

**Evaluation of the Effect of Protective Genetic Variants on cART
Success in HIV-1-infected Patients**

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LIST OF ABBREVIATIONS

ART - Antiretroviral Therapy

AS ERAP2 - Alternatively Spliced Endoplasmic Reticulum Associated Aminopeptidase
type2

ERAP2 - Endoplasmic Reticulum Associated Aminopeptidase type2

FL ERAP2 - Full-Length Endoplasmic Reticulum Associated Aminopeptidase type2

Hap A - GG homozygous ERAP2

Hap B - TT homozygous ERAP2

HESN - HIV-Exposed Sero-Negatives

HIV-1 - Human Immunodeficiency Virus 1

MX2 – Myxovirus Resistance 2

PBMCs - Peripheral Blood Mononuclear Cells

SNP - Single Nucleotide Polymorphism

TLR3 - Toll-like Receptor 3

VL - Viral Load

INTRODUCTION

HIV-1 infection is not a genetic disease; nevertheless, genetic factors could influence its susceptibility and progression. First evidence of this assumption dates back to 1996, when a homozygous variant for CCR5 chemokine receptor, $\Delta 32$, has been described showing a strong association with HIV-1 sexually transmission (1). Indeed, different phenotypes associated with non-classical outcomes to HIV-1 exposure and infection have been observed, leading to the development of the "immunological advantage" concept; a condition where the genetic background allows to control HIV-1 infection/replication in fortunate patients (2, 3). Some of the genes so far associated to this peculiar state include the ones briefly described hereafter. Endoplasmic Reticulum Associated Aminopeptidase type 2 (ERAP2) plays a key role in antigen presentation mechanisms. Trimming the N-terminal region of peptides ERAP2 adjusts the final peptide length for optimal binding to HLA class I molecules and modulates specific CTL responses (4). Haplotype-specific alternative splicing of ERAP2 gene results in either full-length (FL, hapA) or alternatively spliced (AS, haplotype B) mRNA; HapA is observed to be higher in HIV-Exposed Sero-Negative (HESN) individuals suggesting its involvement in conferring natural resistance to HIV-infection (5, 6). APOBEC3H, part of the APOBEC3 family, controls HIV-1 resistance at different levels (7). HAPI APOBEC3H haplotype is associated with sexually protection in HIV-1 transmission (7). Myxovirus resistance 2 (MX2) interferes with the viral replication decreasing nuclear viral DNA accumulation and integration (8). The G allele of rs2074560 in MX2 gene reveals protection from HIV-1 infection regardless of the transmission route (9). Regarding Toll-like receptor 3 (TLR3), in two HESN cohorts, a higher frequency of rs3775291 SNP (Leu412Phe) was observed suggesting protection against HIV-1 infection (10). Taking in mind that none of

the above-mentioned polymorphisms provides an absolute protection over HIV-1 infection, we decided to verify if these genetic variants - individually or in combination- might modulate the initial immunological and virological responses to antiretroviral therapy (ART).

MATERIALS AND METHODS

Sample collection

300 HIV-infected patients from the ICONA Foundation Study cohort undergoing a first ART regimen were enrolled in the study. These were a random sample of the cohort for which there were at least one year of virological follow-up and a full blood sample was collected at some point in the study. Blood samples were collected from each patient in EDTA Vacutainer® tubes. Immediately after collection, peripheral blood mononuclear cells (PBMCs) were isolated and stored at -80°C. Subsequently, cells were transferred to the laboratories of the University of Milan for genotyping analyses. Periodically (6 months and 1 year) HIV-infected patients' clinical parameters (genotype, gender, CD4+ cell count, viral load, therapies, year of infection, clinical evolution) were analyzed and inserted into a predisposed electronic database. The study was designed and conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the hospitals involved in the study. After thoroughly explaining the project and clarifying any doubt concerning the research, all subjects signed an informed consent and the biological material collected was anonymized to ensure the privacy of each individual.

Genotyping

DNA was extracted by the Maxwell® RSC Instrument automated following a nucleic acid purification method (Promega, Madison, Wisconsin, USA) associated with Maxwell® RSC Blood DNA Kit Technical Manual (Promega, Madison, Wisconsin, USA). Genomic DNA was used as template for PCR amplification using TaqMan probes specifically designed to perform a SNP genotyping assay (Applied Biosystems, Foster City, CA, USA). The amplification and fluorescence reading were carried out as previously described. The list of the gene variants analyzed by this strategy and the code of the TaqMan assay used are listed in Supplementary Table I.

Statistical analyses

The allelic discrimination method was used to determine the frequency of each single nucleotide polymorphism (SNP). The main characteristics of the study population at the time of ART initiation were described and tabulated (Table I). Separate models were employed to evaluate the association between the detection of the genetic variants and HIV-RNA, CD4+ cell count and CD8+ cell count (as well as their ratio) responses to ART. For the HIV-RNA response, the time to achieving a value ≤ 50 copies/mL after ART initiation was evaluated by means of standard survival analysis and Kaplan-Meier curves. The date of the first HIV-RNA value ≤ 50 copies/mL after ART initiation (a single value) was used to define the time of achievement of viral suppression. Participants' follow-up was censored at the date of their last available viral load. For each gene and all four endpoints (HIV-RNA, CD4+ cell count, CD8+ cell count and CD4/CD8 ratio) a linear mixed model was used to compare intercept and slopes according to variants detection. Variability between patients was controlled using random intercepts and slopes. Viral load (VL) was fitted in the log₁₀ scale to achieve distribution symmetry. Besides testing the associations with each gene variant, also a genotypic score was constructed by

allocating 1-point every time a variant was detected and summing all points per participants (i.e. a patient in which ERAP2 rs2549782 G/G was detected as well as rs3775291 C/T or T/T for TLR3 and none of the others would have a value of 2 for the score, etc.). In detail, participants were stratified in 3 groups for the survival analysis (with score values >3; 2-3 and 0-1) and comparing those with extremes scores in the mixed linear regression from fitting a binary variable. The score is referred as ‘supergenotype’ throughout the results of this chapter. All models were fitted on the whole dataset of 300 participants and after restricting to 255 participants of Caucasian origins.

RESULTS

Analysis of allelic variants in the ICONA cohort

Analysis of SNPs prevalence in the selected genes did not show any difference compared with the European population distribution reported in U.S. National Library of Medicine Database (Table IIA-B). The presence of single and combined polymorphisms is reported in Table IIC.

Correlation between single allelic variants and clinical parameters at baseline and 1 year after first ART administration

We did not observe any significant association between the analyzed allelic variants and clinical parameters of HIV+ patients enrolled in the ICONA cohort at baseline (Supplementary Tables II-IV). No correlation between APOBEC3H, TLR3 and MX2

SNPs and either HIV-1 viral load or CD4+ or CD8+ T cell count slopes was observed in our cohort 12 months post cART administration (data not shown). Conversely, we observed an association between the rs2549782 GG variant of ERAP2 and the HIV viral load decay after first cART start (Figure 1A). After adjusting for age, gender, CD4+ T cell count and HIV-RNA at cART start, use of INSTI or >3 drugs, ART-naive and year of cART starting, a non-significant steeper decline in HIV VL was detected for GG genotype compared to GT and TT variants: difference in adjusted HIV-RNA mean change/year (GT/TT – GG): +0.36 log₁₀ copies/mL; p=0.09. (26).

Caucasian patients carrying this genotype showed a trend towards a steeper increase in CD4+ T cell count as well (difference in adjusted CD4+ mean change/year (GT/TT - GG)= -49 cells/mm³; p=0.19) (Figure 1 B). As for CD8+ data in GG patients, TT/GT genotyped patients showed a significant decrease in CD8+ T cell count over the year (adjusted CD8+ mean change/year=-133 cells/mm³ (95%CI: -205,-62.4), while the same marker in GG patients remained constant (as supported by the analysis showing a the significant difference in slope) (p=0.14) (Figure 1 C). Indeed, we observed no differences in CD8+ T cell count over 1 year-treatment (adjusted CD8+ mean change/year=-12.2 cells/mm³) (95%CI: -160,135.6). Nevertheless, independently from the genotype no significant differences in CD4+/CD8+ cell ratio were observed over time in our cohort (p=0.34) (Figure 1 D).

Correlation between combined allelic variants and clinical parameters

One of the aims of this study was also to identify polymorphism combinations (a “supergenotype”) in genes that are considered to be protective in viral

infection/replication control and response to ART by monitoring the clinical parameters of interest (VL and CD4 or CD8 cell count). Therefore, a score (SNP score) was assigned to each SNP (Figure 2 A-C) in order to identify a combination of variants that could provide an immunological benefit in terms of infection progression and response to therapy. The analysis, however, did not show significant correlations between variant combinations and response to therapy either in terms of VL (Figure 2 D-E) or of CD4+ or CD8+ T cell count (Figure 2 F-G).

DISCUSSION

As per definition, the immune system of HIV-infected individuals is not characterized by the "immunological advantage" observed in HESN (2). However, one or more protective genetic variants could confer a better control over disease progression if compared with patients who do not express such haplotypes. Aim of this study was to investigate a possible correlation between response to ART and selected host genetic variants in HIV-infected patients, with the goal to optimize pharmacological intervention in HIV-1-infected subjects. Results showed that, allelic variants in ERAP2 gene result into a more favorable virologic and immunologic response to therapy. Indeed, a significant reduction in viral load and a trend in CD4+ cells recovery was observed at 12 months post ART initiation in GG homozygous (Hap A), compared to TT homozygous (Hap B) or G/T heterozygous patients at ERAP2 SNP rs2549782. Considering the key role played by ERAP2 in the activation of antiviral (11) and HIV-specific immune response (5, 6, 12),

it is possible to speculate that HapA ERAP2 generates more immunogenic peptides able to trigger a more efficient CTL HIV-specific response, even in ART-treated HIV patients. As corollary of our results, a "super genotype" associated with a better response to ART response and characterized by the presence of several "protective" allele variants was not yet observed. Nevertheless, the preliminary results obtained upon analyzing ERAP2, warrant the usefulness of investigating the role of Hap A in a case/control study, both in the progression of the infection and in response to treatment.

FIGURE LEGENDS

Figure 1. Mean intercept and slope of log₁₀ Viral Load (A), CD4⁺ T Cells (B), CD8⁺ T Cells (C) and ratio of CD4/CD8 + T cells (D) from fitting a mixed-linear model in Caucasian subjects of ICONA cohort according to ERAP2 genotype (data adjusted for age, gender, ratio and VL at cART, use of INSTI or >3 drugs, ART-naïve and year of cART starting

Figure 2. SNP score assigned to each variant (A) and frequency-percent of total cohort subjects (B) and Caucasian subjects (C) related to the score. Mean intercept and slope of log₁₀ Viral Load (D), CD4⁺ T Cells (F) and CD8⁺ T cells (G) after cART start from fitting a mixed-linear model in Caucasian subjects according to SNPs score strata (data adjusted for age, gender, ratio and VL at cART, use of INSTI or >3 drugs, ART-naïve and year of starting ART) and Kaplan-Meier curves estimating the cumulative probability of virological success (E) according to SNPs score strata.

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AUTHORS' CONTRIBUTIONS

M Clerici and M Biasin, conceived the study; EM Lor. wrote the paper; V Mercurio and SV Ibba performed the experiments; A Cozzi-Lepri analysed the data; S Lo Caputo revised the paper; A d'Arminio Monforte coordinate the Icona Foundation; A Tavelli contributed to the data collection; C Venditti was involved in the bio-bank storage; F Castelli, A Castagna, A Gori, G Marchetti, and selected and enrolled the subjects included in the study.

Conflict of interest statement

All authors report no competing interest.

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CONFLICT OF INTEREST DECLARATION

No competing financial interests exist