

# Evaluation of Novel Immunological Biomarkers as Potential Determinants of Chronic Inflammation in Virologically Suppressed HIV-1 Positive Patients Receiving Antiretroviral Therapy

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## Research

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# Abstract

**Background:** Although HIV-related deaths have decreased dramatically following the introduction of antiretroviral therapy (ART), HIV infection itself causes increased morbidity and mortality for both non-AIDS-related events or chronic inflammation and immune activation. The use of certain antiretroviral drugs can contribute to this process.

**Methods:** We investigated 26 potential biomarkers in serum samples from HIV-1 infected patients virologically suppressed under ART. The main objective of our study was to evaluate if virological suppression achieved with a triple drug regimen containing tenofovir disoproxil fumarate co-formulated with emtricitabine (TDF/FTC) as backbone, could correlate with a better immunological and inflammatory profile in relation to the third class of antiretroviral drug administered. The eligible patients were then divided into 3 groups in relation to the third drug associated with TDF/FTC: NNRTI (Group 1, n=18), + PI (Group 2, n=18) and INI (Group 3, n=18).

**Results:** Traditionally, inflammatory cytokines and chemokines were more represented in Group 2 than in Group 3 (IL-1Ra, p=0.013; IL-12p70 p=0.039; TNF- $\alpha$  p=0.041; IL-8, p=0.027; MIP1  $\beta$ , p=0.033). Eotaxin showed lower levels in Group 1 compared to Group 2 (p=0.010), while IP-10 was significantly lower in Group 1 compared to both Group 2 and Group 3 (p=0.003 and p=0.007, respectively).

**Conclusions:** our results seem to discourage the administration of PI as a third drug in a virologically effective antiretroviral regimen, as its use is linked to the detection of higher levels of pro-inflammatory mediators than INI and NNRTI.

## Background

The introduction of antiretroviral therapy (ART) has contributed to the reduction of AIDS-related mortality among HIV-positive patients (1) and the induced chronicity of HIV infection. Triple drug regimens that include two nucleoside reverse transcriptase inhibitors (NRTI) as the backbone plus a base agent of another class: protease inhibitors (PI), integrase inhibitors (INI) or nucleoside reverse transcriptase inhibitors (NNRTI) (3), have been shown to control viral replication (2) and represent the gold standard for the treatment of HIV infection in both antiretroviral-naïve and antiretroviral experienced patients. Now, these patients live longer, although with a higher prevalence of non-infectious comorbidities including cardiovascular disease, diabetes, renal dysfunction and liver damage, common among the general population (5), but increasingly frequent in patients with ART (4), due to the inflammatory process related to HIV and the adverse pharmacological effects of ART (6, 7, 8, 8, 9, 9, 10, 11).

In this regard, the international guidelines on the management of HIV-positive patients recommend a change in lifestyle (smoking cessation, changes in diet and physical activity) and, in case of high LDL and/or hypertriglyceridemia values, the administration of statins. In addition to these measures, it is highly recommended to move to an antiretroviral regime that contains drugs with a better profile of lipid metabolism, suggesting the adoption of a therapy "tailored" to the needs of the patient.

Clinical studies have also shown that the transition from therapies such as PI or NNRTI (efavirenz) to integration inhibitors (INI) (e.g. raltegravir, dolutegravir) leads to an improvement in the lipid profile, inflammatory pattern and chronic immune activation. In fact, HIV infection combined with ART is directly associated with immune activation (12, 13), even in virologically suppressed patients. It is known, in fact, that the dysregulation of inflammatory processes induced by HIV occurs through multiple pathways (14), including microbial translocation (15).

To better understand this phenomenon and to optimize therapeutic management, some authors have recently been stimulated to seek and validate new immunological and inflammatory predictive biomarkers.

So far, most studies examining changes in levels of inflammatory biomarkers in HIV-infected individuals have been limited by small study populations, cross-drawings and/or a small number of biomarkers (16). In this regard, recent technical advances, including the development of multiplexed cytokine tests, have contributed to a more efficient measurement of multiple inflammatory biomarkers (17).

In this study, we investigated 26 potential biomarkers in HIV patients under ART for at least one year and virologically suppressed (HIV-RNA < 20 copies/ml) for at least 6 months. Strict inclusion criteria were represented by: ART with a triple drug regimen containing tenofovir disoproxil fumarate fumarate co-formulated with emtricitabine (TDF/FTC) as backbone; 2. CVR  $\geq$  7.5%, calculated according to the ASCVD algorithm (18), against which a therapeutic regimen with statin and/or aspirin has not yet been established. The main aim of our study was to assess whether virological suppression can be related to a better immunological and inflammatory profile, in relation to the third antiretroviral drug administered.

## Methods

### Study patients and design

Forty-nine caucasian HIV-1 positive patients aged  $\geq$  40 years, who have taken ART for at least one year, were enrolled at the Infectious Diseases Department (HIV Section) University-Hospital of Ferrara. The inclusion criteria were: 1) virological suppression for at least 6 months obtained with a triple drug regimen containing TDF/FTC as a backbone, 2) RCV  $\geq$  7.5%, calculated according to the ACC-AHA algorithm (18) in the absence of a regimen with statin and / or aspirin. The presence of comorbidities (chronic inflammatory diseases, neoplasms, diabetes, obesity, etc) and coinfections represented a strict exclusion criterion.

Eligible patients were then divided into groups according to the third drug associated with TDF/FTC at the time of enrollment: NNRTI (efavirenz) (Group 1, n = 16), PI (atazanavir/r or darunavir/r) (Group 2, n = 17) and INI (raltegravir) (Group 3, n = 16).

This study is in line with the Code of Ethics of the World Medical Association (Declaration of Helsinki) and was conducted according to the guidelines for Good Clinical Practice (European Medicines Agency).

The study was approved by The Local Ethic Committee and written informed consent was obtained from each patient prior to inclusion in the study.

## **Serum sample collection**

Serum samples were obtained from the 49 enrolled subjects. The whole blood of each subject was collected in a covered tube without anticoagulants and left to clot undisturbed at room temperature for 20 minutes. The clot was then removed by centrifuging at 1500 x g for 10 minutes in a refrigerated centrifuge. After centrifugation, the serum was immediately transferred to a clean polypropylene tube. All sera were maintained at -80 °C until cytokines analysis.

## **Chemokines and cytokines analysis**

The main outcome measures were the quantification of cytokine concentrations and growth factors in biological samples based on magnetic bead multiplex immunoassays (Bio-Plex, BIO-RAD Laboratories, Milano, Italy). Luminex multiplex panel technology was used for simultaneous measurement of a panel of 26 analytes including cytokines, chemokines and growth factors (IL-1, IL-2, IL-1ra, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-13, IL-15, TNF- $\alpha$ , IL-17, IL-18, IFN- $\gamma$ , MIP1 $\alpha$ , MIP1 $\beta$ , IL-8, IP-10, RANTES, MCP-1, GM-CSF, G-CSF, IL-7, VEGF, PDGF-bb) according to the method of Comar et al 2014. Briefly, 50  $\mu$ l of diluted serum samples (1:4) and reaction standards were added, in duplicate, to a 96 multiwells plate containing analyte beads followed by incubation for 30 minutes at room temperature. After washing, the antibody-biotin reporter was added and incubated for 10 minutes with streptavidin-phycoerythrin. Cytokines levels were determined using the Bio-Plex array reader (Luminex, Austin, TX). The Bio-Plex Manager software automatically optimized the standard curves and returned the reading data as Median Fluorescence Intensity (MFI) and concentration (pg/mL). An ELISA set (Quantikine ELISA-Human CCL5/Rantes immunoassay, RnD system, Minneapolis, MN) with a mean minimum detectable dose of 2.0 pg/ml was used as confirmatory test according to manufacturer's instruction (17).

## **Statistical Analysis**

The normality of distribution of continuous variables was assessed by Kolmogorov–Smirnov test; since variables were not normally distributed, group comparisons were made by Kruskal-Wallis followed by the Mann-Whitney U test corrected for multiple comparisons (Bonferroni). To correct for possible confounding factors, such as age, sex, smoke and disease duration, group comparisons were performed on natural logarithm-transformed variables by ANCOVA, including the variables listed as covariates. The Spearman's Rank Test was used to analyze bivariate correlations. The categorical variables were compared by Chi Square Test.

Data analysis was performed using SPSS Statistics for Windows, version 21.0 (SPSS, Inc., Chicago, IL, USA). Two-tailed probability values < 0.05 were considered statistically significant

## **Results**

The demographic and clinical characteristics of the population studied are summarized in Table 1.

Of the 49 patients enrolled, 14 were women (28.57%) and 35 were males (71.43%). A first exploratory statistical survey showed that one patient, belonging to Group 1, showed extreme values (absolute value of z-score > 1.6) for 8 (30.7%) of the 26 cytokines analysed and, consequently, was excluded from the analysis.

Although statistical significance was achieved for only few mediators, probably because of the small sample size, our results showed that 7 of the 26 immune mediators (18.2%), analyzed between the different groups, are influenced by the type of third drug administered (Fig. 1).

As for the group of innate immunity mediators, some traditionally inflammatory cytokines and chemokines were more represented in PI-treated serum samples with higher levels than those treated with INI (IL-1Ra,  $p = 0.013$ ; IL-12p70  $p = 0.039$ ; TNF- $\alpha$   $p = 0.041$ ; IL-8,  $p = 0.027$ ; MIP1  $\beta$ ,  $p = 0.033$ ). Eotaxin showed lower levels in Group 1 compared to Group 2 ( $p = 0.010$ ), while IP-10 was significantly lower in Group 1 compared to both Group 2 and Group 3 ( $p = 0.003$  and  $p = 0.007$ , respectively).

As shown in Table 2, statistically significant correlations between disease duration and levels of some immune mediators were found in the overall population.

The univariate analysis (ANOVA) showed that there were no statistical differences in disease duration between groups, although INI patients showed a tendency to have lower disease duration values. In order to correct cytokine values for possible confusion factors (age, sex, smoking status, duration of disease), we used an ANCOVA approach on natural logarithm-transformed result variables. As summarized in Table 3, some of the immune mediators tested were influenced by confounding factors, in particular IL-1ra, IL12p70 and TNF- $\alpha$ . The other mediators were not affected by the correction.

## Discussion

Although HIV-related deaths have declined dramatically since the introduction of ART, HIV infection is becoming increasingly chronic and people infected with HIV continue to experience risen morbidity and mortality often due to events unrelated to AIDS (19). In fact, due to the inability of antiretroviral drugs to eradicate the virus from infected reservoir cells, treatment of HIV infection requires permanent systemic therapy. However, even when successfully treated, HIV patients still show higher incidence of age-associated co-morbidities than non-infected individuals. Chronic Immune Activation and Senescence (CIADIS), is a process characterized by a progressive decline of immune system function, usually detected by the expression of cellular or soluble markers derived from innate or adaptive immune responses. Immune activation is associated with progression of HIV disease and increased morbidity and mortality in HIV-infected patients despite ART (20). In this regard, the validation of a "CIADIS score" based on activation, senescence, and differentiation markers, could help physicians to identify patients at high risk for non-AIDS-related comorbidities (20).

Although ART has significantly improved both the quality and lifespan of patients, the life expectancy of treated patients is even shorter than that of uninfected individuals. In particular, while ART may

counteract some features of HIV-associated immunosenescence, several anti-HIV drugs may themselves help to amplify other aspects of immune ageing and chronic inflammation (21).

The analysis conducted in the present study reveals that some categories of antiretroviral drugs emphasize the residual inflammation, partly linked to HIV infection itself (12, 13).

According to our findings, some inflammatory cytokines of innate immunity are more represented in patients taking PI than those receiving INI (Fig. 1A,C,G). Among these, IL-1ra, TNF- $\alpha$ , and IL-12p70, interfere directly with the HIV virus itself, stimulating viral replication (22). Other literature data correlate high cytokine levels with an increase in CVR in HIV positive patients, despite an effective ART regimen (23).

With regard to the pro-inflammatory chemokines tested, a statistical significant difference ( $p < 0.05$ ) was achieved between "PI Group" vs "INI Group" for IL-8 and MIP-1 $\beta$  (Fig. 1B,F). The importance of this finding is strengthened by the fact that these 2 chemokines derived from monocytes interact with CXCR4 and CCR5, both of which are HIV co-receptors, and play an important role in inducing HIV-related inflammation (24). Furthermore, since monocytes are key cells in the pathogenesis of atherosclerosis, it can be assumed that high levels of these molecules may be predictable markers of CVR in HIV positive patients.

The IFN- $\gamma$  induced protein 10 (IP-10 or CXCL-10) was significantly lower in patients treated with NNRTI compared to those treated with PI and INI ( $p = 0.003$  and  $p = 0.007$ , respectively) (Fig. 1E). Alteration in IP-10 expression levels has been related with inflammatory diseases, immune dysfunction and tumor development in the general population (25).

The IFN-induced protein 10 (IP-10 or CXCL-10) was significantly lower in patients treated with NNRTI than in patients treated with PI and INI ( $p = 0.003$  and  $p = 0.007$ , respectively) (Fig. 1E). The alteration of IP-10 expression levels was generally related to inflammatory diseases, immune dysfunction and tumor development in the general population (25). Among HIV-positive patients, IP-10 also known to be one of the first chemokines to increase following HIV infection (26) it is involved in immune cell trafficking to inflammatory sites. In particular, the continued production of IFN- $\gamma$  in the lymphoid organs is responsible for a prolonged increase in IP-10 during chronic HIV infection, even in patients taking ART. In fact, IP-10, by increasing the susceptibility of naive T CD4 + T lymphocytes to HIV infection, improves the production (constitution) of HIV reserves.

As for eotaxin, also known as C-C chemokine ligand 11, CCL11, it showed lower levels in the "NNRTI Group" than in the "PI Group" ( $p = 0.010$ ) (Fig. 1D). Recent studies have shown how high levels of this cytokine are related to cellular senescence, necroinflammation and hepatic fibrogenesis. These effects have been demonstrated in patients with liver disease, where a high level of eotaxin may also be a predictive marker of an adverse clinical course (27). Other results, carried out in experimental models, have shown that eotaxin triggered the infiltration of cardiac mast cells contributing to myocardial fibrosis after transplantation (28). In addition, IP-10 and Eotaxin have been reported to be higher in HIV patients who

have experienced a recent HCV infection than in those monoinfected with HIV. These results may provide a potential explanation for accelerated liver fibrosis related to the high levels of these two immune mediators responsible for respectively pro-inflammatory and pro-fibrogenic action (29).

The present study is not without limitations, first of all with regard to the small sample size, followed by a cross sectional design and the lack of comparison of the immune mediators tested with other conventional inflammatory or procoagulant markers such as CRP and D-dimer respectively (30).

However, although with limitations, our study highlights the importance of how the choice of the third drug in an antiretroviral regimen can promote the imbalance of inflammation-related immune mediators. In particular, our results seem to discourage the administration of PI as a third drug in a virologically effective antiretroviral regimen, as its use is linked to the detection of higher levels of pro-inflammatory mediators than INI and NNRTI.

Further studies in larger cohorts are needed to confirm the results found and to provide their usefulness as a significant clinical tool to help clinicians in choosing a “tailored” antiretroviral regimen.

## **Declarations**

## **Ethics approval and consent to participate**

Ethical approval for this study was obtained from the Regional Ethical Committee. The research was carried out in accordance with the ethical principles of the Declaration of Helsinki.

## **Consent for publication**

Not applicable.

### **Availability of data and materials**

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

### **Competing interests**

The authors declare that they have no competing interests.

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The authors received no specific funding for this work.

# Authors' contributions

Martina Maritati and Carlo Contini equally contributed to the design of the study and the writing of the manuscript; Manola Comar and Tiziana Bellini contributed to the revision of the article and its significant intellectual content; Nunzia Zanotta and Alessandro Trentini contributed to the elaboration of the statistical analysis and interpretation of data; Laura Sighinolfi contributed to the collection of patient's data and the creation of graphics.

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## Tables

**Table 1. Demographic and clinical characteristic of the study population.**

	Group 1	Group 2	Group 3
<b>Age (years)</b>	49.7±9.5	52.9±7.9	53.7±5.8
<b>CD4+</b>	569±130	500±228	603±157
<b>Sex (female, %)</b>	44.4	50.0	33.3
<b>Smoking status (smokers, %)</b>	55.6	50.0	50.0
<b>Disease Duration (years)</b>	16.0±8.5	15.5±7.7	10.4±3.3

**Table 2. Spearman's correlation coefficients between Interleukins levels and HIV diseases duration**

Interleukin	r	P-value
IL-1ra	0.434	0.002
IL-7	0.371	0.010
IL-8	0.321	0.028
IL-12	0.295	0.044
G-CSF	0.325	0.026
IFNg	0.314	0.032
MCP-1	0.330	0.023
TNFa	0.402	0.005

**Table 3. Crude and adjusted means of ILs values determined in study population**

Interleukin	Crude geometric means (95% CI)				Adjusted means (95% CI)			
	Group 1	Group 2	Group 3	P value	Group 1	Group 2	Group 3	P value
IL-1ra	7.7 (6.2-9.5)	7.9 (6.4-9.8)	5.9 (4.8-7.4)	0.017	6.7 (5.6-8.1)	7.9 (6.7-9.4)	6.1 (5.0-7.3)	0.105
IL-8	5.7 (4.4-7.6)	7.2 (5.5-9.3)	4.0 (3.0-5.2)	0.030	5.7 (4.2-7.7)	7.1 (5.3-9.3)	4.1 (3.0-5.5)	0.042
IL-12p70	7.3 (4.3-12.3)	5.0 (3.0-8.3)	2.5 (1.7-4.8)	0.020	6.8 (3.8-12.2)	4.9 (2.9-8.6)	2.9 (1.6-5.2)	0.149
Eotaxin	898 (671-1202)	1620 (1232-2130)	1603 (1208-2125)	0.009	912 (679-1226)	1626 (1237-2137)	1573 (1170-2113)	0.011
IP10	14646 (10950-19591)	28679 (21822-37689)	28906 (21611-38664)	0.001	13723 (10215-18430)	27639 (21018-36362)	32179 (23717-43624)	0.0004
MIP1b	48.3 (37.6-62.1)	64.7 (51.2-81.8)	41.0 (32.2-52.2)	0.0034	49.1 (37.7-63.9)	64.8 (50.8-82.8)	40.4 (30.9-52.6)	0.039
TNF-alpha	31.0 (21.7-44.3)	30.6 (21.6-43.2)	19.4 (13.5-27.7)	0.041	26.7 (18.6-38.2)	30.8 (22.1-42.9)	19.7 (13.8-28.3)	0.212
Covariates appearing in the model: age = 52.4 years; sex = 0.67; smoking status; 0.56; HIV duration = 13.9 years.								

# Figures

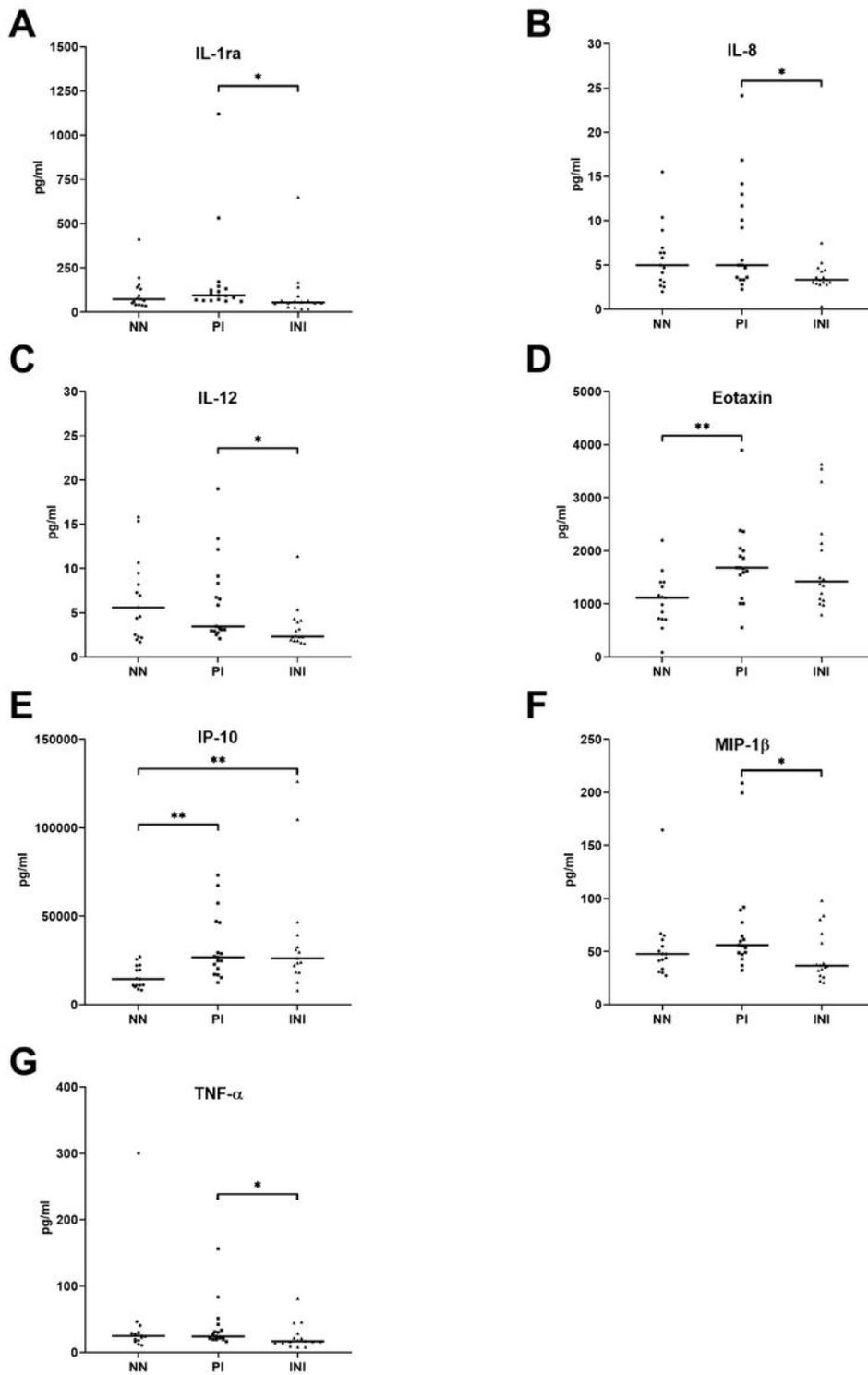


Figure 1

Differences in cytokine concentrations (pg/ml) measured in patients following the therapy simplification regimen. Statistical comparisons between groups were performed with the Kruskal-Wallis test followed by Mann-Whitney U test corrected for multiple comparisons. Panel A: IL1ra; Panel B: IL-8; Panel C: IL-12; Panel D: Eotaxin; Panel E: IP10; Panel F: MIP1b; Panel G: TNF- $\alpha$ . \* P<0.05; \*\* P<0.01.