HIV is associated with thrombophilia and high D-dimer in children and adolescents

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Background and objective: Atherosclerosis and other cardiovascular diseases associated with thrombosis appear more relevant and anticipated in HIV-infected patients after combination antiretroviral therapy (cART) has reduced AIDS-related diseases and has improved survival. The association between viral replication and coagulation abnormalities in a cohort of HIV-infected children and adolescents was investigated here.

Methods: Protein S, protein C anticoagulant and antithrombin activity, together with fibrinogen, D-dimer, high-sensitive C-reactive protein and homocysteine were assayed in a cross-sectional study among a cohort of HIV-infected children and adolescents. Results in patients with high viral load (HVL, HIV-RNA > 1000 copies/ml) were compared with those in patients with a lower replication (LVL), adjusting for other demographic, clinical and therapeutic covariates.

Results: Eighty-eight patients (mean age 13.5 years, CD4 30%, 72% with LVL) were enrolled. A prevalence of protein S and protein C deficiency of 51 and 8% was, respectively, found. HVL group compared to LVL showed a significant reduction of protein S, protein C and antithrombin activities, and an increase of D-dimer levels. The independent association of HVL with decreased protein S activity (−11.2%, $P = 0.04$) and increased D-dimer levels (+0.13 μg/ml, $P = 0.004$) was confirmed in the multivariate model.

Conclusions: HIV-infected children and adolescents present high prevalence of thrombophilic abnormalities. The multivariate model confirmed that high viral replication is independently associated with decrease of protein S and increase of D-dimer, suggesting the advantage of suppressive therapy on coagulation homeostasis and the opportunity of an active control of cardiovascular risk factors starting at a younger age.

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Introduction

The introduction of effective combination antiretroviral therapy (cART) preserving immune function and decreasing acquired immune-deficiency syndrome (AIDS)-related morbidity has changed the natural history of human immunodeficiency virus 1 (HIV-1) infection in adults and children from a lethal to a chronic disease [1,2]. Improved survival has been followed by an increased and anticipated prevalence of non-AIDS-related conditions

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such as atherosclerosis and other cardiovascular diseases (CVDs) associated with arterial and venous thrombosis [3,4]. Atherosclerosis and CVD are multifactorial diseases, whose associated histological lesions begin early in life [5]. The role of cART in increasing the risk of CVD acting on traditional risk factors (impaired glucose metabolism, hypercholesterolemia, hypertriglyceridaemia), or as an independent non-mediated risk factor, has been extensively investigated in adults [3]. Duration of exposure to cART [6] and to protease inhibitors [7] were associated to myocardial infarction, as the recent use of nucleoside analogue reverse transcriptase inhibitors (NRTI) abacavir (ABC) or didanosine (ddI) [8], even if their exact role is currently a matter of debate [9]. The strong and independent role of HIV replication has been recently highlighted by the unexpected results of Strategies for Management of Anti-Retroviral Therapy (SMART) trial. This study showed an increased mortality from all causes and from CVD in patients with CD4-guided treatment interruptions vs. those with continuous ART and viral suppression [10,11].

Viral replication could possibly have a pathogenic role by increasing thrombotic risk. A sub-study of the trial showed that higher D-dimer, a fibrin degradation product currently used in diagnosis of acute thrombosis, was associated with high HIV viral load, and baseline D-dimer levels above 0.18 μg/ml were significantly associated with all-cause and CVD-related mortality [12].

HIV-infected adults have an increased risk of venous and arterial thrombosis, with thrombophilic abnormalities more frequently associated with AIDS progression [13–18]. Protein S and protein C resulted deficient in adult HIV patients [19], and only one study was conducted in children, in a small cohort before the introduction of cART, showing similar results [20].

The aim of this study was to investigate, for the first time in HIV-infected children and adolescents in the cART era, the alterations of anticoagulant and fibrinolysis markers, and a specific role of HIV replication.

Patients and methods

Study population

A cross-sectional study was conducted between December 2007 and October 2008 among the cohort of HIV-infected children and adolescents regularly followed at Bambino Gesù Children Hospital of Rome, Italy. The study was approved by the local Ethical Committee, and informed consent obtained from all participants. Detailed information about demographic data (age, sex, ethnicity), clinical status, HIV treatment history, previous episodes of venous and arterial thrombosis, exposures to risk factors for thrombosis and atherosclerosis (hypertension, oral contraceptives, smoke, pregnancy, surgery in the previous 6 months), were collected by physicians via questionnaire and review of medical records. Treatment history referred to the whole life of the patient, defining cART as any treatment combination comprising at least two different antiretroviral classes. Patients were considered eligible if they had a documented HIV infection and were not co-infected with hepatitis B (HBV) or hepatitis C virus (HCV). Clinical stage of HIV infection has been assessed according to the classification of the Centres for Disease Control (CDC) for adults and adolescents [21] and for children [22]. Blood samples for HIV RNA viral load and thrombophilic tests were simultaneously collected.

Since pregnancy and puerperium are important risk factors for thrombotic events for HIV-negative and HIV-infected women [23], we also purposely investigated the prevalence of thrombophilia in all the girls at procreative age (above 16 years) of our cohort.

Laboratory investigation

HIV infection has been documented by two different samples: positive for serology and/or HIV-RNA. Plasma HIV-RNA has been determined using a quantitative b-DNA assay (Quantiplex HIVRNA 2.0 bDNA Assay; Chiron Diagnostic Corporation, USA) with a lower limit quantification of 50 copies/ml. Lymphocyte subsets have been analysed using flow cytometry on peripheral blood mononuclear cells (PBMCs) in accordance with standard protocols with a flow cytometer (FACScan; Becton Dickinson, USA).

Total cholesterol, low-density lipoproteins (LDLs), high-density lipoproteins (HDLs) and triglycerides have been measured by standard methods.

Thrombophilic tests including protein S, protein C, antithrombin and fibrinogen, have been evaluated by means of automated immunometric and functional assays (Protein S clotting, Protein C chromogen, Antithrombin III, Fibrinogen; STA Compact, Roche Diagnostics, Milan). D-dimer was evaluated by automated latex-enhanced immunoturbidimetric assay (STA Liatest, Roche Diagnostics, Basel, Switzerland). Homocysteine was analysed by chemiluminescence assay (Advia Centaur; Siemens Diagnostics, Deerfield, Illinois, USA) and high-sensitive C-reactive protein (hsCRP) was measured using a latex-enhanced immunoturbidimetric method (Advia 2400, Siemens Diagnostics).

We defined protein S deficiency as a decreased protein S activity less than 70%, and protein C deficiency as a decreased protein C activity less than 70%. Deficiency of antithrombin was defined as percentage of activity less than 75%. We considered increased D-dimer values higher than 0.18 and 0.34 μg/ml, levels which were associated with mortality [odds ratio (OR) > 3] in HIV

adults [12]. Factor V Leiden has been demonstrated by polymerase chain reactions.

Statistical analysis

Data for continuous variables are expressed as mean ± standard deviation (SD), and categorical data as counts and percentages.

Values of coagulation and inflammatory biomarkers (protein S, protein C, D-dimer, antithrombin, fibrinogen, homocysteine, hsCRP) were compared between high viral load (HVL, >1000 copies/ml) and low viral load (LVL, <1000 copies/ml) population. Since we wanted to assess primarily a role of high uncontrolled viral replication in determining coagulation alterations, and adherence problems with episodes of missing pills occur frequently in children and adolescents resulting in isolated low-level rebounds or 'blips', we considered 1000 copies/ml as appropriate cut-off between low and high-level viraemia, as also suggested by recent guidelines [24]. Univariate analysis used either ANOVA or Wilcoxon rank sum test for continuous data, whereas chi-squared test or Fisher's exact test were used for categorical data.

A multivariate model was developed in order to exclude were other factors that could have acted as confounding variables and affected the association between viral load and biomarker levels. Demographic factors (age, sex, smoke, ethnicity), laboratory (total cholesterol and HDL), HIV-related (history of clinical AIDS, current value of CD4 cells, current use of ART, current use of protease inhibitors, ABC and ddI, total duration of cART and protease inhibitor usage) were considered. A two-step process was used: in the multivariate linear regression analysis, difference of every coagulation or inflammatory biomarker level (protein S, protein C, antithrombin, D-dimer, fibrinogen, hsCRP, homocysteine) in HVL group vs. LVL group was adjusted for all the covariates that firstly were associated with the same biomarker with a P value less than 0.20 at the univariate analysis. Levels of biomarkers whose alteration were significantly associated with HVL were plotted in a linear regression graph and related to continuous values of HIV viral load.

A two-tailed P value of less than 0.05 was considered statistically significant in all the analyses. Statistical analysis was done with Stata 10.1 (Stata Corporation, College Station, Texas, USA).

Results

Description of the cohort

The study has been proposed to 90 patients, only two patients refused due to the additional amount of blood extraction required. A total number of 88 patients were evaluated, and the 90% acquired HIV infection by vertical transmission. No patient had hypertension, diabetes, hepatic impairment, a history of surgery within 6 months from the study visit and/or any history of symptomatic thrombosis, or other clinical and laboratory evidence of CVD; no girl was pregnant or used oral contraceptives. Demographic, clinical, therapeutic, and laboratory characteristics of the population at enrolment are summarized in Table 1. Fifty-nine percent were female, and the mean age of the cohort was 13.5 years (range 3–25). The mean value of CD4+ T-lymphocytes was

Table 1. Characteristics of patients, overall and in high and low viraemic group.

	Total ($n=88$)	HVL ($n=25$)	LVL ($n=63$)	P value
Sex female *n* (%)	52 (59.1%)	18 (72.0%)	34 (54.0%)	0.12
Age mean years (±SD)	13.5 (±4.8)	14.3 (±4.3)	13.1(±5.0)	0.31
Smoke	18 (20.5%)	7 (28.0%)	11 (17.5%)	0.27
Ethnicity (white)	73 (83.0%)	23 (92.0%)	50 (79.4%)	0.26
Vertical infection	79 (89.8%)	21 (84.0%)	58 (92.1%)	0.45
History of AIDS (CDC class C)	27 (30.7%)	6 (24.0%)	21 (33.3%)	0.56
CD4% mean (±SD)	29.9 (±9.0)	25.1 (±9.5)	31.8 (±8.2)	0.002
On ART *n* (%)	76 (86.4%)	14 (56.0%)	62 (98.4%)	<0.001
With PI *n* (%)	46 (52.3%)	10 (40.0%)	36 (57.1%)	0.15
With ABC *n* (%)	48 (54.5%)	5 (20.0%)	43 (68.3%)	<0.001
With ddI *n* (%)	11 (12.5%)	2 (8.0%)	9 (14.3%)	0.68
Years of cART mean (±SD)	5.1 (±3.0)	3.3 (±3.2)	5.8 (±2.7)	<0.001
Years of PI mean (±SD)	4.3 (±3.3)	2.5 (±3.2)	5.0 (±3.3)	<0.001
HIV-RNA mean Log10 (±SD)	2.5 (±1.2)	4.3 (±0.6)	1.8 (±0.3)	<0.001
Lipid profile				
Total cholesterol mean (mg/dl) (±SD)	158 (±35)	133 (±27)	168 (±33)	<0.001
LDL mean (mg/dl) (±SD)	81 (±27)	68 (±23)	86 (±26)	0.003
HDL mean (mg/dl) (±SD)	53 (±18)	44 (±15)	57 (±19)	0.001
TG mean (mg/dl) (±SD)	117 (±86)	107 (±102)	121 (±79)	0.12

ABC, abacavir; ART, antiretroviral therapy; cART, combination antiretroviral therapy; ddI, didanosine; HDL, high-density lipoprotein; HVL, higher viral load group: HIV-RNA > 1000 copies/ml; LDL, low-density lipoprotein; LVL, lower viral load group: HIV-RNA < 1000 copies/ml; PI, protease inhibitor; TG, triglyceride.

29.9% (623 cells/μl); 31% of patients had a history of clinical AIDS (CDC class C). The large majority of patients were on ART therapy (86%), of whom 79% were on cART and the rest on a simplification regimen based only on NRTI [25]. Seventy-two percent of the whole cohort had a LVL (HIV RNA < 1000 copies/ml) and 58% (67% of the treated patients) had a viral load less than 50 copies/ml. The HVL group had a significant lower value of CD4 cells (mean% 25.1 vs. 31.8; $P < 0.001$ and was less frequently on ART (56.0 vs. 98.4%; $P < 0.001$); moreover, patients in HVL group were less frequently on treatment with ABC (20.0 vs. 68.3%; $P < 0.001$), had a lower level of total cholesterol (133 vs. 168 mg/dl; $P < 0.001$), LDL (68 vs. 86 mg/dl; $P = 0.003$), HDL (44 vs. 57 mg/dl; $P = 0.001$). On the contrary, the LVL group presented a longer cART treatment history (5.8 vs. 3.3 years; $P < 0.001$) and a longer treatment duration with protease inhibitor (5.0 vs. 2.5 years; $P < 0.001$).

Markers of thrombophilia and inflammation

Results of assays on coagulation and inflammation markers are shown in Table 2. In the whole cohort the prevalence of protein S and protein C deficiency was 51 and 8%, respectively. Only one patient had antithrombin deficiency. D-dimer mean level was 0.24 μg/ml. Three patients (two in the LVL and one in the HVL group) had the factor V Leiden mutation.

We evaluated 23 women at procreative age (mean age 18 years, range 16–23): they showed a frequency of protein S, protein C and antithrombin deficiency of 70, 9, and 4%, respectively.

Protein S activity in the HVL group was significantly lower than in the LVL group (mean activity 57.6 vs. 75.3%; $P < 0.001$), and with a higher prevalence of deficiency (76.0 vs. 41.3%; $P = 0.003$).

In HVL group protein C (92.0 vs. 101.9%; $P = 0.007$) and antithrombin (107.5 vs. 115.5%; $P = 0.006$) mean activity was lower than amongst the LVL patients. The HVL group presented a significantly higher level of D-dimer (0.34 vs. 0.21 μg/ml, $P = 0.02$), with a prevalence of values greater than 0.18 μg/ml (72.0 vs. 49.2%; $P = 0.05$) and greater than 0.34 μg/ml (40.0 vs. 11.1%; $P = 0.004$), significantly higher than LVL. No significant variation between the two groups was observed regarding fibrinogen, homocysteine, hsCRP.

Higher viral replication was associated with reduction of protein S activity even in the multiple regression model (−11.2% in HVL group, $P = 0.04$); in the same model even patients with CD4 less than 25% had a significant reduction of protein S activity (−10.7%, $P = 0.05$).

Higher viral replication was also found to be associated with higher D-dimer in the multivariate model (+0.13 μg/ml in HVL group, $P = 0.004$). Other parameters did not show any significant difference between the two groups in the multivariate model.

Figures 1 and 2 graphically show the association of protein S and D-dimer values with HIV RNA considered as continuous variable, after adjustment for variables associated with a $P < 0.20$ at univariate analysis.

Table 2. Thrombophilic and inflammation markers in the whole cohort, and in higher and lower viraemic group.

	Total ($N = 88$)	Viraemia		Univariate analysis *P* value[a]	Multivariate analysis Adjusted difference (HVL-LVL) and *P* value[c]
		HVL ($n = 25$)	LVL ($n = 63$)		
Protein S					
Mean activity (±SD)	70.2 (±20.8)	57.6 (±21.7)	75.3 (±18.2)	**<0.001**	**−11.2 *P* = 0.04**
n (%) with deficiency	45 (51.1%)	19 (76.0%)	26 (41.3%)	**0.003**	
Protein C					
Mean activity (±SD)	99.1 (±23.7)	92.0 (±14.7)	101.9 (±26.0)	**0.007**	−6.2 *P* = 0.35
n (%) with deficiency	7 (8.0%)	0 (0.0%)	7 (11.1%)	0.53[b]	
Antithrombin					
Mean activity (±SD)	113.3 (±13.0)	107.5 (±9.2)	115.5 (±13.6)	**0.006**	−4.2 *P* = 0.31
n (%) with deficiency	1 (1.1%)	0 (0.0%)	1 (1.6%)	0.99[b]	
D-dimer					
Mean μg/ml (±SD)	0.24 (±0.17)	0.34 (±0.25)	0.21 (±0.10)	**0.02**	**+0.13 *P* = 0.004**
n (%) of increased (>0.18 μg/ml)	49 (55.7%)	18 (72.0%)	31 (49.2%)	**0.05**	
n (%) of increased (>0.34 μg/ml)	17 (19.3%)	10 (40.0%)	7 (11.1%)	**0.004**	
Fibrinogen (mg/dl) mean (±SD)	315.9 (± 67.2)	328.8 (± 58.3)	310.7 (± 70.2)	0.25	N/A[d]
Homocysteine (μmol/l) mean (±SD)	11.1 (± 9.9)	10.4 (± 4.1)	11.4 (± 11.4)	0.96	+0.44 *P* = 0.90
hsCRP (mg/dl) mean (±SD)	0.24 (± 0.35)	0.27 (± 0.47)	0.23 (± 0.30)	0.98	+0.04 *P* = 0.57

hsCRP, high-sensitive C-reactive protein; HVL, higher viral load group: HIV-RNA > 1000 copies/ml; LVL, lower viral load group: HIV-RNA < 1000 copies/ml; SD, standard deviation. Statistically significant differences ($P < 0.05$) are reported in bold.

[a]Unadjusted two-tailed *P* value.

[b]Two-tailed *P* value obtained by Fisher's exact test corrected (adding 0.5 in each cell of the table, since a value resulted null).

[c]Linear regression was performed with adjustment for variables associated with a $P < 0.20$ at univariate analysis among demographic (age, sex, smoke, ethnicity), laboratory (total cholesterol and HDL), HIV-related (history of clinical AIDS, current value of CD4 cells, current use of ART, current use of PI, ABC and ddI, total duration of cART and PI usage) variables.

[d]N/A, multivariate analysis not conducted since no other variable resulted associated with a $P < 0.20$ at univariate analysis.

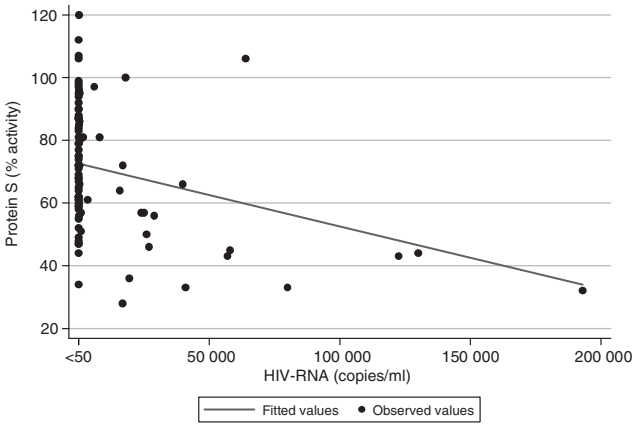

Fig. 1. Levels of protein S activity related with viral load as continuous variable. Association of protein S anticoagulant with viral load. Pearson correlation coefficient $R^2 = 0.22$ ($P < 0.001$). Linear regression was performed with adjustment for variables associated with a $P < 0.20$ at univariate analysis.

Discussion

The introduction of effective cART has been followed by a dramatic change of the spectrum of HIV clinical presentation, with improved survival and emergent relevance of non-AIDS diseases, including atherosclerosis and other thrombosis-associated CVD. Arterial and venous thrombosis are multifactorial diseases that share common risk factors and are often linked together [26,27]: they usually become clinically evident during middle and late adulthood, but are related to histological alterations beginning earlier in lifetime [5]. Thrombophilic abnormalities, like deficiency of protein S and protein C anticoagulant, are among the major factors associated not only with venous, but also with arterial thrombo-embolism in younger people [28,29].

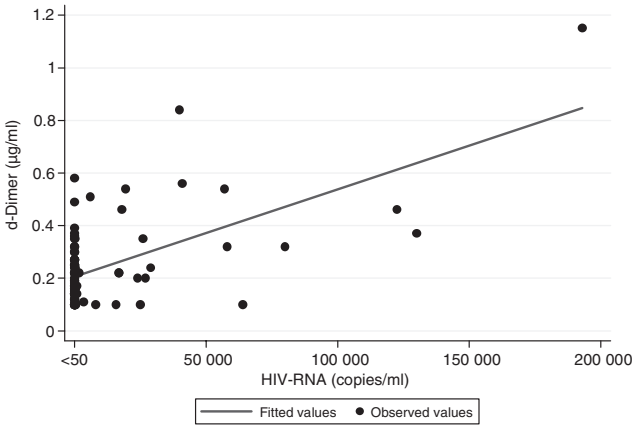

Fig. 2. Levels of D-dimer related with viral load as continuous variable. Association of D-dimer with viral load. Pearson correlation coefficient $R^2 = 0.37$ ($P < 0.001$). Linear regression was performed with adjustment for variables associated with a $P < 0.20$ at univariate analysis.

The role of ART therapy and HIV replication as CVD risk factors has been extensively studied in adults. ART has been associated with an increased risk of atherosclerosis in several studies. Large observational studies have shown that duration of exposure to cART [6], to protease inhibitors [7], and recent use of abacavir (ABC) or didanosine (ddI) [8] increase the risk of myocardial infarction. The exact role of ddI and ABC is, however, still a matter of debate since possible channelling biases have been proposed [9].

The unexpected results of the SMART trial re-focused the attention on the role of HIV replication on CVD. The study showed an increased mortality, from all causes and from CVD, in patients in CD4-guided treatment interruptions arm compared with patients in viral suppression arm [10]. In patients discontinuing ART the levels of the inflammation markers increased and the restarting of treatment was associated with their reduction [12].

The observation is consistent with several recent studies highlighting the role of chronic infections and inflammation on the pathogenesis of CVD and coagulation disorders [30,31]. HIV replication itself is in fact a cause of chronic immune activation, inflammation, endothelium and lipid metabolism dysfunction, and HIV-infected adults have an increased risk for venous and arterial thrombosis, as well as for thrombophilic abnormalities [13–19]. Protein S and protein C were deficient in 60–67% and 9–25%, respectively, of HIV adults, with protein S reduction also associated with advanced disease [19]. A sub-study of the SMART trial showed that high levels of D-dimer, a fibrin degradation product currently used for the diagnosis of acute thrombosis, were related to high HIV viral load; moreover patients with high baseline D-dimer levels had an increased risk for all-cause and CVD-associated mortality [12].

The role of HIV infection in children and adolescents as a risk factor for coagulation alteration, occurrence of thrombosis, and CVD, is still unknown. A study conducted in a small cohort of children, before the introduction of cART, showed a 76% prevalence of protein S deficiency [20]. The aim of this study was to investigate the prevalence of thrombophilia and the role of viral replication on coagulation abnormalities in a cohort of HIV-infected children and adolescents during cART era. The studied population presented a high prevalence of thrombophilic abnormalities. Protein S deficiency has emerged as a highly frequent alteration (about half of the patients involved), whereas protein C and antithrombin deficiency has been less common. These frequencies were slightly less than those observed in adults [19]. Moreover, mean D-dimer levels observed in our cohort (0.24 µg/ml) were higher than the value (0.18 µg/ml) associated in adults with a 3.5 OR-mortality [12]. High HIV viral load was related with a

significant decrease of all the anticoagulant proteins (protein S, protein C, antithrombin) and an increase of D-dimer levels. Patients with higher viraemia (HVL, HIV-RNA > 1000 copies/ml) presented a hazard ratio for having D-dimer higher than 0.18 μg/ml; and 0.34 μg/ml, respectively, of 1.46 ($P = 0.05$) and 3.60 ($P = 0.004$) in comparison with patients with lower viraemia (LVL). Homocysteine and hsCRP were unchanged. The role of HIV replication was also confirmed in a multiple regression model, considering as covariates demographic, laboratory, and HIV-related factors: patients with higher viraemia showed a 11.2% decrease of protein S activity ($P = 0.04$) and a 0.13 μg/ml increase of D-dimer levels ($P = 0.004$) compared to patients with low viral replication (LVL).

The study, given the low mean age of the cohort, highlights the direct role of HIV replication on coagulation disorders excluding the possible confounding role of major known risk factors for thrombosis and CVD, like hypertension, diabetes, and history of clinical thrombotic event. Furthermore, our analysis took into account the putative confounding action of other factors associated with an increased risk of thrombosis and CVD disease both in the general population (smoke, age, dyslipidaemia), and HIV-infected population (cumulative use of cART and protease inhibitor, actual use of ABC and ddI, dyslipidaemia). Nevertheless the study has some limitations since it is a cross-sectional study and the power for analysis of all variables considered has been limited by the relative small amount of observations. Prospective studies are needed to confirm and investigate the clinical implications of our observations.

The pathogenetic mechanism of viral replication on thrombosis remains a matter of study. A link between viral replication, chronic inflammation and thrombosis via endothelial activation has been proposed [32]. HIV can increase the risk of thrombosis directly and indirectly eliciting the release of pro-coagulant factors like von Willebrand factor, tumor necrosis factor alpha, plasminogen activator inhibitor-1, IL-1, IL-6, and tissue factor [33–36]. A possible role in the pathogenesis of acquired protein S deficiency could be also played by antiphospholipid antibodies and increased lipidaemia [37,38]. In our study, patients with higher viral replication have presented lower levels of total cholesterol, LDL and HDL, and had a significant reduction of protein S. In addition, the effect of viral replication on protein S activity has been confirmed in the multivariate model in which lipid levels have been also included as covariates.

In conclusion, our study shows a high prevalence of thrombophilia in HIV-infected children and adolescents and suggests a protective role of full suppressive ART on maintenance of coagulation homeostasis. Continuous viral suppression is essential for all ages, but it is particularly difficult during adolescence, when lower adherence is frequent and often associated with HIV replication and treatment failures. Furthermore, young women should warrant particular attention since pregnancy and puerperium are important risk factors for thrombotic events [23], with an ascertained role of protein S deficiency, and potential indication for anticoagulant preventive interventions [39]. Our observations underline the importance of reinforcing preventive lifestyle measures against CVD (smoking cessation, dietary changes, aerobic physical activity) starting from childhood and adolescence, together with interventions strengthening correct intake of therapy and adherence. Further long-term studies investigating the role of antiretrovirals and HIV on coagulation are required, as well as studies evaluating other therapeutic approaches able to reduce inflammation and/or improve coagulation homeostasis, especially in patients not maintaining viral suppression. Paediatric and adolescent HIV patients, even if they have less concomitant CVD risk factors than adults, are in fact expected to have a longer duration of exposure to both antiretrovirals and viral replication.

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