Current HIV Research, 2021, *19*, 260-268

RESEARCH ARTICLE

Pidotimod and Immunological Activation in Individuals Infected with HIV

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Abstract: ***Background*:** The improvements in HIV infection therapy and the large availability of antiretroviral drugs have led to an increased survival among HIV infected people, and simultaneously to a raised morbidity and mortality due to not-AIDS-related events in this group compared to the general population. An increased systemic inflammation and a persistent immune activation play a pivotal role in determining high rates of non-AIDS comorbidities. In the last years, many natural or synthetic immunomodulatory molecules acting by different mechanisms have been conceived. Pidotimod is a synthetic dipeptide molecule showing immunomodulatory properties. The aim of this pilot study was to evaluate the effects of Pidotimod supplementation on residual inflammation in HIV infected population.

***Methods*:** Forty HIV positive individuals under cART were enrolled: 30 were treated with Pidotimod supplementation (study group) and 10 served as control group (without Pidotimod supplementation). For all participants, Cystatin C, PCR, ESR, microalbuminuria, TNF-α, INF-γ, IL-4, IL-10, IL1β, IL-18 and IL-2 were measured at enrolment (T0), 4 weeks after of Pidotimod supplementation (T1), and 4 weeks after completing supplementation (T2).

***Results*:** In HIV positive participants treated with Pidotimod, the evaluation of cytokine levels showed that IL-10, IFN gamma, and IL-4 were significantly higher at enrolment compared to the control group. The increase under Pidotimod treatment persisted after supplementation suspension, while the pro-inflammatory cytokines levels were reduced. Salivary IgA also increased during 4 weeks of supplementation and persisted at 4 weeks after completing supplementation. On the other hand, the Cystatin C and microalbuminuria levels decreased over time, at a greater extent the Cystatin C serum levels.

***Conclusion*:** The study findings showed that the HIV population receiving Pidotimod achieved a rebalancing of pro-inflammatory and anti-inflammatory cytokines as well as a significant reduction in cystatin C levels. The treatment further allowed for an increase in salivary IgA levels at all the analyzed times, as a secondary event to a remodulation of the immunological status obtained with pidotimod. This approach could represent a new way to design new intervention strategies aimed at improving the persistent immune activation status in the virologically suppressed HIV population.

ARTICLE HISTORY

Received: May 29, 2020
Revised: November 18, 2020
Accepted: November 28, 2020

DOI:
10.2174/1570162X18666210111102046

Keywords: Supplementation, AIDS, cytokines, immunostimulatory, inflammation, immunological rebalancing.

1. INTRODUCTION

The improvements in HIV infection treatment and the increasing availability of drugs have led to an increased survival of HIV infected individuals. Despite the reduced risk of death following cART, HIV positive subjects continue to have an increased morbidity and mortality compared to the general population due to not-AIDS-related events [1-4], including cardiovascular disease (CVD), hypertension, diabetes, neurocognitive dysfunction, osteoporosis, cancer [5-10]. The HIV infected population significantly show CVD risk factors, including low levels of high-density lipoprotein (HDL) cholesterol and increased rates of smoking [11-13] and treatment with several antiretroviral drugs [14-17], which have been associated with high risk of myocardial infarction (MI) [18]. There is evidence that an increased systemic inflammation and a persistent immune acti-

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vation, with predominance of non-classical and intermediate monocytes play an important role in the pathogenesis of atherosclerosis in this population group [5, 19]. The theory that persistent immune activation and inflammation contribute to high rates of non-AIDS comorbidities, such as cardiovascular, liver, kidney and neurologic diseases, is not recent [20, 21]. The immune activation may be considered as a normal and positive event following infection, and T cell activation levels may be predictive of prognosis in the infected patients. The underlying mechanism of immune activation is poorly understood; however, some studies have demonstrated that HIV replication below clinically detectable levels might contribute to persistent immune activation [22, 23]. Various strategies have been attempted to reduce inflammation and restore the immunological balance using different molecules, drugs, probiotics, *etc.* [24-27].

In the last years, different natural or synthetic immunomodulatory molecules, acting on different mechanisms, have been conceived. Among these, Pidotimod (3-L-pyroglutamyl-L-thiaziolidine-4 carboxylic acid) is a synthetic dipeptide molecule with recognized immunomodulatory properties [28].

In vivo and *in vitro* studies have reported that Pidotimod immunomodulatory activity is focused on both adaptive and innate immunity [29, 30]. In particular, Pidotimod is able to induce dendritic cells (DCs) maturation, up-regulate HLA-DR and co-stimulatory molecules expression, to stimulate DCs for pro-inflammatory molecules release driving T-cells proliferation and differentiation towards Th1 phenotype, enhance natural killer (NK) cells functions, and promote phagocytosis [31]. Pidotimod can also increase the production of interferon gamma (IFN-γ), IgG and IgA salivary, and down-regulate production of IL-4 and IgE through Th2 activity modulation [32]. The use of pidotimod has been studied for various diseases, such as community pneumonia, recurrent respiratory infections, allergic states and recently in Covid -19 infection [33-37].

However, currently, there are few available data to demonstrate an immunomodulatory effect of Pidotimod in HIV positive population. The aim of this study was to evaluate the role of Pidotimod supplementation in the immune system modulation and residual inflammation in HIV infected population.

2. MATERIALS AND METHODS

2.1. Study Design

This was a single-center, open-label, prospective pilot study. The protocol was approved by the Ethics Committee at the University "G. d'Annunzio" Chieti-Pescara, Italy (n. richytw2g). No animals were used in this research. All humans research procedures were in accordance with the standards set forth in the Declaration of Helsinki principles of 1975, as revised in 2013. The volunteers were informed about the study objectives concerning the evaluation of Pidotimod supplementation effects on the immune system and were enrolled after signing the informed consent.

The participants in the study group received 800 mg of Pidotimod every 12 hours fasting for 4 weeks.

Overnight fasting venous blood samples were collected at enrolment (T0), 4 weeks after Pidotimod administration (T1), and 4 weeks after completing supplementation (T2) to evaluate biochemical and immunological parameters.

The control group were sex and age-matched with the group of individuals under Pidotimod treatment, selecting among the partecipants who have refused Pidotimod supplementation.

2.2. Study Participants

This pilot study included Caucasian participants with HIV infection who attended the Clinics of Infectious Diseases at the Department of Medicine and Science of Ageing, "G. d'Annunzio" University of Chieti-Pescara, Italy. All participants were under continuous ART, and the study group treatment was further supplemented with Pidotimod (AX-IL® Valeas – Milano) 800 mg twice a day for 30 days, while HIV positive in viro-immunological stability participants were enrolled as a control group (3:1 -treated group: control group ratio).

The inclusion criteria for participation to the study were: (1) patients with plasma viral load <40 copies HIV RNA/mL and CD4+ cell count >200 cells/mL during six months preceding the study; (2) the absence of any opportunistic infections during previous six months and continuous cART for 12 months preceding the study.

During the routine medical examinations of participants, blood was collected for routine biochemical and hematological measurements. A questionnaire was *ad hoc* developed and further administered to participants to acquire demographic information and medical data (any respiratory infection, gastrointestinal symptoms, urinary infection, fever, pain, adverse events) and clinical assessment, including anthropometric measurements and physical examination was conducted as well. Thus, participants were encouraged to report any adverse events to report a self-assessment of their perception of well-being, diet, social life and daily activities.

Exclusion criteria were: (1) using steroids, growth hormone, testosterone or any anabolic agent in the previous six months; (2) drug abuse; (3) to have an acute infection in the previous three months, (4) preexisting hepatic disease or HCV/HBV infection (5), and kidney disease and reduced glomerular filtration rate before the beginning of the study.

The subjects were invited to participate in the study. Those who accepted, prior to participation, gave us informed consent signed by themselves. All participants were similar with respect to smoking status, family income, level of physical activity, socio-economic status and educational levels. All participants were asked to follow their normal diet and lifestyle during the study.

The participants were informed about the present research, and a written consent form was taken from all of them before their enrollment.

2.3. Biochemical Analysis

Fasting venous blood samples were collected from the antecubital vein of all participants at the first clinic examination (T0), at the end of Pidotimod administration (T1), and 4 weeks after completing supplementation (T2). Venipuncture was performed in the morning between 08:00 and 10.00h, using a sterile collecting system consisting of a butterfly needle connected to a syringe (Becton Dickinson, Rutherford, NJ). Anticoagulation was obtained using endotoxin-free heparin (Leo Pharmaceutical Products, Weesp, The Netherlands; final concentration 10 U/mL blood). Plasma was obtained by centrifugation for 10 minutes at 1,000–2,000 x g using a refrigerated centrifuge. Tubes were kept at room temperature and transported to the laboratory for processing within 1 h of collection. These samples were used to determine plasma levels of glucose, triglycerides, total cholesterol, high-density lipoprotein (HDL)-cholesterol, low-density lipoprotein (LDL)-cholesterol, aspartate aminotransferase (AST), alanine aminotransferase (ALT), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), creatinine, urea nitrogen, serum cystatin C and microalbuminuria. Routine laboratory tests were performed at the Division of Clinical Pathology at the "SS. Annunziata Hospital", Chieti, Italy.

2.4. Virologic and Immunologic Markers

CD4+ and CD8+ T cell counts were performed through flow cytometry analysis (BD FACSCanto II), using specimens with fresh cells. Plasma viral load (HIV-RNA) was detected using "Amplicor" method (Roche Molecular Diagnostics, Milan, Italy) with a detection limit higher than 40 HIV RNA copies/mL plasma.

2.5. Serum Samples Preparation

Blood was collected in serum separator vacutainer tubes (BD Biosciences, Oxford, UK). The whole blood, allow the blood to clot by leaving it undisturbed. After 15–30 minutes at room temperature, the clot was removed by centrifuging at 3000 rpm for 10 min minutes in a refrigerated centrifuge. The serum was aliquoted and stored at -20°C for subsequent analysis. Hemolyzed or lipemic samples were discarded.

2.6. Saliva sampling and IgA assay

Salivary samples were collected from all participants in the morning between 08:00 and 10.00h in 5 ml sterile tubes. To prevent sample contamination, participants were not allowed to eat or drink 15 min before saliva collection. After collection, samples were centrifuged at 5000 g/min for 5 minutes to collect supernatant that was stored at -80 °C until processing. Salivary IgA concentration was carried out using a high-sensitivity enzyme-linked immunosorbent assays (ELISA) kit (BioVendor, Czech Republic). The assay was performed according to the manufacturer's instructions, performing the read at 450 nm using a microplate reader (GloMax-Promega, Italy). The analytical sensitivity (detection limit) for salivary IgA assay was 1 ng/mL, and intra- and inter-assay coefficients of variation (CVs) were 8.0% and 8.7%, respectively within acceptable ranges (*i.e.*, accuracy ≤15%; intra-assay CV ≤10%; inter-assay CV ≤15%).

2.7. Cytokine Measurements

Human cytokine levels in serum, including TNF-α, INF-γ, IL-4, IL-10, IL1β, IL-18 and IL-2, were quantified in both study groups through ELISA carried out according to the manufacturer's instructions (Endogen, Woburn, MA, USA). The specificity and the sensitivity for the cytokines were defined according to the manufacturer's instructions. Minimum detectable dose (MDD) was 1.6 pg/ mL for TNF-α and INF-γ, 1 pg/mL for IL-1β, <15 pg/μL for IL-2, 12.5 pg/mL for IL-18, 2.25 pg/mL for IL-10, and 15.4 pg/ mL for IL-4. All samples were analyzed in duplicate at the same time. In brief, a monoclonal antibody specific for each cytokine was precoated onto a microtiter plate. Standards and samples were pipetted into the wells; thus, any cytokine present was bound by the immobilized antibody. After washing away any unbound substance, a specific enzyme-linked polyclonal antibody was added to the wells. After washing, a substrate solution was added to the wells, and color developed in proportion to the amount of cytokine bound in the initial step. The color development was stopped after 30 minutes, and the intensity of the color was measured at 450 nm and the absorbance, assessed using a Glomax multidetection reader spectrophotometer (Promega, Milan, Italy), was transformed into pg/mL, using the calibration curves prepared with cytokine standards included in the kit. Concerning cytokine measurements, the intra- and inter-assay reproducibility was >90%. Duplicate values differing from the mean of more than 10% were not considered for further analysis.

2.8. Statistical Analysis

Demographic and patient-related characteristics were reported as a relative frequency in percentage for qualitative variables, and as mean (± standard deviation) or median for the quantitative ones. Comparison between continuous variables and dependent abovementioned variables was performed using *t*-Student test or Mann-–Whitney test when indicated. Furthermore, the statistical correlation of inflammatory and anti-inflammatory cytokines was assessed in both groups at the different time intervals through Pearson (r) correlation analysis.

Data analysis was performed using statistical software package SPSS for windows, version 25.0 Chicago, SPSS Inc, and p values less than 0.05 were considered as statistically significant.

3. RESULTS

In this study, patients treated with Pidotimod and 10 participants without this supplementation as a control group were enrolled. Only 26 patients treated with Pidotimod completed the study (response rate 86.7%), while four patients were excluded because they did not attend the control visits. All patients, with and without Pidotimod supplementation, were treated with combined cART according to the currently accepted guidelines [38]. The data at the time of enroll-

ment are shown in Table **1**. Most patients (n=21, 80.0%) in the Pidotimod group (mean age 39.2±10.1) were males; the control showed similar data. In all patients, viral load was persistently undetectable at T0. In Pidotimod group, L_TCD4+ cell count was 736.0±286.5 cells/μl, L_TCD8+ cell count was 1102.7±821.9 cells/μl, and CD4+/CD8 ratio was 0.8±0.4. Laboratory parameters on liver, kidney and metabolic functions did not show substantial changes compared to normal parameters (Table **1**). Furthermore, both groups did not show significant differences at T0, especially for viro-immunological, hepatic, renal, metabolic and cytokine parameters (Table **1**).

In the treatment group, all the individuals well tollerated Pidotimod supplementation to the daily diet; only three (11.5%) patients reported grade 1 of gastrointestinal symptoms. Furthermore, most patients (94.1%) reported being satisfied with the supplementation and that Pidotimod taste was acceptable.

The analysis showed that both CD4 and CD8 T lymphocytes were progressively and significantly reduced following 4 weeks of Pidotimod treatment (T1), and this finding was observed at a greater extent, 4 weeks after the end of supplementation (T2). However, CD4/CD8 ratio significantly increased during the study period (T0: 0,81±0,52; T1: 0,87±0,52; T2: 0,98± 0,95), particularly 4 weeks after the end of Pidotimod supplementation; while no significant changes into CD4/CD8 ratio in the control group were found (Table **2**).

In the HIV positive individuals receiving Pidotimod treatment, an alteration of pro-inflammatory cytokines was observed. After 4 weeks of Pidotimod supplementation (T1), both IL-1beta and TNF-alpha significantly increased, but at the end of supplementation (T2), TNF-alpha levels decreased compared to T0 and T1, while IL-1β serum levels were lower than those detected after 4 weeks of supplementation. IL-18 levels were progressively reduced (T0: 680,3±467,1; T1: 625,3±388,3; T2: 547,3±333,9 pg/ml), with a significant reduction at T2 ($p<0.05$). No significant changes were observed in the control group. On the other hand, in the treatment group, an increase in IL-10, IFN gamma, IL-4 and IL-2 values were observed during follow-up, but only IL-10 levels significantly raised 4 weeks after the end of the Pidotimod supplementation (T0: 10,7±20; T1: 11,9±15,5; T2: 17,3±23,4 pg/ml). No significant changes were observed for the anti inflammatory cytokine in the control group (Table **2**).

In the Pidotimod group, salivary IgA levels significantly increased during 4 weeks of supplementation and after the end of supplementation (T0: 220,3±172,1, T1 233,6±196,5; T2 302,5±266,1μg/ml), while no changes were observed in the control group (Table **2**).

Table 1. Clinical and biochemical characteristics of the two groups of the study.

-	HIV-Positive Patients		-
Variables	**Pidotimod Supplementation**	**Control Group**	**-**
-	**(n=26)**	**(n=10)**	**p**
Male gender, %	80	70	n.s.
Age, years	39.2 ± 10.1	40.3 ± 8.6	n.s.
BMI, kg/m2	25.1 ± 2.7	24.9 ± 2.0	n.s.
Duration of cART years	4.7 ± 2.8	4.7 ± 2.9	n.s.
Currently using IP, %	46.2	30	n.s.
Currently using NRTI, %	100	100	n.s.
Currently using NNRTI, %	53.8	70	n.s.
Undetectable viral load, %	100	100	n.s.
Total cholesterol, mg/dL	185 ± 69	177 ± 79	n.s.
Triglycerides, mg/dL	157 ±99	169 ± 104	n.s.
Systolic blood pressure, mmHg	133 ±18	125 ± 9	n.s.
Diastolic blood pressure, mmHg	83 ± 12	81 ± 15	n.s.
Smokers, %	61.5	60	n.s.
Fasting glucose, mg/dL	92 ± 14	93 ± 15	n.s.
AST, U/L	28 ± 16	22 ± 18	n.s.
Creatinine, mg/dl	0.8 ± 0.2	0.9 ± 0.1	n.s.

BMI: body mass index; HAART: Highly active antiretroviral therapy; IP: inhibitors of protease; NRTI: Nucleoside Reverse-Transcriptase Inhibitors. NNRTI: Non Nucleoside Reverse-Transcriptase Inhibitors; AST: aspartate-aminotransferase.
n.s.: not significant.

Table 2. Determination of inflammatory and anti-inflammatory cytokines at enrollment (T0), after 4 weeks with Pidotimod supplementation (T1) and after 4 weeks at Pidotimod discontinuance (T2).

-	Control Group (10 Patients)	-	-	Treatment Group with Pidotimod Supplementation (26 Patients)		
Pro-inflammatory Cytokines	**T0**	**T1**	**T2**	**T0**	**T1**	**T2**
TNF-α (pg/mL)	93.86 ± 152.80	268.31±617.67	102.43±142.81	99.04 ± 155.17*	278.00 ± 428.34**	80.58 ± 158.90
IL-1β (pg/mL)	32.76 ± 78.38	45.89±93.72	46.48±61.44	29.08 ± 42.56*	63.35 ± 91.64	42.46 ± 94.18
IL-18 (pg/mL)	624.58 ± 316.15	622.37±315.98	610.94±373.5	680.46 ± 467.12	625.31 ± 388.35	547.38 ± 333.97***
Anti-inflammatory cytokines	-	-	-	-	-	-
IL-10 (pg/mL)	12.78 ± 18.48	14.13±31.18	14.03±21.59	10.77 ± 20.24	11.88 ± 15.51	17.35 ± 23.44***
IFN-γ (pg/mL)	10.08 ± 8.80	8.73±7.49	12.34±10.02	13.31 ± 20.52	13.62 ± 20.36	15.19 ± 28.05
IL-4 (pg/mL)	56.08 ± 43.76	60.96±47.52	63.58±43.11	68.73 ± 87.78	75.69 ± 117.22	81.42 ± 140.70
IL-2 (pg/mL)	82.41 ± 30.5	33.86±65.57	45.75±67.28	84.08 ± 242.79	67.54 ± 208.69	82.92 ± 276.11
Other parameters	-	-	-	-	-	-
Salivary IgA (μg/mL)	182.51 ± 126.34	177.48±142.95	191.63±111.08	220.31 ± 172.07	233.65 ± 196.51	302.5 ± 266.13***
Cystatin C (mg/L)	0.96 ± 0.26	0.95±0.22	0.93±0.21	1.01 ± 0.25	0.98 ± 0.22**	0.87 ± 0.32***
Microabuminuria (mg/dL)	1.89 ± 1.61	1.76±1.23	1.92±1.25	2.81± 4.04*	2.19 ± 3.38	2.21 ±3.539
CD4 (cell/dL)	699.9 ± 168.51	707.7±230.75	691.1±167.00	736.04 ± 286.56*	690.73 ± 279.59	624.73 ± 309.99***
CD8 (cell/dL)	805.23 ± 316.44	821.7±305.73	740.5±277.46	1102.73 ± 821.92	987.08 ± 666.64	883.88 ± 888.35***
CD4/CD8 ratio	0.93 ± 0.29	0.88±0.29	0.94±0.31	0.81 ±0.52	0.87 ± 0.52**	0.98 ± 0.55***

Legend: * T0 *vs* T1 p <0.05; ** T1 *vs* T2 p <0.05; *** T0 *vs* T2 p < 0.05 using t-test.
The values are expressed in mean and SD.

A significant reduction of Cystatin C was detected after 4 weeks of Pidotimod supplementation, as well as, 4 weeks after the end of supplementation (T0: 1,01±0,2; T1: 0,98±0,2; T2: 0,87±0,3), whereas levels were stable in the control individuals (Table **2**). In the Pidotimod group, microalbuminuria levels further decreased over time, although the reduction was not statistically significant (Table **2**). Among inflammatory cytokines in the treatment group, a significant correlation was found between TNFα and IL-1β at T0 (Pearson correlation r=0.547, p=0.004), as well as at T1 after 4 weeks with Pidotimod supplementation (r=0.825, p<0.01); furthermore, TNFα correlated with IL-18 at T0 (r=0.558, p=0,003). In the control group, TNFα correlated with IL-1β at both T1 and T2 (r=0.901 and r=0.805, respectively p<0.01). Among the anti-inflammatory cytokines in the treated group, a significant correlation was found between IL-2 and IL-4 at T0 (r=0.961, p<0.01), T1 (r=0.971, p<0.01) and at T2 after 4 weeks at Pidotimod discontinuance (r=0.981, p<0.01). Moreover, a significant correlation was found at all intervals between INFγ and IL-4 (r=0.834, p<0.01 at T0; r=0.825, p<0.01 at T1; r=0.921, p<0.01 at T2), as well as between INFγ and IL-2 (r=0.870, p<0.01 at T0; r=0.894, p<0.01 at T1; r=0.962, p<0.01 at T2). In the control group, INFγ correlated with IL-2 at T0 (r=0.680, p=0.03), and with IL-10 at T2 (r=0.780 p<0,01).

4. DISCUSSION

The main findings observed in HIV positive participants on cART with Pidotimod supplementation were an increase in anti-inflammatory cytokines, and a parallel decrease in pro-inflammatory cytokines leading to a reduction in inflammation and an improvement in immunological status.

Pidotimod is a dipeptide able to act on the immune activities, as demonstrated in the previous studies [32, 39], improving the function of macrophages and an increased secretion of certain cytokines [40, 41]. Pidotimod was previously analyzed in elderly people and it was demonstrated that its immunostimulatory effect was able to improve T cells proliferation [42]; this finding is important due to the association of the immune status of HIV positive subjects with inflammaging and immunosenescence of aging [43].

The results further showed that Pidotimod administration in HIV positive and viro-immunological stable subjects can lead to an immunological rebalancing, since a CD4+ cells decrease and a CD4/CD8 increase was observed. The improvement in CD4/CD8 ratio is the target of the cART, and there was a slight rebalancing of the immunological status of HIV positive treated individuals. In HIV negative subjects, a CD4/CD8 ratio <0.3 correlates with immunosenes-

cence and mortality; before the start of therapy, a low CD4/CD8 ratio predicts the risk of clinical progression, which is associated with cell activation and different markers of aging (*i.e*., carotid stiffness, renal low filtration, wasting syndrome) [44].

It has been shown that in treated and viro-suppressed HIV positive individuals, CD4/CD8 ratio is associated independently with serious non-AIDS events and mortality [45-47]. The relative risk of disease progression in patients with a low CD4/CD8 ratio (<0.3) compared to high (<0.45) was reported to be 1.51 (95%CI: 1.09-2.09), with a similar effect to that of progression risk factors, including IV drug addiction, hypertension or HCV co-infection) [48, 49].

The cART accessibility and availability are continuously improving [50], allowing to achieve a comprehensive information on morbidity and mortality compared to the general population due to non-AIDS-related events. In addition, the residual inflammation and immune activation have a pivotal role in non-AIDS-related events [51, 52]. An unbalanced level of pro-inflammatory and anti-inflammatory cytokines was also reported in HIV positive virological suppressed individuals [53]. Furthermore, HIV controllers individuals, compared to chronically HIV-infected individuals taking cART, showed a low inflammatory status and a better balancing in antiflammatory and proinflammatory cytokines [54]. For these reasons, many strategies were attempted to modulate the inflammatory reaction in HIV positive population [55].

There are promising results with the microbiota associated to cART [25], although data are not yet available for immunomodulant supplementation.

Studies on Pidotimod have shown that its immunomodulatory activity is focused on both adaptive and innate immunity [56]. In particular, Pidotimod was demonstrated to induce dendritic cells (DCs) maturation, up-regulate HLADR and co-stimulatory molecules expression, stimulate DCs to release pro-inflammatory molecules driving T-cells proliferation and differentiation towards a Th1 phenotype, thereby, enhancing natural killer (NK) cells functions and promoting phagocytosis [57, 58]. Moreover, Pidotimod was reported to increase production in IFN gamma, IgA on saliva by Th lymphocyte's action [59]. Furthermore, studies in the general population have shown that Pidotimod administration can improve the immunological response to acute infection, reducing the exacerbation in chronic obstructive pulmonary disease [60, 61].

In the present study, Pidotimod supplementation in HIV positive individuals showed an improvement of the immunological activation, mainly reducing the IL18 levels. On the other hand, there was a significant increase in IL-10 levels. The continuous effect observed after the end of supplementation was unexpected. The Pidotimod supplementation partially reduced the immunological unbalance with a reduction in proinflammatory cytokines, mainly IL-18 and cytokines associated with cardiovascular risk [8, 62, 63].

An interesting finding was found in the correction between cytokines in the pidotimod group.

In fact, inflammatory cytokines and anti-inflammatory cytokines correlate with each other, demonstrating how the activity of Pidotimod occurs mainly through a rebalancing of the patient's inflammatory status. Furthermore, these findings could be valuable to support the significant data found for each cytokine, allowing to partially overcome the limits related to the limited size of the study.

As expected, an increase in salivary IgA levels was found at the end of administration (T1) and 4 weeks after the end of supplementation (T2). These data are in agreement with those reporting an enhanced production of secretory IgA after Pidotimod administration in HIV negative adult population [39]. Secretory IgA is the predominant antibody with biologic activity found in saliva and mucosa, which serves as a major source of antigenic material in the oral cavity and bowel. Salivary IgA levels have been found to be significantly low among HIV positive children compared with HIV negative control [64]. Low salivary IgA concentration in HIV positive patients has been associated with the presence of infections [65]; therefore, the increase in IgA levels is necessary for the good health status of people living with HIV.

Furthermore, the reduction in Cystatin C levels demonstrated the switch of the inflammatory balance towards an increased anti-inflammatory status. Indeed, Cystatin C acts as a cysteine protease inhibitor, and its plasmatic levels provide useful indication not only for the renal function status, but also for high cardiovascular risk [7, 63]. The Cystatin C levels have been reported to be associated with all causes of death, particularly due to cardiovascular diseases in HIV negative [66, 67] and HIV positive population [7, 68]. Recent data have shown that Cystatin C can predict all-cause mortality in the population living with HIV [69] and is likely a marker of inflammation and a predictor of mortality in HIV infected people, reflecting inflammation and immune activation [70]. The reduction in Cystatin C levels occurring in patients receiving Pidotimod is of great interest that supports the immunological rebalancing data shown for the other analyzed biomarkes.

There are some limitations of this pilot study, mainly represented by the small sample size of participants and the limited time based follow-up. Thus, additional studies enrolling a great number of patients and with a long-term monitoring after the end of supplementation are necessary to understand the duration and/or reversibility of the immunostimulatory effect of Pidotimod in HIV positive subjects.

CONCLUSION

The results of this study demonstrate that Pidotimod supplementation 800 mg twice a day, improves the immunological status in HIV infected population due to a re-balancing of pro-inflammatory and antiinflammatory cytokines. In addition, the treatment contributes to significantly reduce the Cystatin C levels and increase the salivary IgA levels. These data are important to design novel intervention strategies aimed at improving the persistent immune activation status in this vulnerable population.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The protocol was approved by the Ethics Committee at the University "G. d'Annunzio" Chieti-Pescara, Italy (n. richytw2g).

HUMAN AND ANIMAL RIGHTS

No animals were used in this research. All human research procedures were in accordance with the standards set forth in the Declaration of Helsinki principles of 1975, as revised in 2013. (http://ethics.iit.edu/ecodes/node/3931).

CONSENT FOR PUBLICATION

The participants were informed about the present research, and a written consent form was taken from all of them before their enrollment.

STANDARD OF REPORTING

The study conforms to the STROBE guidelines.

AVAILABILITY OF DATA AND MATERIALS

The authors confirm that the data supporting the results and findings of this study are available within the article.

FUNDING

This research was supported by funds from the G'D'annunzio University, Italy, (Fondi ex 60%).

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

The authors would like to thank all participants who made this research possible.

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