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CD4 recovery is associated with genetic variation in *IFN*γ and *IL19* genes☆

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ABSTRACT

Not all HIV-infected patients receiving cART are able to recover optimal CD4-T cell levels despite achieving undetectable viremia. We evaluated the potential association between polymorphisms (SNPs) in cytokines involved in immune response (*IL15*, *IFN*γ and *IL19)* and the failure to achieve optimal CD4 T-cells restoration after cART. For this, we carried out a retrospective study in 412 HIV-infected patients starting cART with CD4 < 200 cells/μL. These patients were classified as immunological non-responders (INR) if having a CD4 increase (ΔCD4) below 200 cells/μL after two years on successful cART. *IL15, IFN*γ and *IL19* polymorphisms were genotyped using Sequenom's MassARRAY platform. We found 134 INR patients with a median [IQR] ΔCD4 = 133[73–174] cells/μL. In the multivariate analysis adjusted for age, sex, infection route, ethnic origin, hepatitis co-infection and HIV infection length, the AA genotype of the SNP rs2430561 in *IFN*γ (OR:2.01[1.13–3.56], p = 0.017) and the TT genotype of polymorphism rs2243191 in *IL19* (OR:2.58 [1.17–5.68], p = 0.019) showed significant association with the INR status. Our results show that polymorphisms in *IFN*γ and *IL19* genes significantly impacts in the probability of not achieving an optimal immune recovery in HIV-patients starting cART with CD4 T-cells < 200 cells/μL. Thus, these SNPs could represent potential predictive markers of the immunodiscordant response.

Abbreviations: AIDS, acquired immune deficiency syndrome. cART; combination antiretroviral therapy. HBV, hepatitis B virus. HCV; hepatitis C virus. HIV, human immunodeficiency virus. HWE; Hardy-Weinberg equilibrium. IBS, Iberian population in Spain. IDU; injected drug users. INR, immunological non-responders. IR; immunological responders. MAF, minimum allele frequency. PBMC; peripheral blood mononuclear cell. SNP, Single-nucleotide polymorphism. Tregs: regulatory T-cells

☆ The clinical centers and research groups that contribute to CoRIS are shown in Supplementary Material 1.

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Although combination antiretroviral therapy (cART) is able to block viral replication in HIV-infected patients leading to undetectable HIV levels (Perelson et al., 1997) and immune reconstitution in the majority of patients (Guihot et al., 2010), some HIV-patients present a low increase in CD4 T-cells despite successful viral suppression (Cenderello and De Maria, 2016). These patients are called immunological non-responders (INR) and criteria to define them have varied across the different studies, either a certain level of CD4 T-cells gain or a threshold of CD4 T-cell counts after a certain time on successful cART, with prevalence of INR phenotype as high as 30% of patients starting cART (Cenderello and De Maria, 2016).

The main factor related to this discordant status is a low CD4 T-cell count at beginning of cART, with nadir CD4 T-cell < 200 cells/μL predominantly associated to INR group (Moore and Keruly, 2007; Negredo et al., 2010; Massanella et al., 2010). This represents an important problem because of the increasing proportion of patients with delayed diagnosis (Mocroft et al., 2013) that implies a greater probability of impaired CD4 T-cell recovery associated to increased risk of suffering AIDS and non-AIDS defining clinical events (Lapadula et al., 2013, 2015) or death (Engsig et al., 2014).

Other immunological factors influencing the suboptimal reconstitution of CD4 T-cells include high levels of regulatory T-cells (Tregs) (Horta et al., 2013; Saison et al., 2015) and Th17 cells (Rosado-Sánchez et al., 2018), as well as increased T-cell activation (Negredo et al., 2010; Massanella et al., 2010; Goicoechea et al., 2006; Li et al., 2011), high rate of CD4 T-cell apoptosis (Negredo et al., 2010; Massanella et al., 2010, 2015), low level of naïve CD4 T-cell levels (Goicoechea et al., 2006; Li et al., 2011; Massanella et al., 2015) and of baseline CD4/CD8 T-cell ratio (Rosado-Sánchez et al., 2017). Moreover, gut microbiota profile (Tincati et al., 2016; Lee et al., 2018; Lu et al., 2018) has also been associated to INR status. However, these factors do not fully explain the variability in the level of immune reconstitution. Single-nucleotide polymorphisms (SNPs) in different genes related to proliferation, survival and apoptosis pathways have been shown associated to CD4 T-cell recovery during cART (Haas et al., 2006). Among them a SNP in *IL15*, a Th1-type cytokine with an important role in T-cell homeostasis, was associated to the extent of increase in CD4 T-cell counts during cART (Haas et al., 2006). Similarly, genetic variations in other cytokines with relevant functions in immune response such as *IFN*γ and IL19 could affect the CD4 T-cell gains after cART and be associated to the INR phenomenon. Genetic variations in *IFN*γ have been associated to risk of HIV infection (Sobti et al., 2010; Freitas et al., 2015), higher *CCR5* expression (Liptrott et al., 2011), reduced virological suppression (de Carvalho et al., 2016) and rapid AIDS progression (Freitas et al., 2015; An et al., 2003; Singh et al., 2016). SNPs in *IL19* seem to play a role in resistance to HIV acquisition (Li et al., 2015) and in CD4 T-cell dynamics (Shrestha et al., 2010).

Nevertheless, potential association between genetic variants in these cytokines and the inmunodiscordant phenotype is not totally understood. Thus, this is the first study analyzing whether polymorphisms in the cytokines *IL15, IFN*γ and *IL19* could be associated to the phenomenon of immunological discordance observed in HIV patients with low CD4 T-cell counts (< 200 cells/μL) at the beginning of cART.

We conducted a retrospective case-control study using samples from 412 HIV-infected patients starting cART, kindly provided by the AIDS Research Institute IrsiCaixa-HIVACAT (Badalona, Spain) and the Spanish HIV BioBank integrated in the Spanish AIDS Research Network cohort (CoRIS) (García-Merino et al., 2009) from the Spanish AIDS Research Network (RIS). A flow chart with the inclusion criteria and the steps in the selection process is depicted in Fig. 1. Patients meeting inclusion criteria and accepting to participate in the study were included. All patients participating in the study gave their informed

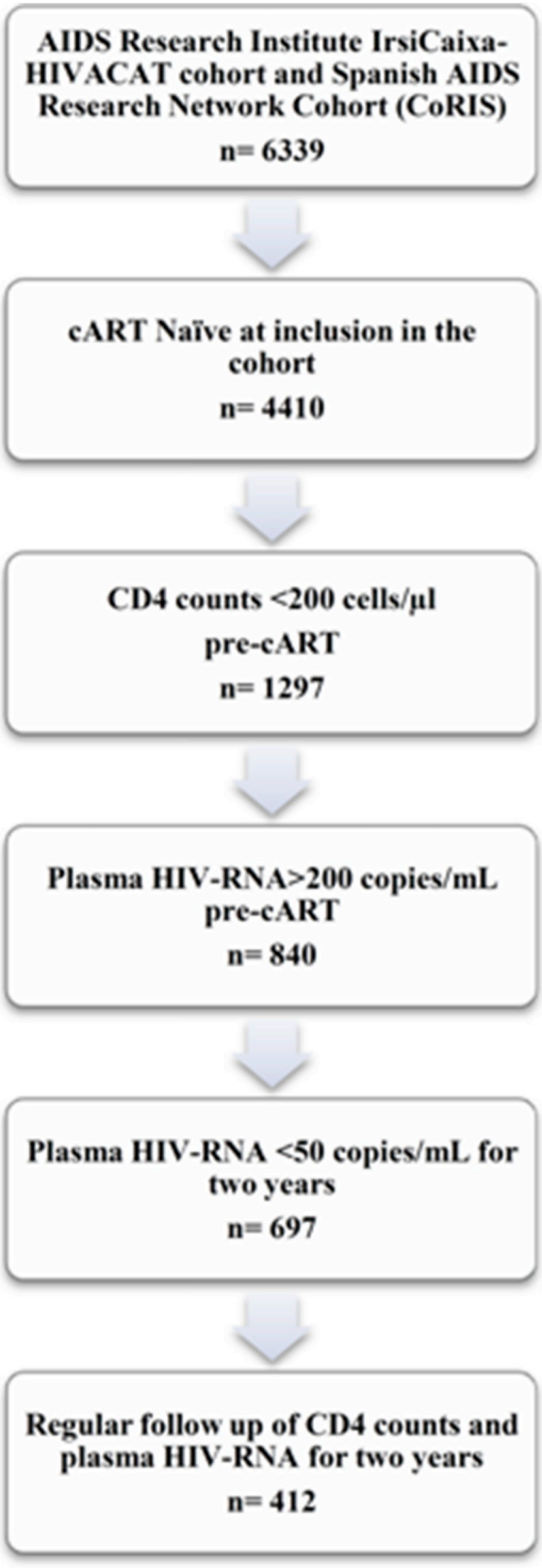

Fig. 1. Flow chart showing the inclusion criteria and the sequential strategy of selection of patients included in the study. Numbers inside the boxes indicate the number of patients selected after each step in the selection process.

consent and protocols were approved by institutional ethics committees. Epidemiological and clinical data were registered from medical records. The characteristics for all 412 HIV-infected patients are shown in Table 1.

Polymorphisms in *IL15*, *IFN*γ and *IL19* were selected based on a minimum allele frequency (MAF) higher than 5% in the CEU (Utah residents with ancestry from Northern and Western Europe) and Tuscan (Italy) from 1000 genomes project data (http://browser.1000genomes.org/index.html). Finally, rs1493013 *IL15*, rs2430561 *IFN*γ and rs2243191 *IL19* polymorphisms were selected. DNA samples of 412 HIV-infected patients were genotyped at the Spanish National Genotyping Center (CeGen; http://www.cegen.org) using Sequenom's MassARRAY platform (Agena Biosciences, USA). Moreover, a control healthy population was obtained at the 1000 Genomes Project's website (http://www.1000genomes.org/home), the Iberian population in Spain (IBS) with 107 individuals.

The primary outcome variable was the increase in CD4 count (ΔCD4) after two years on successful cART. Patients were stratified into two groups according to ΔCD4: INR patients (ΔCD4 < 200 cells/μL; cases) and IR patients (ΔCD4 ≥ 200 cells/μL; controls).

Statistical analyses were performed using the SPSS software (version 15.0, SPSS Inc., Chicago, IL, USA) using two-tailed non-parametric tests. We considered significant p-values < 0.05. Allelic and genotypic frequencies and association studies for individual polymorphisms were performed using the web tool SNPStats (https://www.snpstats.net/start.htm). SNPs were evaluated for Hardy-Weinberg equilibrium (HWE), considering $p > 0.05$. The genetic association was tested using five different models: recessive, dominant, over-dominant, log-additive and co-dominant. Univariate and multivariate logistic regression analysis were used to analyze the association between *IL15*, *IFN*γ and *IL19* SNPs and INR status.

After 2 years of successful cART, 134 (32%) patients showing ΔCD4 < 200 cells/μL were classified as INR. The remaining 278 patients (68%) achieved ΔCD4 ≥ 200 cells/μL and were classified as IR. We found significant differences in ΔCD4 after two years on cART between INR and IR groups ($p < 0.001$), whereas no differences were observed in baseline CD4 T-cell counts pre-cART. Moreover, significant differences were observed in age, years of infection pre-cART and HIV transmission category: INR subjects were older ($p = 0.009$), presented a slightly higher length of HIV infection ($p = 0.029$) and showed more prevalence of injected drug users (IDU) ($p = 0.003$) than IR patients (Table 1).

Table 2
Alleles and genotypes frequencies for IL15, IL19 and IFNγ polymorphisms related to immune restoration after cART.

	All patients (n = 412)	IR[a] (n = 278)	INR[b](n = 134)	p-values*
rs1493013 *IL15*				
C	343 (42%)	219 (40%)	124 (46%)	0.072
T	477 (58%)	333 (60%)	144 (54%)	
CC	78 (19%)	48 (17%)	28 (21%)	0.192
TC	191 (46%)	125 (45%)	68 (51%)	
TT	143 (35%)	105 (38%)	38 (28%)	
HWE[c] p-value	0.42	0.26	0.86	
rs2430561 *IFN*γ				
T	465 (57%)	322 (58%)	143 (53%)	0.197
A	357 (43%)	232 (42%)	125 (47%)	
TT	131 (32%)	86 (31%)	42 (31%)	0.089
TA	206 (50%)	150 (54%)	59 (44%)	
AA	75 (18%)	42 (15%)	33 (25%)	
HWE[c] p-value	0.69	0.14	0.22	
rs2243191 *IL19*				
C	609 (74%)	419 (75%)	190 (71%)	0.172
T	215 (26%)	137 (25%)	78 (29%)	
CC	229 (55%)	157 (56%)	72 (54%)	0.088
CT	151 (37%)	105 (38%)	46 (34%)	
TT	32 (8%)	16 (6%)	16 (12%)	
HWE[c] p-value	0.31	0.87	0.06	

* p-values for the comparison between INR and IR groups (Chi-Square test).
[a] **IR:** immunological responder.
[b] **INR:** immunological non-responder.
[c] **HWE:** Hardy-Weinberg equilibrium.

Previous studies addressing the phenomenon of immunological discordance in patients receiving cART have used different criteria to define INR patients. Studies using a threshold of CD4 T-cell counts have found a clear influence of pre-cART baseline CD4 counts on the risk of INR status (Negredo et al., 2010; Massanella et al., 2010, 2015). In fact, this same association was also observed in our study when a threshold of CD4 counts was used to define INR status (data not shown). However, using the absolute increase of CD4 counts (ΔCD4) to define INR status, our results revealed no association between baseline CD4 T-cell counts and INR status, in agreement with other previous studies (Goicoechea et al., 2006; Haas et al., 2006; Nakanjako et al., 2011). Thus, our data suggest that the absolute increase of CD4 T-cell counts could be more accurate to define INR status, since this parameter was not influenced by the baseline level of CD4 counts and could better

Table 1
Characteristics of study population.

Characteristics	All patients (n = 412)	INR patients (n = 134)	IR patients (n = 278)	p-values*
Gender (n = 412), nº (%)				0,089
Male	324/412 (79)	112/134 (84)	212/278 (76)	
Female	88/412 (21)	22/134 (16)	66/278 (24)	
cART regimen (n = 411), nº (%)				0,721
PI-based	127/411 (31)	40/134 (30)	87/277 (31)	
Non PI-based	284/411 (69)	94/134 (70)	190/277 (69)	
HIV transmission route (n = 385), nº (%)				**0,003**
Sexual	329/385 (85)	93/120 (78)	236/265 (89)	
IDU	56/385 (15)	27/120 (22)	29/265 (11)	
Ethnic Origin (n = 394), nº (%)				0.062
Caucasian	318/394 (81)	108/124 (87)	210/270 (78)	
Latin American	51/394 (13)	9/124 (7)	42/270 (15)	
African	25/394 (6)	7/124 (6)	18/270 (7)	
Age (years)	40 [34–47]	42 [36–48]	39 [32–47]	**0,009**
Length of HIV infection before cART (years)	1 [1-1]	1 [1–2]	1 [1-1]	**0,029**
CD4 counts at baseline (cells/μL)	104 [41–159]	99 [41–148]	107 [41–162]	0,405
CD4 increase after 2 years on cART (cells/μL)	268 [177–400]	133 [73–174]	342 [267–467]	< **0,001**
Coinfetion with hepatitis viruses, n (%)	52/412 (13)	24/134 (18)	28/278 (10)	**0,018**

Note: For continuous variables, data are median (interquartile range, [IQR]).
INR: immunological non responders, **IR:** immunological responders; **IDU**: Injection Drug Users.
* p-values for the comparison between INR and IR groups (Mann-Whitney *U* test or Chi-square test as appropriate).

reflect the patient's ability to reconstitute not only the pool of CD4 T-cells but also different parameters associated to T-cell homeostasis as a recent study has suggested (Pérez-Santiago et al., 2016).

The allele and genotype frequencies of rs1493013 *IL15*, rs2430561 *IFN*γ and rs2243191 *IL19* SNPs in our study population are shown in Table 2. All the three polymorphisms analyzed had a minimum allele frequency (MAF) > 0.05 and satisfied the HWE (p > 0.05). Overall, allele frequencies for the three SNPs were similar between IR and INR groups, except for the rs1493013 *IL15* SNP that showed a trend for lower proportion of T allele in INR (p = 0.072). Regarding genotypes distribution, there was a trend towards a higher proportion of rs2430561 I*FN*γ AA genotype (p = 0.089) and of rs2243191 IL19 TT genotype (p = 0.088) in INR compared to IR group.

Moreover, we compared the alleles and genotypes frequency of the three SNPs in our patients with genotypic data from a group of Spanish reference subjects (Iberian populations in Spain, IBS) (see Supplementary Material 2). We did not find any significant difference between this reference population and our cohort of HIV patients.

Using SNPstats web tool, we tested the best association model with INR status for the different SNPs analyzed. For rs2430561 *IFN*γ and rs2243191 *IL19* SNPs the recessive genetic model was the best model fitting our data. Thus, we compared patients carrying AA genotype (homozygote for minor allele) versus those patients carrying AT or TT genotypes for rs2430561, and patients carrying genotype TT (homozygote for minor allele) were compared with those carrying TC or CC genotypes for rs2243191. For rs1493013 *IL15* SNP the dominant genetic model was the best model fitting our data and thus we compared patients carrying CT or CC genotypes with those patients carrying TT genotype.

The univariate logistic regression analysis (odds ratio; 95% confidence interval; p-value) showed that rs2430561 *IFN*γ AA genotype (OR: 1.83 [1.10–3.05]; p = 0.021) and rs2243191 *IL19* TT genotype (OR: 2.22 [1.07–4.59]; p = 0.031) were significantly associated to INR status. The rs1493013 *IL15* CT/CC genotype presented a clear trend for association with INR status (OR: 1.55 [0.99–2.43]; p = 0.054) (Table 3). However, as shown in Table 1, some clinical and epidemiological aspects were also significantly associated with INR status (age, p = 0.009; length of infection, p = 0.029; transmission route, p = 0.003; presence of coinfection with hepatitis viruses, p = 0.018), or showed a clear trend (gender, p = 0.089; ethnic origin, p = 0.062). Thus, a multivariate logistic regression adjusting by age, length of infection, HIV transmission route, presence of coinfection with hepatitis viruses, ethnic origin and gender was also performed. As shown in Fig. 2, *IFN*γ rs2430561 AA genotype (OR: 2.01 [1.13–3.56]; p = 0.017), and *IL19* rs2243191 TT genotype (OR: 2.58 [1.17–5.68]; p = 0.019) remained strong predictors of INR status. For *IL15* the trend of association with INR in the univariate analysis disappeared after adjusting by these covariables (OR: 1.35 [0.82–2.23]; p = 0.235) (Table 3 and Fig. 2).

The rs2430561 *IFN*γ polymorphism has already been associated with HIV pathogenesis (Sobti et al., 2010; Freitas et al., 2015; Singh et al., 2016). Moreover, the A allele has been shown to contribute to failure to efficiently suppress viral replication after cART (de Carvalho et al., 2016). In our study, AA genotype was associated with a greater risk of being INR. This rs2430561 polymorphism seems to be associated with the ability to produce *IFN*γ, with T allele increasing *IFN*γ expression (Queiroz et al., 2018; Zhang et al., 2018) likely as a consequence of the presence of a *NFKβ* binding site (Pravica et al., 2000), probably disrupted in individuals carrying the A variant. This reduction in *IFN*γ expression in subjects carrying the A allele could limit the anti-viral function of *IFN*γ (de Carvalho et al., 2016), not only facilitating CD4 T-cell loss (Freitas et al., 2015) and disease progression (Sobti et al., 2010; Freitas et al., 2015; Singh et al., 2016), but also impeding the optimal CD4 recovery after successful cART.

Regarding rs2243191 *IL19* SNP, a previous study found several polymorphisms in *IL19* associated to changes in CD4 T-cell levels over time in untreated HIV patients (Shrestha et al., 2010). Moreover, this study also found that genotypes TT and TC of rs2243191 *IL19* were related with a higher increase of CD4 T-cell counts during cART (Shrestha et al., 2010). This result contrasts with our data, since we found that genotype TT was significantly associated to INR status. Several differences in the study cohorts may explain these seemingly contradictory results. First, in the above mentioned study baseline CD4 T-cell counts were very heterogeneous ranging from < 200 to > 500 cels/μL, and the follow-up period was also variable with a median of 1.5 years. Second, differences in the amount of CD4 increase between different genotypes were analyzed and a threshold of increase in CD4 counts was not established to define INR status. Lastly, ethnic differences in the cohorts analyzed in this previous study (African-Americans patients) and in our study (mainly Caucasians).

The rs2243191 SNP results in a non-synonymous amino acid substitution from Phe (allele T) to Ser (allele C) at the position 175 of the IL19 protein, generating a missense variant with function still unclear. Analyzing this SNP in the rSNPBase (http://rsnp.psych.ac.cn/index.do), a database of regulatory annotations on rSNPs, we observed that allele C of rs2243191 was associated with post-transcriptional regulation of *IL19* through RNA-binding protein ELAVL1. This ELAVL1 protein has been described to stabilize mRNAs and regulate their expression (Mukherjee et al., 2011). Interestingly, IL19 has a pivotal role in T cell maturation and homeostasis, promoting Th2 responses (Gallagher et al., 2004). Thus, better regulation of *IL19* expression by rs2243191 polymorphism could lead to increased levels of this cytokine and improved CD4 homeostasis, what could explain the association we found between IL19 rs2243191 genotype and the probability of presenting an

Table 3
Association of polymorphisms in *IL15, IFN*γ and *IL19* with the risk of immunodiscordant response in HIV-patients on successful cART.

			UNIVARIATE		MULTIVARIATE[d]	
rs1493013 *IL15* (dominant model)	IR[a]	INR[b]	OR[c] (95%CI)	p-value	OR[c] (95%CI)	p-value
TT	105 (38%)	38 (28%)	1	0.054	1	0.235
CC/CT	173 (62%)	96 (72%)	1.55 [0.99–2.43]		1.35 [0.82–2.23]	
rs2430561 *IFN*γ (recessive model)						
TT/TA	236 (85%)	101 (75%)	1	0.021*	1	0.017*
AA	42 (15%)	33 (25%)	1.83 [1.10–3.05]		2.01 [1.13–3.56]	
rs2243191 *IL19* (recessive model)						
CC/CT	262 (94%)	118 (88%)	1	0.031*	1	0.019*
TT	16 (6%)	16 (12%)	2.22 [1.07–4.59]		2.58 [1.17–5.68]	

*: p-value < 0.05.
[a] **IR:** immunological responder.
[b] **INR:** immunological non-responder.
[c] OR: Odds Ratio (95% Confidence Interval).
[d] Adjusted by age, gender, length of infection pre-cART, hepatitis coinfection, origin and HIV transmission category.

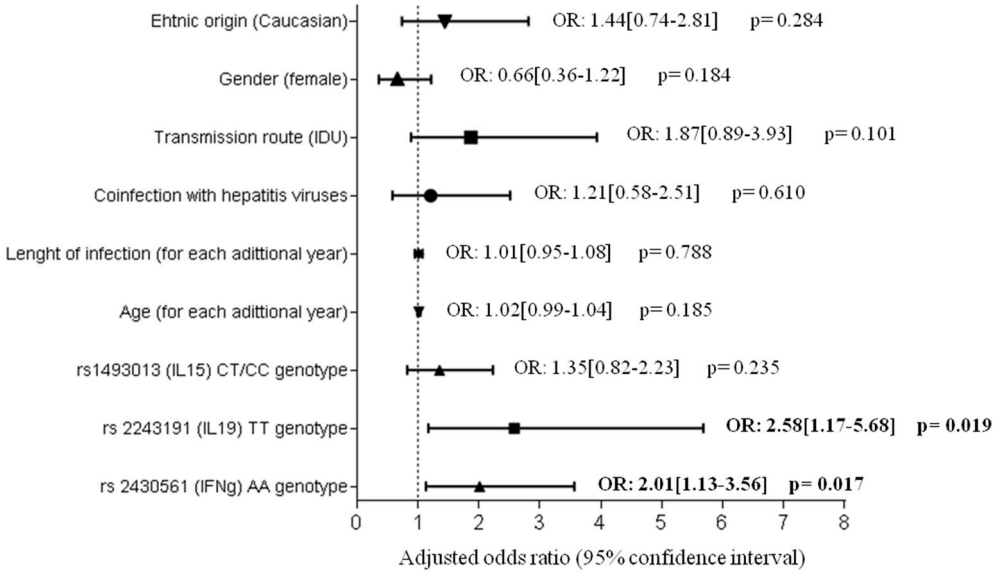

Fig. 2. Predictors of immunological non-response in HIV patients on cART.

INR phenotype after cART. Functional experiments would help to confirm the underlying mechanisms associating rs2243191 SNP with *IL19* expression and the role in CD4 dynamics.

Some limitations of our study need to be taken into account to correctly interpret the results. First, the retrospective design may impose a selection bias and prevent the inclusion of other potential confounding variables. Second, the size of our cohort (although relatively large) may not be enough to achieve statistical significance for rs1493013 *IL15* SNP in the multivariate analysis. Third, in our cohort the majority of patients were Caucasian and thus our conclusions only truly apply to this ethnic group. Lastly, the unavailability of biological samples, other than DNA, prevented the evaluation of the functional association between the studied SNPs and plasma levels of IFNγ and IL19 cytokines, what should be evaluated as part of subsequent steps in future experimental studies to gain insight into the underlying mechanisms that can explain the genetic associations with INR phenotype. However, it has to be noted that the scientific literature addressing such type of associations has shown that, even without founding a biological link between a certain SNP in a certain gene with the expression of the corresponding protein, such genetic associations are by themselves important markers of particular disease conditions, or even predictive markers of the development of a specific disease condition in a determined population of subjects (Pineda-Tenor et al., 2014; Ekenberg et al., 2019; Jiménez-Sousa et al., 2019). A paradigmatic example is the case of SNP rs12979860 in *IL28b* gene that was associated with better response to PEG-IFNa/ribavirin treatment for chronic hepatitis C (Ge et al., 2009) and represented a great advance for the clinical management of the population of HCV-infected patients, and HIV/HCV-coinfected patients (Medrano et al., 2010). In contrast with our results, the IL28b finding was derived from a GWAS analysis which allows a very robust association, and moreover, it was confirmed in several other cohorts (Tanaka et al., 2009; Suppiah et al., 2009; Rallón et al., 2010). Thus, further studies validating our results are warranted.

In conclusion, among the different mechanisms underlying the phenomenon of immunological discordance, our study has demonstrated the relevance of genetic factors related to anti-viral immunity. We found that in HIV-patients starting cART < 200 CD4 cells/μL, rs2430561 *IFN*γ and rs2243191 *IL19* SNPs significantly impact on the patient's ability to reconstitute CD4 cells. Patients carrying AA genotype at rs2430561 and/or TT genotype at rs2243191 had a two-fold greater risk of being INR, representing potential predictive markers of the immunodiscordant response. Thus, these results represent an important progress in the understanding of factors underlying the phenomenon of poor CD4 restoration in this special population of patients, a population that has substantially increased in the last years due to the increasing rate of late presenters among the new HIV diagnoses.

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Conflicts of interest

There is no conflict of interest for any of the authors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.104577.

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