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Thrombophilia, systemic inflammation and prevention of cardiovascular disease in children and adolescents with HIV infection

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Abstract

Atherosclerosis begins early in the lifetime and, as for other cardiovascular diseases associated with thrombosis, appears more relevant and anticipated in HIV-infected patients after combination antiretroviral therapy (cART) has reduced AIDS-related diseases and has improved survival.

Systemic chronic inflammation, a condition associated with HIV, could have a role in determining anticipated cardiovascular diseases since it is a risk factor for atherosclerosis. The association between viral replication, inflammation and coagulation abnormalities in a cohort of HIV-infected children and adolescents was investigated as aim of this research project.

In a first part of the research, prevalence of metabolic syndrome (hyper-cholesterolemia, hyper-trigliceridemia, hyper-glicemia), well characterized in adults on antiretroviral treatment, was studied in the cohort of children and adolescents. In the second part, assays on thrombophilia (protein S, protein C anticoagulant and antithrombin activity), were done together with fibrinogen, D-dimer, high-sensitive C-reactive protein, homocysteine and metabolic exams.

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. 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 (+0.13mg/ml, P=0.004) was confirmed in the multivariate model.

In conclusion 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.

Sommario

La malattia aterosclerotica inizia già dalla seconda decade di vita. Insieme ai tradizionali fattori di rischio associati (fumo, colesterolo, ipertensione) negli ultimi anni si è andata caratterizzando il ruolo dell'infiammazione, risultata, da studi di base confermati da numerose osservazioni cliniche, una componente patogenetica rilevante nell'insorgenza e progressione della lesione aterosclerotica.

Patologie associate ad infiammazione sistemica come il Lupus Eritematoso o la malattia di Kawasaki sono considerate condizioni a rischio moderato di aterosclerosi in età pediatrica. Anche l'infezione da HIV si associa ad alti livelli di infiammazione sistemica, fattore con un ruolo riconosciuto da tempo nell'accelerare l'immunodeficienza caratteristica della malattia. L'introduzione della cART (terapia antiretrovirale di combinazione) ha consentito negli ultimo 15 anni una riduzione straordinaria dell'incidenza di malattie opportunistiche associate all'AIDS (Sindrome da Immunodeficienza Acquisita), e una riduzione della mortalità, ma si è contemporaneamente assistito ad un aumento dell'incidenza di malattie non associate ad immunodeficienza, tra cui un ruolo principale è costituito dall'aterosclerosi e alle altre malattie cardiovascolari associate alla trombosi.

Nell'adulto un recente trial randomizzato (SMART trial) ha mostrato che la replicazione virale incontrollata risulta essere un fattore di rischio non solo per la progressione dell'immunodeficienza, ma anche per l'incidenza e mortalità da malattia cardiovascolare. Il ruolo dell'infezione da HIV come fattore di rischio per l'aterosclerosi e le malattie cardiovascolari è poco conosciuto in età pediatrica, popolazione in cui, anche se è minore l'incidenza di manifestazioni cliniche, tale indagine è resa più appropriata per il minore ruolo concomitante di altri fattori confondenti (es. ipertensione, iperglicemia).

In una prima fase del progetto di ricerca è stata indagata la prevalenza in bambini ed adolescenti con infezione da HIV seguiti presso l'Ospedale Pediatrico Bambino Gesù di Roma, della sindrome metabolica (ipercolesterolemia, iperglicemia, ipertrigliceridemia), già descritta e caratterizzata nei pazienti adulti con infezione da HIV in trattamento con farmaci antiretrovirali.

Successivamente, e per la prima volta, è stata indagata l'associazione tra replicazione virale, infiammazione sistemica e anomalie della coagulazione (trombofilia) in una coorte di bambini e adolescenti con infezione da HIV, producendo dati originali pubblicati nel Febbraio 2010[1] (Pontrelli et al, AIDS).

Sono stati studiati i livelli di attività della proteina S e proteina C anticoagulante e dell'antitrombina, insieme con il fibrinogeno, il D-dimero, la proteina C-reattiva e l'omocisteina. L'ipotesi che la viremia HIV determinasse

aumento dei markers di infiammazione e alterazione dei markers di coagualazione è stata verificata confrontando i valori in pazienti con alta viremia (HVL, High Viral Load: HIV-RNA>1000 copie/mL) con i valori dei pazienti a bassa viremia (Low Viral Load, LVL: HIV-RNA<1000 copie/mL).

All'analisi univariata è seguita un'analisi multivariata che ha valutato il ruolo di altre variabili demografiche, cliniche e terapeutiche potenzialmente confondenti. Sono stati arruolati un totale di ottantotto pazienti (età media 13.5 anni, CD4 medi 30%, 72% pazienti nel gruppo LVL).

Lo studio ha evidenziato un deficit di proteina S e C anticoagulante rispettivamente del 51 e 8%. I pazienti nel gruppo HVL hanno presentato una riduzione significativa dei livelli di proteina S, proteina C e antitrombina, ed un aumento dei livelli di D-dimero.

La riduzione di proteina S (-11.2%, P=0.04) e l'aumento di D-dimero (+0.13mg/ml, P=0.004) sono risultati associati in maniera indipendente con l'alta viremia anche nell'analisi multivariata.

In conclusione lo studio ha dimostrato che in era cART, bambini ed adolescenti con infezione da HIV presentano un'alta prevalenza di alterazioni trombofiliche. L'analisi multivariata ha mostrato che la replicazione virale si associa in maniera significativa ed indipendente alla diminuizione della proteina S anticoagulante e all'aumento di D-dimero, suggerendo il vantaggio

della terapia soppressiva sul mantenimento dell'omeostasi coagulativa e l'opportunità di una prevenzione attiva di tutti i fattori di rischio cardiovascolari a partire dall'età pediatrica.

Introduction

Inflammation, thrombosis and cardiovascular disease

Atherosclerosis and other cardiovascular diseases, are currently the leading cause of death and illness in developed countries. They are the prototype of multifactorial diseases; furthermore pathological alterations started early during lifetime, just from the second decade, but clinical manifestations appears later, most frequently in the adult and elderly age.

Formerly, starting from the 1970s, it was considered mainly a metabolic and lipid disease[2]. Recently, in the past decade, attention was driven to a prominent role of inflammatory response[3].

Basic science have highlighted that inflammation mediate all stages of disease from initiation through progression and, finally, the thrombotic complications of atherosclerosis. Lesion starts with the adhesion of leukocytes to the endothelial layer, followed by leukocyte activation in intima, accumulation of lipids, and formation of a fibrotic cap. If the cap become instable, could be susceptible to rupture, leading to the exposition of tissue factor and thrombosis (Fig.1).

Leukocyte recruitment and expression of pro-inflammatory cytokines characterize early atherogenesis, and malfunction of inflammatory mediators

mutes atheroma formation in mice. Soon after initiating an atherogenic diet, light microscopy reveals attachment of blood leukocytes to the endothelial cells that line the intima, the innermost layer of arteries[4]. VCAM-1, P- and E-selectin seems to have a central role in leukocyte recruitment in atherosclerosis-susceptible mice[5,6].

Pro-inflammatory cytokines such as interleukin (IL)-1b or tumour-necrosis factor-a (TNF-a) induce VCAM-1 expression in endothelial cells by this pathway. Human atherosclerotic lesions contain these cytokines. Nitric oxide arising from endothelial nitric oxide synthase, another shear stress-regulated gene, can inhibit VCAM gene expression through a novel pathway involving inhibition of the activation of NF-kB, the central transcriptional control point in vascular inflammation[7].

At the end, inflammatory pathways promote thrombosis, a late complication of atherosclerosis responsible for myocardial infarctions and most strokes. For instance IFN-gamma, inhibits collagen synthesis, and allow the formation of instable plaque, susceptible to rupture.

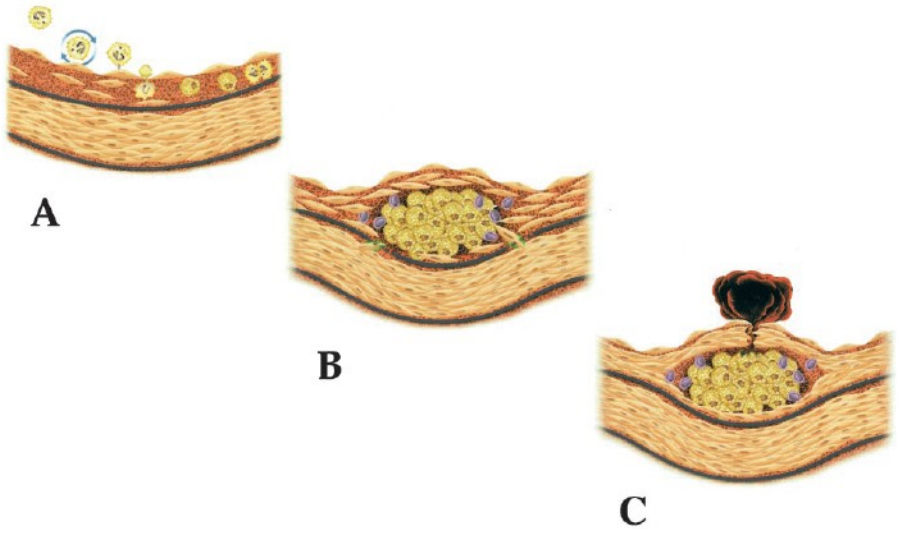

Fig1 Life History of an atheroma. (from Maseri A, Circulation 2002)

Role of inflammation in all stages of atherosclerosis. A) Leukocyte recruitment are elicited by adhesion molecules like VCAM-1 when the endothelial monolayer becomes inflamed. Proinflammatory cytokines expressed within atheroma provide a chemotactic stimulus to the adherent leukocytes, directing their migration into the intima. M-CSF, a inflammatory mediator promote the replication of macrophages within the intima. B) T lymphocytes join macrophages in the intima during lesion evolution. and secrete cytokines and growth factors that promote the migration and proliferation of Smooth Muscle Cells (SMCs) which can degrade the collagen in response to inflammatory stimulation. C, Ultimately, inflammatory mediators such INF-gamma inhibit collagen synthesis rendering weak the fibrous cap and susceptible to rupture. Cross-talk between T lymphocytes and macrophages heightens the expression of the potent procoagulant tissue factor and inhibit anticoagulant factors, allowing thrombosis and clinical consequences of atherosclerosis.

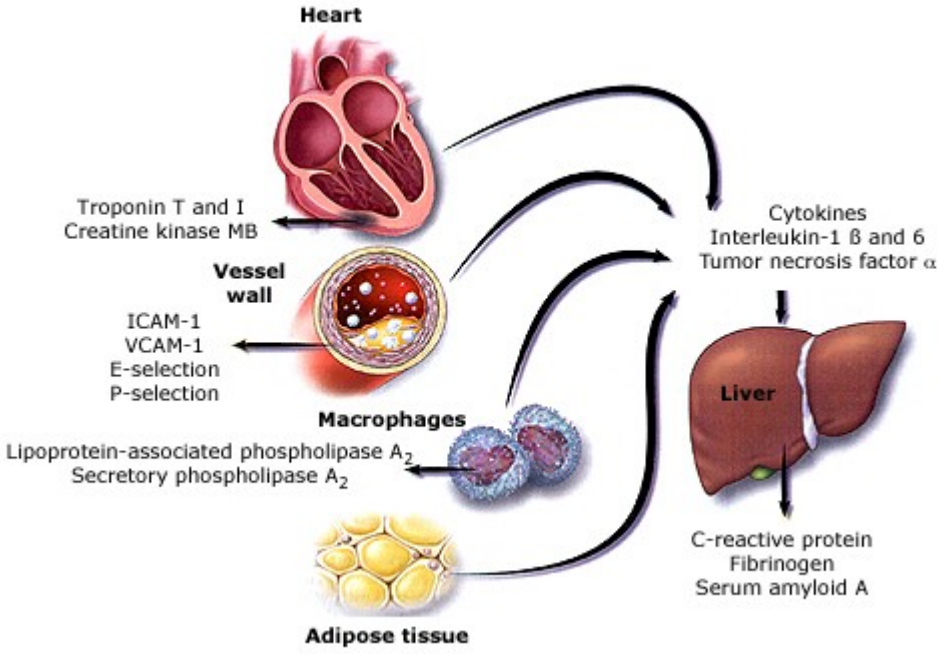

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Fig.2 The cytokine cascade (from Rader, NEJM 2000[8]).

Activated immune cells in the plaque produce inflammatory cytokines (IFN-*g*, IL-1, and tumor necrosis factor [TNF]), which induce the production of substantial amounts of interleukin-6. These cytokines are also produced in various tissues in response to infection and in the adipose tissue of patients with the metabolic syndrome. Interleukin-6, in turn, stimulates the production of large amounts of acute-phase reactants, including C-reactive protein (CRP), serum amyloid A, and fibrinogen, especially in the liver. Although cytokines at all steps have important biologic effects, their amplification at each step of the cascade makes the measurement of downstream mediators such as CRP particularly useful for clinical diagnosis.

Clinical data from large epidemiological studies has confirmed the role of chronic inflammation in atherosclerosis and cardiovascular diseases. Elevation in markers of inflammation predicts outcomes of patients with acute coronary syndromes, independently of myocardial damage. In addition, low-grade chronic inflammation, as indicated by levels of the inflammatory marker C-reactive protein or other markers prospectively defines risk of atherosclerotic complications, thus adding to prognostic information provided by traditional risk factors.

Moreover, certain treatments that reduce coronary risk also limit inflammation. In the case of lipid lowering agents statins, this anti-inflammatory effect does not appear to correlate with reduction in low-density lipoprotein levels, and a "pleiotropic" effect active in reducing inflammation pathway has been proposed[9].

Many studies have focused the role of serum markers of inflammation also as markers of atherosclerotic risk in order to add to the information available from traditional measures such as the lipid profile.

The better characterized marker is C-reactive protein (CRP). Plasma CRP is an acute phase reactant produced primarily by the liver in response to inflammatory cytokines such as IL-6 and TNF-alpha (Fig.2).

Despite a lack of specificity for the cause of inflammation, data from more than 30 epidemiologic studies have shown a significant association between elevated serum or plasma concentrations of CRP and the prevalence of

underlying atherosclerosis, the risk of recurrent cardiovascular events among patients with established disease, and the incidence of first cardiovascular events among individuals at risk for atherosclerosis[10, 11]. These studies have proposed risk categories for predicting atherosclerosis based on value of CRP (low, average, and high risk values defined as serum CRP <1, 1 to 3, and >3 mg/L), Furthermore CRP appears also effector of progression of atherosclerotic lesions: a clinical trial showed that infusion of CRP was associated with marked elevations in markers of both inflammation (IL-6, IL-8, and serum amyloid A) and coagulation (von Willebrand factor antigen, prothrombin F1+2, D-dimer, and plasminogen activator inhibitor type 1)[12]. In addition, a number of drugs used in the treatment of cardiovascular disease reduce serum CRP. It is therefore possible that reduced inflammation contributes to the beneficial effects of these medications. In contrast to these experimental and clinical observations, other reports based on genetical studies have not supported a direct role of CRP in atherogenesis. Alternative explanations for the strong relationship between elevated CRP and ischemic heart disease include the possibility that CRP is a marker of other factors which promote cardiovascular disease (eg, inflammation), or that ischemic cardiovascular disease increases CRP levels[13]. Clinical trials that test the ability to guide preventive therapy in apparently well individuals are ongoing.

Coagulation homeostasis

Several fine molecular process concur to maintain fluidity of the blood in the normal vessels, and induce a clot when damage occurs. A homeostatic balance exists between procoagulant forces and anticoagulant and fibrinolytic forces (Fig.3).

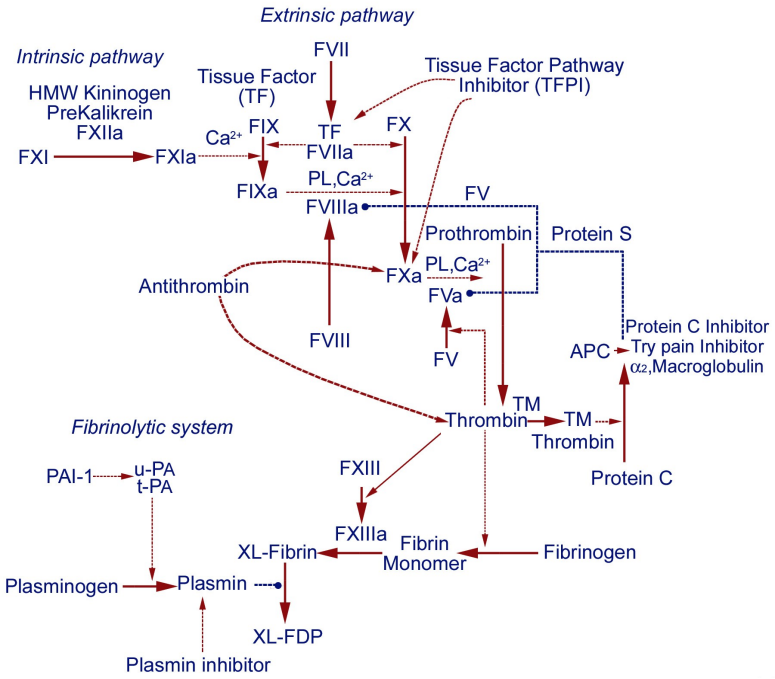


Fig.3 Coagulation cascade. A fine regulated process with anticoagulant mechanisms active at different levels.

Several inhibitory mechanisms prevent activated coagulation reactions from amplifying uncontrollably, causing extensive local thrombosis or disseminated intravascular coagulation. These mechanisms include inactivation of

procoagulant enzymes, fibrinolysis, and hepatic clearance of activated clotting factors.

Antithrombin, inactivate coagulation enzymes. Antithrombin inhibits coagulation process by acting on several targets (thrombin, factor Xa, factor XIa, and factor IXa). Heparin, a widely used life-saving drug, act by enhancing antithrombin activity.

Two vitamin K–dependent proteins, protein C and protein S, form a complex that inactivates factors VIIIa and Va by proteolysis. Thrombin, when bound to a receptor on endothelial cells (thrombomodulin), activates protein C. Activated protein C, in combination with protein S and phospholipid cofactors, proteolyzes and inactivates factors VIIIa and Va.

Genetic defects that increase the propensity for venous thromboembolism include the factor V Leiden mutation, which causes resistance to activated protein C (APC); the prothrombin 20210 gene mutation; and a deficiency of protein C, protein S, or antithrombin.

In 1845, Virchow postulated that 3 factors were important in the development of thrombosis: impairment of blood flow (stasis), vascular injury, and alterations of the blood (thrombophilia). Stasis associated with surgery or orthopedic or paralytic immobilization; heart failure; pregnancy; and obesity increase the risk of venous thrombosis. Neoplastic cells may activate coagulation by secreting a factor X–activating protease, by expressing tissue

factor on exposed membrane surfaces, or both. Sepsis and other severe infections associated with increased tissue factor exposure on monocytes and macrophages can increase the risk of venous thrombosis. Oral contraceptives that contain estrogen increase the risk of arterial and venous thromboembolism even if the risk with modern low-dose regimens is low. Hyperhomocysteinemia may also predispose to arterial thrombosis and venous thromboembolism, possibly because of injury to vascular endothelial cells.

D-dimer, is generated as a result of plasmin mediated clot dissolution processes, and is an indicator of recent clot formation and subsequent fibrinolysis. For this reason it is currently used in diagnosis of acute thrombosis (deep vein thrombosis, pulmonary embolism, and disseminated intravascular coagulation), and is a factor strongly and positively related to the future occurrence of venous thrombosis[14]. More recently, D-dimer levels have been correlated with atherosclerotic cardiovascular disease. Venous and arterial thrombosis appear independently associated, not because they share similar risk factors, such as obesity, hypertension, smoking, and diabetes/hyperglycemia[15].

Risk conditions for anticipated atherosclerosis

Although cardiovascular disease (CVD) is generally manifest in adulthood, the process of atherosclerosis can begin early in childhood. For most children,

atherosclerotic vascular changes are minor and can be minimized or even prevented with adherence to a healthy lifestyle. However, in some children, the process is accelerated because of the presence of identifiable risk factors including overweight/obesity, hypertension, dyslipidemia, a positive family history for premature cardiovascular disease (CVD), and smoking exposure. The presence of multiple risk factors increases the likelihood of accelerated atherosclerosis. Furthermore some specific diseases are associated with premature CVD (eg, diabetes mellitus and Kawasaki disease, metabolic syndrome).

Identification of children who are at-risk for atherosclerosis may allow timely intervention to decrease the atherosclerotic process, thereby preventing or delaying CVD.

Specific diseases are associated with early CVD and accelerated atherosclerosis. A panel of experts of the American Heart Association (AHA) reviewed the literature on premature cardiovascular disease in children and established the following three-tier disease risk stratification schema for coronary artery disease (CAD) (table 1). Eight diseases associated with an increase risk of CAD were also identified and classified based upon their risk for CVD[16]:

Non-pharmacologic interventions, is indicated for children who are judged to be at increased risk for CVD because they have multiple risk factors and/or an

underlying high-risk primary disease. In those who are at a high risk for CVD experts suggest initiating pharmacologic therapy at lower threshold levels.

Tab.1 Risk for cardiovascular disease and pediatric diseases

	Risk category	Rationale	Disease process/condition
Tier I	High risk	Manifest CAD <30 years of age: Clinical evidence	Homozygous familial hypercholesterolemia (FH) Diabetes mellitus, type 1 Chronic kidney disease (CKD)/end-stage renal disease (ESRD) Post-orthotopic heart transplantation (OHT) Kawasaki Disease, current coronary aneurysms
Tier II	Moderate risk	Accelerated atherosclerosis: Pathophysiological evidence	Heterozygous FH Kawasaki disease with regressed coronary artery aneurysms Diabetes mellitus, type 2 Chronic inflammatory disease
Tier III	At risk	High-risk for accelerated atherosclerosis: Epidemiological evidence	Post-cancer treatment Congenital heart disease Kawasaki disease without detected coronary involvement

CAD: Coronary artery disease.

Tab 1 Cardiovascular risk in high-risk pediatric populations

HIV and cardiovascular diseases in adults

The introduction of effective combination antiretroviral therapy (cART) preserving immune function and decreasing AIDS (Acquired Immune-Deficiency Syndrome)-related morbidity has changed in the last fifteen years the natural history of HIV-1 (Human Immunodeficiency Virus 1) infection in adults and children from a lethal to a chronic disease[17, 18].

Improved survival has in fact been followed by an increased and anticipated prevalence of non-AIDS related conditions such as atherosclerosis and other cardiovascular diseases (CVD) associated with arterial and venous thrombosis [19, 20] .The role of cART in increasing the risk of CVD acting on traditional risk factors (impaired glucose metabolism, hyper-cholesterolemia, hyper-triglyceridemia), or as independent non-mediated risk factor has been extensively investigated in adults[18].

Duration of exposure to cART[21] and to protease-inhibitors (PI)[22] resulted associated to myocardial infarction, as the recent use of Nucleoside Analogue Reverse Transcriptase Inhibitors (NRTI) abacavir (ABC) or didanosine (ddI)[23], even if their exact role is still matter of debate[24]. The strong and independent role of HIV replication has been recently highlighted by the unexpected results of SMART (Strategies for Management of Anti-Retroviral Therapy) trial. This study showed an increased mortality from all-causes and from CVD in patients

with CD4-guided treatment interruptions versus those with continuous ART and viral suppression[25, 26].

Viral replication could have a possible pathogenic role by increasing thrombotic risk. A sub-study of the trial showed that higher D-dimer was associated to high HIV viral load, and baseline D-dimer levels above 0.18 µg/ mL were significantly associated with all cause- and CVD- related mortality [27].

HIV infected adults have an increased risk of venous and arterial thrombosis, with thrombophilic abnormalities more frequently associated with AIDS progression[28, 29, 30, 31, 32, 33] , and protein S and protein C resulted deficient in adult HIV patients[34].

HIV and cardiovascular diseases in children

There are very few studies on risk of cardiovascular diseases in children HIV infected.

A quarter of children and adolescents have signs of lipodystrophy, and dyslipidemia was present in 31% of all children[35].

HIV infected children presented higher resistance to insulin, waist-to-hip ratio, cholesterol, triglycerides, myeloperoxidase and lower homocysteine levels in comparison with healthy controls. Atherosclerosis measured echographic as

carotid Intima Media Tickness (IMT) resulted significantly higher in the HIV infected group[36].

Regarding thrombophilia, only one study was conducted in children investigating prevalence of Protein C and Protein S deficiency in a small cohort before the introduction of cART, showing results similar to the adults[37].

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 inflammatory markers together with anticoagulant and fibrinolysis markers, and a specific role of HIV replication.

Methods

Study Population

At Bambino Gesù Children Hospital of Rome, Italy a cohort of HIV-infected children and adolescents of almost one hundred individuals is regularly followed. Follow-up visits are scheduled every three or four months, and included physical examination, emato-chemistry, immunophenotype (comprehending CD4 cell count), HIV-RNA, drug adherence assessment and psychological consult.

The protocol of the study, focused on inflammation, metabolic and cardiovascular complication of HIV infection and antiretroviral treatment, has been approved by the local Ethical Committee before initiating the study, and then 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 six months), have been 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 comprehensive of at least two different antiretroviral classes.

For analysis of thrombophilic markers, patients have been considered eligible if they were not co-infected with hepatitis B (HBV) or hepatitis C virus (HCV), since hepatitis could hamper the synthesis of this proteins by the liver. Clinical stage of HIV infection has been assessed according to the classification of the Centers for Diseases Control (CDC) for adults and adolescents[38] and for children[39]. Blood samples for HIV RNA viral load and thrombophilic tests have been simultaneously collected.

Since pregnancy and puerperium are important risk factors for thrombotic events for HIV negative and HIV infected women[40], the prevalence of thrombophilia in all the girls at procreative age (above 16 years) of our cohort have also purposely investigated.

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. Lymphocytes subsets have been analyzed using flow cytometry on Peripheral Blood Mononuclear Cells (PBMC) in accordance with standard protocols with a flow cytometer (FACScan, Becton Dickinson, U.S.A.).

Total cholesterol, low density lipoproteins (LDL), high density lipoproteins (HDL) 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 has been evaluated by automated latex-enhanced immunoturbidimetric assay (STA Liatest, Roche Diagnostics, Basel, Switzerland). Homocysteine has been analyzed by chemiluminescence assay (Advia Centaur, Siemens Diagnostics, Deerfield, IL) and high sensitive C reactive protein (hsCRP) has been

measured using a latex-enhanced immunoturbidimetric method (Advia 2400, Siemens Diagnostics, Deerfield, IL).

Protein S deficiency was defined as a decreased protein S activity <70%, and protein C deficiency as a decreased protein C activity <70%. Deficiency of antithrombin has been defined as percentage of activity <75%. We have considered increased D-dimer values higher than 0.18 and 0.34 μg/mL, levels which resulted associated with mortality-OR >3 in HIV adults[27]. 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) have been compared between high viral load (>1000 cp/mL, HVL) and low viral load (<1000 cp/mL, LVL) population. In order 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 cp/mL as appropriate cut-off between low and high level

viremia, as also suggested by recent guidelines[41]. Univariate analysis used either ANOVA or Wilcoxon rank sum test for continuous data, while chi-square test or Fisher's exact test have been used for categorical data.

A multivariate model has been developed in order to exclude that other factors could have acted as confounding variables and affected the association between viral load and biomarker levels. Demographic covariants (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) have been considered A two-step process has been 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 versus LVL group has been adjusted for all the covariates that firstly resulted associated with the same biomarker with a p-value <0.20 at the univariate analysis. Levels of biomarkers whose alteration resulted significantly associated with HVL were plotted in a linear regression graph and related to continuous values of HIV viral load.

A 2-tailed p-value of <0.05 has been considered statistically significant in all the analyses. Statistical analysis has been done with Stata 10.1 (Stata corporation, Texas).

Results

Preliminary results on prevalence of metabolic syndrome

In the first part of the project a preliminary cross-sectional study focused on metabolic syndrome and antiretroviral therapy was performed. On the 83 children and adolescents enrolled (mean age 14 years) 94% was on cART. 8.4% had hypercholesterolemia. Interestingly all of them were on cART containing Protease Inibitors (PI). About 30% had hyper-triglyceridemia, and 96% of them were on PI. Only one patient had hyper-glicemia.

Description of the cohort studied for thrombophilia

The study on thrombophilia 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 has been 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 29.9% (623 cells/mm^3); 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% on cART and the rest on a simplification regimen based only on NRTI[42]. 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 other hand, the LVL group presented a longer cART treatment history (5.8 vs 3.3 years; p<0.001) and a longer treatment duration with PI (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 resulted 0.24 μg/mL. Three patients (two in the LVL and one in the HVL group) had the factor V Leiden mutation.

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

Protein S activity in HVL group has resulted significantly lower than in 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 has resulted lower than amongst the LVL patients. The HVL group has presented a significant higher level of D-dimer (0.34 vs 0.21 μg /mL, $p=0.02$), with a prevalence of values >0.18 μg / mL (72.0% vs 49.2%, $p=0.05$) and >0.34 μg /mL (40.0% vs 11.1%, $p=0.004$) significantly higher than LVL. No significant variation between the two groups has been observed regarding fibrinogen, homocysteine, high sensitive C reactive protein.

Higher viral replication has resulted 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<25% have had a significant reduction of protein S activity (-10.7%, $p=0.05$).

Higher viral replication has been found also associated with higher D-dimer in the multivariate model (+0.13 μg/mL in HVL group, p=0.004). Other parameters have not shown any significant difference between the two groups in the multivariate model.

Figure 3 and 4 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.

Tab2 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 (mg/dL) mean (mg/dL) (±SD)	81 (±27)	68 (±23)	86 (±26)	**0.003**
HDL (mg/dL) mean (mg/dL) (±SD)	53 (±18)	44 (±15)	57 (±19)	**0.001**
TG (mg/dL) mean (mg/dL) (±SD)	117 (±86)	107 (±102)	121 (±79)	0.12

HVL, Higher Viral Load group: HIV-RNA>1000 cp/mL; LVL, Lower Viral Load group: HIV-RNA<1000 cp/mL; ART, Antiretroviral Therapy; PI, Protease Inhibitors; ABC, Abacavir; ddI,

Didanosine; cART, combination Antiretroviral Therapy; LDL, Low-density lipoproteins; HDL, High-density lipoproteins; TG, triglycerides.

Table 3. Thrombophilic and inflammation markers in the whole cohort, and in higher and lower viraemic group.

		Viremia			
	Total n=88	**HVL n=25**	**LVL n=63**	**Univariate Analysis p-value[a]**	**Multivariate Analysis Adjusted difference (HVL-LVL) and p-value[c]**
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**	
n (%) of increased (>0.18 µg /mL)	49 (55.7%)	18 (72.0 %)	31(49.2 %)	**0.05**	**+0.13 p=0.004**
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

HVL, Higher Viral Load group: HIV-RNA>1000 cp/mL; LVL, Lower Viral Load group: HIV-RNA<1000 cp/mL; SD, Standard deviation; hsCRP, high-sensitive C-Reactive Protein; statistically significant differences (p<0.05) are reported in bold.

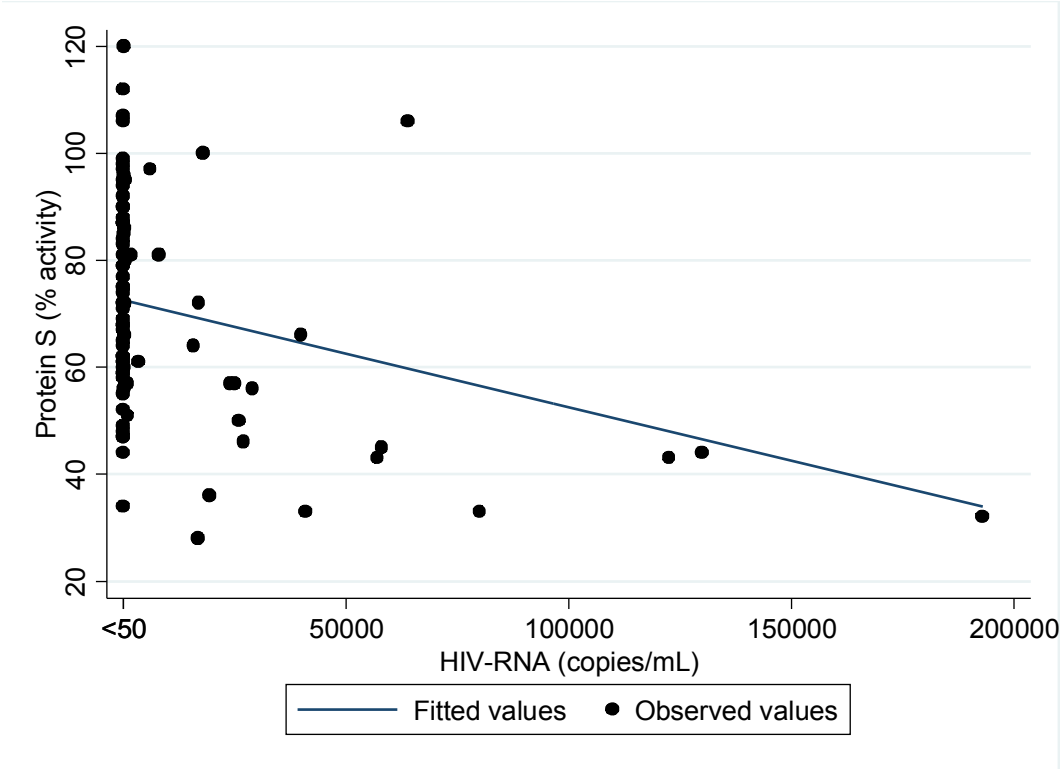
[a] unadjusted 2-tailed p-value

[b] 2-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.

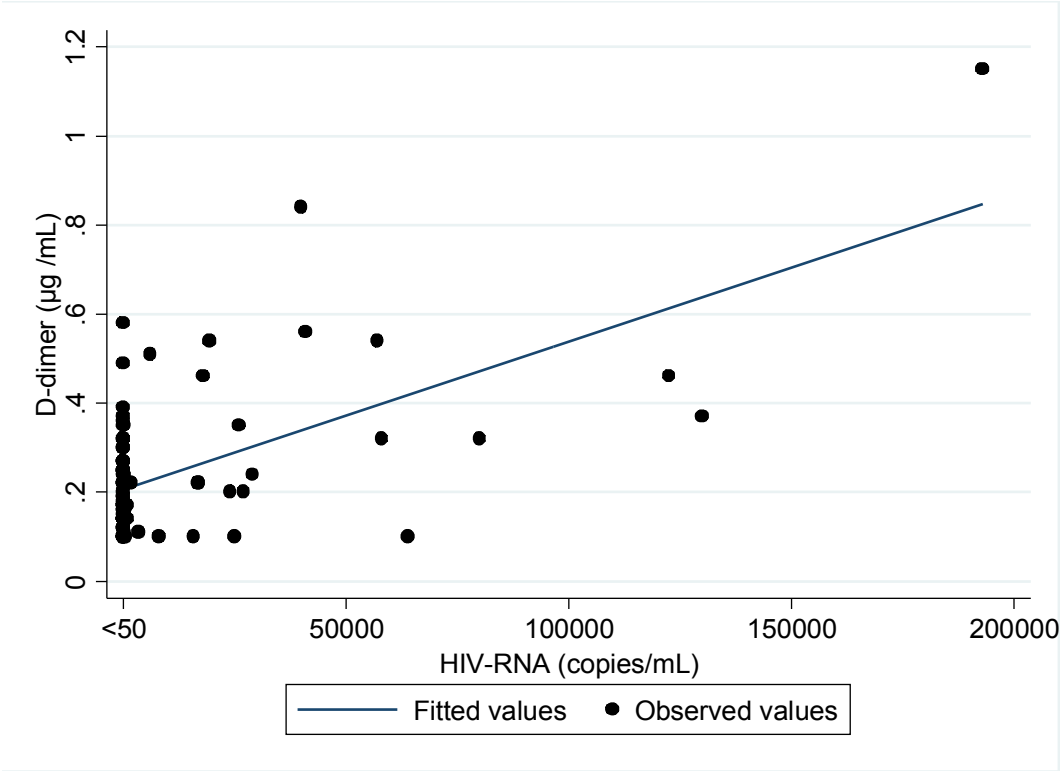
[d] N/A, multivariate analysis not conducted since no other variable resulted associated with a $p<0.20$ at univariate analysis

Figure 4. Levels of protein S activity related with viral load as continuous variable



Association of protein S anticoagulant with viral load. Pearson correlation coefficient r=-0.47, p<0.001. Linear regression was performed with adjustment for variables associated with a p<0.20 at univariate analysis.

Figure 5. Levels of D-dimer related with viral load as continuous variable



Association of D-dimer with viral load. Pearson correlation coefficient r=0.61, 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[43 44]: they usually become clinically evident during the middle and late adulthood, but are related to histological alterations beginning earlier in lifetime. 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 young age[45 46]

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[21], to protease-inhibitors[22], and recent use of abacavir (ABC) or didanosine (ddI)[23] 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 [24].

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 [25]. In patients discontinuing ART the levels of the inflammation markers resulted increased and the restarting of treatment was associated with their reduction [27].

This observation is coherent with recent several studies highlighting the role of chronic infections and inflammation on the pathogenesis of CVD and coagulation disorders[47 48]. 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 for thrombophilic abnormalities [28-34]. Protein S and protein C resulted respectively deficient in 60-67% and 9-25% of HIV adults, with protein S reduction also associated to advanced disease. 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-causes and CVD-associated mortality [27].

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. The aim of this project 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 has presented a high prevalence of thrombophilic abnormalities. Protein S deficiency has emerged as a highly frequent alteration (about half of the patients involved), while protein C and antithrombin deficiency has been less common. These frequencies were slightly less than those observed in adults[24]. Moreover, mean D-dimer levels observed in our cohort (0.24 μg/mL) have resulted higher than value (0.18 μg/mL) associated in adults with a 3.5 OR-mortality [27]. High HIV viral load resulted 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 viremia (HVL, HIV-RNA>1000 copies/ mL) have 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) if compared with patients with lower viremia (LVL).

The levels of homocysteine and hsCRP were unchanged. This is coherent with other studies, and suggest the opportunity to repeat measures or use other, more specific markers of inflammation.

The role of HIV replication has been also confirmed in a multiple regression model, considering as covariates demographic, laboratory, and HIV-related factors: patients with higher viremia 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).

This study, given the low mean age of the cohort, allows to highlight the direct role of HIV replication on coagulation disorders excluding 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 general population (smoke, age, dyslipidemia), and HIV infected population (cumulative use of cART and PI, actual use of ABC and ddI, dyslipidemia). Nevertheless the study has some limits 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, and the clinical meaning of the demonstrated alterations, needs further studies. A link between viral replication, chronic inflammation and thrombosis via endothelial activation has been proposed[49]. 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 [50] [51] [52] [53]. A possible role in the pathogenesis of acquired protein S deficiency could be also played by antiphospholipid antibodies and increased lipidemia [54] [55] . 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 where lipids 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 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 , with an ascertained role of protein S deficiency, and potential indication for anticoagulant preventive interventions[56].

Our observations underline the importance to reinforce preventative 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. Long term studies investigating the role of

antiretrovirals and HIV on coagulation are needed, 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.

Future studies and prospective

Together with prevention based on reduction of traditional risk factors (smoke, inactivity, lipid rich diet) our results have suggest us to investigate other approach for reduction of thrombophilia, inflammation and cardiovascular risk.

In the last period a lot of evidences have come out on the efficacy of polynsatured fatty acids in reduction of triglyceridaemia and other risk factors (hypertension, Thrombophilia, inflammation).

Role of polynsatured fatty acids is under investigational in adults with HIV or other inflammation and atherosclerotic associated condition like Lupus Eritematosus Systemicus.

We are going to investigate efficacy of polynsatured fatty acids in reduction of triglyceridemia, systemic inflammation, and Thrombophilia in children and adolescents in the same cohort of HIV infected and in a cohort of LES patients.

Polynsatured fatty acids are approved for reduction of trygliceridemia, are lacking of major interactions with other drugs, factor important in the context of a chronic and daily complex regimen as antiretrovirals.

The study, submitted to AIFA research program, is the first to investigate the long term effect of polynsatured fatty acids.

The primary outcome will be the reduction of trygliceridemia after 48 weeks from supplementation with polynsatured fatty acids. Inflammatory (IL-6, TNF-alpha), coagulation (Protein S, Protein C, antithrombin III), endothelial activation (ICAM-1, E-selectin), and immune-activation (MCP-1, IL-8, FOXP3) markers will be used as secondary endpoints.

Intima media thickness and pulse wave velocity will be utilized to measure atherosclerosis.

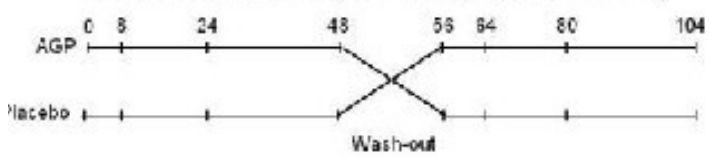


Fig.6 Scheme of a cross over study of efficacy of polynsatured fatty acids in children and adolescents with HIV infection.

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