DOI: 10.1111/hiv.12789
HIV Medicine (2019)

ORIGINAL RESEARCH

Circulating CD40 ligand, Dickkopf-1 and P-selectin in HIV-infected patients

K Falasca [1] M Reale,[2] M Di Nicola,[3] C Ucciferri,[1,4] IA Zecca,[5] F Santilli,[6] M Pontolillo,[1] R Liani,[6] C D'Angelo,[2] E Costantini[2] and J Vecchiet[1]

[1]Clinic of Infectious Diseases, Department of Medicine and Science of Aging, University 'G. d'Annunzio' Chieti-Pescara, Chieti, Italy, [2]Unit of Immunodiagnostic and Molecular Pathology, Department of Medical, Oral and Biotechnological Sciences, University 'G. d'Annunzio' Chieti-Pescara, Chieti, Italy, [3]Laboratory of Biostatistics, Department of Medical, Oral and Biotechnological Sciences, University 'G. d'Annunzio' Chieti-Pescara, Chieti, Italy, [4]Department of Medicine and Health Sciences, University of Molise, Campobasso, Italy, [5]Division of Hygene, Epidemiology and Public Health, Department of Medicine and Science of Aging, University 'G. d'Annunzio' Chieti-Pescara, Chieti, Italy and [6]Department of Medicine and Aging, Center of Aging Science and Translational Medicine (CESI-Met), University 'G. d'Annunzio' Chieti-Pescara, Chieti, Italy

Objectives

The aim of this study was to evaluate the circulating levels of CD40 ligand (CD40 L), Dickkopf-1 (DKK-1) and P-selectin, their relationships and their contributions to cardiovascular risk in subjects with HIV infection.

Methods

The study population included 80 HIV-infected patients, 14 (17.5%) of whom had diabetes mellitus (DM) and 32 (40.0%) of whom had arterial hypertension (AH). The HIV-infected patients were compared with a control group with similar demographic and clinical features. CD40L, DKK-1 and P-selectin levels were measured using an enzyme-linked immunosorbent assay.

Results

The HIV-infected patients showed higher levels of all the cardiovascular disease (CVD) markers. Both serum CD40L and DKK-1 were significantly higher in HIV-infected patients than in the HIV-negative controls ($P < 0.001$), while soluble P-selectin showed no significant between-group difference ($P = 0.133$), reflecting the role of HIV infection in CVD. In the HIV-infected group, patients with DM showed lower levels of CD40L and DKK-1 in comparison with the nondiabetic patients and patients with AH ($P < 0.05$, with Bonferroni correction). In contrast, patients with AH showed higher levels of CD40L and DKK-1 in comparison to patients without DM or AH ($P < 0.05$, with Bonferroni correction). Patients with AH showed higher levels of CD40L and DKK-1 than patients with DM ($P < 0.05$, with Bonferroni correction).

Conclusions

In this study, we found that HIV-infected patients displayed significantly higher circulating levels of both CD40L and DKK-1, which were linearly and directly correlated, when compared to HIV-negative patients. The presence of diabetes was associated with lower levels of both CD40L and DKK-1, whereas the presence of hypertension was associated with higher levels of CD40L.

Keywords: cardiovascular, CD40 ligand, Dickkopf-1, inflammation, P-selectin

Accepted 4 July 2019

Correspondence: Prof. Katia Falasca, Clinic of Infectious Diseases, Department of Medicine and Science of Aging, University 'G. d'Annunzio' Chieti-Pescara, Via dei Vestini, 31 Chieti, Italy. Tel: +39 0871 3557562; fax: +390871358372; e-mail: k.falasca@unich.it

Introduction

The introduction of combination antiretroviral therapy (cART) has greatly increased the life expectancy of HIV-infected individuals, but it has concurrently increased

noninfectious morbidity and mortality, in particular for cardiovascular (CV) ischaemic events [1].

Drivers of cardiovascular disease (CVD) in HIV infection may include a combination of factors, such as a higher prevalence of traditional [e.g. smoking, arterial hypertension (AH), diabetes mellitus (DM) and hyperlipidaemia] and nontraditional (e.g. stress) risk factors, and the effects of cART and of HIV itself [2,3]. The prevalence of metabolic syndrome, which ultimately increases the risk of CVD, ranges globally from 17.0 to 45.4% among HIV-infected patients [4].

Hypertension and DM are the main CV risk factors in the general population and also in HIV-infected patients [5–7]. Hypertension is common among HIV-infected people, with a prevalence ranging from 4 to 54% and, among HIV-infected persons with metabolic syndrome, being as high as 96% in some studies [8–10]. Hypertension is a key feature of metabolic syndrome in HIV-infected patients, and an important risk factor for CVD in this population [11]. The Multicenter AIDS Cohort Study and Veterans Aging Cohort Study Virtual Cohort reported a 14% prevalence of DM [12,13].

Persistent systemic inflammation and immune activation play a central role in the pathogenesis of CVD in HIV-infected patients, despite adequate suppression of viral replication. Oxidative stress, platelet activation [14], endothelial activation/damage [15,16], increased thrombotic activity and metabolic disorders [17] are important features of the pathophysiology of atherothrombosis in this setting. The extent to which these abnormalities are driven by HIV infection *per se* or by concurrent CV risk factors, particularly DM and hypertension, has never been explored.

The role of CD40 ligand (CD40L) in the initiation and progression of atherosclerosis and atherothrombosis has become the focus of much attention [18]. CD40L is a 39-kDa transmembrane glycoprotein structurally related to tumour necrosis factor-α (TNF-α). Originally thought to be restricted to stimulated CD4 T cells, mast cells and basophils, its expression is prominent on platelets, which carry preformed CD40L which is rapidly translocated onto the cell surface following activation [19]. Through its receptor, which is constitutively expressed on monocyte/macrophages, endothelial cells, smooth muscle cells and platelets, CD40L orchestrates cell–cell interactions during inflammation in several clinical settings [20], such as type 1 and 2 diabetes [21], hypertension [22] and infectious diseases [23] including HIV infection [24,25].

Dickkopf-1 (DKK-1), an inhibitory molecule of the Wingless-related integration site (Wnt) signalling pathway, is another soluble marker associated with inflammation and atherogenesis. High levels of DKK-1 have been found in patients with unstable angina and in patients with stents at the site of plaque rupture [26]. In patients with type 2 DM, DKK-1 circulating levels are increased, are correlated with soluble CD40L and are reduced by an improvement in glycaemic control and low-dose aspirin treatment, suggesting that the release of DKK-1 occurs, at least in part, during platelet activation. The Wnt signalling pathway has recently also been implicated in the pathogenesis of certain infectious disorders [27] including HIV infection [28], where circulating DKK-1 is associated with neurocognitive impairment [29].

Platelets bind HIV-1 via the fibronectin receptor expressed on their surface, and via C-X-C chemokine receptor type 4 (CXCR4), the receptor for stromal cell-derived factor 1, one of the chemokine coreceptors essential for HIV entry into cells. In this way, platelets are activated and release α-granule content, including P-selectin. HIV thereby enhances the response to low doses of platelet agonists, thus providing a mechanistic explanation for the increased platelet activation in HIV infection [30].

In the general HIV-negative population, increased circulating levels of C-reactive protein (CRP), a marker of inflammation, P-selectin, a marker of *in vivo* platelet activation, and von Willebrand factor, a marker of endothelial dysfunction, have been demonstrated in patients with essential hypertension, and may help to identify those at very high CV risk [22]. In the setting of type 2 diabetes, a paradigm of low-grade inflammation and accelerated atherosclerosis, an interplay between platelet-derived inflammation and endothelial dysfunction, has been revealed, as reflected by increased circulating levels of both CD40L and DKK-1 versus healthy controls. Both markers are significantly reduced after improvements in metabolic control, suggesting a cause and effect relationship between the metabolic derangement and inflammation, endothelial dysfunction and platelet activation, which together lead to atherothrombosis. Whether this interplay may also apply to HIV infection, as an additional model of increased CV risk, has still not been determined. Although elevated levels of proinflammatory molecules such as soluble CD40 ligand (sCD40L) and DKK-1 have been reported in subjects with diabetes and hypertension, and decreased levels of plasma DKK-1 have been reported during improvement of metabolic control, no study has assessed the interaction between the CD40/CD40L and Wnt/DKK-1 systems in HIV-infected patients. Not even the contribution of HIV infection *per se* versus concurrent risk factors to circulating levels of the above-mentioned CV risk markers has been investigated. Thus, we aimed to assess: (i) circulating levels of CD40L, DKK-1 and P-selectin in a cohort of HIV-infected patients with undetectable viraemia as compared to HIV-negative subjects; (ii) the relationships between soluble markers in this setting; (iii) the

contribution of traditional CV risk factors to their circulating concentrations, determined by comparing HIV-infected patients with and without comorbidities; and (iv) the relationships of subsets of lymphocytes with these soluble markers.

Materials and methods

Design of the study

This was a cross-sectional, nonpharmacological comparison study for the evaluation of soluble factors reflecting the extent of inflammation in HIV-infected patients treated at the Infectious Diseases Clinic, University 'G. D'Annunzio', SS Annunziata Hospital of Chieti, Chieti, Italy. The HIV-infected patients underwent routine clinical control at our Day Hospital (DH) and routine technical and laboratory tests were carried out.

Study population

From March 2017 to October 2017 at the DH of the Infectious Diseases Clinic in Chieti, 80 HIV-infected patients were consecutively enrolled in the study using inclusion and exclusion criteria, of whom 14 (17.5%) were diabetic and 32 (40.0%) were hypertensive. No HIV-infected patients had both diabetes and hypertension. Key demographic data (age, gender and nationality), clinical data [body mass index (BMI), year of HIV infection, presence of coinfection with hepatitis B or C virus (HBV/HCV) and Centers for Disease Control and Prevention (CDC) category], viroimmunological data [CD4 count and HIV RNA, measured using polymerase chain reaction (PCR)], and therapeutic and laboratory data (hepatic and renal function, glucose concentration and lipid profile) were assessed in all subjects. Microalbuminuria was measured on single-day urine samples using particle-enhanced immunonephelometric assays (BN II System; Siemens Healthcare Diagnostics, Inc., NY, USA) [31]. The groups were homogeneous for gender and CDC classification.

The HIV-negative group consisted of 80 patients, frequency-matched with our study population by gender and age and clinical features, who were enrolled at the Department of Clinical Medicine of the SS Annunziata Hospital of Chieti. Of these 80 control subjects, 58 were male (72.5%) and 22 female (27.5%). Their mean [± standard deviation (SD)] age was 52.2 ± 11.3 years and their mean (± SD) BMI was 25.9 ± 3.1 kg/m^2. Thirty of these control subjects (37.5%) had a diagnosis of AH, 16 (20.0%) had type 2 DM and 34 (42.5%) had neither AH nor type 2 DM.

The study protocol was approved by the Ethics Committee at the University 'G. d'Annunzio' Chieti-Pescara (Ethics Committee Project No. 14; 21/07/2016) and the study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

Patient selection

For HIV-infected subjects, the inclusion criteria were: provision of written informed consent; age > 18 years; and HIV infection without HCV infection. Enrolled patients were treated with cART, in accordance with applicable guidelines; in fact, all patients were treated with two nucleoside reverse transcriptase inhibitors (NRTIs) plus either a protease inhibitor (PI) or an integrase inhibitor (INI). They had not had any therapeutic changes for > 12 months. All the HIV-infected subjects had had undetectable viral loads for > 12 months. The mean (± SD) CD4 count was 746 ± 585 cells/μL (mean CD4 percentage 29%), the mean (± SD) CD8 count was 957 ± 466 cells/μL (mean CD8 percentage 42%) and the mean CD4:CD8 ratio was 0.8. In the HIV-negative control group, the inclusion criteria were as follows: provision of written informed consent; age > 18 years; and HIV test negative.

Routine laboratory tests, including an HIV test and tests for HBV markers and HCV antibodies, were performed at the Division of Clinical Pathology in the same hospital for all patients.

The exclusion criteria for enrolment in the study were age < 18 years; being a pregnant or breast-feeding woman; clinically significant hepatic, renal, cardiac or pulmonary insufficiency; a history of malignant neoplasms (diagnosed and treated within the last 5 years); autoimmune disorders and type 1 DM; a recent history (< 6 months) of thrombotic events; regular use of oestroprogestin, iron, antioxidants, nonsteroidal anti-inflammatory drugs or antiplatelet agents; organ transplants (except those of the cornea or hair); abuse of alcohol (≥ 80 g/day) and/or drugs in the past 6 months; and current or recent acute infection (< 6 months).

The criteria for diagnosis of essential hypertension and type 2 DM followed the criteria given in guidelines [32,33]. At the time of the study, all patients had good glycaemic control (Glycated Haemoglobin around 7%) while on oral antihyperglycaemic agents (approximately 50% were on metformin and 20% on sulfonylureas) or insulin (approximately 10% of patients). Essential hypertension and type 2 DM were defined according to diagnostic criteria [33,34]. At the time of the study, all patients with diabetes were being treated with dietary interventions, with or without an antihyperglycaemic

agent, and had grade 1 hypertension with lifestyle interventions, as the guidelines suggest.

Sample collection and CD40L, DKK-1 and P-selectin level assays

Venipuncture was performed, in each clinic, in the morning between 08:00 and 10:00 h. Serum samples were obtained after centrifugation at 1792 *g* for 5 min, divided into four aliquots of 0.5 mL each and immediately frozen (–20°C), and then stored at the same temperature until use. All the assays were performed blind with no prior knowledge of the patients' clinical status.

Determination of serum levels of CD40L, DKK-1 and P-selectin was performed using commercial enzyme-linked immunosorbent assay (ELISA) kits (Quantikine TM; R&D Systems, Minneapolis, MN). The specificity and sensitivity were defined according to the manufacturer's instructions. The minimum detectable dose (MDD) was 10.1 pg/mL for CD40L, 0.948 pg/mL for DKK-1 and 0.5 ng/mL for P-selectin.

Paired samples were measured simultaneously on the same plate, and if samples had coefficients of variation > 10% the measurements were repeated.

Statistical analysis

Continuous parameters were summarized as mean and standard deviation (SD). Categorical variables were summarized as absolute value and percentage. To test the normality of the data, a Shapiro–Wilk test was performed.

The enrolled HIV-infected patients were stratified in three subgroups: group A contained patients with only diabetes, group B contained patients with only hypertension, and group C contained patients without diabetes or hypertension.

A χ^2 test was applied to evaluate differences in qualitative variables between groups (HIV-infected and HIV-negative) and subgroups (groups A, B and C). Student's *t*-test for unpaired data or one-way analysis of variance (ANOVA) was applied to evaluate differences in quantitative variables.

Two different two-way ANOVA models were applied in order to assess, separately, the effects of diabetes and hypertension and the effect of HIV on CVD marker levels. Partial correlation analysis, controlling for diabetes and hypertension, was applied to examine the relationship between CVD marker levels.

In all the statistical tests, the threshold of statistical significance was assumed to be $P = 0.05$. Data were analysed using SPSS ADVANCED STATISTICAL TM 13 (IBM SPSS Advanced Statistics, Chicago, IL, USA).

Results

We enrolled a total of 80 patients [80.0% male and 20.0% female; mean (± SD) age 49.5 ± 9.5 years] in the study. The body mass index (BMI) was calculated, and the mean (± SD) BMI was 26.5 ± 5.0 kg/m^2. All HIV-infected patients were being treated with cART according to current guidelines. Viral load was persistently undetectable and the median nadir CD4 cell count was 184 [interquartile range (IQR) 49–384] cells/μL; 23 (28.7%) patients were classified as CDC class C. Clinical characteristics of the HIV-infected patients are summarized in Table 1. Tests of liver and kidney functions and metabolic parameters did not show any substantial variation from normal ranges, as all patients had a stable clinical status (data not show).

Comparison of soluble CVD markers between HIV-infected and HIV-negative patients

Both serum CD40L and DKK-1 were significantly higher in HIV-infected versus HIV-negative patients (mean ± SD: 3839.3 ± 188.4 versus 843.8 ± 163.0 pg/mL and 3348.0 ± 209.6 versus 346.4 ± 29.2 pg/mL, respectively) ($P < 0.001$), while soluble P-selectin showed no significant difference between groups (mean ± SD: 122.1 ± 3.8 versus 108.2 ± 8.4 ng/mL, respectively; $P = 0.133$), reflecting the role of HIV infection in CVD (Fig. 1).

Comparison of soluble CVD markers among subgroups of HIV-infected patients

When we compared group A with group C, lower levels of CD40L as well as of DKK-1 were found in group A ($P < 0.05$ with Bonferroni correction). However, when we compared group B with group C, higher levels of CD40L as well as of DKK-1 were found in group B ($P < 0.05$ with Bonferroni correction). By contrast, P-selectin was lower in group C compared to both groups A and B (Table 2). Patients of group B showed higher levels of CD40L and DKK-1 than patients of group A ($P < 0.05$ with Bonferroni correction). In patients with hypertension (group B), counts of CD3 T lymphocytes, CD45 B lymphocytes and platelets were statistically significantly lower compared to group C [mean ± SD: 1516.7 ± 484.7 versus 1887.4 ± 760.9 ($P < 0.05$), 2130.6 ± 598.8 versus 2552.1 ± 936.3 ($P < 0.05$) and 209.3 ± 48.6 versus 247.3 ± 63.4 × 10^3/μL ($P < 0.05$), respectively]. HIV-infected patients with diabetes (group A) showed a lower mean (± SD) platelet count compared to group C (174.3 ± 84.4 × 10^3/μL versus 247.3 ± 63.4 × 10^3/μL; $P < 0.05$), while levels of CD3 T lymphocytes and CD45 B

Table 1 Demographic and clinical characteristics of the 80 HIV-infected patients and 80 HIV-negative patients enrolled in the study

	HIV-infected patients					HIV-negative patients	
Variable	Overall	Group A (only diabetes)	Group B (only hypertension)	Group C (without diabetes and hypertension)	*P*-value*		*P*-value†
Number of subjects	80	14	32	34		80	
Gender [*n* (%)]					0.625		0.353
Male	64 (80.0)	12 (85.7)	26 (81.3)	26 (76.5)		58 (72.5)	
Female	16 (20.0)	2 (14.3)	6 (18.8)	8 (23.5)		22 (27.5)	
Age (years) (mean ± SD)	49.5 ± 9.5	52.2 ± 7.7	50.2 ± 9.7	47.1 ± 8.4	0.146	52.2 ± 11.3	0.104
Nationality [*n* (%)]					0.711		0.134
Italian	63 (78.8)	12 (85.7)	24 (75.0)	27 (79.4)		71 (88.7)	
Foreign	17 (21.2)	2 (14.3)	8 (25.0)	7 (20.6)		9 (11.3)	
Current smoker [*n* (%)]	27 (33.8)	5 (35.7)	9 (28.1)	13 (38.2)	0.676	20 (25.0)	0.298
Drug abuser [*n* (%)]	14 (17.5)	3 (21.4)	5 (15.6)	6 (17.6)	0.892	–	–
BMI (kg/m^2) (mean ± SD)	26.5 ± 5.0	25.2 ± 3.9	27.7 ± 5.1	26.0 ± 5.1	0.235	25.9 ± 3.1	0.363
AIDS [*n* (%)]	23 (28.7)	7 (50.0)	6 (18.8)	10 (29.4)	0.098	–	–
Anti-HCV-positive [*n* (%)]	19 (23.8)	4 (30.8)	6 (18.8)	9 (27.3)	0.610	–	–
HBsAg-positive [*n* (%)]	20 (25.0)	6 (42.8)	8 (25.0)	6 (17.6)	0.186	–	–
Anti-HDV-positive [*n* (%)]	1 (1.3)	–	1 (1.3)	–	–	–	–
Diabetes [*n* (%)]	14 (17.5)	14 (17.5)	–	–	–	16 (20.0)	0.931
Hypertension [*n* (%)]	32 (40.0)	–	32 (40.0)	–	–	30 (37.5)	0.871
Microalbuminuria [*n* (%)]	36 (45.0)	8 (61.5)	18 (56.3)	10 (29.4)	0.055	35 (43.7)	0.999

**P*-value for comparison among the three HIV-infected subgroups. The significance of differences in qualitative variables was evaluated using the χ^2 test, while the significance of differences in quantitative variables was assessed using one-way analysis of variance.
†*P*-value for comparison between the HIV-infected and HIV-negative groups. The significance of differences in qualitative variables was evaluated using the χ^2 test, while the significance of differences in quantitative variables was assessed using the Student *t*-test for unpaired data.BMI, body mass index; HBsAg, hepatitis B virus surface antigen; HCV, hepatitis C virus; HDV, hepatitis D virus; SD, standard deviation.

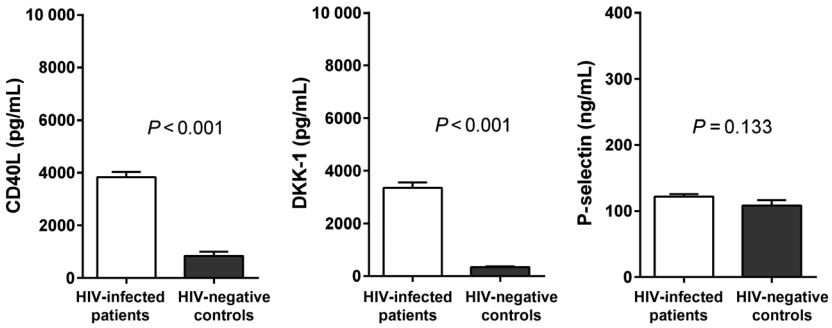

Fig. 1 Bar histogram showing mean (and standard error of the mean) cardiovascular disease marker levels in HIV-infected and HIV-negative patients.

lymphocytes were not significantly different in comparison with group C. Monocytes, neutrophils and natural killer (NK) cells did not show statistically significant differences among groups (Table 3).

Relationships among soluble protein concentrations

In our HIV-infected patients, the correlation analysis, adjusted for the presence of diabetes and hypertension, showed a linear positive correlation between circulating levels of CD40L and DKK-1 ($R = 0.404$; $P < 0.001$). No significant correlation was found between either CD40L or DKK-1 and P-selectin (Fig. 2).

A significant positive correlation of CD40L and P-selectin with the total T lymphocyte number ($R = 0.225$; $P = 0.047$ and $R = 0.223$; $P = 0.048$, respectively) was found. In addition, a positive correlation was found also between CD40L and DKK-1 and platelet count ($R = 0.379$; $P < 0.001$ and $R = 0.514$; $P < 0.001$, respectively).

No significant correlation was found between CD45 B lymphocytes and the examined CVD markers ($R = 0.047$;

Table 2 Effects of diabetes and hypertension on cardiovascular disease (CVD) marker levels in HIV-infected patients

	HIV-infected patients			
CVD marker	Group A (only diabetes)	Group B (only hypertension)	Group C (without diabetes and hypertension)	ANOVA*P*-value
Number of subjects	14	32	34	
CD40L (pg/mL) (mean ± SD)	2450.1 ± 1012.2*	4590.3 ± 1758.0*†	3573.9 ± 1534.1	**0.002**
DKK-1 (pg/mL) (mean ± SD)	**2760.4 ± 1068.7***	3503.8 ± 1768.6	3372.6 ± 1053.7	**0.050**
P-selectin (ng/mL) (mean ± SD)	122.6 ± 38.1	126.0 ± 28.6	112.8 ± 25.7	0.867

*$P < 0.05$ versus group C; †$P < 0.05$ versus group A.ANOVA, analysis of variance; CD40L, CD40 ligand; DKK-1, Dickkopf-1; SD, standard deviation. The bold values are stastistically significant.

Table 3 Effects of diabetes and hypertension on lymphocyte subpopulations in HIV-infected patients

	HIV-infected patients			
Variable	Group A (only diabetes)	Group B (only hypertension)	Group C (without diabetes and hypertension)	ANOVA*P*-value
CD3 T lymphocytes (cells/μL) (mean ± SD)	1594.8 ± 552.3	1516.7 ± 484.7*	1887.4 ± 760.9	**0.049**
CD45 B lymphocytes (cells/μL) (mean ± SD)	2152.4 ± 105.6	2130.6 ± 598.8*	2552.1 ± 936.3	**0.047**
Platelets ($\times 10^3$ cells/μL) (mean ± SD)	174.3 ± 84.4	209.3 ± 48.6	247.3 ± 63.4†	**0.001**
Monocytes ($\times 10^3$ cells/μL) (mean ± SD)	0.51 ± 0.20	0.51 ± 0.13	0.57 ± 0.19	0.303
Neutrophils ($\times 10^3$ cells/μL) (mean ± SD)	3.7 ± 2.0	3.3 ± 1.2	3.6 ± 1.6	0.625
CD16 NK cells (cells/μL) (mean ± SD)	366.6 ± 321.7	329.6 ± 226.0	307.9 ± 197.7	0.706

*$P < 0.05$ versus group C; †$P < 0.05$ versus group A.ANOVA, analysis of variance; NK, natural killer; SD, standard deviation. The bold values are stastistically significant.

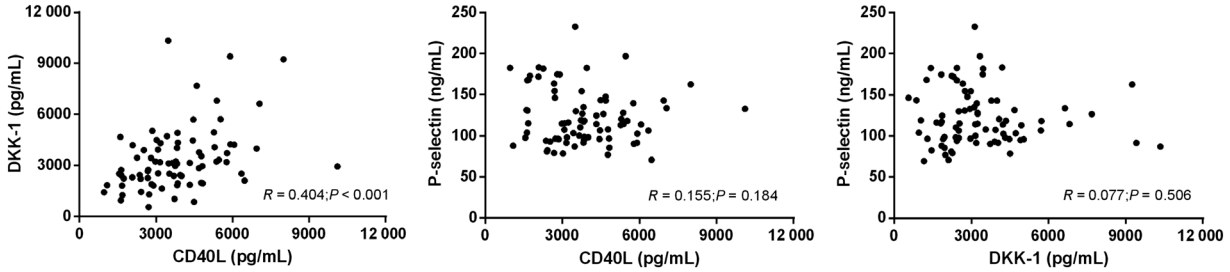

Fig. 2 Correlations between cytokine levels in the HIV-infected group (hypertension and diabetes adjusted model).

$P = 0.683$, $R = 0.099$; $P = 0.387$, and $R = 0.072$; $P = 0.527$ for CD40L, P-selectin and DKK-1, respectively).

Discussion

In this study, we found that HIV-infected patients displayed significantly higher circulating levels of both CD40L and DKK-1, which were linearly and directly correlated, as compared to HIV-negative patients. The presence of diabetes was associated with lower levels of CD40L and DKK-1, whereas the presence of hypertension was associated with higher levels of CD40L.

To our knowledge, this is the first study evaluating the relationship between two soluble inflammatory proteins (in this case CD40L and DKK-1) strongly implicated in the pathophysiology of atherothrombosis in HIV-infected patients. [34], suggesting that these two soluble molecules may interact.

There is a single previous study on DKK-1 in HIV infection, in which this marker was found to be correlated to bone metabolism [28], while many studies have been performed on CD40L in HIV infection. CD40L expression contributes to the immunological control of viral replication by inducing HIV-suppressive chemokines and supporting the production of anti-HIV antibodies and cytotoxic T cells. However, by activating antigen-presenting cells, such as dendritic cells and macrophages, CD40L can also lead to increased CD4 T-cell activation, which

promotes the replication of HIV in these lymphocytes. Later, with the development of AIDS, CD40L-expressing CD4 T cells become selectively depleted, perhaps as a result of a glycoprotein 120 (gp120)-induced signal through CD4 that down-regulates CD40L expression [24].

Thus, as our data show, neither virological suppression nor cART appears to be able to normalize this biochemical imbalance. In this study, we confirmed that, in HIV-infected patients, in contrast to HIV-negative subjects, CD40L, DKK-1 and P-selectin cannot be modulated by drug-induced metabolic improvement and blood pressure control [35,36].

In this study, we found that the concentration of soluble DKK-1 was higher in HIV-infected subjects than in controls, and there was a trend for soluble DKK-1 concentration to be influenced by the presence of CV comorbidity, such as microalbuminuria, diabetes and hypertension.

In the hypertensive HIV-infected population, we observed an increase in CD40L and DKK-1, probably as a result of the increased inflammation caused by HIV and by the presence of endothelial damage related to hypertension and renal disease, as indicated by the presence of microalbuminuria [7,17]. Several studies showed an increase in these biomarkers as a consequence of the activation of different inflammatory pathways in the progression of renal and CV atherosclerotic disease [10,37].

Moreover, in diabetic HIV-infected patients, although levels of soluble markers were increased as compared to HIV-negative controls, these values were lower compared to the HIV-infected group without diabetes, suggesting that, in our HIV-infected patients, drug-induced glycaemic control contributed to a reduction in the inflammatory burden, leading to CD40L and DKK-1 release.

This hypothesis is supportedby previous studies in HIV-negative diabetic subjects, showing that improved glycaemic control is associated with reduced levels of both circulating CD40L and DKK-1 [23,34].

CVD is the second leading non-HIV-related cause of death in the USA (~ 15%) and the third leading non-HIV-related cause of death in Europe (~ 8%) [38,39]. A number of factors may contribute to CVD risk in untreated HIV infection, including inflammation, platelet activation, endothelial dysfunction and immune activation [40]. Thus, HIV infection requires a multidisciplinary approach, with attention to the long-term effects of antiretroviral therapies and the consequences of HIV-related chronic inflammation which can lead to complications such as metabolic and CV diseases.

Another interesting finding that emerged from the study is that soluble inflammatory proteins examined in HIV-infected patients were associated with circulating levels of T and B lymphocytes and with platelet count. A previous study showed that high levels of CD40L were present in the activated T cells of HIV-infected patients, and this was explained by chronic inflammatory activation in these patients [25].

Platelets have been shown to circulate in an activated state in HIV infection [41]. Along with their role in vessel wall adhesion and aggregation, activated platelets can interact with monocytes, resulting in platelet–monocyte complexes (PMCs), which are found at increased levels in myocardial infarction (MI) in the general population [42]. Increased levels of PMCs have also been demonstrated in HIV infection, where their percentage correlates with platelet activation [43]. P-selectin, which interacts with P-selectin glycoprotein ligand-1 (PSGL-1) on leucocytes, preferentially monocytes, and CD40 ligand, which promotes platelet–leucocyte adhesion, play an important role in the formation of these PMCs [44], with platelet activation rather than monocyte activation correlating with PMC formation *in vitro* [43].

CD40L, which was identified as a co-stimulatory molecule, is expressed on T cells and platelets. Soluble CD40L, which is mainly produced by platelets after activation, degranulation and cleavage, modulates platelet–platelet interactions in an auto-amplification loop and activates leucocytes, enhancing platelet–leucocyte and leucocyte–endothelium interactions. In this study, a correlation has been shown between CD40L and DKK-1 and platelet count and between CD40L and P-selectin and T lymphocyte count, in HIV-infected subjects with undetectable viraemia on stable cART, further reinforcing the interplay between immune and platelet activation and inflammation in this setting.

In HIV infection, low CD4 cell count and traditional CVD risk factors are predictive of primary MI; in fact, the relative risk increases as CD4 count declines. Also, a detectable viral load is associated with a higher risk of MI [45]. However, biomarkers of inflammation and immune activation such as CRP, microalbuminuria, cystatin C and soluble CD14 are elevated in HIV-infected patients even when on cART [31,46–48], reflecting residual inflammatory/immune activation. Consistent with these findings, we did not find any correlation between the evaluated parameters and viral load or CD4 count, suggesting that, after viral suppression, the detrimental factors leading to atherothrombotic events are not completely normalized, and may reflect the excess CV risk observed in HIV-infected patients even when successfully treated with ART [6,7,49,50].

The present study has some limitations, such as the cross-sectional nature of the main study and the fact that it was monocentric, the low sample size and the lack of evaluation of other markers with *in vivo* effects that may provide

further insight into the mechanisms linking DKK-1, CD40 and P-selectin with DM and hypertension development and their complications. Finally, additional mechanistic studies are needed to further elucidate the roles of these markers and their signalling in populations with severe immunodeficiency such as HIV-positive patients.

In conclusion, we found that HIV infection was associated with higher CD40L and DKK-1 levels, which were related to a higher degree of inflammation in HIV-infected patients than in HIV-negative controls. In addition, we showed that two major risk factors, diabetes and hypertension, combined to worsen inflammatory marker levels in this population. The relevance of these soluble molecules as markers of residual CV risk should be assessed, and novel therapeutic strategies should be explored to target and possibly reverse these biochemical abnormalities and the underlying pathophysiology.

Acknowledgements

Conflicts of interest: The authors have no conflicts of interest to report.

Financial disclosure: No financial support was received for the study.

References

1 Antiretroviral Therapy Cohort Collaboration. Causes of death in HIV-1-infected patients treated with antiretroviral therapy, 1996–2006: collaborative analysis of 13 HIV cohort studies. *Clin Infect Dis* 2010; **50**: 1387–1396.

2 Freiberg MS, Chang CC, Kuller LH *et al.* HIV infection and the risk of acute myocardial infarction. *JAMA Intern Med* 2013; **173**: 614–622.

3 Islam FM, Wu J, Jansson J, Wilson DP. Relative risk of cardiovascular disease among people living with HIV: a systematic review and meta-analysis. *HIV Med* 2012; **13**: 453–468.

4 Santilli F, D'Ardes D, Guagnano MT, Davi G. Metabolic syndrome: sex-related cardiovascular risk and therapeutic approach. *Curr Med Chem* 2017; **24**: 2602–2627.

5 Falasca K, Ucciferri C, Manzoli L *et al.* Metabolic syndrome and cardiovascular risk in HIV-infected patients with lipodystrophy. *Int J Immunopathol Pharmacol* 2007; **20**: 519–527.

6 Falasca K, Ucciferri C, Mancino P *et al.* Cystatin C, adipokines and cardiovascular risk in HIV infected patients. *Curr HIV Res* 2010; **8**: 405–410.

7 Vecchiet J, Ucciferri C, Falasca K, Mancino P, Di Iorio A, De Caterina R. Antihypertensive and metabolic effects of telmisartan in hypertensive HIV-positive patients. *Antivir Ther* 2011; **16**: 639–645.

8 De Socio GV, Ricci E, Maggi P *et al.* Time trend in hypertension prevalence, awareness, treatment, and control in a contemporary cohort of HIV-infected patients: the HIV and Hypertension Study. *J Hypertens* 2017; **35**: 409–416.

9 Boyd MA, Mocroft A, Ryom L *et al.* Cardiovascular disease (CVD) and chronic kidney disease (CKD) event rates in HIV-positive persons at high predicted CVD and CKD risk: a prospective analysis of the D:A:D observational study. *PLoS Med* 2017; **14**: e1002424.

10 Ucciferri C, Falasca K, Vecchiet J. Hypertension in HIV: management and treatment. *AIDS Rev* 2017; **19**: 198–211.

11 Martin-Iguacel R, Negredo E, Peck R, Friis-Moller N. Hypertension is a key feature of the metabolic syndrome in subjects aging with HIV. *Curr Hypertens Rep* 2016; **18**: 46.

12 Brown TT, Cole SR, Li X *et al.* Antiretroviral therapy and the prevalence and incidence of diabetes mellitus in the multicenter AIDS cohort study. *Arch Intern Med* 2005; **165**: 1179–1184.

13 Armah KA, Chang CC, Baker JV *et al.* Prehypertension, hypertension, and the risk of acute myocardial infarction in HIV-infected and -uninfected veterans. *Clin Infect Dis* 2014; **58**: 121–129.

14 Gresele P, Falcinelli E, Sebastiano M, Baldelli F. Endothelial and platelet function alterations in HIV-infected patients. *Thromb Res* 2012; **129**: 301–308.

15 Teofili L, Iachininoto MG, Capodimonti S *et al.* Endothelial progenitor cell trafficking in human immunodeficiency virus-infected persons. *AIDS* 2010; **24**: 2443–2450.

16 Vecchiet J, Iachininoto MG, Capodimonti S *et al.* Effect of antiviral therapy on pro-angiogenic hematopoietic and endothelial progenitor cells in HIV-infected people. *Thromb Res* 2013; **131**: 238–243.

17 Ucciferri C, Falasca K, Mancino P, Di Iorio A, Vecchiet J. Microalbuminuria and hypertension in HIV-infected patients: a preliminary study of telmisartan. *Eur Rev Med Pharmacol Sci* 2012; **16**: 491–498.

18 Santilli F, Basili S, Ferroni P, Davi G. CD40/CD40L system and vascular disease. *Intern Emerg Med* 2007; **2**: 256–268.

19 Henn V, Steinbach S, Buchner K, Presek P, Kroczek RA. The inflammatory action of CD40 ligand (CD154) expressed on activated human platelets is temporally limited by coexpressed CD40. *Blood* 2001; **98**: 1047–1054.

20 Ferroni P, Santilli F, Guadagni F, Basili S, Davi G. Contribution of platelet-derived CD40 ligand to inflammation, thrombosis and neoangiogenesis. *Curr Med Chem* 2007; **14**: 2170–2180.

21 Cipollone F, Chiarelli F, Davi G *et al.* Enhanced soluble CD40 ligand contributes to endothelial cell dysfunction *in vitro* and monocyte activation in patients with diabetes mellitus: effect of improved metabolic control. *Diabetologia* 2005; **48**: 1216–1224.

22 Ferroni P, Guagnano MT, Falco A *et al*. Association of low-grade inflammation and platelet activation in patients with hypertension with microalbuminuria. *Clin Sci (Lond)* 2008; **114**: 449–455.
23 Lattanzio S, Santilli F, Vitale G *et al*. Soluble CD40L in Mediterranean spotted fever: relation to oxidative stress and platelet activation. *Thromb Res* 2010; **125**: 362–364.
24 Kornbluth RS. The emerging role of CD40 ligand in HIV infection. *J Leukoc Biol* 2000; **68**: 373–382.
25 Sipsas NV, Sfikakis PP, Kontos A, Kordossis T. Levels of soluble CD40 ligand (CD154) in serum are increased in human immunodeficiency virus type 1-infected patients and correlate with CD4(+) T-cell counts. *Clin Diagn Lab Immunol* 2002; **9**: 558–561.
26 Sorensen LL, Kjoller-Hansen L, Jensen GV, Poulsen B, Fisker D, Carstensen S. Percutaneous coronary intervention without onsite cardiac surgery. *Dan Med Bull* 2010; **57**: A4202.
27 Pereira C, Schaer DJ, Bachli EB, Kurrer MO, Schoedon G. Wnt5A/CaMKII signaling contributes to the inflammatory response of macrophages and is a target for the antiinflammatory action of activated protein C and interleukin-10. *Arterioscler Thromb Vasc Biol* 2008; **28**: 504–510.
28 Mora S, Puzzovio M, Giacomet V *et al*. Sclerostin and DKK-1: two important regulators of bone metabolism in HIV-infected youths. *Endocrine* 2015; **49**: 783–790.
29 Yu C, Seaton M, Letendre S, Heaton R, Al-Harthi L. Plasma dickkopf-related protein 1, an antagonist of the Wnt pathway, is associated with HIV-associated neurocognitive impairment. *AIDS* 2017; **31**: 1379–1385.
30 Nkambule BB, Davison G, Ipp H. The value of flow cytometry in the measurement of platelet activation and aggregation in human immunodeficiency virus infection. *Platelets* 2015; **26**: 250–257.
31 Falasca K, Di Nicola M, Porfilio I *et al*. Predictive factors and prevalence of microalbuminuria in HIV-infected patients: a cross-sectional analysis. *BMC Nephrol* 2017; **18**: 255.
32 Piepoli MF, Hoes AW, Agewall S *et al*. 2016 European Guidelines on cardiovascular disease prevention in clinical practice: the Sixth Joint Task Force of the European Society of Cardiology and Other Societies on Cardiovascular Disease Prevention in Clinical Practice (constituted by representatives of 10 societies and by invited experts) Developed with the special contribution of the European Association for Cardiovascular Prevention & Rehabilitation (EACPR). *Eur Heart J* 2016; **37**: 2315–2381.
33 Akhabue E, Rittner SS, Carroll JE *et al*. Implications of American College of Cardiology/American Heart Association (ACC/AHA) Cholesterol Guidelines on Statin Underutilization for Prevention of Cardiovascular Disease in Diabetes Mellitus Among Several US Networks of Community Health Centers. *J Am Heart Assoc* 2017; **6**: pii:e005627.
34 Lattanzio S, Santilli F, Liani R *et al*. Circulating dickkopf-1 in diabetes mellitus: association with platelet activation and effects of improved metabolic control and low-dose aspirin. *J Am Heart Assoc* 2014; **3**: pii: e001000.
35 Santilli F, Davi G, Consoli A *et al*. Thromboxane-dependent CD40 ligand release in type 2 diabetes mellitus. *J Am Coll Cardiol* 2006; **47**: 391–397.
36 Santilli F, Paloscia L, Liani R *et al*. Circulating myeloid-related protein-8/14 is related to thromboxane-dependent platelet activation in patients with acute coronary syndrome, with and without ongoing low-dose aspirin treatment. *J Am Heart Assoc* 2014; **3**: pii: e000903.
37 Ucciferri C, Falasca K, Vignale F, Di Nicola M, Vecchiet J. Long term effect of telmisartan in HIV-positive male patients with high blood pressure. *Braz J Infect Dis* 2015; **19**: 668–669.
38 Palella FJ Jr, Baker RK, Moorman AC *et al*. Mortality in the highly active antiretroviral therapy era: changing causes of death and disease in the HIV outpatient study. *J Acquir Immune Defic Syndr* 2006; **43**: 27–34.
39 Lewden C, May T, Rosenthal E *et al*. Changes in causes of death among adults infected by HIV between 2000 and 2005: the "Mortalite 2000 and 2005" surveys (ANRS EN19 and Mortavic). *J Acquir Immune Defic Syndr* 2008; **48**: 590–598.
40 Baker JV, Lundgren JD. Cardiovascular implications from untreated human immunodeficiency virus infection. *Eur Heart J* 2011; **32**: 945–951.
41 Mayne E, Funderburg NT, Sieg SF *et al*. Increased platelet and microparticle activation in HIV infection: upregulation of P-selectin and tissue factor expression. *J Acquir Immune Defic Syndr* 2012;**59**:340–346.
42 Sarma J, Laan CA, Alam S, Jha A, Fox KA, Dransfield I. Increased platelet binding to circulating monocytes in acute coronary syndromes. *Circulation* 2002; **105**: 2166–2171.
43 Singh MV, Davidson DC, Kiebala M, Maggirwar SB. Detection of circulating platelet-monocyte complexes in persons infected with human immunodeficiency virus type-1. *J Virol Methods* 2012; **181**: 170–176.
44 Blann AD, Nadar SK, Lip GY. The adhesion molecule P-selectin and cardiovascular disease. *Eur Heart J* 2003; **24**: 2166–2179.
45 Drozd DR, Kitahata MM, Althoff KN *et al*. Increased risk of myocardial infarction in HIV-infected individuals in North America compared with the general population. *J Acquir Immune Defic Syndr* 2017; **75**: 568–576.
46 Wada NI, Jacobson LP, Margolick JB *et al*. The effect of HAART-induced HIV suppression on circulating markers of inflammation and immune activation. *AIDS* 2015; **29**: 463–471.
47 Falasca K, Ucciferri C, Teofili L *et al*. Short communication: proangiogenic hematopoietic cells in acute HIV infection. *AIDS Res Hum Retroviruses* 2013; **29**: 307–310.

48 Falasca K, Vecchiet J, Ucciferri C, Di Nicola M, D'Angelo C, Reale M. Effect of probiotic supplement on cytokine levels in HIV-infected individuals: a preliminary study. *Nutrients* 2015; **7**: 8335–8347.

49 Petoumenos K, Reiss P, Ryom L *et al.* Increased risk of cardiovascular disease (CVD) with age in HIV-positive men: a comparison of the D:A: D CVD risk equation and general population CVD risk equations. *HIV Med* 2014; **15**: 595–603.

50 Viskovic K, Zidovec Lepej S, Gorenec A *et al.* Cardiovascular markers of inflammation and serum lipid levels in HIV-infected patients with undetectable viremia. *Sci Rep* 2018; **8**: 6113.

