

6.9.1.3.- Subclonación de los insertos CR2_S y CR2_L.

Para clonar los insertos *CR2_S* y *CR2_L* amplificados mediante PCR en el vector final pFLAP-CMV-WPRE fue necesario realizar una primera clonación en otro vector. La enzima ADN polimerasa con la que se realizó la amplificación de los insertos (descrita en el apartado 6.9.1.1.) deja

Pacientes, materiales y métodos/Patients, materials and methods

extremos romos en los amplicones. De esta forma, se le agrega a los fragmentos de los insertos una adenina extra en los extremos 3', dejando de ser romos. Posteriormente, se realizó la subclonación y luego las restricciones con las enzimas seleccionadas, haciendo posible la correcta ligación con el vector final.

Para realizar este proceso se utilizó el Kit TOPO TA Cloning® Kit Dual Promoter (Thermo Fisher Scientific, Carlsbad, CA, EE.UU.) y se siguieron las especificaciones del fabricante.

6.9.1.4.- Restricciones de los vectores.

Las reacciones de restricción se llevaron a cabo con enzimas de la misma casa comercial, por lo que se usó para todas las reacciones el mismo tampón de reacción. También coincidían en que tenían la misma temperatura de restricción.

Solo se llevó a cabo una restricción por cada reacción. En el caso de los vectores pCR II-TOPO_CR2_S y pCR II-TOPO_CR2_L, la primera restricción se llevó a cabo con la enzima Bgl II y la segunda con Sal I. En el caso del vector pFLAP-CMV-EGFP-WPRE, se realizó la primera restricción con BamH I y la segunda con Xho I.

Material:

- Enzima de restricción Bgl II 20000 U/ml (New England Biolabs, Ipswich, MA, EE.UU.).
- Enzima de restricción Sal I 20000 U/ml (New England Biolabs, Ipswich, MA, EE.UU.).
- Enzima de restricción BamH I HF 20000 U/ml (New England Biolabs, Ipswich, MA, EE.UU.).
- Enzima de restricción Xho I 20000 U/ml (New England Biolabs, Ipswich, MA, EE.UU.).
- Tampón de reacción 3.1 10X concentrado (New England Biolabs, Ipswich, MA, EE.UU.).
- H₂O destilada autoclavada.
- Vectores.

- Baño maría a 37 °C.

Método:

Mezcla de reacción de restricción 1	
Material	Volumen por muestra (μl)
H ₂ O	16
Tampón de reacción 3.1	3
Enzima Bgl II/BamH I	1
Vector	10
Volumen final de reacción	30 μl/pocillo

Se incubaron los vectores a 37 °C durante 1 hora.

Mezcla de reacción de restricción 2	
Material	Volumen por muestra (μl)
H ₂ O	8
Tampón de reacción 3.1	1
Enzima Sal I/Xho I	1
Mezcla de reacción de restricción 1	30
Volumen final de reacción	40 μl/pocillo

Se incubaron los vectores a 37 °C durante 1 hora.

Se realizó una primera electroforesis de verificación y tras eso, se purificaron las bandas de interés según se ha descrito en el apartado 6.9.1.2.

6.9.1.5.- Ligación de los insertos CR2_S y CR2_L con el vector pFLAP-CMV-WPRE.

Material:

- Enzima ligasa T4 DNA Ligase (Thermo Fisher Scientific, Waltham, MA, EE.UU.).

- Tampón de reacción 10X T4 DNA Ligase Buffer con ATP (Thermo Fisher Scientific, Waltham, MA, EE.UU.).

Pacientes, materiales y métodos/Patients, materials and methods

- H₂O destilada autoclavada.
- Insertos (CR2_S y CR2_L) y vector pFLAP-CMV-WPRE.
- Baño maría a 37 °C.

Método:

Mezcla de reacción de ligación	
Material	Volumen por muestra (μl)
H ₂ O	7
Tampón de reacción 10X T4 DNA Ligase	1,2
Enzima T4 DNA Ligase	1
Inserto CR2_S/CR2_L	6
Vector pFLAP-CMV-WPRE	1
Volumen final de reacción	16,2 μl/pocillo

Se incubó a 4 °C durante toda la noche.

Tras esto, se realizaron transformaciones bacterianas y purificaciones de los vectores según la metodología descrita en el apartado 6.8.

Se mandaron los vectores a secuenciar a un laboratorio externo para verificar que eran correctos.

6.9.2.- Generación de los vectores virales VSV-CR2 S y VSV-CR2 L.

Para generar los dos vectores virales necesarios para crear las dos líneas celulares estables, se utilizaron las células 293T. Esta línea celular está derivada de células de riñón embrionario humano. Se escogieron, debido a que su eficiencia de transfección con fosfato cálcico y de empaquetamiento es muy elevada, por lo que son muy apropiadas para la producción de vectores virales.

6.9.2.1.- Transfección con fosfato cálcico.

La transfección por fosfato cálcico es un método químico de transfección mediante el cual, en una solución salina de fosfatos, el ADN forma agregados con el cloruro cálcico que penetran en la célula por

endocitosis. Esta técnica se utilizó para transfectar la línea 293T con los vectores pFLAP-CMV-CR2_S-WPRE y pFLAP-CMV-CR2_L-WPRE.

Material:

- H₂O estéril de Gibco (Thermo Fisher Scientific, Carlsbad, CA, EE.UU.).
- CaCl₂ 1 M (Sigma-Aldrich, ST. Louis, MO, EE.UU.).
- Tampón salino HEPES (Sigma-Aldrich, ST. Louis, MO, EE.UU.).
- Fosfato de sodio dihidratado (Na₂HPO₄ • 2 H₂O) (Sigma-Aldrich, ST. Louis, MO, EE.UU.).
- NaCl (Sigma-Aldrich, ST. Louis, MO, EE.UU.).
- Plásmidos a una concentración de 1 µg/µl (se necesitan tres plásmidos diferentes: uno que porte los genes *gag* y *pol* del VIH-1, otro que porte la envuelta del virus que se quiera crear y el tercero, será el vector lentiviral que aportará el material genético que se quiera introducir en la célula).
- Células 293T (nº ATCC: CRL-3216 (ATCC, Manassas, VA, EE.UU.)).
- Medio DMEN completo (500 ml de medio DMEN de Gibco (Thermo Fisher Scientific, Carlsbad, CA, EE.UU.) al que se le añade 50 ml de suero de vaca fetal descomplementado por calor durante 1 hora a 56 °C y penicilina y estreptomina a una concentración final de 100 UI/ml de penicilina y 100 µg/ml de estreptomina).
- Estufa a 37 °C con una atmósfera de 5 % de CO₂ y una humedad del 100 %.

Método:

24 horas antes de realizar la transfección por fosfato cálcico, se pusieron en cultivo 8 placas de Petri por condición de transfección, a una densidad de 6.500.000 células/placa de Petri en 8 ml de medio DMEN completo. Una hora antes de empezar la transfección, se cambió el medio añadiendo esta vez, 6 ml de medio DMEN completo.

Pacientes, materiales y métodos/Patients, materials and methods

Para transfectar 8 placas de Petri, se añadieron en un tubo de 50 ml: 2800 µl de H₂O estéril, 1000 µl de CaCl₂ 1 M, 20 µl de plásmido que contenía la envuelta viral, 80 µl de plásmido con los genes *gag* y *pol* y 80 µl del vector con el gen de interés (tubo 1). En otro tubo de 50 ml se añadieron 4 ml de HEPES (tubo 2). Posteriormente, se agregó gota a gota el contenido del tubo 1 en el tubo 2 mientras se agitaba suavemente. Se incubó a temperatura ambiente entre 5 y 10 minutos. Transcurrido este tiempo, se añadió 1 ml de la mezcla final a cada placa de Petri y se incubó en la estufa a 37 °C durante 24 horas. Luego, se les cambio el medio añadiéndoles 8 ml de medio DMEN completo.

A las 48 horas de realizar las transfecciones, se recogieron los sobrenadantes celulares que es donde se encuentran los vectores virales producidos.

6.9.2.2.- Recogida de vectores virales.

El protocolo seguido para recoger los vectores virales producidos por las 293T fue el siguiente:

Material:

- Células 293T transfectadas con fosfato cálcico.
- Tubos de 50 ml.
- Centrífuga para tubos de 50 ml.
- Criotubos.
- Congelador -80 °C.

Método:

A las 48 horas de haber realizado la transfección de las células con fosfato cálcico, se recogieron los sobrenadantes de las placas de Petri y se pasaron a un tubo de 50 ml para su centrifugación a 2500 rpm durante 10 minutos. Tras la centrifugación, se alicuotaron los sobrenadantes en criotubos y se congelaron a -80 °C para su conservación.

6.9.2.3.- Cuantificación de partículas virales generadas.

Se titraron los vectores virales generados para poder crear la línea estable. Para ello, en primer lugar, se realiza una transducción sobre la línea celular 293T con diluciones seriadas de los vectores. Posteriormente, se lisan estas células y luego se cuantifican las partículas virales mediante PCR a tiempo real.

6.9.2.3.1.- Transducción de células 293T.

Material:

- Células 293T.
- Vectores virales: VSV-CR2_S y VSV-CR2_L. Se puso en este caso un tercer vector viral que portaba EGFP como control positivo.
- Agitador rotativo para placas de cultivo celular.
- Estufa a 37 °C con una atmósfera del 5 % de CO₂ y 100 % de humedad relativa.
- Medio DMEN completo.

Método:

24 horas antes de realizar la transducción, se pusieron en cultivo 1 placa de 6 pocillos por condición de transducción, a una densidad de 200.000 células_{293T}/pocillo en 2 ml de medio DMEN completo.

Se hicieron tres diluciones de cada vector. En un primer tubo (tubo 1), se añadió: 1600 µl de medio DMEN completo y 400 µl de sobrenadante viral puro recogido. Se homogeneizó y se añadió 500 µl del tubo 1 a un tubo 2 que contenía 500 µl de medio DMEN completo. Se homogeneizó el tubo 2 y se añadió 500 µl del tubo 2 a un tubo 3 que contenía 500 µl de medio DMEN completo.

Posteriormente, se quitó el medio a las células y se añadió 500 µl del tubo 1 en el pocillo 1, 500 µl del tubo 2 en el pocillo 2 y 500 µl del tubo 3 en el pocillo 3. El volumen sobrante del tubo 1, se incubó a 70 °C durante 30 minutos para inactivar las partículas virales (control negativo); pasado este tiempo, se añadió 500 µl del tubo 1 tratado por calor al pocillo 4. La placa de 6 pocillos se incubó en un

Pacientes, materiales y métodos/Patients, materials and methods

agitador rotativo en la estufa a 37 °C durante 2 horas. Luego, se añadió 1500 µl de medio DMEN completo a cada pocillo y se incubó la placa en la estufa durante 48 horas,

Pasado este tiempo, se procedió a la lisis celular.

6.9.2.3.2.- Lisis de las células transducidas.

Material:

- Células 293T transducidas.
- Tampón fosfato salino de Gibco (Thermo Fisher Scientific, Carlsbad, CA, EE.UU.).
- Tampón de lisis celular: H₂O estéril de Gibco (Thermo Fisher Scientific, Carlsbad, CA, EE.UU.), tampón de lisis 20X (Eurobio AbCys, París, Francia) y RNasas (Thermo Fisher Scientific, Carlsbad, CA, EE.UU.).
- Proteinasa K (Eurobio AbCys, París, Francia).
- Baños a 37 y 56 °C.

Método:

Se inicia el protocolo pasadas las 48 horas de incubación tras la transducción. Se lavaron las células 2 veces con 700 µl de tampón fosfato salino. Tras estos lavados, se hicieron otros 2 lavados más con 700 µl de tampón fosfato salino pero esta vez, se añadió directamente sobre las células para despegarlas. Se pasaron a un tubo de 1,5 ml.

Se centrifugaron los tubos a 10000 rpm durante 1 minuto y se quitó el sobrenadante.

Se preparó el tampón de lisis celular (según el siguiente cuadro) y se añadió 200 µl a cada tubo. El sobrante del tampón de lisis se reservó y se le añadió 3 µl/muestra de Proteinasa K.

Se incubaron los tubos a 37 °C durante 30 minutos. Luego, se añadió 100 µl de tampón de lisis con proteinasa K. Posteriormente, se incubaron a 56 °C durante 4 horas.

Pacientes, materiales y métodos/Patients, materials and methods

Los lisados celulares se guardaron a -20 °C.

Tampón de lisis celular	
Material	Volumen por muestra (μl)
H ₂ O estéril	285
Tampón de lisis 20X	15
RNAsas	1,5
	200 μl/pocillo
+ Proteinasa K	3
	100 μl/pocillo

6.9.2.3.3.- PCR a tiempo real de cuantificación de partículas virales.

Para cuantificar el número de copias que hay en las células de los vectores virales, se realizó una PCR en tiempo real en el equipo Mastercycler ep Realplex Gradient S (Eppendor, Hamburgo, Alemania). El objetivo fue detectar el fragmento *U5R* de la LTR de los vectores: pFLAP-CMV-CR2_S-WPRE, pFLAP-CMV-CR2_L-WPRE y pFLAP-CMV-EGFP-WPRE. Como normalizador se utilizó *CD3*, correceptor de las células T que se expresa en la línea celular 293T.

Material:

- Cebadores:

Cebador_sentido_*CD3*: 5'-GGCTATCATTCTTCTTCAAGGTA-3'.

Cebador_antisentido_*CD3*: 5'-CCTCTCTTCAGCCATTTAAGTA-3'.

Cebador_sentido_*U5R*: 5'-GGCTAACTAGGGAACCCACTG-3'.

Cebador_antisentido_*U5R*: 5'-GCTAGACATTTTCCACACTGACTA A-3'.

- Kit Platinum® SYBR® Green qPCR SuperMix-UDG (thermo Fisher Scientific, Carlsbad, CA, EE.UU.).

- H₂O destilada autoclavada.

- Diluciones de los lisados celulares.

Pacientes, materiales y métodos/Patients, materials and methods

- Muestras para hacer la recta patrón.

Método:

Se prepararon las diluciones de los lisados celulares añadiendo a un tubo de 1,5 ml: 45 μ l de H₂O estéril y 5 μ l de lisado celular, previamente homogeneizado con un mezclador.

Se prepararon las diluciones seriadas con las que se calculará la recta patrón, que servirá para calcular el número de copias que hay en los lisados celulares. Se tomaron 7 tubos de 1,5 ml y se les añadió 90 μ l de lisado de células 3T3 a cada uno de ellos. Luego, de un tubo que contiene 10^9 número de copias, se tomaron 10 μ l y se añadieron al tubo 1, homogeneizándolo correctamente (10^8 número de copias). De ese tubo, se tomaron otros 10 μ l y se añadieron al siguiente tubo homogeneizándolo (10^7 número de copias) y así se realiza sucesivamente hasta alcanzar la dilución de 10^2 . Las diluciones que se utilizaron para realizar la PCR y la posterior recta patrón fueron, desde la 10^7 hasta la 10^2 número de copias.

Mezcla de reacción de PCR de cuantificación de partículas virales – U5R	
Material	Volumen por muestra (μ l)
H ₂ O	2,7
SybrGreen	10,3
Cebador_sentido_U5R	1
Cebador_antisentido_U5R	1
Diluciones lisado celular	5
Volumen final de reacción	20 μ l/pocillo

Pacientes, materiales y métodos/Patients, materials and methods

Mezcla de reacción de PCR de cuantificación de partículas virales – <i>CD3</i>	
Material	Volumen por muestra (μl)
H ₂ O	2,7
SybrGreen	10,3
Cebador_sentido_ <i>CD3</i>	1
Cebador_antisentido_ <i>CD3</i>	1
Diluciones lisado celular	5
Volumen final de reacción	20 μl/pocillo

Condiciones de la PCR de cuantificación de partículas virales			
Etapas de la PCR		Tª (°C)	Tiempo (hh:mm:ss)
Activación de la Taq polimerasa (1 ciclo)		95	00:05:00
Amplificación (35 ciclos)	Desnaturalización	95	00:00:05
	Hibridación	59	00:00:10
	Extensión	72	00:00:08 (medición de fluorescencia)
Curva de disociación		95	00:01:00
	Medida de fluorescencia con cada incremento de 0,5 °C para la curva de disociación	55	00:00:15 hasta
		95	00:00:15

Se analizaron los resultados de la PCR calculando las medias de las réplicas y sus correspondientes desviaciones estándar, para luego realizar la normalización con *CD3*.

6.9.3.- Transducción de células HeLa p4 CCR5-CXCR4 con VSV-CR2 S y VSV-CR2 L.

La línea celular HeLa p4 CCR5-CXCR4 (Clavel & Charneau 1994) son células de cáncer cervico-uterino. En su interior portan el gen *LacZ* bacteriano flanqueado por dos secuencias LTR. En presencia del

Pacientes, materiales y métodos/Patients, materials and methods

transactivador *Tat* del VIH, se activan produciendo β -galactosidasa y acumulándose esta en el citoplasma celular. Se escogió esta línea celular para crear las dos líneas celulares estables que expresen CR2_S y CR2_L debido a que ya expresan en su membrana la glicoproteína CD4 (Receptor del VIH) y los correceptores virales CCR5 y CXCR4. De esta forma, una vez creadas las líneas estables, se procedería a la infección midiéndose esta a través de β -galactosidasa.

6.9.3.1.- Transducción de las células HeLa p4 CCR5-CXCR4 con los vectores virales VSV-CR2_S y VSV-CR2_L.

Tras la cuantificación mediante PCR a tiempo real del número de partículas virales que se tienen por el de sobrenadante recogido, se procedió a la realización de la transducción de las células HeLa p4 CCR5-CXCR4.

La multiplicidad de infección o MOI, es la relación que existe entre el número de partículas virales y el número de células diana presentes en un espacio definido. En este caso y debido a la experiencia previa del grupo de investigación de Pierre Charneau, se seleccionaron dos MOI diferentes: 20 MOI y 50 MOI. Esto quiere decir que, por cada célula HeLa p4 CCR5-CXCR4 en cultivo habría 20 y 50 partículas virales respectivamente.

Se realizaron 4 transducciones diferentes y por duplicado: dos con VSV-CR2_S y otras dos con VSV-CR2_L, ambos con los MOI previamente citados. Pasadas las 24 horas de la primera transducción, se realizó una segunda sobre los duplicados, con las mismas condiciones que la anterior. Tras 24 horas, se midió la expresión del receptor en membrana mediante citometría de flujo (protocolo explicado en el apartado 6.9.3.2.). Como la expresión tras la segunda transducción aun no llegaba a los niveles deseados, se realizó una tercera y cuarta transducción.

El protocolo seguido para la realización de las transducciones es muy similar al que ha sido explicado previamente en el apartado 6.9.2.3.1. En este caso, se calculó el volumen de cada vector viral a añadir según la cantidad de partículas virales que se obtuvo en la titración. Se añadió dicho volumen tras quitar el medio a las células y se incubó en un agitador rotativo en la estufa a 37 °C durante 2 horas. Luego, se completó el volumen hasta los 2000 μ l con medio DMEN completo. Se incubó en la

estufa durante 24 horas. Pasado este tiempo, se realizó la siguiente transducción de las mismas.

6.9.3.2.- Medición de la expresión de CR2_S y CR2_L mediante citometría de flujo.

Material:

- H₂O estéril de Gibco (Thermo Fisher Scientific, Carlsbad, CA, EE.UU.).
- Tampón fosfato salino de Gibco (Thermo Fisher Scientific, Carlsbad, CA, EE.UU.).
- Tampón fosfato salino de Gibco con EDTA 1 mM (Thermo Fisher Scientific, Carlsbad, CA, EE.UU.).
- Tampón fosfato salino de Gibco (Thermo Fisher Scientific, Carlsbad, CA, EE.UU.) con BSA al 1 % (Sigma-Aldrich, ST. Louis, MO, EE.UU.)
- Solución fijadora de células, BD Cell Fix 10X (Becton, Dickinson and Company, Franklin Lakes, NJ, EE.UU.).
- Anticuerpo primario, CR2 clone LT21 IgG1 de antibodies-online.com La dilución realizada de este anticuerpo es de: 1 µl de anticuerpo en 999 µl de PBS-BSA 1 %. Se añaden 150 µl de dilución de anticuerpo primario por cada 200.000 células.
- Anticuerpo secundario, Goat Anti-Mouse IgG1 marcado con FITC de Becton, Dickinson and Company, Franklin Lakes, NJ, EE.UU.). La dilución realizada de este anticuerpo es de: 2,5 µl de anticuerpo en 997,5 µl de PBS-BSA 1%. Se añaden 150 µl de dilución de anticuerpo secundario por cada 200.000 células.

Método:

Pasadas 24 horas de la última transducción, se despegaron las células con tampón fosfato salino que contenía EDTA 1 mM. Se pasaron a un tubo, se les añadió 3 ml de tampón fosfato salino y se centrifugaron a 1600 rpm durante 6 minutos.

Pacientes, materiales y métodos/Patients, materials and methods

Se les quitó el sobrenadante, se les añadió 500 µl de solución fijadora y se incubaron 15 minutos a temperatura ambiente. Se lavaron las células dos veces añadiéndoles 2,5 ml de PBS y centrifugándolas a 1600 rpm durante 6 minutos.

Tras quitarles el sobrenadante, se preparó la dilución del anticuerpo primario y se añadió a las muestras. Se incubaron a 4 °C durante 1 hora. Posteriormente, se lavaron 2 veces las muestras con PBS-BSA 1 %. Se preparó la dilución del anticuerpo secundario marcado con FICT y se añadió a las muestras. Se incubaron a 4 °C durante 1 hora. Se añadió 3 ml de PBS-BSA 1% y se centrifugaron a 1600 rpm durante 6 minutos.

Se les quitó de nuevo el sobrenadante, se le añadió 300 µl de PBS-BSA 1% y se midió en el citómetro de flujo.

6.9.3.3.- Aislamiento de células, selección de clones y creación de línea estable.

Tras realizar las transducciones consecutivas, se consiguió una población de células que expresaban niveles muy heterogéneos de receptor. Se procedió al aislamiento de células para llegar a obtener clones que expresaran los niveles de receptores homogéneos.

Para ello, se realizó una dilución de las células obtenidas tras la tercera y cuarta transducción de CR2_S y CR2_L respectivamente. La dilución fue de 0,2 células/pocillo, cultivándose en 6 placas de 96 pocillos de cada receptor, en un volumen de 200 µl/pocillo de medio DMEN completo.

Se dejaron crecer aproximadamente durante 15 días en una estufa a 37 °C con una atmosfera de 5 % de CO₂ y un 100 % de humedad. Se seleccionaron aquellos pocillos en los que solo había una colonia celular. Esas colonias se pusieron en placas de 6 pocillos y se dejaron crecer 15 días en las mismas condiciones anteriormente mencionadas.

Transcurrido este tiempo, se midieron los clones mediante citometría de flujo siguiendo el procedimiento descrito en el apartado 6.9.3.2. Se seleccionaron los clones que expresaban los niveles aproximados de receptor de interés y se pasaron a cultivar en frascos de 25 cm².

6.10.- Producción de pseudovirus.

6.10.1.- Transfección con fosfato cálcico y recogida de pseudovirus.

Para la generación y recogida de los pseudovirus se siguieron los mismos procedimientos descritos en los apartados 6.9.2.1. y 6.9.2.2.

En este caso, para la transfección por fosfato cálcico, se usaron solo dos plásmidos: uno que aportó la envuelta viral (VSV, HIV-1 CCR5) y otro que aportó los genes *gag-pol* del VIH-1 así como, el material genético que va en el interior de pseudovirus. En esta ocasión ese material incluía la β -*galactosidasa*, a través de la cual se midió la infección. Se generaron 3 pseudovirus detallados a continuación.

Vectores usados en la generación de pseudovirus			
Vectores		Procedencia y envuelta que aportan	
Vectores de expresión de envuelta	pHEF-VSVG (Anexo 6)	Vector del NIH, REF:4693 Envuelta VSV	
	pCAGGS SF162 gp160	Vector del NIH, REF:10463 Envuelta HIV-1_CCR5	
	HIV clone BaL.01	Vector del NIH, REF:11445 Envuelta HIV_CCR5	
	Vector de expresión de <i>gag-pol</i> y <i>β-galactosidasa</i>	pBRU-ΔEnv	Vector de la Unidad de Virología Molecular y Vacunas del Instituto Pasteur de París similar al pSG3Δenv, vector del NIH, REF: 11051 (Anexo 7)
Pseudovirus producidos			
Pseudovirus Env-VSV		pHEF-VSVG + pBRU-ΔEnv	
Pseudovirus Env-VIH-1_CCR5 (1)		pCAGGS SF162 gp160 + pBRU-ΔEnv	
Pseudovirus Env-VIH-1_CCR5 (2)		HIV-1 clone BaL.01 + pBRU-ΔEnv	

6.10.2.- Titración de los pseudovirus producidos.

Para determinar la concentración adecuada a añadir de los pseudovirus en los experimento de infección, se realizó la titración de los

Pacientes, materiales y métodos/Patients, materials and methods

pseudovirus producidos sobre las células HeLa p4 CCR5-CXCR4. Previamente, se cuantificó la proteína de la cápsida viral (p24) en los sobrenadantes recogidos. El objetivo de esta cuantificación fue determinar el volumen de sobrenadante viral a añadir en la titración.

6.10.2.1.- Cuantificación de la proteína p24.

La cuantificación de la proteína p24 se realizó mediante el Kit comercial HIV-1 p24 Antigen Capture Assay (Advanced Biosciences laboratorios, Rockville, MD, EE.UU.) siguiendo las especificaciones del fabricante.

6.10.2.2.- Titración de los pseudovirus sobre las células HeLa p4 CCR5-CXCR4:

24 horas antes de realizar la titración, se pusieron en cultivo 10.000 células_{HeLa p4 CCR5-CXCR4}/pocillo en una placa de 96 pocillos en un volumen de 200 µl/pocillo de medio DMEN completo.

Las concentraciones de p24 con las que se realizó la titración de cada pseudovirus fueron: 5 ng, 2,5 ng, 1 ng, 0,5 ng y 0,25 ng. La titración se hizo por triplicado. Una vez que se infectó las células con esas concentraciones en un volumen final de 200 µl de medio DMEN completo, se incubaron durante 48 horas.

6.10.2.3.- Medición de β-galactosidasa y proteínas.

Transcurridas las 48 horas de la infección, se procedió a la medición de β-galactosidasa mediante el Kit comercial β-Gal Reporter Gene Assay (Chemiluminiscent) (Sigma-Aldrich, ST. Louis, MO, EE.UU.). Para normalizar los valores obtenidos se procedió a la cuantificación de proteínas con el Kit comercial DC Protein Assay reagents Package (BIO-RAD, Hercules, CA, EE.UU.). En ambos casos, se siguieron las especificaciones del fabricante.

Las concentraciones seleccionadas para cada pseudovirus fueron aquellas que proporcionaban un nivel de infección similar en las células. En el caso del pseudovirus sin envuelta (control negativo de infección), se seleccionó la concentración mayor.

6.11.- Experimentos funcionales *CD209*.

Para determinar las diferencias de expresión *in vitro* entre el haplotipo protector XAGA y otras variantes de este haplotipo, se amplificó 1,4 Kb de la 5'UTR del promotor de *CD209* de dos pacientes que portaban: uno, el haplotipo protector XAGA; y el otro, el haplotipo XGAG. Posteriormente, se clonaron en el vector final pd2EGFP-1 de Clontech (Takara Bio Inc, Kusatsu, Japón) (Anexo 8).

Para ello, se diseñaron unos cebadores específicos que amplificaran dichos fragmentos, se subclonaron en el plásmido pSC-A-Amp/Kan de Strataclone PCR Cloning Kit (Stratagene, La Jolla, CA, EE.UU.) (Anexo 9), se cortaron con las enzimas de restricción adecuadas y se ligaron los productos de estas restricciones con el vector final anteriormente citado.

6.11.1.- Amplificación del fragmento de la 5'UTR del promotor de *CD209*, purificación y clonación en el vector.

6.11.1.1.- Diseño de oligonucleótidos de amplificación de la 5'UTR del promotor de *CD209*.

El diseño de los oligonucleótidos se llevó a cabo con el programa SerialCloner V2.4 (Franck Perez, SerialBasics, instituto Curie, París, Francia).

El termociclador en el que se realizó la PCR de amplificación fue T-Gradient Thermocycler (Biometra GmbH, Göttingen, Alemania).

Material:

- Cebadores, todos ellos de la misma casa comercial (Eurofins Scientific, Luxemburgo, Luxemburgo):

Cebador_sentido_1: 5'-AGGTCATTGCCATGGCATCGGT-3'.

Cebador_sentido_2: 5'-AGGAGTCCAGGAGTCTCTCGTC-3'.

Cebador_antisentido_1: 5'-TACCTCATTACCATGTGAAATG-3'.

Cebador_antisentido_2: 5'-GGCTGGCCATCCCTTCCCCCTT-3'.

- Kit Expand™ High Fidelity PCR System (Sigma-Aldrich, ST. Louis, MO, EE.UU.) que incluye la enzima ADN polimerasa Expand High

Pacientes, materiales y métodos/Patients, materials and methods

Didelity Enzyme Mix y el tampón de reacción Expand High Fidelity Buffer (10X).

-dNTPs 25 mM (Sigma-Aldrich, ST. Louis, MO, EE.UU.).

- H₂O destilada autoclavada.

- ADN genómico humano (10 ng/μl).

Método:

Mezcla de reacción de PCR para la amplificación de 5'UTR de CD209	
Material	Volumen por muestra (μl)
H ₂ O	39,8
Tampón de reacción	5
dNTPs	0,4
Cebador_sentido_1/2	1
Cebador_antisentido_1/2	1
ADN polimerasa	1,8
ADN	1
Volumen final de reacción	50 μl/pocillo

Condiciones de la PCR para amplificación de 5'UTR de CD209		
Etapas de la PCR		Tª (°C)
		Tiempo (hh:mm:ss)
Activación de la Taq polimerasa (1 ciclo)		94
Amplificación (30 ciclos)	Desnaturalización	94
	Hibridación (Gradiente de temperatura)	51
		53
		55
		57
		60
		62
	Extensión	72
Extensión final		72

Pacientes, materiales y métodos/Patients, materials and methods

Para realizar la verificación de la amplificación, se cargaron las muestras en un gel de agarosa al 1% con bromuro de etidio (solución de bromuro de etidio (10 mg/ml) (Sigma-Aldrich, ST. Louis, MO, EE.UU.)) a una concentración de 0,014 mg/ml aproximada y se corrió durante 1 hora y 30 minutos a 100 V.

6.11.1.2.- Purificación de bandas en geles de agarosa.

Para realizar las purificaciones de fragmentos de PCR amplificados, se corrieron los 45 µl de dos de los productos de la PCR de amplificación de los haplotipos en un gel de agarosa sin bromuro durante 2 horas y 30 minutos a 50 V. Posteriormente, se tiñó con azul de metileno.

Se cortaron las bandas y se realizó la purificación de las mismas mediante el Kit NucleoSpin® Gel and PCR Clean up (Macherey-Nagel, Düren, Alemania) siguiendo las instrucciones detalladas por el fabricante.

6.11.1.3.- Subclonación de los insertos, haplotipos XAGA y XGAG.

Se subclonaron los dos insertos amplificados en el vector pSC-A-Amp/Ken. Para esto, se utilizó el Kit Strataclone PCR Cloning (Stratagene, La Jolla, CA, EE.UU.) con las condiciones especificadas por el fabricante.

Para verificar que las amplificaciones y las clonaciones eran correctas, se secuenciaron los insertos usando un secuenciador CEQ 2000XL DNA Analysis System Sequencer (Beckman Coulter Inc, Brea, CA, EE.UU.) mediante el método enzimático de terminación de la cadena.

6.11.1.4.- Restricciones de los vectores:

Las reacciones de restricción se llevaron a cabo con enzimas de la misma casa comercial, por lo que se usó para todas las reacciones el mismo tampón de reacción. También coincidían en que tenían la misma temperatura de restricción.

Se realizaron las restricciones sobre los vectores: pSC-A-AGA y pSC-A-GAG de los que se extrajeron los insertos y sobre el vector final, pd2EGFP-1. Solo se llevó a cabo una restricción por cada reacción. La primera restricción se realizó con la enzima Sac I y la segunda con Kpn I, ambas de 20000 U/ml (New England Biolabs, Ipswich, MA, EE.UU.).

Pacientes, materiales y métodos/Patients, materials and methods

Material:

- Enzima de restricción Sac I 20000 U/ml (New England Biolabs, Ipswich, MA, EE.UU.).
- Enzima de restricción Kpn I 20000 U/ml (New England Biolabs, Ipswich, MA, EE.UU.).
- Tampón de reacción 3.1 10X concentrado (New England Biolabs, Ipswich, MA, EE.UU.).
- H₂O destilada autoclavada.
- Vectores.
- Baño maría a 37 °C.

Método:

Mezcla de reacción de restricción 1	
Material	Volumen por muestra (μl)
H ₂ O	16
Tampón de reacción 3.1	3
Enzima Sac I	1
Vector	10
Volumen final de reacción	30 μl/pocillo
Se incubaron los vectores a 37 °C durante 1 hora.	
Mezcla de reacción de restricción 2	
Material	Volumen por muestra (μl)
H ₂ O	8
Tampón de reacción 3.1	1
Enzima Kpn I	1
Mezcla de reacción de restricción 1	30
Volumen final de reacción	40 μl/pocillo
Se incubaron los vectores a 37 °C durante 1 hora.	

Pacientes, materiales y métodos/Patients, materials and methods

Se realizó una primera electroforesis de verificación y tras eso, se purificaron las bandas de interés según se ha descrito en el apartado 6.11.1.2.

6.11.1.5.- Ligación de los insertos XAGA y XGAG con el vector pd2EGFP-1.

Para realizar la ligación de los insertos (XAGA y XGAG) con el vector final pd2EGFP-1, se siguió el procedimiento descrito en el apartado 6.9.1.5.

6.11.2.- Medición de la actividad de los distintos promotores de CD209.

Para realizar los experimentos *in vitro* de expresión de los diferentes haplotipos de *CD209*, se co-transfectaron, mediante electroporación, las células THP-1 y LC5 con los vectores generados: pd2EGFP-1_AGA y pd2EGFP-1_GAG y un plásmido control de β -galactosidasa.

La línea celular THP-1 es una línea derivada de leucemia monocítica aguda; mientras que las LC5 son células derivadas de fibroblastos de pulmón embrionario. La primera línea celular, son células no adherentes y su capacidad de transfección es mucho menor que la segunda línea celular. Las células LC5 son adherentes.

6.11.2.1.- Transfección por electroporación.

Materiales:

- Células THP-1 y LC5.
- Medio RPMI con glutamax de Gibco (Thermo Fisher Scientific, Carlsbad, CA, EE.UU.) completo (500ml de medio RPMI con glutamax al que se le añade 50 ml de suero de vaca fetal descomplementado por calor durante 1 hora a 56 °C y penicilina y estreptomycin a una concentración final de 100 UI/ml de penicilina y 100 µg/ml de estreptomycin).
- Cubetas de electroporación (BIO-RAD, Hercules, CA, EE.UU.).
- Equipo de electroporación, Gene Pulser II Electroporation SystemM (BIO-RAD, Hercules, CA, EE.UU.).

Pacientes, materiales y métodos/Patients, materials and methods

- Medio de electroporación: medio compuesto por 50 % de medio RPMI completo y 50 % de suero de vaca fetal descomplementado.
- Dithiothreitol (Sigma-Aldrich, ST. Louis, MO, EE.UU.).
- Plásmido control de β -Galactosidasa, pVS- β -Galactosidase reporter plasmid (Promega Corporation, Madison, WI, EE.UU.).
- Vectores pd2EGFP-1_AGA y pd2EGFP-1_GAG.

Método:

Condiciones para la electroporación		
	THP-1	LC5
Nº de células transfectadas	5×10^6	10^6
Voltaje	400 V	260 V
Capacitancia	1,075 μ F	1200 μ F
Resistencia	∞	∞
Pd2EGFP-1_AGA/pd2EGFP-1_GAG	2 μ g por cada 10^6	2 μ g por cada 10^6
pVS- β -Galactosidase	0,5 μ g	0,5 μ g

LC5: Se despegaron las células, se contaron y se electroporaron.

THP-1: Se contaron, se centrifugaron y se pusieron en medio RPMI libre de suero bovino descomplementado, con dextrosa a 10 mM y a 0,1 mM. Se electroporaron.

Posteriormente, ambas células se incubaron 20 minutos en medio RPMI completo. Luego se centrifugaron y se pusieron en cultivo con medio RPMI completo.

6.11.2.2.- Medición de la expresión de los promotores de CD209.

La medición de la expresión de los diferentes promotores se realizó mediante citometría de flujo en el equipo Epic Elite XL Flow Cytometer (Beckman Coulter Inc, Brea, CA, EE.UU.).

La preparación de las muestras se realizó siguiendo el protocolo descrito en el apartado 6.9.3.2. El anticuerpo usado fue FICT Mouse Anti-Human CD209 (Becton, Dickinson and Company, Franklin Lakes, NJ, EE.UU.). La dilución realizada de este anticuerpo es de: 1 μ l de anticuerpo

en 999 µl de PBS-BSA 1 %. Se añaden 150 µl de dilución de anticuerpo primario por cada 200.000 células.

También se midió la actividad de la β -galactosidasa y se normalizó los datos obtenidos mediante cuantificación de proteínas. Para realizar este procedimiento se utilizó el protocolo descrito en el apartado 6.10.2.3.

6.12.- Bioestadística y bioinformática.

Los SNPs de los genes reguladores del complemento se seleccionaron en base a dos criterios: por un lado, se descargaron los datos de los genotipos poblacionales de la base de datos Hapmap Database (www.hapmap.org) y se identificaron los Tag SNPs mediante el programa Haploview (www.broad.harvard.edu/haploview/haploview, Broad Institute, Cambridge, MA, EE.UU.); por otro lado, se completó la lista de los SNPs con polimorfismos funcionales (www.ncbi.nlm.nih.gov/omim/).

Para calcular el equilibrio de Hardy-Weinberg y determinar los bloques de ligamiento de los polimorfismos genéticos, se utilizó el programa informático Haploview V4.1. Con el mismo programa, se calcularon los valores de Desequilibrio de Ligamiento de los marcadores genéticos (que son cuantificados a través del coeficiente estandarizado de desequilibrio de Lewontin (D')) considerándose un bloque aquellos pares de marcadores que tuvieran una $D' > 0,8$.

Las frecuencias de los SNPs y de los haplotipos fueron estimadas y comparadas con el programa PLINK V1.7, que se complementó con el gPLINK (<http://pngu.mgh.harvard.edu/Bpurcell/plink/gplink.shtml>). Los test de asociación estadística se corrigieron por múltiple testado mediante “SNP spectral decomposition approach” (Nyholt 2004), que consiste en una corrección del test de Bonferroni en función del grado de desequilibrio de ligamiento de los SNPs. En el estudio del complemento e *IFNL4* este valor se situó en p -valor $< 0,005$ y para el estudio de *CD209* fue de p -valor $> 0,012$.

Para realizar el diseño de cebadores, de sondas y las clonaciones de los vectores y plásmidos, se utilizaron los software: Bioedit V7.2.5 (Tom Hall, Ibis Biosciences, An Abbott Company, Carlsbad, CA, EE.UU.), Beacon

Pacientes, materiales y métodos/Patients, materials and methods

Designer V5.01 (PREMIER Biosoft, Palo Alto, CA, EE.UU.) y SerialCloner V2.5 (Franck Perez, Serial Basics, Instituto Curie, París, Francia).

Para comparar las variables continuas, se utilizó el test de la T de Student y Mann-Whitney U según tuvieran distribución normal o no respectivamente.

Para estos análisis y otros complementarios (medias, desviaciones estándar, intervalos de confianza, errores estadísticos, etc.), se utilizó el programa SPSS V14.0.

7. Resultados/Results

Artículo 1/Research paper 1:

Association of complement receptor 2 polymorphisms with innate resistance to HIV-1 infection.



ORIGINAL ARTICLE

Association of complement receptor 2 polymorphisms with innate resistance to HIV-1 infection

R Herrero¹, LM Real², A Rivero-Juárez³, JA Pineda², Á Camacho³, J Macías², M Laplana⁴, P Konieczny¹, FJ Márquez¹, JC Souto⁵, JM Soria⁵, I Saulle⁶, S Lo Caputo⁷, M Biasin⁶, A Rivero³, J Fibla⁴ and A Caruz¹

HIV-1 induces activation of complement through the classical and lectin pathways. However, the virus incorporates several membrane-bound or soluble regulators of complement activation (RCA) that inactivate complement. HIV-1 can also use the complement receptors (CRs) for complement-mediated antibody-dependent enhancement of infection (C'-ADE). We hypothesize that hypofunctional polymorphisms in RCA or CRs may protect from HIV-1 infection. For this purpose, 139 SNPs located in 19 RCA and CRs genes were genotyped in a population of 201 Spanish HIV-1-exposed seronegative individuals (HESN) and 250 HIV-1-infected patients. Two SNPs were associated with infection susceptibility, rs1567190 in *CR2* (odds ratio (OR) = 2.27, $P = 1 \times 10^{-4}$) and rs2842704 in *C4BPA* (OR = 2.11, $P = 2 \times 10^{-4}$). To replicate this finding, we analyzed a cohort of Italian, sexually HESN individuals. Although not significant ($P = 0.25$, OR = 1.57), similar genotypic proportions were obtained for the *CR2* marker rs1567190. The results of the two association analyses were combined through a random effect meta-analysis, with a significant P -value of 2.6×10^{-5} (OR = 2.07). Furthermore, we found that the protective *CR2* genotype is correlated with lower levels *CR2* mRNA as well as differences in the ratio of the long and short *CR2* isoforms.

Genes and Immunity advance online publication, 8 January 2015; doi:10.1038/gene.2014.71

INTRODUCTION

HIV-1 is able to activate the classical pathway of complement in the absence of antibodies. Such activation depends on the direct binding of C1q envelope protein¹ and the attachment of C3b on the viral membrane. This fact is enhanced in the presence of specific anti-HIV-1 antibodies.² In addition, HIV-1 interacts with mannose binding lectin through direct binding to gp120, triggering the activation of the complement lectin pathway.³ However, the virus appears to be resistant to complement.^{4,5} The biological model to explain this fact is based on the active viral exploitation of the regulators of complement activation (RCA) protein family. The exploitation of RCA proteins by several families of viruses is a clear example of adaptive convergence.⁶ In the case of HIV-1, the absorption of membrane-bound RCA proteins in virions has been demonstrated.^{4,7,8} These proteins enhance virus resistance to complement-mediated lysis.^{4,9,10} This incorporation contributes to the survival of HIV-1 in blood or other tissues as both virions and infected cells are resistant to neutralizing antibodies.¹⁰ Other soluble proteins such as Complement Factor H (CFH) interacts with viral envelope.¹¹ Depletion of CFH in human serum increased destruction of cells infected by HIV-1 and free virus *in vitro*.^{11,12} Similarly, viruses produced in cells defective for any of the RCA proteins are extremely sensitive to killing by serum in the presence or absence of neutralizing antibodies. Interestingly, *Streptococcus* spp. has developed a similar strategy of resistance to serum by addition of CFH to its surface by the M protein.¹³ Both complement and the RCA genes are extremely

polymorphic, and in some cases, a functional effect of the polymorphisms has been found.¹⁴

Complement-coated virions are concentrated in several tissues; especially the lymphatic system and can interact with complement receptors (CRs) that bind C3 fragments. Although viral inactivation through opsonization and lysis was observed,¹⁵ CRs expression in T/B lymphocytes or erythrocytes can also boost viral infectivity in a process known as complement-mediated antibody-dependent enhancement of infection (C'-ADE).¹⁶ The CRs implicated in C'-ADE include CR1, CR2 and CR3. Both *cis* and *trans* C'-ADE were observed on cell lines as well as on primary cells and with primary isolates of HIV-1.¹⁷

Regarding above interactions of HIV-1 with RCA and CRs, we hypothesized that individuals carrying polymorphisms that reduce the functional capacity of the CRs or RCA proteins or their site of interaction with gp120/gp41 could display a natural resistance to HIV-1 infection mediated by an increased probability of virolysis mediated by full complement activation or lower complement-mediated enhancement of infection. To address this hypothesis, we have performed a genetic association analysis as well as a phenotypic characterization of RCA and CRs in two independent cohorts of HIV-1-exposed seronegative (HESN) and HIV-1-positive individuals.

RESULTS

Genotyping statistics

Four SNPs did not pass the genotyping quality controls of Illumina Veracode Golden Gate protocol rs7084554 (*MBL2*), rs12406509

¹Immunogenetics Unit, Department of Experimental Biology, University of Jaen, Jaen, Spain; ²Infectious Diseases and Microbiology Clinical Unit, Valme Hospital, Seville, Spain; ³Maimonides Institute for Research in Biomedicine of Cordoba (IMIBIC)/Reina Sofia University Hospital, Cordoba, Spain; ⁴Human Genetics Unit, Department of Basic Medical Sciences, University of Lleida IRBLleida, Lleida, Catalonia, Spain; ⁵Institut d'Investigació Biomèdica Sant Pau (IIB-Sant Pau), Hospital de la Santa Creu i de Sant Pau, Barcelona, Spain; ⁶Department of Biomedical and Clinical Sciences, University of Milan, Milan, Italy and ⁷Maria Annunziata Hospital, Florence, Italy. Correspondence: Dr A Caruz, Immunogenetics Unit, University of Jaen, Campus Las Lagunillas SN, Edificio B3 344, Jaén, 23071, Spain. E-mail: caruz@ujaen.es

Received 5 June 2014; revised 10 November 2014; accepted 12 November 2014

(*CFHR4*), rs7046 (*CD59*) and rs9837104. Additionally, we found significant deviation from Hardy-Weinberg equilibrium ($P=1.3 \times 10^{-5}$) in the marker rs9331949 (*CLU*). Mean genotyping call rate for the remaining SNPs was 99.6%.

Single SNP association analyses

We selected 143 polymorphic markers (Table 1) within *RCA* and *CRs* genes for susceptibility to HIV-1 association in the injection drug use (IDU)-Spain cohort. Two intronic SNPs positioned in the same region of the chromosome 1 (Figure 1) showed experiment-wide significant allelic or genotypic association with resistance to HIV-1 infection. The G allele of rs2842704 in *C4BPA* was associated with decreased risk of infection under dominant model ($P=2.1 \times 10^{-4}$; odds ratio (OR) = 0.47 (0.31–0.70)). Subjects carrying GG+AG genotypes were significantly over-represented in HESN group (43%) compared with HIV-1-infected one (26%) (Tables 2A and 2B). Regarding *CR2* gene, rs1567190-T allele was associated with increased risk of HIV infection under a dominant model ($P=1.9 \times 10^{-4}$; OR = 2.27 (1.46–3.51)) (Tables 2A and 2B). Subjects carrying TT+CT genotypes were significantly over-represented in the HIV-1-positive group (81%) compared with HESN (65.5%) (Tables 2A and 2B). Three other SNPs showed a trend to association with HIV-1 infection protection but did not reach statistical significance after correction for multiple testing; rs419137 in *CFH* ($P=6 \times 10^{-3}$), rs831618 in *CD59* ($P=7 \times 10^{-3}$) and rs6690037 in *C4BPB* ($P=8 \times 10^{-3}$) (Supplementary Table 1).

Additionally, to replicate our previous findings, rs2842704 in *C4BPA* and rs1567190 in *CR2* were genotyped in a second population (Sexual-Italy) exposed to the virus through sexual route. Both genetic markers were consistent with Hardy-Weinberg proportions. Genotype distribution of rs2842704 marker differs to that observed in the IDU-Spain sample therefore not supporting the observed association for this SNP in the IDU-Spain cohort (Tables 2A and 2B).

Similarly to what was observed in the Sexual-Italy sample, *CR2* rs1567190 TT+TC genotype was over-represented in HIV-1 (77.5%) compared with HESN (68.5%), although it did not reach statistical significance ($P=0.25$; OR = 1.57 (0.7–3.4)) (Tables 2A and 2B). The results of the two association analyses were combined through a random effect meta-analysis, with a significant P -value of 2.6×10^{-5} (Tables 2A and 2B). These observations suggest that the rs1567190-T allele confers risk to HIV-1 infection with a dominant effect, independently of the infection route.

Haplotype association analysis in Spanish intravenous drug users A haplotype-based association test was performed by multimer regression analysis using PLINK and Haploview software. Haplotype frequencies were estimated and compared between the two groups of the IDU-Spain cohort. This analysis identified three haplotypes associated with HIV-1 resistance with a significant multiple-testing P -value $< 5 \times 10^{-3}$. Haplotypes from *CFH*, *C4BPA* and *CR2* genes included SNPs previously identified as associated in the univariate analysis (Table 3). Using PLINK software, we calculated the chromosome phases for HESN and HIV groups and performed haplotype copy number calculation in HIV-1-positive and HESN individuals. The presence of two copies of the CGGGG haplotype encompassing the promoter, and the first 12 exons of *CR2* (rs1567190, rs1507764, rs1048971, rs17615 and rs4317805) showed the strongest association with protection under a recessive model ($P=4.2 \times 10^{-5}$) (Table 3). The other two haplotypes associated with innate resistance to HIV-1 infection followed a dominant model and included the presence of one or two copies of the haplotype GCT (rs2842704, rs4425986 and rs9943077) located in the fourth intron of *C4BPA* ($P=1.1 \times 10^{-3}$) (Table 3), as well as the haplotype AAAGTGGC (rs2019727, rs6695321, rs10737680, rs419137, rs395544, rs2284664,

rs11582939, rs16840658) that spans from the intron 9 of *CFH* to the 5' end of *CFHR1* gene (Table 3).

Sequencing of *C4BPA*, *CD55*, *CR2* and *CR1* chromosome region

To identify new functional SNPs linked with the protective genotypes in *CR2* and *C4BPA* in the candidate region and perform a sequence haplotyping, a mean of 900 000 bp were sequenced confidently with a $30 \times$ mean coverage. The target region included *C4BPB*, *C4BPA*, *CD55*, *CR2*, *CR1* and *CR1L* genes (207,224,455–207,740,299; GRCh37/hg19). Five pools of HIV-1-positive homozygous for the risk (T) allele of the SNP rs1567190 and three pools of HESN patients homozygous for the protective (C) allele of the SNP rs1567190 were sequenced. The main statistics for pool sequencing can be found in Supplementary Table 2. We observed a perfect match between the two genotyping systems Veracode Golden Gate and Next Generation Sequencing. The individuals homozygous for the C allele of rs1567190 are also homozygous for 52 polymorphisms spanning 45 Kb of the *CR2* gene (Figure 2). In particular, these individuals are homozygous for the coding SNPs rs1048971, rs17615, rs4308977 and rs17616 (G-G-T-G). On the contrary, individuals homozygous for the risk allele of rs1567190 displayed a higher level of heterozygosity along the *CR2* gene with only 11 homozygous polymorphisms and only 1 of them (rs17258982) located in the *CR2* coding region (Figure 2). No new coding SNPs were detected in the region.

CR2 and *C4BPA* phenotyping

Next, we validated whether the observed effect could be associated with differential expression of *CR2* mRNA in peripheral blood mononuclear cells (PBMCs) from healthy donors. Nine rs1567190-CC and nine rs1567190-TT homozygous samples were examined by Taqman real-time quantitative PCR. The relative amount of the *CR2* expression of different genetic backgrounds was normalized to *CD19* (a specific B-lymphocyte marker). We found a lower level of expression of the protective genotype CC (mean = 0.89 ± 0.34) compared with the risk genotype TT (mean = 1.36 ± 0.38 ; $P=0.029$, student T -test, Figure 3). These data suggest that the global level of *CR2* expression is increased in B-lymphocytes from rs1567190 TT carriers. These data represent the mean of two independent experiments performed in triplicate. The levels of the short and long *CR2* mRNA splicing isoforms were also quantified by real-time PCR in both genetic backgrounds. The *CD19*-normalized short *CR2* isoform expression was significantly reduced in PBMCs from homozygous CC individuals compared with TT (mean = 0.37 ± 0.04 vs 1.25 ± 0.89 ; $P=0.02$, student T -test, Figure 3). Finally, we have found that the expression of long *CR2* isoform in rs1567190-CC carriers is higher compared with the other genotype (mean = 5.0 ± 3 vs 0.21 ± 0.23 ; $P=0.0026$, student T -test, Figure 3).

DISCUSSION

In this hypothesis-driven, low-scale, candidate gene association study, we have genotyped 139 SNPs in 19 genes related to complement regulatory proteins or CRs. Our discovery population is highly uniform: all are male, white Caucasians of Spanish ancestry, Hepatitis C virus-positive and intravenous drug users; including 201 HESN and 250 HIV-1-positive. We found two SNPs that are significantly associated after Bonferroni's correction with resistance to HIV-1 infection; rs1567190 located in *CR2* and rs2842704 in *C4BPA*. In the replication cohorts from Italy, we did not detect significant association probably because of the small size and lack of power. However, similar proportions in rs1567190 *CR2* genotypes were observed in this population compared with the Spanish one; HESN subjects are more likely to be homozygous for the C allele than HIV-1-infected patients. Consequently,

Table 1. Chromosomal location and genomic characteristics of selected markers

SNP	Chr	Coordinate	Gene	Location	Coding status
rs17573790	1	194876018	CFH	5UTR	NS
rs3753394	1	194887539		5UTR	
rs7524776	1	194889959		intron	
rs800292	1	194908855		coding	
rs12405238	1	194928235		intron	
rs10922096	1	194929081		intron	
rs4658046	1	194937379		intron	
rs2019727	1	194941336		intron	
rs6695321	1	194942483		intron	
rs10737680	1	194946077		intron	
rs419137	1	194963497		intron	S
rs395544	1	194964894		intron	
rs2284664	1	194969147		intron	
rs11582939	1	194976779		intron	
rs16840658	1	195092250	CFHR1	3UTR	
rs12406509	1	195140814	CFHR2	5UTR	
rs1409153	1	195146627		5UTR	
rs10494745	1	195154079		5UTR	
rs7538501	1	195168375		5UTR	
rs12723250	1	195196716		3UTR	
rs6428379	1	195204158	CFHR5	5UTR	NS
rs6675769	1	195208283		5UTR	
rs9427657	1	195209943		5UTR	
rs12116643	1	195239805		intron	
rs6663083	1	195247282		3UTR	
rs7555070	1	195248776		3UTR	
rs2336597	1	195259961		3UTR	
rs6673422	1	205320109	C4BPB	3UTR	
rs2842754	1	205323925		3UTR	
rs6690037	1	205330451		intron	
rs11120211	1	205343001	C4BPA	5UTR	S
rs2808470	1	205353809		intron	
rs17020993	1	205355014		intron	
rs2842704	1	205356058		intron	
rs4425986	1	205360402		intron	
rs9943077	1	205361920		intron	
rs1126618	1	205364302		coding	
rs2491393	1	205366881		intron	
rs4571969	1	205372210		intron	
rs11120277	1	205386192		3UTR	
rs2782842	1	205550198	CD55	5UTR	NS
rs2564978	1	205561038		5UTR	
rs1507765	1	205601868		3UTR	
rs17044576	1	205701292	CR2	intron	
rs1567190	1	205702411		intron	
rs1507764	1	205708917		intron	
rs1048971	1	205712944		coding	
rs17615	1	205713084		coding	
rs7549152	1	205717560		intron	
rs4317805	1	205719614		intron	
rs12032512	1	205719800		intron	NS
rs6690215	1	205722672		intron	
rs12021671	1	205724767		intron	
rs2182913	1	205727000		intron	
rs7519408	1	205727911		intron	
rs4618971	1	205732683		3UTR	
rs1410408	1	205732930		3UTR	
rs11117913	1	205734857	CR1	5UTR	
rs1571344	1	205737550		intron	
rs4844597	1	205737891		intron	
rs2025935	1	205742277		intron	NS
rs677066	1	205840613		intron	
rs6701713	1	205852911		intron	
rs10429953	1	205855200		intron	
rs11576522	1	205855891		intron	
rs12734030	1	205860586		intron	
rs12034383	1	205870217		intron	
rs17048254	1	205875072		intron	
rs10779339	1	205881073		3UTR	
rs6696840	1	205886351	CR1L	intron	
rs12080578	1	205887234		intron	NS
rs1323720	1	205889783		intron	
rs2796265	1	205987410	CD46	5UTR	

Table 1. (Continued)

SNP	Chr	Coordinate	Gene	Location	Coding status
rs2796266	1	205989567		5UTR	NS
rs2796267	1	205991528		5UTR	
rs2796269	1	206003508		intron	
rs7545126	1	206004858		intron	
rs2796278	1	206022445		intron	
rs7541230	1	206024177		intron	
rs14374	1	206034879		3UTR	
rs2724377	1	206041440		3UTR	
rs761277	1	206059633		5UTR	
rs7512286	1	206059724		5UTR	
rs2235768	1	206059961		5UTR	S
rs9683303	3	95071253	PROS1	3UTR	
rs6123	3	95075808		coding	
rs8178610	3	95129085		intron	
rs8178607	3	95136681		intron	
rs13062355	3	95171200		intron	
rs7439493	4	110876178	CFI	3UTR	
rs10033900	4	110878515		3UTR	
rs11726949	4	110884078		intron	
rs13104777	4	110884929		intron	
rs6822976	4	110885740		intron	NS
rs9998151	4	110886793		intron	
rs4541508	4	110901935		intron	
rs11929767	4	110912983		intron	
rs4698788	4	110938982		intron	
rs7675460	4	110940036		intron	
rs884711	4	110945770		5UTR	
rs4698792	4	110948752		5UTR	
rs10503814	8	27510491	CLU	3UTR	
rs9331949	8	27510602		3UTR	NS
rs9331942	8	27511030		3UTR	
rs3087554	8	27511358		3UTR	
rs9331930	8	27514210		intron	
rs9331926	8	27517011		intron	
rs9331908	8	27519534		intron	
rs1532278	8	27522231		intron	
rs9314349	8	27530120		5UTR	
rs569205	8	27532937		5UTR	
rs492638	8	27535145		5UTR	
rs499046	8	27538788		5UTR	NS
rs1493521	8	27539185		5UTR	
rs10824792	10	54196211	MBL2	3UTR	
rs11595876	10	54196767		3UTR	
rs1838065	10	54199262		intron	
rs1982266	10	54200641		intron	
rs1800450	10	54201240		coding	
rs7096206	10	54201690		5UTR	
rs7084554	10	54201818		5UTR	
rs11003125	10	54202019		5UTR	
rs7046	11	33683555	CD59	3UTR	NS
rs831618	11	33685189		3UTR	
rs11032350	11	33686635		3UTR	
rs12272807	11	33698323		intron	
rs704700	11	33700109		intron	
rs831627	11	33702622		intron	
rs831626	11	33702925		intron	
rs17760694	11	33713667		intron	
rs2273121	11	33714345		intron	
rs11032362	11	33715667		5UTR	
rs28362934	11	57120118	SERPING1	5UTR	NS
rs11603020	11	57130907		intron	
rs2508443	11	57134567		intron	
rs2511988	11	57135745		intron	
rs2072449	12	8101903	C3AR1	3UTR	
rs11567785	12	8109919		3UTR	
rs11567782	12	8111186		5UTR	
rs17728664	12	8115730		5UTR	
rs7253663	19	52500667	C5AR1	5UTR	
rs4427917	19	52507554		intron	
rs4577202	19	52507761		intron	

Abbreviations: NS, non-synonymous; S, synonymous; SNP, single nucleotide polymorphism; UTR, untranslated region.

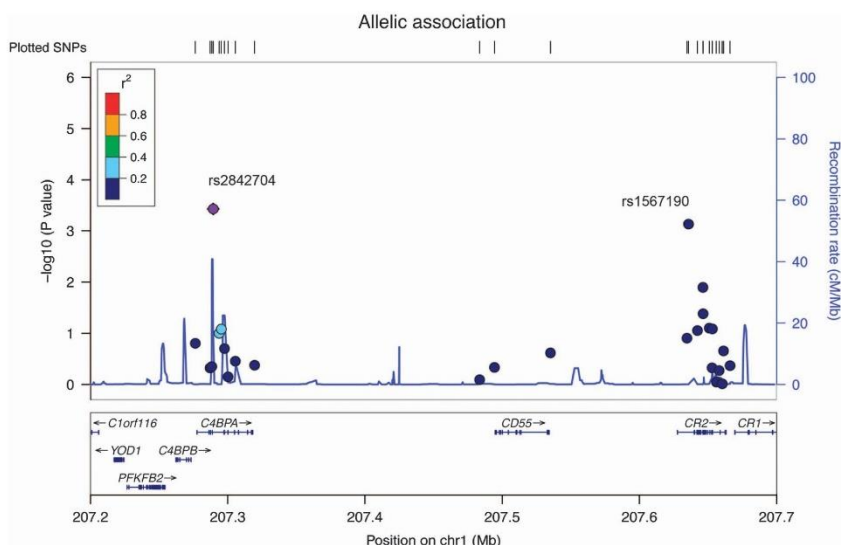


Figure 1. Scatter plot of genetic association results. Negative log₁₀-transformed *P*-values are plotted against physical position in the chromosome 1. SNPs rs2842704 rs1567190 are statistically significant after Bonferroni's correction (Fisher's exact test).

although statistical analysis was significant for the Spanish cohort only, the meta-analysis that we performed revealed no heterogeneity in the effect of rs1567190 among the two populations and yielded a robust significant association.

This study has several limitations; first, the list of genes related to complement system is not exhaustive. Second, our phenotypic analysis is limited to mRNA expression, and third, the replication cohort from Italy is significantly smaller than the Spanish one.

The *CR2* gene encodes a membrane protein also known as CD21, which functions as a receptor for C3d/iC3b among other molecules.^{18–20} *CR2* gene contains 18 exons and there exist two main isoforms that result from alternative splicing of exon 11: *CR2-L* and *CR2-S*. The first form is expressed mainly in follicular dendritic cells and contains 16 short consensus repeats; the second isoform contains 15 short consensus repeats and is expressed mainly in B-lymphocytes. The specific functions of each isoform as well as its binding capacity to the natural ligands are unknown.

CR2 function can be subverted by some viruses that use antibodies and/or complement for a highly efficient entry into target cells in a process known as C'-ADE of viral infection. Although C'-ADE is not a well-established factor in acquisition of lentiviruses infections in either humans or animals, two types of ADE have been suggested in the context of HIV-1. The first type of ADE is dependent on the interaction between antibody and FcγRIIIa. Polymorphisms in this gene were associated with outcomes in preventive HIV-1 vaccination trial.²¹ Second, complement alone can boost infection of dendritic cells as well as monocytes/macrophages and antibodies can augment this process by a more efficient recruitment of complement on the viral surface. This mechanism is dependent on the *CR2*/*CR1* expression in the target cells^{17,22} and probably is mediated by an increased adhesion of the virus-antibody-complement (C3d) complexes through binding with *CR2* on the target surface.^{23,24}

The protective SNP identified in this study belongs to an extended haplotype that displays strong linkage disequilibrium and includes other 52 genetic polymorphisms that span 45 Kb

from the promoter to 3' downstream region. The protective *CR2* haplotype displays lower levels of total *CR2* mRNA as well as reduced short isoform expression. In view of the data about the role of *CR2* in C'-ADE, we hypothesize that the ratio of short/long *CR2* isoform expression and/or the global *CR2* level may contribute to the likelihood of HIV-1 acquisition through the parenteral as well as sexual routes. ADE is an important factor affecting other viruses as Dengue, Ross River or Ebola;¹⁶ we conjecture that the *CR2* haplotypes could have also an influence in the susceptibility to these viruses. Although the main SNP identified in this study has not been previously related with any pathology, the protective haplotype has been associated with susceptibility to systemic lupus erythematosus.^{25,26}

C4BPA gene encodes a plasma protein that forms an oligomer complex with *C4BPB*, producing three isoforms. *C4BP* is a plasmid regulator of the classical and alternative complement pathways. Flaviviruses actively recruit *C4BP* through the NS1 protein, reducing the functional capacity of C4 and complement activation.²⁷ Bacterial infections such as *Streptococcus*,²⁸ *Yersinia*,²⁹ *Lepstospira*³⁰ or *Neisseria*³¹ among others have also developed active protein systems to capture *C4BP* in the surface to escape complement-mediated lysis. The observed association of *C4BPA* haplotypes with HIV-1 resistance in HESN at risk through the parenteral route suggests that this gene could be a candidate for further functional phenotypic characterization.

Several observations may underlie the absence of association of *C4BPA* marker in the Italian cohort. First, this cohort is mainly constituted by women³² and the Spanish one is exclusively constituted by males. There are significant gender differences in the innate and adaptive immune responses, the pathogenesis of infectious diseases, response to vaccines and the prevalence of autoimmune diseases.^{33,34} In the case of *C4BP*, the synthesis appears to be influenced by sex hormones.^{35–37}

CFH regulates the function of the alternative complement pathway in fluid phase and on cellular surfaces. SNPs in the *CFH* region that appears to be related with HIV-1 resistance at the haplotypic level were previously associated with several

Cohort	Genotype counts rs2842704_C48PA GG/AG/AA (%)				Genotype counts (dominant) rs2842704_C48PA GG+AG/AA (%)			
	HESN	HIV+	Genotypic P-value	Combined P-value	HESN	HIV+	Dominant P-value	OR (95% CI)
IDU-Spain	7/79/114 (3.5/39.5/57)	5/58/177 (2/24/74)	1 × 10 ⁻³	ND	86/114 (43/57)	63/177 (26/74)	2.1 × 10 ⁻⁴	0.47 (0.3–0.7)
Sexual-Italy	4/23/43 (5.7/32.8/61.4)	3/24/31 (5.1/41.3/53.4)	0.36		27/43 (38.5/61.4)	27/31 (46.5/53.5)	0.36	1.38 (0.6–2.8)
								ND

Abbreviations: CI, confidence interval; HESN, HIV-1-exposed seronegative; ND, Not determined (because of opposite effects in the two cohorts); OR, odds ratio. Underlined: % of allele or genotypes with significant differences between HESN and HIV+ cohorts.

Cohort	Genotype counts rs1567190 CR2 TT/CT/CC (%)				Genotype counts (dominant) rs1567190 CR2 TT+CT/CC (%)			
	HESN	HIV+	Genotypic P-value	Combined P-value	HESN	HIV+	Dominant P-value	OR (95% CI)
IDU-Spain	35/96/69 (17.5/48/34.5)	59/135/45 (24.5/56.5/19)	7 × 10 ⁻⁴	1.8 × 10 ⁻⁴	131/69 (65.5/34.5)	194/45 (81/19)	1.9 × 10 ⁻⁴	2.27 (1.4–3.5)
Sexual-Italy	14/34/22 (20/48.5/31.5)	9/39/14 (14.5/63/22.5)	0.26		48/22 (68.5/31.5)	48/14 (77.5/22.5)	0.25	1.57 (0.7–3.4)
								2.07

Abbreviations: CI, confidence interval; HESN, HIV-1-exposed seronegative; ND, Not determined (because of opposite effects in the two cohorts); OR, odds ratio. Underlined: % of allele or genotypes with significant differences between HESN and HIV+ cohorts.

Table 3. Haplotype distribution of selected markers at *CFH*, *C4BPA* and *CR2* genes in Spanish cohorts

GENE	SNPs in the haplotype	Haplotypes	Freq HIV-1, HESN	Freq n° copies HIV-1, HESN	Fisher P-value	OR (CI 95%)
<i>CFH</i>	rs2019727	AAAATGCG	0.281, 0.283	0.09, 0.20^a	$P = 1.4 \times 10^{-3}$	0.38 (0.2–0.6)
	rs6695321	AGCACACG	0.202, 0.222			
	rs10737680	TACACGCG	0.215, 0.172			
	rs419137	AGAACGTA	0.154, 0.167			
	rs395544	AAACTGCG	0.054, 0.110			
	rs2284664	AGAACGTG	0.052, 0.038			
	rs11582939					
	rs16840658 (CFHR1)					
<i>C4BPA</i>	rs2842704	ATC	0.463, 0.407	0.27, 0.46^a	$P = 1.1 \times 10^{-3}$	0.51 (0.3–0.7)
	rs4425986	ACT	0.250, 0.225			
	rs9943077	GCT	0.133, 0.222			
		ACC	0.142, 0.136			
<i>CR2</i>	rs1567190	CGGGG	0.464, 0.585	0.18, 0.35^b	$P = 4.2 \times 10^{-5}$	0.42 (0.2–0.6)
	rs1507764	TAAAG	0.227, 0.190			
	rs1048971	TGAGG	0.103, 0.080			
	rs17615	TGGGA	0.092, 0.080			
	rs4317805	TGAAG	0.050, 0.035			
		TGGGG	0.049, 0.030			

Abbreviations: CI, confidence interval; HESN, HIV-1-exposed seronegative; OR, odds ratio; SNP, single nucleotide polymorphism. Associated haplotypes are in bold. ^aIndividuals harboring one or two copies of the protective haplotype (haplotype dominant model). ^bIndividuals harboring two copies of the protective haplotype (haplotype recessive model).

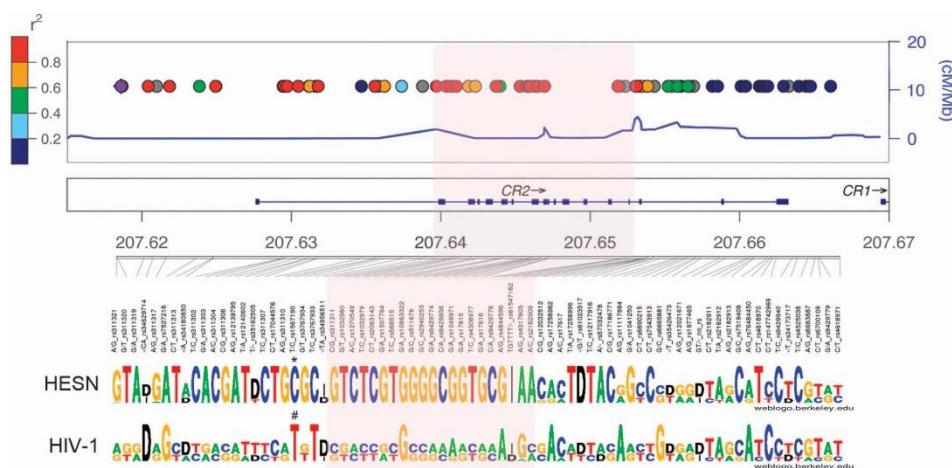


Figure 2. Haplotypes inferred from genomic sequence of chromosome 1 region (207.61 kb to 207.66) in DNA pools from 33 HESN and 46 HIV-1-infected patients, selected as homozygous at rs1567190_C protective allele (labelled as *) and rs1567190_T risk allele (labelled as #), respectively. On top, decorated genomic region including CR2 gene structure, recombination frequency (black line, cM/Mb) and linkage disequilibrium (greys scheme circles, r²). Shaded region corresponds to the homozygous track on HESN subjects that expand exon 2 to exon 12 of the CR2 gene. (D = deletion allele, I = insertion allele).

autoimmune diseases;¹⁴ as well as susceptibility to meningitis³⁸ and Dengue.³⁹ High levels of CFH in human serum can protect cells infected by HIV-1 and free virus *in vitro*.¹² We hypothesize that the protective haplotype of *CFH* may be related to lower levels of CFH expression by the liver or altered splicing efficacy.

The inhibition of membrane-bound RCA has been proposed as new therapeutic target against HIV-1. In the case of CD59,⁴⁰ the presence of specific inhibitors of this protein renders HIV-1 virions highly sensitive to complement-mediated lysis activated by antibodies. The results presented here, if confirmed, could

lead to developm a similar approach to combat the viral infection by the means of development of new antagonists of CR2, C4BPA or CFH.

MATERIALS AND METHODS

Patients and controls

We recruited 451 Hepatitis C virus-seropositive white males exposed to HIV-1 by IDU (IDU-Spain) that had shared needles for > 3 months. Of the subjects, 250 were HIV-1-seropositive and 201 HIV-1-seronegative (HESN).

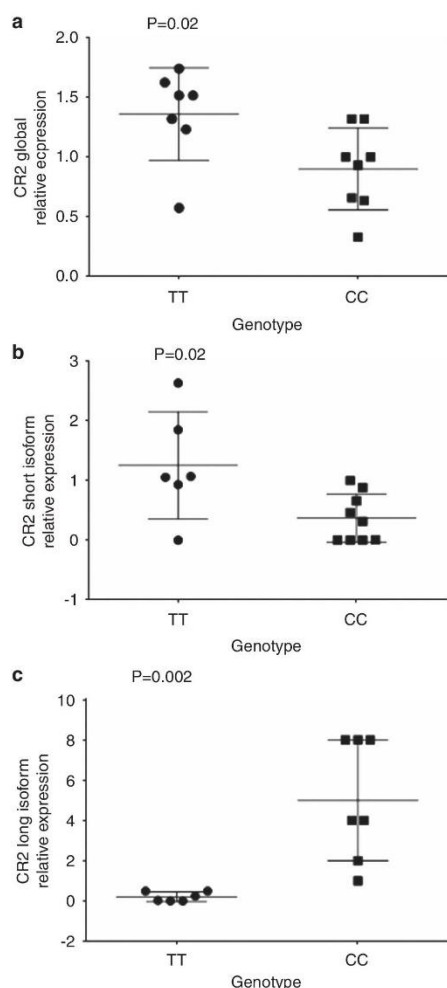


Figure 3. CR2 mRNA expression normalized by CD19 according to the genotype for rs1567190. (a) Global CR2 expression levels ($P=0.02$). (b) Short CR2 isoform ($P=0.02$) and (c) Long CR2 isoform ($P=0.002$) (student *t*-test).

This cohort was enrolled in prospective studies in Spain and their epidemiological and clinical characteristics were described in previous studies. Additionally, a second population exposed to HIV-1 infection by sexual route, all of whom are part of a serodiscordant heterosexual couple that had unprotected sexual events, was analyzed as replication population; both HIV-1-infected (62 subjects) and HESN (80 subjects). This cohort was enrolled in prospective studies in Italy (Sexual-Italy). The main characteristics of this population were previously described.³²

Ethical aspects

The study was designed and performed according to the Helsinki declaration and was approved by the Human Subject Institutional Review Boards to the Ethics Committee of the participating hospitals. The University of Jaen's Bioethics Institutional Review Board approved the

transfer and use of materials for this research. All patients and donors provided written informed consent to participate in this study.

Polymorphisms selection and genotyping

SNPs from complement regulatory genes (Table 1) were selected according to two criteria. First, genotype data from Hapmap-CEU population were directly downloaded from the Hapmap database (www.hapmap.org) and the Tag SNPs were identified using Haploview's V4.1 software tagger option (<https://www.broad.harvard.edu/haploview/haploview>, Broad Institute, Cambridge, MA, USA). Second, SNPs list were complemented with potentially functional polymorphisms previously associated or not with disease (<http://www.ncbi.nlm.nih.gov/omim/>). DNA was extracted from fresh PBMC or frozen whole blood using the Quick Pure Blood DNA extraction Kit (Macherey-Nagel, Düren, Germany) or Magna Pure system (Roche, Basel, Switzerland). The SNPs were genotyped using a custom Veracode Golden Gate genotyping assay (Illumina, San Diego, California, USA). Original genotyping data are available upon request.

Sequencing of C4BPA, CD55, CR2 and CR1 chromosome region

DNAs from 33 HESN individuals, homozygous CC for the rs1567190 SNP associated with the resistant phenotype, were mixed in 3 independent pools. In parallel, DNA from 46 HIV positive individuals homozygous TT associated with the risk phenotype were mixed in 5 independent pools. Sequencing of each pool DNA was done by custom capture and Next Generation Sequencing using Illumina HiSeq2000 sequencer (Illumina, San Diego, CA, USA). The resequencing strategy was focused on 515,844 bp from chromosome 1 region including the genes *C4BPA*, *CD55*, *CR2* and *CR1* (coordinates from 207,224,455 bp to 207,740,299 bp, NCBI buildGRCh37/hg19). Raw data generated were analyzed using the SNP discovery tool of DNAnexus cloud computing software (www.dnanexus.com, Mountain View, CA, USA). We calculated a score for each polymorphic position identified (frequency of reads equal to the consensus sequence/number of total reads). The original sequencing data are available upon request. Sequence variations were plotted by WebLogo facilities (<http://weblogo.berkeley.edu/>, University of California, Berkeley, CA, USA) according to allele information content.

Biostatistics and bioinformatics

Hardy-Weinberg equilibrium and pairwise linkage disequilibrium (D') were calculated using Haploview V4.1 software. Block structure was considered for marker pairs showing $D' > 0.8$, following the solid-spine block definition implemented in Haploview. Single marker and haplotype frequencies were estimated and compared using PLINK software. Complementary analysis was performed by use of SPSS software (version 14.0, IBM Corporation, Armonk, NY, USA). Single-marker association P -values were corrected for multiple testing following the SNP spectral decomposition approach,⁴¹ experiment-wide significance threshold required to keep type I error rate at 5% was 5×10^{-4} . Haplotype association tests were adjusted using Bonferroni's correction for multiple testing by dividing the significance level (0.05) by the number of major haplotypes (that is, $> 5\%$), a P -value $< 5 \times 10^{-3}$ was considered statistically significant.

CR2 mRNA quantification

PBMCs from healthy blood donors were purified by centrifugation in Ficoll gradient, DNA was extracted as previously described and genotyped for the SNPs rs1567190 of *CR2* with Taqman probes. Nine individuals harboring CC genotype and nine with TT genotype for *CR2* were randomly selected for gene expression analyses. Then, from each sample, total RNA from 5×10^6 PBMCs was isolated using Tripure reagent (Roche), quantified by spectrophotometry and dissolved at $1 \mu\text{g } \mu\text{L}^{-1}$. Reverse transcription of RNA was performed using the Superscript III (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. All the RNA samples were also subjected to control reactions without reverse transcriptase to check for contamination. The normalized *CR2* expression was determined in a multiplexed, one tube PCR reaction on a MX3005P thermocycler (Stratagene, La Jolla, CA, USA). *CR2* transcript levels were quantified using the Applied Biosystems Assay Hs00153398_m1 (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions. A CD19 assay was used as endogenous control (Hs01047410_g1, Applied Biosystems). Each donor cDNA was amplified in triplicate wells and repeated in two independent days. At the same time, negative control

experiments were done with reverse transcriptase-negative cDNAs. The relative expression of *CR2* mRNAs compared with endogenous *CD19* (*CR2*) were calculated with MX-pro-V4.0 software (Stratagene). The Comparative quantitation of short and long isoform of *CR2* transcripts was done as previously described.²⁵

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by Spanish Health Ministry [PI021476, PI051778 and PI10/01232 to JF, JAP and ACa]; Instituto de Salud Carlos III-RETIC (RD06/006 to JAP); Fundació Marató TV3 [020730 and 020732 to JF and ACa]; Junta de Andalucía [PI-0335/2009 to ACa]; Fundación Progreso y Salud de la Consejería de Salud de la Junta de Andalucía [AI-0021 to JAP]; and Universidad de Jaén [UJA2013/10/03 to ACa].

REFERENCES

- Thielens NM, Tacnet-Delorme P, Arlaud GJ. Interaction of C1q and mannan-binding lectin with viruses. *Immunobiology* 2002; **205**: 563–574.
- Prohaszka Z, Hidvegi T, Ujhelyi E, Stoiber H, Dierich MP, Susal C et al. Interaction of complement and specific antibodies with the external glycoprotein 120 of HIV-1. *Immunology* 1995; **85**: 184–189.
- Ji X, Gewurz H, Spear GT. Mannose binding lectin (MBL) and HIV. *Mol Immunol* 2005; **42**: 145–152.
- Stoiber H, Clivio A, Dierich MP. Role of complement in HIV infection. *Annu Rev Immunol* 1997; **15**: 649–674.
- Banki Z, Stoiber H, Dierich MP. HIV and human complement: inefficient virolysis and effective adherence. *Immunol Lett* 2005; **97**: 209–214.
- Tortorella D, Gewurz BE, Furman MH, Schust DJ, Ploegh HL. Viral subversion of the immune system. *Annu Rev Immunol* 2000; **18**: 861–926.
- Saifuddin M, Hedayati T, Atkinson JP, Holguin MH, Parker CJ, Spear GT. Human immunodeficiency virus type 1 incorporates both glycosyl phosphatidylinositol-anchored CD55 and CD59 and integral membrane CD46 at levels that protect from complement-mediated destruction. *J Gen Virol* 1997; **78** (Pt 8): 1907–1911.
- Montefiori DC, Cornell RJ, Zhou JY, Zhou JT, Hirsch VM, Johnson PR. Complement control proteins, CD46, CD55, and CD59, as common surface constituents of human and simian immunodeficiency viruses and possible targets for vaccine protection. *Virology* 1994; **205**: 82–92.
- Schmitz J, Zimmer JP, Kluxen B, Aries S, Bogel M, Gigli I et al. Antibody-dependent complement-mediated cytotoxicity in sera from patients with HIV-1 infection is controlled by CD55 and CD59. *J Clin Invest* 1995; **96**: 1520–1526.
- Saifuddin M, Parker CJ, Peeples ME, Gorny MK, Zolla-Pazner S, Ghassemi M et al. Role of virion-associated glycosylphosphatidylinositol-linked proteins CD55 and CD59 in complement resistance of cell line-derived and primary isolates of HIV-1. *J Exp Med* 1995; **182**: 501–509.
- Stoiber H, Schneider R, Janatova J, Dierich MP. Human complement proteins C3b, C4b, factor H and properdin react with specific sites in gp120 and gp41, the envelope proteins of HIV-1. *Immunobiology* 1995; **193**: 98–113.
- Stoiber H, Pinter C, Siccardi AG, Clivio A, Dierich MP. Efficient destruction of human immunodeficiency virus in human serum by inhibiting the protective action of complement factor H and decay accelerating factor (DAF, CD55). *J Exp Med* 1996; **183**: 307–310.
- Haapasalo K, Vuopio J, Syrjänen J, Suvihehto J, Massinen S, Karpellin M et al. Acquisition of complement factor H is important for pathogenesis of Streptococcus pyogenes infections: evidence from bacterial *in vitro* survival and human genetic association. *J Immunol* 2012; **188**: 426–435.
- Rodríguez de Córdoba S, Esparza-Gordillo J, Goicoechea de Jorge E, Lopez-Trascasa M, Sanchez-Corral P. The human complement factor H: functional roles, genetic variations and disease associations. *Mol Immunol* 2004; **41**: 355–367.
- Willemseder D, Banki Z, García E, Pruenster M, Pfister G, Muellauer B et al. IgG opsonization of HIV impedes provirus formation in and infection of dendritic cells and subsequent long-term transfer to T cells. *J Immunol* 2007; **178**: 7840–7848.
- Takada A, Kawaoaka Y. Antibody-dependent enhancement of viral infection: molecular mechanisms and *in vivo* implications. *Rev Med Virol* 2003; **13**: 387–398.
- Willey S, Aasa-Chapman MM, O'Farrell S, Pellegrino P, Williams I, Weiss RA et al. Extensive complement-dependent enhancement of HIV-1 by autologous non-neutralising antibodies at early stages of infection. *Retrovirology* 2011; **8**: 16.

- Frank MM. CD21 deficiency, complement, and the development of common variable immunodeficiency. *J Allergy Clin Immunol* 2012; **129**: 811–813.
- Asokan R, Hua J, Young KA, Gould HJ, Hannan JP, Kraus DM et al. Characterization of human complement receptor type 2 (CR2/CD21) as a receptor for IFN- α : a potential role in systemic lupus erythematosus. *J Immunol* 2006; **177**: 383–394.
- Asokan R, Banda NK, Szakonyi G, Chen XS, Holers VM. Human complement receptor 2 (CR2/CD21) as a receptor for DNA: implications for its roles in the immune response and the pathogenesis of systemic lupus erythematosus (SLE). *Mol Immunol* 2013; **53**: 99–110.
- Forthal DN, Gabriel EE, Wang A, Landucci G, Phan TB. Association of Fc γ receptor IIIa genotype with the rate of HIV infection after gp120 vaccination. *Blood* 2012; **120**: 2836–2842.
- Huber G, Banki Z, Lengauer S, Stoiber H. Emerging role for complement in HIV infection. *Curr Opin HIV AIDS* 2011; **6**: 419–426.
- Lund O, Hansen J, Soerensen AM, Mosekilde E, Nielsen JO, Hansen JE. Increased adhesion as a mechanism of antibody-dependent and antibody-independent complement-mediated enhancement of human immunodeficiency virus infection. *J Virol* 1995; **69**: 2393–2400.
- Fust G. Enhancing antibodies in HIV infection. *Parasitology* 1997; **115** (Suppl): S127–S140.
- Douglas KB, Windels DC, Zhao J, Gadeliya AV, Wu H, Kaufman KM et al. Complement receptor 2 polymorphisms associated with systemic lupus erythematosus modulate alternative splicing. *Genes Immun* 2009; **10**: 457–469.
- Wu H, Boackle SA, Hanvivadhanakul P, Ulgiate D, Grossman JM, Lee Y et al. Association of a common complement receptor 2 haplotype with increased risk of systemic lupus erythematosus. *Proc Natl Acad Sci USA* 2007; **104**: 3961–3966.
- Avirutnan P, Hauhart RE, Somnuk P, Blom AM, Diamond MS, Atkinson JP. Binding of flavivirus nonstructural protein NS1 to C4b binding protein modulates complement activation. *J Immunol* 2011; **187**: 424–433.
- Erment D, Weckel A, Agarwal V, Frick IM, Björck L, Blom AM. Binding of complement inhibitor C4b-binding protein to a highly virulent Streptococcus pyogenes M1 strain is mediated by protein H and enhances adhesion to and invasion of endothelial cells. *J Biol Chem* 2013; **288**: 32172–32183.
- Kirjavainen V, Jarva H, Biedka-Sarek M, Blom AM, Skurnik M, Meri S. Yersinia enterocolitica serum resistance proteins YadA and ail bind the complement regulator C4b-binding protein. *PLoS Pathog* 2008; **4**: e1000140.
- Castiblanco-Valencia MM, Fraga TR, Silva LB, Monaris D, Abreu PA, Strobel S et al. Leptospiral immunoglobulin-like proteins interact with human complement regulators factor H, FHL-1, FHR-1, and C4BP. *J Infect Dis* 2012; **205**: 995–1004.
- Ram S, Cullinane M, Blom AM, Gulati S, McQuillen DP, Monks BG et al. Binding of C4b-binding protein to porin: a molecular mechanism of serum resistance of Neisseria gonorrhoeae. *J Exp Med* 2001; **193**: 281–295.
- Sironi M, Biasin M, Cagliani R, Forni D, De Luca M, Saulle I et al. A common polymorphism in TLR3 confers natural resistance to HIV-1 infection. *J Immunol* 2012; **188**: 818–823.
- Fish EN. The X-files in immunity: sex-based differences predispose immune responses. *Nat Rev Immunol* 2008; **8**: 737–744.
- Pennell LM, Galligan CL, Fish EN. Sex affects immunity. *J Autoimmun* 2012; **38**: J282–J291.
- Marcovina SM, Zoppo A, Viganò D'Angelo S, Di Cola G, D'Angelo A. Determination of serum levels of complement component C4b-binding protein: influence of age and inflammation. *Int J Clin Lab Res* 1991; **21**: 171–175.
- Comp PC, Thurnau GR, Welsh J, Esmon CT. Functional and immunologic protein S levels are decreased during pregnancy. *Blood* 1986; **68**: 881–885.
- Melissari E, Kakkar VV. The effects of oestrogen administration on the plasma free protein S and C4b-binding protein. *Thromb Res* 1988; **49**: 489–495.
- Davila S, Wright VJ, Khor CC, Sim KS, Binder A, Breunis WB et al. Genome-wide association study identifies variants in the CFH region associated with host susceptibility to meningococcal disease. *Nat Genet* 2010; **42**: 772–776.
- Pastor AF, Rodrigues Moura L, Neto JW, Nascimento EJ, Calzavara-Silva CE, Gomes AL et al. Complement factor H gene (CFH) polymorphisms C-257T, G257A and haplotypes are associated with protection against severe dengue phenotype, possible related with high CFH expression. *Hum Immunol* 2013; **74**: 1225–1230.
- Hu W, Yu Q, Hu N, Byrd D, Amet T, Shikuma C et al. A high-affinity inhibitor of human CD59 enhances complement-mediated virolysis of HIV-1: implications for treatment of HIV-1/AIDS. *J Immunol* 2010; **184**: 359–368.
- Nyholt DR. A simple correction for multiple testing for single-nucleotide polymorphisms in linkage disequilibrium with each other. *Am J Hum Genet* 2004; **74**: 765–769.

Supplementary Information accompanies this paper on Genes and Immunity website (<http://www.nature.com/gene>)

Supplementary table 1

CHR	SNP_GENE	Allele 1	Allele 2	TEST	Genotype	Genotype	CHISQ	DF	P
					counts HIV-	counts			
1	rs1567190_CR2	T	C	DOM	194/45	131/69	13,91	1	0.0001918
1	rs2842704_C4BPA	G	A	DOM	63/177	86/114	13,67	1	0.0002184
1	rs2842704_C4BPA	G	A	TREND	68/412	93/307	12,56	1	0.0003933
1	rs1567190_CR2	T	C	TREND	253/225	166/234	12,06	1	0.0005152
1	rs2842704_C4BPA	G	A	ALLELIC	68/412	93/307	12,04	1	0.00052
1	rs1567190_CR2	T	C	ALLELIC	253/225	166/234	11,4	1	0.0007338
1	rs1567190_CR2	T	C	GENO	59/135/45	35/96/69	14,41	2	0.0007415
1	rs2842704_C4BPA	G	A	GENO	5/58/177	7/79/114	13,67	2	0.001076
8	rs9331930_CLUS	C	A	REC	24/214	6/193	8,47	1	0.00361
1	rs419137_CFH	C	A	ALLELIC	27/455	43/353	8.188	1	0.004216
1	rs419137_CFH	C	A	TREND	27/455	43/353	7.892	1	0.004966
11	rs831618_CD59	T	G	DOM	179/62	125/75	7.075	1	0.007816
1	rs6690037_C4BPB	G	A	DOM	166/74	114/86	6.978	1	0.00825
8	rs9331930_CLUS	C	A	GENO	24/91/123	6/83/110	8,48	2	0.01441
1	rs1048971_CR2	A	G	REC	39/200	17/183	5,98	1	0.01447
4	rs13104777_CFI	C	T	DOM	151/90	147/53	5.866	1	0.01544
1	rs9427657_CFHR5	G	A	ALLELIC	61/417	31/369	5.831	1	0.01575
1	rs1048971_CR2	A	G	ALLELIC	183/295	122/278	5.821	1	0.01584
11	rs831618_CD59	T	G	GENO	51/128/62	43/82/75	8,25	2	0.01616
1	rs1409153_CFHR2	G	A	DOM	129/109	131/69	5.751	1	0.01648
1	rs395544_CFH	T	C	DOM	131/108	132/68	5.675	1	0.01721
1	rs1048971_CR2	A	G	TREND	183/295	122/278	5.649	1	0.01746
1	rs9427657_CFHR5	G	A	TREND	61/417	31/369	5.537	1	0.01861
1	rs7545126_CD46	T	C	ALLELIC	22/406	34/332	5.182	1	0.02283
1	rs6690037_C4BPB	G	A	GENO	42/124/74	33/81/86	7.425	2	0.02442
1	rs1409153_CFHR2	G	A	TREND	156/320	160/240	4.972	1	0.02577
1	rs1409153_CFHR2	G	A	ALLELIC	156/320	160/240	4.923	1	0.02651
1	rs7545126_CD46	T	C	TREND	22/406	34/332	4.808	1	0.02833
1	rs1048971_CR2	A	G	GENO	39/105/95	17/88/95	6.729	2	0.03458
1	rs12021671_CR2	A	G	GENO	32/120/87	35/76/89	6.622	2	0.03648
1	rs395544_CFH	T	C	GENO	31/100/108	25/107/68	6.558	2	0.03767
8	rs1532278_CLUS	T	C	DOM	142/99	137/63	4.315	1	0.03778
1	rs17615_CR2	A	G	ALLELIC	138/344	90/310	4.286	1	0.03842
1	rs10737680_CFH	C	A	REC	46/193	24/176	4.266	1	0.03887
1	rs17615_CR2	A	G	TREND	138/344	90/310	4,21	1	0.04018
1	rs6428379_CFHR5	T	C	DOM	150/89	144/56	4.201	1	0.04039
1	rs7512286_LOC14865	G	A	ALLELIC	54/428	29/371	4.008	1	0.0453
4	rs13104777_CFI	C	T	TREND	187/295	181/219	3.996	1	0.0456
4	rs4541508_CFI	T	C	ALLELIC	143/339	144/256	3.993	1	0.0457
1	rs7549152_CR2	T	G	ALLELIC	28/454	12/388	3.984	1	0.04593
1	rs12032512_CR2	G	C	DOM	167/73	121/79	3.981	1	0.04603
1	rs6690037_C4BPB	G	A	ALLELIC	208/272	147/253	3.929	1	0.04746
4	rs4541508_CFI	T	C	TREND	143/339	144/256	3,9	1	0.0483
1	rs7512286_LOC14865	G	A	TREND	54/428	29/371	3.894	1	0.04846
8	rs499046_SCARA3	A	G	DOM	149/91	142/58	3.873	1	0.04908
1	rs6690037_C4BPB	G	A	TREND	208/272	147/253	3.807	1	0.05104
1	rs7549152_CR2	T	G	TREND	28/454	12/388	3.769	1	0.05223
4	rs13104777_CFI	C	T	GENO	36/115/90	34/113/53	5.887	2	0.05268
4	rs13104777_CFI	C	T	ALLELIC	187/295	181/219	3.744	1	0.053
1	rs1409153_CFHR2	G	A	GENO	27/102/109	29/102/69	5.807	2	0.05483
1	rs4658046_CFH	C	T	DOM	128/113	124/76	3.526	1	0.06043
1	rs1507764_CR2	A	G	REC	20/221	8/192	3.397	1	0.06533
1	rs2019727_CFH	T	A	ALLELIC	108/372	70/330	3,38	1	0.06598
1	rs1567190_CR2	T	C	REC	59/180	35/165	3.342	1	0.06755
8	rs1532278_CLUS	T	C	TREND	175/307	169/231	3.311	1	0.06882
1	rs2019727_CFH	T	A	TREND	108/372	70/330	3.288	1	0.06979

Resultados/Results

Supplementary table 1

CHR	SNP_GENE	Allele 1	Allele 2	TEST	Genotype counts		CHISQ	DF	P
					1 +	HESN			
4	rs7675460_CFI	A	C	ALLELIC	169/313	164/236	3.279	1	0.07015
1	rs4425986_C4BPA	T	C	DOM	177/64	131/69	3.275	1	0.07036
8	rs1532278_CLUS	T	C	ALLELIC	175/307	169/231	3.245	1	0.07163
1	rs17615_CR2	A	G	DOM	117/124	80/120	3.231	1	0.07226
4	rs4698792_CFI	T	C	REC	15/225	22/178	3.196	1	0.07383
8	rs9331930_CLUS	C	A	TREND	139/337	95/303	3.193	1	0.07395
4	rs7675460_CFI	A	C	TREND	169/313	164/236	3.154	1	0.07576
8	rs9331930_CLUS	C	A	ALLELIC	139/337	95/303	3.144	1	0.07622
12	rs2072449_C3AR	T	C	REC	19/222	26/174	3.122	1	0.07723
1	rs9943077_C4BPA	T	C	TREND	188/294	179/221	3.066	1	0.07993
1	rs12032512_CR2	G	C	ALLELIC	220/260	160/240	3.026	1	0.08194
4	rs4541508_CFI	T	C	DOM	121/120	117/83	3.025	1	0.08197
1	rs12021671_CR2	A	G	DOM	152/87	111/89	2.973	1	0.08466
1	rs9943077_C4BPA	T	C	ALLELIC	188/294	179/221	2.97	1	0.0848
1	rs4425986_C4BPA	T	C	TREND	229/253	167/233	2.956	1	0.08555
1	rs17044576_CR2	C	T	TREND	50/428	29/371	2.951	1	0.08585
1	rs4425986_C4BPA	T	C	ALLELIC	229/253	167/233	2.932	1	0.08685
1	rs4658046_CFH	C	T	GENO	28/100/113	20/104/76	4.886	2	0.08692
11	rs831618_CD59	T	G	ALLELIC	230/252	168/232	2.886	1	0.08935
1	rs1507764_CR2	A	G	ALLELIC	114/368	76/324	2.798	1	0.09436
1	rs10429953_CR1L	G	A	REC	5/234	10/190	2.79	1	0.09485
11	rs831618_CD59	T	G	TREND	230/252	168/232	2.779	1	0.0955
1	rs12032512_CR2	G	C	TREND	220/260	160/240	2.77	1	0.09602
1	rs395544_CFH	T	C	TREND	162/316	157/243	2.756	1	0.09687
1	rs17044576_CR2	C	T	ALLELIC	50/428	29/371	2.741	1	0.09781
1	rs6428379_CFHR5	T	C	TREND	186/292	177/223	2.712	1	0.09957
1	rs395544_CFH	T	C	ALLELIC	162/316	157/243	2.703	1	0.1001
1	rs2235768_LOC14865	C	G	DOM	50/190	55/145	2.669	1	0.1023
1	rs1048971_CR2	A	G	DOM	144/95	105/95	2.665	1	0.1026
1	rs6428379_CFHR5	T	C	ALLELIC	186/292	177/223	2.559	1	0.1097
1	rs10737680_CFH	C	A	GENO	46/117/76	24/110/66	4.404	2	0.1106
1	rs1507764_CR2	A	G	TREND	114/368	76/324	2.541	1	0.1109
8	rs499046_SCARA3	A	G	GENO	43/106/91	36/106/58	4.328	2	0.1148
8	rs1532278_CLUS	T	C	GENO	33/109/99	32/105/63	4.316	2	0.1156
1	rs17615_CR2	A	G	GENO	21/96/124	10/70/120	4.266	2	0.1185
1	rs2235768_LOC14865	C	G	ALLELIC	56/424	61/339	2.43	1	0.119
1	rs6428379_CFHR5	T	C	GENO	36/114/89	33/111/56	4.25	2	0.1195
1	rs17573790_CFH	C	A	ALLELIC	55/425	33/365	2.42	1	0.1198
1	rs7538501_CFHR2	T	C	ALLELIC	74/408	47/353	2.397	1	0.1216
1	rs17573790_CFH	C	A	TREND	55/425	33/365	2.384	1	0.1226
1	rs7538501_CFHR2	T	C	TREND	74/408	47/353	2.321	1	0.1276
1	rs17615_CR2	A	G	REC	21/220	10/190	2.306	1	0.1288
1	rs9943077_C4BPA	T	C	REC	34/207	39/161	2.301	1	0.1293
1	rs1507765_CD55	A	C	DOM	168/70	154/46	2.295	1	0.1298
4	rs4698792_CFI	T	C	ALLELIC	129/351	126/274	2.268	1	0.1321
4	rs4698792_CFI	T	C	TREND	129/351	126/274	2.266	1	0.1322
1	rs2235768_LOC14865	C	G	TREND	56/424	61/339	2.243	1	0.1342
4	rs7675460_CFI	A	C	REC	31/210	36/164	2.238	1	0.1346
1	rs12080578_CR1L	G	A	ALLELIC	24/456	12/388	2.224	1	0.1359
1	rs12032512_CR2	G	C	GENO	53/114/73	39/82/79	3.988	2	0.1361
1	rs1323720_CR1L	C	T	DOM	151/87	113/87	2.189	1	0.139
12	rs17728664_C3AR	C	T	TREND	11/471	4/396	2.187	1	0.1391
1	rs11120211_C4BPA	A	G	TREND	31/451	36/364	2.162	1	0.1414
4	rs4541508_CFI	T	C	GENO	22/99/120	27/90/83	3.905	2	0.1419
12	rs17728664_C3AR	C	T	ALLELIC	11/471	4/396	2.15	1	0.1426
1	rs7541230_CD46	C	T	GENO	12/111/117	16/76/108	3.878	2	0.1439

Supplementary table 1

CHR	SNP_GENE	Allele 1	Allele 2	TEST	Genotype	Genotype	CHISQ	DF	P
					counts HIV-	counts			
4	rs4541508_CFI	T	C	REC	22/219	27/173	2.115	1	0.1459
1	rs10779339_CR1	C	T	ALLELIC	229/249	172/228	2.114	1	0.146
11	rs28362934_SER	A	G	TREND	31/451	36/364	2.091	1	0.1481
4	rs7675460_CFI	A	C	DOM	138/103	128/72	2.073	1	0.1499
1	rs12080578_CR1L	G	A	TREND	24/456	12/388	2.073	1	0.15
1	rs10779339_CR1	C	T	TREND	229/249	172/228	2.068	1	0.1504
1	rs111120211_C4BPA	A	G	ALLELIC	31/451	36/364	2.054	1	0.1518
11	rs28362934_SER	A	G	ALLELIC	31/451	36/364	2.054	1	0.1518
1	rs10922096_CFH	T	C	GENO	60/111/69	42/111/47	3.743	2	0.1539
1	rs2336597_CFHR5	T	C	ALLELIC	52/430	32/368	1.972	1	0.1602
1	rs1507764_CR2	A	G	GENO	20/74/147	8/60/132	3.632	2	0.1627
1	rs761277_LOC148696	G	A	TREND	191/289	141/259	1.927	1	0.1651
1	rs761277_LOC148696	G	A	ALLELIC	191/289	141/259	1.916	1	0.1663
8	rs1493521_SCARA3	T	G	TREND	26/456	14/386	1.902	1	0.1679
1	rs2796265_CD46	C	T	TREND	83/399	83/317	1.884	1	0.1699
12	rs11567785_C3AR	G	A	ALLELIC	26/454	14/386	1.847	1	0.1741
12	rs11567785_C3AR	G	A	TREND	26/454	14/386	1.839	1	0.1751
1	rs9943077_C4BPA	T	C	DOM	154/87	140/60	1.83	1	0.1761
1	rs2336597_CFHR5	T	C	TREND	52/430	32/368	1.828	1	0.1764
10	rs11003125_MBL2	C	G	ALLELIC	154/322	112/286	1.817	1	0.1777
8	rs499046_SCARA3	A	G	ALLELIC	192/288	178/222	1.813	1	0.1781
8	rs1493521_SCARA3	T	G	ALLELIC	26/456	14/386	1.812	1	0.1783
8	rs499046_SCARA3	A	G	TREND	192/288	178/222	1.793	1	0.1806
1	rs2796265_CD46	C	T	ALLELIC	83/399	83/317	1.783	1	0.1818
1	rs4425986_C4BPA	T	C	GENO	52/125/64	36/95/69	3.406	2	0.1822
3	rs8178607_PROS1	T	C	REC	8/231	12/188	1.762	1	0.1844
4	rs4698792_CFI	T	C	GENO	15/99/126	22/82/96	3.367	2	0.1858
1	rs761277_LOC148696	G	A	DOM	154/86	116/84	1.75	1	0.1859
10	rs11003125_MBL2	C	G	TREND	154/322	112/286	1.732	1	0.1882
1	rs10737680_CFH	C	A	TREND	209/269	158/242	1.704	1	0.1918
1	rs1126618_C4BPA	T	C	TREND	82/400	81/319	1.674	1	0.1958
12	rs2072449_C3AR	T	C	GENO	19/106/116	26/80/94	3.244	2	0.1975
1	rs7541230_CD46	C	T	REC	12/228	16/184	1.648	1	0.1993
4	rs7675460_CFI	A	C	GENO	31/107/103	36/92/72	3.211	2	0.2008
4	rs11726949_CFI	T	C	TREND	41/441	25/375	1.632	1	0.2014
4	rs6822976_CFI	A	G	REC	60/179	40/160	1.613	1	0.2041
4	rs7439493_CFI	A	G	REC	40/199	43/157	1.612	1	0.2043
1	rs10429953_CR1L	G	A	GENO	5/73/161	10/54/136	3.174	2	0.2045
4	rs11726949_CFI	T	C	ALLELIC	41/441	25/375	1.607	1	0.2049
1	rs10737680_CFH	C	A	ALLELIC	209/269	158/242	1.597	1	0.2063
1	rs9943077_C4BPA	T	C	GENO	34/120/87	39/101/60	3.151	2	0.2069
11	rs11603020_SER	C	T	TREND	137/345	129/271	1.59	1	0.2073
1	rs10922096_CFH	T	C	DOM	171/69	153/47	1.549	1	0.2133
10	rs1838065_MBL2	G	A	ALLELIC	153/321	113/285	1.542	1	0.2144
1	rs1323720_CR1L	C	T	TREND	182/294	137/263	1.538	1	0.215
1	rs1507765_CD55	A	C	TREND	213/263	195/205	1.525	1	0.2168
1	rs1126618_C4BPA	T	C	ALLELIC	82/400	81/319	1.521	1	0.2175
11	rs11603020_SER	C	T	ALLELIC	137/345	129/271	1.52	1	0.2177
1	rs10779339_CR1	C	T	DOM	173/66	134/66	1.502	1	0.2204
1	rs1323720_CR1L	C	T	ALLELIC	182/294	137/263	1.491	1	0.2221
10	rs1838065_MBL2	G	A	TREND	153/321	113/285	1.471	1	0.2252
1	rs7519408_CR2	G	C	ALLELIC	62/420	41/359	1.447	1	0.229
8	rs9314349_CLUS	G	A	DOM	142/99	129/71	1.436	1	0.2308
1	rs12021671_CR2	A	G	REC	32/207	35/165	1.423	1	0.2329
1	rs677066_CR1	G	A	GENO	7/90/143	10/62/128	2.905	2	0.234
1	rs1507765_CD55	A	C	ALLELIC	213/263	195/205	1.399	1	0.2369

Resultados/Results

Supplementary table 1

CHR	SNP_GENE	Allele 1	Allele 2	TEST	Genotype counts		CHISQ	DF	P
					1 +	HESN			
1	rs7519408_CR2	G	C	TREND	62/420	41/359	1.387	1	0.239
8	rs9314349_CLUS	G	A	TREND	169/313	155/245	1.366	1	0.2425
11	rs17760694_CD59	C	T	ALLELIC	68/412	68/332	1.341	1	0.2469
1	rs4658046_CFH	C	T	TREND	156/326	144/256	1.327	1	0.2493
11	rs17760694_CD59	C	T	REC	6/234	9/191	1.325	1	0.2497
10	rs7096206_MBL2	G	C	DOM	79/162	76/124	1.307	1	0.253
1	rs4658046_CFH	C	T	ALLELIC	156/326	144/256	1.287	1	0.2566
12	rs11567782_C3AR	T	C	TREND	17/465	9/391	1.285	1	0.257
8	rs9314349_CLUS	G	A	ALLELIC	169/313	155/245	1.279	1	0.2581
1	rs677066_CR1	G	A	REC	7/233	10/190	1.275	1	0.2589
1	rs2235768_LOC14865	G	G	GENO	6/44/190	6/49/145	2,7	2	0.2593
1	rs10779339_CR1	C	T	REC	56/183	38/162	1,27	1	0.2597
10	rs1838065_MBL2	G	A	REC	28/209	17/182	1.251	1	0.2634
12	rs11567782_C3AR	T	C	ALLELIC	17/465	9/391	1.246	1	0.2643
11	rs17760694_CD59	C	T	TREND	68/412	68/332	1.243	1	0.2648
1	rs2796269_CD46	T	C	TREND	199/277	152/246	1.232	1	0.2671
8	rs569205_CLUS	T	A	GENO	39/135/66	39/97/64	2,64	2	0.2671
10	rs11003125_MBL2	C	G	REC	28/210	17/182	1.218	1	0.2697
1	rs2782842_CD55	A	G	REC	11/230	14/186	1.213	1	0.2708
1	rs7541230_CD46	C	T	DOM	123/117	92/108	1.203	1	0.2727
11	rs11603020_SER	C	T	DOM	120/121	110/90	1.188	1	0.2758
1	rs2796269_CD46	T	C	REC	40/198	26/173	1.183	1	0.2767
1	rs2796269_CD46	T	C	ALLELIC	199/277	152/246	1.179	1	0.2775
1	rs1507764_CR2	A	G	DOM	94/147	68/132	1.178	1	0.2778
10	rs11003125_MBL2	C	G	DOM	126/112	95/104	1.174	1	0.2787
11	rs704700_CD59	C	T	ALLELIC	67/415	66/334	1.154	1	0.2828
10	rs7096206_MBL2	G	C	ALLELIC	92/390	88/312	1.142	1	0.2853
8	rs9331949_CLUS	G	A	ALLELIC	21/461	12/388	1.117	1	0.2905
11	rs704700_CD59	C	T	TREND	67/415	66/334	1.096	1	0.2952
1	rs11120277_C4BPA	C	T	TREND	23/447	14/386	1.079	1	0.299
8	rs569205_CLUS	T	A	DOM	174/66	136/64	1.061	1	0.3029
10	rs7096206_MBL2	G	C	TREND	92/390	88/312	1.045	1	0.3066
1	rs11120277_C4BPA	C	T	ALLELIC	23/447	14/386	1.031	1	0.31
1	rs1507765_CD55	A	C	GENO	45/123/70	41/113/46	2.296	2	0.3173
12	rs2072449_C3AR	T	C	ALLELIC	144/338	132/268	0,9925	1	0.3191
1	rs10922096_CFH	T	C	REC	60/180	42/158	0,9802	1	0.3222
12	rs2072449_C3AR	T	C	TREND	144/338	132/268	0,9739	1	0.3237
1	rs1409153_CFHR2	G	A	REC	27/211	29/171	0,9704	1	0.3246
11	rs704700_CD59	C	T	DOM	61/180	59/141	0,9682	1	0.3251
1	rs2796266_CD46	A	G	DOM	136/104	104/96	0,9582	1	0.3276
11	rs11032350_CD59	C	A	REC	21/220	23/177	0,9447	1	0.3311
1	rs1323720_CR1L	C	T	GENO	31/120/87	24/89/87	2.209	2	0.3314
8	rs492638_CLUS	T	G	REC	20/221	22/178	0,9255	1	0.336
1	rs2025935_CR1	T	C	DOM	123/118	93/107	0,9004	1	0.3427
1	rs677066_CR1	G	A	DOM	97/143	72/128	0,8996	1	0.3429
4	rs4698792_CFI	T	C	DOM	114/126	104/96	0,8837	1	0.3472
3	rs8178610_PROS1	A	G	DOM	137/103	123/77	0,8803	1	0.3481
10	rs1838065_MBL2	G	A	DOM	125/112	96/103	0,8769	1	0.349
1	rs4425986_C4BPA	T	C	REC	52/189	36/164	0,8754	1	0.3495
11	rs11603020_SER	C	T	REC	17/224	19/181	0,8723	1	0.3503
1	rs4618971_CR2	C	T	DOM	99/142	91/109	0,8712	1	0.3506
3	rs8178610_PROS1	A	G	ALLELIC	169/311	153/247	0,87	1	0.351
1	rs7524776_CFH	C	T	DOM	72/169	68/132	0,8581	1	0.3543
4	rs6822976_CFI	A	G	GENO	60/118/61	40/111/49	2.075	2	0.3544
10	rs1982266_MBL2	A	G	DOM	170/71	149/51	0,8567	1	0.3547
1	rs10779339_CR1	C	T	GENO	56/117/66	38/96/66	2.069	2	0.3554

Supplementary table 1

CHR	SNP_GENE	Allele 1	Allele 2	TEST	Genotype counts HIV-	Genotype counts	CHISQ	DF	P
					1 +	HESN			
3	rs8178610_PROS1	A	G	TREND	169/311	153/247	0,8444	1	0.3581
8	rs9331949_CLUS	G	A	TREND	21/461	12/388	0,8346	1	0.361
11	rs2511988_SERPING	T	C	DOM	127/111	98/102	0,8275	1	0.363
1	rs2842704_C4BPA	G	A	REC	5/235	7/193	0,8253	1	0.3636
1	rs761277_LOC148696	G	A	GENO	37/117/86	25/91/84	1.976	2	0.3723
8	rs569205_CLUS	T	A	REC	39/201	39/161	0,7901	1	0.3741
1	rs17020993_C4BPA	G	A	DOM	78/162	73/127	0,7744	1	0.3789
1	rs4571969_C4BPA	T	C	TREND	151/331	136/264	0,7725	1	0.3795
1	rs761277_LOC148696	G	A	REC	37/203	25/175	0,7666	1	0.3813
1	rs7524776_CFH	C	T	ALLELIC	83/399	78/322	0,7616	1	0.3828
1	rs6701713_CR1	A	G	REC	11/229	6/194	0,7363	1	0.3909
11	rs17760694_CD59	C	T	DOM	62/178	59/141	0,7356	1	0.3911
1	rs11117913_CR1	T	C	ALLELIC	27/455	28/372	0,731	1	0.3926
1	rs11117913_CR1	T	C	TREND	27/455	28/372	0,723	1	0.3952
1	rs2025935_CR1	T	C	GENO	20/103/118	20/73/107	1.856	2	0.3954
1	rs4571969_C4BPA	T	C	ALLELIC	151/331	136/264	0,7111	1	0.3991
1	rs6696840_CR1L	C	T	REC	47/193	33/167	0,6972	1	0.4037
1	rs7524776_CFH	C	T	TREND	83/399	78/322	0,695	1	0.4045
1	rs4618971_CR2	C	T	ALLELIC	116/366	106/294	0,6874	1	0.4071
10	rs11003125_MBL2	C	G	GENO	28/98/112	17/78/104	1.792	2	0.4083
4	rs7439493_CFI	A	G	GENO	40/126/73	43/96/61	1.787	2	0.4093
8	rs492638_CLUS	T	G	GENO	20/117/104	22/86/92	1.767	2	0.4132
3	rs8178607_PROS1	T	C	GENO	8/95/136	12/77/111	1.763	2	0.4141
11	rs831626_CD59	G	A	TREND	90/388	84/316	0,658	1	0.4173
1	rs4618971_CR2	C	T	TREND	116/366	106/294	0,6553	1	0.4182
11	rs831626_CD59	G	A	ALLELIC	90/388	84/316	0,6462	1	0.4215
11	rs831626_CD59	G	A	DOM	82/157	76/124	0,6437	1	0.4224
4	rs9998151_CFI	C	T	TREND	15/467	9/391	0,6313	1	0.4269
4	rs9998151_CFI	C	T	ALLELIC	15/467	9/391	0,6137	1	0.4334
1	rs4571969_C4BPA	T	C	REC	19/222	20/180	0,6072	1	0.4359
10	rs1982266_MBL2	A	G	ALLELIC	232/250	203/197	0,599	1	0.4389
1	rs2796269_CD46	T	C	DOM	159/79	126/73	0,582	1	0.4455
3	rs13062355_PROS1	A	G	DOM	171/69	149/51	0,5809	1	0.4459
10	rs10824792_MBL2	C	T	ALLELIC	201/281	177/223	0,5798	1	0.4464
11	rs11603020_SER	C	T	GENO	17/103/121	19/91/90	1,61	2	0.4471
11	rs17760694_CD59	C	T	GENO	6/56/178	9/50/141	1.608	2	0.4475
10	rs1838065_MBL2	G	A	GENO	28/97/112	17/79/103	1.607	2	0.4478
8	rs9331930_CLUS	C	A	DOM	115/123	89/110	0,563	1	0.4531
11	rs2511988_SERPING	T	C	ALLELIC	154/322	120/280	0,5598	1	0.4543
10	rs1982266_MBL2	A	G	TREND	232/250	203/197	0,5551	1	0.4562
10	rs10824792_MBL2	C	T	TREND	201/281	177/223	0,5542	1	0.4566
1	rs2796266_CD46	A	G	GENO	21/115/104	20/84/96	1,55	2	0.4607
11	rs11032350_CD59	C	A	GENO	21/113/107	23/84/93	1.541	2	0.4627
3	rs8178607_PROS1	T	C	TREND	111/367	101/299	0,5257	1	0.4684
11	rs12272807_CD59	T	C	ALLELIC	47/435	45/355	0,5257	1	0.4684
11	rs2511988_SERPING	T	C	TREND	154/322	120/280	0,5255	1	0.4685
10	rs10824792_MBL2	C	T	REC	44/197	42/158	0,5238	1	0.4692
4	rs7439493_CFI	A	G	TREND	206/272	182/218	0,5234	1	0.4694
1	rs2284664_CFH	A	G	DOM	88/152	80/120	0,5135	1	0.4736
8	rs9314349_CLUS	G	A	GENO	27/115/99	26/103/71	1.492	2	0.4742
11	rs12272807_CD59	T	C	TREND	47/435	45/355	0,5108	1	0.4748
4	rs7439493_CFI	A	G	ALLELIC	206/272	182/218	0,5102	1	0.4751
1	rs6701713_CR1	A	G	ALLELIC	88/392	66/334	0,5079	1	0.476
8	rs3087554_CLUS	G	A	ALLELIC	77/405	57/343	0,5049	1	0.4774
1	rs2782842_CD55	A	G	GENO	11/91/139	14/69/117	1.477	2	0.4779
8	rs3087554_CLUS	G	A	TREND	77/405	57/343	0,4977	1	0.4805

Resultados/Results

Supplementary table 1

CHR	SNP_GENE	Allele 1	Allele 2	TEST	Genotype counts		CHISQ	DF	P
					1 +	HESN			
1	rs4317805_CR2	A	G	ALLELIC	45/437	32/368	0,4897	1	0.484
1	rs2564978_CD55	T	C	ALLELIC	111/367	101/299	0,4891	1	0.4843
3	rs8178607_PROS1	T	C	ALLELIC	111/367	101/299	0,4891	1	0.4843
3	rs6123_PROS1	G	A	DOM	133/108	117/83	0,4887	1	0.4845
1	rs2796278_CD46	A	C	DOM	163/77	142/58	0,4876	1	0.485
1	rs17020993_C4BPA	G	A	TREND	85/395	78/322	0,4868	1	0.4854
1	rs6701713_CR1	A	G	TREND	88/392	66/334	0,4812	1	0.4879
1	rs2564978_CD55	T	C	TREND	111/367	101/299	0,4807	1	0.4881
1	rs17020993_C4BPA	G	A	ALLELIC	85/395	78/322	0,4641	1	0.4957
11	rs2273121_CD59	T	C	TREND	82/394	76/324	0,464	1	0.4958
1	rs4571969_C4BPA	T	C	DOM	132/109	116/84	0,4628	1	0.4963
8	rs1532278_CLUS	T	C	REC	33/208	32/168	0,4629	1	0.4963
11	rs2273121_CD59	T	C	ALLELIC	82/394	76/324	0,4622	1	0.4966
1	rs2796278_CD46	A	C	TREND	203/277	178/222	0,461	1	0.4971
1	rs2564978_CD55	T	C	REC	13/226	14/186	0,4595	1	0.4979
1	rs14374_CD46	C	T	TREND	17/463	11/389	0,459	1	0.4981
1	rs4317805_CR2	A	G	TREND	45/437	32/368	0,4555	1	0.4997
1	rs2796269_CD46	T	C	GENO	40/119/79	26/100/73	1.385	2	0.5002
1	rs2808470_C4BPA	T	C	DOM	87/152	79/121	0,4445	1	0.505
1	rs14374_CD46	C	T	ALLELIC	17/463	11/389	0,4439	1	0.5053
1	rs12032512_CR2	G	C	REC	53/187	39/161	0,4402	1	0.507
1	rs6695321_CFH	G	A	REC	49/190	36/164	0,4366	1	0.5088
1	rs2796278_CD46	A	C	ALLELIC	203/277	178/222	0,4334	1	0.5103
11	rs2508443_SERPING	T	G	DOM	146/95	115/85	0,4295	1	0.5122
10	rs7096206_MBL2	G	C	GENO	13/66/162	12/64/124	1.319	2	0.517
1	rs11582939_CFH	T	C	REC	10/231	6/194	0,413	1	0.5205
19	rs4427917_C5AR	C	G	REC	8/233	9/191	0,411	1	0.5215
19	rs7253663_C5AR	G	T	REC	8/232	9/191	0,3998	1	0.5272
3	rs13062355_PROS1	A	G	ALLELIC	225/255	196/204	0,3948	1	0.5298
3	rs13062355_PROS1	A	G	TREND	225/255	196/204	0,3938	1	0.5303
1	rs1410408_CR2	T	C	TREND	62/420	46/354	0,3913	1	0.5316
11	rs704700_CD59	C	T	REC	6/235	7/193	0,39	1	0.5323
19	rs4577202_C5AR	G	A	TREND	30/452	21/379	0,3884	1	0.5332
1	rs2025935_CR1	T	C	REC	20/221	20/180	0,3835	1	0.5357
1	rs1571344_CR1	C	T	ALLELIC	54/424	40/360	0,3832	1	0.5359
1	rs6690215_CR2	C	T	REC	48/191	45/155	0,3808	1	0.5372
19	rs4577202_C5AR	G	A	ALLELIC	30/452	21/379	0,3807	1	0.5372
4	rs6822976_CFI	A	G	TREND	238/240	191/209	0,3796	1	0.5378
1	rs1410408_CR2	T	C	ALLELIC	62/420	46/354	0,378	1	0.5387
1	rs2808470_C4BPA	T	C	TREND	96/382	87/313	0,3773	1	0.5391
1	rs1571344_CR1	C	T	TREND	54/424	40/360	0,3746	1	0.5405
1	rs12021671_CR2	A	G	ALLELIC	184/294	146/254	0,369	1	0.5436
1	rs2808470_C4BPA	T	C	ALLELIC	96/382	87/313	0,3665	1	0.5449
4	rs6822976_CFI	A	G	ALLELIC	238/240	191/209	0,363	1	0.5469
1	rs6696840_CR1L	C	T	ALLELIC	204/276	162/238	0,3593	1	0.5489
8	rs492638_CLUS	T	G	DOM	137/104	108/92	0,3587	1	0.5493
1	rs12021671_CR2	A	G	TREND	184/294	146/254	0,352	1	0.553
4	rs13104777_CFI	C	T	REC	36/205	34/166	0,3481	1	0.5552
1	rs6696840_CR1L	C	T	TREND	204/276	162/238	0,3466	1	0.556
1	rs12734030_CR1	T	C	TREND	82/400	74/326	0,3364	1	0.5619
8	rs9314349_CLUS	G	A	REC	27/214	26/174	0,3337	1	0.5635
8	rs10503814_CLUS	T	C	ALLELIC	40/442	29/371	0,3334	1	0.5637
1	rs12734030_CR1	T	C	ALLELIC	82/400	74/326	0,3322	1	0.5643
8	rs10503814_CLUS	T	C	TREND	40/442	29/371	0,3203	1	0.5714
1	rs6675769_CFHRS5	G	A	TREND	222/256	193/207	0,3109	1	0.5771
11	rs704700_CD59	C	T	GENO	6/55/180	7/52/141	1.097	2	0.5778

Supplementary table 1

CHR	SNP_GENE	Allele 1	Allele 2	TEST	Genotype counts HIV-		CHISQ	DF	P
					1 +	HESN counts			
1	rs2796266_CD46	A	G	TREND	157/323	124/276	0,3053	1	0.5806
3	rs6123_PROS1	G	A	ALLELIC	165/317	144/256	0,3001	1	0.5838
1	rs800292_CFH	T	C	DOM	101/140	89/111	0,2993	1	0.5843
1	rs6675769_CFHRS5	G	A	DOM	175/64	151/49	0,2957	1	0.5866
1	rs4658046_CFH	C	T	REC	28/213	20/180	0,2951	1	0.587
1	rs2796266_CD46	A	G	ALLELIC	157/323	124/276	0,293	1	0.5883
1	rs6695321_CFH	G	A	GENO	49/108/82	36/100/64	1.059	2	0.589
3	rs6123_PROS1	G	A	TREND	165/317	144/256	0,2862	1	0.5927
1	rs6675769_CFHRS5	G	A	ALLELIC	222/256	193/207	0,2851	1	0.5934
1	rs2564978_CD55	T	C	DOM	98/141	87/113	0,2782	1	0.5979
10	rs10824792_MBL2	C	T	DOM	157/84	135/65	0,2709	1	0.6027
1	rs17020993_C4BPA	G	A	GENO	7/71/162	5/68/127	1.009	2	0.6039
1	rs6695321_CFH	G	A	DOM	157/82	136/64	0,2617	1	0.609
1	rs2284664_CFH	A	G	ALLELIC	100/380	89/311	0,2596	1	0.6104
1	rs2284664_CFH	A	G	TREND	100/380	89/311	0,2572	1	0.612
4	rs11929767_CFI	A	G	ALLELIC	8/474	5/395	0,2527	1	0.6152
3	rs8178610_PROS1	A	G	REC	32/208	30/170	0,2503	1	0.6168
10	rs11595876_MBL2	C	T	TREND	24/456	23/377	0,2456	1	0.6202
1	rs11595876_MBL2	C	T	ALLELIC	24/456	23/377	0,2428	1	0.6222
4	rs4698788_CFI	C	T	ALLELIC	23/459	22/378	0,2394	1	0.6246
4	rs4698788_CFI	C	T	TREND	23/459	22/378	0,2302	1	0.6314
3	rs8178610_PROS1	A	G	GENO	32/105/103	30/93/77	0,9186	2	0.6317
1	rs12034383_CR1	G	A	ALLELIC	179/301	143/257	0,2235	1	0.6364
4	rs11929767_CFI	A	G	TREND	8/474	5/395	0,2215	1	0.6379
1	rs6701713_CR1	A	G	DOM	77/163	60/140	0,2208	1	0.6384
1	rs4618971_CR2	C	T	GENO	17/82/142	15/76/109	0,8874	2	0.6417
1	rs12034383_CR1	G	A	TREND	179/301	143/257	0,2149	1	0.643
1	rs2025935_CR1	T	C	ALLELIC	143/339	113/287	0,2134	1	0.6441
11	rs2511988_SERPING	T	C	GENO	27/100/111	22/76/102	0,873	2	0.6463
1	rs7524776_CFH	C	T	GENO	11/61/169	10/58/132	0,8671	2	0.6482
1	rs2025935_CR1	T	C	TREND	143/339	113/287	0,2069	1	0.6492
10	rs1982266_MBL2	A	G	GENO	62/108/71	54/95/51	0,8586	2	0.651
1	rs2796266_CD46	A	G	REC	21/219	20/180	0,2017	1	0.6533
11	rs2508443_SERPING	T	G	ALLELIC	183/299	146/254	0,2011	1	0.6538
1	rs12034383_CR1	G	A	REC	36/204	27/173	0,2001	1	0.6547
1	rs4571969_C4BPA	T	C	GENO	19/113/109	20/96/84	0,8422	2	0.6563
1	rs800292_CFH	T	C	REC	17/224	12/188	0,1976	1	0.6567
11	rs11032350_CD59	C	A	DOM	134/107	107/93	0,1948	1	0.659
1	rs10429953_CR1L	G	A	ALLELIC	83/395	74/326	0,1914	1	0.6618
11	rs2508443_SERPING	T	G	TREND	183/299	146/254	0,1889	1	0.6638
1	rs10429953_CR1L	G	A	TREND	83/395	74/326	0,1886	1	0.6641
1	rs677066_CR1	G	A	TREND	104/376	82/318	0,1849	1	0.6672
1	rs7555070_CFHRS5	C	T	REC	28/212	26/174	0,1801	1	0.6713
8	rs9331908_CLUS	A	G	DOM	141/100	113/87	0,1801	1	0.6713
1	rs677066_CR1	G	A	ALLELIC	104/376	82/318	0,1782	1	0.673
1	rs3753394_CFH	T	C	REC	22/219	16/184	0,1768	1	0.6741
1	rs6701713_CR1	A	G	GENO	11/66/163	6/54/140	0,7866	2	0.6748
1	rs1507765_CD55	A	C	REC	45/193	41/159	0,1746	1	0.676
1	rs6428379_CFHRS5	T	C	REC	36/203	33/167	0,1698	1	0.6803
1	rs12405238_CFH	T	G	TREND	142/340	113/287	0,1666	1	0.6831
8	rs9331908_CLUS	A	G	TREND	170/312	136/264	0,1572	1	0.6917
1	rs12405238_CFH	T	G	REC	18/223	13/187	0,157	1	0.6919
1	rs12405238_CFH	T	G	ALLELIC	142/340	113/287	0,1559	1	0.693
1	rs2284664_CFH	A	G	GENO	12/76/152	9/71/120	0,733	2	0.6931
8	rs9331908_CLUS	A	G	ALLELIC	170/312	136/264	0,1555	1	0.6933
1	rs3753394_CFH	T	C	TREND	148/334	118/282	0,1543	1	0.6945

Resultados/Results

Supplementary table 1

CHR	SNP_GENE	Allele 1	Allele 2	TEST	Genotype counts HIV-	Genotype counts	CHISQ	DF	P
					1 +	HESN			
1	rs2491393_C48PA	G	A	REC	33/208	30/170	0,1525	1	0.6962
1	rs3753394_CFH	T	C	ALLELIC	148/334	118/282	0,1508	1	0.6978
1	rs6690215_CR2	C	T	GENO	48/118/73	45/91/64	0,717	2	0.6987
1	rs800292_CFH	T	C	GENO	17/84/140	12/77/111	0,7114	2	0.7007
1	rs7541230_CD46	C	T	TREND	135/345	108/292	0,1475	1	0.701
4	rs10033900_CFI	T	C	DOM	153/87	131/69	0,146	1	0.7024
1	rs6696840_CR1L	C	T	GENO	47/110/83	33/96/71	0,706	2	0.7026
1	rs2491393_C48PA	G	A	TREND	193/289	165/235	0,1456	1	0.7028
19	rs7253663_C5AR	G	T	GENO	8/91/141	9/70/121	0,694	2	0.7068
11	rs831627_CD59	T	C	TREND	14/468	10/390	0,1391	1	0.7092
19	rs7253663_C5AR	G	T	DOM	99/141	79/121	0,1387	1	0.7096
1	rs7541230_CD46	C	T	ALLELIC	135/345	108/292	0,1381	1	0.7101
1	rs2182913_CR2	A	G	REC	24/215	18/182	0,1366	1	0.7117
1	rs2796278_CD46	A	C	REC	40/200	36/164	0,1357	1	0.7126
11	rs831627_CD59	T	C	ALLELIC	14/468	10/390	0,1352	1	0.7131
11	rs831626_CD59	G	A	GENO	8/74/157	8/68/124	0,6696	2	0.7155
1	rs2491393_C48PA	G	A	ALLELIC	193/289	165/235	0,1324	1	0.716
11	rs831626_CD59	G	A	REC	8/231	8/192	0,1321	1	0.7163
1	rs11576522_CR1	A	G	REC	36/201	28/172	0,1228	1	0.726
1	rs6675769_CFHRS	G	A	REC	47/192	42/158	0,12	1	0.729
1	rs12034383_CR1	G	A	DOM	143/97	116/84	0,1129	1	0.7368
10	rs10824792_MBL2	C	T	GENO	44/113/84	42/93/65	0,6045	2	0.7391
1	rs6690215_CR2	C	T	DOM	166/73	136/64	0,1075	1	0.743
1	rs6663083_CFHRS	C	T	DOM	175/66	148/52	0,1071	1	0.7435
1	rs1323720_CR1L	C	T	REC	31/207	24/176	0,104	1	0.747
3	rs13062355_PROS1	A	G	GENO	54/117/69	47/102/51	0,581	2	0.7479
1	rs2235768_LOC14865	C	G	REC	6/234	6/194	0,1028	1	0.7485
1	rs2796267_CD46	G	A	DOM	135/106	109/91	0,1017	1	0.7498
1	rs2564978_CD55	T	C	GENO	13/85/141	14/73/113	0,5749	2	0.7502
11	rs11032362_CD59	A	G	ALLELIC	49/425	44/356	0,1001	1	0.7517
19	rs4427917_C5AR	C	G	GENO	8/91/142	9/71/120	0,5684	2	0.7526
1	rs12405238_CFH	T	G	DOM	124/117	100/100	0,09223	1	0.7614
10	rs1982266_MBL2	A	G	REC	62/179	54/146	0,09149	1	0.7623
11	rs11032362_CD59	A	G	TREND	49/425	44/356	0,09122	1	0.7626
3	rs8178607_PROS1	T	C	DOM	103/136	89/111	0,08719	1	0.7678
3	rs6123_PROS1	G	A	GENO	32/101/108	27/90/83	0,5222	2	0.7702
11	rs2508443_SERPING	T	G	GENO	37/109/95	31/84/85	0,516	2	0.7726
1	rs2796278_CD46	A	C	GENO	40/123/77	36/106/58	0,5145	2	0.7732
8	rs9331942_CLUS	C	T	ALLELIC	15/461	14/386	0,08258	1	0.7738
10	rs1800450_MBL2	A	G	DOM	69/171	60/140	0,08226	1	0.7743
1	rs2782842_CD55	A	G	TREND	113/369	97/303	0,07828	1	0.7796
1	rs2782842_CD55	A	G	ALLELIC	113/369	97/303	0,07828	1	0.7796
1	rs2796267_CD46	G	A	ALLELIC	167/315	135/265	0,07817	1	0.7798
1	rs6690037_C48PB	G	A	REC	42/198	33/167	0,07715	1	0.7812
10	rs7096206_MBL2	G	C	REC	13/228	12/188	0,07501	1	0.7842
8	rs9331942_CLUS	C	T	TREND	15/461	14/386	0,07451	1	0.7849
1	rs2796267_CD46	G	A	TREND	167/315	135/265	0,0735	1	0.7863
1	rs3753394_CFH	T	C	DOM	126/115	102/98	0,07195	1	0.7885
1	rs10737680_CFH	C	A	DOM	163/76	134/66	0,07175	1	0.7888
10	rs1800450_MBL2	A	G	ALLELIC	76/404	66/334	0,07166	1	0.7889
1	rs7555070_CFHRS	C	T	DOM	147/93	120/80	0,07144	1	0.7892
1	rs17020993_C48PA	G	A	REC	7/233	5/195	0,07139	1	0.7893
10	rs1800450_MBL2	A	G	TREND	76/404	66/334	0,06985	1	0.7916
1	rs800292_CFH	T	C	ALLELIC	118/364	101/299	0,0692	1	0.7925
1	rs11582939_CFH	T	C	GENO	10/81/150	6/70/124	0,4607	2	0.7943
1	rs800292_CFH	T	C	TREND	118/364	101/299	0,06771	1	0.7947

Supplementary table 1

CHR	SNP_GENE	Allele 1	Allele 2	TEST	Genotype counts HIV-	Genotype counts	CHISQ	DF	P
					1 +	HESN			
1	rs2808470_C4BPA	T	C	GENO	9/78/152	8/71/121	0,4467	2	0.7999
3	rs13062355_PROS1	A	G	REC	54/186	47/153	0,06168	1	0.8039
1	rs2491393_C4BPA	G	A	DOM	160/81	135/65	0,0608	1	0.8052
4	rs6822976_CFI	A	G	DOM	178/61	151/49	0,06068	1	0.8054
1	rs2284664_CFH	A	G	REC	12/228	9/191	0,06001	1	0.8065
1	rs16840658_CFHR1	A	G	TREND	78/404	67/333	0,05784	1	0.8099
1	rs2724377_CD46	C	T	DOM	170/71	139/61	0,0563	1	0.8124
12	rs2072449_C3AR	T	C	DOM	125/116	106/94	0,05623	1	0.8126
19	rs4427917_C5AR	C	G	DOM	99/142	80/120	0,05275	1	0.8183
1	rs16840658_CFHR1	A	G	ALLELIC	78/404	67/333	0,05123	1	0.8209
1	rs7524776_CFH	C	T	REC	11/230	10/190	0,04575	1	0.8306
1	rs17048254_CR1	A	G	ALLELIC	32/450	28/372	0,04493	1	0.8321
1	rs17048254_CR1	A	G	TREND	32/450	28/372	0,04344	1	0.8349
1	rs6663083_CFHR5	C	T	REC	55/186	44/156	0,04238	1	0.8369
1	rs7555070_CFHR5	C	T	GENO	28/119/93	26/94/80	0,3518	2	0.8387
1	rs6696840_CR1L	C	T	DOM	157/83	129/71	0,04029	1	0.8409
8	rs9331926_CLUS	C	G	ALLELIC	19/455	17/381	0,03778	1	0.8459
1	rs6675769_CFHR5	G	A	GENO	47/128/64	42/109/49	0,3332	2	0.8465
8	rs569205_CLUS	T	A	TREND	213/267	175/225	0,03716	1	0.8471
4	rs10033900_CFI	T	C	REC	40/200	32/168	0,03543	1	0.8507
8	rs569205_CLUS	T	A	ALLELIC	213/267	175/225	0,03457	1	0.8525
1	rs10922096_CFH	T	C	TREND	231/249	195/205	0,03447	1	0.8527
1	rs10922096_CFH	T	C	ALLELIC	231/249	195/205	0,03413	1	0.8534
8	rs9331926_CLUS	C	G	TREND	19/455	17/381	0,03341	1	0.855
1	rs4618971_CR2	C	T	REC	17/224	15/185	0,03231	1	0.8573
1	rs11576522_CR1	A	G	ALLELIC	171/303	142/258	0,0313	1	0.8596
1	rs2782842_CD55	A	G	DOM	102/139	83/117	0,03045	1	0.8615
8	rs9331908_CLUS	A	G	REC	29/212	23/177	0,02987	1	0.8628
1	rs10494745_CFHR2	A	G	ALLELIC	45/437	36/364	0,02961	1	0.8634
1	rs11576522_CR1	A	G	TREND	171/303	142/258	0,02901	1	0.8648
1	rs11582939_CFH	T	C	TREND	101/381	82/318	0,02862	1	0.8657
1	rs10494745_CFHR2	A	G	TREND	45/437	36/364	0,02788	1	0.8674
1	rs11582939_CFH	T	C	ALLELIC	101/381	82/318	0,02744	1	0.8684
3	rs9683303_PROS1	T	A	REC	19/221	15/185	0,02656	1	0.8705
4	rs10033900_CFI	T	C	TREND	193/287	163/237	0,02658	1	0.8705
4	rs10033900_CFI	T	C	ALLELIC	193/287	163/237	0,02657	1	0.8705
1	rs4844597_CR1	C	T	REC	10/229	9/191	0,02624	1	0.8713
4	rs10033900_CFI	T	C	GENO	40/113/87	32/99/69	0,2561	2	0.8798
1	rs2182913_CR2	A	G	ALLELIC	142/336	117/283	0,02188	1	0.8824
1	rs395544_CFH	T	C	REC	31/208	25/175	0,02168	1	0.8829
1	rs2182913_CR2	A	G	TREND	142/336	117/283	0,02101	1	0.8848
1	rs6690215_CR2	C	T	ALLELIC	214/264	181/219	0,02028	1	0.8867
1	rs10429953_CR1L	G	A	DOM	78/161	64/136	0,02013	1	0.8872
1	rs12034383_CR1	G	A	GENO	36/107/97	27/89/84	0,2381	2	0.8878
1	rs6690215_CR2	C	T	TREND	214/264	181/219	0,01954	1	0.8888
1	rs6663083_CFHR5	C	T	GENO	55/120/66	44/104/52	0,2162	2	0.8975
1	rs2808470_C4BPA	T	C	REC	9/230	8/192	0,01606	1	0.8992
1	rs3753394_CFH	T	C	GENO	22/104/115	16/86/98	0,1994	2	0.9051
1	rs12405238_CFH	T	G	GENO	18/106/117	13/87/100	0,1986	2	0.9055
11	rs2511988_SERPING	T	C	REC	27/211	22/178	0,01298	1	0.9093
1	rs12116643_CFHR5	C	T	TREND	76/406	62/338	0,01246	1	0.9111
3	rs9683303_PROS1	T	A	ALLELIC	130/350	107/293	0,01232	1	0.9116
3	rs9683303_PROS1	T	A	TREND	130/350	107/293	0,01203	1	0.9127
11	rs11032350_CD59	C	A	TREND	155/327	130/270	0,01197	1	0.9129
8	rs9331908_CLUS	A	G	GENO	29/112/100	23/90/87	0,1819	2	0.9131
1	rs12116643_CFHR5	C	T	ALLELIC	76/406	62/338	0,01186	1	0.9133

Resultados/Results

Supplementary table 1

CHR	SNP_GENE	Allele 1	Allele 2	TEST	Genotype	Genotype	CHISQ	DF	P
					counts	HIV- counts			
11	rs11032350_CD59	C	A	ALLELIC	155/327	130/270	0,01171	1	0.9138
1	rs2724377_CD46	C	T	REC	52/189	44/156	0,0115	1	0.9146
19	rs7253663_C5AR	G	T	TREND	107/373	88/312	0,01146	1	0.9148
19	rs7253663_C5AR	G	T	ALLELIC	107/373	88/312	0,01076	1	0.9174
1	rs2491393_C48PA	G	A	GENO	33/127/81	30/105/65	0,1722	2	0.9175
1	rs2182913_CR2	A	G	GENO	24/94/121	18/81/101	0,1612	2	0.9225
1	rs2724377_CD46	C	T	ALLELIC	222/260	183/217	0,00836	1	0.9272
1	rs2724377_CD46	C	T	TREND	222/260	183/217	0,00813	1	0.9281
1	rs11576522_CR1	A	G	GENO	36/99/102	28/86/86	0,1435	2	0.9308
11	rs831618_CD59	T	G	REC	51/190	43/157	0,00745	1	0.9312
1	rs2796267_CD46	G	A	REC	32/209	26/174	0,0074	1	0.9315
1	rs6663083_CFHRS	C	T	TREND	230/252	192/208	0,0071	1	0.9328
1	rs6663083_CFHRS	C	T	ALLELIC	230/252	192/208	0,00697	1	0.9334
1	rs12723250_CFHRS	T	C	TREND	16/450	14/384	0,0047	1	0.9453
3	rs6123_PROS1	G	A	REC	32/209	27/173	0,00465	1	0.9456
1	rs12723250_CFHRS	T	C	ALLELIC	16/450	14/384	0,00453	1	0.9463
1	rs2796267_CD46	G	A	GENO	32/103/106	26/83/91	0,1025	2	0.9501
4	rs884711_CFI	T	G	TREND	5/475	4/396	0,00378	1	0.951
4	rs884711_CFI	T	G	ALLELIC	5/475	4/396	0,00374	1	0.9512
1	rs2724377_CD46	C	T	GENO	52/118/71	44/95/61	0,09686	2	0.9527
3	rs9683303_PROS1	T	A	DOM	111/129	92/108	0,00274	1	0.9582
1	rs11582939_CFH	T	C	DOM	91/150	76/124	0,00269	1	0.9586
10	rs1800450_MBL2	A	G	REC	7/233	6/194	0,00264	1	0.959
10	rs1800450_MBL2	A	G	GENO	7/62/171	6/54/140	0,083	2	0.9593
1	rs4844597_CR1	C	T	DOM	77/162	64/136	0,00236	1	0.9612
11	rs2508443_CERPING	T	G	REC	37/204	31/169	0,00182	1	0.966
1	rs6695321_CFH	G	A	ALLELIC	206/272	172/228	0,00082	1	0.9771
1	rs6695321_CFH	G	A	TREND	206/272	172/228	0,0008	1	0.9775
1	rs2182913_CR2	A	G	DOM	118/121	99/101	0,00071	1	0.9788
8	rs492638_CLUS	T	G	TREND	157/325	130/270	0,00055	1	0.9813
1	rs4844597_CR1	C	T	GENO	10/67/162	9/55/136	0,03702	2	0.9817
8	rs492638_CLUS	T	G	ALLELIC	157/325	130/270	0,00053	1	0.9817
8	rs499046_SCARA3	A	G	REC	43/197	36/164	0,00051	1	0.9819
1	rs4844597_CR1	C	T	ALLELIC	87/391	73/327	0,00035	1	0.985
19	rs4427917_C5AR	C	G	TREND	107/375	89/311	0,00035	1	0.9851
1	rs4844597_CR1	C	T	TREND	87/391	73/327	0,00033	1	0.9855
19	rs4427917_C5AR	C	G	ALLELIC	107/375	89/311	0,00033	1	0.9856
3	rs9683303_PROS1	T	A	GENO	19/92/129	15/77/108	0,02656	2	0.9868
1	rs7555070_CFHRS	C	T	TREND	175/305	146/254	0,00017	1	0.9896
1	rs7555070_CFHRS	C	T	ALLELIC	175/305	146/254	0,00016	1	0.9898
4	rs7439493_CFI	A	G	DOM	166/73	139/61	9,91E-05	1	0.9921
1	rs11576522_CR1	A	G	DOM	135/102	114/86	6,38E-02	1	0.9936
1	rs17573790_CFH	C	A	GENO	3/49/188	2/29/168	NA	NA	NA
1	rs17573790_CFH	C	A	DOM	52/188	31/168	NA	NA	NA
1	rs17573790_CFH	C	A	REC	3/237	2/197	NA	NA	NA
1	rs2019727_CFH	T	A	GENO	16/76/148	4/62/134	NA	NA	NA
1	rs2019727_CFH	T	A	DOM	92/148	66/134	NA	NA	NA
1	rs2019727_CFH	T	A	REC	16/224	4/196	NA	NA	NA
1	rs419137_CFH	C	A	GENO	1/25/215	3/37/158	NA	NA	NA
1	rs419137_CFH	C	A	DOM	26/215	40/158	NA	NA	NA
1	rs419137_CFH	C	A	REC	1/240	3/195	NA	NA	NA
1	rs16840658_CFHRS	A	G	GENO	3/72/166	2/63/135	NA	NA	NA
1	rs16840658_CFHRS	A	G	DOM	75/166	65/135	NA	NA	NA
1	rs16840658_CFHRS	A	G	REC	3/238	2/198	NA	NA	NA
1	rs10494745_CFHRS	A	G	GENO	4/37/200	2/32/166	NA	NA	NA
1	rs10494745_CFHRS	A	G	DOM	41/200	34/166	NA	NA	NA

Supplementary table 1

CHR	SNP_GENE	Allele 1	Allele 2	TEST	Genotype counts HIV-	Genotype counts	CHISQ	DF	P
					1 +	HESN			
1	rs10494745_CFHR2	A	G	REC	4/237	2/198	NA	NA	NA
1	rs7538501_CFHR2	T	C	GENO	7/60/174	3/41/156	NA	NA	NA
1	rs7538501_CFHR2	T	C	DOM	67/174	44/156	NA	NA	NA
1	rs7538501_CFHR2	T	C	REC	7/234	3/197	NA	NA	NA
1	rs12723250_CFHR2	T	C	GENO	0/16/217	0/14/185	NA	NA	NA
1	rs12723250_CFHR2	T	C	DOM	16/217	14/185	NA	NA	NA
1	rs12723250_CFHR2	T	C	REC	0/233	0/199	NA	NA	NA
1	rs9427657_CFHR5	G	A	GENO	5/51/183	2/27/171	NA	NA	NA
1	rs9427657_CFHR5	G	A	DOM	56/183	29/171	NA	NA	NA
1	rs9427657_CFHR5	G	A	REC	5/234	2/198	NA	NA	NA
1	rs12116643_CFHR5	C	T	GENO	6/64/171	2/58/140	NA	NA	NA
1	rs12116643_CFHR5	C	T	DOM	70/171	60/140	NA	NA	NA
1	rs12116643_CFHR5	C	T	REC	6/235	2/198	NA	NA	NA
1	rs2336597_CFHR5	T	C	GENO	4/44/193	3/26/171	NA	NA	NA
1	rs2336597_CFHR5	T	C	DOM	48/193	29/171	NA	NA	NA
1	rs2336597_CFHR5	T	C	REC	4/237	3/197	NA	NA	NA
1	rs11120211_C4BPA	A	G	GENO	1/29/211	0/36/164	NA	NA	NA
1	rs11120211_C4BPA	A	G	DOM	30/211	36/164	NA	NA	NA
1	rs11120211_C4BPA	A	G	REC	1/240	0/200	NA	NA	NA
1	rs11120277_C4BPA	C	T	GENO	0/23/212	0/14/186	NA	NA	NA
1	rs11120277_C4BPA	C	T	DOM	23/212	14/186	NA	NA	NA
1	rs11120277_C4BPA	C	T	REC	0/235	0/200	NA	NA	NA
1	rs17044576_CR2	C	T	GENO	1/48/190	0/29/171	NA	NA	NA
1	rs17044576_CR2	C	T	DOM	49/190	29/171	NA	NA	NA
1	rs17044576_CR2	C	T	REC	1/238	0/200	NA	NA	NA
1	rs7549152_CR2	T	G	GENO	2/24/215	0/12/188	NA	NA	NA
1	rs7549152_CR2	T	G	DOM	26/215	12/188	NA	NA	NA
1	rs7549152_CR2	T	G	REC	2/239	0/200	NA	NA	NA
1	rs4317805_CR2	A	G	GENO	3/39/199	3/26/171	NA	NA	NA
1	rs4317805_CR2	A	G	DOM	42/199	29/171	NA	NA	NA
1	rs4317805_CR2	A	G	REC	3/238	3/197	NA	NA	NA
1	rs7519408_CR2	G	C	GENO	4/54/183	4/33/163	NA	NA	NA
1	rs7519408_CR2	G	C	DOM	58/183	37/163	NA	NA	NA
1	rs7519408_CR2	G	C	REC	4/237	4/196	NA	NA	NA
1	rs1410408_CR2	T	C	GENO	3/56/182	2/42/156	NA	NA	NA
1	rs1410408_CR2	T	C	DOM	59/182	44/156	NA	NA	NA
1	rs1410408_CR2	T	C	REC	3/238	2/198	NA	NA	NA
1	rs11117913_CR1	T	C	GENO	1/25/215	1/26/173	NA	NA	NA
1	rs11117913_CR1	T	C	DOM	26/215	27/173	NA	NA	NA
1	rs11117913_CR1	T	C	REC	1/240	1/199	NA	NA	NA
1	rs1571344_CR1	C	T	GENO	4/46/189	2/36/162	NA	NA	NA
1	rs1571344_CR1	C	T	DOM	50/189	38/162	NA	NA	NA
1	rs1571344_CR1	C	T	REC	4/235	2/198	NA	NA	NA
1	rs12734030_CR1	T	C	GENO	3/76/162	10/54/136	NA	NA	NA
1	rs12734030_CR1	T	C	DOM	79/162	64/136	NA	NA	NA
1	rs12734030_CR1	T	C	REC	3/238	10/190	NA	NA	NA
1	rs17048254_CR1	A	G	GENO	1/30/210	2/24/174	NA	NA	NA
1	rs17048254_CR1	A	G	DOM	31/210	26/174	NA	NA	NA
1	rs17048254_CR1	A	G	REC	1/240	2/198	NA	NA	NA
1	rs2796265_CD46	C	T	GENO	1/81/159	11/61/128	NA	NA	NA
1	rs2796265_CD46	C	T	DOM	82/159	72/128	NA	NA	NA
1	rs2796265_CD46	C	T	REC	1/240	11/189	NA	NA	NA
1	rs14374_CD46	C	T	GENO	0/17/223	0/11/189	NA	NA	NA
1	rs14374_CD46	C	T	DOM	17/223	11/189	NA	NA	NA
1	rs14374_CD46	C	T	REC	0/240	0/200	NA	NA	NA
1	rs7512286_LOC14865	G	A	GENO	5/44/192	0/29/171	NA	NA	NA

Resultados/Results

Supplementary table 1

CHR	SNP_GENE	Allele 1	Allele 2	TEST	Genotype counts HIV-	Genotype counts	CHISQ	DF	P
					1 +	HESN			
1	rs7512286_LOC14865	G	A	DOM	49/192	29/171	NA	NA	NA
1	rs7512286_LOC14865	G	A	REC	5/236	0/200	NA	NA	NA
1	rs1126618_C4BPA	T	C	GENO	5/72/164	4/73/123	NA	NA	NA
1	rs1126618_C4BPA	T	C	DOM	77/164	77/123	NA	NA	NA
1	rs1126618_C4BPA	T	C	REC	5/236	4/196	NA	NA	NA
1	rs12080578_CR1L	G	A	GENO	1/22/217	1/10/189	NA	NA	NA
1	rs12080578_CR1L	G	A	DOM	23/217	11/189	NA	NA	NA
1	rs12080578_CR1L	G	A	REC	1/239	1/199	NA	NA	NA
1	rs7545126_CD46	T	C	GENO	2/18/194	2/30/151	NA	NA	NA
1	rs7545126_CD46	T	C	DOM	20/194	32/151	NA	NA	NA
1	rs7545126_CD46	T	C	REC	2/212	2/181	NA	NA	NA
4	rs11726949_CFI	T	C	GENO	2/37/202	0/25/175	NA	NA	NA
4	rs11726949_CFI	T	C	DOM	39/202	25/175	NA	NA	NA
4	rs11726949_CFI	T	C	REC	2/239	0/200	NA	NA	NA
4	rs9998151_CFI	C	T	GENO	0/15/226	0/9/191	NA	NA	NA
4	rs9998151_CFI	C	T	DOM	15/226	9/191	NA	NA	NA
4	rs9998151_CFI	C	T	REC	0/241	0/200	NA	NA	NA
4	rs11929767_CFI	A	G	GENO	1/6/234	0/5/195	NA	NA	NA
4	rs11929767_CFI	A	G	DOM	7/234	5/195	NA	NA	NA
4	rs11929767_CFI	A	G	REC	1/240	0/200	NA	NA	NA
4	rs4698788_CFI	C	T	GENO	2/19/220	0/22/178	NA	NA	NA
4	rs4698788_CFI	C	T	DOM	21/220	22/178	NA	NA	NA
4	rs4698788_CFI	C	T	REC	2/239	0/200	NA	NA	NA
4	rs884711_CFI	T	G	GENO	0/5/235	0/4/196	NA	NA	NA
4	rs884711_CFI	T	G	DOM	5/235	4/196	NA	NA	NA
4	rs884711_CFI	T	G	REC	0/240	0/200	NA	NA	NA
8	rs10503814_CLUS	T	C	GENO	3/34/204	1/27/172	NA	NA	NA
8	rs10503814_CLUS	T	C	DOM	37/204	28/172	NA	NA	NA
8	rs10503814_CLUS	T	C	REC	3/238	1/199	NA	NA	NA
8	rs9331949_CLUS	G	A	GENO	6/9/226	0/12/188	NA	NA	NA
8	rs9331949_CLUS	G	A	DOM	15/226	12/188	NA	NA	NA
8	rs9331949_CLUS	G	A	REC	6/235	0/200	NA	NA	NA
8	rs9331942_CLUS	C	T	GENO	2/11/225	0/14/186	NA	NA	NA
8	rs9331942_CLUS	C	T	DOM	13/225	14/186	NA	NA	NA
8	rs9331942_CLUS	C	T	REC	2/236	0/200	NA	NA	NA
8	rs3087554_CLUS	G	A	GENO	8/61/172	3/51/146	NA	NA	NA
8	rs3087554_CLUS	G	A	DOM	69/172	54/146	NA	NA	NA
8	rs3087554_CLUS	G	A	REC	8/233	3/197	NA	NA	NA
8	rs9331926_CLUS	C	G	GENO	0/19/218	3/11/185	NA	NA	NA
8	rs9331926_CLUS	C	G	DOM	19/218	14/185	NA	NA	NA
8	rs9331926_CLUS	C	G	REC	0/237	3/196	NA	NA	NA
8	rs1493521_SCARA3	T	G	GENO	0/26/215	0/14/186	NA	NA	NA
8	rs1493521_SCARA3	T	G	DOM	26/215	14/186	NA	NA	NA
8	rs1493521_SCARA3	T	G	REC	0/241	0/200	NA	NA	NA
10	rs11595876_MBL2	C	T	GENO	0/24/216	1/21/178	NA	NA	NA
10	rs11595876_MBL2	C	T	DOM	24/216	22/178	NA	NA	NA
10	rs11595876_MBL2	C	T	REC	0/240	1/199	NA	NA	NA
11	rs12272807_CD59	T	C	GENO	3/41/197	3/39/158	NA	NA	NA
11	rs12272807_CD59	T	C	DOM	44/197	42/158	NA	NA	NA
11	rs12272807_CD59	T	C	REC	3/238	3/197	NA	NA	NA
11	rs831627_CD59	T	C	GENO	0/14/227	0/10/190	NA	NA	NA
11	rs831627_CD59	T	C	DOM	14/227	10/190	NA	NA	NA
11	rs831627_CD59	T	C	REC	0/241	0/200	NA	NA	NA
11	rs2273121_CD59	T	C	GENO	4/74/160	10/56/134	NA	NA	NA
11	rs2273121_CD59	T	C	DOM	78/160	66/134	NA	NA	NA
11	rs2273121_CD59	T	C	REC	4/234	10/190	NA	NA	NA

Supplementary table 1

CHR	SNP_GENE	Allele 1	Allele 2	TEST	Genotype	Genotype	CHISQ	DF	P
					counts HIV-	counts			
11	rs11032362_CD59	A	G	GENO	6/37/194	3/38/159	NA	NA	NA
11	rs11032362_CD59	A	G	DOM	43/194	41/159	NA	NA	NA
11	rs11032362_CD59	A	G	REC	6/231	3/197	NA	NA	NA
11	rs28362934_SER	A	G	GENO	1/29/211	1/34/165	NA	NA	NA
11	rs28362934_SER	A	G	DOM	30/211	35/165	NA	NA	NA
11	rs28362934_SER	A	G	REC	1/240	1/199	NA	NA	NA
12	rs11567785_C3AR	G	A	GENO	1/24/215	0/14/186	NA	NA	NA
12	rs11567785_C3AR	G	A	DOM	25/215	14/186	NA	NA	NA
12	rs11567785_C3AR	G	A	REC	1/239	0/200	NA	NA	NA
12	rs11567782_C3AR	T	C	GENO	0/17/224	0/9/191	NA	NA	NA
12	rs11567782_C3AR	T	C	DOM	17/224	9/191	NA	NA	NA
12	rs11567782_C3AR	T	C	REC	0/241	0/200	NA	NA	NA
12	rs17728664_C3AR	C	T	GENO	0/11/230	0/4/196	NA	NA	NA
12	rs17728664_C3AR	C	T	DOM	11/230	4/196	NA	NA	NA
12	rs17728664_C3AR	C	T	REC	0/241	0/200	NA	NA	NA
19	rs4577202_C5AR	G	A	GENO	1/28/212	0/21/179	NA	NA	NA
19	rs4577202_C5AR	G	A	DOM	29/212	21/179	NA	NA	NA
19	rs4577202_C5AR	G	A	REC	1/240	0/200	NA	NA	NA

Supplementary table 2

	HIV-1 POSITIVE POOLS										HESN POOLS					
	POOL1A	POOL1B	POOL2A	POOL2B	POOL3A	POOL3B	POOL5A	POOL5B	POOL6A	POOL6B	POOL4A	POOL4B	POOL7A	POOL7B	POOL8A	POOL8B
Read length	100 bp	100 bp	100 bp	100 bp	100 bp	100 bp	100 bp	100 bp	100 bp	100 bp	100 bp	100 bp	100 bp	100 bp	100 bp	100 bp
Paired	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
Bottlenecking	none	none	none	none	none	none	none	none	none	none	none	none	none	none	none	none
Bottleneck score	16.56	16.96	14.34	14.58	16.87	17.17	18.96	19.33	17.27	17.5	16.05	16.31	16.09	16.43	17.8	18.1
Mapped reads	702,357	681,292	893,851	871,813	772,981	752,572	954,816	929,452	1,038,668	1,011,084	879,709	857,925	959,819	944,694	1,049,940	1,020,419
	59.44%	57.66%	49.88%	48.65%	49.08%	47.79%	75.46%	73.45%	77.44%	75.39%	57.17%	55.76%	57.93%	57.02%	78.70%	76.48%
Mapped confidently	673.13	652,369	858,543	836,806	741,845	721,844	916.7	891,437	997,719	970,651	844,598	822,968	921,656	906,309	1,009,737	980,952
	56.97%	55.21%	47.91%	46.70%	47.11%	45.84%	72.45%	70.45%	74.39%	72.37%	54.89%	53.48%	55.63%	54.70%	75.68%	73.53%
Mapped repetitively	29,227	28,923	35,308	35,007	31,136	30,728	38,116	38,015	40,949	40,433	35,111	34,957	38,163	38,385	40,203	39,467
	2.47%	2.45%	1.97%	1.95%	1.98%	1.95%	3.01%	3.00%	3.05%	3.01%	2.28%	2.27%	2.30%	2.32%	3.01%	2.96%
Reads not mapped	479,189	500,254	898,186	920,224	801,876	822,285	310,555	335,919	302,524	330,108	659,021	680,805	697,048	712,173	284,233	313,754
	40.56%	42.34%	50.12%	51.35%	50.92%	52.21%	24.54%	26.55%	22.56%	24.61%	42.83%	44.24%	42.07%	42.98%	21.30%	23.52%
no mapping	432,492	393,437	794,039	760,079	711,111	681,908	263,251	251,632	252,598	245,053	581,55	557,783	631,19	588,804	233,38	274,371
	36.60%	33.30%	44.31%	42.41%	45.15%	43.30%	20.80%	19.89%	18.83%	18.27%	37.79%	36.25%	38.10%	35.54%	17.49%	16.82%
low quality	46,554	86,422	103,918	153,894	90,616	136,088	47,007	77,109	49,62	81,827	77,23	116,238	65,58	102,54	50,355	84,993
	3.94%	7.31%	5.80%	8.59%	5.75%	8.64%	3.71%	6.09%	3.70%	6.10%	5.02%	7.55%	3.96%	6.19%	3.77%	6.37%
ribosomal RNA	143	131	228	222	149	109	296	277	305	295	240	231	278	241	488	441
	0.01%	0.01%	0.01%	0.01%	0.01%	0.01%	0.02%	0.02%	0.02%	0.02%	0.02%	0.02%	0.02%	0.01%	0.04%	0.03%
primer	0	20,262	0	6,027	0	4,178	0	6,901	0	2,932	0	6,552	0	20,588	0	3,939
	0.00%	1.71%	0.00%	0.34%	0.00%	0.27%	0.00%	0.55%	0.00%	0.22%	0.00%	0.43%	0.00%	1.24%	0.00%	0.30%
control (phiX-174)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
poly-A	0	1	0	2	0	2	1	0	1	1	1	0	0	0	9	10
	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
poly-C	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
poly-G	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
poly-T	0	1	1	0	0	0	0	0	0	0	0	1	0	0	1	0
	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Total reads	1,181,546	1,181,546	1,792,037	1,792,037	1,574,857	1,574,857	1,265,371	1,265,371	1,341,192	1,341,192	1,538,730	1,538,730	1,656,867	1,656,867	1,334,173	1,334,173
	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%	100.00%
Total bases	118,154,600	118,154,600	179,203,700	179,203,700	157,485,700	157,485,700	126,537,100	126,537,100	134,119,200	134,119,200	153,873,000	153,873,000	165,686,700	165,686,700	133,417,300	133,417,300

Supplementary table 3: New polymorphic sites discovered in the *C4BPB-C4BPA-CD55-CR2-CR1-CR1L* region. *SNP: single nucleotide polymorphism; MNP: multiple nucleotide polymorphism, DEL/INS: insertion/deletion polymorphism. **GRCh37/hg19 version

Polymorphism type*	zygosity	Variation	Consensus sequence	Gene name	Where in transcript	Coding change type	Chromosome Position**
SNP	Het	t	c	C4BPB	Upstream		207258427
MNP	Het	aa	tc	C4BPB	Upstream		207260289
SNP	Het	c	t	C4BPB	Upstream		207260970
MNP	Het	at	tt	C4BPB	Intron		207263333
DEL	Het	g	tg	C4BPB	Intron		207265992
SNP	Het	a	c	C4BPB	Intron		207266055
SNP	Het	g	a	C4BPB	Intron		207270082
MNP	Het	aa	tc	C4BPB	Intron		207270909
SNP	Het	t	c	C4BPB	Intron		207270960
SNP	Het	g	t	C4BPB	Intron		207272203
MNP	Het	gga	atc	C4BPB	Intron		207272392
SNP	Het	c	t	C4BPB	3' UTR		207273312
SNP	Het	a	g	C4BPB	Downstream		207273434
SNP	Het	a	g	C4BPB	Downstream		207273434
MNP	Het	gtgtgtgtg	gtgtatgtg	C4BPB	Downstream		207273444
MNP	Het	gtgtgtgtg	gtatgtgtg	C4BPB	Downstream		207273445
SNP	Het	c	t	C4BPB	Downstream		207274464
SNP	Het	a	g	C4BPB	Downstream		207274656
SNP	Het	t	a	C4BPA	Intron		207278827
DEL	Het	g	ag	C4BPA	Intron		207278920
DEL	Het	t	tt	C4BPA	Intron		207279803
DEL	Het	g	tg	C4BPA	Intron		207279804
DEL	Het		at	C4BPA	Intron		207285547
DEL	Het	a	ta	C4BPA	Intron		207285715
MNP	Het	tt	ga	C4BPA	Intron		207287859
DEL	Het		tatc	C4BPA	Intron		207291463
SNP	Het	a	g	C4BPA	Intron		207291491
MNP	Het	gtctg	gtcta	C4BPA	Intron		207291515
MNP	Het	gtct	atct	C4BPA	Intron		207291518
DEL	Het		tatc	C4BPA	Intron		207291539
SNP	Het	g	a	C4BPA	Intron		207291552
INS	Het	t		C4BPA	Intron		207291554
MNP	Het	cc	ag	C4BPA	Intron		207292083
INS	Het	ga		C4BPA	Intron		207292363
MNP	Het	ca	gc	C4BPA	Intron		207292368
MNP	Het	tg	ga	C4BPA	Intron		207293183
SNP	Het	g	a	C4BPA	Intron		207293185
SNP	Het	a	t	C4BPA	Intron		207293187
SNP	Het	c	a	C4BPA	Intron		207293189
DEL	Het		g	C4BPA	Intron		207293194
SNP	Het	a	c	C4BPA	Intron		207294807
SNP	Het	t	c	C4BPA	Intron		207296011
MNP	Het	ga	ct	C4BPA	Intron		207298652
SNP	Het	t	c	C4BPA	Intron		207302253
MNP	Het	ct	gg	C4BPA	Intron		207312035
DEL	Het	t	tt	C4BPA	Intron		207313862
SNP	Het	c	a	C4BPA	Intron		207313894
MNP	Het	gat	atg	C4BPA	Intron		207315300
SNP	Het	a	g	C4BPA	Intron		207315359
SNP	Het	c	a	C4BPA	Intron		207315376

Resultados/Results

SNP	Het	a	t	C4BPA	Intron	207315379
SNP	Het	a	g	C4BPA	Intron	207315470
SNP	Het	c	a	C4BPA	Intron	207315832
SNP	Het	g	t	C4BPA	Intron	207315834
SNP	Het	t	c	C4BPA	Intron	207315837
DEL	Het	g	tg	C4BPA	DOWNST	207321537
SNP	Het	c	g	C4BPA	Downstream	207321718
INS	Het	a		CD55	Intron	207503690
SNP	Het	t	a	CD55	Intron	207503946
MNP	Het	tt	aa	CD55	Intron	207503954
DEL	Het		tatttac	CD55	Intron	207508265
MNP	Het	aa	gt	CD55	Intron	207514902
MNP	Het	ag	ct	CD55	Intron	207514907
INS	Het	t		CD55	Intron	207518070
MNP	Het	ga	tc	CD55	Intron	207518793
MNP	Het	at	gg	CD55	Intron	207519524
MNP	Het	ccg	tta	CD55	Intron	207526227
MNP	Het	ttttg	ttttt	CD55	Intron	207526411
INS	Het	agatc		CD55	Intron	207530912
SNP	Het	g	a	CD55	Downstream	207537784
SNP	Het	c	t	CD55	Downstream	207538083
SNP	Het	t	a	CD55	Downstream	207539040
SNP	Het	t	a	CD55	Downstream	207539040
DEL	Het		att	CD55	Downstream	207539044
DEL	Het	ta	tga	CD55	Downstream	207539061
MNP	Het	ga	tg	CD55	Downstream	207540364
SNP	Het	t	c	CD55	Downstream	207541929
SNP	Het	a	g	CD55	Downstream	207542038
MNP	Het	aga	tac	CD55	Downstream	207542858
MNP	Het	gg	ca	CD55	Downstream	207542986
SNP	Het	g	a	CD55	Downstream	207542989
SNP	Het	g	a	CD55	Downstream	207543003
SNP	Het	t	c	CD55	Downstream	207543819
SNP	Het	t	a	CD55	Downstream	207544200
SNP	Het	t	c	CR2	Upstream	207619668
SNP	Het	c	t	CR2	Upstream	207620843
MNP	Het	ga	at	CR2	Upstream	207620907
SNP	Het	c	t	CR2	Upstream	207620973
MNP	Het	cat	tac	CR2	Upstream	207620975
SNP	Het	t	c	CR2	Upstream	207620977
DEL	Het		ac	CR2	Upstream	207620995
MNP	Het	cc	tt	CR2	Upstream	207622054
MNP	Het	gca	ttt	CR2	Upstream	207622058
DEL	Het	ttt	tttttt	CR2	Upstream	207622169
SNP	Het	c	g	CR2	Upstream	207622648
MNP	Het	cc	tt	CR2	Upstream	207625000
SNP	Het	a	t	CR2	Upstream	207626577
SNP	Het	a	c	CR2	Upstream	207626614
SNP	Het	g	c	CR2	Upstream	207626863
SNP	Het	g	t	CR2	Upstream	207627294
SNP	Het	c	t	CR2	Intron	207627875
SNP	Het	a	g	CR2	Intron	207629074
SNP	Het	t	c	CR2	Intron	207629554
SNP	Het	g	a	CR2	Intron	207629864
DEL	Hom	t	t	CR2	Intron	207631636
SNP	Het	t	a	CR2	Intron	207631811
INS	Het	t		CR2	Intron	207632678
SNP	Het	c	t	CR2	Intron	207638118

SNP	Het	t	a	CR2	Intron	207638765
DEL	Het		ta	CR2	Intron	207638769
SNP	Het	t	g	CR2	Intron	207641426
SNP	Het	t	c	CR2	Intron	207643983
MNP	Het	tg	cc	CR2	Intron	207649328
SNP	Het	a	g	CR2	Intron	207651894
INS	Het	a		CR2	Intron	207652242
DEL	Het	g	aaaaag	CR2	Intron	207659603
DEL	Het		gt	CR2	Intron	207659919
SNP	Het	t	c	CR2	Intron	207661862
SNP	Het	a	g	CR2	Downstream	207665664
DEL	Het		t	CR1	Upstream	207668301
MNP	Het	aa	tc	CR1	Intron	207672223
SNP	Het	a	c	CR1	Intron	207681295
SNP	Het	c	t	CR1	Intron	207687079
SNP	Het	t	c	CR1	Intron	207687122
MNP	Het	aa	cc	CR1	Intron	207687155
SNP	Het	a	c	CR1	Intron	207687157
MNP	Het	ca	ta	CR1	Intron	207688341
MNP	Het	caca	cata	CR1	Intron	207688375
SNP	Het	t	c	CR1	Intron	207688524
SNP	Het	c	t	CR1	Intron	207690804
SNP	Het	t	c	CR1	Intron	207692848
DEL	Het		t	CR1	Intron	207694309
SNP	Het	t	g	CR1	Intron	207694310
MNP	Het	ttt	tg	CR1	Intron	207694311
SNP	Het	t	g	CR1	Intron	207694314
MNP	Het	ac	at	CR1	Intron	207694358
DEL	Het	tt	ttttt	CR1	Intron	207694996
MIXED	Het	at	atgt	CR1	Intron	207695173
SNP	Het	a	g	CR1	Intron	207695174
DEL	Het		t	CR1	Intron	207695195
SNP	Het	t	a	CR1	Intron	207698088
MNP	Het	at	ca	CR1	Intron	207717659
SNP	Het	a	c	CR1	Intron	207717954
MNP	Het	ac	gg	CR1	Intron	207726426
SNP	Het	c	t	CR1	Intron	207727285
SNP	Het	a	c	CR1	Intron	207727948
SNP	Het	g	a	CR1	Intron	207736562
SNP	Het	t	g	CR1	Intron	207736723
DEL	Het		ac	CR1	Intron	207737691
SNP	Het	t	a	CR1	Intron	207739958
DEL	Het		t	CR1	Intron	207753846
SNP	Het	t	g	CR1	CDS	207785100
					Non synonymous	
MNP	Het	gc	tt	CR1	Intron	207787742
SNP	Het	c	t	CR1	Intron	207842742
SNP	Het	t	a	CR1	Intron	207842745
SNP	Het	c	t	CR1	Intron	207842752
SNP	Het	t	g	CR1	Intron	207842755
MNP	Het	tt	ga	CR1	Intron	207842756
MIXED	Het	t	ag	CR1	Intron	207842757
SNP	Het	t	g	CR1	Intron	207847708
SNP	Het	c	t	CR1	Intron	207847892
SNP	Het	t	a	CR1	Intron	207847894
SNP	Het	g	t	CR1	Intron	207847897
SNP	Het	c	g	CR1	Intron	207848704
SNP	Het	t	g	CR1	Intron	207848915

Resultados/Results

DEL	Het		t	CR1	Intron	207852358
SNP	Het	t	a	CR1	Intron	207852908
SNP	Het	c	t	CR1	Intron	207853240
MNP	Het	tt	aa	CR1	Intron	207853889
SNP	Het	a	g	CR1	Intron	207859075
SNP	Hom	c	c	CR1	Intron	207859503
SNP	Hom	c	c	CR1	Intron	207859511
SNP	Hom	c	c	CR1	Intron	207859519
SNP	Het	a	c	CR1	Intron	207860270
SNP	Het	a	g	CR1	Intron	207860798
SNP	Het	t	g	CR1	Intron	207862719
SNP	Het	a	t	CR1	Intron	207863747
SNP	Het	a	c	CR1	Intron	207864648
SNP	Het	t	a	CR1	Intron	207865034
SNP	Het	t	a	CR1	Intron	207867668
SNP	Het	a	c	CR1	Intron	207874755
DEL	Het		aga	CR1	Intron	207875123
SNP	Het	g	a	CR1	Intron	207875983
SNP	Het	t	g	CR1	Intron	207876827
SNP	Het	t	c	CR1	Intron	207877297
MNP	Het	tg	ca	CR1	Intron	207877300
SNP	Het	a	g	CR1	Intron	207884232
SNP	Het	g	t	CR1	Intron	207891178

Supplementary table 4

Chromosome position	dbSNP	Pools	% of reads with consensus sequence	position
207629417	rs311302	HESN	0	intron
207629417	rs311302	HIV-1	52	intron
207629515	rs311303	HESN	0	intron
207629515	rs311303	HIV-1	45	intron
207629703	rs311304	HESN	0	intron
207629703	rs311304	HIV-1	56	intron
207630445	rs311306	HESN	0	intron
207630445	rs311306	HIV-1	51	intron
207631159	rs12140602	HESN	100	intron
207631159	rs12140602	HIV-1	63	intron
207631206	rs12139795	HESN	100	intron
207631206	rs12139795	HIV-1	60	intron
207631779	rs311307	HESN	0	intron
207631779	rs311307	HIV-1	59	intron
207634671	rs17044576	HESN	100	intron
207634671	rs17044576	HIV-1	76	intron
207635574	rs311310	HESN	0	intron
207635574	rs311310	HIV-1	60	intron
207635790	rs1567190	HESN	0	intron
207635790	rs1567190	HIV-1	100	intron
207636209	rs3767934	HESN	100	intron
207636209	rs3767934	HIV-1	51	intron
207639696	rs311311	HESN	0	intron
207639696	rs311311	HIV-1	58	intron
207640346	rs1032980	HESN	0	intron
207640346	rs1032980	HIV-1	59	intron
207640674	rs1270549	HESN	100	intron
207640674	rs1270549	HIV-1	54	intron
207641024	rs1032979	HESN	100	intron
207641024	rs1032979	HIV-1	43	intron
207641841	rs2063143	HESN	100	intron
207641841	rs2063143	HIV-1	55	intron
207642296	rs1507764	HESN	100	intron
207642296	rs1507764	HIV-1	52	intron
207643674	rs568515	HESN	100	intron
207643674	rs568515	HIV-1	41	intron
207643950	rs10863322	HESN	0	intron
207643950	rs10863322	HIV-1	100	intron
207645283	rs511678	HESN	100	intron
207645283	rs511678	HIV-1	45	intron
207645744	rs2940253	HESN	100	intron
207645744	rs2940253	HIV-1	46	intron
207645896	rs9429774	HESN	100	intron
207645896	rs9429774	HIV-1	44	intron
207645957	rs9429936	HESN	100	intron
207645957	rs9429936	HIV-1	40	intron

Resultados/Results

Supplementary table 4

Chromosome position	dbSNP	Pools	% of reads with consensus sequence	position
207646323	rs1048971	HESN	100	synonymous
207646323	rs1048971	HIV-1	20	synonymous
207646463	rs17615	HESN	100	Non synonymous
207646463	rs17615	HIV-1	49	Non synonymous
207646899	rs4308977	HESN	100	Non synonymous
207646899	rs4308977	HIV-1	47	Non synonymous
207646924	rs17616	HESN	100	Non synonymous
207646924	rs17616	HIV-1	48	Non synonymous
207651836	rs9429778	HESN	100	intron
207651836	rs9429778	HIV-1	46	intron
207651936	rs4844596	HESN	0	intron
207652348	rs61547182	HIV-1	59	intron
207652993	rs4317805	HESN	0	intron
207652993	rs4317805	HIV-1	10	intron
207653107	rs2182909	HESN	100	intron
207653107	rs2182909	HIV-1	45	intron
207653179	rs12032512	HESN	85	intron
207653179	rs12032512	HIV-1	40	intron
207653365	rs17258982	HESN	80	Non synonymous
207653365	rs17258982	HIV-1	100	Non synonymous
207653396	rs6540433	HESN	19	Non synonymous
207653396	rs6540433	HIV-1	15	Non synonymous
207653445	rs17258996	HESN	100	intron
207653445	rs17258996	HIV-1	51	intron
207653794	rs61023517	HESN	100	intron
207653794	rs61023517	HIV-1	61	intron
207653815	rs12117916	HESN	100	intron
207653815	rs12117916	HIV-1	62	intron
207654275	rs57032478	HESN	100	intron
207654275	rs57032478	HIV-1	55	intron
207655194	rs17186771	HESN	100	intron
207655194	rs17186771	HIV-1	50	intron
207655473	rs11117884	HESN	40	intron
207655816	rs1041250	HIV-1	48	intron
207656051	rs6690215	HESN	71	intron
207656051	rs6690215	HIV-1	45	intron
207656503	rs7543913	HESN	100	intron
207656503	rs7543913	HIV-1	48	intron
207656606	rs6665881	HESN	45	intron
207656606	rs6665881	HIV-1	100	intron
207656910	rs35426473	HESN	53	intron
207656910	rs35426473	HIV-1	55	intron
207658146	rs12021671	HESN	41	intron
207658146	rs12021671	HIV-1	43	intron
207658508	rs1977465	HESN	34	intron
207658508	rs1977465	HIV-1	50	intron

Supplementary table 4

Chromosome position	dbSNP	Pools	% of reads with consensus sequence	position
207660072	rs2182911	HESN	24	intron
207660072	rs2182911	HIV-1	20	intron
207660345	rs2182912	HESN	14	intron
207660345	rs2182912	HIV-1	17	intron
207660379	rs2182913	HESN	43	intron
207660379	rs2182913	HIV-1	30	intron
207661290	rs7519408	HESN	0	intron
207661290	rs7519408	HIV-1	22	intron
207661486	rs76484450	HESN	53	intron
207661486	rs76484450	HIV-1	100	intron
207661641	rs4618970	HESN	0	intron
207661641	rs4618970	HIV-1	22	intron

Artículo 2/Research paper 2:

Common haplotypes in *CD209* promoter and susceptibility to HIV-1 infection intravenous drug users. (*Submitted*)

**Common haplotypes in CD209 promoter and susceptibility to HIV-1 infection
intravenous drug users**

Rocio Herrero¹, Juan Antonio Pineda², Antonio Rivero-Juarez³, Meriem Echbarhi^{1, 5}, Luis-Miguel Real², Angela Camacho³, Juan Macias², Joan Fibla⁴, Antonio Rivero³ and Antonio Caruz^{1§}

¹Immunogenetics Unit, Department of Experimental Biology, University of Jaen, 23071, Jaen, Spain.

²Infectious Diseases and Microbiology Clinical Unit. Valme Hospital, 41014, Seville, Spain.

³Maimonides Institute for Research in Biomedicine of Cordoba (IMIBIC)/Reina Sofia University Hospital, 14004, Cordoba, Spain.

⁴Human Genetics Unit, Department of Basic Medical Sciences, University of Lleida IRBLleida, 25003, Lleida, Catalonia, Spain.

⁵Present address: Department of Chemistry and Molecular Biology, University of Gothenburg, S-405 30 Gothenburg, Sweden.

[§]Corresponding author.

Corresponding author:

Antonio Caruz, PhD. Immunogenetics Unit, University of Jaen, Campus Las Lagunillas SN, 23071, Jaén, Spain. Telephone: +34 606352046. Fax: +34 953211875. e-mail: caruz@ujaen.es

Email addresses of authors:

RH: rocio.herrero.delrio@gmail.com

JAP: japineda@telefonica.net

ARJ: arjvet@gmail.com

ME: echbarhi.meriem@gmail.com

LMR: lmreal67b@gmail.com

AnC: acamachoespejo@gmail.com

JM: juan.macias.sanchez@gmail.com

JF: joan.fibla@cmb.udl.cat

AR: ariveror@gmail.com

AC: caruz@ujaen.es

Key words: HIV-1, CD209, DC-SIGN, HCV, HESN, Polymorphism, Haplotypes, IDU

1 Abstract

2 **Introduction:** CD209 is a receptor expressed on dendritic cells involved in recognition of
3 oligosaccharides present in several pathogens with huge impact on human health. An SNP
4 located in the promoter region has been associated with HIV-1 susceptibility although it has
5 not been replicated in other populations. The objective of this study is to evaluate the
6 association of CD209 promoter haplotypes of with risk of HIV-1 infection in a cohort of male
7 Spanish injection drug users (IDU) infected with hepatitis C virus (HCV) and characterize the
8 phenotypic effects of the associated variants.

9 **Methods:** We genotyped 4 SNPs of CD209 promoter in 295 HCV males exposed to HIV-1
10 infection by IDU, 165 HIV-1 infected and 130 exposed uninfected (EUI) and 142 healthy
11 controls (HC). We have cloned the promoter variants in a reporter vector and evaluated the
12 promoter activities in a cell culture model, CD209 mRNA were quantified in PBMC.

13 **Results:** Single-marker analysis revealed no significant allelic association with risk of HIV-1
14 infection by parenteral route. Nevertheless, we have identified one haplotype that is
15 significantly overrepresented in EUI compared with HIV-1 positive and associated with
16 protection ($P=0.0008$; OR: 0.43). Functional experiments suggested that promoter variants
17 including the protective haplotype displayed lower transcriptional activity *in vitro* ($P<0.001$)
18 and was associated with lower CD209 mRNA expression in PBMC ($P=0.014$).

19 **Conclusions:** This study suggests that the promoter haplotypes of CD209 influence the risk
20 of HIV-1 acquisition in IDU and this association is correlated with the mRNA expression
21 levels.

22

23 Introduction

24 DC-SIGN or CD209 is an adhesion receptor expressed on dendritic cells (DCs) that
25 mediates the interaction between DCs and resting T cells through ICAM-2/3 [1] [2]. The
26 encoded protein is also involved in recognizing mannose N-linked oligosaccharides [3], an
27 evolutionary selected structures present in polyphyletic virus families with a huge impact on
28 human health as Retroviruses, Flavivirus, Filovirus, and Herpesvirus [4]. Alternative splicing
29 results in multiple variants some of them are probably secreted or intracellular isoforms [5,6].
30 Two SNPs in CD209 promoter (-336G and -871A) have been previously associated to
31 tuberculosis susceptibility [7]. The -336G allele has been found to confer protection against
32 HIV-1 in Caucasians [8] while other studies observed the opposite effect in Indians [9] and no
33 effect in Thais or Africans [8,10]. Despite continuous daily high-risk behavior and multiple
34 exposures to blood as consequence of needle sharing during drug consumption, most of
35 injection drug users (IDU) in Spain become co-infected with HCV and HIV-1. Nevertheless a
36 small subgroup of them, identified as exposed uninfected (EUI) remains HIV-1 seronegative
37 despite of markers of HCV infection. Although significant genetic association was found with
38 CCR5 genotype when compared the EUI vs. HIV-1 infected individuals [11,12], the majority
39 of EUI individuals harbor wild type CCR5 alleles. In the present work, we have examined the
40 contribution of 4 polymorphisms in the promoter region of CD209 gene to the susceptibility to
41 HIV-1 infection. We have identified differences in haplotype prevalence between 2 extreme
42 populations, HIV-1 exposed uninfected and HIV-1–infected subjects, from which we infer that
43 CD209 haplotypes might influence the risk of HIV-1 acquisition. Functional experiments
44 revealed that CD209 promoter harboring the protective haplotype displays lower
45 transcriptional activity in a cell culture model, and lower expression in PBMCs suggesting
46 that reduced expression of this receptor can increase the resistance to HIV-1 infection in
47 IDUs.

48

49 Methods

50 Patients and controls

51 We recruited 295 white males exposed to HIV-1 infection by injection drug use (IDU), both
52 infected (165 subjects) and at-risk HIV-1 uninfected (130 subjects), enrolled in prospective
53 cohort studies in Spain (Arnau de Vilanova Hospital, Lleida; Valme Hospital, Sevilla and
54 Reina Sofía Hospital, Córdoba) who had shared needles for >3 months. The main
55 epidemiological and clinical characteristics of the series studied were described previously
56 [11] [12,13]. Concurrent markers of hepatitis C virus (HCV) infection, one of the most
57 prevalent blood borne virus, was present in 100% of EUI and HIV-1 positive subjects. These
58 values are significantly higher than the reported HCV prevalence of 1%–2% for the general
59 population in Spain. In addition, a group of 142 healthy white males recruited from
60 anonymous blood donors from City of Jaen Hospital testing HIV-1 and HCV negative was
61 used as healthy controls (HC). HIV-1 and HCV diagnostics were performed as previously
62 described [11]. A subset of 140 HIV/HCV coinfectd patients received Peg-IFN alfa-2a at a
63 dose of 180 µg once per week or Peg-IFN alfa-2b at a dose of 1.5 µg/kg once per week, both
64 in combination with RBV at a daily dose of 800 mg to 1200 mg. Subjects harboring HCV
65 genotype 2-3 received HCV therapy during 24 weeks, if showed undetectable plasma HCV-
66 RNA load at week 4. Therapy was given for 48 weeks in the remaining patients. At weeks 12
67 and 24, HCV therapy was prematurely discontinued in non-responders.

68 **Ethical approval:** The study was designed and performed according to the Helsinki
69 declaration and was approved by the Ethics Committee of the three participating hospitals.
70 All patients provided written informed consent to participate in this study.

71 Polymorphism identification and genotyping

72 DNA was extracted from frozen whole blood using the Quick Pure Blood DNA extraction Kit
73 (Macherey-Nagel). The polymorphic markers located in the CD209 genomic region:
74 rs2287886, rs4804803, rs735239, rs735240 (positions -139, -336, -871 and -939

75 respectively) were genotyped using commercial TAQMAN genotyping assay (Applied
76 Biosystems), according to the manufacturer's instructions on the MX3005 thermocycler
77 (Stratagene). All subjects were also genotyped for the rs333 (Δ 32) polymorphism in the
78 CCR5 gene. Two EU1 were homozygous for CCR5 Δ 32 polymorphism and were not included
79 in this study, CCR5 Δ 32 polymorphism was determined as previously described [12].

80 **Biostatistics and bioinformatics**

81 Hardy-Weinberg equilibrium and pairwise linkage disequilibrium (D') were calculated using
82 Haploview V4.1 software. Block structure was considered for marker pairs showing $D' > 0.8$,
83 following the solid-spine block definition. Single marker and haplotype frequencies were
84 estimated and compared using PLINK software. Single-marker and haplotype association P
85 values were corrected for multiple testing following the SNP spectral decomposition
86 approach [14], a P value < 0.012 was considered statistically significant. The Student's t -test
87 and the Mann-Whitney U were used for comparing continuous variables.

88 **CD209 promoter variants cloning and functional assays**

89 A 1.4 Kb fragment of CD209 promoter and 5'UTR was PCR amplified by nested PCR using
90 Long Expand PCR Kit (Roche) from genomic DNAs of two patients harboring the XAGA or
91 the XGAG haplotypes. The primers used were, DC-forward1: 5'AG
92 GTCATTGCCATGGCATCGGT3', DC-reverse1: 5'AGGAGTCCAGGAGTCTCTCGTC3', DC-
93 forward2: 5'TACCTCATTACCATGTGAAATG3', DC-reverse2: 5'GGCTGGCCATCCCTTCC
94 CCCTT3'. The PCR products were cloned in the plasmid pSC-A-amp/kan using the
95 Strataclone PCR cloning kit (Stratagene). The fidelity of PCR amplifications and subcloning
96 were confirmed by Sanger DNA sequencing. The eGFP (destabilized protein variant)
97 reporter plasmids were constructed by digestion of the pSC-A-AGA and pSC-A-GAG
98 plasmids with the restriction enzymes Sac-I and Kpn-I and the products ligated to the
99 promoterless pd2EGFP-1 plasmid (Clontech).

100 **Cells, transient transfection, flow cytometry and beta-galactosidase assays**

101 LC5 and THP-1 cells were propagated and transfected as previously described [15]. The
102 promoter activities were measured by flow cytometry (Epics Elite XL, Coulter). In parallel, the
103 cells were washed with PBS and processed for β -Galactosidase Gene chemiluminescent
104 Assay (Roche) and Bradford Protein Assay (Bio-Rad). The transfection efficiency was
105 calculated after normalization by chemiluminescent β -Galactosidase and μ g of protein. The
106 experiments were repeated three times in independent days. The CD209 promoter
107 expression plasmids names were codified and researchers responsible for functional
108 experiments were blinded to the genotypes of the plasmids.

CD209 mRNA quantification

110 PBMC from healthy blood donors (City of Jaen, Blood Bank) were purified by centrifugation
111 in Ficoll gradient, DNA was extracted as previously described and genotyped for the four
112 SNPs of CD209 promoter. Six individuals harboring XAGA haplotype and six without this
113 haplotype were randomly selected for CD209 gene expression analyses. From each sample,
114 total RNA from 5×10^6 PBMCs was isolated using Tripure reagent (Roche), quantified by
115 spectrophotometry and dissolved at 1 μ g/ μ l. Reverse transcription of RNA was performed
116 using the Superscript III (Invitrogen), following manufacturer's instructions. All the RNA
117 samples were also subjected to control reactions without reverse transcriptase to check for
118 contamination. The normalized CD209 expression were determined in a multiplexed, one
119 tube PCR reaction using the human β -actin as endogenous control (Applied Biosystems) and
120 CD209 Gene Expression Assays (Assay ID: Hs01588349_m1, Applied Biosystems). Each
121 donor cDNA were amplified in duplicate wells, and repeated in two independent days.
122 Parallel negative control experiments were done with reverse transcriptase negative cDNAs.
123 The relative expression of CD209 mRNAs compared to endogenous β -actin controls were
124 calculated using the MX-pro V4.0 software (Stratagene) according to manufacturer's
125 instructions.

126

Results

Single marker analysis

We selected four previously described polymorphic markers within CD209 promoter for association analysis rs2287886, rs4804803, rs735239, rs735240 (positions -139, -336, -871 and -939 respectively). They are in linkage disequilibrium our series ($D' > 0.85$, $P < .001$ for 828 chromosomes). We found no deviation from Hardy-Weinberg equilibrium for these markers in our population (whole series, $P > .02$) or any case or control subgroup ($P > .0125$) (Table 1). The observed allelic frequencies in our series are similar to previously reported data for series of European ancestry (www.ncbi.nlm.nih.gov/snp). Single genetic marker association studies between all individuals with HIV-1 infection versus EUI or HC are summarized in table 1. These analyses revealed no evidences for association in HIV-1 compared to EUI or HC (table 1). If all the HIV-1 negative individuals were considered as a single group, a tendency to significance was found for the rs4804803 (position -336; $P = 0.07$).

Table 1: CD209 allelic distribution in EUI, HIV-1 and healthy controls (HC). Fisher's exact test. HW: Hardy-Weinberg Equilibrium. EUI: Exposed Uninfected, HC: Healthy Controls.

SNP (position)	HW <i>P</i> value	Alleles	Major allele frequency				<i>P</i> (HIV-1 positive vs negative)
			Global	EUI	HC	HIV positive	
rs2287886 (-139)	0.6	G:A	0.70	0.74	0.69	0.68	0.25
rs4804803 (-336)	1	A:G	0.76	0.75	0.73	0.78	0.07
rs735239 (-871)	0.92	A:G	0.64	0.61	0.66	0.66	0.28
rs735240 (-939)	0.48	G:A	0.55	0.53	0.58	0.56	0.86

Haplotype analyses

At the haplotype level the global distribution of frequencies in the three groups is represented in table 2.

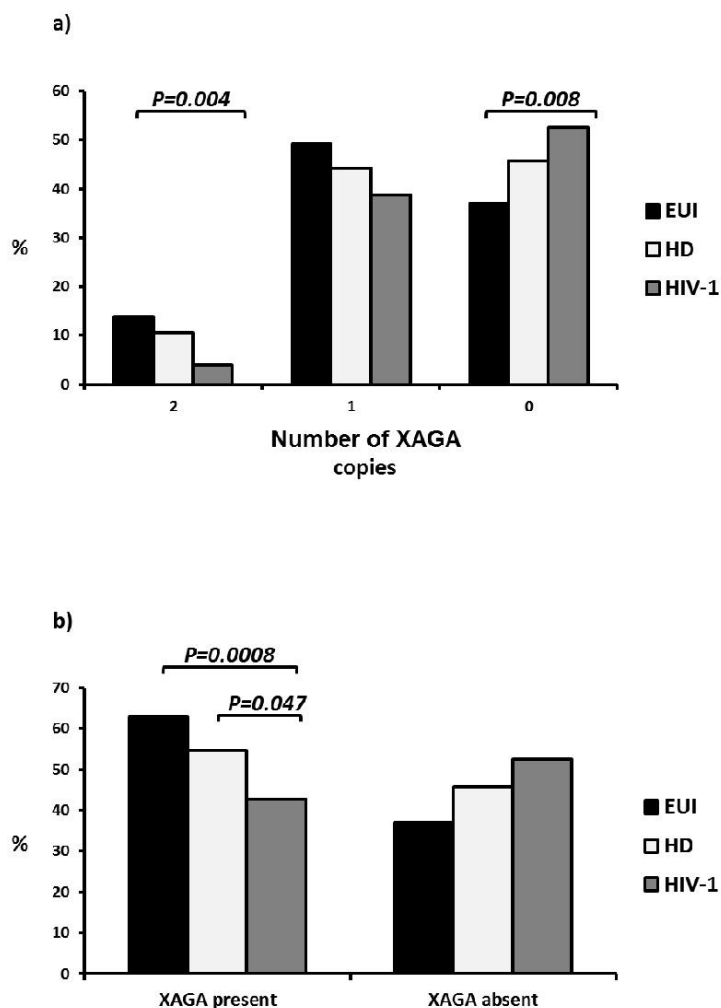
Resultados/Results

Table 2: CD209 haplotype frequencies in EUI, HIV-1 and healthy controls (HC), including the SNPs rs4804803, rs735239, rs735240 only haplotypes with a frequency over 5%. EUI: Exposed Uninfected, HC: Healthy Controls.

Haplotype	EUI	HC	HIV-1
AGA	0.38	0.32	0.28
AAA	0.08	0.08	0.13
GAG	0.24	0.25	0.20
AAG	0.26	0.31	0.32

We identified a protective haplotype and dominant and recessive models were tested to the different haplotype combinations. The haplotype XAGA containing the three markers (rs4804803, 735239, rs735240) turned out to be the main genotype block responsible for such discordant frequency distribution (Figure 1).

Two copies of this haplotype was found to be associated with the EUI group and protection from HIV-1 infection ($P=0.004$; OR: 0.25 [0.09-0.66]). But the highest level of statistical significance was found when a dominant model was tested, XAGA carriers vs. non carriers ($P=0.0008$; OR: 0.43 [0.27-0.70]). The association with this haplotype remained significant even after correction for multiple testing using SNP spectral decomposition approach (threshold level $P<0.012$). A borderline significant differences were observed when dominant model were tested in healthy individuals group vs. HIV-1 positive group ($P=0.047$). No other haplotypes with frequency over 1% displayed significant association with the tested phenotype (Figure 1).



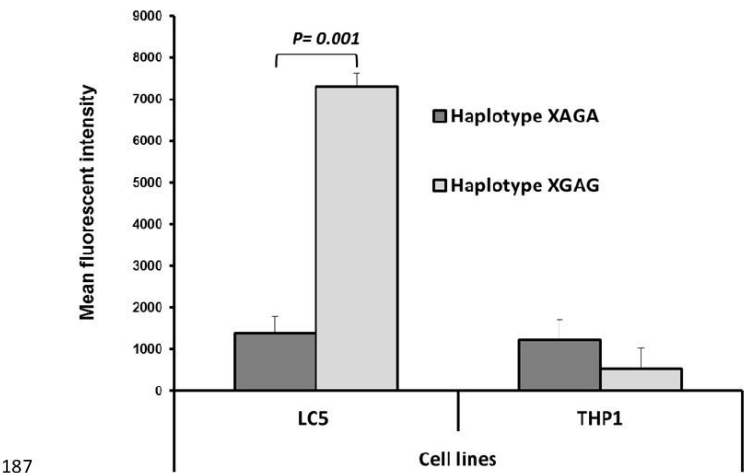
166

167 **Figure 1:** CD209 promoter haplotypes and susceptibility to parenterally HIV-1 acquired
 168 infection. a) Comparison of number of copies (2, 1 and 0) of XAGA haplotypes (%) in EUI,
 169 healthy controls (HC) and HIV-1. Fisher's exact test. P values in *italics* were significant after
 170 correction by multiple testing (SNP spectral decomposition approach). b) Comparison of the
 171 presence vs. absence of the XAGA haplotype (dominant model).
 172

173

174 **Functional characterization**

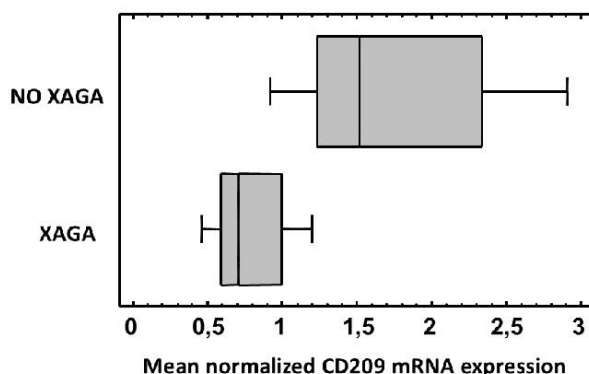
175 With the purpose of characterize the functional effects of these promoter variants in basal
176 transcription of CD209; we have cloned approximately 1400 bp of the promoter region of a
177 XAGA and XGAG variants in a GFP-based reporter plasmid. These constructions were
178 transfected in two independent cell lines, THP1 and LC5. The basal promoter activity
179 displayed cell-dependent transcriptional differences, the LC5 cells showed higher levels of
180 promoter activity compared to THP-1 cells (Figure 2). The protective XAGA promoter
181 haplotype promoter exhibited significantly lower normalized activity compared to the
182 reference haplotype XGAG (mean fluorescent intensity 7305[SD±325] vs. 1390 [SD±400],
183 $P<0.001$, Student T test) but only in LC5 cells. In THP-1 cells we do not observed statistically
184 significant differences 1237[SD±480] vs. 524[SD±500] between both haplotypes ($p>0.1$)
185 (Figure 2). There were no differences in transfection efficiency measured by β -galactosidase
186 activity between XAGA and XGAG plasmids in both cell types. ($p>0.2$).



187
188 **Figure 2:** Flow cytometry analysis of CD209 promoter haplotypes activities (mean
189 fluorescent intensity) in transfected LC5 and THP1 cells, the graphics represents the mean of
190 three independent experiments.
191

192

193 Next, we validated whether the observed effect could be associated with differential
 194 expression of CD209 mRNA in PBMC from healthy donors. Six XAGA positive vs. XAGA
 195 absent samples were examined by Taqman real-time quantitative PCR. The overall
 196 expression levels of CD209 mRNA in PBMC were low, with the Ct values over the 30 cycles.
 197 The relative amount of the XAGA containing haplotypes normalized to β -actin revealed a
 198 lower level of expression compared to the other haplotypes (No XAGA mean 1,73 95% IC
 199 [0,96; 2,51], XAGA mean 0,77 [0,48; 1,07] ($P=0.014$, Mann-Whitney U) (Figure 3). These
 200 data indicate that the expression of CD209 is reduced in PBMC from XAGA carriers under
 201 our experimental conditions.



202

203 **Figure 3:** CD209 mRNA quantification normalized with β -actin in PBMC according to the
 204 presence or absent of XAGA haplotype ($P=0.014$, Mann-Whitney U).
 205

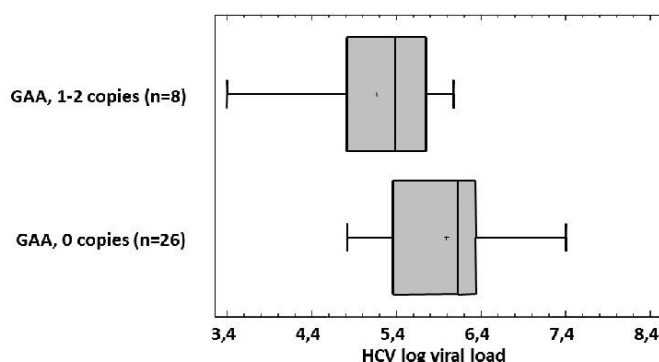
206

207

208 **Impact of CD209 promoter polymorphisms in HCV infection**

Resultados/Results

We have explored the potential impact of CD209 polymorphisms in the clinical course of HCV infection in 140 HIV-1/HCV coinfecting patients. None of the CD209 SNPs or haplotypes appeared to be associated with HCV pretreatment viral load neither with sustained viral response to interferon plus ribavirin in patients infected with HCV genotypes 1 or 4. However, in patients infected with HCV genotypes 2-3 the presence of the haplotype GAA (including the SNPs rs2287886, rs4804803 and rs735239) was associated with pretreatment viral load 5.16 [4.45-5.88] vs. 5.99 [5.70-6.27] ($P=0.009$, Student T test) (Figure 4).



216

Figure 4: Association between CD209 haplotype GAA and HCV viral load in genotype 2-3 infected patients ($P=0.009$, Student T test)

219

Discussion

The role of CD209 promoter polymorphisms in HIV-1 infection susceptibility has been analyzed in three previous studies [8–10] although a clear heterogeneity exists among them. One study [8] claimed that -336G allele would confer susceptibility to HIV-1 infection by the parenteral route in individuals of European ancestry, however although the -336G allele is quite frequent in African-Americans, no significant association with risk of HIV infection was

226 detected in this group [8]. Other paper found that the same allele would confer protection [9]
227 and another one reported no effect [10]. Having these conflicting results, the involvement of
228 CD209 promoter polymorphisms in HIV-1 infection remained controversial to date. In the
229 present study, our single-marker analysis does not confirm in the Spanish population the
230 previously reported association of HIV-1 infection by the parenteral route for the -336 SNP in
231 Europeans.

232 Discordance among our results and those previously reported could be explained if the
233 haplotype structure of CD209 in the Spanish population is closer to Africans than to
234 Europeans, the effect detected in Europeans is not probably due directly to -336 SNP but
235 rather to another functional SNP that is in linkage disequilibrium with -336 only in Europeans.
236 Other possibility is that the sex might have an influence in CD209 phenotypes; this option
237 cannot be ruled out because our experimental strategy has included only men in order to
238 reduce the immunological variation of the series and is well known the influence of sex
239 hormones on dendritic cell activity [16]. In parallel, we cannot exclude that HCV infection
240 could be an important modifier factor influencing the resistance to HIV-1 infection in IDU.
241 Chronic HCV infection blocks the ability of dendritic cells to secrete IFN- α and T-cell
242 proliferation [17]. HCV core and NS3 proteins inhibit the expression of CD1 and CD209
243 during DCs differentiation, while markers of macrophages (CD14 and HLA-DR) are
244 increased [18] and the G allele of rs4804803 of CD209 has been found associated with
245 higher liver fibrosis and transaminases in HCV infection [17]. In our cohort of HIV-1/HCV
246 coinfecting patients, we have observed an association between CD209 polymorphisms and
247 HCV viral load in genotype 2-3 infected individuals, suggesting a potential genetic interaction
248 between HCV and CD209.

249 The -336G allele has been identified as a marker of tuberculosis susceptibility in a South-
250 African cohort [7] but as a protector in other African cohorts [19], as well as a susceptibility
251 factor in Flavivirus infections [20,21]. Regarding -871 marker no previous studies have
252 analyzed its impact in HIV-1 infection. A previous work have reported association of the -

253 871A allele with tuberculosis susceptibility [7] but results were not confirmed in other
254 populations [19]. No indication of association with HIV-1 susceptibility was found for this
255 isolate marker, although the region including the -871 marker appeared to be an important
256 genetic signal correlated with infectious diseases susceptibility. Remarkably, the XAGA
257 haplotype, identified here as a protective for HIV-is also protective for tuberculosis [7] and
258 associated with lower levels of transcription and mRNA expression.

259 This study has several limitations; first, our phenotypic analysis was limited to global CD209
260 mRNA expression in PBMC not in dendritic cells. Second, the mRNA expression was
261 measured in healthy donors not in HCV or HCV/HIV infected patients. And third, this gene
262 has at least 11 protein-coding transcript variants, the molecular phenotype of the genetic
263 association reported here, could result from haplotype-specific mRNA splicing pattern.

264 Interestingly, CD209 promoter polymorphisms have been associated with vaccine-induced
265 cytokine secretion in Measles Virus and specific neutralizing antibody levels in Measles and
266 Rubella Virus vaccinees [22,23]24], probably due to an enhanced CD209 expression
267 correlated with improved antigen capture by DC. We hypothesize that CD209 haplotypes
268 might be major pharmacogenetic trait influencing the response to HIV-1 vaccination. In this
269 context, we would expect that the haplotype having lower promoter activity would be
270 associated with impaired immunological response to vaccine antigens that interact with
271 CD209.

272 Conclusions

273 The present study suggests that CD209 promoter haplotypes are associated with protection
274 from HIV-1 infection through parenteral route; additionally our data suggest that this
275 association is correlated with decreased CD209 mRNA expression and lower promoter
276 activity.

277

278 **Competing interest:** The authors declare no conflicts of interest.

279 **Acknowledgements**

280 Funding: This study was supported by the grants from the Consejería de Salud de la Junta
281 de Andalucía (PI-0335/2009, PI-0118-2013, PI-0481-2012 and AC-0095-2013), Gilead
282 (GLDL13-00145), the Ministerio de Sanidad (EC11-2086, PI021476 and PI10/01232), the
283 Red de Investigación en SIDA (ISCIII-RETIC RD06/006 and RD12/0017), the Fundació
284 Marató TV3 (020730 and 020732), the Universidad de Jaén (2015/00064/001,
285 2014/00026/001) and Consejería de Economía, Innovación Ciencia y Empleo de la Junta de
286 Andalucía (2014/00210/001). JAP has received a research extension grant from the
287 Programa de Intensificación de la Actividad de Investigación del Servicio Nacional de Salud
288 Carlos III (I3SNS). JM is the recipient of a grant from the Servicio Andaluz de Salud de la
289 Junta de Andalucía (B-0037).

290

291 **Author's contributions**

292 AC, RH and JF contributed to this study's concept. RH and ME performed the genetic
293 analysis. RH completed the mRNA expression experiments, CD209 promoter cloning,
294 transfections and reporter gene analysis. ARJ, AR, AnC, JAP, LMR, JM, and JF. followed up
295 patients enrolled in the study. AC, JF and RH contributed to the analysis and interpretation of
296 the data. AC and JF contributed to writing the article. All authors contributed to the critical
297 review of the article.

298

299

References

1. Geijtenbeek TB, Torensma R, van Vliet SJ, van Duijnhoven GC, Adema GJ, van Kooyk Y, et al. Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. *Cell*. 2000;100(5):575–85.
2. van Kooyk Y, Geijtenbeek TBH. A novel adhesion pathway that regulates dendritic cell trafficking and T cell interactions. *Immunol Rev*. 2002;186:47–56.
3. Pomerantz RJ, Feinberg MB, Trono D, Baltimore D. Lipopolysaccharide is a potent monocyte/macrophage-specific stimulator of human immunodeficiency virus type 1 expression. *J Exp Med*. 1990;172(1):253–61.
4. Khoo U-S, Chan KYK, Chan VSF, Lin CLS. DC-SIGN and L-SIGN: the SIGNs for infection. *J Mol Med (Berl)*. 2008;86(8):861–74.
5. Hladik F, Liu H, Speelman E, Livingston-Rosanoff D, Wilson S, Sakchalathorn P, et al. Combined effect of CCR5-Delta32 heterozygosity and the CCR5 promoter polymorphism -2459 A/G on CCR5 expression and resistance to human immunodeficiency virus type 1 transmission. *J Virol*. 2005;79(18):11677–84.
6. Bleiber G, May M, Martinez R, Meylan P, Ott J, Beckmann JS, et al. Use of a combined ex vivo/in vivo population approach for screening of human genes involved in the human immunodeficiency virus type 1 life cycle for variants influencing disease progression. *J Virol*. 2005;79(20):12674–80.
7. Barreiro LB, Neyrolles O, Babb CL, Tailleux L, Quach H, McElreavey K, et al. Promoter variation in the DC-SIGN-encoding gene CD209 is associated with tuberculosis. *PLoS Med*. 2006;3(2):e20.
8. Martin MP, Lederman MM, Hutcheson HB, Goedert JJ, Nelson GW, van Kooyk Y, et al. Association of DC-SIGN promoter polymorphism with increased risk for parenteral,

- 325 but not mucosal, acquisition of human immunodeficiency virus type 1 infection. *J Virol.*
326 2004;78(24):14053–6.
- 327 9. Selvaraj P, Alagarasu K, Swaminathan S, Harishankar M, Narendran G. CD209 gene
328 polymorphisms in South Indian HIV and HIV-TB patients. *Infect Genet Evol.*
329 2009;9(2):256–62.
- 330 10. Wichukchinda N, Kitamura Y, Rojanawiwat A, Nakayama EE, Song H, Pathipvanich P,
331 et al. The polymorphisms in DC-SIGNR affect susceptibility to HIV type 1 infection.
332 *AIDS Res Hum Retroviruses.* 2007;23(5):686–92.
- 333 11. de la Torre MS, Torres C, Nieto G, Vergara S, Carrero AJ, Macías J, et al. Vitamin D
334 Receptor Gene Haplotypes and Susceptibility to HIV-1 Infection in Injection Drug
335 Users. *J Infect Dis.* 2008;197(3):405–10.
- 336 12. Real LM, Herrero R, Rivero-Juárez A, Camacho Á, Macías J, Vic S, et al. IFNL4
337 rs368234815 polymorphism is associated with innate resistance to HIV-1 infection.
338 *AIDS.* 2015;29(14):1895–7.
- 339 13. Herrero R, Real LM, Rivero-Juárez A, Pineda JA, Camacho Á, Macías J, et al.
340 Association of complement receptor 2 polymorphisms with innate resistance to HIV-1
341 infection. *Genes Immun.* 2015;16(2):134–41.
- 342 14. Nyholt DR. A simple correction for multiple testing for single-nucleotide polymorphisms
343 in linkage disequilibrium with each other. *Am J Hum Genet.* 2004;74(4):765–9.
- 344 15. García-Morúa C, Alonso-Lobo JM, Rueda P, Torres C, González N, Bermejo M, et al.
345 Functional Characterization of SDF-1 Proximal Promoter. *J Mol Biol.* 2005;348(1):43–
346 62.
- 347 16. Meier A, Chang JJ, Chan ES, Pollard RB, Sidhu HK, Kulkarni S, et al. Sex differences
348 in the Toll-like receptor-mediated response of plasmacytoid dendritic cells to HIV-1.

- 349 Nat Med. 2009;15(8):955–9.
- 350 17. Ryan EJ, Stevenson NJ, Hegarty JE, O’Farrelly C. Chronic hepatitis C infection blocks
351 the ability of dendritic cells to secrete IFN- α and stimulate T-cell proliferation. J Viral
352 Hepat. 2011;18(12):840–51.
- 353 18. Tu Z, Hamalainen-Laanaya HK, Nishitani C, Kuroki Y, Crispe IN, Orloff MS. HCV core
354 and NS3 proteins manipulate human blood-derived dendritic cell development and
355 promote Th 17 differentiation. Int Immunol. 2012;24(2):97–106.
- 356 19. Vannberg FO, Chapman SJ, Khor CC, Tosh K, Floyd S, Jackson-Sillah D, et al.
357 CD209 genetic polymorphism and tuberculosis disease. PLoS One. 2008;3(1):e1388.
- 358 20. Sakuntabhai A, Turbpaiboon C, Casadémont I, Chuansumrit A, Lowhnoo T, Kajaste-
359 Rudnitski A, et al. A variant in the CD209 promoter is associated with severity of
360 dengue disease. Nat Genet. 2005;37(5):507–13.
- 361 21. Barkhash A V, Perelygin AA, Babenko VN, Brinton MA, Voevoda MI. Single nucleotide
362 polymorphism in the promoter region of the CD209 gene is associated with human
363 predisposition to severe forms of tick-borne encephalitis. Antiviral Res. 2012;93(1):64–
364 8.
- 365 22. Clifford HD, Richmond P, Khoo S-K, Zhang G, Yerkovich ST, Le Souëf PN, et al.
366 SLAM and DC-SIGN measles receptor polymorphisms and their impact on antibody
367 and cytokine responses to measles vaccine. Vaccine. 2011;29(33):5407–13.
- 368 23. Ovsyannikova IG, Haralambieva IH, Vierkant RA, O’Byrne MM, Jacobson RM, Poland
369 GA. The association of CD46, SLAM and CD209 cellular receptor gene SNPs with
370 variations in measles vaccine-induced immune responses: a replication study and
371 examination of novel polymorphisms. Hum Hered. 2011;72(3):206–23.
- 372 24. Haralambieva IH, Lambert ND, Ovsyannikova IG, Kennedy RB, Larrabee BR,
373 Pankratz VS, et al. Associations between single nucleotide polymorphisms in cellular

374 viral receptors and attachment factor-related genes and humoral immunity to rubella
375 vaccination. PLoS One. 2014 Jan;9(6):e99997.
376
377

Artículo 3/Research paper 3:

***IFNL4* rs368234815 polymorphism is associated with innate resistance to HIV-1 infection.**

Research Letters

AIDS 2015, 29:1895–1898

***IFNL4* rs368234815 polymorphism is associated with innate resistance to HIV-1 infection**

Luis M. Real^{1a}, Rocío Herrero^{2b}, Antonio Rivero-Juárez^{2c}, Ángela Camacho^{2c}, Juan Macías^{2a}, Sandra Vic^{2b}, Vincent Soriano^{2d}, Sergio Viedma^{2b}, Josep M. Guardiola^{2e}, Joan Fibla^{2f}, Antonio Rivero^{2c}, Juan A. Pineda^{2g} and Antonio Caruz^{2b}

The interferon (*IFN*)*L4* polymorphism rs368234815 is associated with hepatitis C virus (HCV) spontaneous clearance and response to IFN-based treatments. The role of this polymorphism in HIV-1 infection is controversial. We investigated whether genetic variation at *IFNL4* is associated to HIV-1 acquisition. The HCV protective allele TT was associated with decreased likelihood of HIV-1 infection in male intravenous drug users [odds ratio (OR): 0.3; $P = 0.006$], and this association was not modified by the genotype of *CCR5*. These results suggest that genetic susceptibility to HCV and HIV-1 infection shares common molecular pathways.

Type III IFNs induce antiviral activity through binding to the IFN-L-R1/interleukin (IL)10R2 receptor complex and activation of the JAK-STAT pathway [1]. This process leads to upregulation of IFN-stimulated genes including among others *IFIT1*, *MX1* and *OASL*. The complementary use of Genome Wide Association Studies and RNA-sequencing technologies allowed the identification of single nucleotide polymorphism within *IFNL4* gene, including the functional rs368234815 one, associated with both the spontaneous clearance of HCV and the response to the treatment against HCV [2–4].

Nevertheless, there is an apparently contradictory phenotype regarding this association. The protective allele of rs368234815 marker includes a frameshift polymorphism that introduces a premature stop codon that blocks the correct protein translation. Individuals harbouring the homozygous protective allele (TT/TT) are natural knockouts for this gene. In-vitro studies reported that the protective genotype induces higher levels of IL28B as well as interferon gamma-induced protein 10 [5]. These observations are paradoxical. It has been proposed that *IFNL4* pretreatment can induce IFNA desensitization or activate a genetic algorithm that impairs effective HCV eradication [1].

Although IFNL family members can inhibit HIV-1 infection of macrophages and T-cells *in vitro*, their effect

on the viral replication is limited *in vivo* [6,7]. In spite of this, several studies have analyzed the association between the *IFNL4* genetic markers and HIV-1 infection susceptibility and/or disease progression obtaining contradictory results [7–11]. For that reason, it is necessary to perform additional association studies using the functional polymorphism in order to clarify the role of this gene in the HIV-1 infection susceptibility. Therefore, the objective of this study was to analyze the association of the *IFNL4* rs368234815 polymorphism with the susceptibility to HIV-1 infection in a well characterized cohort of intravenous drug users including both highly exposed seronegative (HESN) and HIV-1-infected individuals.

We enrolled 401 HCV seropositive white men exposed to HIV-1 by injection drug use who had shared needles for more than 3 months. Among them, 213 men were HIV-1 seropositive and 188 individuals were HESN. All of them were 100% white Caucasian of Spanish ancestry. Three individuals homozygous for the D32 deletion in *CCR5* were excluded from this study. DNA purification and *IFNL4* polymorphism genotyping were performed as previously described [8]. Hardy–Weinberg equilibrium and genetic association were calculated using the online tool (<https://ihg.gsf.de/cgi-bin/hw/hwa1.pl>). This study was designed and performed according to the principles of the Helsinki Declaration and was approved by the Ethical Committee of the participating hospitals and the University of Jaen. All patients provided written informed consent to participate in this study. This cohort was enrolled in prospective studies aimed to study innate resistance to HIV-1 as well as pharmacogenetics of IFNA plus along with ribavirin in the context of HCV infection. The epidemiological and clinical characteristics of these patients were described in the previous studies [8–10].

The genotypic distributions of *IFNL4* rs368234815 were in accordance with the Hardy–Weinberg equilibrium law ($P > 0.05$) in both HESN and HIV-1-infected groups. The HCV-protective genotype rs368234815-TT/TT was significantly more frequent in the HESN individuals (TT/TT $n = 92$, TT/ ΔG $n = 85$, $\Delta G/\Delta G$ $n = 11$) compared with the HIV-1-infected patients (TT/TT $n = 88$, TT/ ΔG $n = 96$, $\Delta G/\Delta G$ $n = 29$); the frequency of TT/TT genotype was 0.49 vs. 0.41 [$P = 0.006$; OR: 0.36, 95% confidence interval: 0.1–0.7] (Table 1). The frequency of the well-known HIV-1 protective allele D32 in the HESN cohort were significantly higher compared with HIV-1-infected (0.11 vs. 0.6, $P = 0.005$). However, we did not detect interaction between *IFNL4* with *CCR5* (analysis of variance $P = 0.7$). In addition, the

Table 1. rs368234815 genotypic and allelic distribution in HIV-1-infected and highly exposed seronegative individuals.

Cohorts	Alleles TT ^a	Genotypes TT/TT	TT/ΔG	ΔG/ΔG
HIV-1, n (%)	272 (64)	88 (41)	96 (45)	29 (14)
HESN, n (%)	269 (72)	92 (49)	85 (45)	11 (6)
Comparisons	TT vs. ΔG	TT/TT vs. ΔG/ΔG	TT/TT vs. TT/ΔG	TT/TT + TT/ΔG vs. ΔG/ΔG
OR (95% CI)	0.70 (0.5–0.9)	0.36 (0.1–0.7)	0.42 (0.2–0.9)	0.39 (0.1–0.8)
P value	0.02	0.006	0.024	0.009

Data are genotype. CI, confidence interval; HESN, highly exposed seronegative; OR, odds ratio.

^aallele counts (%).

analysis restricted to homozygous *CCR5* wt patients gave rise to similar results (TT/TT vs. DG/DG, $P=0.005$; odds ratio: 0.30, 95% confidence interval: 0.13–0.73). The number of individuals harbouring both *IFNL4* GG and *CCR5* D32 were very limited and did not allow any statistical comparison (data not shown).

In a multiracial cohort at risk through parenteral as well as sexual routes, Martin *et al.* [11] did not find any relationship between the *IFNL4* rs12979860 genotypes and likelihood of acquiring HIV-1 infection neither the rate of progression to AIDS in seroconverters. Similar results were observed by Rallon *et al.* [12] in Spaniards and Salgado *et al.* [13] in HIV-1 elite controllers/suppressors of African American ancestry. On the contrary, Machmach *et al.* [14] studied the prevalence of *IFNL4* genotypes (rs12979860) among HIV controllers compared with HIV-1-infected progressors and found that this SNP was associated with spontaneous HIV control. The same group communicated the association of the *IFNL4* frameshift variant rs368234815-ΔG with higher prevalence of AIDS-defining illnesses and lower CD4 lymphocytes levels [15].

The use of the rs12979860 polymorphism as a genetic marker and the small sample size could be the reasons why other similar studies did not reach the same results. Thus, Martin *et al.* [11] analyzed the association of the rs12979860 with the susceptibility to HIV-1 infection in a sample containing a high percentage of African American patients (26%). The rs12979860 marker is in strong LD with the rs368234815 functional polymorphisms in Europeans and Asians ($r^2>0.85$) but not in Africans ($r^2=0.64$). This fact could reduce the statistical power to detect any association. Similarly, Rallon *et al.* [12] also obtained negative results probably because of the small sample size included in this study.

In conclusion, we found an association between the functional rs368234815 polymorphism within *IFNL4* gene and HIV-1 infection susceptibility. This finding suggests the existence of a shared innate resistance mechanism for HCV, HIV-1 and, probably, other viruses. More studies, including the analysis of the pattern of

HIV-1 restriction factors expression as well as in-vitro HIV-1 infection experiments in the context of protective and risk *IFNL4* genetic backgrounds, are necessary to shed light on the molecular mechanisms underlying this association.

Acknowledgements

This study was supported by grants from the Consejería de Salud de la Junta de Andalucía (PI-0335/2009, PI-0118-2013, PI-0481-2012 and AC-0095-2013), Gilead (GLDL13-00145), the Ministerio de Sanidad (EC11-2086, PI021476 and PI10/01232), the Red de Investigación en SIDA (ISCIII-RETIC RD06/006 and RD12/0017), the Fundació Marató TV3 (020730 and 020732), the Universidad de Jaén (2015/00064/001, 2014/00026/001) and Consejería de Economía, Innovación Ciencia y Empleo de la Junta de Andalucía (2014/00210/001). JAP has received a research extension grant from the Programa de Intensificación de la Actividad de Investigación del Servicio Nacional de Salud Carlos III (3SNS). JM is the recipient of a grant from the Servicio Andaluz de Salud de la Junta de Andalucía (B-0037).

LMR, RH, JAP and AC contributed to this study's concept. RH, SeV and SaV performed the genetic analysis. ARJ, AR, AnC, JAP, JM, LMR, JMG, VS and JF followed up patients enrolled in the study. LMR, AC, RH and SeV contributed to the analysis and interpretation of the data. LMR and AC contributed to writing the article. All authors contributed to the critical review of the article.

Conflicts of interest

There are no conflicts of interest.

^aInfectious Diseases and Microbiology Clinical Unit, Valme Hospital, Seville; ^bImmunogenetics Unit, Department of Experimental Biology, University of Jaén, Jaén; ^cMaimonides Institute for Research in Biomedicine of Cordoba (IMIBIC)/Reina Sofia University Hospital, Cordoba; ^dDepartamento de Enfermedades

Infecciosas, Hospital Carlos III, Madrid; ^eServicio de Medicina Interna, Hospital de la Santa Creu i Sant Pau, Barcelona; and ^fHuman Genetics Unit, Department of Basic Medical Sciences, University of Lleida IRBLleida, Lleida, Catalonia, Spain.

Correspondence to Antonio Caruz, PhD, Immunogenetics Unit, University of Jaen, Campus Las Lagunillas SN, 23071, Jaén, Spain.
Tel: +34 606352046; fax: +34 953211875;
e-mail: caruz@ujaen.es

Received: 12 February 2015; revised: 28 May 2015; accepted: 10 June 2015.

References

- O'Brien TR, Prokunina-Olsson L, Donnelly RP. IFN- λ 4: the Paradoxical New Member of the Interferon Lambda Family. *J Interferon Cytokine Res* 2014; **34**:829–838.
- Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature* 2009; **461**:399–401.
- Thomas DL, Thio CL, Martin MP, Qi Y, Ge D, O'Huigin C, et al. Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. *Nature* 2009; **461**:798–801.
- Prokunina-Olsson L, Muchmore B, Tang W, Pfeiffer RM, Park H, Dickensheets H, et al. A variant upstream of IFNL3 (IL28B) creating a new interferon gene IFNL4 is associated with impaired clearance of hepatitis C virus. *Nat Genet* 2013; **45**:164–171.
- Bibert S, Roger T, Calandra T, Bochud M, Cerny A, Semmo N, et al. IL28B expression depends on a novel TT/-G polymorphism which improves HCV clearance prediction. *J Exp Med* 2013; **210**:1109–1116.
- Liu MQ, Zhou DJ, Wang X, Zhou W, Ye L, Li JL, et al. IFN- λ 3 inhibits HIV infection of macrophages through the JAK-STAT pathway. *PLoS One* 2012; **7**:e35902.
- Tian RR, Guo HX, Wei JF, Yang CK, He SH, Wang JH. IFN- λ 3 inhibits HIV-1 integration and posttranscriptional events *in vitro*, but there is only limited *in vivo* repression of viral production. *Antiviral Res* 2012; **95**:57–65.
- Real LM, Neukam K, Herrero R, Guardiola JM, Reiberger T, Rivero-Juarez A, et al. IFNL4 rs469415590 variant shows similar performance to rs12979860 as predictor of response to treatment against Hepatitis C Virus genotype 1 or 4 in Caucasians. *PLoS One* 2014; **9**:e95515.
- de la Torre MS, Torres C, Nieto G, Vergara S, Carrero AJ, Macías J, et al. Vitamin D receptor gene haplotypes and susceptibility to HIV-1 infection in injection drug users. *J Infect Dis* 2008; **197**:405–410.
- Caruz A, Neukam K, Rivero-Juarez A, Herrero R, Real LM, Camacho A, et al. Association of low-density lipoprotein receptor genotypes with hepatitis C viral load. *Genes Immun* 2014; **15**:16–24.
- Martin MP, Qi Y, Goedert JJ, Hussain SK, Kirk GD, Hoots WK, et al. IL28B polymorphism does not determine outcomes of hepatitis B virus or HIV infection. *J Infect Dis* 2010; **202**:1749–1753.
- Rallon NI, Restrepo C, Naggie S, Lopez M, Del Romero J, Goldstein D, et al. Interleukin-28B gene polymorphisms do not influence the susceptibility to HIV-infection or CD4 cell decline. *AIDS* 2011; **25**:269–271.
- Salgado M, Kirk GD, Cox A, Rutebemberwa A, Higgins Y, Astemborski J, et al. Protective interleukin-28B genotype affects hepatitis C virus clearance, but does not contribute to HIV-1 control in a cohort of African-American elite controllers/suppressors. *AIDS* 2011; **25**:385–387.
- Machmach K, Abad-Molina C, Romero-Sánchez MC, Abad MA, Ferrando-Martínez S, Genebat M, et al. IL28B single-nucleotide polymorphism rs12979860 is associated with spontaneous HIV control in white subjects. *J Infect Dis* 2013; **207**:651–655.
- Machmach K, Abad-Molina C, Romero-Sánchez MC, Dominguez-Molina B, Moyano M, Rodríguez MM, et al. IFNL4 rs469415590 polymorphism is associated with unfavourable clinical and immunological status in HIV-infected individuals. *Clin Microbiol Infect* 2015; **21**:289.e1–289.e4.
DOI:10.1097/QAD.0000000000000773

Time of HIV diagnosis in infants after weaning from breast milk

Athena P. Kourtis^a, Caroline C. King^a, Julie Nelson^b, Denise J. Jamieson^a and Charles van der Horst^c

Of 28 infants diagnosed with HIV infections after the recommended time of breastfeeding cessation in a large clinical trial (the Breastfeeding, Antiretrovirals and Nutrition Study), 19 (68%) were first detected more than 6 weeks (and up to 168 days) after recommended weaning. The current WHO recommendation for HIV testing of infants is at 6 weeks or more after weaning; these data argue for repeat HIV testing of infants up to 6 months after breastfeeding cessation, so that no infant HIV infections are missed.

We previously conducted a clinical trial in 2369 mother–infant pairs randomized to either maternal combination antiretroviral agents (zidovudine/lamivudine/lopinavir–ritonavir), daily infant nevirapine, or neither, during 24 weeks of exclusive breastfeeding followed by 4 weeks of weaning (the Breastfeeding, Antiretrovirals and Nutrition [BAN] Study) [1,2]. The study demonstrated that both maternal and infant antiretrovirals significantly reduced HIV transmission during breastfeeding. In the BAN study, infants were tested for HIV infection at birth, 2, 12, 28, and 48 weeks, and the window of transmission was later narrowed by testing dry blood spot specimens from all interim visits (around/postweaning these were at 24, 32, 36, and 42 weeks) [1,2]. The median time of weaning for infants in the BAN study was 26.6 weeks (interquartile range 24.3–28.9 weeks). As previously reported, there were 28 infants in the study whose HIV infection was first detected after 28 weeks, time that all mothers should have weaned by study protocol [2]. The median (interquartile range) time of first detection of HIV infection in these infants from their time of weaning was 83.5 (32.5–121) days. By study arm, this time was 57 (29–84) days for the infant nevirapine arm, 89 (33–125) days for the maternal antiretroviral arm, and 121 (38–125) days for the control arm. In three infants (two in the infant nevirapine arm and one in the maternal antiretroviral arm), HIV infection was detected 42, 85, and 84 days prior to actual weaning, respectively (these infants continued breastfeeding past the time of recommended weaning at 28 weeks). In another infant with reported weaning at 26 days and missed visits since 40 days of age, HIV was first detected at 258 days, upon return to the clinic. For all the rest, HIV infection was first

Resultados complementarios/Complementary results

Nuestro grupo ha descrito cómo el SNP rs1567190, localizado en el primer intron de *CR2*, se encuentra asociado con un incremento en la resistencia a la infección por VIH-1 por vía parenteral y muy probablemente, por vía sexual. Además, correlacionamos el genotipo protector de *CR2* con menores niveles de ARNm, así como con diferencias en los ratios de expresión de las variantes del gen (Herrero et al. 2015).

CR2 puede ser usado por algunos virus para entrar de forma más eficiente en la célula diana en un proceso llamado C´ADE. Además, la presencia de anticuerpos no neutralizantes, pueden contribuir a que se produzca un aumento de la opsonización sobre la superficie de los virus. El proceso de C´ADE, es dependiente de la expresión en las células dianas de *CR2/CR1*, produciendo un aumento en la adhesión del virus-anticuerpo-complemento (C3d) con *CR2* en la superficie celular (Willey et al. 2011).

En base a todo esto, queremos caracterizar funcionalmente las diferentes isoformas de este receptor (isoforma corta e isoforma larga). Para esto, se utilizarán diferentes condiciones, con el fin de comprobar si existe alguna diferencia en el papel que juegan las dos isoformas en la infección por VIH-1.

En este trabajo se han desarrollado las herramientas necesarias para poder llevar a cabo esta caracterización. Por un lado, se han desarrollado dos líneas estables: una, que exprese la isoforma corta de *CR2* (*CR2_S* o *CR2_SHORT*) y la otra, la isoforma larga (*CR2_L* o *CR2_LONG*). Las células elegidas para esto han sido las HeLa p4 CCR5 CXCR4 ya que expresan, el receptor viral CD4 y los correceptores virales: CCR5 y CXCR4. Por otro lado, se han generado los pseudovirus con los que infectar estas células.

Fue en la Unidad de Virología Molecular y Vacunas del Instituto Pasteur de Paris, bajo la dirección de PhD. Pierre Charneau (Director de la unidad) y la supervisión de Philippe Souque y Catherine Blanc donde se llevó a cabo todo este proceso.

Creación de las líneas estables: HELA p4 CCR5-CXCR4-CR2 S y HELA p4 CCR5-CXCR4-CR2 L.

Las dos isoformas de *CR2*: transcrito 1 (larga) y transcrito 2 (corta) (*CD21*; NP_001006659.1 y NP_001868.2 respectivamente), fueron

amplificadas a partir de los plásmidos pCDNA31_CR2_S y pCDNA31_CR2_L (GenScript EE.UU. Inc., NJ, EE.UU.). En esta amplificación, se les incluyó a través de los cebadores, los sitios de corte para las enzimas de restricción: Bgl II y Sal I. Tras la purificación de las dos isoformas amplificadas, se realizó una subclonación en el vector TOPO_TA (Thermo Fisher Scientific, Carlsbad, CA, EE.UU.). Posteriormente, se realizó la restricción de los dos plásmidos con las enzimas de restricción Bgl II-Sal I.

De forma paralela, se realizó la restricción con las enzimas BamH I y Xho I del vector lentiviral pFLAP_CMV_EGFP_WPRE (Zennou et al. 2000), para extraer EGFP y poder realizar la posterior ligación de los dos insertos. Los vectores pFLAP_CMV_CR2_S_WPRE y pFLAP_CMV_CR2_L_WPRE se secuenciaron para verificar que la clonación había sido la adecuada.

Las células 293T se transfectaron mediante fosfato cálcico: una transfección, se realizó con el vector pFLAP_CMV_CR2_S_WPRE y la otra, con pFLAP_CMV_CR2_L_WPRE. En ambos casos, los vectores se cotransfectaron con dos plásmidos más: uno, con la envuelta VSV y otro, con los genes *gag-pol*. De esta forma, se generaron las partículas lentivirales con ambas isoformas. Las concentraciones de las partículas virales se determinaron mediante PCR a tiempo real, detectándose el fragmento *U5R* de la LTR de los vectores y normalizando con *CD3*, correceptor que se expresa en la línea celular 293T.

Para generar las líneas estables que expresasen ambas isoformas, se hicieron reiteradas transducciones con las partículas lentivirales generadas sobre las células HeLa p4 CCR5-CXCR4. Se utilizaron dos MOI para realizar estas transducciones: 20 y 50 MOI, siendo esta última, la más eficaz para conseguir una mayor expresión de las isoformas. Mediante citometría de flujo, se midió la expresión de ambos receptores. Finalmente, se aislaron células de la tercera transducción realizada con las partículas de la isoforma corta y de la cuarta transducción de la isoforma larga.

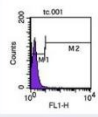
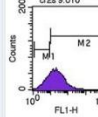
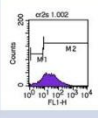
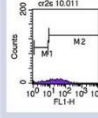
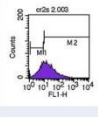
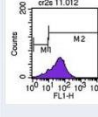
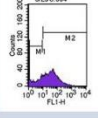
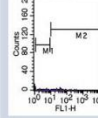
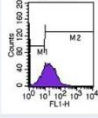
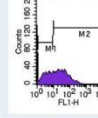
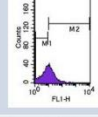
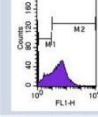
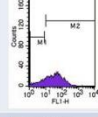
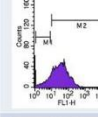
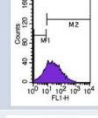
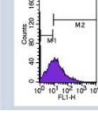
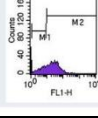
Expresión de CR2_S de los diferentes clones			Expresión de CR2_S de los diferentes clones		
Clon	Media de fluorescencia (M2)	Gráfica de citometría	Clon	Media de fluorescencia (M2)	Gráfica de citometría
HeLa control	1,84 (M1)		CR2_S – Clon 9	23,93	
CR2_S – Clon 1	30,23		CR2_S – Clon 10	31,77	
CR2_S – Clon 2	22,07		CR2_S – Clon 11	43,32	
CR2_S – Clon 3	38,20		CR2_S – Clon 12	26,18	
CR2_S – Clon 4	22,67		CR2_S – Clon 13	31,34	
CR2_S – Clon 5	15,54		CR2_S – Clon 14	36,85	
CR2_S – Clon 6	41,79		CR2_S – Clon 15	35,87	
CR2_S – Clon 7	31,62		CR2_S – Clon 16	21,87	
CR2_S – Clon 8	25,71				

Figura 2 | Expresión de los clones 1-16 de CR2_S medida mediante FACS.

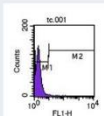
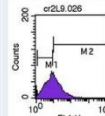
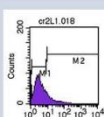
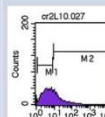
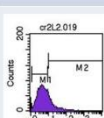
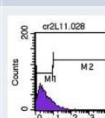
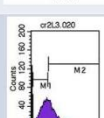
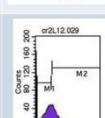
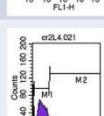
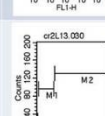
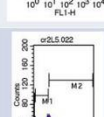
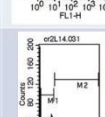
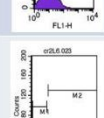
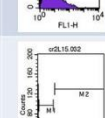
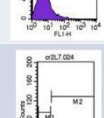
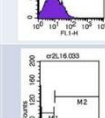
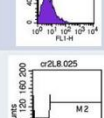
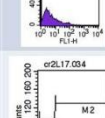
Expresión de CR2_L de los diferentes clones			Expresión de CR2_L de los diferentes clones		
Clon	Media de fluorescencia (M2)	Gráfica de citometría	Clon	Media de fluorescencia (M2)	Gráfica de citometría
HeLa control	1,84 (M1)		CR2_L – Clon 9	16,40	
CR2_L – Clon 1	16,40		CR2_L – Clon 10	16,25	
CR2_L – Clon 2	13,82		CR2_L – Clon 11	13,22	
CR2_L – Clon 3	17,15		CR2_L – Clon 12	16,55	
CR2_L – Clon 4	16,70		CR2_L – Clon 13	15,26	
CR2_L – Clon 5	17,78		CR2_L – Clon 14	18,60	
CR2_L – Clon 6	14,59		CR2_L – Clon 15	18,43	
CR2_L – Clon 7	14,86		CR2_L – Clon 16	18,11	
CR2_L – Clon 8	14,20		CR2_L – Clon 17	17,00	

Figura 3 | Expresión de los clones 1-17 de CR2_L medida mediante FACS.

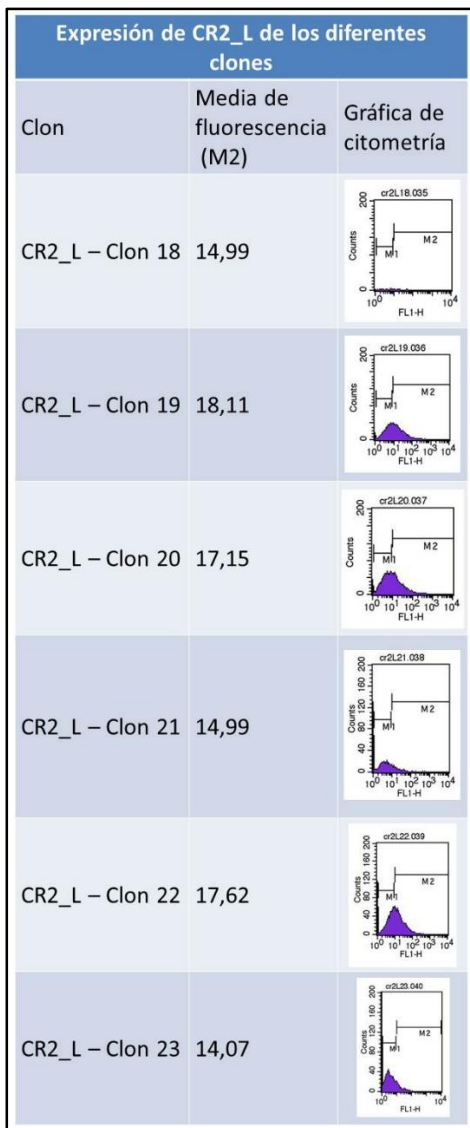


Figura 4 | Expresión de los clones 18-23 de CR2_L medida mediante FACS.

Se obtuvieron 15 y 23 clones aislados que expresaban CR2_S y CR2_L respectivamente (Figura 2-4). Sin embargo, estas células aisladas morían tras realizar el pase para expandirlas. Es por eso que los experimentos de infección y de caracterización se realizaran con las líneas estables sin realizar aislamiento y clonación de las mismas.

Producción de virus y pseudovirus.

Se generaron tres pseudovirus diferentes mediante la transfección con fosfato cálcico de las células 293T: dos pseudovirus con envuelta VIH-1, ya que se quería testar la eficacia de infección de los dos plásmidos de la envuelta VIH-1, y otro con envuelta VSV.

Para generar el pseudovirus envuelta VIH-1_CCR5 (1), se cotransfectó el plásmido pCAGGS SF162 gp160 con pBRU-ΔEnv; para el pseudovirus envuelta VIH-1_CCR5 (2), el plásmido HIV-1 clone BaL.01 y pBRU-ΔEnv. Para el pseudovirus con envuelta VSV, se cotransfectó el plásmido pHEF-VSVG con pBRU ΔEnv (Figura 5).

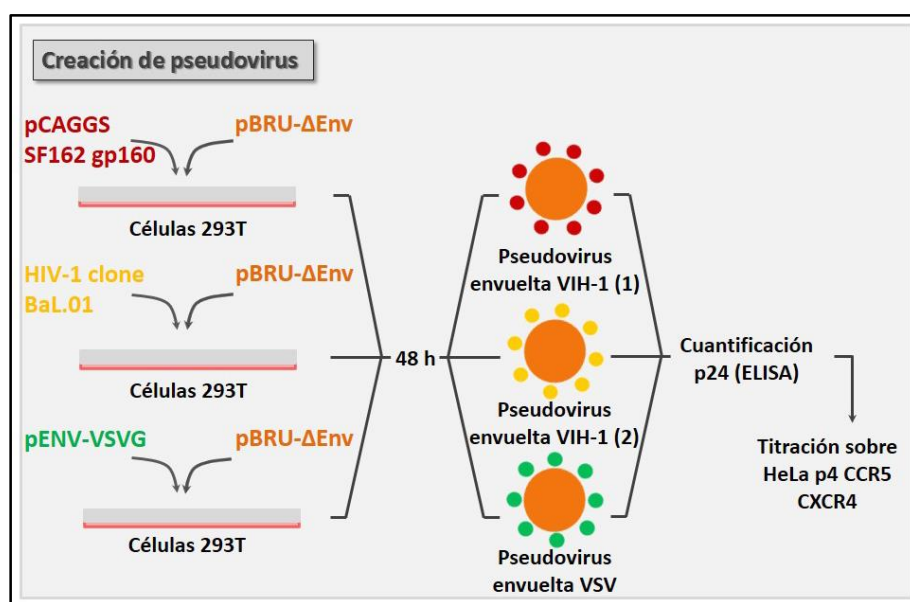


Figura 5 | Proceso de creación de pseudovirus.

Una vez producidos y recogidos los pseudovirus, se realizó un ELISA para detectar la proteína p24 y así, cuantificar la producción viral. En cuanto a la infectividad de los pseudovirus, se determinó a través la infección de las células HeLa p4 CCR5-CXCR4. La cuantificación se llevó a cabo mediante β -galactosidasa y se normalizó mediante la cuantificación de proteínas (Figura 6).

Con los datos obtenidos en la cuantificación de p24 mediante el ELISA y en la titulación de los tres pseudovirus generados, se determinó que el plásmido más eficaz para la producción del pseudovirus con

envuelta VIH-1 y para la posterior infección de las células HeLa p4 CCR5-CXCR4, es el HIV-1 clone BaL.01. El pseudovirus VIH-1 (1) originado con el plásmido pCAGGA SF162 gp160, pese a los valores de p24 generados, el ratio de β -galactosidasa/proteína producido, es muy inferior al originado por el pseudovirus VIH-1 (2) (Figura 6).

También se determinó, las concentraciones de pseudovirus con las que realizar las infecciones de caracterización de las dos isoformas de *CR2* mediante las dos líneas estables creadas. Las concentraciones adecuadas de los pseudovirus para realizar la infección, son aquellas que provocan un nivel de infección similar y por ende, aquellas concentraciones en las que el ratio β -galactosidasa/proteína sea semejante. Es por eso, que las concentraciones más apropiadas de p24 para realizar dichas infecciones son: 5 ng para el pseudovirus VIH-1 (2) y 0,5 ng para el pseudovirus VSV (Figura 6).

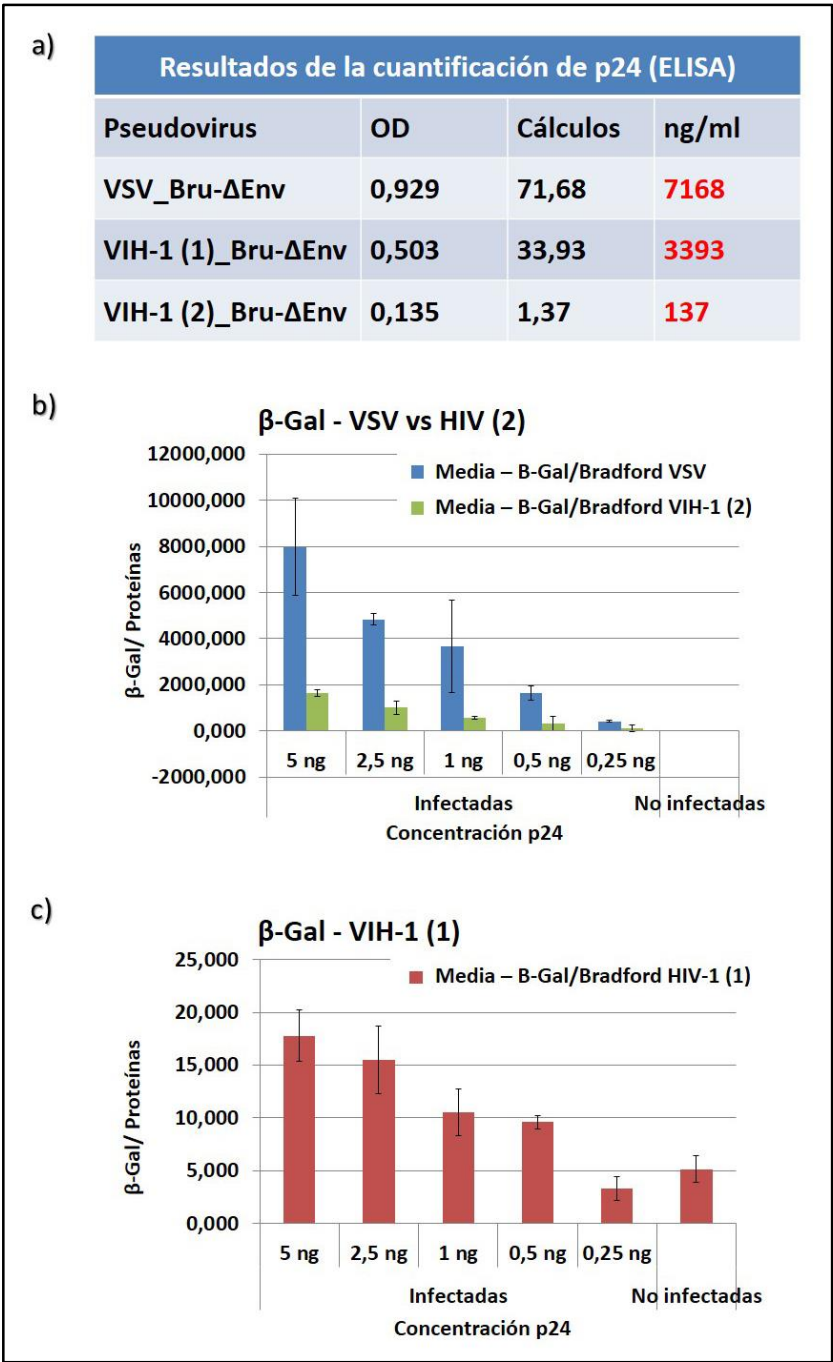


Figura 6 | Titulación de los pseudovirus generados. a) Resultados de la cuantificación de la proteína p24 de los pseudovirus generados mediante ELISA. b) Resultados del nivel de infectividad de los pseudovirus con envuelta VSV y virus VIH-1 (2)-Bru sobre las células HeLa p4 CCR5-CXCR4. c) Resultados del nivel de infectividad del pseudovirus VIH-1 (1)-Bru sobre las células HeLa p4 CCR5-CXCR4.

8. Discusión/Discussion

Dos SNPs ubicados en genes del sistema del complemento se han relacionado con la infección por VIH-1: el alelo G del rs2842704 de *C4BPA*, se asoció en modelo dominante con una disminución del riesgo de infección por vía parenteral, formando parte con un haplotipo (GCT) constituido por los marcadores rs2842704, rs4425986 y rs9943077 y asociado también en modelo dominante a resistencia a la infección. Sin embargo, estas asociaciones se perdieron cuando se intentó replicar los resultados por vía sexual. Por otro lado, el alelo T del rs1567190 de *CR2*, se asoció en modelo dominante con un aumento del riesgo de infección independientemente de la vía de transmisión; además, este marcador forma parte de un haplotipo protector (CGGGG), constituido por los marcadores rs1567190, rs1507764, rs1048971, rs17615 y rs4317805, que en homocigosis, se asocia a protección a la infección. El rs1567190 de *CR2* se encuentra ubicado en el promotor del gen y determina el empalme alternativo del exón 11 generando 2 isoformas: *CR2_S* y *CR2_L*. Fenotípicamente, el genotipo de este marcador, determina el nivel de expresión global del gen, así como el de sus isoformas. Los homocigotos CC, tienen una menor expresión de ARNm que los TT. Además, existe una clara diferencia entre las ratios de *CR2_S* y *CR2_L* entre los genotipos, habiendo una menor expresión de la isoforma corta y mayor expresión de la isoforma larga en homocigotos CC que TT. Se encontró también, un haplotipo ubicado en el *CFH* (formado por los marcadores: rs2019727, rs6695321, rs10737680, rs419137, rs395544, rs2284664, rs11582939 y rs16840658) que pese a perder la significación al realizar el análisis por multiple testing, queda en el umbral de la significación; es posible que al aumentar el número de individuos de la población de estudio, el nivel de significación se vuelva a alcanzar.

También se halló un haplotipo en el promotor de *CD209*, *XAGA*, que se asoció a protección por VIH-1 por vía parenteral. Este haplotipo estaba compuesto por los marcadores: rs2287886 (-139), rs4804803 (-336), rs735239 (-871) y rs735240 (-939). Además, se comprobó que portadores de este haplotipo en homocigosis, tienen una menor expresión de ARNm frente a portadores de haplotipos no *XAGA*. Cuando se comprobó la repercusión de estos SNPs y haplotipos en la infección por VHC, se asoció el haplotipo GAA (formado por los marcadores: rs2287886, rs4804803 y rs735239) con menor carga viral en aquellos individuos infectados con genotipos virales 2 y 3.

En cuanto al efecto que tiene el rs368234815 de *IFNL4* sobre la infección por VIH-1, se comprobó que el genotipo favorable para VHC (TT/TT), también lo es para la infección por VIH-1 por vía parenteral.

Todos estos estudios están realizados en población masculina, de rasgos genéticos similares y de un rango de edad establecido. Solo en la cohorte italiana de parejas serodiscordantes, usada para la replicación de los datos ya obtenidos, existe población femenina. Además, todos los individuos (menos las parejas serodiscordantes), están infectados con el VHC, tanto los VIH-1 positivos como los ENIS, aportando mayor homogeneidad y siendo en el caso de los ENIS, un marcador de exposición al VIH-1.

Las características de nuestra población son muy importantes y nos dan ventajas frente a otros estudios realizados. Por un lado, los rasgos genéticos similares, aportan mayor consistencia a los datos obtenidos frente a otros estudios donde la población es más heterogénea como los estudios de GWAS realizados en VIH-1 y ENIS (Joubert et al. 2010; Petrovski et al. 2011; Lingappa et al. 2011; Luo et al. 2012; Lane et al. 2013).

Así, al haber realizado los estudios sobre individuos varones, la inmunidad innata y adaptativa no se encontraran estimuladas debido a hormonas sexuales femeninas, como el estrógeno. La mayoría de las células del sistema inmune expresan receptores de estrógenos (células T y B, DCs, NK) y se ha demostrado que este, entre otros efectos, aumenta el número de linfocitos B, reduce la producción de citoquinas pro-inflamatorias, estimulan la producción de anticuerpos, mejoran en células T CD4+ la expresión de CCR1 y CCR5, etc. También se ha demostrado que existen respuestas diferentes entre ambos sexos en respuestas a vacunas (Giefing-Kröll et al. 2015).

Por otro lado, se sabe que la infección por VIH-1 afecta al curso clínico del VHC y a la inversa, habiendo cierta controversia en cómo afecta realmente el VHC a la infección por VIH-1 (Kang et al. 2014; Younas et al. 2015). Es importante tener esto en cuenta, debido a que los resultados detallados aquí, se obtienen bajo las condiciones de infección o co-infección con VHC.

Una limitación de nuestro estudio es el bajo número de individuos sobre todo, la de la cohorte italiana de parejas serodiscordantes. Esto

podría explicar, la no significación inicial de los datos obtenidos solo por vía sexual, en el estudio realizado con genes del complemento. A pesar de ello, el aumento en la significación estadística en el meta-análisis realizado cuando se engloban ambas poblaciones confirma, que el tamaño de efecto de la asociación y sobre todo, su dirección es similar en ambas poblaciones.

Otra limitación de este mismo estudio, es la selección de los marcadores testados. En este estudio no se han tenido en cuenta polimorfismos raros que pudieran tener una alta penetrancia en el fenotipo. Sería interesante tras los resultados obtenidos, realizar un estudio de secuenciación de exomas en individuos VIH-1 y ENIS, ya que esto, nos aportaría una visión mucho más amplia y a la vez, más específica de las diferencias existentes entre ambos grupos.

Es importante señalar aquí, que casi todos los marcadores que se han asociado del sistema del complemento (rs1567190 de *CR2* y rs2842704 de *C4BPA*) y de *CD209* (rs2287886, rs735239, rs735240) con la infección por VIH-1 en nuestro trabajo, están incluidos en casi todos los estudios realizados de GWAS sobre ENIS (Joubert et al. 2010; Petrovski et al. 2011; Lingappa et al. 2011; Lane et al. 2013). A pesar de ello, no fueron reportados como asociados a la resistencia a la infección en estos estudios masivos. Esto, sin embargo, puede ser el efecto que los rigurosos controles de calidad tienen sobre los marcadores genéticos en los GWAS, haciendo que muchos de ellos no lleguen a la fase de análisis, o bien, que sean descartados por no superar las correcciones por múltiple testado, a pesar de que sea verdadera la asociación. Otras posibles causas de estas discrepancias, pueden ser las diferencias intrínsecas que nuestra población pueda tener con las poblaciones donde estos estudios se han realizado (principalmente africanos de ambos sexos), las diferencias en las rutas de transmisión del VIH (principalmente sexual o vertical) y la heterogeneidad de los individuos incluidos en algunos de estos estudios. Además, en los estudios de GWAS no se han realizado estudios de haplotipos.

CR2 ha sido identificado como el ligando principal *in vivo* de la cubierta del virus, que interacciona con CD23 y que se une a IFN- α (Braun et al. 1998). También se conoce que a través de este, se produce el fenómeno de C'ADE, viéndose implicados también los FcRs y pudiendo ser determinante el balance de CRs y FcRs (Willey et al. 2011). En el caso

de DC-SIGN y el Virus del Dengue, ocurre de forma parecida. Se han descrito fenómenos de mejora de infección en los que están implicados los FcRs y como, los ratios de DC-SIGN y FcRs influyen en esa mejora (Boonnak et al. 2008).

Sin embargo, se desconoce cuál es el papel de las dos isoformas de CR2 en la infección por VIH-1 y en este fenómeno de mejora de la infección. Para evaluar esto en el futuro, hemos desarrollado las líneas celulares que expresen ambas isoformas y puesto a punto el proceso de generación de pseudovirus. También sería necesario evaluar relaciones sinérgicas entre CRs, FcRs y DC-SIGN y sus efectos sobre el virus del VIH-1 ya que quizás, pueda existir una relación entre los genotipos de *CD209* y *CR2* y el fenotipo de protección/susceptibilidad a la infección.

Las variantes incluidas en el haplotipo de *CD209* descrito en este trabajo, se ha asociado de forma aislada al desarrollo o al agravamiento de diferentes patologías como: aspergilosis pulmonar invasiva en pacientes inmunodeprimidos (rs2287886, posición -139) (Sainz et al. 2010), diabetes mellitus tipo 1 en población brasileña (rs735239 y rs4804803, posiciones -871 y -336 respectivamente) (da Silva et al. 2014). La variante rs4804803 (-336), ha sido sin duda la que se ha asociado a un mayor número de patologías: tuberculosis (Vannberg et al. 2008), enfermedad de Kawasaki (Yu et al. 2012), mayor gravedad en la enfermedad hepática en infectados por VHC (Ryan et al. 2010).

En un estudio, un haplotipo formado por 3 de los 4 marcadores de *CD209* descritos en este trabajo (rs2287886, rs4804803, rs735239), es asociado con el agravamiento y la aparición de trombocitopenia en el curso clínico del Dengue (Alagarasu et al. 2013). Clifford y colaboradores, relacionaron los marcadores rs2287886, rs4804803 y rs735239 de *CD209*, con las respuestas a la vacuna del sarampión. Y es que, DC-SIGN captura a este virus en la superficie celular y luego lo transmite a los recetores de entrada del virus (SLAM) (Clifford et al. 2011). En otro estudio, este sí relacionado con la resistencia a la infección por VIH-1, se asocia un haplotipo compuesto por 7 SNPs ubicados en el promotor de *CD209*, de los cuales 3 forman parte del haplotipo que hemos encontrado en nuestro trabajo (rs2287886, rs4804803, y rs735240), con riesgo de infección por VIH-1 por vía parenteral, aunque tras la corrección por multiple-testing, esa asociación se perdió (Martin et al. 2004). Si bien es

cierto, en este estudio no se determinó el efecto fenotípico del haplotipo hallado.

Hemos relacionado una menor expresión del receptor DC-SIGN con resistencia a la infección por VIH-1 por vía parenteral. Sin embargo, se necesitan estudios adicionales, para corroborar si este haplotipo confiere resistencia también por vía sexual y comprobar, si el efecto de estos haplotipos es solo a nivel cuantitativo, ya que podría tener otros efectos como por ejemplo, la generación de variantes que hagan menos funcional al receptor o incluso pueden tener alguna relación con otros SNPs de la región codificante.

En el caso de *IFNL4*, corroboramos en cierta medida lo que otros estudios han mostrado, y es que el genotipo TT/TT del rs368234815 tiene un efecto protector ya sea, como defendemos nosotros en la infección por VIH-1 por vía parenteral o como se publica en otros trabajos, con una mejora en el estado inmune del paciente con VIH-1 (Machmach et al. 2015).

En cuanto al genotipo ΔG de rs368234815 *IFNL4*, hace que se exprese IFNL4, proteína muy parecida a IFNL3, que podrían competir por la unión al receptor IFNLR1. Una vez unido IFNL4 a este receptor, provocaría una preactivación de la señalización de los interferones, reduciéndose la respuesta de los IFN tipo I y tipo III (Prokunina-Olsson et al. 2013).

También se han relacionado a los IFNLs con enfermedades autoinmunes, como el lupus eritematoso sistémico. Hasta hace poco tiempo se pensaba, que el IFN-α era el que progresivamente rompía la tolerancia inmunológica y contribuía al desarrollo de la enfermedad, por lo que siempre ha sido una diana terapéutica. Sin embargo, la falta de eficacia clínica de los fármacos desarrollados, ha dirigido las miradas hacia los IFNLs ya que pueden compartir las mismas rutas biológicas. Se ha detectado mayores niveles de IFNL2 en suero de pacientes con lupus que de donantes sanos y se ha correlacionado los niveles de IFNL1 con esta enfermedad y además, con la artritis y la glomerulonefritis (Amezcu-Guerra et al. 2015; Lasfar et al. 2015).

Se tendrán que realizar más estudios para determinar realmente el papel que tiene el genotipo ΔG de rs368234815 *IFNL4*. En nuestro caso,

Discusión/Discussion

se procederá en futuros experimentos a replicar los datos obtenidos por vía parenteral en la cohorte de parejas serodiscordantes.

Los datos obtenidos aquí, muestran que nos encontramos ante dianas terapéuticas potenciales. Si bien es cierto, estos genes son difícilmente drugables debido a que participan en procesos esenciales de la inmunidad. Es por esto que pueden ser utilizados con otros fines, bien como marcadores que indiquen la respuesta a un tratamiento eficaz, como puede ser *IFNL4* o *IFNL3*, o en farmacogenética de vacunas como marcador de respuesta inmunológica eficiente a las mismas.

9. Conclusiones/Conclusions

Conclusión global: Se han identificado factores genéticos en los genes *CR2*, *C4BPA*, *CD209* e *INFL4* que han sido asociados a resistencia innata al VIH-1.

Conclusión 1.1: Hemos identificado dos polimorfismos en genes del complemento que han sido asociados con riesgo de infección del VIH-1 en drogadictos por vía parenteral: el rs2842704 ubicado en *C4BPA* y el rs1567190 localizado en *CR2*. En el caso de *CR2*, el alelo protector se asoció con niveles más bajos de ARNm; además, la expresión de la isoforma larga era mayor en el genotipo protector. Estas diferencias sugieren que los genotipos de *CR2* así como sus variantes, podrían jugar un papel importante en las etapas iniciales de la infección por VIH-1.

Conclusión 1.2: Los datos obtenidos no se pudieron replicar por vía sexual, probablemente debido al tamaño más reducido de la cohorte sexual. Sin embargo, la distribución genotípica encontrada en la cohorte por vía sexual, es similar a la de la población de usuarios de drogas por vía parenteral. Al realizar un meta-análisis con los resultados de los dos análisis, se obtuvo un *p*-valor altamente significativo lo que indica que *CR2* podría ser un factor genético que influye en el riesgo de infección por VIH-1 tanto por vía parenteral como por vía sexual.

Conclusión 1.3: Se crearon las líneas celulares estables que expresan la isoforma larga y la isoforma corta del *CR2* para evaluar, en futuros experimentos de infección, la actividad funcional de ambos receptores durante el acoplamiento y la infección por VIH-1. Se generaron también los pseudovirus con envuelta VIH-1-R5 y VSV, poniendo a punto de este modo, el proceso de creación de pseudovirus.

Conclusión 2.1: El haplotipo (XAGA) formado por rs2287886, rs4804803, rs735239 y rs735240 respectivamente, en homocigosis se asocia con protección en la adquisición de la infección por VIH-1 por vía parenteral.

Conclusiones/Conclusions

Conclusión 2.2: El haplotipo protector XAGA, se correlaciona con una menor expresión de ARNm de *CD209* en células mononucleares de sangre periférica de donantes sanos, así como con menor actividad en el modelo *in vitro* de células humanas

Conclusión 3.1: La variante de *IFNL4* que genera un marco de lectura abierto, se asocia con una mayor resistencia a la infección por VIH-1 en usuarios de drogas por vía parenteral. Este alelo impide la producción adecuada de IFNL4. Parece ser, que la expresión de *IFNL4* en la infección por VIH-1 podría ser un factor negativo.

Global conclusion: Polymorphisms in *CR2*, *C4BPA*, *CD209* and *IFNL4* genes was associated to innate resistance to HIV-1.

Conclusion 1.1: We have identified two polymorphisms in the complement genes associated with risk of HIV-1 infection in intravenous drug users: rs2842704 (*C4BPA*) and rs1567190 (*CR2*). In the case of *CR2*, the protective allele was associated with lower levels of mRNA. Furthermore, the long isoform displayed increased expression in the protective genotype background. These differences suggest that *CR2* genotypes and protein variants play an important role in initial phases of HIV-1 infection.

Conclusion 1.2: These results were not replicated in a cohort of patients at risk of infection through sexual route, probably due to the small size of this cohort. However, the distribution of *CR2* genotypes in the sexual cohort was similar compared to the IDU cohort. The results of the two association analyses were combined through a random effect meta-analysis with a highly significant p-value. This indicates that *CR2* could be a major genetic factor influencing the risk of HIV-1 infection by parenteral as well as sexual route.

Conclusion 1.3: Two cell stable cell lines expressing the long and short variants of *CR2* were created to evaluate in upcoming experiments, the functional activity of both receptors during HIV-1 attachment and infection. R5-HIV-1 and VSV envelope pseudo-viruses were created and tuning the process of generated pseudo-viruses.

Conclusion 2.1: Two copies of XAGA haplotype, including the SNPs rs2287886, rs4804803, rs735239 and rs735240 respectively, were associated with protection from HIV-1 infection by parenteral route.

Conclusion 2.2: The protective XAGA haplotype were correlated with lower expression of *CD209* mRNA in PBMCs from healthy donors as well as lower promoter activity *in vitro*.

Conclusiones/Conclusions

Conclusion 3.1: A frameshift variant in *IFNL4* is associated with increased resistance from HIV infection in IDU. This allele impedes the correct IFNL4 protein production. The expression of *IFNL4* appears to be a negative factor in the context of HIV-1 infection.

10. Referencias bibliográficas/Bibliography

- Aguilar-Jiménez, W. et al., 2013. High transcript levels of vitamin D receptor are correlated with higher mRNA expression of human beta defensins and IL-10 in Mucosa of HIV-1-exposed seronegative individuals. *PLoS ONE*, 8(12), pp.1–9.
- Alagarasu, K. et al., 2013. Association of promoter region polymorphisms of CD209 gene with clinical outcomes of dengue virus infection in Western India. *Infection, Genetics and Evolution*, 17, pp.239–242.
- Amezcu-Guerra, L.M. et al., 2015. Limited effectiveness for the therapeutic blockade of interferon α in systemic lupus erythematosus: A possible role for type III interferons: Type III interferons in lupus. *Rheumatology (United Kingdom)*, 54(2), pp.203–205.
- An, P. et al., 2002. Modulating influence on HIV/AIDS by interacting RANTES gene variants. *Proceedings of the National Academy of Sciences of the United States of America*, 99(15), pp.10002–7.
- An, P. et al., 2007. Polymorphisms of CUL5 are associated with CD4+ T cell loss in HIV-1 infected individuals. *PLoS genetics*, 3(1), p.e19.
- Assil, S., Webster, B. & Dreux, M., 2015. Regulation of the Host Antiviral State by Intercellular Communications. *Viruses*, 7(8), pp.4707–4733.
- Ballegaard, V. et al., 2014. The lectin pathway of complement: Advantage or disadvantage in HIV pathogenesis? *Clinical Immunology*, 154(1), pp.13–25.
- Bánki, Z., Stoiber, H. & Dierich, M.P., 2005. HIV and human complement: Inefficient virolysis and effective adherence. *Immunology Letters*, 97(2 SPEC. ISS.), pp.209–214.
- Biasin, M. et al., 2010. TLR Activation Pathways in HIV-1-Exposed Seronegative Individuals. *The Journal of Immunology*, 184(5), pp.2710–2717.
- Boonnak, K. et al., 2008. Role of Dendritic Cells in Antibody-Dependent Enhancement of Dengue Virus Infection. *Journal of Virology*, 82(8), pp.3939–3951.
- Braun, M. et al., 1998. Human B and T lymphocytes have similar amounts of CD21 mRNA, but differ in surface expression of the CD21

Referencias bibliográficas/Bibliography

- glycoprotein. *International immunology*, 10(8), pp.1197–202.
- Celerino da Silva, R., Segat, L. & Crovella, S., 2011. Role of DC-SIGN and L-SIGN receptors in HIV-1 vertical transmission. *Human Immunology*, 72(4), pp.305–311.
- Chaaithanya, I.K. et al., 2016. Association of Oligoadenylate Synthetase Gene Cluster and DC-SIGN (CD209) Gene Polymorphisms with Clinical Symptoms in Chikungunya Virus Infection. *DNA and cell biology*, 35(1), pp.44–50.
- Chaudhary, O. et al., 2008. Polymorphic variants in DC-SIGN, DC-SIGNR and SDF-1 in high risk seronegative and HIV-1 patients in Northern Asian Indians. *Journal of Clinical Virology*, 43(2), pp.196–201.
- Chung, K.M. et al., 2006. West Nile virus nonstructural protein NS1 inhibits complement activation by binding the regulatory protein factor H. *Proceedings of the National Academy of Sciences of the United States of America*, 103(50), pp.19111–6.
- Clavel, F. & Charneau, P., 1994. Fusion from without directed by human immunodeficiency virus particles. *J Virol*, 68(2), pp.1179–1185.
- Clifford, H.D. et al., 2011. Slam and dc-sign measles receptor polymorphisms and their impact on antibody and cytokine responses to measles vaccine. *Vaccine*, 29(33), pp.5407–5413.
- Cruickshank, M.N. et al., 2012. Transcriptional effects of a lupus-associated polymorphism in the 5' untranslated region (UTR) of human complement receptor 2 (CR2/CD21). *Molecular Immunology*, 52(3-4), pp.165–173.
- Diop, G. et al., 2006. Exhaustive genotyping of the interferon alpha receptor 1 (IFNAR1) gene and association of an IFNAR1 protein variant with AIDS progression or susceptibility to HIV-1 infection in a French AIDS cohort. *Biomedicine & pharmacotherapy = Biomédecine & pharmacothérapie*, 60(9), pp.569–577.
- Donaldson, P. et al., 2015. *Genetics of Complex Disease* First edit. Garland Science, ed., New York, USA.
- Douglas, K.B. et al., 2010. Complement receptor 2 polymorphisms associated with systemic lupus erythematosus modulate alternative

- splicing. *Genes Immun.*, 10(5), pp.457–469.
- Duarte, R. & Frank, D., 2000. [The JAK-STAT signaling pathway and its role in oncogenesis, immunomodulation and development]. *Med Clin (Barc)*, 114, pp.227–34.
- Essomba, E.N. et al., 2015. Facteurs associés à la non observance thérapeutique des sujets adultes infectés par le VIH sous antirétroviraux dans un hôpital de référence à Douala. *Pan African Medical Journal*, 20, pp.1–8.
- Frank, M.M., Hester, C. & Jiang, H., 2014. Complement and the control of HIV infection: an evolving story. *Current opinion in HIV and AIDS*, 9(3), pp.278–290.
- Garcia-moruja, C. et al., 2009. Molecular Phenotype of CXCL12beta 3'UTR G801A Polymorphism (rs1801157) Associated to HIV-1 Disease Progression. *Current HIV research*, 7, pp.384–389.
- Giefing-Kröll, C. et al., 2015. How sex and age affect immune responses, susceptibility to infections, and response to vaccination. *Aging Cell*, 14(3), pp.309–321.
- Haapasalo, K. et al., 2008. Complement factor H allotype 402H is associated with increased C3b opsonization and phagocytosis of *Streptococcus pyogenes*. *Molecular Microbiology*, 70(3), pp.583–594.
- Herrero, R. et al., 2015. Association of complement receptor 2 polymorphisms with innate resistance to HIV-1 infection. *Genes and immunity*, 16(2), pp.134–141.
- Hou, W. et al., 2009. Lambda interferon inhibits human immunodeficiency virus type 1 infection of macrophages. *Journal of virology*, 83(8), pp.3834–42.
- Hütter, G. et al., 2015. CCR5 Targeted Cell Therapy for HIV and Prevention of Viral Escape. *Viruses*, 7(8), pp.4186–4203.
- Javier, A. et al., 2015. Costs and cost-efficacy analysis of the 2014 GESIDA/Spanish National AIDS Plan recommended guidelines for initial antiretroviral therapy in HIV-infected adults. *Enferm Infecc Microbiol Clin.*, 33(3), pp.156–65.

Referencias bibliográficas/Bibliography

- Joubert, B. et al., 2010. A whole genome association study of mother-to-child transmission of HIV in Malawi. *Genome Medicine*, 2(3), p.17.
- Kagone, T.S. et al., 2014. A variant of DC-SIGN gene promoter associated with resistance to HIV-1 in serodiscordant couples in Burkina Faso. *Asian Pacific journal of tropical medicine*, 7S1(Suppl 1), pp.S93–6.
- Kang, W. et al., 2014. Hepatitis C virus infection in patients with HIV-1: epidemiology, natural history and management. *Expert review of gastroenterology & hepatology*, 8(3), pp.247–66.
- Kaur, G. et al., 2013. Genomic architecture of HIV-1 infection: Current status & challenges. *Indian Journal of Medical Research*, 138(Nov), pp.663–681.
- Kenedy, M.R. et al., 2009. CspA-mediated binding of human factor H inhibits complement deposition and confers serum resistance in *Borrelia burgdorferi*. *Infection and Immunity*, 77(7), pp.2773–2782.
- de la Torre, M.S. et al., 2008. Vitamin D receptor gene haplotypes and susceptibility to HIV-1 infection in injection drug users. *The Journal of infectious diseases*, 197(3), pp.405–10.
- Lama, J. & Planelles, V., 2007. Host factors influencing susceptibility to HIV infection and AIDS progression. *Retrovirology*, 4(1), p.52.
- Lander, E. et al., 2001. Initial sequencing and analysis of the human genome. *Nature*, 409(6822), pp.860–921.
- Lane, J. et al., 2013. A genome-wide association study of resistance to HIV infection in highly exposed uninfected individuals with hemophilia A. *Human Molecular Genetics*, 22(9), pp.1903–1910.
- Laplana, M. et al., 2013. Association of BST-2 gene variants with HIV disease progression underscores the role of BST-2 in HIV type 1 infection. *Journal of Infectious Diseases*, 207(3), pp.411–419.
- Lasfar, A., Zloza, A. & Cohen-Solal, K.A., 2015. IFN-lambda therapy: Current status and future perspectives. *Drug Discovery Today*, 21(1), pp.167–71.
- Lin, F. & Young, H.A., 2014. Interferons: Success in anti-viral immunotherapy. *Cytokine & growth factor reviews*, 25(4), pp.369–

76.

Lingappa, J.R. et al., 2011. Genomewide Association Study for Determinants of HIV-1 Acquisition and Viral Set Point in HIV-1 Serodiscordant Couples with Quantified Virus Exposure. *PLoS ONE*, 6(12), p.e28632.

Liu, H. et al., 2004. Analysis of genetic polymorphisms in CCR5, CCR2, stromal cell-derived factor-1, RANTES, and dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin in seronegative individuals repeatedly exposed to HIV-1. *The Journal of infectious diseases*, 190(6), pp.1055–1058.

Luo, M. et al., 2012. A Genetic Polymorphism of FREM1 Is Associated with Resistance against HIV Infection in the Pumwani Sex Worker Cohort. *Journal of Virology*, 86(21), pp.11899–11905.

Machmach, K. et al., 2015. IFNL4 ss469415590 polymorphism is associated with unfavourable clinical and immunological status in HIV-infected individuals. *Clinical microbiology and infection: the official publication of the European Society of Clinical Microbiology and Infectious Diseases*, 21(3), pp.289.e1–4.

Mackelprang, R.D. et al., 2014. Toll-like Receptor Polymorphism Associations With HIV-1 Outcomes Among Sub-Saharan Africans. *Journal of Infectious Diseases*, 209(10), pp.1623–1627.

Martin, M.P. et al., 2004. Association of DC-SIGN promoter polymorphism with increased risk for parenteral, but not mucosal, acquisition of human immunodeficiency virus type 1 infection. *Journal of virology*, 78(24), pp.14053–14056.

Martin, M.P. et al., 2010. IL28B polymorphism does not determine outcomes of hepatitis B virus or HIV infection. *The Journal of infectious diseases*, 202(11), pp.1749–53.

Martin, M.P. & Carrington, M., 2013. Immunogenetics of HIV disease. *Immunological Reviews*, 254(1), pp.245–264.

Nakayama, E. et al., 2011. Antibody-dependent enhancement of Marburg virus infection. *J.Infect.Dis.*, 204 Suppl(1537-6613 (Electronic)), pp.S978–S985.

Referencias bibliográficas/Bibliography

- Nyholt, D.R., 2004. A simple correction for multiple testing for single-nucleotide polymorphisms in linkage disequilibrium with each other. *American journal of human genetics*, 74(4), pp.765–9.
- Ogunniyi, A.D. et al., 2009. Pneumococcal histidine triad proteins are regulated by the Zn²⁺-dependent repressor AdcR and inhibit complement deposition through the recruitment of complement factor H. *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 23(3), pp.731–738.
- Oliver, M.A. et al., 2008. Binding of complement regulatory proteins to group A Streptococcus. *Vaccine*, 26 Suppl 8, pp.175–8.
- Petrovski, S. et al., 2011. Common human genetic variants and HIV-1 susceptibility: a genome-wide survey in a homogeneous African population. *AIDS (London, England)*, 25(4), pp.513–8.
- Price, H. et al., 2011. A TRIM5alpha exon 2 polymorphism is associated with protection from HIV-1 infection in the Pumwani sex worker cohort. *AIDS*, 24(12), pp.1813–1821.
- Prokunina-Olsson, L. et al., 2013. A variant upstream of IFNL3 (IL28B) creating a new interferon gene IFNL4 is associated with impaired clearance of hepatitis C virus. *Nature genetics*, 45(2), pp.164–71.
- Ram, S. et al., 1998. A novel sialic acid binding site on factor H mediates serum resistance of sialylated Neisseria gonorrhoeae. *The Journal of experimental medicine*, 187(5), pp.743–752.
- Rathore, A. et al., 2008. Risk for HIV-1 infection is not associated with Repeat-Region polymorphism in the DC-SIGN neck domain and Novel Genetic DC-SIGN Variants among North Indians. *Clinica Chimica Acta*, 391(1-2), pp.1–5.
- Real, L.M. et al., 2014. IFNL4 ss469415590 Variant Shows Similar Performance to rs12979860 as Predictor of Response to Treatment against Hepatitis C Virus Genotype 1 or 4 in Caucasians. *PLoS ONE*, 9(4), p.e95515.
- Ryan, E.J. et al., 2010. Variant in CD209 promoter is associated with severity of liver disease in chronic hepatitis C virus infection. *Human immunology*, 71(8), pp.829–32.

- Sainz, J., Segura-Catena, J. & Jurado, M., 2010. [Association between genetic polymorphism in the promotor region of CD209 and propensity to develop invasive pulmonary aspergillosis]. *Methods and findings in experimental and clinical pharmacology*, 32 Suppl A, pp.9–13.
- Selvaraj, P. et al., 2009. CD209 gene polymorphisms in South Indian HIV and HIV-TB patients. *Infection, genetics and evolution*, 9(2), pp.256–62.
- Serra, C. et al., 2008. Type III and I interferons increase HIV uptake and replication in human cells that overexpress CD4, CCR5, and CXCR4. *AIDS research and human retroviruses*, 24(2), pp.173–180.
- da Silva, R.C. et al., 2014. DC-SIGN polymorphisms are associated to type 1 diabetes mellitus. *Immunobiology*, 219(11), pp.859–865.
- Sironi, M. et al., 2012. A common polymorphism in TLR3 confers natural resistance to HIV-1 infection. *Journal of immunology (Baltimore, Md. : 1950)*, 188(2), pp.818–23.
- Sironi, M. et al., 2014. Evolutionary analysis identifies an MX2 haplotype associated with natural resistance to HIV-1 infection. *Molecular Biology and Evolution*, 31(9), pp.2402–2414.
- Stieh, D.J. et al., 2015. Discrete partitioning of HIV - 1 Env forms revealed by viral capture. *Retrovirology*, 12, pp.1–15.
- Stoiber, H., Clivio, A. & Dierich, M.P., 1997. Role of complement in HIV infection. *Annual review of immunology*, 15, pp.649–74.
- Thames, A.D. et al., 2015. The role of chemokine C-C motif ligand 2 genotype and cerebrospinal fluid chemokine C-C motif ligand 2 in neurocognition among HIV-infected patients. *AIDS (London, England)*, 29(12), pp.1483–91.
- Tortorella, D. et al., 2000. Viral Subversion of the Immune System. *Annu. Rev. Immunol.*, 18, pp.861–926.
- Vannberg, F.O. et al., 2008. CD209 genetic polymorphism and tuberculosis disease. *PLoS ONE*, 3(1), pp.1–6.
- Willey, S. et al., 2011. Extensive complement-dependent enhancement of

Referencias bibliográficas/Bibliography

- HIV-1 by autologous non-neutralising antibodies at early stages of infection. *Retrovirology*, 8(1), p.16.
- Xu, N., Li, X. & Zhong, Y., 2015. Inflammatory cytokines: potential biomarkers of immunologic dysfunction in autism spectrum disorders. *Mediators of inflammation*, 2015, p.531518.
- Xu, Y. et al., 2009. A novel approach to inhibit HIV-1 infection and enhance lysis of HIV by a targeted activator of complement. *Virology journal*, 6, p.123.
- Yi, L. et al., 2015. The association between CD209 gene polymorphisms and pulmonary tuberculosis susceptibility: a meta-analysis. *Int J Clin Exp Pathol.*, 8(10), pp.12437–12445.
- Younas, M. et al., 2015. Immune activation in the course of HIV-1 infection: Causes, phenotypes and persistence under therapy. *HIV Med*, 17(2), pp.89–105.
- Yu, H.-R. et al., 2012. DC-SIGN (CD209) promoter -336 A/G (rs4804803) polymorphism associated with susceptibility of Kawasaki disease. *TheScientificWorldJournal*, 2012, p.634835.
- Zapata, W. et al., 2015. Identification of innate immune antiretroviral factors during in vivo and in vitro exposure to HIV-1. *Microbes and Infection*, (November), pp.1–9.
- Zennou, V. et al., 2000. HIV-1 genome nuclear import is mediated by a central DNA flap. *Cell*, 101(2), pp.173–85.
- Zhang, J. et al., 2008. Protective role of DC-SIGN (CD209) neck-region alleles with <5 repeat units in HIV-1 transmission. *The Journal of infectious diseases*, 198(1), pp.68–71.
- Zhao, K. et al., 2012. Evidence for selection at HIV host susceptibility genes in a West Central African human population. *BMC evolutionary biology*, 12, p.237.

11. Anexos/Annexes



UNIVERSIDAD DE JAÉN
Vicerrectorado de Investigación,
Desarrollo Tecnológico e Innovación

Comité de Bioética

- Informe -

Tipo de actividad: Investigadora – proyecto de investigación.

Título de la actividad: POLIMORFISMOS GENÉTICOS DE LA RUTA DEL
COMPLEMENTO Y RESISTENCIA A LA INFECCIÓN POR VIH-1 EN DROGADICTOS
POR VIA PARENTERAL

Convocatoria y/o entidad a la que se presenta: Junta de Andalucía. Consejería de
Salud 2009.

Investigador principal: Caruz Arcos, Antonio José.

Departamento: Biología Experimental.

Tipo de documentación examinada: Memoria del proyecto y formulario.

Tipo de experimentación o actividad sometida a informe: Experimentación con
muestras de origen humano.

Informe que se emite: Favorable.

Jaén, 9 de noviembre de 2009



María de los Ángeles Peinado Herreros
Vicerrectora de Investigación, Desarrollo Tecnológico e Innovación

Vicerrectorado de Investigación Desarrollo Tecnológico e Innovación

Campus Las Lagunillas, s/n. Edificio B-1 Rectorado – Telf. 953 212597 – Fax 953 211968 – E-mail: vicinv@ujaen.es

Anexo 1 | Informe del Comité de bioética de la Universidad de Jaén.

CONSENTIMIENTO INFORMADO – CONSENTIMIENTO POR ESCRITO DEL PACIENTE

Título de proyecto de investigación: Caracterización funcional de polimorfismos genéticos del complemento asociados a resistencia a la infección por VIH-1.

Yo (Nombre y Apellidos):

- He leído el documento informativo que acompaña a este consentimiento (Información al Paciente)

He podido hacer preguntas sobre el estudio **Caracterización funcional de polimorfismos genéticos del complemento asociados a resistencia a la infección por VIH-1.**

He recibido suficiente información sobre el estudio **Caracterización funcional de polimorfismos genéticos del complemento asociados a resistencia a la infección por VIH-1.**

He hablado con el profesional sanitario informador:

- Dr/Dra.
 - Comprendo que mi participación es voluntaria y soy libre de participar o no en el estudio.
 - Se me ha informado que todos los datos obtenidos en este estudio serán confidenciales y se tratarán conforme establece la Ley Orgánica de Protección de Datos de Carácter Personal 15/99.
 - Se me ha informado de que la donación/información obtenida sólo se utilizará para los fines específicos del estudio.
 - He sido informado/a de que los tejidos que cedo serán utilizados exclusivamente con finalidad de investigación sin ánimo de lucro
 - He sido informado de que no se utilizarán las muestras para otros estudios diferentes a los de este proyecto y a no traspasar las muestras a otros posibles proyectos o equipos de investigación sin solicitar previamente la autorización de la comisión de bioética de los centros participantes
 - **Deseo** ser informado/a de mis datos genéticos y otros de carácter personal que se obtengan en el curso de la investigación, incluidos los descubrimientos inesperados que se puedan producir, siempre que esta información sea necesaria para evitar un grave perjuicio para mi salud o la de mis familiares biológicos.
- Si
No

Comprendo que puedo retirarme del estudio:

- Cuando quiera
 - Sin tener que dar explicaciones
 - Sin que esto repercuta en mis cuidados médicos
- Presto libremente mi conformidad para participar en el *proyecto titulado* : **Caracterización funcional de polimorfismos genéticos del complemento asociados a resistencia a la infección por VIH-1.**

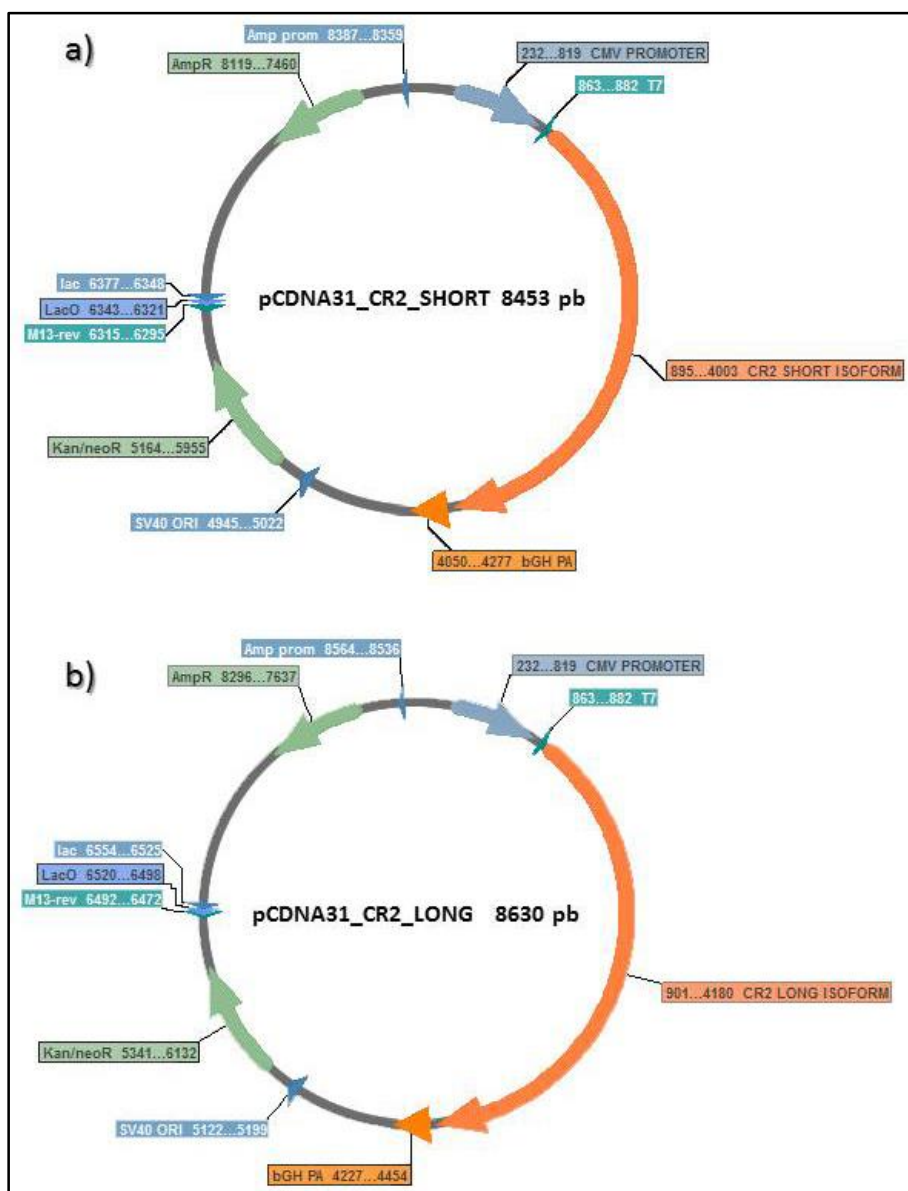
Firma del paciente
(o representante legal en su caso)

Firma del profesional sanitario informador

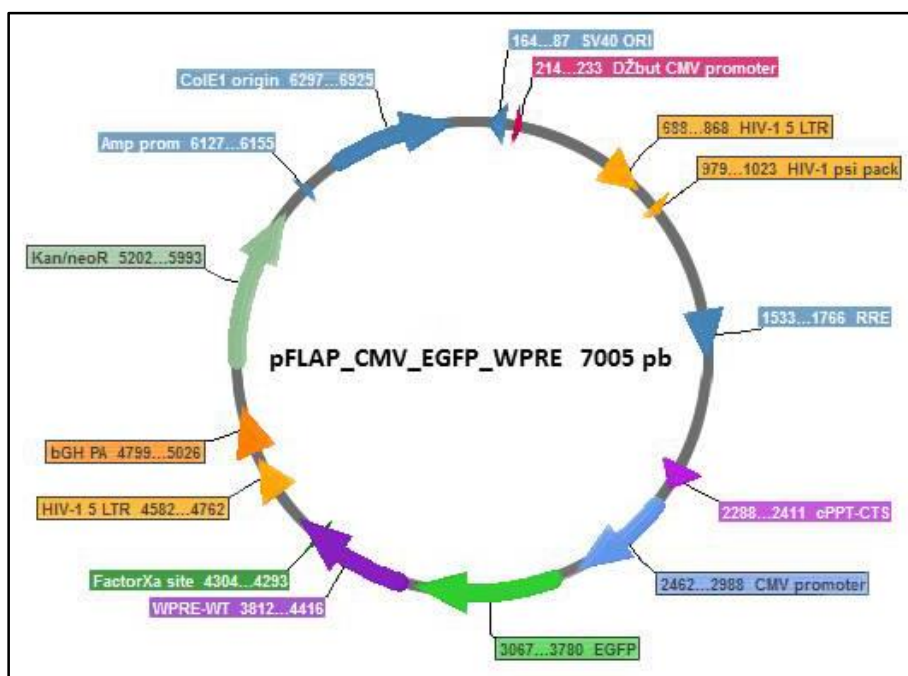
Nombre y apellidos:.....
Fecha:

Nombre y apellidos:
Fecha:

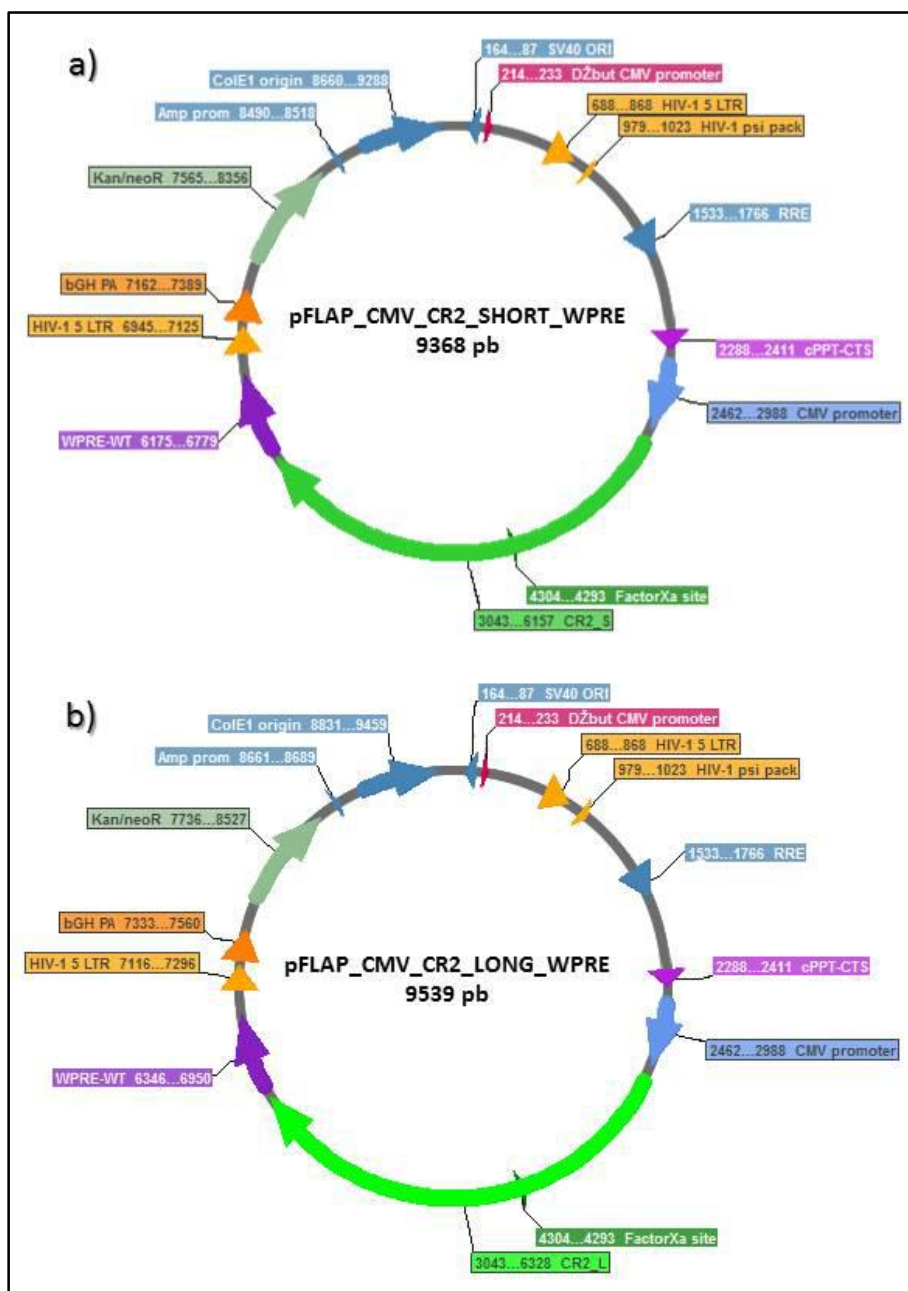
Anexo 2 | Ejemplo de consentimiento informado.



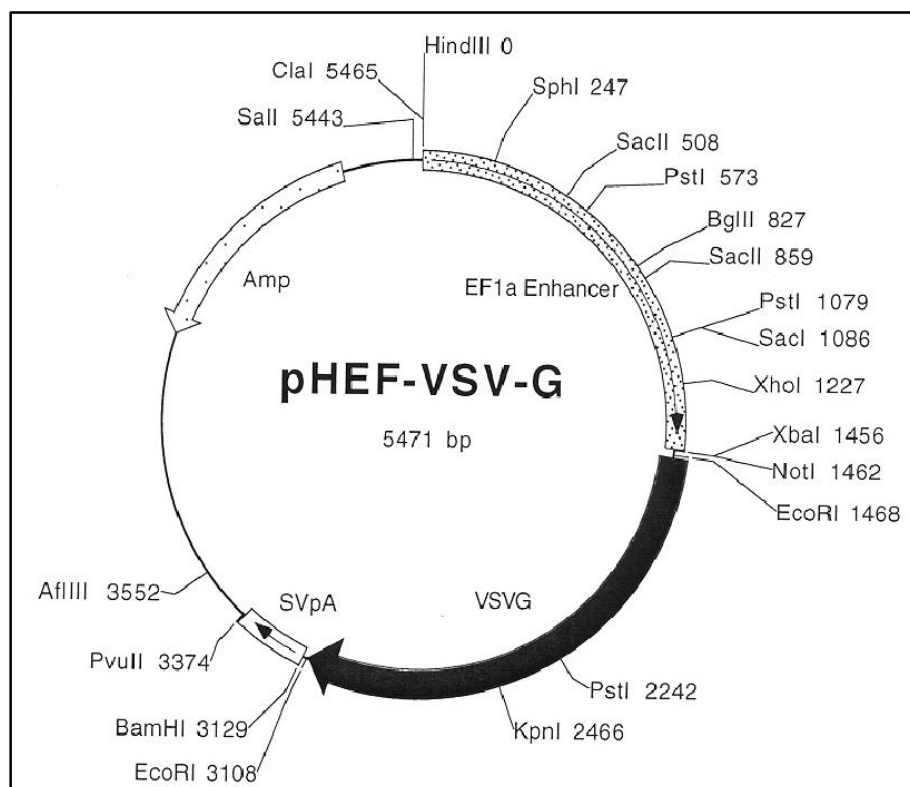
Anexo 3 | Mapa de los plásmido pCDNA31_CR2_SHORT y pCDNA31_CR2_LONG (GenScript EE.UU. Inc., NJ, EE.UU.).



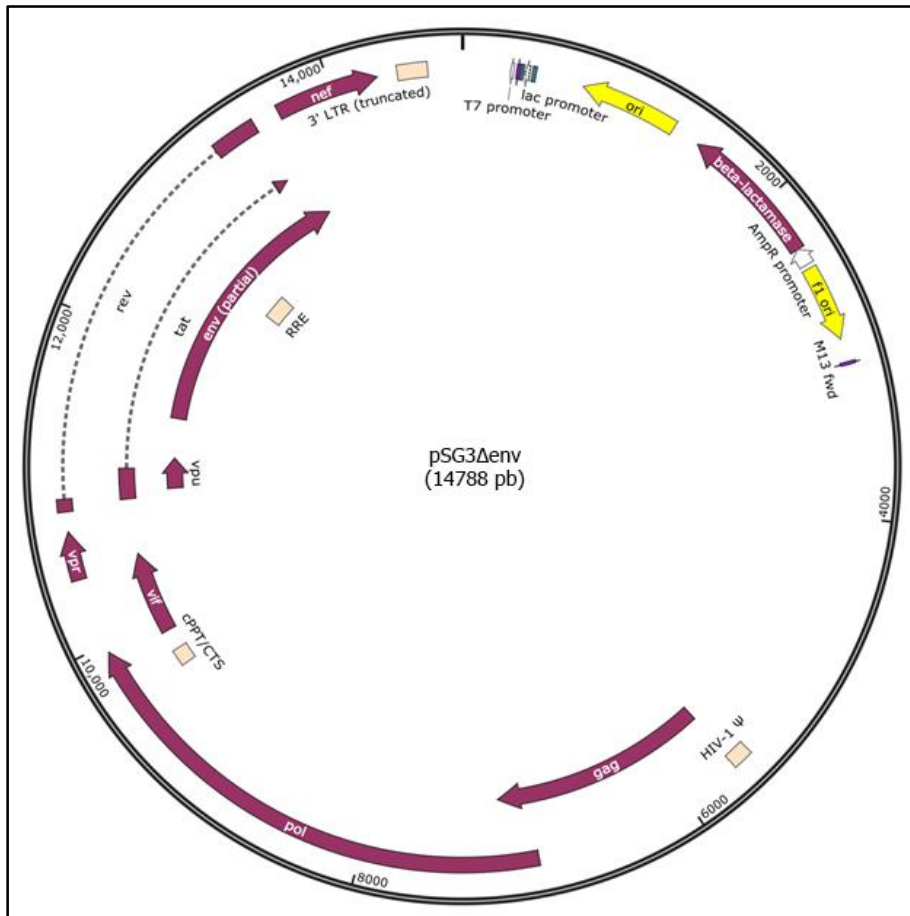
Anexo 4 | Mapa del vector pFLAP-CMV-EGFP-WPRE (Zennou et al. 2000).



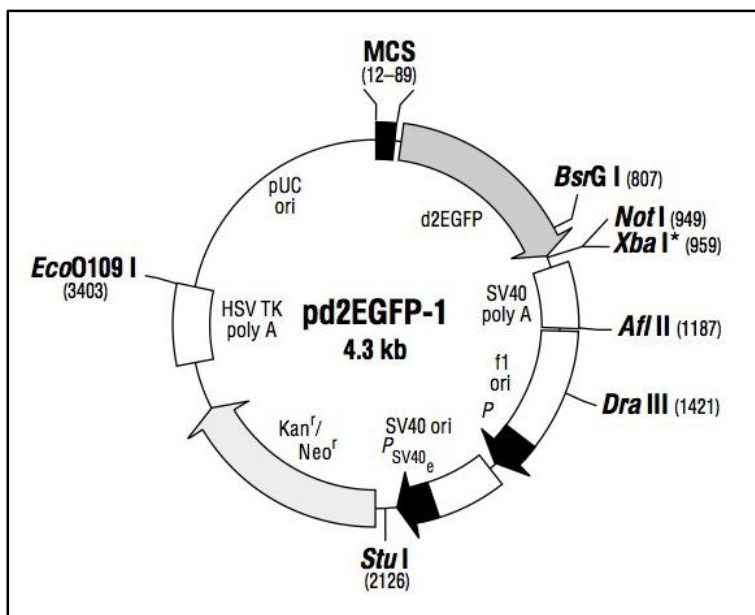
Anexo 5 | Mapa de los vectores pFLAP-CMV-CR2_SHORT-WPRE y pFLAP-CMV-CR2_LONG-WPRE.



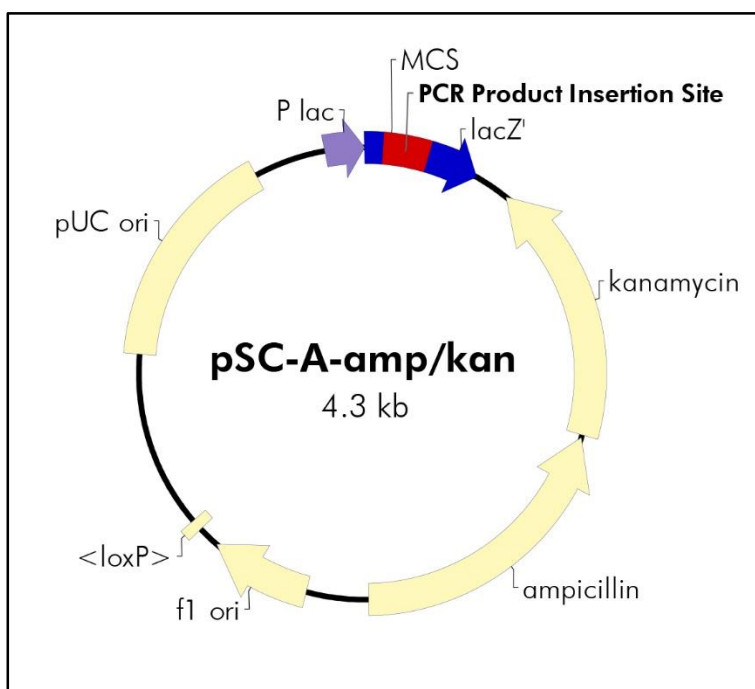
Anexo 6 | Mapa del vector pHEF-VSV-G (REF NIH: 4693).



Anexo 7 | Mapa del vector pSG3Δenv (REF NIH: 11051)



Anexo 8 | Mapa del plásmido pd2EGFP-1 de Clontech (Takara Bio Inc, Kusatsu, Japón).



Anexo 9 | Mapa del vector StrataClone PCR Cloning vector pSC-A-amp/kan (Stratagene, La Jolla, CA, EE.UU.).

12. Otros trabajos publicados/Other published papers



IFNL4 ss469415590 Variant Shows Similar Performance to rs12979860 as Predictor of Response to Treatment against Hepatitis C Virus Genotype 1 or 4 in Caucasians

Luis M. Real^{1,2}, Karin Neukam^{1,2}, Rocío Herrero³, Josep M. Guardiola⁴, Thomas Reiberger^{5,6}, Antonio Rivero-Juarez^{7,8}, Juliana Salazar⁴, Mattias Mandorfer^{5,6}, Dolores Merino⁹, Vicente Soriano¹⁰, Antonio Rivero^{7,8}, Juan Macías^{1,2}, Juan A. Pineda^{1,2}, Antonio Caruz^{3*}

1 Unidad Clínica de Enfermedades Infecciosas y Microbiología, Hospital Universitario de Valme, Sevilla, Spain, **2** Instituto de Biomedicina de Sevilla (IBIS), Sevilla, Spain, **3** Unidad de Inmunogenética, Universidad de Jaén, Jaén, Spain, **4** Servicio de Medicina Interna, Hospital de la Santa Creu i Sant Pau, Barcelona, Spain, **5** Division of Gastroenterology and Hepatology, Department of Internal Medicine III, Medical University of Vienna, Vienna, Austria, **6** Vienna HIV & Liver Study Group, Vienna, Austria, **7** Unidad de Enfermedades Infecciosas, Hospital Universitario Reina Sofía, Córdoba, Spain, **8** Instituto Maimónides de Investigación Biomédica de Córdoba (IMIBIC), Córdoba, Spain, **9** Unidad de Enfermedades Infecciosas, Hospital Juan Ramón Jiménez, Huelva, Spain, **10** Departamento de Enfermedades Infecciosas, Hospital Carlos III, Madrid, Spain

Abstract

Objectives: The rs12979860 variant, linked to *IL28B* gene, predicts sustained viral response (SVR) to pegylated-interferon/ribavirin (pegIFN/RBV) therapy in Hepatitis C Virus genotype 1 or 4 (HCV-1/4)-infected patients. Recently, a functional variant, ss469415590, in linkage disequilibrium (LD) with rs12979860, has been discovered. Our objective was to assess the value of ss469415590 to predict SVR to pegIFN/RBV in Caucasian HCV-1/4-infected individuals and to compare its performance with that of rs12979860.

Methods: 272 Caucasian HCV-1/4-infected patients who completed a course of pegIFN/RBV were genotyped for both rs12979860 and ss469415590 markers. Logistic regression models including factors with univariate association with SVR and each genetic marker were elaborated. The area under the receiver operating-characteristic curve (AUROC) was calculated for each model and both were compared.

Results: Both markers were in LD ($r^2 = 0.82$). For rs12979860, 66 (64.0%) CC versus 56 (33.1%) T allele carriers achieved SVR (Adjusted OR = 4.156, 95%CI = 2.388–7.232, $p = 4.647 \times 10^{-7}$). For ss469415590, 66 (66.0%) TT/TT versus 56 (32.5%) –G allele carriers (Adjusted OR = 4.783, 95%CI = 2.714–8.428, $p = 6.153 \times 10^{-8}$) achieved SVR. The AUROC of the model including rs12979860 was 0.742 (95%CI = 0.672–0.813) and of that based on ss469415590 was 0.756 (95%CI = 0.687–0.826) ($p = 0.780$).

Conclusions: The ss469415590 variant shows an equivalent performance to predict SVR to pegIFN/RBV than the rs12979860 in Caucasian HCV-1/4-infected patients.

Citation: Real LM, Neukam K, Herrero R, Guardiola JM, Reiberger T, et al. (2014) *IFNL4* ss469415590 Variant Shows Similar Performance to rs12979860 as Predictor of Response to Treatment against Hepatitis C Virus Genotype 1 or 4 in Caucasians. PLoS ONE 9(4): e95515. doi:10.1371/journal.pone.0095515

Editor: Maria Eugenia Saez, CAEBi, Spain

Received: February 11, 2014; **Accepted:** March 27, 2014; **Published:** April 18, 2014

Copyright: © 2014 Real et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported in part by the Red de Investigación en SIDA (ISCIII-RETIC RD12/0017), the Fundación Progreso y Salud, Consejería de Salud de la Junta de Andalucía (grant numbers PI-0247-2010, PI-0157/2011, PI-0430/2012), the Fondo de Investigaciones Sanitarias (grant numbers PI10/01664), the Ministerio de Sanidad y Servicios Sociales (EC11-304) and the Fundación para la Investigación y la Prevención del Sida en España (grant number 121004/10). Sara Borrell postdoctoral perfection grant from the Instituto de Salud Carlos III (grant number SCO/523/2008 to KN). Human resources grant from the Servicio de Salud de la Junta de Andalucía (grant number B-0037 to JMS). Intensification grant from the Instituto de Salud Carlos III (grant number Programa-13SNS to J.A.P.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: caruz@ujaen.es

Introduction

Since the association of the rs12979860 polymorphism with viral clearance and response to pegylated interferon and ribavirin (pegIFN/RBV) treatment was reported [1], the genotype of this marker has been widely used as a pharmacogenetic tool. rs12979860 genotyping provides useful information for individualizing the management of patients with chronic hepatitis C [2]. This genetic marker mainly predicts the outcome of therapy of HCV genotype 1 or 4 (HCV-1/4)-infections [3,4], both in HCV-

monoinfected and in HIV/HCV-co-infected individuals [5,6]. Moreover, this pharmacogenetic marker continues showing impact on the outcome of direct-acting antiviral (DAA)-based regimens, particularly in treatment-naïve patients [7,8].

The rs12979860 genetic marker is located upstream of *IL28B* gene (also known as *IFNL3* gene) suggesting a prominent role of this locus in the processes involved in viral clearance [1]. *IL28B* has been implicated in the inhibition of HCV replication in vitro and in vivo [9–15]. In addition, it also shows a synergistic anti-HCV effect in combination with IFN- α [16]. However,

rs12979860 variant has not been consistently associated with hepatic IL28B mRNA expression [17–19]. That suggests that this polymorphism should be in linkage disequilibrium (LD) with other functional variants.

Recently, a new variant, denoted ss469415590 (TT/–G) (rs368234815), in strong LD with rs12979860, has been reported, which seems to have functional effects on IL28B expression [20]. Thus, the ss469415590 TT/TT carriers show higher expression of IL28B than –G carriers, independently of the rs12979860 genotype [20]. Interestingly, it has also been found that the –G allele of this new variant introduces a frame shift in the DNA sequence, which induces mRNA expression of an IFN analogue (IFNL4) in stimulated human hepatocytes [21]. In fact, both ss469415590 and rs12979860 polymorphisms are located within the *IFNL4* gene [21]. IFNL4 could limit the action of type I and type III IFNs, reducing the capacity of HCV clearance [21]. According to these findings, ss469415590 might be a better predictor of sustained viral response (SVR) than the rs12979860 variant, but only in African populations, where both markers are not so strongly linked [21]. However, other authors have reported differences between these markers in the performance to predict SVR among Caucasians populations [20].

The contradictory results observed in Caucasians require new validation studies in order to clarify and validate which genetic marker yields the best performance to predict SVR to IFN-based therapies in this population. Considering the relevance of rs12979860 genotype in treatment decisions in HCV-infected patients [2], it is necessary to clarify if this pharmacogenetic tool should be replaced by the ss469415590 genotyping in our geographical area.

The objective of this study was to assess the value of ss469415590 genotyping to predict SVR to pegIFN/RBV among Caucasian HCV-1/4-infected individuals and to compare its performance to that of rs12979860 testing.

Patients and Methods

Ethics Statement

This study was in compliance with the national legislation and it was performed according to the ethical guidelines of the Declaration of Helsinki. The study was approved by the Ethics Committee of the Hospital Universitario de Valme. Written consent was obtained from all individuals before sampling.

Patients and Follow Up

This study was carried out on Caucasian HCV-1/4-infected patients who consecutively completed a course of pegIFN/RBV treatment at the infectious diseases units of six hospitals in Spain and one in Austria between May 2001 and December 2011. Patients were prospectively followed and all of them were seen, at least, at week 4, 12, 24 and 48 while on treatment and 24 weeks after completing therapy. Clinical, biochemical and hematological evaluations were performed at each visit. In all patients, a whole blood sample was collected, frozen and stored for subsequent genetic determinations. Patients in whom SVR could be assessed as an on-treatment setting, i.e. excluding those who voluntarily dropped out or discontinued therapy due to adverse events, were included in this study.

Treatment Strategies and Response Definition

pegIFN alfa-2a or alfa-2b was administered at doses of 180 µg or 1.5 µg/kg once weekly, respectively, in combination with weight-adjusted RBV (1000 mg/day for <75 kg and 1200 mg/day for ≥75 kg). The scheduled treatment duration was 48 weeks

for all patients. SVR was defined as undetectable plasma HCV-RNA 24 weeks after the completion of treatment.

Genotyping

DNA was extracted from frozen whole blood samples using the Quick Pure Blood DNA extraction Kit (Macherey-Nagel, Düren, Germany) or Magna Pure system (Roche Diagnostics, Mannheim, Germany). The rs12979860 and ss469415590 genetic markers were genotyped by real-time PCR techniques as previously reported [5,21]. Samples that showed genetic recombination between these markers were re-genotyped for both markers as a quality control.

Statistical Analyses

The online resource at the Institute for Human Genetics, Munich, Germany (<http://ihg.gsf.de>) was used for the comparison of rs12979860 and ss469415590 genotypic frequencies between groups to determine p values, odds-ratios (OR) and confidence intervals (C.I.). This resource was also used to carry out the Hardy-Weinberg equilibrium (HWE) test and to calculate the allelic frequencies for each marker. Pair-wise LD estimate (r^2) was determined using the Plink software [22].

Group comparisons of categorical variables were performed using Pearson chi-square test or Fisher's exact test. To compare age among groups we used the Mann-Whitney U tests (data not normally distributed). Multivariate logistic regression models were elaborated including variables associated with SVR in univariate analysis ($p < 0.05$), as well as age and gender, to obtain adjusted p and OR values. All these calculations, as well as those performed to obtain the area under the receiver operating-characteristic curve (AUROC) of models, were carried out using the SPSS software 19.0 (IBM Corporation, Somers, NY, USA). Comparisons between the AUROCs were performed using the MedCalc Statistical Software version 12.7.7 (MedCalc Software bvba, Ostend, Belgium; <http://www.medcalc.org>; 2013).

Results

Features of the Studied Population

Two-hundred and seventy-two HCV-1/4-infected individuals, most of them men (79.4%), were included in this study. Among them, 209 (76.8%) patients were co-infected with HIV. The main characteristics of the population studied are depicted in table 1.

General Genotyping Data Analysis

rs12979860 and ss469415590 were in LD ($r^2 = 0.82$). Consequently, the minor allelic frequency was similar for both markers (0.38 for rs12979860 T allele and 0.39 for ss469415590–G allele). The rs12979860 genotypes were distributed as follow: 37 (13.6%) TT, 132 (48.5%) CT and 103 (37.9%) CC. The ss469415590 genotypic distribution was 39 (14.3%) –G/–G, 133 (48.9%) TT/–G and 100 (36.7%) TT/TT. Both genotypic distributions were in accordance with the HWE law ($p > 0.500$ in both cases). Fifteen individuals (5.5%) carrying one of the favorable genotypes (rs12979860 CC or ss469415590 TT/TT) showed genetic recombination between these markers. Thus, 6 of them were rs12979860 CT ss469415590 TT/TT and, 9 individuals were rs12979860 CC ss469415590 –G/–G or TT/–G.

Response to Therapy

One hundred and twenty two patients (44.8%) achieved SVR. The median age (quartile 1–quartile 3) of these patients was 42.0 (37.8–46.6) years versus 43.0 (38.7–46.7) years of those who did not reach SVR ($p = 0.263$). The rates of patients achieving SVR

Table 1. Main characteristics of the study population.

Variables	
Age ¹ , years	43.0 (38.7–46.7)*
Male gender, no. (%)	216 (79.4)
HIV infection, no. (%)	209 (76.8)
HCV genotype 4, no (%)	56 (20.6)
BMI, Kg/m ²	23.7 (21.9–26.5)*
Plasma HCV RNA, log ₁₀ IU/mL	6.2 (5.6–6.7)*
Advanced fibrosis ¹ , no (%)	97 (39.3)

BMI: body mass index.

*Median (quartile 1–quartile 3).

¹Determined by liver biopsy (F $\geq$ 3 according to the Scheuer Index) or a liver stiffness value $\geq$ 11 kPa.

doi:10.1371/journal.pone.0095515.t001

according to the age, gender, HIV coinfection, viral genotype, body mass index, baseline viral load and advanced fibrosis are depicted in table 2.

For rs12979860, 66 (64.0%) CC versus 56 (33.1%) T allele carriers achieved SVR (OR = 3.599, 95% CI = 2.152–6.021, $p = 6.459 \times 10^{-7}$). For ss469415590, 66 (66.0%) TT/TT versus 56 (32.5%) –G allele carriers showed SVR (OR = 4.021, 95% CI = 2.385–6.780, $p = 8.940 \times 10^{-8}$) (Table 2). Figure 1 shows the rates of SVR according to genetic markers in groups of patients stratified by HCV genotype.

Regarding to the recombinant individuals, 3 (33.3%) among 9 patients bearing the rs12979860 CC genotype, and 3 (50%) among 6 patients bearing the ss469415590 TT/TT genotype achieved SVR ($p = 0.622$).

Predictive Performance of Markers

The univariate and multivariate associations between the well-established predictive factors, plus age and gender, as well as the analyzed genetic factors and SVR are shown in Table 2. The ROCs of the models obtained with each marker are displayed in

Table 2. Univariate and multivariate analysis (one model for each genetic marker) of factors associated with SVR.

Variables		Univariate		rs12979860 model	ss469415590 model
		SVR n (%)	p value	AOR (95% CI)/p value	AOR (95% CI)/p value
Age	≤ 43.0 years	65 (48.1)	0.214	0.992 (0.956–1.029)/0.677	0.990 (0.954–1.027)/0.591
	> 43.0 years	54 (40.6)			
Gender	Male	98 (45.4)	0.736	0.855 (0.442–1.654)/0.642	0.792 (0.407–1.543)/0.494
	Female	24 (42.9)			
HIV infection	No	36 (57.1)	0.025	0.461 (0.245–0.866)/0.016	0.443 (0.235–0.838)/0.012
	Yes	86 (41.1)			
HCV genotype	1	94 (43.5)	0.385	NI	NI
	4	28 (50.0)			
BMI	< 25 Kg/m ²	61 (44.9)	0.241	NI	NI
	≥ 25 Kg/m ²	44 (53)			
Plasma HCV ARN	$\leq 6 \times 10^5$ IU/ml	49 (57.0)	0.004	0.526 (0.371–0.745)/2.923 $\times 10^{-4}$	0.522 (0.367–0.742)/2.903 $\times 10^{-4}$
	$> 6 \times 10^5$ IU/mL	67 (38.1)			
Advanced fibrosis	No	71 (47.3)	0.207	NI	NI
	Yes	38 (39.2)			
rs12979860 [†]	TT	10 (27.0)	6.459×10^{-7}	*4.156 (2.388–7.232)/ 4.647×10^{-7}	NI
	CT	46 (34.8)			
	CC	66 (64.0)			
ss469415590 [‡]	–G/–G	10 (25.6)	8.940×10^{-8}	NI	*4.783 (2.714–8.428)/ 6.153×10^{-8}
	TT/–G	46 (34.6)			
	TT/TT	66 (66.0)			

SVR, Sustained viral response; BMI, body mass index; NI, not included in the model; CI, confidence interval; AOR, adjusted odds ratio.

* p values and AOR are for a recessive model (CC vs CT+TT for rs12979860 and TT/TT vs TT/–G+–G/–G for ss469415590).

[†]Count for non responders were 27, 86 and 37 for TT, CT and CC genotypes respectively.

[‡]Counts for non responders were 29, 87 and 34 for –G/–G, TT/–G and TT/TT genotypes respectively.

doi:10.1371/journal.pone.0095515.t002

Comparison of HCV Treatment Response Pharmacogenetic Markers

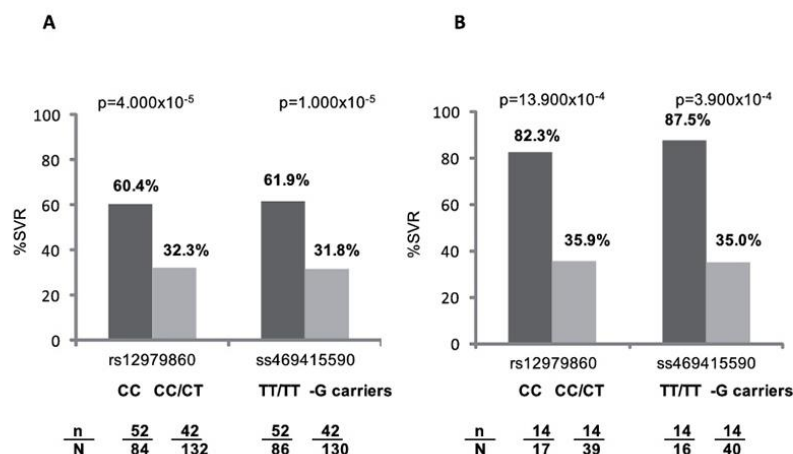


Figure 1. Rates of SVR according to marker genotypes. A: in HCV-1 infected patients and B: in HCV-4 infected patients. n/N: number of patients who achieved SVR/total number of patients (for each genotype group). For each polymorphism, the p values depicted were obtained from the comparison of SVR rates between genotype groups.
doi:10.1371/journal.pone.0095515.g001

Figure 2. HIV coinfection, HCV-RNA viral load and the genetic marker included were independently associated with SVR in both models (Table 2). The AUROC of the model that included the rs12979860 marker was 0.742 (95% CI = 0.672–0.813) and of that based on ss469415590 was 0.756 (95% CI = 0.687–0.826) (Figure 2). Both AUROCs were comparable ($p = 0.780$).

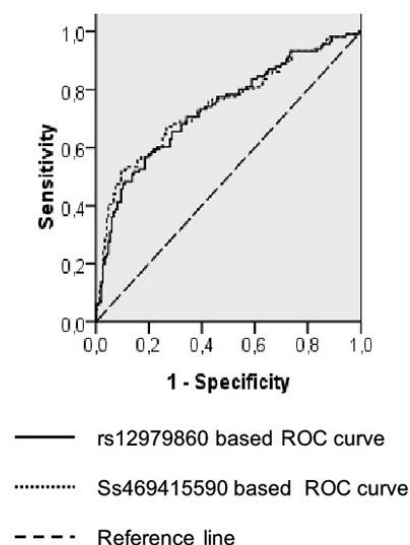


Figure 2. Receiver operating-characteristic curves obtained from each logistic regression model.
doi:10.1371/journal.pone.0095515.g002

In order to investigate whether these markers could have a different performance as SVR predictors in patients infected with HCV-1 and HCV-4, separate substudies were carried out in these particular subsets. Logistic regression models for each marker, introducing the same variables as in the overall study, were constructed. In the HCV-1 infected patient subgroup the adjusted OR (AOR) (95% CI) obtained for rs12979860 was 3.491 (1.899–6.419) ($p = 5.734 \times 10^{-5}$), and that for ss469415590 was 3.935 (2.118–7.310) ($p = 1.460 \times 10^{-5}$). In the HCV-4 infected patient subgroup, these values were 18.802 (3.342–105.791) ($p = 8.725 \times 10^{-5}$) for rs12979860 and 29.147 (4.215–201.559) ($p = 6.305 \times 10^{-5}$) for ss469415590.

In the HCV-1 infected patient subgroup, The AUROC of the model including the rs12979860 marker was 0.717 (95% CI = 0.656–0.798) and of that including ss469415590 was 0.726 (95% CI = 0.656–0.797). In the HCV-4 infected patient subgroup, these values were 0.830 (95% CI = 0.716–0.945) and 0.847 (95% CI = 0.736–0.958), respectively. There were not significant differences between the AUROCs obtained for each marker neither in the HCV-1 infected patient subgroup ($p = 0.859$) nor in the HCV-4 infected patient subgroup ($p = 0.833$).

Discussion

Our results indicate that ss469415590 and rs12979860 variants perform similarly to predict SVR to pegIFN/RBV in Caucasian HCV-1/4-infected patients. This finding implies that replacing rs12979860 genotype determination with ss469415590 as pharmacogenetic predictive tool in clinical practice would not provide further benefit.

The ss469415590 TT allele seems to increase the IL28B expression [20], and also seems to prevent the IFNL4 expression [21]. Both effects are involved in the inhibition of HCV replication. By the contrary, rs12979860 has not a known functional effect. Accordingly, a higher predictive performance of ss469415590 TT was expected. However, the results of this study do not confirm such a hypothesis.

Both ss469415590 and rs2979860 markers are located within the exon 1 and intron 1 of *IFNL4* gene respectively, separated by 367 pairs of bases and, therefore, in high LD. However, some inter ethnic variations exist in the correlation level between ss469415590 TT and rs2979860 C alleles with stronger correlation in Caucasian than in Africans [21]. This data might explain why we did not observe any differences in the performance of these markers to predict SVR in our Caucasian population, as it has also previously been reported [21], and why these differences are found in Africans [21]. Similar conclusions were obtained by Stattemayer et al. [23] recently. They studied a large sample of HCV mono-infected patients, mainly Caucasians, and concluded that there is no benefit in additional testing for ss469415590 for treatment prediction in these patients. In spite of this fact, a study conducted in the Swiss Hepatitis C Cohort, which included Caucasians HCV-1/4 and HCV-2/3 infected patients [20], reported small, but statistically significant, differences between these markers for predicting SVR achievement. Two additional studies reported contradictory results in Caucasian HIV-HCV-1/4 coinfected patients [24,25], although none of these studies carried out a formal statistical comparison of the results obtained with each marker. Taken together, all these data suggest that the difference between these markers in terms of performance to predict SVR in Caucasians, if any, is negligible.

Because both markers are in high LD, the possible differences in the performance to predict SVR may be more evident in the recombinant individuals. In accordance with the possible functional role of the ss469415590, a higher rate of SVR was expected in the recombinant individuals bearing the favorable ss469415590 TT/TT genotype than in those bearing the rs12979860 CC genotype. In our study, 15 individuals were recombinants, more than those reported by Stattemayer et al. [23] in their larger sample. In spite of this, that assumption was not proven in the population analyzed herein.

The impact of ss469415590 variations on the likelihood of achieving SVR had been proven in patients bearing HCV-1/4. However, there was little information on subjects infected with HCV-4 so far [23]. This study confirms that ss469415590 genotype has also a robust impact on the response to therapy to pegIFN plus RBV in subjects harboring HCV-4, which might be

even stronger than in patients infected with HCV-1 (figure 1). This fact is in agreement with that found with rs12979860 genotype here and in previous studies, where it has been found that this SNP is a reliable SVR predictor in HCV-4 infection [26], with an impact even stronger than on patients infected with HCV-1. But again, ss469415590 genotyping did not show a better performance than rs12979860 variation in HCV-4 carriers either.

This study has two limitations. First, the population analyzed is mostly made up by HIV coinfected patients. Consequently, our results could be mainly applicable to this population. However, as the population analyzed here is the largest HIV/HCV 1/4-coinfected patients reported so far, this study provides the strongest evidence to date on the fact that ss469415590 TT/TT genotype does not perform better than rs12979860 genotype for SVR prediction in this specific subset. Second, this study has been performed on pegIFN/RBV treated patients. This regimen is being replaced with DAA-based combinations. Moreover, the interferon-free regimens will likely be the standard of care in the next few years. Because of this, it could be thought that the future relevance of these findings might be low. However, it has been shown that rs12979860 genotype also impacts on the outcome of most DAA-based regimens including pegIFN [7]. Also, rs12979860 genotype has a role in the viral kinetics and response in interferon-free regimens [8]. These facts suggest that our conclusions could be extended to scenarios of DAA-based therapy.

In conclusion, our results indicate that the ss469415590 marker shows equivalent performance to predict SVR to pegIFN/RBV than the rs12979860 variant in Caucasian HCV-1/4-infected patients. The strong LD between both genetic variants may explain why the possible functional one, i.e. ss469415590, does not perform better than the rs2979860 marker. Consequently, there is no evidence to support a replacement of rs12979860 by ss469415590 genotyping in clinical practice.

Author Contributions

Conceived and designed the experiments: AC JAP VS AR LMR. Performed the experiments: AC RH ARJ JS. Analyzed the data: LMR KN AC JAP JM. Contributed reagents/materials/analysis tools: DM JMG TR MM. Wrote the paper: LMR AC KN JAP. Acquisition and analysis of clinical data: DM JMG TR MM JM.

References

- Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, et al. (2009) Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature* 461: 399–401.
- Mandorfer M, Neukam K, Reiberger T, Payer BA, Rivero A, et al. (2013) The impact of interleukin 28B rs12979860 single nucleotide polymorphism and liver fibrosis stage on response-guided therapy in HIV/HCV-coinfected patients. *AIDS* 27: 2707–2714.
- Sarrasin C, Süsser S, Doehring A, Lange CM, Müller T, et al. (2011) Importance of IL28B gene polymorphisms in hepatitis C virus genotype 2 and 3 infected patients. *J Hepatol* 54: 415–421.
- Thompson AJ, Muir AJ, Sulkowski MS, Ge D, Fellay J, et al. (2010) Interleukin-28B polymorphism improves viral kinetics and is the strongest pretreatment predictor of sustained virologic response in genotype 1 hepatitis C virus. *Gastroenterology* 139: 120–129 e118.
- Pineda JA, Carraz A, Rivero A, Neukam K, Salas I, et al. (2010) Prediction of response to pegylated interferon plus ribavirin by IL28B gene variation in patients coinfected with HIV and hepatitis C virus. *Clin Infect Dis* 51: 788–795.
- Rallon NI, Naggie S, Benito JM, Medrano J, Restrepo C, et al. (2010) Association of a single nucleotide polymorphism near the interleukin-28B gene with response to hepatitis C therapy in HIV/hepatitis C virus-coinfected patients. *AIDS* 24: F23–29.
- Bota S, Sporea I, Sirli R, Neghina AM, Popescu A, et al. (2013) Role of interleukin-28B polymorphism as a predictor of sustained virologic response in patients with chronic hepatitis C treated with triple therapy: a systematic review and meta-analysis. *Clin Drug Investig* 33: 325–331.
- Chu TW, Kulkarni R, Gane EJ, Roberts SK, Stedman C, et al. (2012) Effect of IL28B genotype on early viral kinetics during interferon-free treatment of patients with chronic hepatitis C. *Gastroenterology* 142: 790–795.
- Ank N, West H, Bartholdy C, Eriksson K, Thomsen AR, et al. (2006) Lambda interferon (IFN-lambda), a type III IFN, is induced by viruses and IFNs and displays potent antiviral activity against select virus infections in vivo. *J Virol* 80: 4501–4509.
- Hou W, Wang X, Ye L, Zhou L, Yang ZQ, et al. (2009) Lambda interferon inhibits human immunodeficiency virus type 1 infection of macrophages. *J Virol* 83: 3834–3842.
- Kotenko SV, Gallagher G, Bairin IV, Lewis-Antes A, Shen M, et al. (2003) IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. *Nat Immunol* 4: 69–77.
- Meager A, Visvalingam K, Däiger P, Bryan D, Wadliwa M (2005) Biological activity of interleukins-28 and -29: comparison with type I interferons. *Cytokine* 31: 109–118.
- Robek MD, Boyd BS and Chikari IV (2005) Lambda interferon inhibits hepatitis B and C virus replication. *J Virol* 79: 3851–3854.
- Sheppard P, Kindsvogel W, Xu W, Henderson K, Schlottmeyer S, et al. (2003) IL-28, IL-29 and their class II cytokine receptor IL-28R. *Nat Immunol* 4: 63–68.
- Zhang L, Jilg N, Shao RX, Lin W, Fusco DN, et al. (2011) IL28B inhibits hepatitis C virus replication through the JAK-STAT pathway. *J Hepatol* 55: 289–298.
- Shindo H, Maekawa S, Komase K, Miura M, Kadokura M, et al. (2013) IL-28B (IFN-lambda3) and IFN-alpha synergistically inhibit HCV replication. *J Viral Hepat* 20: 281–289.
- Dill MT, Duong FH, Vogt JE, Bilezikci S, Bocharov PY, et al. (2011) Interferon-induced gene expression is a stronger predictor of treatment response than IL28B genotype in patients with hepatitis C. *Gastroenterology* 140: 1021–1031.
- Honda M, Sakai A, Yamashita T, Nakamoto Y, Mizukoshi E, et al. (2010) Hepatic ISG expression is associated with genetic variation in interleukin 28B

Comparison of HCV Treatment Response Pharmacogenetic Markers

- and the outcome of IFN therapy for chronic hepatitis C. *Gastroenterology* 139: 499–509.
19. Marukian S, Andrus I, Sheahan TP, Jones CT, Charles ED, et al. (2011) Hepatitis C virus induces interferon-lambda and interferon-stimulated genes in primary liver cultures. *Hepatology* 54: 1913–1923.
 20. Bibert S, Roger T, Calandra T, Bochud M, Cerny A, et al. (2013) IL28B expression depends on a novel TT/–G polymorphism which improves HCV clearance prediction. *J Exp Med* 210: 1109–1116.
 21. Prokunina-Olsson L, Muchmore B, Tang W, Pfeiffer RM, Park H, et al. (2013) A variant upstream of IFNL3 (IL28B) creating a new interferon gene IFNL4 is associated with impaired clearance of hepatitis C virus. *Nat Genet* 45: 164–171.
 22. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, et al. (2007) PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet* 81: 559–575.
 23. Stattemayer AF, Strassl R, Maieron A, Rutter K, Stauber R, et al. (2014) Polymorphisms of interferon-lambda4 and IL28B - effects on treatment response to interferon/ribavirin in patients with chronic hepatitis C. *Aliment Pharmacol Ther* 39: 104–111.
 24. Franco S, Aparicio E, Parera M, Clotet B, Tural C, et al. (2013) IFNL4 rs469415590 variant is a better predictor than ILF3 (IL28B) rs12979860 of pegylated interferon-alpha/ribavirin therapy failure in hepatitis C virus/HIV-1 coinfecting patients. *AIDS* 28: 133–136.
 25. Kramer B, Nischalke HD, Boesecke C, Ingiliz P, Voigt E, et al. (2013) Variation in IFNL4 genotype and response to interferon-based therapy of hepatitis C in HIV-positive patients with acute and chronic hepatitis C. *AIDS* 27: 2817–2819.
 26. Mira JA, Rivero A, de Los Santos-Gil I, Lopez-Cortes LF, Giron-Gonzalez JA, et al. (2012) Hepatitis C virus genotype 4 responds better to pegylated interferon with ribavirin than genotype 1 in HIV-infected patients. *AIDS* 26: 1721–1724.



ORIGINAL ARTICLE

Association of low-density lipoprotein receptor genotypes with hepatitis C viral load

A Caruz¹, K Neukam², A Rivero-Juárez³, R Herrero¹, LM Real², Á Camacho³, P Barreiro⁴, P Labarga⁴, A Rivero³ and JA Pineda²

Several data suggest that low-density lipoprotein receptor (LDLR) is a co-receptor for hepatitis C virus (HCV). Soluble LDLR can inhibit HCV infectivity; greater plasma low-density lipoprotein levels are associated with treatment success; *LDLR* genotypes have a synergistic impact on the likelihood of achieving SVR with Peg-IFN plus RBV, as well as on viral kinetics after starting treatment. The objective of this study was to assess the impact of genetic polymorphisms in genes related to cholesterol synthesis and transport pathways on pre-treatment plasma HCV viral load (VL). A total of 442 patients infected with HCV and treatment naive were prospectively recruited. One hundred forty-four SNPs located in 40 genes from the cholesterol synthesis/transport and *IL28B* were genotyped and analyzed for genetic association with pre-treatment plasma HCV VL. SNPs rs1433099 and rs2569540 of *LDLR* showed association with plasma HCV VL ($P = 4 \times 10^{-4}$ and $P = 2 \times 10^{-3}$) in patients infected with genotypes 1 and 4. A haplotype including the last three exons of *LDLR* showed association with the cutoff level of 600 000 IU ml⁻¹ VL for genotypes 1 and 4 (OR = 0.27; $P = 8 \times 10^{-9}$), as well as a quantitative VL (mean $\pm$ s.d.: 6.19 ± 0.9 vs CC + CG 5.58 ± 1.1 log IU ml⁻¹, $P = 8 \times 10^{-9}$). *LDLR* genotypes are a major genetic factor influencing HCV VL in patients infected with genotypes 1 and 4.

Genes and Immunity (2014) 15, 16–24; doi:10.1038/gene.2013.56; published online 31 October 2013

Keywords: hepatitis C virus; viral load; low-density lipoprotein receptor; SNP; haplotypes; LDL

INTRODUCTION

Hepatitis C virus (HCV) viral replication cycle is affected by cholesterol and fatty acid biosynthesis.^{1–3} HCV infection increases lipid biosynthesis and storage in the hepatocytes,⁴ contributing to the HCV-associated steatosis, hypobetalipoproteinemia and insulin resistance.^{1–3} The inhibition of cholesterol production by means of blocking of HMG CoA reductase or 3-beta-hydroxysterol Δ^7 reductase induces a marked reduction in HCV RNA levels *in vitro*.^{4,5}

The assembly of HCV virus is tightly linked to very-low-density lipoprotein (VLDL) synthesis and the secretory pathway.⁶ HCV particles in serum are complexed with VLDL; although the exact molecular structure is not resolved, indirect data suggest that HCV may hide in the lipid nucleus of VLDL, making HCV undetectable to antibodies and serum complement. This association with VLDL allows the virus to bind target cells through lipoprotein receptors⁷ after partial processing by lipoprotein and hepatic lipase, which hydrolyze the triglycerides in the VLDL giving rise to IDL and low-density lipoprotein (LDL). Experimental⁸ and clinical⁹ data suggest that LDL receptor (LDLR) is a docking molecule or a co-receptor for HCV: (1) the levels of HCV RNA in primary hepatocytes correlate with *LDLR* mRNA expression and LDL uptake efficiency;¹⁰ (2) soluble LDLR can inhibit HCV infectivity;¹⁰ (3) greater plasma LDL levels are associated with sustained virological response (SVR) after treatment with Peg-IFN plus RBV, likely due to competitive block of the HCV entry;¹¹ (4) polymorphisms in *LDLR*, which are associated with plasma levels of LDL, have a synergistic impact on the likelihood of achieving SVR with Peg-IFN plus RBV in patients infected with viral genotypes 1 and 4^{11,12} as well as on viral kinetics after treatment.¹³

However, some discrepancies remain. Thus, 'time of addition' experiments with a monoclonal antibody against LDLR reveal that LDLR has a major role in post-entry phases of viral life cycle, probably increasing lipoprotein uptake, an essential factor for HCV replication.¹⁴ In addition, changes in the intracellular distribution of cholesterol from plasma membrane to membranous web can disrupt the cholesterol-rich surface lipid rafts leading to repression of the interferon-regulated JAK-STAT signaling pathway. In the case of the West Nile virus, the exogenous addition of cholesterol can rescue interferon signaling and restrict viral replication.¹⁵

Four prior studies reported data regarding HCV clinical evolution and genetic polymorphisms of *LDLR*;^{11–13,16,17} nevertheless, the role of genetic polymorphisms in cholesterol metabolism in HCV replication is not clear. The main advantages of the present study are the in-depth genetic analysis of the most essential genes of the cholesterol transport pathway, a dense coverage of SNPs in *LDLR* gene and the fact that no prior studies have studied an association of *LDLR* with pre-treatment viral load (VL). Drug target identification in virology has been limited to the few genes encoded by viral genomes. However, several cases show that inhibiting host cell proteins can prevent viral infection. The paradigm of this strategy is the inhibitors of CCR5 that have already reached the clinical practice for HIV infection,¹⁸ but others, such as Emzetimibe, an inhibitor of the NPC1L1 cholesterol transporter (used as cholesterol-lowering drug), have been proposed as an alternative drug target against HCV infection.¹⁹ The identification of critical host factors that modulate HCV replication and propagation has the potential to deliver new

¹Immunogenetics Unit, Department of Experimental, Biology Faculty of Sciences, Universidad de Jaén, Jaén, Spain; ²Unit of Infectious Diseases and Microbiology, Hospital Universitario de Valme, Instituto de Biomedicina de Sevilla (IBIS), Seville, Spain; ³Unit of Infectious Diseases, Maimonides Institute for Biomedical Research (IMBIB), Hospital Universitario Reina Sofía, Córdoba, Spain and ⁴Department of Infectious Diseases, Hospital Carlos III, Madrid, Spain. Correspondence: Dr JA Pineda, Unit of Infectious Diseases and Microbiology, Hospital Universitario de Valme, Instituto de Biomedicina de Sevilla (IBIS), Avenida de Bellavista s/n, Seville 41014, Spain. E-mail: japineda@telefonica.net

Received 22 July 2013; revised 26 September 2013; accepted 27 September 2013; published online 31 October 2013

antiviral drug targets focused in the inhibition of cellular machinery subverted by the viral replication.

In this context, the objective of this study was to assess the impact of genetic polymorphisms in genes related to cholesterol synthesis and transport pathways on pre-treatment HCV VL.

RESULTS

Features of the study population

The main characteristics of the population studied are depicted in Table 1. Patients were selected from a cohort of HCV-infected patients who were prospectively followed up at five units of Infectious Diseases in Spain. The inclusion criteria for this cohort were as follows: (i) older than 18 years, (ii) prior treatment naive and (iii) initiation of dual therapy with Peg-IFN plus RBV. Among the 442 selected individuals, 332 (75.2%) were coinfecting with HIV and HCV, 80.2% were male individuals and 68% were infected with genotypes 1 or 4. One patient infected with genotype 2 was removed from the subsequent analysis.

Genotyping statistics

The 5 SNPs rs1800796 (IL6), rs5742911 (LDLR), rs673 (TNFA), rs7412 (APOE) and rs7491 (LDLRAP1) did not pass the genotyping quality control tests of Illumina Veracode Golden Gate protocol. In addition, eight SNPs were monomorphic in our population with only one allele being detected: rs12713911, rs12720791, rs12720794, rs1801703, rs7589300 (APOB), rs2069830 (IL6), rs6880589 (HAVCR1) and rs71638390 (LDLRAP1). The SNPs rs4418583 (LDLRAP1) and rs1131454 (OAS1) were discarded due to a significant deviation from Hardy–Weinberg equilibrium. Mean genotyping call rates for the remaining SNPs were 99.2% (s.d. $\pm$ 2.09). Allele counts and frequencies of the SNPs included in this study are represented in Supplementary Table 1.

Univariate VL genetics association analysis

The pre-treatment plasma HCV VL values were categorized according to the cutoff level 600 000 IU ml⁻¹; similarly, quantitative association analysis was also carried out. When analyzed as continuous variable, VL values were expressed as Log10. Only the marker rs2569540 of LDLR showed a borderline association under dominant model in using the cutoff level of 600 000 IU ml⁻¹ (OR = 1.74; P = 0.009). When patients were considered classified according to the HCV genotype (Table 2), we found association in patients infected with HCV genotypes 1 and 4 under the dominant model with the genotypes AA + AG of rs11672123 located in the distal promoter region of LDLR (P = 0.001; OR = 2.3; 95% CI (1.359–3.917)), the genotypes AA + AG of rs1433099 positioned in the 3' untranslated region (UTR) of LDLR (P = 0.0004; OR = 0.38;

95% CI (0.22–0.66)) and rs2569540 GG + GC (P = 0.0023; OR = 0.45; 95% CI (0.277–0.761)) located in an intron near the 3' end of the gene (Figure 1a). The C allele of rs2738459 from LDLR is a risk factor and correlated with a higher VL in genotypes (OR = 4.43; P = 0.00017; 95% CI (1.98–9.88)). Associations of rs1433099 and rs2738459 with the VL reached statistical significance after correction for multiple testing. The locations of the SNPs in the LDLR gene are represented in the Figure 1.

Quantitative association analysis of the log10 VL displayed association only with rs2569540 from LDLR (mean $\pm$ s.d.; CC 6.22 $\pm$ 0.9 vs GG + CG 5.8 $\pm$ 0.9, P = 0.0007, Mann–Whitney U -test) and rs1433099 (mean $\pm$ s.d.; GG 6.2 $\pm$ 0.9 vs AA + AG 5.8 $\pm$ 0.9, P = 0.0001, Mann–Whitney U -test) but only for viral genotypes 1 and 4 (Figure 2). The statistical interaction between viral and host genotypes was confirmed by the multivariate lineal regression test (P = 0.0002). The association of rs2738459 with the VL stratified according to a cutoff level of 600 000 IU ml⁻¹ for genotype 3 was not confirmed in the quantitative analysis (P = 0.02). The coinfection with HIV-1 did not significantly influence the VL levels in the population infected with HCV genotypes 1 and 4 (HIV-1 positive n = 204, mean $\pm$ s.d.: 6.06 $\pm$ 1.01 vs HIV-1 negative n = 74, mean $\pm$ s.d.: 6.10 $\pm$ 0.73, P = 0.73, Student's t -test). The effect of the most significant SNPs is not affected by the HIV-1 status: rs1433099 (HIV-1 positive P = 7×10^{-3} , HIV-1 negative, P = 8.6×10^{-3} , Mann–Whitney U -test); rs2569540 (HIV-1 positive P = 2.3×10^{-2} , HIV-1 negative, P = 1.5×10^{-3} , Mann–Whitney U -test). No other covariates displayed significant interaction with the host genotype.

None of the other studied genes (Table 3) appear to be related with HCV VL phenotype.

Haplotypic analyses

A haplotype-based association test was performed by multimer regression analysis using Plink software. Haplotype frequencies were estimated and independently compared for patients infected with genotypes 1 and 4, or 2 and 3 (Supplementary Table 2). The LDLR SNPs included in this study span 92.6 kb (from rs1529729 to rs4804146). A pairwise linkage disequilibrium (LD) of LDLR gene was computed in patients infected with viral genotypes 1 and 4, and 2 and 3 and showed that in both populations the SNPs with univariate experiment-wide significant association with the VL were independently informative and were contained within a three haplotype blocks, which were similar to the block structure in the current HapMap Phase II database (Figure 1b). Supplementary Table 2 displays the frequencies of major haplotypes in our cohort for the three haplotypes identified. The presence (1 or 2 copies) of an extended haplotype encompassing the last three exons of LDLR (block 3,

Table 1. Main characteristics of the study population

Variables	All patients, n = 442	VL < 600 000, n = 153	VL > 600 000, n = 269	P -value
Age (years) ^a	41.8 (38.2–45.4)	41.7 (37.8–44.8)	41.8 (38.5–46)	0.494
Male gender, no. (%)	354 (80.2)	129 (84.3)	210 (78.1)	0.121
HIV infection, no. (%)	332 (75.2)	113 (73.9)	203 (75.5)	0.714
Undetectable HIV RNA, no. (%) ^b	167 (78.8)	61 (83.6)	106 (76.8)	0.251
Alcohol intake > 50 g per day, no. (%) ^c	33 (7.7)	16 (17.4)	17 (10.1)	0.089
HCV genotype 1 and 4 (%) ^a and 2 and 3 (%)	301 (68.0)–141 (31.9)	98 (32.5)–60 (42.5)	196 (65.1)–79 (56.0)	0.047
BMI (kg m ⁻²) ^a	24.2 (22.1–27.2)	24.3 (21.9–26.7)	24.2 (22.1–27.2)	0.950
T-Chol (mg dl ⁻¹) ^a	165 (141–193)	166 (145–195)	164 (137–190)	0.147
HDL-Chol (mg dl ⁻¹) ^a	43.5 (35–55.8)	47 (37.5–60)	42 (33–53.3)	0.019
LDL-Chol (mg dl ⁻¹) ^a	91 (71.6–115.0)	92.7 (75.5–118)	90 (69.9–113)	0.061
TGL (mg dl ⁻¹) ^a	110 (79.8–166.0)	108 (68.5–159)	112 (82–167)	0.247

Abbreviations: BMI, body mass index; HCV, hepatitis C virus; HDL-Chol, HDL-cholesterol; LDL-Chol, LDL-cholesterol; T-Chol, total cholesterol; TGL, triglyceride.
^aMean (interquartile range). ^bAvailable in 212 patients. ^cAvailable in 261 patients.

Table 2. Single-marker association analysis for VL in *LDLR* gene. Genotype and allele distribution among patients grouped by the VL cutoff level of 600 000 IU ml⁻¹

HCV genotypes	Gene	SNP	Alleles 1/2	Allele distribution by VL (allele 1), n (%)		Genotype distribution by VL, n (%)						Model association with VL	
				>600 000 n=380	<600 000 n=182	>600 000 n=190 (genotypes 1 + 4) n=75 (genotype 3)			<600 000 n=91 (genotypes 1 + 4) n=59 (genotype 3)			Allele OR (95%CI) P-value	Genotype OR (95%CI) P-value
						Homozygote A1	Heterozygote	Homozygote A2	Homozygote A1	Heterozygote	Homozygote A2		
1 + 4	<i>LDLR</i>	rs1433099	A/G	90 (24.0)	63 (34.6)	14 (7.4)	62 (32.6)	114 (60.0)	7 (7.7)	49 (53.8)	35 (38.5)	0.006	OR=0.38 (0.22–0.66) P=0.0004
			A/G	114 (31.0)	35 (19.7)	17 (9.2)	80 (43.5)	87 (47.3)	6 (6.7)	23 (25.8)	60 (67.4)		
		rs2569540	G/C	82 (21.6)	59 (32.1)	11 (5.8)	60 (31.6)	119 (62.6)	7 (7.6)	45 (48.9)	40 (43.5)	0.007	OR=0.45 (0.27–0.76) P=0.0023
			C/A	77 (51.3)	42 (36.7)	14 (18.7)	49 (65.3)	12 (16.0)	10 (18.3)	22 (36.7)	27 (45.0)		
2 + 3	<i>LDLR</i>	rs2738459	C/A	77 (51.3)	42 (36.7)	14 (18.7)	49 (65.3)	12 (16.0)	10 (18.3)	22 (36.7)	27 (45.0)	0.016	OR=4.43 (1.98–9.88) P=0.00017

Abbreviations: HCV, hepatitis C virus; *LDLR*, low-density lipoprotein receptor; SNP, single-nucleotide polymorphism; VL, viral load.

Figure 1), including the combination AGGCGGAG of the SNPs rs2738456, rs2738457, rs2569540, rs2738459, rs2738460, rs2116898, rs6413504 and rs14158, showed a strong association with the cutoff level of 600 000 IU ml⁻¹ VL for genotypes 1 and 4 (OR=0.27; $P=3 \times 10^{-6}$, Figure 3a), as well as quantitative VL (mean $\pm$ s.d.: 6.19 $\pm$ 0.9 vs 5.58 $\pm$ 1.1 log IU ml⁻¹, $P=1 \times 10^{-4}$, Figure 3b). However, this haplotype has no multiple-testing significant effect on viral genotype 3. The presence of the haplotype GAA including the Tag SNPs rs2738465, rs1433099 and rs2738466 located in the block 4 is also associated with VL with the cutoff level of 600 000 IU ml⁻¹ VL (OR=0.41; $P=6 \times 10^{-4}$) and quantitative VL (mean $\pm$ s.d.: 5.87 $\pm$ 0.9 vs 6.25 $\pm$ 0.9, $P=1 \times 10^{-3}$).

Phenotypic analysis

The potential functional effects of the *LDLR* SNPs associated with VL on the levels of LDL, high-density lipoprotein (HDL), total cholesterol (tChol) and triglycerides were analyzed by quantitative genetic association in the two HCV genotype groups. The most important genetic factor influencing the serum lipid values in all HCV infected patients is the SNP rs28416813 located near *IL28B* gene, LDL (mean $\pm$ s.d.: CC=79.9 $\pm$ 29.7, CG=91.2 $\pm$ 26.9, GG=107.2 $\pm$ 30.4, $P=3 \times 10^{-6}$, Mann-Whitney *U*-test) and tChol (mean $\pm$ s.d.: CC=155.2 $\pm$ 30.8, CG=168.1 $\pm$ 32.0, GG=181.0 $\pm$ 37.0, $P=8 \times 10^{-5}$ Mann-Whitney *U*-test). The genotypes of the SNP rs11672123 located in *LDLR* distal promoter associated with the HCV VL cutoff level of 600 000 IU ml⁻¹ also displayed association with LDL (mean $\pm$ s.d.: AA+AG=89.8 $\pm$ 30.0, GG=99.8 $\pm$ 30.1, $P=6 \times 10^{-3}$ Mann-Whitney *U*-test) but not with tChol or HDL. The genotypes associated with a low serum HCV VL are correlated with higher lipid values (Figure 4a).

Next, we have tested the potential genetic interaction between the SNPs in *LDLR* and *IL28B*, the SNP rs11672123 of *LDLR* modified the impact of *IL28B* SNP in serum LDL (Figure 4b), individuals with homozygous GG+GG (*IL28B* rs28416813 + *LDLR* rs11672123) were compared with GG+AA, a difference was observed in serum LDL levels (mean $\pm$ s.d.: 104 $\pm$ 7 mg dl⁻¹ vs 82 $\pm$ 15, $P=0.015$) and the same profile was observed when CC/CG (*IL28B* rs28416813) was tested in *LDLR* rs11672123 GG or AA genetic backgrounds (92.8 $\pm$ 5.4 vs 71.5 $\pm$ 15.4; $P=0.010$). The remaining SNPs (Figure 4b) or haplotypes (data not shown) associated with HCV VL showed no other significant interactions with serum lipids. The HIV-1 status is not associated in our population with significant differences in tChol, LDL, HDL or triglycerides levels, nor has it showed interaction with *IL28B* or *LDLR* SNPs on the lipid values ($P>0.05$ in all comparisons).

Finally, we validated whether the observed effect could be associated with the differential expression of *LDLR* mRNA in PBMC. rs1433099 AA+AG vs GG individuals were examined using the Taqman real-time quantitative PCR. The relative amount of the *LDLR* mRNA normalized to β -actin revealed a lower level of expression of genotypes AA+AG compared with GG (AA+AG, $n=10$, mean = 2.3 $\pm$ 1.74 (0.59–4.09) vs GG, $n=7$, mean = 7.6 $\pm$ 6.26 (1.38–13.91) ($P=0.036$, Student's *t*-test). These data indicate that the expression of *LDLR* mRNA is reduced in PBMCs from AA+AG carriers under our experimental conditions.

DISCUSSION

HCV VL is not linked to disease progression, and its significance as a predictor of treatment failure in the era of direct-acting antivirals is limited; however, the identification of genetic factors affecting the viral replication could shed light on the physiopathology of the infection and could eventually lead to the identification of new drug targets acting on the host proteins needed by the virus to achieve optimal replication. In this low-scale study, we have

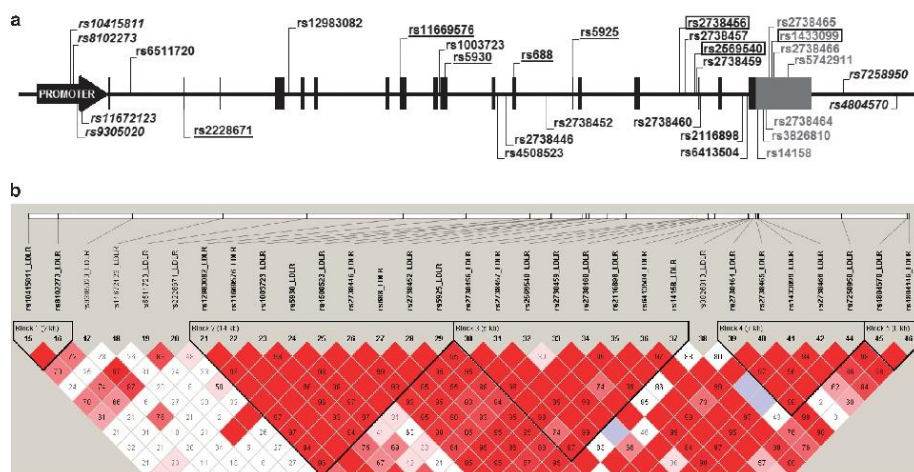


Figure 1. Gene structure of the *LDLR* gene and linkage-disequilibrium pattern of population analyzed. (a) Graphical representation of the *LDLR* gene with the positions of the genotyped polymorphisms. Coding SNPs are underlined; SNPs associated with VL are in boxes. 3' UTR in gray and the rest of exons in black. (b) Linkage-disequilibrium patterns in the study population. D' values were calculated and plotted by use of Haploview. Numbers inside boxes correspond to $D' \times 100$; $D' = 100$ is not represented. A block structure was considered for marker pairs showing $D' > 0.8$, following the solid-spine block definition implemented in Haploview.

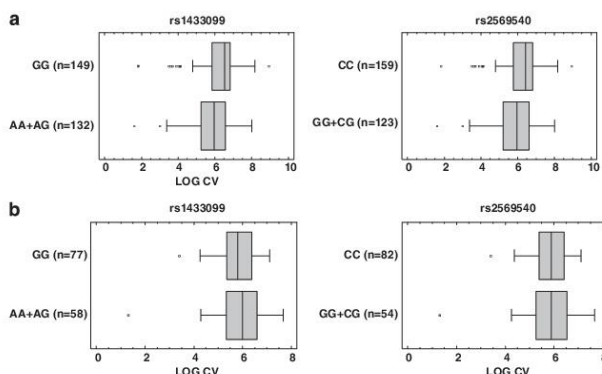


Figure 2. Comparison of VL between patients carrying the *LDLR* polymorphisms rs1433099 (left side) and rs2569540 (right side). (a) Patients infected with HCV genotypes 1 and 4 ($P = 0.0001$ for rs1433099; $P = 0.007$ for rs2569540 Mann-Whitney test). (b) Patients infected with HCV genotype 3 ($P = 0.18$ for rs1433099; $P = 0.82$ for rs2569540 Mann-Whitney U-test).

genotyped 144 SNPs in 40 gene regions that are candidates for having a role in cholesterol metabolism and transport as well as other SNPs previously associated with HCV clinical evolution.^{20–22} We found two SNPs in *LDLR* that had an association with significant P -values after Bonferroni's correction for pre-treatment HCV VL. Interestingly, the effect of *LDLR* SNPs is restricted to patients infected with viral genotypes 1 and 4, and no quantitative associations were observed in patients infected with genotype 3. However, we should take into account that the sample size analyzed is different between the two groups of HCV genotypes. Considering a minor allele frequency of 0.3 and the

ratio of high VL vs low VL, our study had an 80% power to detect an OR = 2 (and 95% for OR = 2.5) in genotypes 1 and 4 compared with 55% power to detect an OR = 2 (and 78% for OR = 2.5) in genotype 3. At least four prior studies reported data regarding HCV disease evolution and the genetic marker rs14158 of *LDLR*;^{11,16,17,23} however, the main advantages of the current study are the in-depth genetic analysis including the most important genes of cholesterol pathways, a high coverage of *LDLR* gene with 33 SNPs in the region and the fact that no prior studies have studied an association of *LDLR* with pre-treatment VL. Three *LDLR* SNPs identified in this study related with HCV VL



Table 3. SNPs included in this study

SNP name	Chr	Coordinate	Gene	Location	Coding status
rs71638390	1	25889161	LDLRAP1	CODING	NONSYN
rs6687605	1	25889632	LDLRAP1	CODING	SYNON
rs28969504	1	25890189	LDLRAP1	CODING	NONSYN
rs4075184	1	25890835	LDLRAP1	INTRON	
rs4418583	1	25893242	LDLRAP1	INTRON	
rs11563	1	25894853	LDLRAP1	UTR	
rs7491	1	25895238	LDLRAP1	UTR	
rs17111503	1	55503448	PCSK9	INTERGENIC	
rs2479408	1	55504188	PCSK9	INTERGENIC	
rs2479409	1	55504650	PCSK9	INTERGENIC	
rs11591147	1	55505647	PCSK9	CODING	NONSYN
rs10888896	1	55509213	PCSK9	INTRON	
rs4927193	1	55509872	PCSK9	INTRON	
rs4997118	1	55512549	PCSK9	INTRON	
rs10888897	1	55513061	PCSK9	INTRON	
rs676297	1	55514611	PCSK9	INTRON	
rs572512	1	55517344	PCSK9	INTRON	
rs2495477	1	55518467	PCSK9	INTRON	
rs2479413	1	55518682	PCSK9	INTRON	
rs7552841	1	55518752	PCSK9	INTRON	
rs557435	1	55520864	PCSK9	INTRON	
rs483462	1	55525400	PCSK9	INTRON	
rs603247	1	55525868	PCSK9	INTRON	
rs10465832	1	55528807	PCSK9	INTRON	
rs505151	1	55529187	PCSK9	CODING	NONSYN
rs662145	1	55529828	PCSK9	UTR	
rs2254002	1	65301669	JAK1	INTRON	
rs2236876	1	173017486	TNFSF18	INTRON	
rs486907	1	182554557	RNASEL	CODING	NONSYN
rs4290029	1	224400640	DEGS1/NVL	INTERGENIC	
rs1801702	2	21225485	APOB	CODING	NONSYN
rs1801703	2	21225912	APOB	CODING	NONSYN
rs12713771	2	21235565	APOB	INTRON	
rs3749054	2	21237238	APOB	INTRON	
rs673548	2	21237544	APOB	INTRON	
rs2854725	2	21237786	APOB	INTRON	
rs12713844	2	21238413	APOB	CODING	NONSYN
rs11126598	2	21240364	APOB	INTRON	
rs12713911	2	21240538	APOB	INTRON	
rs10199768	2	21244000	APOB	INTRON	
rs11676704	2	21244358	APOB	INTRON	
rs12720791	2	21244617	APOB	INTRON	
rs7589300	2	21244760	APOB	INTRON	
rs12720804	2	21254997	APOB	INTRON	
rs12720832	2	21256713	APOB	INTRON	
rs12720842	2	21257927	APOB	INTRON	
rs579826	2	21261507	APOB	INTRON	
rs12720796	2	21261998	APOB	INTRON	
rs10205849	2	21262190	APOB	INTRON	
rs12720794	2	21262375	APOB	INTRON	
rs531819	2	21263639	APOB	INTRON	
rs512535	2	21267782	APOB	INTERGENIC	
rs7575840	2	21273490	APOB	INTERGENIC	
rs1267059	2	162018643	TANK	INTRON	
rs5742909	2	204732347	CTLA4	INTERGENIC	
rs231775	2	204732714	CTLA4	CODING	SYNON
rs919178	2	231044464	SP110	INTRON	
rs4571051	2	234091158	INPP5D	INTRON	
rs17740066	3	121100283	STXBPSL	CODING	SYNON
rs12654264	5	74648603	HMGCR	INTRON	
rs2070721	5	131825842	IRF1	INTRON	
rs6880589	5	156460440	HAVCR1	INTRON	
rs953569	5	156477400	HAVCR1	INTRON	
rs1800629	6	31543031	TNF	INTERGENIC	
rs673	6	31543095	TNF	INTERGENIC	
rs3093665	6	31545391	TNF	UTR	
rs1800796	7	22766246	IL6	INTERGENIC	
rs1800795	7	22766645	IL6	INTERGENIC	
rs2069830	7	22767137	IL6	CODING	SYNON
rs13242262	7	128591364	IRF5	INTERGENIC	
rs10488631	7	128594183	IRF5	INTERGENIC	
rs2280714	7	128594725	IRF5	INTERGENIC	
rs328	8	19819724	LPL	CODING	NONSYN
rs1454626	9	2621030	VLDLR	INTERGENIC	
rs7043199	9	2621145	VLDLR	INTERGENIC	
rs7852409	9	2621482	VLDLR	INTERGENIC	
rs7032549	9	2623615	VLDLR	INTERGENIC	
rs7022122	9	2624186	VLDLR	INTERGENIC	
rs10967213	9	2625172	VLDLR	INTERGENIC	

Table 3. (Continued)

SNP name	Chr	Coordinate	Gene	Location	Coding status
rs1545566	9	2626031	VDLDR	INTERGENIC	
rs4741747	9	2626654	VDLDR	INTERGENIC	
rs12551418	9	2627365	VDLDR	INTERGENIC	
rs1869592	9	2632250	VDLDR	INTERGENIC	
rs10812379	9	2633548	VDLDR	INTERGENIC	
rs4740698	9	2637551	VDLDR	INTRON	
rs3890182	9	107647655	ABCA1	INTRON	
rs4986791	9	120475602	TLR4	UTR	
rs886277	11	2439767	TRPM5	CODING	NONSYN
rs2298455	11	71710478	IL18BP	INTRON	
rs187238	11	112034988	IL18	INTERGENIC	
rs1946518	11	112035438	IL18	INTERGENIC	
rs2878771	12	50352393	AQP2	UTR	
rs7303577	12	64874102	TBK1	INTRON	
rs1131454	12	113348870	OAS1	COMPLEX	
rs2295977	14	24657226	IPOM	INTRON	
rs1800588	15	58723675	LIPC	INTERGENIC	
rs2290351	15	90374781	AP3S2	UTR	
rs1800775	16	56995236	CETP	INTERGENIC	
rs1529729	19	11163562	LDLR	INTRON	
rs10415811	19	11177408	LDLR	INTERGENIC	
rs8102273	19	11180047	LDLR	INTERGENIC	
rs9305020	19	11186711	LDLR	INTERGENIC	
rs11672123	19	11194823	LDLR	INTERGENIC	
rs6511720	19	11202306	LDLR	INTRON	
rs2228671	19	11210912	LDLR	CODING	NONSYN
rs12983082	19	11216561	LDLR	INTRON	
rs11669576	19	11222300	LDLR	CODING	SYNON
rs1003723	19	11224181	LDLR	INTRON	
rs55930	19	11224265	LDLR	CODING	NONSYN
rs4508523	19	11226944	LDLR	INTRON	
rs2738446	19	11227326	LDLR	INTRON	
rs688	19	11227602	LDLR	CODING	NONSYN
rs2738452	19	11229218	LDLR	INTRON	
rs5925	19	11230881	LDLR	CODING	NONSYN
rs2738456	19	11236804	LDLR	INTRON	
rs2738457	19	11237269	LDLR	INTRON	
rs2569540	19	11238239	LDLR	INTRON	
rs2738459	19	11238473	LDLR	INTRON	
rs2738460	19	11238807	LDLR	INTRON	
rs2116898	19	11241810	LDLR	INTRON	
rs6413504	19	11241915	LDLR	INTRON	
rs14158	19	11242044	LDLR	UTR	
rs3826810	19	11242133	LDLR	UTR	
rs2738464	19	11242307	LDLR	UTR	
rs2738465	19	11242496	LDLR	UTR	
rs1433099	19	11242658	LDLR	UTR	
rs2738466	19	11242765	LDLR	UTR	
rs5742911	19	11243445	LDLR	UTR	
rs7258950	19	11250139	LDLR	INTERGENIC	
rs4804570	19	11256059	LDLR	INTERGENIC	
rs4804146	19	11256176	LDLR	INTERGENIC	
rs28416813	19	39735644	IL28B	INTERGENIC	
rs8099917	19	39743165	IL28B	INTERGENIC	
rs1800471	19	41858876	TGFB1	CODING	NONSYN
rs1800469	19	41860296	TGFB1	INTERGENIC	
rs405509	19	45408836	APOE	INTERGENIC	
rs429358	19	45411941	APOE	CODING	NONSYN
rs7412	19	45412079	APOE	CODING	NONSYN
rs439401	19	45414451	APOC1	INTERGENIC	
rs445925	19	45415640	APOC1	INTERGENIC	
rs4420638	19	45422946	APOC1	INTERGENIC	
rs12104272	19	50148052	SCAF1/IRF3	INTERGENIC	
rs2229207	21	34614250	IFNAR2	CODING	NONSYN
rs2243592	21	34707808	IFNAR1	INTRON	
rs217429	7	44555199	NPC1L1	INTRON	
rs2072183	7	44579180	NPC1L1	CODING	SYNON
rs74597329	19	39739155	INFL4	CODING	NONSYN

Abbreviations: LDLR, low-density lipoprotein receptor; SNP, single-nucleotide polymorphism; UTR, untranslated region.

have been previously associated with SVR after treatment with interferon² (rs11672123, $P=0.0067$; rs1433099, $P=0.0019$; rs2569540, $P=0.0044$); all are located in introns or regulatory regions as promoter or 3' UTR. The marker rs11672123 is placed in the promoter region of LDLR (position -5215 bp upstream of the

transcription start site) and no other previous studies have linked this SNP with any phenotype. rs11672123 is not linked to other SNPs included in this study; however, this SNP displays a strong LD in CEU population ($D'=1$) with rs6511720, an SNP located in the first intron of LDLR and previously identified as a risk factor for

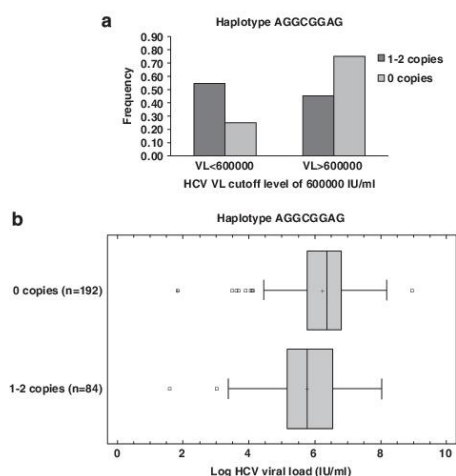


Figure 3. Association between HCV VL and the presence of 1 or 2 copies of the haplotype AGGCGGAG from the SNPs rs2738456, rs2738457, rs2569540, rs2738459, rs2738460, rs2116898, rs6413504 and rs14158 in patients infected with HCV genotypes 1 and 4. (a) The association of the haplotype AGGCGGAG with the VL cutoff level of 60000 IU ml⁻¹ ($P = 9 \times 10^{-6}$, Fisher exact test). (b) The association of the haplotype AGGCGGAG with log₁₀ HCV viral load ($P = 8 \times 10^{-5}$, Mann-Whitney *U*-test).

myocardial infarction²⁴ as well as elevated levels of LDL²⁵ and tChol.²⁵ The allele G of rs11672123 associated with lower VL is in disequilibrium with the allele G of rs6511720 associated with elevated LDL and tChol²⁵ and risk of myocardial infarction.²⁴ In this study, the allele G is associated with a lower VL and a higher LDL in the same *IL28B* genetic background, suggesting that the protective *LDLR* genotypes have lower *in vivo* activity or expression. On the contrary, the allele A of rs1433099 located in the 3' UTR is a genetic marker identified in this study with a lower VL and has been previously strongly associated with a lower LDL and tChol in the *ARIC* cohort,²⁶ and it has been described as a risk factor for myocardial infarction.²⁴ The molecular basis of this phenotype is probably related to the alterations in mRNA half-life as the genotypes AA + AG associated with a lower VL and higher levels of SVR¹² displayed a significantly lower mRNA expression compared with individuals carrying the GG genotypes. Our results are in agreement with a previous report²⁶ that used a tissue culture-based system to measure the expression of a reporter gene carrying two main haplotypes of *LDLR* mRNA 3' UTR. The rs2569540 marker has not been previously associated with any phenotype but it is in LD with SNPs located in the 3' UTR, a well-known regulatory region of *LDLR* mRNA stability. The SNP rs14158 located in the 3' UTR of *LDLR* has been previously associated with viral kinetics during the first days of treatment initiation with PEG-IFN/RBV in HIV/HCV genotype 1 coinfecting patients, as well as SVR response after treatment with Pegylated interferon plus ribavirin.¹³ Although rs14158 is in LD with the SNPs identified here associated with the VL, we found no association with the pre-treatment VL for this marker at the univariate analysis. We also found no association between the *IL28B* SNP rs28416813 or the INF.4 rs74597329 and the pre-treatment VL.

LDLR haplotypes display a higher correlation with the VL compared with the effects of single SNPs; however, at the

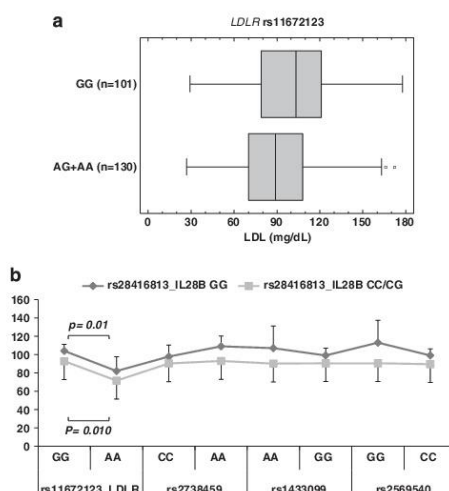


Figure 4. Phenotypic effect of *LDLR* polymorphisms associated with VL on LDL plasma levels in patients infected with HCV genotypes 1 and 4. (a) The association of rs11672123 genotypes with plasma LDL ($P = 0.006$ Mann-Whitney *U*-test). (b) The interaction between *IL28B* genotypes and SNPs associated with HCV VL on plasma LDL. Only the SNPs rs11672123 showed association with LDL levels in homogeneous *IL28B* genetic backgrounds ($P = 0.01$) (Mann-Whitney *U*-test).

haplotype level, we have not identified association with serum lipids, probably because of the dominant effect of *IL28B* genotype on these parameters and the limited size of our cohort. Two observations deserve careful attention, first the effect of *LDLR* on VL is limited to patients infected with genotypes 1 and 4, and our data indicate that the VL of genotype 3 is less sensitive to genetic variations in *LDLR* and probably to the levels of expression of this gene. We propose two hypotheses to explain our results, first that the viral genotype 3 does not use *LDLR* as co-receptor or docking molecule in its interaction with the target cell. Second, core and NS4B proteins from genotype 3 viruses appear to be better inducers of hepatocytes lipogenesis compared with genotype 1 proteins;^{27,28} as a consequence, genotype 3 strains could depend mainly on intrahepatic biosynthesis of cholesterol and fatty acids for replication and they could be more autonomous of plasma cholesterol uptake through *LDLR*. In contrast, strains with genotypes 1 and 4 could need supplemental lipid intake passing through *LDLR*. According to this hypothesis, we predict that hepatocytes from patients with protective *LDLR* genotypes could contain lower levels of intracellular cholesterol and fatty acids. Previous data,^{11,29} additionally, suggest a possible epistatic interaction between *LDLR* and *IL28B*; this could be explained by a cross-talk between the *IL28B* and *LDLR* pathways. It has been described that an interferon induces increased expression of *LDLR*, and this phenomenon may explain previous reports on the reduction of serum cholesterol in interferon treated patients.³⁰ Interferon also stimulates the production of an extracellular soluble N-terminal ligand-binding domain of *LDLR* with antiviral activities against Vesicular stomatitis virus and Rhinoviruses^{31,32} and also can inhibit HCV produced in cell culture.³³ The higher levels of LDL and tChol observed in patients with protective *IL28B* genotype could be related to an impaired function of *LDLR* in this genetic context.

This study had several limitations. First, our significant findings are limited to viral genotypes 1 and 4, we cannot rule out an effect of *LDLR* polymorphism on viral genotype 3 due to the limited size and statistical power in this group, and the results await verification in other major cohort. A second concern was that the main *LDLR* polymorphism associated with VL rs1433099 does not exhibit a clear phenotype on the levels of lipids, although it affects the levels of mRNA expression. Third, other genes previously associated with HCV disease evolution as *HLA*, *KIR* and *TLR2* have not been included in this study; however, we have included other genes associated with viral clearance as *TNFSF18*, *TANK*, *HAVCR1* and *IL18BP*,²⁰ but they do not appear to be related to the HCV VL under our conditions. Fourth, the list of genes and SNPs related to cholesterol metabolism/receptors is not exhaustive; polymorphisms in other genes not included in this study, such as *SCARB1*, the main receptor for HDL and known co-receptor of HCV,³⁴ could be considered as potential modulators of HCV replication. Finally, our study contains both HCV monoinfected and HIV/HCV coinfecting patients with clearly different HCV disease evolution; a more homogeneous approach including only HCV monoinfected patients could be beneficial to discriminate specific genetic patterns associated with HCV VL in the absence of the confounding effect of HIV coinfection.

The epistatic interaction between *IL28B* and the SNPs located in *LDLR* rs14158, rs11672123, rs1433099 and rs2569540 of *LDLR* on SVR has been previously communicated by our group,^{11,12} and this study clarifies the influence of *LDLR* genetics in HCV replication.

PATIENTS AND METHODS

Study population

A population of 442 patients infected with HCV was prospectively recruited in the infectious diseases units of five tertiary-care hospitals in Spain (Seville, Cordoba, Madrid, Malaga and Huelva) from May 2000 to December 2010. All of them were previously HCV treatment naive. Clinical and laboratory controls were performed at the initial visit and a whole blood and/or plasma sample was collected and stored at -70°C for subsequent genetic, biochemical and virological determinations. Two hundred and thirty-nine patients were infected with HCV genotype 1, 62 with genotype 4, 141 patients with genotype 3 and 1 patient with genotype 2. The ethnicity of all patients was Caucasian (white Spanish).

Ethical aspects

The study was designed and performed according to the Helsinki declaration and was approved by the Ethics Committee of the University Hospital of Valme (Sevilla, Spain). All patients provided a written informed consent to participate in this study.

Clinical laboratory determinations

Serum HCV antibodies were determined by an enzyme immunoassay (ADVIA Centaur XP, Siemens Healthcare Diagnostics S.L. Tarrytown, NY, USA). Plasma HCV-RNA load was measured by quantitative real-time PCR assays according to the available technique at the time when the patient was treated (Cobas Amplicor HCV Monitor; Roche Diagnostic Systems Inc., Branchburg, NJ, USA: detection limit of 600 IU ml⁻¹; Cobas AmpliPrep-Cobas TaqMan; Roche Diagnostic Systems Inc., Meylan, France: detection limit of 50 IU ml⁻¹; Cobas TaqMan; Roche Diagnostic Systems Inc., Pleasanton, CA, USA: detection limit of 10 IU ml⁻¹). The HCV genotypes were determined by Versant HCV genotype 2.0 LIPA; Siemens. tChol, triglycerides, HDL-cholesterol and calculated LDL were measured in fasting plasma by standard routine procedures in the clinical biochemistry departments of the participating hospitals.

Polymorphism selection and genotyping

SNPs from cholesterol metabolism and transport or previously associated with SVR or liver fibrosis (Table 3) were selected on the basis of two criteria: first, genotype data from Hapmap-CEU population were directly

downloaded from the Hapmap database (www.hapmap.org), and the Tag SNPs were identified using Haploview's V4.1 software tagger option (<https://www.broad.harvard.edu/haploview/haploview/>); second, the list of SNPs was completed with potentially functional polymorphisms previously associated either with HCV spontaneous clearance or with liver fibrosis. DNA was extracted from fresh peripheral blood mononuclear cells or frozen whole blood using the Quick Pure Blood DNA extraction Kit (Macherey-Nagel, Düren, Germany) or the Magna Pure system (Roche, Basel, Switzerland). The SNPs were genotyped using a custom Golden Gate Veracode genotyping assay (Illumina, San Diego, CA, USA) in the Department of Structural Genomics of Neocodex Inc. (Seville, Spain) according to the manufacturer's instructions. *Interferon Lambda-4* ins/del polymorphism rs74597329 was genotyped as previously described.³⁵ Two SNPs rs2072183 and rs217429 in *NPC1L1*, a cholesterol transporter and an HCV receptor, respectively,¹⁹ were genotyped using the Taqman system (Applied Biosystems, Foster City, CA, USA).

LDLR mRNA quantification

PBMCs were purified by centrifugation in the Ficoll gradient. Ten individuals harboring AA + AG genotypes and seven with GG genotype were randomly selected for *LDLR* gene expression analyses. From each sample, total RNA from 5×10^6 PBMCs was isolated using the Tripure reagent (Roche, Basel, Switzerland), quantified by spectrophotometry and dissolved at $1 \mu\text{g} \mu\text{l}^{-1}$. Reverse transcription of RNA was performed using the Superscript III (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. All the RNA samples were also subjected to control reactions without reverse transcriptase to check for contamination. The normalized *LDLR* expression was determined in a multiplexed, one-tube PCR reaction using the human β -actin as endogenous control (Applied Biosystems) and *LDLR* Gene Expression Assays (Applied Biosystems). Each individual cDNA was amplified in duplicate wells and repeated in two independent days. Parallel negative control experiments were performed with reverse transcriptase negative cDNAs. The relative expression of *LDLR* mRNAs compared with endogenous β -actin controls was calculated using MX-pro V4.0 software (Stratagene, Santa Clara, CA, USA) according to the manufacturer's instructions.

Biostatistics and bioinformatics

Single-marker association *P*-values were corrected for multiple testing following the SNP spectral decomposition approach,³⁶ experiment-wide significance threshold required to keep type I error rate at 5% was 0.0005. The LD values between the genetic markers studied (measured in Lewontin's standardized disequilibrium coefficient D'), haplotype frequencies and haplotype-based association analyses were calculated using Haploview V4.1, Plink V1.07 and SPSS v14 software. Haplotype effects are expressed as odds ratios (for case-control binary VL analysis) or mean differences (for numeric VL values). Haplotype association tests were adjusted using Bonferroni's correction for multiple testing by dividing the significance level (0.05) by the number of major haplotypes (that is, $>5\%$). A *P*-value <0.0025 was considered statistically significant (corrected for 20 tests) for haplotype analyses assuming a risk of underestimation, given the inherent lack of independence of haplotype analyses. Hardy-Weinberg equilibrium was calculated using Haploview V4.1 software, and the test³⁷ at the online resource of the Institute for Human Genetics, Munich, Germany (<http://ihg.gsf.de/>). Single marker, haplotype frequencies and chromosome phases were estimated and compared using PLINK software, complemented with the Java-based implementation software gPLINK (<http://pngu.mgh.harvard.edu/~purcell/plink/gplink.shtml>). Standard case/control allelic association (1 degree of freedom) as well as different genetic models, such as dominant, recessive and general (2 degrees of freedom), was tested using Fisher's exact test; in addition, the Cochran-Armitage trend test was used to test for genotypic association. The additive genetic models were tested using Armitage's test for trend. Log10 HCV VLs were tested for association using the asymptotic likelihood ratio test and the Wald test (Plink software) as well as the Mann-Whitney *U*-test. Complementary analysis was performed using SPSS software (version 14.0). The original genotype data are available upon request.

CONFLICT OF INTEREST

The authors declare no conflict of interest.



ACKNOWLEDGEMENTS

This study has been supported in part by grants from the Spanish Health Ministry (ISCIII-RETIC RD06/006 and projects PI10/01664 and PI10/01232) from the Consejería de Salud de la Junta de Andalucía (PI-0247-2010) and from the Fundación para la Investigación y la Prevención del Sida en España (reference 360799/09). JAP is the recipient of an extension grant from the Instituto de Salud Carlos III (grant number Programa-135NS). KN is the recipient of a 'Sara Borrell' postdoctoral perfection grant from the Instituto de Salud Carlos III (SCO/523/2008).

REFERENCES

- Jones DM, McLauchlan J. Hepatitis C virus: assembly and release of virus particles. *J Biol Chem* 2010; **285**: 22733–22739.
- Georgel P, Schuster C, Zeisel MB, Stoll-Keller F, Berg T, Bahram S et al. Virus-host interactions in hepatitis C virus infection: implications for molecular pathogenesis and antiviral strategies. *Trends Mol Med* 2010; **16**: 277–286.
- Negro F. Abnormalities of lipid metabolism in hepatitis C virus infection. *Gut* 2010; **59**: 1279–1287.
- Woodhouse SD, Narayan R, Latham S, Lee S, Antrobus R, Gangadharan B et al. Transcriptome sequencing, microarray, and proteomic analyses reveal cellular and metabolic impact of hepatitis C virus infection in vitro. *Hepatology* 2010; **52**: 443–453.
- Ikedo M, Abe K, Yamada M, Dansako H, Naka K, Kato N. Different anti-HCV profiles of statins and their potential for combination therapy with interferon. *Hepatology* 2006; **44**: 117–125.
- Bartenschlager R, Penin F, Lohmann V, Andre P. Assembly of infectious hepatitis C virus particles. *Trends Microbiol* 2011; **19**: 95–103.
- Burlone ME, Budkowska A. Hepatitis C virus cell entry: role of lipoproteins and cellular receptors. *J Gen Virol* 2009; **90**: 1055–1070.
- Agnello V, Abel G, Elfahal M, Knight GB, Zhang QX. Hepatitis C virus and other flaviviridae viruses enter cells via low density lipoprotein receptor. *Proc Natl Acad Sci USA* 1999; **96**: 12766–12771.
- Gopal K, Johnson TC, Gopal S, Walfish A, Bang CT, Suwandhi P et al. Correlation between beta-lipoprotein levels and outcome of hepatitis C treatment. *Hepatology* 2006; **44**: 335–340.
- Molina S, Castet V, Fournier-Wirth C, Pichard-Garcia L, Avner R, Harats D et al. The low-density lipoprotein receptor plays a role in the infection of primary human hepatocytes by hepatitis C virus. *J Hepatol* 2007; **46**: 411–419.
- Pineda JA, Caruz A, Di Lello FA, Camacho A, Mesa P, Neukam K et al. Low-density lipoprotein receptor genotyping enhances the predictive value of IL28B genotype in HIV/hepatitis C virus-coinfected patients. *AIDS* 2011; **25**: 1415–1420.
- Neukam K, Caruz A, Rivero-Juarez A, Barreiro P, Merino D, Real LM et al. Variations at multiple genes improve interleukin 28b genotype predictive capacity for response to therapy against hepatitis C genotype 1 or 4 infection. *AIDS* (e-pub ahead of print 9 July 2013; doi:10.1097/01.aids.0000432459.36970.a9).
- Rivero-Juarez A, Camacho A, Caruz A, Neukam K, Gonzalez R, Di Lello FA et al. LDLr genotype modifies the impact of IL28B on HCV viral kinetics after the first weeks of treatment with PEG-IFN/RBV in HIV/HCV patients. *AIDS* 2012; **26**: 1009–1015.
- Albecka A, Belouzard S, Op de Beeck A, Descamps V, Goueslain L, Bertrand-Michel J et al. Role of low-density lipoprotein receptor in the hepatitis C virus life cycle. *Hepatology* 2012; **55**: 998–1007.
- Mackenzie JM, Khromykh AA, Parton RG. Cholesterol manipulation by West Nile virus perturbs the cellular immune response. *Cell Host Microbe* 2007; **2**: 229–239.
- Mas Marques A, Mueller T, Welke J, Taube S, Sarrazin C, Wiese M et al. Low-density lipoprotein receptor variants are associated with spontaneous and treatment-induced recovery from hepatitis C virus infection. *Infect Genet Evol* 2009; **9**: 847–852.
- Hennig BJ, Hellier S, Frodsham AJ, Zhang L, Klennerman P, Knapp S et al. Association of low-density lipoprotein receptor polymorphisms and outcome of hepatitis C infection. *Genes Immun* 2002; **3**: 359–367.
- Hagani AA, Tilton JC. Entry inhibitors and their use in the treatment of HIV-1 infection. *Antiviral Res* 2013; **98**: 158–170.
- Sainz Jr B, Barretto N, Martin DN, Hiraga N, Imamura M, Hussain S et al. Identification of the Niemann-Pick C1-like 1 cholesterol absorption receptor as a new hepatitis C virus entry factor. *Nat Med* 2013; **19**: 281–285.
- Mosbrugger TL, Duggal P, Goedert JJ, Kirk GD, Hoots WK, Tobler LH et al. Large-scale candidate gene analysis of spontaneous clearance of hepatitis C virus. *J Infect Dis* 2010; **201**: 1371–1380.
- Agundez JA, Garcia-Martin E, Devesa MJ, Carballo M, Martinez C, Lee-Brunner A et al. Polymorphism of the TLR4 gene reduces the risk of hepatitis C virus-induced hepatocellular carcinoma. *Oncology* 2012; **82**: 35–40.
- Danilovic DL, Mendes-Correa MC, Lima EU, Zambirini H, R KB, Marui S. Correlations of CTLA-4 gene polymorphisms and hepatitis C chronic infection. *Liver Int* 2012; **32**: 803–808.
- Li H, Liu Z, Han Q, Li Y, Chen J. Association of genetic polymorphism of low-density lipoprotein receptor with chronic viral hepatitis C infection in Han Chinese. *J Med Virol* 2006; **78**: 1289–1295.
- Anand SS, Xie C, Pare G, Montpetit A, Rangarajan S, McQueen MJ et al. Genetic variants associated with myocardial infarction risk factors in over 8000 individuals from five ethnic groups: the INTERHEART genetics study. *Circ Cardiovasc Genet* 2009; **2**: 16–25.
- Teslovich TM, Musunuru K, Smith AV, Edmondson AC, Stylianou IM, Koseki M et al. Biological, clinical and population relevance of 95 loci for blood lipids. *Nature* 2010; **466**: 707–713.
- Muallem H, North KE, Kakoki M, Wojcynski MK, Li X, Grove M et al. Quantitative effects of common genetic variations in the 3'UTR of the human LDL-receptor gene and their associations with plasma lipid levels in the Atherosclerosis Risk in Communities study. *Hum Genet* 2007; **121**: 421–431.
- Park CY, Jun HJ, Wakita T, Cheong JH, Hwang SB. Hepatitis C virus nonstructural 4B protein modulates sterol regulatory element-binding protein signaling via the AKT pathway. *J Biol Chem* 2009; **284**: 9237–9246.
- Waris G, Felmlee DJ, Negro F, Siddiqui A. Hepatitis C virus induces proteolytic cleavage of sterol regulatory element binding proteins and stimulates their phosphorylation via oxidative stress. *J Virol* 2007; **81**: 8122–8130.
- Rivero-Juarez A, Camacho Espejo A, Perez-Camacho I, Neukam K, Caruz A, Mira JA et al. Association between the IL28B genotype and hepatitis C viral kinetics in the early days of treatment with pegylated interferon plus ribavirin in HIV/HCV co-infected patients with genotype 1 or 4. *J Antimicrob Chemother* 2012; **67**: 202–205.
- Schechtman G, Kaul S, Mueller RA, Borden EC, Kissebah AH. The effect of interferon on the metabolism of LDLs. *Arterioscler Thromb* 1992; **12**: 1053–1062.
- Fischer DG, Novick D, Cohen B, Rubinstein M. Isolation and characterization of a soluble form of the LDL receptor, an interferon-induced antiviral protein. *Proc Soc Exp Biol Med* 1994; **206**: 228–232.
- Fischer DG, Tal N, Novick D, Barak S, Rubinstein M. An antiviral soluble form of the LDL receptor induced by interferon. *Science* 1993; **262**: 250–253.
- Albecka A, Belouzard S, Op de Beeck A, Descamps V, Goueslain L, Bertrand-Michel J et al. Role of low-density lipoprotein receptor in the hepatitis C virus life cycle. *Hepatology* 2012; **55**: 998–1007.
- Scarselli E, Ansuini H, Cerino R, Roccasecca RM, Acali S, Filocomo G et al. The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. *Embo J* 2002; **21**: 5017–5025.
- Prokunina-Olsson L, Muchmore B, Tang W, Pfeiffer RM, Park H, Dickensheets H et al. A variant upstream of IFNL3 (IL28B) creating a new interferon gene IFNL4 is associated with impaired clearance of hepatitis C virus. *Nat Genet* 2009; **41**: 164–171.
- Nyholt DR. A simple correction for multiple testing for single-nucleotide polymorphisms in linkage disequilibrium with each other. *Am J Hum Genet* 2004; **74**: 765–769.
- Sasieni PD. From genotypes to genes: doubling the sample size. *Biometrics* 1997; **53**: 1253–1261.

Supplementary Information accompanies this paper on Genes and Immunity website (<http://www.nature.com/gene>)

Variations at multiple genes improve interleukin 28b genotype predictive capacity for response to therapy against hepatitis c genotype 1 or 4 infection

Karin Neukam^{a,b}, Antonio Caruz^c, Antonio Rivero-Juárez^d, Pablo Barreiro^e, Dolores Merino^f, Luis M. Real^{a,b}, Rocío Herrero^c, Angela Camacho^d, Vicente Soriano^e, Federico A. Di Lello^{a,b}, Juan Macías^{a,b}, Antonio Rivero^d and Juan A. Pineda^{a,b}

Objective: To identify genetic factors that predict sustained virological response (SVR) to pegylated interferon (Peg-IFN)/ribavirin (RBV) in HIV/hepatitis C virus (HCV) genotype 1 or 4-coinfected patients and that enhance the predictive capacity of *IL28B* genotype in this population.

Design: Prospective cohort study.

Setting: Five tertiary care centers in Spain.

Subjects: 205 HIV/HCV genotype 1 or 4-coinfected patients who received a complete course of Peg-IFN/RBV for 48 weeks.

Main outcome measures: All individuals were genotyped for 144 SNPs.

Results: One hundred sixty-two (79%) patients bore HCV genotype 1. Overall SVR was achieved by 73 (36%) individuals. SNPs at the following genes were associated with SVR: *IL28B*, low-density lipoprotein receptor (*LDLR*), transforming growth factor β (*TGF- β*), aquaporine 2 (*AQP-2*), very-low-density lipoprotein receptor, Sp110 nuclear body protein, interferon alpha/beta receptor 1, 2'-5'-oligoadenylate synthase 1 and apolipoprotein B. There was a strong synergy between SNPs at *IL28B*, *TGF- β* and *AQP-2* genes: the number of patients reaching SVR with all three favorable genotypes versus unfavorable genotypes were 22 (78.6%) versus 1 (7.1%) ($p = 2.1 \times 10^{-3}$). HCV baseline viral load, *IL28B*, *TGF- β* , *AQP-2* and *LDLR* haplotypes were independently associated with SVR.

Conclusion: A number of genetic factors modify the predictive capacity of *IL28B* genotype. These can be used to identify HCV genotype 1 or 4 infected patients with a very high or a very low probability to respond to bitherapy with Peg-IFN/RBV. Predictive models based on these factors could be helpful to tailor direct acting antiviral-based therapy.

© 2013 Wolters Kluwer Health | Lippincott Williams & Wilkins

AIDS 2013, 27:000–000

Keywords: aquaporine 2, hepatitis C virus, HIV, low density lipoprotein receptor, pegylated interferon, ribavirin, transforming growth factor β

^aUnit of Infectious Diseases and Microbiology. Hospital Universitario de Valme. Sevilla, Spain, ^bInstituto de Biomedicina de Sevilla (IBiS). Sevilla, Spain, ^cImmunogenetics Unit. Faculty of Sciences. Universidad de Jaén. Jaén, Spain, ^dUnit of Infectious Diseases, Hospital Universitario Reina Sofía, Instituto Maimónides de Investigación Biomédica de Córdoba (IMIBIC). Córdoba, Spain, ^eDepartment of Infectious Diseases, Hospital Carlos III. Madrid, Spain, and ^fUnit of Infectious Diseases, Hospital Juan Ramón Jiménez. Huelva, Spain.

Correspondence to Dr. Juan A. Pineda, PhD, MD, Unidad de Enfermedades Infecciosas y Microbiología. Hospital Universitario de Valme. Avda. de Bellavista. 41014 Sevilla. Spain.

Tel: +0034 955015684; fax: +0034 955015795; e-mail: japineda@telefonica.net

DOI:10.1097/01.aids.0000432459.36970.a9

ISSN 0269-9370 © 2013 Wolters Kluwer Health | Lippincott Williams & Wilkins
Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

1

Introduction

In European countries, over 50% of the cases of chronic hepatitis C among HIV-infected patients are caused by hepatitis C virus (HCV) genotype 1 [1]. The rates of sustained virological response (SVR) to dual therapy with pegylated interferon (Peg-IFN) and ribavirin (RBV) are very low in this subset under real-life conditions [2]. HIV/HCV genotype 4-infected patients represent approximately 15%–20% of the HIV/HCV-coinfected population [1,2]. The response rates to Peg-IFN plus RBV observed in these individuals are somewhat higher, as compared to HIV/HCV genotype 1 infections; however, approximately two thirds do not achieve SVR [2]. Triple therapy including Peg-IFN, RBV and either telaprevir or boceprevir has recently become the standard of care against chronic hepatitis C by genotype 1 in the HIV-coinfected patient [3]. SVR rates with these regimens observed in clinical trials in treatment-naïve patients have reached up to 74% [4,5] and data obtained under real-life conditions are also promising [6,7]. However, response in all patients is not achieved. In the case of HIV/HCV genotype 4 coinfection, no alternative treatment option has been approved to date. Therefore, predictive tools to select patients with a very high or a very low probability to achieve SVR are necessary in the current clinical practice, especially for those infected with HCV genotype 4.

As it is the case for dual therapy, the standard recommended treatment duration for triple therapy in HIV/HCV-coinfected patients remains 48 weeks [3]. The finding of reliable predictors for SVR could, on the one hand, help us to identify candidates who may benefit from dual therapy and, on the other hand, allow the development of shorter treatment schedules with direct-acting antivirals (DAAs). Since tolerance to Peg-IFN plus RBV is poor especially in the setting of antiretroviral therapy, the benefit of shorter regimens is even higher in HIV-coinfected patients. Likewise, source-limited settings are in high need of predictors of SVR to dual therapy. Pharmacogenetic determinations represent cost-effective tools to predict the probability of SVR. In this context, the single nucleotide polymorphism (SNP) rs12979860 near the interleukin 28B (*IL28B*) gene is a potent predictor for SVR to dual therapy in HIV/HCV genotype 1 or 4-coinfected patients [2,3,9]. Likewise, it has a lower, but evident, impact on the outcome of first-generation protease inhibitor (PI)-based therapy in prior treatment-naïve patients without HIV coinfection [10–12]. The predictive capacity of *IL28B* genotype can be enhanced by its combination with viral and host factors [13–16]. In this context, the determination of genetic variations of the SNP rs14158, at the low-density lipoprotein receptor (*LDLR*) gene, increases the *IL28B* predictive performance [13], which may be caused by the HCV viral replication cycle being affected by cholesterol and fatty acid biosynthesis. However, the predictive value

obtained for HIV/HCV genotype 1 or 4-infected patients even using *IL28B* plus rs14158 genotyping is suboptimal, as the probability of SVR in patients identified as likely responders using these parameters hardly reaches 70% [13–16].

This study aimed to identify genes other than *IL28B* and *LDLR* whose variations predict response to Peg-IFN plus RBV and which may allow us to enhance the predictive value of *IL28B* genotype in HIV/HCV genotype 1 or 4-infected patients.

Methods

Study population

HCV genotype 1 or 4-infected patients were selected from a cohort that was prospectively followed at the units of Infectious Diseases of five Spanish hospitals between June 2000 and December 2010. The inclusion criteria in this cohort were: i) older than 18 years, ii) prior HCV treatment naïve, iii) initiation of dual therapy with Peg-IFN plus RBV, iv) coinfection with HIV and v) stored whole blood samples available for genetic determinations. Responses were analyzed in an on-treatment approach, i.e. those who interrupted treatment due to adverse events or who voluntarily dropped out were excluded. The scheduled visits were at baseline, every 4 weeks during the first six months of treatment and every 12 weeks thereafter. In order to evaluate SVR, a visit 24 weeks after stopping therapy was conducted.

Treatment regimens and definition of response

Peg-IFN alfa-2a or alfa-2b was administered at doses of 180 µg or 1.5 µg/kg once per week, respectively, in combination with weight-adjusted RBV (1000 mg/day for < 75 kg and 1200 mg/day for ≥ 75 kg). The scheduled treatment duration was 48 weeks for all patients. Stopping rules were applied following international guidelines in force at the moment of treatment. SVR was defined as undetectable plasma HCV RNA 24 weeks after the completion of therapy. A decline of plasma HCV RNA of less than 2 log₁₀ at week 12 or lack of undetectable HCV RNA at treatment week 24 after having presented a ≥ 2 log₁₀ decline but without reaching undetectability, was considered as non-response. Undetectable HCV RNA at the end of therapy without achieving SVR was considered as relapse. Viral breakthrough was defined as detectable plasma HCV RNA during treatment after having reached undetectability.

Selection and genotyping of SNPs and HCV RNA quantification

Genomic DNA was isolated from whole blood samples or from isolated peripheral blood mononuclear cells using the automated MagNA Pure DNA extraction method (Roche Diagnostics Corporation, Indianapolis, IN, USA)

or the Quick Pure Blood DNA extraction Kit (Macherey-Nagel, Düren, Germany). Selected SNPs included SNPs belonging to genes involved in the cholesterol metabolism and transport or in the immune response and which were identified as tag SNPs after an analysis of the genotype data obtained from Hapmap CEU population (www.hapmap.org) using Haploview V4.1 software (<https://www.broad.harvard.edu/haploview/haploview>). Likewise, SNPs from genes which had previously been found to be associated with SVR or with liver fibrosis in HCV-infected patients were tested [17–19]. All SNPs were genotyped using a custom Golden Gate Veracode genotyping assay (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Those SNPs with the following characteristics were discarded: i) a minor allele frequency <0.05, ii) a Hardy-Weinberg-Equilibrium $p < 0.01$ and iii) genotyping of less than 80%. Genotyping of *IL28B* and determination of plasmatic HCV-RNA was conducted as described elsewhere [8,9]. The researchers responsible for genotyping were blinded to other patient data.

Data analysis

Haploview V4.1 Software (Haploview V4.1 software (<https://www.broad.harvard.edu/haploview/haploview>) was used to calculate the Hardy-Weinberg equilibrium for all SNPs. A first analysis of standard case/control allelic associations (1 degree freedom), as well as of different genetic models using PLINK software (<http://pngu.mgh.harvard.edu/~purcell/plink/gplink.shtml>) was performed. Those SNPs that were associated with SVR showing a p -value <0.05 were selected for further analysis. Genotypes within one SNP that showed similar rates of SVR were combined. The linkage disequilibrium (LD) values between the genetic markers studied (measured in Lewontin's standardized disequilibrium coefficient D'), haplotype frequencies, and haplotype-based association analyses were calculated using Haploview V4.1 for those genes with ≥ 2 SNPs available. Chromosome phases for LDLR haplotypes were estimated using PLINK software.

Continuous variables were compared by means of the Student's t -test or the Mann-Whitney U-test, when applicable, and are presented as median (interquartile range, IQR). For the analysis of categorical variables, the χ^2 test or the Fisher's exact test was used in 2x2 tables. SVR was the primary outcome variable. A multivariate logistic regression analysis including as covariables age, sex, as well as those factors that were associated with SVR with a p value <0.05 in the univariate analysis, was conducted. For additive genetic factor analysis, a simple unweighted genetic risk score (GRS) was calculated with those SNPs that were independently associated with SVR. In the subpopulation of *IL28B* genotype CC carriers, protective genotypes were added (+1 point for each favourable genotype, unfavourable genotypes were rated as 0). Likewise, in the subpopulation of patients

presenting *IL28B* genotype non-CC, unfavourable genotypes were subtracted (–1 point for each unfavourable genotype, favourable genotypes were rated as 0). Descriptive and inferential analyses were carried out using the SPSS statistical software package release 19.0 (IBM Corporation, Somers, NY, USA).

Ethical issues

The study was designed and performed according to the Helsinki declaration and was approved by the Ethics Committee of the Hospital Universitario de Valme.

Results

Overall study population

A total of 262 patients fulfilled the inclusion criteria for this study. Of these, 57 (21.8%) individuals were excluded because they could not be assessed in an on-treatment approach. Finally, 205 patients were selected (Table 1). One hundred and sixty-two (79%) were infected with HCV genotype 1 and 43 (21%) bore HCV genotype 4. Sixty-seven (32.8%) presented a baseline plasma HCV RNA below 600000 IU/mL.

Response to Peg-IFN plus RBV

SVR was achieved by 73 (35.6%) individuals. Undetectable HCV RNA at week 4 of treatment was observed in 32 (16.8%) of the 190 patients in whom this information was available. Eighty-nine (43.4%) patients were non-responders and 30 (14.6%) subjects relapsed. Thirteen (6.3%) individuals experienced a viral breakthrough. Fifty-five (34%) of the patients infected with HCV genotype 1 versus 18 (41.9%) of those individuals bearing HCV genotype 4 achieved SVR ($p = 0.336$). The rate of patients achieving SVR according to *IL28B* rs12979869 genotype was: 44 (55.7%) for CC, 20 (20.8%) CT and 9

Table 1. Baseline characteristics of the study population.

Parameter	Value
Age (years)*	41.6 (38.5–44.8)
Male gender, no. (%)	170 (82.9)
Body mass index (kg/m ²)*	23.4 (21.6–26.2)
Intravenous drug user, no. (%)	174 (84.9)
HCV genotype 1, no. (%)	162 (79)
<i>IL28B</i> rs12979860 CC, no. (%)	79 (38.5)
Plasma HCV viral load (log ₁₀ IU/mL)*	6.21 (5.52–6.72)
Advanced fibrosis, no. (%)	80 (47.6)
Alanine-aminotransferase(U/L)*	66 (44–99.5)
Total cholesterol (mg/dL)*	168 (148–196)
Low-density lipoprotein cholesterol (mg/dL)*	92.6 (74.9–116)
Triglycerides (mg/dL)*	117 (83–172)
Pegylated interferon alfa 2a, no. (%)	146 (71.2)
Daily dose of ribavirin (mg/kg)*	14.9 (13.1–16.9)
Undetectable plasma HIV RNA, no. (%)	167 (81.5)
CD4 cell count (cells/ μ L)*	521 (388–730)

*median (IQR); †determined by liver biopsy (F ≥ 3 according to the Scheuer Index) or a liver stiffness value ≥ 11 kPa, available in 168 patients.

(30%) TT, respectively ($p = 8.1 \times 10^{-6}$). Individuals who achieved SVR presented a baseline median HCV RNA (IQR) of 5.7 (5.1–6.5) $\log_{10}$ IU/mL versus 6.4 (5.9–6.8) $\log_{10}$ IU/mL for those who did not ($p = 4.5 \times 10^{-5}$). The median (IQR) plasmatic LDL cholesterol levels were 105 (84.6–120) mg/dL among patients with SVR versus 89 (70.5–113) mg/dL for those without SVR ($p = 0.007$).

SNPs selected

One hundred and forty-four SNPs were selected for the primary analysis (Supplement 1). Of these, 26 were excluded due to either a minor allele frequency < 0.05 [20(13.9%) SNPs], a p of the Hardy-Weinberg-Equilibrium below 0.01 [1(0.7%) SNP] or a rate of genotyping below 80% [5(3.5%) SNPs]. Thus, 118 (81.9%) of the previously selected SNPs were analyzed (Supplement 1). SNPs at the following genes were associated with SVR in the analysis conducted using PLINK software: *LDLR* (rs10415811, $p = 0.047$; rs11672123, $p = 0.0067$; rs1433099, $p = 0.0019$; rs2569540, $p = 0.0044$; rs5930; $p = 0.046$), transforming growth factor β (*TGF- β* ; rs1800469, $p = 0.0087$), aquaporin 2 (*AQP-2*; rs2878771, $p = 8 \times 10^{-4}$), very-low-density lipoprotein receptor (*VLDLR*; rs1454626, $p = 0.015$), Sp110 nuclear body protein (*SP110*; rs919178, $p = 0.045$), interferon alpha/beta receptor 1 (*IFNAR1*; rs2243592, $p = 0.036$), 2'-5'-oligoadenylate synthase 1 (*OAS1*; rs1131454, $p = 0.047$) and apolipoprotein B (*APO-B*; rs11126598, $p = 0.0094$). Genotyping was achieved in 100% (*AQP-2*, *VLDLR*, *SP110*, *IFNAR1*, *OAS1* and *APO-B*) and 97.1% (*TGF- β*) of the cases, respectively. The *VLDLR* rs7043199 was discarded due to the low prevalence of its favourable genotype TT (2.9%), so that subsequent analysis exclusively refers to rs1454626.

Treatment response according to newly identified SNPs

According to the SVR rates observed for each genotype, the SNPs genotyping results were classified as favourable and unfavourable. Figure 1 presents the rates of SVR, as well as the distribution of each genotype in the study population. SVR rates according to variations of rs1131454 in the *IFNAR1* gene were 18 (49%) for genotype CC and 55 (33%) for genotype AG/AA ($p = 0.067$).

In spite of the number of SNPs in specific genes identified as predictors for SVR in this study, haplotypic analysis could only be performed for *LDLR*, *VLDLR* and *TGF- β* genes due to the number of SNPs analyzed in these genes (Supplement 1). In the *LDLR* gene, seven LD blocks were detected (Figure 2). An extended haplotype (GGAAG) in LD block number 6 defined by rs2738464, rs2738465, rs1433099, rs2738466 and rs7258950 (Figure 2) was associated with SVR ($p = 0.0046$). After categorization, 31 (50.8%) of the subjects with *LDLR* haplotype GGAAG versus 42 (29.4%) of those with the non-GGAAG haplotype

showed SVR ($p = 0.003$). Among the HCV genotype 1-infected subjects, 22 (50%) of those presenting *LDLR* haplotype GGAAG versus 33 (28.2%) of those with other *LDLR* haplotypes reached SVR ($p = 0.009$). The corresponding figures for HCV genotype 4 carriers were 9 (52.9%) versus 9 (34.6%), respectively ($p = 0.23$). The interaction between *LDLR* haplotype and *IL28B* genotype on SVR rates are shown in Table 3. No haplotype of *VLDLR* or *TGF- β* was associated with SVR.

Interaction of newly identified SNPs with the predictive value of *IL28B* rs12979860

Forty-four (55.7%) of the patients presenting *IL28B* genotype CC versus 29 (23%) of those with genotype CT/TT attained SVR ($p = 2 \times 10^{-6}$). The corresponding figures among the subpopulations infected with HCV genotype 1 and 4 were 34 (51.5%) versus 21 (21.9%; $p = 9 \times 10^{-4}$) and 10 (76.9%) versus 8 (26.7%; $p = 0.002$), respectively. SVR rates according to the newly identified genes stratified for *IL28B* genotype are listed in Table 2. After categorization for *IL28B*, *TGF- β* and *AQP-2* genotypes, the number of patients reaching SVR with favorable genotypes (*IL28B* CC, *TGF- β* non-AA and *AQP-2* non-GG) versus unfavorable genotypes (*IL28B* non-CC, *TGF- β* AA and *AQP-2* GG) were 22 out of 28 (78.6%) versus 1 out of 13 (7.1%) ($p = 2.1 \times 10^{-5}$). According to HCV genotype, the numbers of individuals with the former combinations of genotypes reaching SVR were 16 (76.2%) versus 1 (9.1%) in HCV genotype 1 ($p = 3 \times 10^{-4}$) and 6 (85.7%) versus 0 in HCV genotype 4 ($p = 0.023$), respectively. Furthermore, an interaction between *IL28B* and *VLDLR*, *SP110*, *IFNAR1* and *APO-B*, respectively, was observed (Table 2).

Multivariate analysis

According to the univariate analysis, SNPs in *TGF- β* , *AQP-2*, *VLDLR* and *APO-B* genes, as well as the *LDLR* haplotype GGAAG, were entered into a multivariate logistic regression model adjusted for age, sex, presence of advanced fibrosis, HCV baseline viral load and *IL28B* genotype. *SP110* and *OAS1* were excluded due to their considerably weak association with SVR and, mainly, because of the low prevalence of the unfavourable genotype (Figure 1). In the multivariate analysis *TGF- β* non-AA, *AQP-2* non-GG and *LDLR* GGAAG were independently associated with SVR along with *IL28B* CC genotype and low baseline HCV RNA load (Table 3).

Additive genetic factor analysis

In accordance with the multivariate analysis, *TGF- β* , *AQP-2* and *LDLR* haplotype were selected for this calculation. In patients presenting *IL28B* genotype CC, the mean GRS for patients with SVR was 1.88 versus 1.18 presented by those individuals without SVR ($p = 1.3 \times 10^{-4}$). In the subpopulation of patients with *IL28B* non-CC genotype, mean GRS was -1 in patients presenting SVR

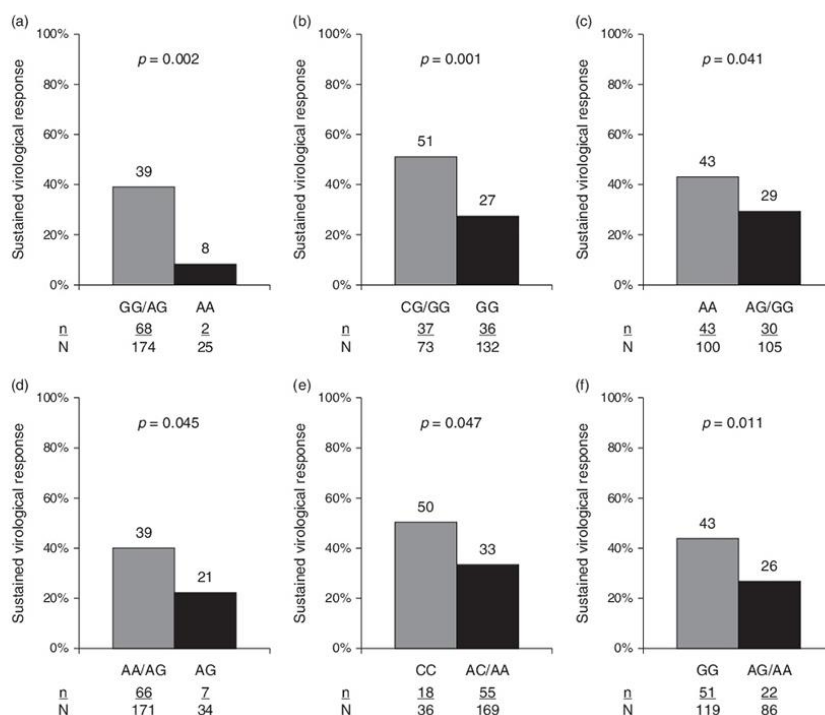


Fig. 1. Rates of sustained virological response (SVR) according to genotypes of the SNPs newly identified and distribution of each genotype in the study. A: transforming growth factor β (TGF- β); B: aquaporin 2 (AQP-2); C: very-low-density lipoprotein receptor (VLDLR); D: Sp110 nuclear body protein (SP110); E: 2'-5'-Oligoadenylate synthase 1 (OAS1); F: apolipoprotein B (APO-B). n = Number of patients achieving SVR for each genotype. N = Number of patients bearing the specific genotype in the overall population.

versus -1.62 in those without SVR ($p = 3.5 \times 10^{-5}$). The rates of SVR according to different GRS among patients with favourable *IL28B* genotype were 0 out of 4 patients with GRS = 0, 38.9% (14 patients) with GRS = 1, 82.6% (19 patients) with GRS = 2 and 75% (9 patients) with GRS = 3, $p = 4 \times 10^{-4}$. Among patients with *IL28B* genotype non-CC, the rates of SVR were 55.6% (5 patients) for GRS = 0, 34.6% (18 patients) for GRS = -1, 8.9% (5 patients) for GRS = -2 and none out of 6 patients of GRS = -3, $p = 0.001$.

Discussion

This study has identified genetic variations in the *TGF-β* and *AQP-2* genes as independent predictors of SVR to Peg-IFN plus RBV in HIV/HCV genotype 1 or 4-

infected patients. Additionally, a haplotype on the *LDLR* gene has also been found to be associated with SVR. These pharmacogenomic parameters improve the predictive capacity of *IL28B* genotype and may therefore play a role in the development of a tool to accurately predict response to therapy against HCV.

The *IL28B* genotype is a potent predictor of response to dual therapy in HIV/HCV-coinfected patients [2,8,9], which is commonly used in daily practice. However, the meaning of a favourable or unfavourable *IL28B* genotype in terms of the likelihood of SVR is very different depending on viral [16] and host factors, such as plasma levels of IP-10 [15] or *LDLR* genotype [13,14]. This study demonstrates that other genomic factors may determine the SVR rates associated with *IL28B* variations and that some of those factors with a greater impact

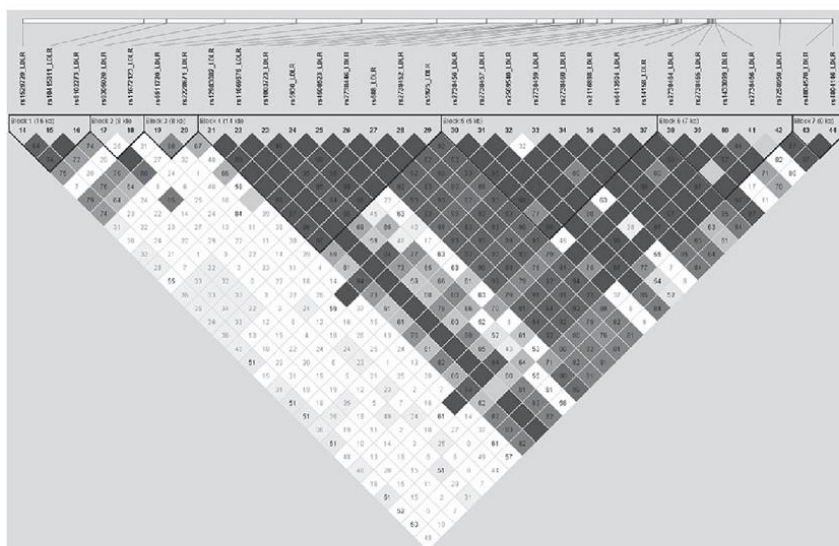


Fig. 2. Genomic position of the SNPs on the LDLR gene and linkage disequilibrium blocks analyzed.

should be considered along with *IL28B* genotype when used in clinical routine.

The findings presented in this study raise a number of questions, since few or no data is available on the association of SVR with the protein encoded by the corresponding genes of some of the herein described SNPs. In this context, TGF- β is a cytokine with multiple functions that has been associated with the development of hepatic fibrosis [20,21]. However, the role of TGF- β on the outcome of treatment against HCV is unclear and data are scarce and contradictory [22–27]. In this regard, high TGF- β levels have been described to diminish response to dual therapy in this population [22]. On the other hand, a study observed a direct relationship between TGF- β levels and response to Peg-IFN in HIV-infected patients with acute hepatitis C [23]. The results of the present study supports that TGF- β is involved in viral clearance. However, it is unknown how this influence is exerted. Similarly, AQP-2 seems to be involved in the development of fibrosis [28], but no data is currently available on its impact on HCV treatment outcome. The main function of AQP-2 is the vasopressin-dependent reabsorption of water by forming water-specific membrane channels in the renal collecting duct. Dysfunction of AQP-2 caused by mutations on the AQP-2 gene can lead to diabetes insipidus [29]. There might be an association between cholesterol metabolism and AQP-2, as statins interfere with its expression [30].

Plasma lipoproteins, including VLDLR [31] and APO-B [32], play an important role on HCV infectivity and on the outcome of therapy against HCV. Genetic variations in the *VLDLR* gene were observed to impact on SVR, herein, although no independent association was observed in the multivariate analysis. The identification of a *LDLR* haplotype associated with SVR supports the findings of a previous study where an influence of a specific SNP (rs14158) in the 3'UTR of the *LDLR* gene on SVR was described [13]. Likewise, this study shows that using this haplotype, the predictive capacity of isolated SNPs in *LDLR* genes is improved.

The predictive performance of *IL28B* genotype can be markedly enhanced by using other genomic predictors concomitantly. This has been proven using the combination of *IL28B* and the rs14158 CC genotype on the *LDLR* gene [13]. However, almost 31% of the carriers of both *IL28B* and *LDLR* favourable genotypes do not respond to therapy, whereas 14% of those harboring both unfavourable genotypes show SVR to Peg-IFN plus RBV [13]. This points out the necessity to optimize this combination of genotypes. In this study, using three genotypes (*IL28B*/AQP-2/TGF- β), the probability of SVR increased to 80% for the favourable combination and it was only 7% for the triple unfavourable genotype. Unfortunately, the clinical utility of this combination is limited because the triple favourable and unfavourable genotypes are relatively uncommon; indeed, they were

Table 2. Interaction of newly identified SNPs and *IL28B* rs12979860 genotype regarding rates of SVR.

Gene	Genotype	IL28B rs12979860			
		CC		CT/TT	
		n SVR (%)	p	n SVR (%)	p
SNPs					
TCF-βrs1800469	GG/AG	42 (63)	0.001	26 (24)	0.251
	AA	0		2 (12)	
AQP-2 rs2878771	CC/CG	22 (73)	0.014	15 (35)	0.023
	GG	22 (45)		14 (17)	
VLDLR rs1454626	AA	29 (71)	0.005	14 (24)	0.858
	AC/CC	15 (40)		15 (22)	
SP110 rs919178	AA/AG	39 (60)	0.171	27 (26)	0.108
	GG	5 (39)		2 (9.5)	
OAS1 rs1131454	GG	13 (72)	0.604	24 (22)	0.108
	AG/AA	31 (51)		5(28)	
IFNAR1 rs2243592	CC	11 (73)	0.127	7 (32)	0.280
	AC/AA	33 (52)		22 (21)	
APO-Brs11126598	GG	33 (66)	0.015	18 (26)	0.368
	AG/AA	11 (38)		11 (19)	
Haplotypes					
LDLR*	GGAAG	15 (71)	0.09	16 (40)	0.002
	non GGAAG	29 (50)		13 (15)	

TGF-β: transforming growth factor β; AQP-2: aquaporine 2; VLDLR: very-low-density lipoprotein receptor; SP110: Sp110 nuclear body protein; OAS1:2'-5'-Oligoadenylate synthase 1; IFNAR1: interferon alpha/beta receptor 1; APO-B: apolipoprotein B; LDLR: low-density lipoprotein receptor.*defined by rs2738464, rs2738465, rs1433099, rs2738466 and rs7258950.

Table 3. Univariate and multivariate analysis to identify factors associated with sustained virologic response (SVR).

Parameter	SVR, n (%)	p univariate	AOR (95% CI)	p multivariate
Age				
≤41.6 years	42 (41.2)	0.098	1 (0.91–1.1)	0.961
>41.6 years	31 (30.1)			
Gender				
Male	61 (35.9)	0.857	0.844 (0.26–2.73)	0.776
Female	12 (34.3)			
Advanced fibrosis ¹				
No	35 (39.8)	0.093	2.11 (0.86–5.15)	0.102
Yes	22 (27.5)			
Baseline HCV RNA load				
≤600000 IU/mL	39 (58.2)	1.7*10 ⁻⁶	5.87 (2.19–15.7)	4.3*10 ⁻⁴
>600000 IU/mL	33 (24.1)			
<i>IL28B</i> rs12979860				
CC	44 (55.7)	1.9*10 ⁻⁶	8.88 (3.32–23.7)	1.3*10 ⁻⁴
CT/TT	29 (23)			
<i>TGF-β</i> rs1800469				
GG/AG	68 (39.1)	0.002	6.927 (1.29–37.3)	0.024
AA	2 (8)			
<i>AQP-2</i> rs2878771				
CC/CG	37 (50.7)	0.001	3.781 (1.51–9.49)	0.005
GG	36 (27.3)			
<i>VLDLR</i> rs1454626				
AA	43 (43)	0.041	1.49 (0.63–3.56)	0.368
AC/CC	30 (28.6)			
<i>APO-B</i> rs11126598				
GG	51 (42.9)	0.011	1.56 (0.65–3.75)	0.321
AG/AA	22 (25.6)			
<i>LDLR</i> haplotype				
GGAAG	31 (50.8)	0.003	5.078 (1.79–14.4)	0.002
non GGAAG	42 (29.4)			

SVR: sustained virological response; AOR: adjusted odds ratio; CI: confidence interval; *IL28B*: interleukin 28B; IU: IU; *LDLR*: low-density lipoprotein receptor; *TGF-β*: transforming growth factor β; *AQP-2*: aquaporine 2; *VLDLR*: very-low-density lipoprotein receptor; *APO-B*: apolipoprotein B; *LDLR*: low-density lipoprotein receptor. ¹available in 168 individuals.

found only in 13.7% and in 6.3% of the population analyzed herein. However, it is probable that the combination of some of the SNPs described in this study with other viral or host predictors of SVR may yield valuable predictive tools. As it can be seen with the GRS calculation, SVR rates vary considerably according to the number of risk factors both among carriers of the favourable and the unfavourable *IL28B* genotype. Importantly, the rates of SVR are higher for those patients bearing *IL28B* non-CC but no other unfavourable genotype than those patients with *IL28B* CC but no other favourable genotype. In the case of HCV genotype 1 infection, this information could be used to select those individuals who may greatly benefit from dual therapy. This is a critical point because PI-based therapy is unlikely to be widely available in many countries due to financial restrictions in the next few years. Likewise, HCV genotype 4-infected patients with a very high probability to respond could be motivated to undergo dual therapy. On the other hand, treatment could be deferred in those patients with a very low likelihood to respond to dual therapy, if they do not present advanced fibrosis.

The duration of dual therapy against HCV may be decided on the basis of HCV kinetics on treatment [3,33,34]. Similarly, in patients with rapid viral decline, DAA-based therapy may be also shortened without reduction of the rate of SVR [35,36]. Viral kinetics in HCV-infected patients strongly depend on the pharmacogenomic host features [37–39]. Consequently, the genomic predictors identified here may correlate with viral kinetics. If so, these predictors could be useful to identify patients who qualify for shorter double or triple treatment durations from baseline, thus avoiding very early viral load determinations. Further studies are required in order to address this topic.

This study has some limitations. First, the number of patients is relatively limited to allow classification into multiple genetic profiles. This led to categories with low numbers of cases. Because of this, these data should be reproduced with a higher number of patients and in other populations. However, the main objective of this study was to identify novel SNPs that may be used to develop a predictive model that allows calculating the individual probability of response for each patient, and, in fact, we have identified SNP potentially candidates to be entered in predictive models along with viral and other host factors. Second, these results should be analyzed in HCV-monoinfected patients, since the predictive value may be different in this population. Third, the analysis presented herein is limited to HCV genotypes 1 and 4. In genotype 3-infected individuals, a higher mortality has been observed for *IL28B* CC carriers [40] and these individuals would benefit from identifying alternative predictors. However, and similar to what is observed for *IL28B* genotype in patients with or without HIV coinfection [8,9,41,42], no association between SVR and the SNPs

described herein could be detected in HCV genotype 2 or 3-infected individuals (data not shown). However, an impact of a SNP on the proprotein convertase subtilisin/kexin type 9 gene on SVR uniquely in genotype 3 infection has been described recently [39], lining out the necessity to distinguish between these genotypes. Finally, in the era of new DAAs genomic predictors may be less important. However, an impact of *IL28B* genetic variations has been observed under interferon-based triple therapy [10–12] in treatment naïve patients. It also seems to play a role in interferon-free regimens [43,44], particularly with specific combinations [44]. Therefore, the value of genomic predictors is likely to remain important in the setting of DAA-based therapy. Furthermore, DAA-based therapy will not be available for all HCV-infected patients in most countries, mainly due to financial restrictions. Because of this, dual therapy may continue to be given in a significant number of patients in these settings. A combination of pharmacogenomic markers with high predictive performance may be very helpful to identify patients to be treated with dual therapy and, among them, those who may benefit from shorter courses of therapy.

In conclusion, there is a number of important genetic factors that modify the predictive capacity of *IL28B* genotype, as *TGF-β*, *AQP-2* and *LDLR* genotype. A combination of these factors can be used to identify HIV/HCV genotype 1 or 4 infected patients with a very high or a very low probability to respond to dual therapy with Peg-IFN/RBV. Furthermore, the predictive ability of models based on these factors should be analyzed in patients on direct acting antivirals.

Financial support: This work was supported in part by the Red de Investigación en SIDA (grant number RETIC RD06/006 / RD12/0017), the Fundación Progreso y Salud, Consejería de Salud de la Junta de Andalucía (grant number PI-0247–2010, PI-0157–2011, PI-0430–2012 PI10/01232), the Fondo de Investigaciones Sanitarias (grant number PI10/01664), the Ministerio de Sanidad y Servicios Sociales (grant number EC11–304) and the Fundación para la Investigación y la Prevención del Sida en España (grant number 121004/10). KN is the recipient of a Sara Borrell postdoctoral perfection grant from the Instituto de Salud Carlos III (grant number SCO/523/2008). JAP is the recipient of an intensification grant from the Instituto de Salud Carlos III (grant number Programa-I3SNS).

Acknowledgements

Author contribution:

K. N.: planning and conducting the study, collecting and interpreting data and drafting the manuscript.

A. Caruz: planning and conducting the study, collecting and interpreting data.

A. R.-J.: collecting and interpreting data.

P.B.: collecting and interpreting data.

L.M.: collecting and interpreting data.

L.M.R.: genotyping, collecting and interpreting data.

R. H.: collecting and interpreting data.

A. Camacho: collecting and interpreting data.

V.S.: planning and conducting the study, interpreting data and drafting the manuscript.

F. A. D. L.: collecting and interpreting data and drafting the manuscript.

J. M.: collecting and interpreting data and drafting the manuscript.

A. R.: planning and conducting the study, interpreting data and drafting the manuscript.

J. A. P.: planning and conducting the study, interpreting data and drafting the manuscript.

Source of Funding: D. M. has received consulting fees from ViiV Healthcare, Bristol-Myers Squibb, Abbott Pharmaceuticals, Merck Sharp & Dohme, Janssen-Cilag and Boehringer Ingelheim. V.S. has received consulting fees from Merck Sharp & Dohme and Boehringer Ingelheim and Janssen. He has received research support from Merck Sharp & Dohme, Janssen and Gilead and Boehringer Ingelheim and received lecture fees from Gilead, ViiV Healthcare, Bristol-Myers Squibb, Merck Sharp & Dohme and Janssen. A.R. has received consulting fees from Bristol-Myers Squibb, Abbott, Gilead, Janssen Cilag, Merck Sharp & Dohme and Boehringer Ingelheim, has received research support from Abbott, GlaxoSmithKline, Roche, Bristol-Myers Squibb, Abbott and Boehringer Ingelheim and has received lecture fees from GlaxoSmithKline, Abbott, Bristol-Myers Squibb, Merck Sharp & Dohme, Janssen Cilag, Gilead, Boehringer Ingelheim and Schering-Plough. J. A. P. has received consulting fees from GlaxoSmithKline, Bristol-Myers Squibb, Abbott Pharmaceuticals, Gilead, Merck Sharp & Dohme, Schering-Plough, Janssen and Boehringer Ingelheim. He has received research support from GlaxoSmithKline, Roche, Bristol-Myers Squibb, Schering-Plough, Abbott Pharmaceuticals, Gilead, Merck Sharp & Dohme, Janssen and Boehringer Ingelheim, and has received lecture fees from GlaxoSmithKline, Roche, Abbott Pharmaceuticals, Bristol-

Myers Squibb, Gilead, Merck Sharp & Dohme, Janssen, Boehringer Ingelheim and Schering-Plough.

Conflicts of Interest

All other authors: none to declare.

References

1. Soriano V, Mocroft A, Rockstroh J, Ledergerber B, Knysz B, Chaplinskas S, Peters L, *et al*. Spontaneous viral clearance, viral load, and genotype distribution of hepatitis C virus (HCV) in HIV-infected patients with anti-HCV antibodies in Europe. *J Infect Dis* 2008; **198**:1337–1344.
2. Mira JA, Rivero A, de Los Santos-Gil I, López-Cortés LF, Girón-González JA, Márquez M, Merino D, *et al*. Hepatitis C virus genotype 4 responds better to pegylated interferon with ribavirin than genotype 1 in HIV-infected patients. *AIDS* 2012; **26**:1721–1724.
3. European AIDS Clinical Society. Guidelines version 6.1 November 2012. Available at: http://www.europeanaidsclinical-society.org/images/stories/EACS-Pdf/EACS_Guidelines-v6.1-English-Nov2012.pdf. Last date accessed March 12, 2012.
4. Sulkowski M, Sherman KE, Soriano V, Rockstroh JK, Dieterich DT, Girard P, Bsharat M, *et al*. Telaprevir in combination with peginterferon alfa-2a/ribavirin in HCV/HIV co-infected patients: SVR24 final study results. 63rd Annual Meeting of the American Association for the Study of Liver Diseases (AASLD 2012). 63rd Annual Meeting of the American Association for the Study of Liver Disease, November 9–13 2012, Boston, USA. Abstract 54.
5. Sulkowski M, Pol S, Cooper C, Fainboim H, Slim J, Rivero A, Thompson S, *et al*. Boceprevir + pegylated interferon + ribavirin for the treatment of HCV/HIV co-infected patients: end-of-treatment (week 48) interim results. In: Program and abstracts of the 19th Conference on Retroviruses and Opportunistic Infections, March 3–6 2012, Seattle, USA. Abstract 47.
6. Moreno A, Bárcena R, Quereda C, Pérez-Ellías MJ, García-Hoz, Casado JL, Rodríguez-Casado MA, *et al*. Does HIV-coinfection reduce initial safety or efficacy of boceprevir (BOC) or telaprevir (TPV)-based triple therapy in cirrhotic patients in the “real-life”? 63rd Annual Meeting of the American Association for the Study of Liver Disease, November 9–13 2012, Boston, US. Abstract 1789.
7. Berg T, Buggisch P, Huettpie D, Mauss S, Wedemeyer H, Decker-Burgard S. Telaprevir-based triple-therapy in patients with chronic hepatitis C in Germany: a 12-week interim analysis of real-life data. *J Int AIDS Soc* 2012; **15**:18424.
8. Pineda JA, Caruz A, Rivero R, Neukam K, Salas I, Camacho A, Palomares JC, *et al*. Prediction of response to pegylated interferon plus ribavirin by IL28B gene variation in patients coinfected with HIV and hepatitis C virus. *Clin Infect Dis* 2010; **51**:788–795.
9. Rallón N, Naggie S, Benito JM, Medrano J, Restrepo C, Goldstein D, Shianna KV, *et al*. Association of a single nucleotide polymorphism near the interleukin-28B gene with response to hepatitis C therapy in HIV/hepatitis C virus co-infected patients. *AIDS* 2010; **24**:F23–29.
10. Bronowicki JP, Hézode C, Bengtsson L, Pol S, Bourlière M, Serfaty L, De Ledinghen V, *et al*. 100% SVR in IL28B SNP rs12979860 C/C patients treated with 12 weeks of telaprevir, peginterferon and ribavirin in the PROVE-2 trial. 47th Annual Meeting of the European Association for the Study of the Liver. April 18–22 2012, Barcelona, Spain. Abstract 1094.
11. Jacobson IM, Catlett I, Marcellin P, Bzowej NH, Muir AJ, Adda N, Bengtsson L, *et al*. Telaprevir substantially improved SVR rates across all IL28B genotypes in the ADVANCE trial. *J Hepatol* 2011; **54**:1369.
12. Poordad F, Bronowicki JP, Gordon SC, Zeuzem S, Jacobson IM, Sulkowski MS, Poyndar T, *et al*. Factors that predict response of patients with hepatitis C virus infection to boceprevir. *Gastroenterology* 2012; **143**:608–618.

13. Pineda JA, Caruz A, Di Lello FA, Camacho A, Mesa P, Neukam K, Rivero-Juárez A, et al. **Low-density lipoprotein receptor genotyping enhances the predictive value of IL28B genotype for the response to pegylated interferon plus ribavirin in HIV/hepatitis C virus-coinfected patients with genotype 1 or 4.** *AIDS* 2011; **25**:1415–1420.
14. Neukam K, Almeida C, Caruz A, Rivero-Juárez A, Rallón N, Di Lello FA, Herrero R, et al. **A model to predict the response to therapy against hepatitis C virus (HCV) including low-density lipoprotein receptor genotype in HIV/HCV-coinfected patients.** *J Antimicrob Chemother* 2012; epub ahead of print. doi:10.1093/jac/dks488.
15. Beinhardt S, Aberle JH, Strasser M, Dulic-Lakovic E, Maieron A, Kreil A, Rutter K, et al. **Serum level of IP-10 increases predictive value of IL28B polymorphisms for spontaneous clearance of acute HCV infection.** *Gastroenterology* 2012; **142**:78–85.
16. Hayes CN, Kobayashi M, Akuta N, Suzuki F, Kumada H, Abe H, Miki D, et al. **HCV substitutions and IL28B polymorphisms on outcome of peg-interferon plus ribavirin combination therapy.** *Gut* 2011; **60**:261–267.
17. Mosbruger TL, Duggal P, Goedert JJ, Kirk GD, Hoos WK, Tobler LH, et al. **Large-scale candidate gene analysis of spontaneous clearance of hepatitis C virus.** *J Infect Dis* 2012; **201**:1371–1380.
18. Agundez JA, García-Martín E, Devesa MJ, Carballo M, Martínez C, Lee-Brunner A, et al. **Polymorphism of the TLR4 gene reduces the risk of hepatitis C virus-induced hepatocellular carcinoma.** *Oncology* 2012; **82**:35–40.
19. Danilovic DL, Mendes-Correa MC, Lima EU, Zambrini H, Barros K, Marui S, et al. **Correlations of CTLA-4 gene polymorphisms and hepatitis C chronic infection.** *Liver Int* 2012; **32**:803–808.
20. Brenner DA. **Molecular pathogenesis of liver fibrosis.** *Trans Am Clin Climatol Assoc* 2009; **120**:361–368.
21. Gewaltig J, Mangasser-Stephan K, Gartung C, Biesterfeld S, Gressner AM. **Association of polymorphisms of the transforming growth factor-beta1 gene with the rate of progression of HCV-induced liver fibrosis.** *Clin Chim Acta* 2002; **316**:83–94.
22. Lee S, Varano J, Flexman JP, Cheng W, Watson MW, Rossi E, Adams LA, et al. **Decreased IP-10 and elevated TGF-beta1 levels are associated with viral clearance following therapy in patients with hepatitis C virus.** *Dis Markers* 2010; **28**:273–280.
23. Nattermann J, Vogel M, Nischalke HD, Danta M, Ahlenstiel G, Michalk M, Sauerbruch T, et al. **The transforming growth factor-beta high-producer genotype is associated with response to hepatitis C virus-specific therapy in HIV-positive patients with acute hepatitis C.** *AIDS* 2008; **22**:1287–1292.
24. Abbas Z, Moatier T, Hussaini A, Jafri W. **Effect of cytokine gene polymorphism on histological activity index, viral load and response to treatment in patients with chronic hepatitis C genotype 3.** *World J Gastroenterol* 2005; **11**:6656–6661.
25. Marek B, Kajdaniuk D, Mazurek U, Janczewska-Kazek E, Kos-Kudla B, Strzalka B, Fila A, et al. **TGF-beta1 mRNA expression in liver biopsy specimens and TGF-beta1 serum levels in patients with chronic hepatitis C before and after antiviral therapy.** *J Clin Pharm Ther* 2005; **30**:271–277.
26. Barrett S, Collins M, Kenny C, Ryan E, Keane CO, Crowe J. **Polymorphisms in tumour necrosis factor-alpha, transforming growth factor-beta, interleukin-10, interleukin-6, interferon-gamma, and outcome of hepatitis C virus infection.** *J Med Virol* 2003; **71**:212–218.
27. Harfouch S, Guiguet M, Valantin MA, Samri A, Ouazene Z, Slama L, Dominguez S, et al. **Lack of TGF-beta production by hepatitis C virus-specific T cells during HCV acute phase is associated with HCV clearance in HIV coinfection.** *J Hepatol* 2012; **56**:1259–1268.
28. Huang H, Shiffman ML, Friedman S, Venkatesh R, Bzowej N, Abar OT, Rowland CM, et al. **A 7 gene signature identifies the risk of developing cirrhosis in patients with chronic hepatitis C.** *Hepatology* 2007; **46**:297–306.
29. Deen PM, Verdijk MA, Knoers NV, Wieringa B, Monnens LA, van Os CH, van Oost BA. **Requirement of human renal water channel aquaporin-2 for vasopressin-dependent concentration of urine.** *Science* 1994; **264**:92–95.
30. Li W, Zhang Y, Bouley R, Chen Y, Matsuzaki T, Nunes P, Hasler U, et al. **Simvastatin enhances aquaporin-2 surface expression and urinary concentration in vasopressin-deficient Brattleboro rats through modulation of Rho GTPase.** *Am J Physiol Renal Physiol* 2011; **301**:F309–318.
31. Maillard P, Walic M, Meuleman P, Roohvand F, Huby T, Le Goff W, Leroux-Roels G, et al. **Lipoprotein lipase inhibits hepatitis C virus (HCV) infection by blocking virus cell entry.** *PLoSOne* 2011; **6**:e26637.
32. Sheridan DA, Price DA, Schmid ML, Toms GL, Donaldson P, Neely D, Bassendine MF. **Apolipoprotein B-associated cholesterol is a determinant of treatment outcome in patients with chronic hepatitis C virus infection receiving anti-viral agents interferon-alpha and ribavirin.** *Aliment Pharmacol Ther* 2009; **29**:1282–1290.
33. Zeuzem S, Buti M, Ferenci P, Sperl J, Horsmans Y, Cianciara J, Ibranyi E, et al. **Efficacy of 24 weeks treatment with peginterferon alfa-2b plus ribavirin in patients with chronic hepatitis C infected with genotype 1 and low pretreatment viremia.** *J Hepatol* 2006; **44**:97–103.
34. Kamal SM, El Kamary SS, Shaddell MD, Hashem M, Ahmed IN, Muhammad M, Sayed K, et al. **Pegylated interferon alpha-2b plus ribavirin in patients with genotype 4 chronic hepatitis C: The role of rapid and early virologic response.** *Hepatology* 2007; **46**:1732–1740.
35. Poordad F, McCone J, Bacon BR, Bruno S, Manns MP, Sulkowski MS, Jacobson IM, et al. **Bocoprevir for untreated chronic HCV genotype 1 infection.** *N Engl J Med* 2011; **364**:1195–1206.
36. Sherman KE, Flamm SL, Afdahl NH, Nelson DR, Sulkowski MS, Everson GT, Fried MW, et al. **Response-guided combination treatment for hepatitis C virus infection.** *N Engl J Med* 2011; **365**:1014–1024.
37. Neukam K, Barreiro P, Rivero-Juárez A, Caruz A, Mira JA, Cifuente C, et al. **Lack of rapid virological response in patients with favorable IL28B genotype is associated with suboptimal sustained response rates to treatment with pegylated interferon plus ribavirin.** 63rd Annual Meeting of the American Association for the Study of the Liver. November 9–13 2012, Boston, USA. Abstract 1828.
38. Rivero-Juarez A, Camacho A, Caruz A, Neukam K, Gonzalez R, Di Lello FA, Pérez-Camacho I, et al. **LDLR genotype modifies the impact of IL28B on HCV viral kinetics after the first weeks of treatment with PEG-IFN/RBV in HIV/HCV patients.** *AIDS* 2012; **26**:1009–1015.
39. Neukam K, Rivero-Juárez A, Caruz A, Labarga P, Márquez M, Real LM, Camacho A, et al. **Variations in Proprotein Convertase Subtilisin/Kexin Type 9 (PCSK9) Gene Are Associated with Response to Therapy Against Hepatitis C Virus Genotype 3 Infection.** 20th Conference on Retroviruses and Opportunistic Infections. March 3–6 2013, Atlanta, USA. Abstract 686.
40. Clausen LN, Astvad K, Ladelund S, Larsen MV, Schønning K, Benfield T. **Hepatitis C viral load, genotype 3 and interleukin-28B CC genotype predict mortality in HIV and hepatitis C-coinfected individuals.** *AIDS* 2012; **26**:1509–1516.
41. Thomas DL, Thio CL, Martin MP, Qi Y, Ge D, O'Huigin C, et al. **Genetic variation in IL28B and spontaneous clearance of hepatitis C virus.** *Nature* 2009; **461**:798–801.
42. Rauch A, Kutalik Z, Descombes P, Cai T, Di Iulio J, Mueller T, et al. **Genetic variation in IL28B is associated with chronic hepatitis C and treatment failure: a genome-wide association study.** *Gastroenterology* 2011; **138**:1338–1345, 1345.e1–7.
43. Chu TW, Kulkarni R, Gane EJ, Roberts SK, Stedman C, Angus PW, Ritchie B, et al. **Effect of IL28B genotype on early viral kinetics during interferon-free treatment of patients with chronic hepatitis C.** *Gastroenterology* 2012; **142**:790–795.
44. Zeuzem S, Soriano V, Asselah T, Bronowicki JP, Lohse AW, Muhlaupt B, Schuchmann M, et al. **SVR4 and SVR12 with an interferon-free regimen of BI 201335 AND BI 207127, +/- ribavirin, in treatment-naïve patients with chronic genotype-1 HCV infection: interim results of SOUND-C2.** 47th Annual Meeting of the European Association for the Study of the Liver. April 18–22 2012, Barcelona, Spain. Abstract 101.

A model to predict the response to therapy against hepatitis C virus (HCV) including low-density lipoprotein receptor genotype in HIV/HCV-coinfected patients

Karin Neukam¹, Carmen Almeida², Antonio Caruz³, Antonio Rivero-Juárez⁴, Norma I. Rallón⁵, Federico A. Di Lello¹, Rocío Herrero³, Angela Camacho⁴, José M. Benito⁵, Juan Macías¹, Antonio Rivero⁴, Vicente Soriano⁵ and Juan A. Pineda^{1*}

¹Unit of Infectious Diseases and Microbiology, Hospital Universitario de Valme, Avenida de Bellavista S/N, 41014 Seville, Spain; ²Unit of Investigation, Hospital Universitario de Valme, Avenida de Bellavista S/N, 41014 Seville, Spain; ³Immunogenetics Unit, Faculty of Sciences, Universidad de Jaén, Campus Las Lagunillas S/N, 23071 Jaén, Spain; ⁴Unit of Infectious Diseases, Maimónides Institute for Biomedical Research (IMIBIC), Hospital Universitario Reina Sofía, Avd. Menéndez Pidal S/N, 14004 Córdoba, Spain; ⁵Department of Infectious Diseases, Hospital Carlos III, Calle Sinesio Delgado 10, 28029 Madrid, Spain

*Corresponding author. Tel: +34955015684; Fax: +34955015795; E-mail: japineda@telefonica.net

Received 26 July 2012; returned 8 September 2012; revised 16 October 2012; accepted 11 November 2012

Objectives: Accurate prediction of sustained virological response (SVR) to pegylated interferon- α (Peg-IFN) plus ribavirin in HIV/hepatitis C virus (HCV)-coinfected patients could improve the management of these patients. We aimed to develop a model to predict SVR to Peg-IFN/ribavirin in HIV/HCV-coinfected individuals combining HCV genotype and baseline HCV RNA load with interleukin 28B and low-density lipoprotein receptor genetic variations.

Methods: Three hundred and twelve treatment-naïve HIV/HCV-coinfected patients receiving Peg-IFN/ribavirin were analysed in an on-treatment approach. One hundred and eighty-one of them were included in the development group and 131 in the validation population. The predictive model was obtained from a logistic regression equation including the above-mentioned variables. The areas under the receiver operating characteristic (AUROC) curves (95% CI), sensitivity and specificity, as well as negative and positive predictive values, were calculated.

Results: SVR was achieved by 88 (48.6%) patients from the development group and 68 (51.9%) individuals from the validation group. The AUROC curve values (95% asymptotic CI) were 0.83 (0.77–0.89) for the development group and 0.84 (0.77–0.91) for the validation group. Using two cut-off values, maximum specificity and sensitivity were 89.7% and 96.6%, respectively, with a negative predictive value for SVR of 88.9% and a positive predictive value of 83.6%. Thirteen (7.2%) individuals were misclassified using these cut-off values.

Conclusions: This model represents a reliable and easily applicable tool to individually evaluate the probability of achieving an SVR to Peg-IFN/ribavirin among HIV/HCV-coinfected patients.

Keywords: interferon- α , ribavirin, interleukin 28B, viruses, HCV genotype, HCV viral load

Introduction

The estimation of the probability of response to treatment against hepatitis C virus (HCV) with pegylated interferon- α (Peg-IFN) plus ribavirin can have a high clinical impact. In particular, special populations with lower response rates could benefit from better selection of candidates for HCV therapy. HIV/HCV-coinfected patients achieve sustained virological response (SVR) rates of <50%.^{1–3} Precise identification of

individuals with a very low likelihood of achieving SVR could help us to more properly manage a treatment with frequent and sometimes severe side effects. Thus, in patients without advanced fibrosis and classified as very unlikely responders, treatment against HCV could be deferred until more effective combinations, including new direct-acting antivirals (DAAs), become approved in HIV/HCV coinfection. Conversely, patients with a high probability of SVR with Peg-IFN/ribavirin should be treated with this drug regimen, given that SVR is associated

with decreases in the incidence of hepatic decompensations and in liver-related mortality in HIV/HCV-coinfected individuals.⁴ Thus, a reliable tool to easily predict the probability of SVR to Peg-IFN/ribavirin could be very useful in current clinical practice.

Among the pre-treatment predictors of SVR that have been identified, HCV genotype, baseline plasma HCV RNA load and host genetic variations are highly predictive of response.⁵⁻⁷ In this context, the single-nucleotide polymorphism (SNP) rs12979860 near the interleukin 28B (*IL28B*) gene^{5,6} and the SNP rs14158 at the low-density lipoprotein receptor (*LDLR*) gene⁷ have recently been independently associated with SVR in HIV/HCV-coinfected patients. In both SNPs, the C allele exerts a beneficial effect and lower response rates are observed among carriers of the homozygous TT or the heterozygous CT genotype compared with CC genotype carriers. Interestingly, a synergistic effect between these SNPs has been observed in HCV genotype 1/4 carriers.⁷ Thus, HIV/HCV-coinfected individuals with both favourable *IL28B* and *LDLR* genotypes show a response rate of ~70%, while only 14% of those who present a combination of both genotypes other than CC achieve SVR.⁷ One approach to enhancing the predictive capacity of isolated factors is the combination of several predictors.^{8,9} However, the use of the predictive tools currently available is limited due to incomplete accuracy or because they use parameters not accessible everywhere.^{8,9} It is therefore important to develop a predictive model that allows reliable determination of the probability of response to Peg-IFN/ribavirin in HIV/HCV-coinfected patients.

To overcome the drawbacks of the currently available predictive tools to forecast SVR to Peg-IFN/ribavirin in HIV/HCV-coinfected patients, we aimed to develop a new model using novel predictors of SVR, such as the genotype of *LDLR*, the determination of which is both simple and inexpensive, in order to facilitate HCV therapy decisions in such a population.

Patients and methods

Study patients

HIV/HCV-coinfected patients who started treatment between May 2000 and May 2010 were included in this prospective study. Data to develop the model were collected from a cohort followed in two university hospitals in southern Spain.⁷ To validate the model, data derived from patients who were seen at a hospital in Madrid were acquired.⁵ The inclusion criteria of the cohorts were: (i) older than 18 years; (ii) written informed consent given; (iii) coinfection with HIV; (iv) previously naive for therapy against chronic hepatitis C; (v) initiation of therapy with Peg-IFN/ribavirin; and (vi) collection and cryopreservation at -70°C of a whole blood sample for genetic determinations. Those who voluntarily dropped out or discontinued therapy due to adverse events were excluded from the study population in order to analyse data using an on-treatment approach. Likewise, patients in whom HCV, *IL28B* or *LDLR* genotype could not be determined were excluded from the analysis. Clinical visits were scheduled every 4 weeks during the first 24 weeks of treatment and every 8–12 weeks afterwards, according to a pre-defined protocol that included a visit 24 weeks after completing therapy in order to evaluate SVR. All patients were prospectively followed and data were collected in real time.

Drug therapy

The daily doses of Peg-IFN 2a and Peg-IFN 2b were 180 µg once per week and 1.5 µg/kg once per week, respectively. Peg-IFN was administered in combination with 800–1200 mg of ribavirin per day. Those patients

bearing HCV genotype 2 or 3 received HCV therapy for 24 weeks if they showed undetectable plasma HCV RNA load at week 4, and 48 weeks otherwise. Carriers of HCV genotype 1 or 4 were treated for 48 weeks. Treatment duration and stopping rules were applied according to international guidelines in force at the moment of treatment.^{10,11} SVR was defined as undetectable plasma HCV RNA 24 weeks after discontinuation of scheduled HCV therapy.

Plasma HCV RNA determination

The plasma HCV RNA was determined by a quantitative PCR assay according to the technique available at the time when the patient was treated [Cobas Amplicor HCV Monitor (Roche Diagnostic Systems Inc., Branchburg, NJ, USA), detection limit of 600 IU/mL; Cobas AmpliPrep-Cobas TaqMan (Roche Diagnostic Systems Inc., Meylan, France), detection limit of 50 IU/mL; Cobas TaqMan (Roche Diagnostic Systems Inc., Pleasanton, CA, USA), detection limit of 10 IU/mL].

rs12979860 and rs14158 SNP genotyping

DNA was extracted from cryopreserved whole blood using the automated MagNA Pure DNA extraction method (Roche Diagnostics Corporation, Indianapolis, IN, USA). The SNP rs14158 in the 3' untranslated region of the *LDLR* gene was genotyped. Likewise, variations in rs12979860, an SNP located 3 kb upstream of the *IL28B* gene, were analysed. Genotyping was carried out using a custom TaqMan assay (Applied Biosystems, Foster City, CA, USA), following the manufacturer's instructions, on a Stratagene MX3005 thermocycler using MXpro software (Stratagene, La Jolla, CA, USA) as described previously.⁷ The researchers responsible for genotyping were blinded to the treatment outcome of the patients.

Statistical analysis

The study population parameters were characterized in a descriptive analysis. Baseline characteristics of the groups for development and validation of the model were compared using the χ^2 test or Fisher's test for categorical variables and Student's *t*-test or the Mann-Whitney *U*-test for continuous variables. The Hardy-Weinberg equilibrium was analysed for both rs12979860 and rs14158 using Haploview software v.4.2.¹² For development of the predictive model, the host genetic profiles were classified into four categories according to the risk alleles (TT=TC<CC): *IL28B* CC/*LDLR* CC, *IL28B* non-CC/*LDLR* CC, *IL28B* CC/*LDLR* non-CC and *IL28B* non-CC/*LDLR* non-CC. Likewise, patients were categorized by HCV genotype as 2/3 or 1/4 carriers. The outcome variable was SVR. The diagnostic performance of the isolated predictors was analysed by binary logistic regression analysis. Multivariate logistic regression analysis adjusted for age and sex was performed in order to confirm that the host genetic profile, the HCV genotype and the baseline HCV RNA load were independently associated with SVR in the study population. Clinically relevant variables for SVR were entered into the model in order to optimize its diagnostic performance. The formula resulting from the logistic regression equation represented the predictive model. The 95% CIs of the SVR rates were calculated. The predictive value of the logistic regression model was assessed via receiver operating characteristic curves, where 1.0 indicates perfect discrimination and 0.5 indicates random prediction. Because receiver operating characteristic curves are only able to incorporate binary outcomes, two cut-off values were selected, a high one and a low one, derived from the formula that maximized the negative predictive value (NPV) and positive predictive value (PPV), as well as sensitivity and specificity in predicting SVR versus non-SVR. The CIs for the predictive values were calculated and diagnostic accuracy was determined on the basis of these values. Statistical analysis was carried out using the SPSS statistical software package release 19.0 (IBM Corporation, Somers, NY, USA) and STATA 9.0 (StataCorp LP, College Station, TX, USA) and Fistera.com (Elsevier 2012; http://www.fistera.com/mbe/investigacion_diagnosticas/pruebas_diagnosticas.asp).

Ethical aspects

The study was designed and performed according to the Helsinki declaration and was approved by the Ethics Committees of the participating hospitals.

Results**Main characteristics of the study population**

Three hundred and twelve patients were included in this study, 181 of them constituting the development group and 131 the validation group. Thirty-six (9.9%) and 17 (4.7%) patients had been excluded due to voluntary drop-out and discontinuation caused by adverse events. In the overall population, 131 (42.0%) of the patients bore *IL28B* genotype CC, 140 (44.9%) genotype CT and 41 (13.1%) genotype TT. The distribution of *LDLR* genotypes was: 178 (57.1%) CC, 120 (38.5%) CT and 14 (4.5%) TT. Both SNPs were in Hardy-Weinberg equilibrium ($P=0.709$ for rs12979860 and $P=0.266$ for rs14158). The main baseline characteristics of the two study groups are presented in Table 1.

Response to HCV therapy

SVR was achieved by 88 (48.6%; 95% CI 41.1%–56.1%) patients in the model development group and by 68 (51.9%; 95% CI 43%–60.7%) individuals in the validation group. In the development

group, 49 (65.3%) of those patients with *IL28B* genotype CC versus 39 (36.8%) patients with *IL28B* genotype CT or TT ($P=2.8 \times 10^{-4}$) achieved SVR. Likewise, 60 (54.5%) individuals with *LDLR* genotype CC versus 28 (39.4%) with genotype CT or TT attained SVR ($P=4.7 \times 10^{-2}$). The numbers of patients who achieved SVR according to HCV genotype in the development group were 29 (30.5%) for genotype 1, 49 (81.7%) for genotype 2/3 and 10 (38.5%) for genotype 4 ($P=3.2 \times 10^{-9}$). In the development group, patients who achieved SVR showed a median (IQR) HCV RNA load at baseline of 5.8 (5.2–6.5) $\log_{10}$ IU/mL compared with 6.2 (5.7–6.7) $\log_{10}$ IU/mL for those who did not reach SVR ($P=5 \times 10^{-3}$). The univariate and multivariate associations of the variables entered into the model are presented in Table 2.

Model development

On the basis of the logistic regression equation, the following formula to determine the individual probability of response to treatment against HCV with Peg-IFN/ribavirin was elaborated:

$$\text{Probability}_{\text{SVR}} = \frac{1}{(1 + e^{-z})}$$

$$z = 5.227 - 2.233 \times \text{GT} - 0.443 \times \text{VL} - 1.327 \times \text{A} - 0.803 \times \text{B} - 2.02 \times \text{C}$$

where GT is HCV genotype (1 or 4 = 1 and 2 or 3 = 0), VL is baseline plasma HCV RNA load ($\log_{10}$ IU/mL), A is *IL28B*/*LDLR* genetic

Table 1. Baseline characteristics of the overall study population and the subgroups

Characteristic	Development group, n=181	Validation group, n=131	P
Age (years) ^a	41.5 (38–45.4)	41.9 (38.9–44.7)	7.7×10^{-1}
Male gender, n (%)	152 (84.0)	93 (71.0)	6×10^{-3}
IDU ^b , n (%)	161 (89.0)	114 (89.8)	8.2×10^{-1}
<i>IL28B</i> / <i>LDLR</i> genotype, n (%)			3.9×10^{-1}
CC/CC	46 (25.4)	32 (24.4)	
non-CC/CC	64 (35.4)	36 (27.5)	
CC/non-CC	29 (16.0)	24 (18.3)	
non-CC/non-CC	42 (23.2)	39 (29.8)	
HCV genotype, n (%)			9.4×10^{-1}
1	95 (52.5)	73 (55.7)	
2	1 (0.6)	1 (0.8)	
3	59 (32.6)	40 (30.5)	
4	26 (14.4)	17 (13.0)	
HCV viral load ($\log_{10}$ IU/mL) ^a	6.06 (5.4–6.6)	6.1 (5.5–6.7)	4×10^{-1}
Undetectable HIV viral load, n (%) ^c	77 (82.8)	100 (76.3)	2.4×10^{-1}
CD4 cell count (cells/mL) ^a	505 (383–708)	496 (368–700)	7.7×10^{-1}
ALT (U/L) ^a	68 (47–100)	69 (48–107)	8.6×10^{-1}
Advanced liver fibrosis, n (%) ^d	73 (41.7)	26 (33.3)	2.1×10^{-1}

^aMedian (IQR).

^bIDU, injection drug user; available in 308 patients.

^cAvailable in 224 patients.

^dDetermined by liver biopsy ($F \geq 3$ according to the Scheuer index) or transient elastometry, using a cut-off value of 11 kPa, if biopsy was not available; available in 253 patients.

Table 2. Associations between SVR and host genetic profile, HCV genotype and baseline HCV RNA load in the univariate and multivariate analysis

Variable	Model development group, n=181			
	SVR, n (%)	P univariate	adjusted OR (95% CI)	P multivariate
<i>IL28B/LDLR</i> genetic profile		3×10^{-4}		2×10^{-3}
non-CC/non-CC	13 (31.0)		0.13 (0.05–0.39)	2.2×10^{-4}
non-CC/CC	26 (40.6)		0.27 (0.11–0.67)	5×10^{-3}
CC/non-CC	15 (51.7)		0.45 (0.45–1.37)	1.6×10^{-1}
CC/CC	34 (73.9)		1	
HCV genotype		1.1×10^{-8}		8.4×10^{-8}
1/4	39 (32.2)		0.11 (0.05–0.24)	
2/3	49 (81.7)		1	
Baseline HCV RNA load (IU/mL)		4×10^{-3}		5×10^{-20}
≥600 000	46 (40.4)		0.64 (0.41–1) ^a	
<600 000	42 (62.7)		1	

^aConducted with the baseline HCV RNA load expressed as a continuous variable ($\log_{10}$ IU/mL); the Nagelkerke r^2 value was 0.38.

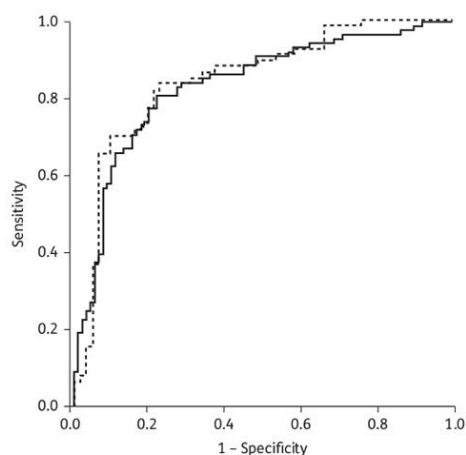


Figure 1. Diagnostic performance of the predictive model. AUROC curve values (95% asymptotic CI) were 0.83 (0.77–0.89) for the development group (continuous line, n=181) and 0.84 (0.77–0.91) for the validation group (broken line, n=131).

profile (*IL28B* non-CC/*LDLR* CC=1 and other=0), B is *IL28B/LDLR* genetic profile (*IL28B* CC/*LDLR* non-CC=1 and other=0) and C is *IL28B/LDLR* genetic profile (*IL28B* non-CC/*LDLR* non-CC=1 and other=0).

Model validation

The areas under the receiver operating characteristic (AUROC) curve values (95% asymptotic CI) were 0.83 (0.77–0.89) for

Table 3. Predictive values of the model within the two groups

	Development group (n=181)	Validation group (n=131)
Cut-off value = 18.8		
sensitivity, % (95% CI)	96.6 (89.7–99.1)	95.4 (86.1–98.9)
specificity, % (95% CI)	25.2 (17.5–36.1)	33.3 (22.3–46.4)
PPV, % (95% CI)	55.2 (47.0–63.1)	60.7 (50.8–69.9)
NPV, % (95% CI)	88.9 (69.7–97.1)	87.5 (66.5–96.7)
misclassified patients, n (%)	3 (1.7)	3 (2.3)
Cut-off value = 62.0		
sensitivity, % (95% CI)	58 (47.0–68.3)	62.5 (50.6–74.4)
specificity, % (95% CI)	89.7 (80.7–94.4)	92.1 (81.7–97.0)
PPV, % (95% CI)	83.6 (71.5–91.5)	89.6 (76.6–96.1)
NPV, % (95% CI)	69.2 (60.0–77.1)	69.9 (58.7–79.2)
misclassified patients, n (%)	10 (5.5)	5 (3.8)

the development group and 0.84 (0.77–0.91) for the validation group (Figure 1). Maximum predictive values were yielded by applying cut-off values of 18.8 and 62. The predictive values obtained are shown in Table 3.

Discussion

We report herein a useful and innovative model for the prediction of response to Peg-IFN/ribavirin in HIV/HCV-coinfected patients based on the combination of two well-known predictors of SVR—HCV genotype and baseline plasma HCV-RNA load—plus the genetic pattern defined by *IL28B* and *LDLR* genotype. Its application allows reliable determination of the probability of achieving SVR by combining host genetic and viral parameters.

Predicting success of treatment against HCV in the setting of HIV coinfection is of the highest relevance in current clinical practice. Although DAAs, such as the protease inhibitors (PIs) boceprevir and telaprevir, are currently available for HIV/HCV-coinfected individuals, their use in the coinfecting population has not been licensed yet and therapy with Peg-IFN/ribavirin still represents the standard of care (SOC) for this population. Unfortunately, the overall response rates that are achieved with SOC are low for these patients.¹⁻³ Treatment combinations with new PIs have yielded higher response rates compared with SOC in HCV-monoinfected patients.^{13,14} Likewise, preliminary results of clinical trials carried out with combinations including PIs in the HIV/HCV genotype 1-coinfecting population are promising.^{15,16} However, the 24 week preliminary results reported in clinical trials give reason to expect that the SVR rates will reach 70% at the most; hence, a considerable percentage of patients may not benefit from triple therapy.^{17,18} Furthermore, boceprevir and telaprevir will not be applicable to all coinfecting patients. Drug-drug interactions between boceprevir or telaprevir and antiretroviral therapy will limit the use of these PIs in HIV/HCV-coinfecting patients^{17,18} and there is a higher probability of side effects with triple therapy.^{15,16} Besides these limitations of the use of PIs that are specific for HIV/HCV coinfection, the first-generation PIs will be limited to patients infected by HCV genotype 1 and the treatment costs will increase considerably. This may result in lack of availability of DAAs in some economically depressed areas, where, as a consequence, bitherapy would remain the only treatment option. Because of these reasons, accurate identification of the likelihood of SVR to SOC among HIV/HCV-coinfecting patients could improve their management. Individuals with a very high probability of SVR to SOC could be treated without delay. In the case of HCV genotype 1-infected patients, this would also imply an important saving of costly PIs. On the other hand, treatment could be deferred in individuals with a very low probability of response to SOC until more effective regimens are available. Finally, the first regimens including a PI will be based on Peg-IFN/ribavirin and it is likely that the predictive value of predictors of SVR to standard therapy will be preserved at least partially.

A number of predictors of SVR have been identified so far. In fact, the determinations of baseline HCV viral load, HCV genotype and *IL28B* genotype are among the standard procedures recommended in recent updates of international guidelines in order to evaluate whether a patient is a candidate for treatment.^{19,20} Furthermore, there is growing evidence of the usefulness of *LDLR* genetic variants to predict SVR to SOC⁷ and it can be speculated that the determination of *LDLR* genotype, which is inexpensive and comparable to the cost of *IL28B* genotype determination, will be incorporated in clinical practice in the future. However, a single predictor usually provides less accurate information about the probability of response than more than one predictor considered together. In the model reported here, parameters that are mostly available in routine practice are combined in order to achieve maximal predictive performance. The model yielded an NPV of 89% and a PPV of 84%, respectively, with considerably high specificity and sensitivity. Importantly, the misclassification rates were low and only 5.5% were erroneously classified as likely to achieve SVR. In contrast, the use of the same parameters as in this study but without including the *LDLR* genotype leads to a considerably higher rate of

misclassification.⁹ Finally, AUROC curve values of both the elaboration and validation groups indicate that the diagnostic performance of the model was good and similar to that described in the Prometheus index, which showed an AUROC curve value (95% CI) of 0.85 (0.76–0.93) in the validation group.⁸

The model presented in this work is not the first approach to the prediction of the treatment response of HIV/HCV-coinfecting patients. For example, the Prometheus index allows the calculation of the probability of SVR to Peg-IFN/ribavirin in HIV/HCV-coinfecting patients as a function of HCV genotype (1/4 versus 2/3), *IL28B* genotype (CC or non-CC), baseline HCV RNA load and baseline liver stiffness as assessed by transient elastometry.⁸ However, this approach has the limitation that transient elastometry is not available as a standard procedure and many sites do not have this method at their disposal. In a more recently published model, patients were classified into anticipated responders and unlikely responders according to HCV genotype, baseline plasma HCV RNA concentration above or below 600000 IU/mL and *IL28B* genotype.⁹ Nevertheless, a considerably high percentage of patients remained unclassified when this algorithm was applied, specifically 42% of genotype 1 carriers and 38% of genotype 4 carriers.⁹ In contrast, the model reported here is applicable in all patients since it enables the calculation of a precise probability for each individual and thus represents a clear advantage over treatment algorithms. In this way, the contribution to individual treatment decisions using this application is higher.

This study has limitations. First, the determination of the *LDLR* genotype is not a standard procedure in clinical practice. However, the determination of *LDLR* SNPs may be incorporated in clinical practice without difficulties wherever a PCR procedure can be carried out. Second, only one SNP of the *LDLR* gene was incorporated in this model. Further investigation is required in order to evaluate whether there is an *LDLR* SNP associated with higher SVR rates or whether the combination of several SNPs in this region results in better diagnostic performance. Finally, the model was not validated in HCV-monoinfected patients. Critical differences in the characteristics of HIV/HCV-coinfecting patients may lead to a different response profile and the effectiveness of the model in a monoinfected population may not be satisfactory. Further studies to validate this model in HCV monoinfection are required.

In conclusion, this model allows reliable determination of the probability of SVR to Peg-IFN/ribavirin in HIV/HCV-coinfecting patients using simple laboratory determinations. Its application in clinical practice could aid treatment decisions and represents a further step towards individualization of therapy against HCV.

Acknowledgements

These data were presented at the Nineteenth Conference on Retroviruses and Opportunistic Infections, Seattle, WA, USA, 2012 (Abstract 764; Themed Discussion #16).

We would like to thank Eugenia Vispo and Pablo Barreiro for their support.

Funding

This work was supported in parts by the Red de Investigación en SIDA (ISCIII-RETIC RD06/006), the Fundación Progreso y Salud, Consejería de

Neukam et al.

Salud de la Junta de Andalucía (PI-0247-2010 and AI-001-2010 to A. R.), the Fondo de Investigaciones Sanitarias (PI10/01664) and the Fundación para la Investigación y la Prevención del Sida en España (121004/10) and the Instituto de Salud Carlos III (Programa-13SNS to J. A. P. and SCO/523/2008 to K. N.).

Transparency declarations

J. M. has been an investigator in clinical trials supported by Roche, Bristol-Myers Squibb and Abbott Pharmaceuticals. He has received lecture fees from Roche, Gilead, Boehringer Ingelheim and Bristol-Myers Squibb, and consulting fees from Boehringer Ingelheim, Bristol-Myers Squibb, Merck Sharp & Dohme and Schering-Plough. A. R. has received consulting fees from Bristol-Myers Squibb, Abbott, Gilead, Janssen-Cilag, Merck Sharp & Dohme and Boehringer Ingelheim, has received research support from Abbott, GlaxoSmithKline, Roche, Bristol-Myers Squibb, Abbott and Boehringer Ingelheim, and has received lecture fees from GlaxoSmithKline, Abbott, Bristol-Myers Squibb, Merck Sharp & Dohme, Janssen-Cilag, Gilead, Boehringer Ingelheim and Schering-Plough. J. A. P. reports having received consulting fees from GlaxoSmithKline, Bristol-Myers Squibb, Abbott Pharmaceuticals, Gilead, Merck Sharp & Dohme, Schering-Plough, Janssen-Cilag and Boehringer Ingelheim. He has received research support from GlaxoSmithKline, Roche, Bristol-Myers Squibb, Schering-Plough, Abbott Pharmaceuticals and Boehringer Ingelheim, and has received lecture fees from GlaxoSmithKline, Roche, Abbott Pharmaceuticals, Bristol-Myers Squibb, Gilead, Merck Sharp & Dohme, Janssen-Cilag, Boehringer Ingelheim and Schering-Plough. All other authors: none to declare.

Author contributions

K. N.: planning and conducting the study, collecting and interpreting data and drafting the manuscript.

C. A.: interpreting data.

A. Caruz: collecting and interpreting data.

A. R.-J.: collecting and interpreting data.

N. I. R.: collecting and interpreting data.

F. A. D. L.: collecting and interpreting data and drafting the manuscript.

R. H.: collecting and interpreting data.

A. Camacho: collecting and interpreting data.

J. M. B.: collecting and interpreting data.

J. M.: collecting and interpreting data and drafting the manuscript.

A. R.: planning and conducting the study, interpreting data and drafting the manuscript.

V. S.: planning and conducting the study, interpreting data and drafting the manuscript.

J. A. P.: planning and conducting the study, interpreting data and drafting the manuscript.

References

- 1 Carrat F, Bani-Sadr F, Pol S et al. Pegylated interferon alfa-2b vs standard interferon alfa-2b, plus ribavirin, for chronic hepatitis C in HIV-infected patients: a randomized controlled trial. *JAMA* 2004; **292**: 2839-48.
- 2 Torriani FJ, Rodriguez-Torres M, Rockstroh JK et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection in HIV-infected patients. *N Engl J Med* 2004; **351**: 438-50.
- 3 Pineda JA, Mira JA, de los Santos Gil I et al. Influence of concomitant antiretroviral therapy on the rate of sustained virological response to pegylated interferon plus ribavirin in hepatitis C virus/HIV-coinfected patients. *J Antimicrob Chemother* 2007; **60**: 1347-54.

- 4 Berenguer J, Alvarez-Pellicer J, Miralles-Martin P et al. Sustained virological response to interferon plus ribavirin reduces liver-related complications and mortality in patients coinfected with human immunodeficiency virus and hepatitis C virus. *Hepatology* 2009; **50**: 407-13.

- 5 Rallón N, Naggie S, Benito JM et al. Association of a single nucleotide polymorphism near the interleukin-28B gene with response to hepatitis C therapy in HIV/hepatitis C virus co-infected patients. *AIDS* 2010; **24**: F23-9.

- 6 Pineda JA, Caruz A, Rivero R et al. Prediction of response to pegylated interferon plus ribavirin by IL28B gene variation in patients coinfected with HIV and hepatitis C virus. *Clin Infect Dis* 2010; **51**: 788-95.

- 7 Pineda JA, Caruz A, Di Lello FA et al. Low-density lipoprotein receptor genotyping enhances the predictive value of IL28B genotype in HIV/hepatitis C virus-coinfected patients. *AIDS* 2011; **25**: 1415-20.

- 8 Medrano J, Neukam K, Rollón N et al. Modeling the probability of sustained virological response to therapy with pegylated interferon plus ribavirin in patients coinfected with hepatitis C virus and HIV. *Clin Infect Dis* 2010; **51**: 1209-16.

- 9 Neukam K, Camacho A, Caruz A et al. Prediction of response to pegylated interferon plus ribavirin in HIV/hepatitis C virus (HCV)-coinfected patients using HCV genotype, IL28B variations, and HCV-RNA load. *J Hepatol* 2012; **56**: 788-94.

- 10 European AIDS Clinical Society (EACS). *European AIDS Clinical Society (EACS) Guidelines for the Clinical Management and Treatment of Chronic Hepatitis B and C Coinfection in HIV-Infected Adults*. <http://www.europeanaidsclinicalociety.org/> (1 July 2012, date last accessed).

- 11 NIH. National Institutes of Health Consensus Development Conference Statement: Management of Hepatitis C: 2002 — June 10-12, 2002. *Hepatology* 2002; **36** Suppl 1: S3-20.

- 12 Barrett JC, Fry B, Maller J et al. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 2005; **21**: 263-5.

- 13 Jacobson IM, McHutchison JG, Dusheiko G et al. Telaprevir for previously untreated chronic hepatitis C virus infection. *N Engl J Med* 2011; **364**: 2405-16.

- 14 Poordad F, McCone J Jr, Bacon BR et al. Boceprevir for untreated chronic HCV genotype 1 infection. *N Engl J Med* 2011; **364**: 1195-206.

- 15 Dieterich DT, Soriano V, Sherman K et al. Telaprevir in combination with pegylated interferon- α -2a+ribavirin in HCV/HIV-co-infected patients: a 24-week treatment interim analysis. In: *Abstracts of the Nineteenth Conference on Retroviruses and Opportunistic Infections*, Seattle, WA, 2012. Abstract 46, p. 93. Foundation for Retrovirology and Human Health, Alexandria, VA, USA.

- 16 Sulkowski M, Pol S, Cooper C et al. Boceprevir+pegylated interferon+ribavirin for the treatment of HCV/HIV co-infected patients: end-of-treatment (week 48) interim results. In: *Abstracts of the Nineteenth Conference on Retroviruses and Opportunistic Infections*, Seattle, WA, 2012. Abstract 47, p. 94. Foundation for Retrovirology and Human Health, Alexandria, VA, USA.

- 17 van Heeswijk R, Vandevoorde A, Boogaerts G et al. Pharmacokinetic interactions between antiretroviral agents and the investigational HCV protease inhibitor telaprevir in healthy volunteers. In: *Abstracts of the Eighteenth Conference on Retroviruses and Opportunistic Infections*, Boston, MA, 2011. Abstract 119, p. 115. Foundation for Retrovirology and Human Health, Alexandria, VA, USA.

- 18 Hulskotte E, Feng HP, Xuan F et al. Pharmacokinetic interactions between the HCV protease inhibitor boceprevir and ritonavir-boosted HIV-1 protease inhibitors atazanavir, lopinavir and darunavir. In: *Abstracts of the Nineteenth Conference on Retroviruses and Opportunistic Infections*, Seattle, WA, 2012. Abstract 771LB, p. 370. Foundation for Retrovirology and Human Health, Alexandria, VA, USA.

Prediction of HCV therapy outcome

JAC

19 Ghany MG, Nelson DR, Strader DB et al. An update on treatment of genotype 1 chronic hepatitis C virus infection: 2011 practice guideline by the American Association for the Study of Liver Diseases. *Hepatology* 2011; **54**: 1433–4.

20 European AIDS Clinical Society (EACS). *Guidelines Version 6.0 – October 2011*. http://www.europeanaidsclicalsociety.org/index.php?option=com_content&view=article&id=59&Itemid=41 (24 November 2012, date last accessed).

13. *Curriculum vitae*

Ma DEL ROCÍO HERRERO DEL RIO

Datos de contacto:

✉ rocio.herrero.delrio@gmail.com

🌐 <https://es.linkedin.com/in/rocío-herrero-3037644b>

ORCID <http://orcid.org/0000-0002-9678-0309>

Capacidades, competencias técnicas y profesionales:

Las capacidades personales que mejor me definen son: trabajo en equipo, responsabilidad, polivalencia y adaptabilidad.

Debido a mi formación multidisciplinar, conozco y realizo correctamente las técnicas más usuales del ámbito de la enfermería (extracción de muestras, curas, administración de tratamientos, sondajes, planes de cuidados, etc.) así como las técnicas más utilizadas en un laboratorio de biología molecular (extracción de ADN y ARN, PCR cuantitativa, RT-PCR, ingeniería genética, manejo de cultivos celulares, etc.). Además, tengo experiencia en la producción y manipulación de virus y he trabajado en el laboratorio de alta seguridad nivel P3 de la Universidad de Jaén y del Instituto Pasteur de París.

Formación académica:

Noviembre 2010 – Máster en Biomedicina y Nivel
Noviembre 2011 Biotecnología. MECU: 3

Universidad de Jaén, Jaén, España.

Septiembre 2007 – Diplomatura en Enfermería. Nivel
Septiembre 2010 MECU: 2

Universidad de Cádiz, Cádiz, España.

Experiencia laboral:

Mayo 2015 – Julio 2015 Estancia predoctoral de investigación.

Unidad de Patogénesis Viral, Instituto Pasteur de París, París, Francia

Curriculum Vitae

(Financiada por: EMBO Short-Term Fellowship Programme).

Creación y manipulación de virus, infecciones virales en cultivos celulares.

Enero 2015 – Abril 2015 Estancia predoctoral de investigación.

Unidad de Virología Molecular y Vacunas, Instituto Pasteur de París, París, Francia (Financiada por: Programa de Actividades y Ayudas al Doctorado EDUJA 2014).

Desarrollo de vectores lentivirales, creación de líneas celulares estables.

Mayo 2014 – Mayo 2015 Técnico de Laboratorio.

Unidad de Inmunogenética, Universidad de Jaén, Jaén, España.

Responsable de la gestión de stocks y materiales, mantenimiento de líneas estables.

Noviembre 2011 – Profesional autónomo en investigación. Noviembre 2013

Universidad de Jaén, Jaén, España.

Ingeniería genética, cultivos celulares, genotipación.

Producción científica:

Publicaciones científicas:

Título: Common haplotypes in CD209 promoter and susceptibility to HIV-1 infection intravenous drug users.

Autores: **Herrero R**, Pineda JA, Rivero-Juárez A, Echbarthi M, Real LM, Camacho A, Macías J, Fibla J, Rivero A, Caruz A.

Fecha de publicación: Submitted.

Título: IFNL4 rs368234815 polymorphism is associated with innate resistance to HIV-1 infection.

Autores: Real LM, **Herrero R**, Rivero-Juárez A, Camacho Á, Macías J, Vic S, Soriano V, Viedma S, Guardiola JM, Fibla J, Rivero A, Pineda JA, Caruz A.

Revista: AIDS Fecha de publicación: Septiembre 2015

Volumen: 29 Issue: 14 Páginas: 1895-7

doi: 10.1097/QAD.0000000000000773

Título: Association of complement receptor 2 polymorphisms with innate resistance to HIV-1 infection.

Autores: **Herrero R**, Real LM, Rivero-Juárez A, Pineda JA, Camacho Á, Macías J, Laplana M, Konieczny P, Márquez FJ, Souto JC, Soria JM, Saulle I, Lo Caputo S, Biasin M, Rivero A, Fibla J, Caruz A.

Revista: Genes Immun. Fecha de publicación: Marzo 2015

Volumen: 16 Issue: 2 Páginas: 134-41

doi: 10.1038/gene.2014.71

Título: IFNL4 ss469415590 variant shows similar performance to rs12979860 as predictor of response to treatment against Hepatitis C Virus genotype 1 or 4 in Caucasians.

Autores: Real LM, Neukam K, **Herrero R**, Guardiola JM, Reiberger T, Rivero-Juarez A, Salazar J, Mandorfer M, Merino D, Soriano V, Rivero A, Macías J, Pineda JA, Caruz A.

Revista: PLoS One. Fecha de publicación: Abril 2014

Volumen: 9 Issue: 4 Páginas: -

doi: 10.1371/journal.pone.0095515

Título: Association of low-density lipoprotein receptor genotypes with hepatitis C viral load.

Autores: Caruz A, Neukam K, Rivero-Juárez A, **Herrero R**, Real LM, Camacho A, Barreiro P, Labarga P, Rivero A, Pineda JA.

Revista: Genes Immun. Fecha de publicación: Enero 2014

Volumen: 15 Issue: 1 Páginas: 16-24

doi: 10.1038/gene.2013.56

Curriculum Vitae

Título: Variations at multiple genes improve interleukin 28B genotype predictive capacity for response to therapy against hepatitis C infection.

Autores: Neukam K, Caruz A, Rivero-Juárez A, Barreiro P, Merino D, Real LM, **Herrero R**, Camacho A, Soriano V, Di Lello FA, Macías J, Rivero A, Pineda JA.

Revista: AIDS.

Fecha de publicación: Noviembre 2013

Volumen: 27

Issue: 17

Páginas: 2715-24

doi: 10.1097/01.aids.0000432459.36970.a9

Título: A model to predict the response to therapy against hepatitis C virus (HCV) including low-density lipoprotein receptor genotype in HIV/HCV-coinfected patients.

Autores: Neukam K, Almeida C, Caruz A, Rivero-Juárez A, Rallón NI, Di Lello FA, **Herrero R**, Camacho A, Benito JM, Macías J, Rivero A, Soriano V, Pineda JA.

Revista: J Antimicrob Chemother.

Fecha de publicación: Abril 2013

Volumen: 68

Issue: 4

Páginas: 915-21

doi: 10.1093/jac/dks488

Aportaciones a congresos:

Título: Genetic variations in proprotein convertase subtilisin/kexin type 9 (PCSK9) gene are associated with response to pegylated interferon/ribavirin in hepatitis C virus genotype-3 infected patients.

Autores: Neukam K, Caruz A, Rivero-Juárez A, Labarga P, Márquez M, Real LM, **Herrero R**, Rivero A, Soriano V, Pineda JA.

Comunicación a congreso internacional (póster): 48th Annual Meeting of the European Association for the Study of the Liver (EASL).

Entidad organizadora: European Association for the Study of the Liver (EASL)

Fecha de celebración: 24/04/2013-28/04/2013. Lugar: Ámsterdam (Bélgica)

Título: IL28B and the LDLr have a synergistic Effect on HCV early viral kinetics during the first weeks of treatment with pegylated interferon + ribavirin in HIV/HCV-co-infected patients.

Autores: Rivero-Juárez A, Di-Lello F, Caruz A, Camacho A, Neukam K, **Herrero R**, Perez-Camacho I, Torre-Cisneros J, Pineda JA, Rivero A.

Comunicacion a congreso internacional (póster): 19th Conference on Retroviruses and Opportunistic Infections.

Entidad organizadora: CROI Foundation

Fecha de celebración: 05/03/2012-08/03/2012. Lugar: Seattle (E.E.U.U.)

Título: IL28B y el receptor de la lipoproteína de baja densidad (LDLr) tienen un efecto sinérgico sobre la cinética viral del VHC tras los primeros días de tratamiento con INFPEG/RBV en pacientes coinfectados por el VIH/VHC.

Autores: Rivero-Juárez A., Di Lello F., Caruz A., Camacho A., Neukam K., **Herrero R.**, Pérez-Camacho I., Torre-Cisneros J., Pineda J.A., Rivero A.

Comunicacion a congreso nacional (oral): XIII Congreso de la Sociedad Andaluza de Enfermedades Infecciosas (SAEI).

Entidad organizadora: Sociedad Andaluza de Enfermedades Infecciosas (SAEI)

Fecha de celebración: 17/11/2011 – 19/11/2011. Lugar: Huelva (España)

Título: Factores de riesgo para recidivas en pacientes coinfectador pos el VIH/VHC tratados con interferon pegilado mas ribavirina.

Autores: Rivero-Juárez A, Mira JA, Caruz A, Camacho A, García-Rey S, Pérez-Camacho I, **Herrero R**, Merchante N, Pineda JA, Rivero A.

Comunicacion a congreso nacional (oral): XIII Congreso de la Sociedad Andaluza de Enfermedades Infecciosas (SAEI).

Entidad organizadora: Sociedad Andaluza de Enfermedades Infecciosas (SAEI)

Fecha de celebración: 17/11/2011 – 19/11/2011. Lugar: Huelva (España)

Título: Resistencia genética a la infección por VIH-1: asociación con genes reguladores del complemento C4BPA, CR2 y CD55.

Autores: Rivero-Juárez A, Di-Lello F, Caruz A, Camacho A, Neukam K, **Herrero R**, Perez-Camacho I, Torre-Cisneros J, Pineda JA, Rivero A.

Comunicacion a congreso nacional (póster): XXXVIII Congreso de la Sociedad Española de Genética.

Entidad organizadora: Sociedad Española de Genética

Fecha de celebración: 21/09/2011-23/09/2011. Lugar: Murcia (España)