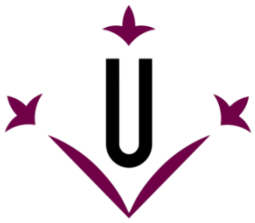

Title:

Genetic associations of the vitamin D and antiviral pathways with natural resistance to HIV-1 infection are influenced by interpopulation variability

Authors:

Wbeimar Aguilar-Jimeneza,*, Wildeman Zapataa,b, Antonio Rivero-Juarezc, Juan A. Pinedad, Marina Laplanae, Natalia A. Tabordaa,f, Mara Biasing, Mario Clericih,i, Antonio Caruzj, Joan Fiblae, Maria T. Rugelesa

a *Grupo Inmunovirología, Facultad de Medicina, Universidad de Antioquia UdeA, 050010 Medellín, Colombia*
b *Grupo Infettare, Facultad de Medicina, Universidad Cooperativa de Colombia, 050012 Medellín, Colombia*
c *Unidad Clínica de Enfermedades Infecciosas, Instituto Maimonides para la Investigación Biomédica de Córdoba (IMIBIC), Hospital Universitario Reina Sofia, 14004 Córdoba, Spain*
d *Unidad Clínica de Enfermedades Infecciosas y Microbiología, Hospital Universitario de Valme, 41014 Seville, Spain*
e *Unitat de Genètica Humana, Departament de Ciències Mèdiques Bàsiques, IRBLleida, Universitat de Lleida, 25198 Lleida, Spain*
f *Grupo de Investigaciones Biomédicas UniRemington, Facultad de Medicina, Corporación Universitaria Remington, 050010 Medellín, Colombia*
g *Dipartimento di Scienze Biomediche e Cliniche-L. Sacco, Università Degli Studi di Milano, 20157 Milan, Italy*
h *Dipartimento di Fisiopatologia Medico-Chirurgica e dei Trapianti, Università degli Studi di Milano, 20100 Milan, Italy*
i *Fondazione Don C, Gnocchi IRCCS, 20100 Milan, Italy*
j *Unidad de Inmunogenética, Departamento de Biología Experimental, Facultad de Ciencias Experimentales, Universidad de Jaén, 23071 Jaén, Spain*

*__Correspondence:__

* Corresponding author at: Grupo Inmunovirologia, Facultad de Medicina, Universidad de Antioquia, Calle 70 No. 52-21, 050010 Medellin, Colombia.
E-mail addresses: wbeimar.aguilar@udea.edu.co (W. Aguilar-Jimenez), mara.biasin@unimi.it (M. Biasin), mario.clerici@unimi.it (M. Clerici), caruz@ujaen.es (A. Caruz), joan.fibla@cmb.udl.cat (J. Fibla), maria.rugeles@udea.edu.co (M.T. Rugeles).

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Abstract

Vitamin D (VitD) may modulate anti-HIV-1 responses modifying the risk to acquire the HIV-1-infection. We performed a nested case-control exploratory study involving 413 individuals; HIV-1-exposed seropositives (cases) and seronegatives (HESN) (controls) from three cohorts: sexually-exposed from Colombia and Italy and parenterally-exposed from Spain. The association and interactions of 139 variants in 9 VitD pathway genes, and in 14 antiviral genes with resistance/susceptibility (R/S) to HIV-1 infection was evaluated. Associations between variants and mRNA levels were also analyzed in the Colombian samples.

Variants and haplotypes in genes of VitD and antiviral pathways were associated with R/S, but specific associations were not reproduced in all cohorts. Allelic heterogeneity could explain such inconsistency since the associations found in all cohorts were consistently in the same genes: *VDR* and *RXRA* of the VitD pathway genes and in TLR2 and RNASE4.

Remarkably, the multi-locus genotypes (interacting variants) observed in genes of VitD and antiviral pathways were present in most HESNs of all cohorts. Finally, HESNs carrying resistance-associated variants had higher levels of VitD in plasma, of VDR mRNA in blood cells, and of ELAFIN and defensins mRNA in the oral mucosa.

In conclusion, despite allelic heterogeneity, most likely due to differences in the genetic history of the populations, the associations were locus dependent suggesting that genes of the VitD pathway might act in concert with antiviral genes modulating the resistance phenotype of the HESNs. Although these associations were significant after permutation test, only haplotype results remained statistically significant after Bonferroni test, requiring further replications in larger cohorts and functional analyzes to validate these conclusions.

1. Introduction

The HIV-1 pandemic is considered a major public health issue, affecting worldwide around 37 million people, for which no cure or effective vaccine has yet been developed (UNAIDS, 2017). However, the presence of HIV-1-exposed but seronegative individuals (HESNs) demonstrates the existence of natural resistance to this infection, which seems to be mediated by a high immune responsiveness (Saulle et al., 2016) but controlled immune activation (Songok et al., 2012), and high expression of antiviral molecules (Iqbal et al., 2009; Zapata et al., 2016), producing a reduction in the susceptibility of target cells while restricting viral infectivity.

Interestingly, vitamin D (VitD) is an immunoregulatory hormone capable to reduce inflammation (Hansdottir et al., 2010; Korf et al., 2012), while inducing the production of antimicrobial peptides (McMahon et al., 2011; Wang et al., 2004) having anti-HIV-1 protective effects as elicited in other viral infections (Gal-Tanamy et al., 2011; Hansdottir et al., 2010). Indeed, as observed by other authors and us, *in vivo* and *in vitro* supplementation with VitD raised peripheral lymphocyte counts and reduced HIV-1 productive infection (Aguilar-Jimenez et al., 2016a; Campbell and Spector, 2011; Coussens et al., 2015; Stallings et al., 2015). Moreover, our previous results support that VitD might be part of the resistance mechanisms of HESNs, since they had higher calcidiol levels in plasma and higher vitamin D receptor (*VDR)* mRNA expression in blood and mucosa compared to healthy controls, which were positively correlated with expression of the anti-inflammatory cytokine IL-10, and the antimicrobial defensins (Aguilar- Jimenez et al., 2013).

Importantly, allelic variants having functional effects in VitD pathway genes and thus in VitD responsiveness (Ahn et al., 2010; Alcina et al., 2013; Uitterlinden et al., 2004; Yin et al., 2016) have been associated with different diseases involving immune disturbances, including multiple sclerosis, asthma and HIV-1 (Alcina et al., 2013; Bosse et al., 2009; de la Torre et al., 2008; Nieto et al., 2004). Notably, the main genome-wide association studies (GWAS) only found the delta-32 mutation, which prevent CCR5 expression in cell surface, as the most consistent resistance mechanism against HIV infection, suggesting that genetic influences on HIV acquisition are either rare or have small effects not detected by GWAS (Lane et al., 2013; McLaren et al., 2013; Petrovski et al., 2011). However, there is little compelling evidence evaluating gene-gene interactions, frequently cited as important components in complex diseases such as HIV-1 infection. Furthermore, due to the difficulty of having large numbers of HESNs and SP individuals with similar exposure rates, such GWAS studies have been performed using general population as controls or cases *vs.* controls with different exposure ways, possibly reducing the probability to detect real and significant associations. More importantly, HIV-1 susceptibility and pathogenicity vary among different populations, which could be partly linked to differences in the genetic and evolutionary origin of such populations as previously reported (Klimentidis et al., 2011) and in accordance with unpublished observations from our group (manuscript in preparation). Accordingly, the study of genetic interactions between VitD and antiviral pathways in HESNs cohorts with different genetic background could further bring to light the participation of the VitD pathway on the resistance phenotype to this infection.

Therefore, we performed a pilot candidate gene study to explore the relationship between VitD and antiviral pathways with resistance/susceptibility (R/S) to HIV-1 infection. We compared frequencies and interactions of single nucleotide polymorphisms (SNPs) and of haplotypes in genes of the VitD and antiviral pathways between chronically HIV-1- infected subjects (SPs, seropositives) and HESNs from two sexuallyexposed cohorts from Colombia and Italy, and a parenterally-exposed cohort from Spain. In addition, associations between SNPs and haplotype genotypes and mRNA of the VitD and antiviral pathway genes from the sexually-exposed Colombian cohort were also explored.

2. Materials and methods

2.1. Population and samples

This is a nested case-control study involving three cohorts of HIV-1- exposed, SPs (cases) and HESNs (controls) with different exposure routes: *i)* a sexually-exposed cohort (serodiscordant couples: 52 SP and 61 HESNs) from Colombia; *ii)* a sexually-exposed cohort (serodiscordant couples: 65 SP and 51 HESNs) from the *S. Maria* Annunziata Hospital (Florence, Italy); and *iii)* a parenterally-exposed cohort (intravenous drug users [IDU]: 93 SP and 91 HESNs) from the hospitals Arnau de Vilanova in Lleida, Valme in Seville and Reina Sofia in Cordoba, Spain.

The inclusion criteria and epidemiological characteristics were previously described for each cohort (Biasin et al., 2010; de la Torre et al., 2008; Zapata et al., 2008). Briefly, these included multiple unprotected sexual episodes for>2 years at the time of the enrolment, with at least 5 episodes of at-risk intercourse within 6 months before study entry with an SP partner with a detectable viral load for sexually exposed cohorts. For parenterally-exposes cohort, syringe and needle sharing for>2 years and diagnostic of Hepatitis C virus (HCV) infection, a marker of blood-borne pathogen exposure were considered. Infection in HESN subjects was ruled-out by plasma HIV RNA and proviral DNA analyses. No Δ32-homozygous subjects were included. The demographic profile of the populations is shown in Table 1.

Genomic DNA was extracted from whole blood of each of the individuals and stored at −20°C until used.

The study was approved by the Institutional Bioethics Committee of the participating hospitals as well as of the Universidad de Antioquia, Colombia; Universidad de Jaen and the Universidad de Lleida, Spain. Written informed consent was obtained from all subjects.

2.2. Genotyping

Twenty-three genes in VitD and antiviral pathways were selected according to literature reports: nine of them involved in the metabolism and function of the VitD, and 14 in the antiviral response with previous evidence of being involved in resistance or immune response against HIV-1. Tag SNPs with minor allele frequency (MAF) > 1% according to the CEU population (Utah residents with ancestry from northern and western Europe) in the HapMap, (International HapMap Project: http://hapmap.ncbi.nlm.nih.gov, Ensemble 36, dbSNP b126, phase III), and with Illumina GoldenGate design scores>0.6 were considered. The SNPs in

coding sequences or 3′- and 5′-untranslated regions (UTRs) of each gene or with previous evidence of association with HIV-1 infection or other immunopathologies (http://www.ncbi.nlm.nih.gov/omim/) were also tested. In total, 139 SNPs were selected, including 25 coding sequence variants (19 non-synonymous and 6 synonymous) in 16 genes, and 22 SNPs located in the 3′- and 5′-UTRs or in putative promoter regions of 10 genes (Table S1).

The 139 SNPs were genotyped in each sample using 300–400 ng of DNA by the Illumina GoldenGate assay at NeoCodex Laboratory, Sevilla, Spain following the manufacturer's protocol.

2.3. Data analysis

Based on differences in the genetic history of the populations studied, the data in each cohort was analyzed independently by using the gPLINK 2.050 software (Purcell et al., 2007). Because of the relatively small sample size within each cohort and the exploratory nature of this study looking for prioritizing which genes might be worth pursuing, allelic associations of SNPs with R/S to HIV-1 infection were identified by Fisher's exact test, followed by adaptive permutation test to correct for multiple comparisons. The permutation test is a method less conservative than Bonferroni, it is useful with small sample sizes and when the distribution is doubtful due to relaxing assumptions about normality (Che et al., 2014). The adaptive permutation test shuffles the response n times and gives up permuting SNPs that are clearly going to be non-significant more quickly than SNPs that look interesting. Thus computational burden could then be reserved for a small subset of SNPs with high associations to phenotype (the SNPs of this study required 600 to 10,000 permutations). A $p < .05$ after the permutation test (referred to as p') was considered statistically significant.

Linkage blocks were inferred by assessing the linkage disequilibrium (LD) between SNPs in the same gene using gPLINK sliding window option that shifts one SNP at a time within a specific chromosome region. Then, haplotype frequencies were estimated by maximum likelihood using the EM (expectation maximization) algorithm and compared between cases and controls using the chi-squared test. Merely principal haplotypes (frequency > 0.05) in cases or controls were considered. Only the haplotype with the most significant association with R/S per gene was analyzed in each population. In addition, a correction for multiple comparisons (referred as p') was performed, based on the number of principal haplotypes in the corresponding linkage block of the haplotype with the most significant association with R/S (frequency > 0.05), as previously described (de la Torre et al., 2008). Within each gene, only haplotypes maintaining multiple testing correction in at least one cohort were associated with R/S. In addition, additive effects of SNPs and haplotype pairs using Model-Based Multifactor Dimensionality Reduction (MB-MDR) software (Calle et al., 2010; Calle et al., 2008) correcting for multiple comparisons by permutation test was also performed. Synthesis-View (Ritchie Lab. https://ritchielab.psu.edu/software/synthesis-viewdownload) were used to plot allelic and haplotype p-values as well as the LD patterns, and EINVIS tool (http://filer.case.edu/yxw407/einvis/ ; (Wu et al., 2013)) were used to visualize the genetic interactions.

The amount of VitD (quantified in plasma by ELISA (Cobas –Roche)) and the mRNA relative levels of vitamin D receptor (VDR), Cytochrome P450 Sterol 27-Hydroxylase (CYP27A1), Human Beta Defensin (HBD)-2, HBD-3, Human Alfa Defensin (HAD)-1, HAD-4, ELAFIN (also known as peptidase inhibitor 3), Apolipoprotein B mRNAediting enzyme catalytic polypeptide-like 3G (APOBEC3G), Tripartite motif-containing protein 5 (TRIM5), Cathelicidin Antimicrobial Peptide (CAMP), and Toll-like Receptor 2 (TLR2) (quantified in blood and mucosa by real time PCR, normalized to actin and phosphoglycerate kinase 1 reference genes expression by using delta-Ct method) which were previously reported in the Colombian cohort (Aguilar-Jimenez et al., 2013; Aguilar-Jimenez et al., 2016b; Gonzalez et al., 2015; Zapata et al., 2016), were compared between the genotypes of SNPs or haplotypes, based on model selection in the association test that showed an association at $p < .05$ with R/S to HIV-1, through a Mann-Whitney U two-tailed test ($p < .05$) using the GraphPad Prism 7.0 software (GraphPad Software, CA, USA). Besides, a p=.0042 was also considered as threshold after correction by multiple comparisons (0.05/12 tests).

3. Results

3.1. Demographic data and quality controls assessments

We analyzed 413 samples distributed in three cohorts. Moderate risk of acquiring HIV-1 infection in HESNs was revealed by the frequency of unprotected sexual intercourse with SP partners with detectable viral loads (sexually-exposed cohorts) and by risk behavior sharing drug injection equipment during at least 3 months and by the presence of HCV infection suggestive of blood-borne pathogen exposure (parenterally-exposed cohort) (Table 1). Out of 413 subjects, 39 (16 Colombians, 11 Italians, and 12 Spaniards) were excluded due to assay failure (call rate < 95%).

After further quality control assessments, 15 SNPs were excluded (Table S1): 5 SNPs due to assay failure (call rate < 95%), 9 SNPs with MAF < 1%, and 1 SNP due to deviation from Hardy-Weinberg equilibrium ($p < .001$), leaving 124 SNPs in 374 subjects for subsequent analyses.

The European ascendancy of the European cohorts and the similar ancestry component (obtained by using the frequency of 17 out of the 139 SNPs being ancestry informative markers) and pair-wise fixation index (FST) values in the Colombian cohort (Zapata et al., 2013) indicated not intra-cohort stratification by ethnicity (Table 1). Moreover, when adjusting our analyzes by ancestry in Colombian cohort (triethnic population) and by sex in Colombian and Italian cohorts using logistic regressions, similar results were obtained with or without adjustment, confirming no intra-cohort stratification (the ancestry-and sex-adjusted *p*-values are shown in the table S1). Since no stratification was found, the permutation-corrected instead of sex- or ancestry-corrected p-values are shown in Fig. 1. Furthermore, the distribution of observed and expected p-values along with genomic inflation factors are shown in Supplementary Fig. 1.

3.2. SNPs and haplotypes in the VitD pathway and antiviral response genes are associated with R/S to HIV-1 infection in a population-specific pattern

Although none of the SNPs remained significant at the Bonferroni threshold in any of the populations (p-value threshold=0.05/124 tests=0.0004), the minor alleles of eight SNPs were associated with R/ S to HIV-1 infection after correction by the permutation test ($p < .05$) in the Colombian cohort. Meanwhile, three SNPs were associated in Italian and three SNPs in Spanish cohorts. However, the association with R/S to HIV-1 of these 14 variants were not replicated among the studied cohorts (Fig. 1. Table S1). Four out of the eight SNPs associated with R/S in the Colombian cohort were in genes of the VitD pathway: rs4240705-G in *RXRA* and rs2189480-T in *VDR* associated with resistance, and rs4674338-A in *CYP27A1* and rs10875695-A in *VDR* associated with susceptibility. The other 4 SNPs: rs1816702-T in *TLR2*, rs7864330-G and rs4986790-G in *TLR4*, and rs352140-T in *TLR9* were associated with susceptibility as previously reported (Beima-Sofie et al., 2013; Kirichenko et al., 2013; Mackelprang et al., 2014). In the Italian cohort, three SNPs were associated with susceptibility: rs12339163-G in *RXRA* and rs2239179-C in *VDR* of the VitD pathway, and rs3748338-T in *RNASE4*. Meanwhile, in the Spanish cohort, three SNPs were associated with resistance and located in *PDIA3* (rs11070410-A, rs1053492- C, and rs3087657-G), a VitD pathway gene.

Remarkably, the T allele in the SNP rs28919570 C/T, producing a change of Arg240Trp in the viral co-receptor CD4, which was previously associated with an increased risk of HIV-1 infection in African commercial sex workers (Oyugi et al., 2009), was not associated with R/S in European (Italian and Spanish) nor ancestry-mixed Colombian cohorts (Fig. 1. Table S1).

Subsequently, as shown in Fig. 1 and Table 2, haplotypes in *VDR, RXRA,* and *LRP2* of the VitD pathway, as well as *TLR2* and *RNASE4* of the antiviral response were associated with R/S to HIV-1 infection in the cohorts studied, meanwhile haplotypes in *CYP24A1* were associated with susceptibility in the Colombian and Italian cohorts. The association of each specific haplotype with R/S to HIV-1 were maintained after multiple comparisons correction in at least one, but not in each analyzed cohort. Although the sequence, and in most cases the SNPs composing those haplotypes, were different among the cohorts, we observed that some SNPs in each haplotype, excluding only the haplotypes in *RXRA*, were shared among the cohorts. These shared SNPs included 4 out of 14 SNPs associated in single maker analysis with R/S to HIV-1 (rs2239179 and rs2189480 in *VDR,* rs1816702 in *TLR2,* and rs3748338 in *RNASE4*). Finally, the haplotype GCTA in *PDIA3* was associated with susceptibility only in the Spanish cohort.

3.3. Interactions of variants in genes of the VitD and antiviral pathways play an additive role in the R/S to HIV-1 infection

We subsequently explored whether multi-locus genotypes were associated with natural resistance to HIV-1 infection to identify potential R/S-related interactions of variants and haplotypes in genes of the VitD and antiviral pathways.

Indeed, we found that some variants in genes of the VitD and antiviral pathways interact with each other and such interactions were associated with R/S to HIV-1 in at least one of the cohorts (Fig. 2. Table S2). Although none of the interactions was replicated in all three

cohorts, the common genotypes of SNPs in VDR and RNASE4, associated in single marker or haplotype analyses with R/S to HIV-1 infection in sexually-exposed cohorts (Fig. 1. Table 2. Table S1), formed multi-locus genotypes significantly associated with resistance to HIV-1 infection as follows: rs2239179-TT in *VDR* with rs3748338-AA in *RNASE4* in the Italian cohort, and rs2189480-TT-TG in *VDR* with rs12895066-GG, rs17114699-GG and rs17516133-GG in *RNASE4* in the Colombian cohort (Fig. 2. Table S2).

Considering the three cohorts, the multi-locus genotypes (interacting variants) associated with resistance were present in 68% (IQR=60%–75%) of HESNs and in 35% (IQR=26%–40%) of SPs, whereas the multi-locus genotypes associated with susceptibility were present in 16% (IQR=12%–20%) of HESNs and in 52% (IQR=45%–55%) of SPs.

A similar distribution of the resistance- and susceptibility-associated multi-locus genotypes were found within each cohort.

Remarkably, additive interactions were also demonstrated between haplotypes significantly associated with R/S in genes of the VitD and antiviral pathways; indeed, variants in *VDR* and *RXRA* form multi-locus genotypes associated with resistance in the three cohorts evaluated. Likewise, additive interactions between *LRP2* and *RNASE4* were observed in the Colombian and Spanish cohorts and between *TLR2* with *VDR, CYP24A1* and *LRP2* were observed in the Colombian population (Table 3).

3.4. Association of SNPs with the transcriptional expression of VDR, HBD-2, HBD-3, ELAFIN, and with VitD plasma levels

In the SNP–mRNA association analysis in the Colombian cohort, the susceptibility AA+AG genotypes of the SNP rs4674338 in the *CYP27A1* gene, were correlated with lower relative levels of VDR mRNA in PBMCs, compared to homozygous rs4674338-GG in HESN individuals (*pAG+AA vs. GG*=0.0243) (Fig. 3A). In addition, the susceptibility genotype AC of the SNP rs10875695 in *VDR* was associated with lower VitD levels in HESNs as compared to homozygous rs10875695-CC individuals (*pAC vs. CC*=0.0095 for VitD) (Fig. 3B).

The resistance genotypes rs2189480-TT-TG in *VDR* and rs4240705-GG+GA in *RXRA* were correlated with higher relative levels of ELAFIN mRNA in oral mucosa, compared to homozygous counterparts in HESN individuals (*pTT+TG vs. GG*=0.0193 and *pGG+GA vs. AA*=0.0072) (Fig. 3C, D).

The rs1816702-T SNP in the *TLR-2* gene, associated with susceptibility independently and included in a haplotype shared region, showed a correlation with lower relative levels of VDR mRNA in PBMCs of HESNs, as compared to homozygous rs1816702-CC individuals (*pTT+TC vs. CC*=0.0445) (Fig. 3E).

Finally, the rs1816702-T SNP was also associated with lower relative levels of HBD-2 and HBD-3 mRNA in the oral mucosa of sexuallyexposed HESNs, as compared to homozygous rs1816702-CC individuals (*pTT+TC vs. CC*=0.0341 for HBD-2 and *pTT+TC vs. CC*=0.0289 for HBD- 3) (Fig. 3F, G).

However, none of the associations found remain significant after multiple comparison correction (*p*-value threshold of 0.0042), requiring further validation before sustains these SNPs can be considered as quantitative trait loci (QTLs).

4. Discussion

In an attempt to explore the role of VitD pathway in R/S to HIV infection, variants in genes of the VitD – antiviral response axis were genotyped in three HIV-1-exposed cohorts from Colombia, Italy and Spain, representing two of the most common routes of HIV-1transmission: sexual and parenteral (De Cock et al., 2012).

Significant, but apparently no consistent allelic associations in *CYP27A1, RXRA, VDR* and *PDIA3* genes of the VitD pathway, and in *TLR2, TLR4, TLR9* and *RNASE4* genes of the antiviral response axis among the three cohorts were found (Fig. 1). However, the allelic frequencies of the SNPs and the LD patterns may depend on the genetic history of the populations (Hamblin and Di Rienzo, 2000; Parra et al., 1998; Philip et al., 2011); making that a functional SNP truly explaining a specific phenotype may be in LD with different SNPs depending on the population. Consequently, the plausibility to find the same SNP associated with R/S to HIV-1 in all cohorts is reduced. This assumption is supported by the fact that from three SNPs, previously associated with susceptibility to HIV-1 infection (rs28919570-T in CD4 and rs352140-T in *TLR9* associated in African (Beima-Sofie et al., 2013; Mackelprang et al., 2014; Oyugi et al., 2009) and rs4986790-G in *TLR4* in European/Asian populations (Kirichenko et al., 2013)), only two of them (rs4986790-G and rs352140-T) were replicated in this study, both being associated with susceptibility only in the Colombian cohort. These results might be explained by differences in ethnic backgrounds among the cohorts.

Allelic heterogeneity, in which different mutations originated as a result of natural selection processes, genetic drift, or genetic migration at the same locus cause a similar phenotype, could be the phenomenon behind our results, as previously reported for other diseases (Chavez- Saldana et al., 2010). Moreover, the gene expression signature of specific phenotypes is influenced not only by the genotype but also by the architecture of the genome, epistasis and genome-ambient interactions (Kaisaki et al., 2016).

In fact, instead of single SNPs, different haplotypes associated with R/S to HIV-1 were observed in the three cohorts studied located at the *VDR, RXRA* and *LRP2 genes* of the VitD pathway, and in *TLR2* and *RNASE4* of the antiviral response axis (Fig. 1, Table 2). Interestingly, it was also observed among the cohorts that these haplotypes shared particular SNPs (Fig. 1. Table 2), where it could be likely found the functional variant responsible for the observed associations. Moreover, the genes with consistent haplotype associations among the cohorts included four of the eight genes with allelic associations; most of them particularly located at the haplotype shared regions. These results support the assumption of heterogeneity in the LD patterns among the cohorts and suggest that functional variants modulating the R/S to HIV- 1 infection may be found in those genes of the VitD and antiviral pathways. Additional studies in larger cohorts and performing deep sequencing and functional assays are encouraged to identify functional variants to confirm this hypothesis.

Another factor that might explain the heterogeneity observed among the populations is the route of HIV-1 exposure. In fact, protective factors can be different depending on the HIV-1 transmission route (Baggaley et al., 2006; Cohen et al., 2000; Martin et al., 2004; Royce et al., 1997). However, some heterogeneity still remains when comparing sexually-exposed Colombian and Italian cohorts making challenging to conclude to this respect.

It is well known that VitD has several immunomodulatory properties and its protective role during HIV-1 infection is being increasingly reported (Aguilar-Jimenez et al., 2016a; Coussens et al., 2015; Jimenez- Sousa et al., 2018; Stallings et al., 2015; Villamor, 2006). Based on our results we cannot ascertain the functional effects of the associations found in the VitD pathway. However, the consistent associations with R/S among the cohorts in genes such as *LRP2 (*encoding megalin) involved in the entrance of VitD to the cells (Abboud et al., 2013) and in *VDR* and *RXRA* involved in the functional role of VitD as inducer of transcription of target genes (Carlberg and Seuter, 2009), reinforce our hypothesis that VitD pathway could influence somehow the resistance to HIV-1 infection.

Previous evidence has shown contrasting results regarding the role of TLR2 on HIV-1 infection (Cote et al., 2013; Schlaepfer et al., 2014), however, the SNP and haplotype association with susceptibility are in the same direction with previous results showing that Colombian HESNs express lower *TLR2* mRNA levels in endocervical mucosa compared to HCs and SPs (Aguilar-Jimenez et al., 2016b). Although no associations between *TLR2* SNP nor haplotypes with mRNA levels were found in this study, the rs1816702-T SNP in the *TLR-2* gene, associated here with susceptibility, was previously associated with higher risk of inflammatory diseases such as Crohn's disease and ulcerative colitis (Bank et al., 2014) and with increased receptor levels on monocytes (Bielinski et al., 2011), favoring our hypothesis.

Meanwhile, the association of *RNASE4* with R/S suggests that this gene could modulate resistance to HIV-1 infection. Interestingly, this gene is part of a cluster encoding ribonucleases, including ANG and EDN, which have anti-HIV-1 activity *in vitro* (Bedoya et al., 2006; Cocchi et al., 2012; Zapata et al., 2016) and have been associated with natural resistance to HIV-1 infection (Bedoya et al., 2008; Rugeles et al., 2003; Zapata et al., 2016). In addition, the deleterious non-synonymous SNP rs3748338 A/T in *RNASE4* (Li et al., 2013) could damper the RNASE4 function and may explain its association with susceptibility to HIV-1 infection in the Italian population.

We hypothesized that VitD might be influencing resistance to the infection by orchestrating anti-HIV-1 responses since it is known that VitD induces antimicrobial molecules (Aguilar-Jimenez et al., 2016a; Wang et al., 2004). Moreover, the fact that in all three cohorts, genes of both, the VitD (particularly in the VDR) and the antiviral pathways were associated to R/S, suggest their interaction for influencing the susceptibility to HIV infection. In fact, some SNPs and haplotypes in the VitD pathway genes displayed epistatic interactions with genes within the same pathway and with antiviral genes, and such interactions were associated with R/S to HIV-1 in all cohorts (Fig. 2. Table 3. Table S2).

Remarkably,>50% of the HESNs from each cohort had a resistance multi-locus genotype (interacting variants) suggesting that interactions involving genes of VitD and antiviral

pathways are quite common despite allelic heterogeneity probably conferred by differences in the populations´ origin (Table S2).

Supporting our hypothesis, the protective actions of VitD by modulating the immune and anti-microbial responses have been previously described in other infections such as *Mycobacterium tuberculosis* (Liu et al., 2006), *Respiratory syncytial virus* (Hansdottir et al., 2010) and even during HIV-1 infection (Aguilar-Jimenez et al., 2016a; Campbell and Spector, 2012). Moreover, the Colombian sexually-exposed HESNs have significantly higher levels of HBD-2 and HBD-3 (Zapata et al., 2008), RNases (1, 2, 5 and 7), APOBEC3G, TRIM5α, SerpinA1, cathelicidin, HAD-1, and Elafin mRNA compared to healthy controls (Aguilar-Jimenez et al., 2016b; Gonzalez et al., 2015); in fact, some of these antiviral molecules were positively correlated with the VDR mRNA (Aguilar-Jimenez et al., 2016b). These results support the potential protective role of these antiviral molecules and of VDR during mucosal exposure to HIV-1.

Forthcoming to a functional approach and taking advantage of the fact that we have previously reported higher transcriptional levels of VitD pathway and antiviral genes in HESNs in the same cohort of Colombian serodiscordant couples (Aguilar-Jimenez et al., 2013; Aguilar-Jimenez et al., 2016b; Gonzalez et al., 2015; Zapata et al., 2016), we performed an SNP–mRNA association analysis (Fig. 3). The associations between SNPs in the *CYP27A1* and *VDR* genes with lower levels of VitD and VDR mRNA in PBMCs, suggested their genetic regulation (Aguilar-Jimenez et al., 2013). In addition, the SNPs in *VDR* and *RXRA*, associated with resistance, were also associated with higher mRNA levels of ELAFIN in the oral mucosa of HESNs. Also, the rs1816702-T allele in *TLR2* was correlated with a significant decrease in VDR, HBD-2 and HBD-3 transcript levels (Fig. 3), further supporting the interaction among genes of these two pathways. Interestingly, except for rs4240705 in *RXRA*, all the SNPs found in this study to be potential trans-QTLs were previously associated as cis-QTLs (online database https://eqtl.onderzoek.io/). However, our results should be interpreted with caution since none of the SNP–mRNA associations found remain significant after multiple comparison correction, and no previous evidence of them acting as trans-QTLs have been reported, requiring further validation before sustains these SNPs can be considered as trans- QTLs.

These results are inclined towards a positive role of the VitD pathway in the context of natural resistance to HIV-1 infection. Nevertheless, our results are limited by the small sample size, affecting the statistical power, requiring to be interpreted with caution and further validation in larger cohorts in particular for the unsolved debate regarding VitD role on HIV infection (Aguilar-Jimenez et al., 2013; Fibla and Caruz, 2010; Villamor, 2006). Likewise, the absence of any significant association in previous GWAS suggested that genetic influences on HIV acquisition are either rare or have small effects (Lane et al., 2013; Limou et al., 2012; McLaren et al., 2013; Petrovski et al., 2011) challenging our conclusions. However, some of the associations reported here were supported by high ORs that could partially compensate for this limitation. In addition, the inclusion of HESNs as controls in this study, increased the probability to detect real and significant associations since the HESNs should have a higher frequency of resistance variants compared to the general population that had been frequently used as controls in such GWAS on HIV-1 susceptibility (Limou et al., 2012; McLaren et al., 2013; Petrovski et al., 2011). It will also be interesting to investigate *in vitro* the effect of

these polymorphisms in the expression and function of these VitD and antiviral factors, and their role in modulating the risk to acquire HIV-1 infection to validate our conclusions.

5. Conclusions

Although requiring confirmation in larger cohorts and with functional analyzes, this study suggests that genes of the VitD pathway may interact with antiviral genes for modulating resistance to HIV-1 infection. Besides, allelic heterogeneity observed in the associations with R/S may greatly depend on the evaluated population, given that a resulting phenotype can be the consequence of complex interactions including the route of HIV exposure or be influenced by the genetic history of the population, where admixture processes become important.

Authors' contributions

WAJ, JF, WZ, AC, and MTR conceived and designed the study. WAJ, WZ, and MTR led Colombian patient recruitment and sample processing. JF, AC, ML, ARJ, and JAP led Spanish patient recruitment and sample processing. MB and MC led the Italian patient recruitment and sample processing. WAJ, JF, AC, and ML participated in variants selection and DNA adequacy for Illumina genotyping assay. WAJ and JF analyzed the data. WZ, AC, and MTR contribute to the data analysis and interpretation of the results. WAJ, WZ, NT, and MTR wrote the manuscript. AC and MTR guided and reviewed the research.

All authors have contributed to editing this paper; they have approved this final submission and declare no conflict of interest.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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FIGURE LEGENDS

Figure 3. Interaction network for resistance to HIV-1.

The illustration corresponds to interaction network for resistance to HIV-1 during sexual-exposure in Colombian (A) and Italian (B) cohorts or during parenteral-exposure in the Spanish cohort (C) involving 20 genes that were found with significant ($p<0.05$) single or multi-locus association results (**green nodes**) and by SNP-SNP interactions, multilocus genotypes as well as mRNA-mRNA {Aguilar-Jimenez, 2013 #1158} and SNP-mRNA correlations (**blue edges**). The genes harboring the best combinations of genetic variants are shown as **red-border green nodes**. **Black nodes** are those with at least one interaction in any of the cohorts, but are not independently associated with R/S to HIV-1 in the represented cohort. **Grey nodes** were inferred by the GeneMANIA plug-in implemented in the Cytoscape 3.2.0. software, as the top genes interacting with the aforementioned 20 genes to find the functional pathways involved; based on gene ontology parameters such as co-expression and co-localization (**grey edges**), functional pathway and physical interactions (**orange edges**) and shared protein domains (**purple edges**). CentiSCaPe tool implemented in the Cytoscape was used to analyze the network topology.

Figure 2.

Association of SNPs with transcriptional expression of VDR, HBD-2, HBD-3 and VitD plasma levels

The SNP rs4674338 in *CYP27A1* were correlated with the expression of VDR mRNA in PBMCs of HESNs (closed squares) and SPs (open triangles) **(A).** The SNP rs10875695 in *VDR* was correlated with both VitD plasma levels of HESNs **(B)** and with VDR mRNA levels in PBMCs of HESNs and SPs **(C)**. Finally, the SNP rs1816702 was correlated with VDR mRNA levels in PBMCs of HESNs **(D),** with expression of HBD-2 **(E)** and HBD-3 **(F)** mRNA in oral mucosa of HESNs. Correlations were evaluated by grouping Colombian individuals into categories according to the genotype of each SNP and comparing the levels of VitD in plasma or mRNA relative units (RU) of VDR, HBD-2 or HBD-3. For these analyses a Mann-Whitney U two-tailed test with a $p<0.05$ as significant. *P*-values, median, and interquartile range are shown in each graph.

Figure S1. Global results of the association study.

Association with resistance or susceptibility to HIV-1 infection was modeled as allelic (blue dots), dominant (red dots), and recessive (green dots). Minus Log_{10} *P* values are plotted according to the genomic coordinate of each tested SNP that was located in genes involved in vitamin D pathway (A) and antiviral response (B). Linkage disequilibrium structure (D' value) of each sample is presented at the botton of each plot.