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Abbreviations

AIDS: acquired immunodeficiency syndrome
BMI: body mass index
CANTOS: Canakinumab Anti-Inflammatory Thrombosis Outcomes Study
cART: combination antiretroviral therapy
CI: confidence interval
CIRT: Cardiovascular Inflammation Reduction Trial
DC: drug conservation
DNA: deoxyribonucleic acid
eGFR: estimated glomerular filtration rate
ESPRIT: the Evaluation of Subcutaneous Proleukin® in a Randomized International Trial
FD: fold difference
HBV: hepatitis B virus
HCV: hepatitis C virus
HDL-c: high-density lipoprotein cholesterol
HIV: human immunodeficiency virus
HPTN 052: the HIV Prevention Trials Network 052 trial
HR: hazard ratios
hsCRP: high-sensitivity C-reactive protein
IL-2: interleukin-2
IL-6: interleukin-6
INSIGHT: International Network of Strategic Initiatives for Global HIV Trials
IQR: interquartile range
IR: incidence rate
IRR: incidence rate ratios
JUPITER: Justification for the Use of Statins in Primary Prevention: An Intervention Trial Evaluating Rosuvastatin
LDL-c: Low-density lipoprotein cholesterol
MCV: mean corpuscular volume
PYFU: person-years of follow-up
REPRIEVE: The Evaluating the Use of Pitavastatin to Reduce the Risk of Cardiovascular Disease in HIV-Infected Adults trial
RNA: Ribonucleic acid
SILCAAT: the Subcutaneous Recombinant, Human Interleukin-2 in HIV-Infected Patients with Low $CD4^+$ Counts under Active Antiretroviral Therapy trial
SMART: the Strategies for Management of Anti-Retroviral Therapy trial
START: the Strategic Timing of AntiRetroviral Treatment trial
TNF-α : tumour necrosis factor- α
VL: viral load
VS: virological suppression

Introduction

The changing epidemiology of HIV infection and the SMART trial

The advent of combination antiretroviral therapy (cART) has turned HIV infection from a uniformly fatal disease into a treatable chronic condition [1-3]. Indeed, the life expectancy of selected subgroups of HIV+ persons may approach that of the general population [4-8]. However, the success of cART in reducing AIDS-related morbidity is tempered by short and long term toxicity [9], problems with adherence and emergence of viral resistance. Moreover, health is not entirely restored by cART. When compared to the general population, treated HIV+ persons, even when fully virologically suppressed, seems to be at increased risk of developing a variety of non-AIDS-defining conditions [10], such as cardiovascular disease [11,12], cancer [13-15], renal impairment [16], hepatic disease and bone demineralization [17,18]. While a share of this increased risk of non-AIDS-morbidity is likely explained by ongoing immunodeficiency [19-24], cART toxicity has also been shown to be a contributor [25-30].

The Strategies for Management of Anti-Retroviral Therapy (SMART) study, the largest international HIV trial to date, compared continuous cART use to maintain virological suppression (virological suppression [VS] arm) with $CD4^+$ cell count guided, structured cART interruptions (drug conservation [DC] arm). In the DC arm, cART was stopped when $CD4^+$ was > 350 cells/mm^3 and re-initiated when $CD4^+$ was < 250 cells/mm^3 [31]. At the time, these $CD4^+$ cell thresholds were considered safe from an immunological point of view with a low risk of progression to AIDS [32]. By reducing exposure to cART, structured treatment interruption was expected to limit toxicity ultimately leading to a reduction in the risk of death from causes other than opportunistic diseases. Surprisingly, however, the data safety monitoring board recommended that the study should be halted prematurely owing to safety issues in the DC arm. Participants who interrupted cART had a significantly higher risk of developing the primary composite outcome of death or opportunistic disease. Even more startling, cART interruption also increased the risk of major cardiovascular, renal and hepatic events combined. The increased risk of opportunistic disease in the DC arm was largely, albeit not entirely, explained by a longer time with lower $CD4^+$ counts and higher viral replication [31,33]. The reasons why cART interruption also turned out to be associated with non-AIDS morbidity were less clear.

Enhanced inflammation, coagulation and mortality risk during HIV infection

On the basis of previous data pointing out a potential role of chronic immune activation on HIV disease progression [34,35], a panel of inflammatory and coagulation biomarkers was compared in SMART between death cases and matched controls who had stored frozen plasma. SMART participants with higher plasma levels of three biomarkers at study entry were found to be at significantly increased of death [36]: 1) interlukin-6 (IL-6), a pro-inflammatory cytokine that is a proximal mediator or upstream inflammatory marker [37]; 2) high-sensitivity C-reactive protein (hsCRP), a downstream acute phase reactant [38] whose hepatic production is stimulated, among other factors, by IL-6; and 3) D-dimer, a degradation product of cross-linked intravascular fibrin that is a marker of hypercoagulation [39,40]. These three biomarkers have similarly been linked to disease in the general population [41-43], remain stable in stored blood samples

and single time point measurements by different assays have shown to be reproducible and representative of extended periods of time [44,45].

Although cART interruption led to increases in the plasma levels of these biomarkers which were positively correlated with the degree of viral replication [36], the association between IL-6, hsCRP, D-dimer with higher risk of death remained the same across both treatment groups of SMART [36]. Therefore, ongoing activation of inflammatory and coagulation pathways seemed to exist even in the presence of suppressive cART. This possibility was further supported by other studies that found significantly higher levels of IL-6, hsCRP and D-dimer among HIV+ persons when compared to general population even when HIV replication was fully suppressed with cART [46,47].

Owing to the strikingly higher risk of death observed in individuals with elevated levels, D-dimer stands out among the biomarkers thus far investigated in the setting of HIV infection [36]. Correlations of D-dimer with markers of endothelial dysfunction and microbial translocation [48] have also been reported. The expression of the pro-coagulant tissue factor in monocytes correlates positively with HIV viraemia and blood levels of D-dimer and soluble CD14, a receptor shed by monocytes after activation by bacterial lipopolysaccharides [48]. This favours the hypothesis that HIV replication and microbial translocation are among the main determinants of the hypercoagulable state seen in HIV+ persons. On the other hand, correlations with other biomarkers may also indicate that, rather than being determined exclusively by HIV infection itself, elevated D-dimer levels could just reflect the presence of co-morbidities or unmeasured demographic confounders. It had been hypothesised that the increase of D-dimer levels with age observed in the general population was mainly due to a higher burden of co-morbidities [40]. However, the interaction of multiple factors leading to inflammation and activated coagulation in persons with HIV is very complex. Prior to our work described in this thesis, there were questions about the potential contribution of HIV infection, demographics and co-morbidities to the variance of D-dimer levels.

Anaemia and thrombocytopenia following cART interruption and their association with enhanced inflammation and coagulation

Anaemia is the most common hematological abnormality seen in HIV disease [49,50] and observational data have consistently demonstrated that individuals with lower haemoglobin levels at baseline are three times more likely to die during follow-up than those with normal haemoglobin [51]. In fact, haemoglobin decreases as small as 1 g/dL are clinically meaningful and identify subsets of HIV+ persons at increased risk of death [51]. In the SMART trial, cART interruption increased the risk of developing new or worsening anaemia [52]. Anaemic participants were subsequently shown to have a significantly higher incidence of AIDS, death and non-AIDS-defining conditions [52].

The morphological classification of red cells based on their mean corpuscular volume (MCV) is a readily available and helpful method to assess the aetiology of anemia. While microcytic (MCV< 80 fL) and macrocytic (MCV> 100 fL) anaemias are usually caused by micronutrient deficiencies, namely iron and folic acid/cobalamin respectively, normocytic anemia (MCV 80-100 fL) is predominately seen in patients with chronic disease [53]. In the general population, chronic inflammation is associated with normocytic anaemia. This effect is mediated by IL-6 [54], which can induce the hepatic production of hepcidin, a hormone that interferes with iron absorption and promotes iron uptake by the reticuloendothelial system, ultimately leading to anaemia [55,56]. It is possible, therefore, that the previously identified link between anaemia and increased morbidity in HIV+ persons may have a causal component involving activated inflammation. Prior to the work presented in this thesis, the relationship between anemia, inflammation and coagulation was poorly understood in the setting of HIV infection. We then set out to determine the relationship between IL-6, hsCRP and D-dimer and presence/ type of anemia at study entry among HIV+ individuals participating in an international trial.

Another clinically meaningful haematological abnormality brought about by cART interruption was thrombocytopenia [57]. Since the beginning of the AIDS pandemic, thrombocytopenia has been commonly reported among HIV+ persons [58]. Despite an apparent prevalence reduction in cART era, thrombocytopenia is still frequently observed, with recent prevalence estimates ranging around 10% [59]. Of particular interest was the finding that the decline in platelets counts following cART interruption in SMART was correlated with the rise in plasma D-dimer levels [57]. This indicates that, at least to a certain extent, platelet decline reflected activated coagulation and inflammatory pathways. Prior to our work, the effect of platelet kinetics on clinical event risk, both in terms of serious non AIDS-defining and AIDS-defining events, was not entirely understood. An attempt to investigate this in SMART was not appropriately powered due to the relatively smaller number of clinical outcomes [57]. We investigated the relationship between platelet counts and risk of clinical events in EuroSIDA, a large cohort of HIV+ persons from across Europe, Argentina and Israel.

The growing burden of cancer during HIV infection and its relationship with enhanced inflammation and coagulation

As HIV+ persons age, the incidence of cancer is expected to increase, as it does in the general population [60]. Furthermore, clinical and experimental evidence indicates that HIV infection leads to both reduced immune surveillance of malignant cells and accelerated viral oncogenesis. Indeed, when compared to the general population, not only classically AIDS-associated and virus-related cancers, but also non-AIDS-defining malignancies, have been shown to occur more frequently in HIV+ persons, particularly in those with lower $CD4^+$ cell counts [21]. As a result, cancer is now a leading cause of death in the setting of HIV infection [61].

The potential contribution of chronic inflammation and activated coagulation to cancer risk during HIV infection is not well understood. In SMART, participants who interrupted cART were found to be at increased risk of developing cancer [62]. Moreover, elevated levels of IL-6 and hsCRP are associated with an increased risk of cancer in the general population [63,64]. Before our work presented here, few systematic studies [65,66] had investigated the interplay among inflammation, coagulation and cancer in the setting of HIV infection. We tried to shed light on this question by assessing the relationship between plasma levels of IL-6, hsCRP and D-dimer and the subsequent risk of cancer among HIV+ persons receiving standard-of-care in the control arms of three international HIV trials. We also attempted to critically appraise the literature and summarise the current evidence on factors associated with cancer risk during HIV infection in a review.

Objectives

The work presented in this PhD thesis had the following objectives:

1) To identify factors independently associated with plasma D-dimer levels during HIV infection (paper *I*);
2) To assess the relationship between inflammatory (IL-6 and hsCRP) and coagulation (D-dimer) biomarkers and the presence/ type of anemia among HIV+ persons (paper *II*);
3) To assess the relationship between platelet counts and the risk of developing AIDS-defining and non-AIDS-defining conditions during HIV infection (paper *III*);
4) To investigate the association between IL-6, hsCRP and D-dimer levels and the risk of developing infection-related and infection-unrelated cancer in the setting of HIV infection (paper *IV*).

Hypotheses

1) The increase of D-dimer levels with age is mainly attributable to a higher burden of co-morbidities and enhanced inflammation. HIV-specific variables (HIV viremia, $CD4^+$ cell count and ART use) are independently associated with higher D-dimer levels and this association remains strong after adjustment for demographics, co-morbidities, smoking, and biomarkers of inflammation and renal function (paper *I*).
2) There is an association between enhanced inflammation/coagulation, as demonstrated by higher plasma levels of IL-6, hsCRP and D-dimer, and the presence of normocytic anemia in treated HIV+ persons (paper *II*).
3) During treated HIV disease, the decline of platelet counts or development of thrombocytopenia is more strongly associated with non-AIDS-defining conditions that with AIDS-defining conditions (paper *III*).
4) Elevated IL-6, hsCRP and D-dimer levels independently predict risk of developing both infection-related and infection-unrelated cancer. This independent association persists after adjustment for $CD4^+$ cell counts, HIV RNA and demographics suggesting a direct link between activated inflammation and coagulation and risk of different types of cancer (paper *IV*).

Methodology

The work presented in this PhD thesis encompassed secondary analyses of existing data from participants in international HIV trials conducted by the International Network of Strategic Initiatives for Global HIV Trials (INSIGHT, http://insight.ccbr.umn.edu/), a National Institute of Heath-funded global network for the conduct of trials and observational studies in infectious disease. The three trials we used data from were: (1) SMART [31], (2) the Evaluation of Subcutaneous Proleukin® in a Randomized International Trial (ESPRIT) and (3) the Subcutaneous Recombinant, Human Interleukin-2 in HIV-Infected Patients with Low $CD4^+$ Counts under Active Antiretroviral Therapy (SILCAAT) [67]. To achieve one of the objectives of this thesis we also used data from participants enrolled in EuroSIDA [2], a heterogeneous international cohort of HIV+ persons.

SMART

The SMART trial (NCT00027352) compared, in 5,472 individuals from 33 countries with $CD4^+ > 350$ cells/mm^3 at study entry, continuous use of ART with structured treatment interruptions guided by $CD4^+$ cell count, involving stopping cART when $CD4^+$ was > 350 cells/mm^3 and re-initiating ART when $CD4^+$ was < 250 cells/mm^3 [31].

ESPRIT

The ESPRIT trial (NCT00004978) compared interleukin-2 (IL-2) plus cART with cART alone in 4,111 individuals from 25 countries with $CD4^+$ counts > 300 cells/mm^3 at study entry [67].

SILCAAT

The SILCAAT trial (NCT00013611) compared IL-2 plus cART with cART alone in 1,695 individuals from 11 countries with $CD4^+$ counts between 50 and 299 cells/mm^3 at study entry [67]. Of all three trials re-analysed in this thesis, SILCAAT was the only one in which information on the mean corpuscular volume (MCV) of red cells was collected at study entry.

Follow-up and ascertainment of clinical events in SMART, ESPRIT and SILCAAT

The median follow-up time in SMART, ESPRIT and SILCAAT was 29, 81 and 91 months, respectively. In ESPRIT, as well as in SMART, all clinical events including cancers were systematically reported to and centrally adjudicated by an Endpoint Review Committee. In SILCAAT, AIDS-defining malignancies (Kaposi sarcoma, invasive cervical cancer and non-Hodgkin lymphoma) and Hodgkin lymphoma were centrally adjudicated, while other malignancies were identified from the adverse event reporting system.

In all three trials, individuals in the control arms received standard of care according to current HIV guidelines and were to be continuously maintained on cART.

EuroSIDA

The EuroSIDA study is a multinational prospective cohort of 18,791 HIV+ persons [2]. Detailed information on clinical, virological and immunological parameters is collected every 6 months with accurate recording of dates of AIDS-defining event (as defined in the 1993 revised AIDS classification from the Centres for Disease Control [68]) and non-AIDS-defining conditions (defined as cardiovascular disease, end-stage hepatic/renal disease, non-AIDS defining malignancies and pancreatitis [69]). In paper *IV*, we included patients aged >16 year with at least one platelet count after 1/1/2005 (when platelets were routinely collected in EuroSIDA) with prospective follow up data and $CD4^+$ count or viral load measured within 6 months of baseline, which was defined as the first platelet measurement.

Ethical considerations

The work included in this PhD thesis represents secondary analyses that used existing data previously collected as part of the SMART, ESPRIT and SILCAAT trials and the EuroSIDA cohort. No additional biological specimens were taken or analysed for the specific purpose of being included in this thesis. Prior to the enrolment of human subjects in the SMART, ESPRIT and SILCAAT trials, as well as in EuroSIDA, the institutional review board at each study site or country had approved the original study protocols, and written informed consent, when required by local or international legislation, was obtained from study participants. SMART, ESPRIT and SILCAAT were conducted in compliance with the Declaration of Helsinki Guidelines, were registered on clinical trials databases and reviewed by independent data and safety interim monitoring boards.

Biomarker measurements

The analyses included in this PhD thesis involved the SMART, ESPRIT and SILCAAT participants who consented to donating blood for future research and whose plasma levels of IL-6, hsCRP and D-dimer were measured at study entry, prior to the randomisation. The results of cross-sectional analyses presented here, therefore, were not biased by effects of cART interruption or IL-2 treatment in increasing plasma levels of biomarkers [70,71].

For SMART participants, measurements were performed at the Laboratory for Clinical Biochemistry Research at the University of Vermont (Burlington). In ESPRIT and SILCAAT, laboratory measurements were performed by SAIC-Frederick (Frederick, Maryland, USA). IL-6 was measured by the same method at each laboratory (Chemiluminescent Sandwich ELISA; R&D Systems, Minneapolis, Minnesota, USA). In SMART, D-dimer levels were measured with immunoturbidometric methods on the Sta-R analyzer, Liatest D-DI (Diagnostic Stago, Parsippany, New Jersey, USA) and high-sensitivity C-reactive protein (hsCRP) was measured with a NBTMII nephelometer, N Antiserum to Human CRP (Siemens Diagnostics, Deerfield, Illinois, USA). Cystatin C was measured in SMART participants a BNII nephelometer (Dade Behring Inc., Deerfield, Illinois, USA). In ESPRIT and SILCAAT, D-dimer was measured using an enzyme-linked fluorescent assay (ELISA) on a VIDAS instrument (bioMerieux Inc., Durham, North Carolina, USA), and hsCRP was measured using ELISA (R&D Systems, Minneapolis, Minnesota, USA). In SMART, lower limits of detection for IL-6, hsCRP, D-dimer and cystatin C were 0.16 pg/mL, 0.16 µg/mL, 0.01 µg/mL and 0.195 mg/dL, respectively. In ESPRIT and SILCAAT, lower limits of detection for IL-6, hsCRP and D-dimer were 0.156 pg/mL, 0.078 µg/mL and 0.045 µg/mL. The assays used to measure D-dimer and hsCRP in the three trials, while different, compared well on duplicates. The median plasma levels measured at each laboratory were comparable and well correlated (Table 1).

Table 1: Summary of biomarker measurements made on 20 samples. SMART, ESPRIT and SILCAAT

Biomarker	U. of Vermont [mean (IQR)]	SAIC-Frederick [mean (IQR)]	Correlation	Average Difference* (U. of Vermont–SAIC-Frederick) (SD)
IL-6 (pg/ml)	2.63 (1.78–3.75)	2.30 (1.50–3.18)	0.91	0.12 (0.38)
D-dimer (µg/ml)	0.29 (0.13–0.59)	0.41 (0.21–0.82)	0.81	-0.44 (0.96)
hsCRP(µg/ml)	2.39 (0.91–5.81)	2.18 (0.87–6.28)	0.99	0.15 (0.27)

* After log-transformation

Source: Reference [72]

Among SMART and ESPRIT participants co-infected with hepatitis B and hepatitis C, levels of HCV RNA and HBV DNA were determined at study entry using branched DNA assays (Versant HCV RNA 3.0 and Versant HBV DNA 3.0, respectively; Bayer Diagnostics). We also measured in co-infected participants the baseline levels of hyaluronic acid, a marker of hepatic fibrosis [73], using an enzyme-linked binding protein assay (Corgenix, Colorado, USA) .

Estimated glomerular filtration rate (eGFR) was calculated using the Cockcroft-Gault formula [74] in ESPRIT and SMART. Total cholesterol, low-density lipoprotein cholesterol (LDLc) and high-density lipoprotein cholesterol (HDLc) were measured in SMART by Quest Diagnostics, Inc. (Madison, NJ) using standard enzymatic methods.

Statistical Analyses

Multivariable linear regression

Linear regression is one of statistical methods more frequently used to determine cross-sectional associations between covariates (or statistical predictors) and a continuous variable. It demands that the outcome variable should be continuous and normally distributed. We deployed this method to determine factors independently associated with plasma D-dimer levels (paper *I*). Because the distribution of IL-6, hsCRP and D-dimer were right skewed, $\log_2$ transformations were used in the analyses. We chose the $\log_2$

transformation for the sake of easy interpretation as, with this approach, the back transformation of the parameter estimates leads to a coefficient which can be interpreted as an increase per doubling of the covariate; i.e. a one $\log_2$ higher increment corresponds to a two-fold higher level of the biomarker in question. Back transformed exponentiated estimates (i.e., corresponding to fold differences [FDs] in D-dimer levels per unit or category difference in the covariates) with 95% confidence intervals (CI) were calculated to assess the contribution of baseline covariates to the variance of D-dimer.

Because the variables of interest were not routinely collected in all three trials, we had no alternative but to fit models to three different datasets:

(1) SMART, ESPRIT and SILCAAT (N=9,864): age, gender, ethnicity, BMI, $CD4^+$ cell counts (nadir and baseline), hsCRP, D-dimer, cART use and cART regimens;
(2) SMART and ESPRIT (N=6,938): co-morbidities (hepatitis B, hepatitis C, diabetes mellitus, cardiovascular disease) and renal function (eGFR);
(3) SMART participants only (N=4,498): smoking, level of education (less than high school, high school and bachelor's degree or above), cystic C and cholesterol levels (total cholesterol and HDLc).

To account for inter-laboratory variability and inherent differences among participants in each trial, all models were adjusted for study type. The adjusted R^2 coefficient, which reflects variance in the outcome variable accounted for by the best-fit line, was used to assess the goodness of fit of the models.

Multivariable logistic regression

This is the statistical method of choice to explore cross-sectional associations between covariates and an outcome measure that is a categorical or binominal variable. Logistic regression estimates the change in the odds of an outcome produced by the increment of one unit in the corresponding covariate. We deployed this method in paper *II* to explore the relationship between IL-6, hsCRP and D-dimer and presence/type of anaemia at baseline in SILCAAT. We assessed whether the associations between biomarkers and anaemia was independent of confounding by adjusting models for multiple covariates: demographics (age, race and gender), HIV RNA levels, $CD4^+$ cell counts (nadir and baseline), Karnofsky score, previous AIDS diagnosis, HBV and HCV co-infections and use of zidovudine. These variables were chosen on the basis of their epidemiological importance and biological plausibility

Poisson regression

Poisson regression is frequently used to analyse counts of the number of outcomes, namely incidence rate ratios (IRR), which occurred in a defined period of time. Overall, it describes the probability that the outcome will occur in a defined time interval when the probability of occurrence of the event in question is very small, but the number of individuals at risk is very large. This method, therefore, lends itself to analyses of factors associated with uncommon outcomes in very big datasets of large cohorts. Another advantage of Poisson regression is that, differently from Cox regression as discussed below, it does not require the hazards proportion assumption and the risk of outcome can vary over follow-up. In multivariable models it is possible to adjust for confounding baseline or time-updated variables.

In paper *III*, we used multivariable Poisson regression to quantify the association between the latest or current platelet count, categorised as $\leq$100, 101-200, 201-300 and >300 x 10^9/L, with the incidence of AIDS-defining conditions and non-AIDS-defining conditions (pancreatitis, end-stage liver/renal disease, cancer and cardiovascular disease [69]) in EuroSIDA. Fatal and non-fatal events were included; causes of death were determined using the Coding Causes of Death in HIV (CoDe) methodology [75]. Adjusted IRRs were calculated after adjustment for baseline (gender, ethnicity, HIV exposure group, baseline date, $CD4^+$ nadir) and time-updated (age, HBV and HCV co-infection, $CD4^+$ counts, HIV-viral load [VL], diabetes, hypertension, smoking status and anaemia) variables, as defined in a previous EuroSIDA report [69]. We also explored the association between platelet counts and the risk of cardiovascular disease and non-AIDS-defining cancer, which were the two most commonly occurring non-AIDS-defining events.

Time to event analyses, Kaplan-Meier curve and Cox proportional hazards regression

In time to event analyses, the outcome variable is the time between the initial exposure to a covariate of interest and the development of an outcome. Here, the assessment of relationship between covariate and outcome is very sensitive because it is possible to look at not only how frequently the outcome was, but also how long the outcome occurred after the exposure to the covariate. The Kaplan-Meier curve is a graphical estimate of the proportion of participants, grouped according to their distribution across baseline covariates of interest, who remain free of the outcome after the start of study.

The Cox proportional hazards regression is a particular subtype of regression analysis that describes associations between covariates of interest with the time that study participants remain free of disease or other clinical outcome. Cox regression has clear advantages over linear regression because the latter cannot distinguish between censored observations, that is the time until lost to follow up or drop out, and the time that study participants remained free of the outcome. It is assumed with the proportional hazards model that the risk of developing the outcome associated with a higher versus lower category of exposure to a baseline covariate is constant during the entirety of follow up period. In multivariable models it is possible to adjust for confounding baseline or time-updated variables.

In paper *IV*, we studied the relationship between IL-6, hsCRP and D-dimer and the risk of infection-related and infection-unrelated cancer using Kaplan-Meier curves and Cox regression. Kaplan-Meier curves were used to graphically display the cumulative percentage of participants with cancer for biomarker quartiles. As biomarker distributions might have differed among studies, quartiles were defined separately for SMART, ESPRIT and SILCAAT. Hazard ratios (HRs) corresponding to one $\log_2$ increase in biomarker and 95% CI were estimated using three models: (1) unadjusted; (2) adjusting for age, sex, race, continent of enrolment, study-entry and time-updated $CD4^+$ counts; and (3) adjusting for the same covariates and all biomarkers simultaneously. In secondary analyses restricted to smaller datasets, we could further adjust models for other traditional risk factors for cancer, such as obesity, diabetes and smoking.

The problem of reverse causality

Analyses of potential predictors of cancer and other clinical events such as cardiovascular and end-stage liver or renal disease should always take into account the possibility of reverse causality. These diseases have a long latency period, which means that they may progress silently for an extended time before being diagnosed. If higher levels of biomarkers measured during the latency period turn out to be associated with a particular disease, this association can be spurious and entirely attributable to reverse causality. In this case, rather that truly preceding the disease, the observed increases in biomarker levels could be due to the disease itself.

In our work we used different approaches to reduce the possibility of bias by reverse causality. In paper *III*, we recalculated incidence rates for AIDS-defining and non-AIDS-defining conditions after lagging platelet counts by one year prior to clinical events. In paper *IV*, we redid analyses excluding all cancer events that occurred in the first two years of follow up. In this case, we explored the associations between cancers and biomarkers measured two years before.

The Wei-Lin-Weissfeld test

In paper *IV*, we also sought to compare the strength of association of each biomarker with infection-related and infection-unrelated cancer. We used the competing risk or marginal Cox model described by Wei, Lin & Weissfeld [76] to model multiple unordered events and to test for equal effects of biomarkers on different types of cancer. With this approach the fit of a model which assumed a common association for each biomarker with infection-related and infection non-related cancers was compared with the fit of a model that allowed the association to vary. The test, therefore, provides a means of performing a significance test to determine what the probability is that the differences in estimated HRs could have arisen by chance if there were no real differences in the actual HRs. A similar approach was taken for assessing the association of the biomarkers with type-specific cancers of interest.

The Strategic Timing of AntiRetroviral Treatment (START) study

Besides the scientific work presented in this thesis, the PhD applicant has also worked as the medical officer of the Copenhagen International Coordinating Centre, one of the four coordinating centres of the INSIGHT network responsible for enrolling and following up study participants from across 13 European countries. He has been actively involved in the ongoing Strategic Timing of AntiRetroviral Treatment (START) trial (NCT00867048) [77,78], which is a multicentric, fully enrolled international trial with 4,685 participants comparing, in individuals with CD4 > 500 cells/mm^3, immediate versus deferred (i.e., until CD4 counts reach 350 cells/mm^3) initiation of cART. START is a clinical endpoint-driven trial with a composite primary study endpoint consisting of AIDS-related and non-AIDS-related morbidity and mortality [77,78]. The study is expected to reach the required 213 endpoints by the end of 2016 and will provide the highest evidence for a reliable assessment of the individual benefit:risk ratio of early initiation of cART. His activities as medical officer have had a broad range, including pharmacovigilance monitoring, review and validation of clinical endpoint report forms, clinical support for site investigators and study monitors and development of strategies to improve enrolment rates and minimise lost to follow up and protocol violation.

Summary of Results

Paper I

The demographic factors independently associated with higher D-dimer levels were age, race and gender. In SMART, adjusted FDs (95% CI) in D-dimer levels were 1.14 (1.10-1.18) per 10 years older, 1.20 (1.12-1.28) for black versus withe race, and 1.51 (1.42-1.61) for female versus male sex. BMI was not associated with D-dimer levels. In analyses stratified by gender, the effect of increasing age on D-dimer was much steeper in men than in women (Figure 1). Not surprisingly, there was a significant interaction between age and gender (p<.001).

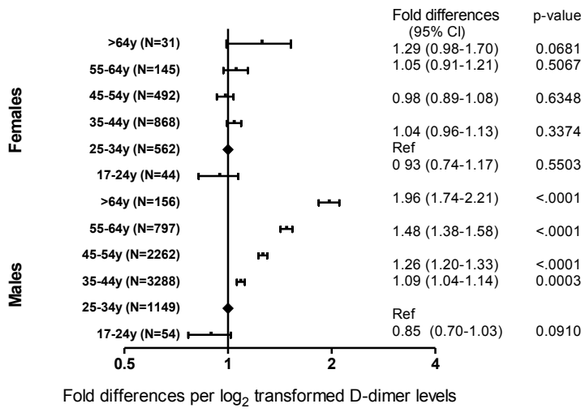

Figure 1: D-dimer levels across age groups. SMART, ESPRIT and SILCAAT; models adjusted for demographics, HIV-specific variables and biomarkers of inflammation

With respect to HIV-specific variables, lower $CD4^+$ cell counts at study entry (adjusted FD [95%CI]: 0.98 [0.97-1.00] per 100 cells/mm^3 in SMART, p=0.004,) and higher HIV RNA (1.12 [1.08-1.17] for those with HIV RNA> 500 copies/mL versus those with ≤ 500 copies/mL in SMART, ESPRIT and SILCAAT) were positively associated with higher D-dimer levels. On the other hand, the higher the nadir $CD4^+$ count, the higher the D-dimer levels (1.02 [1.00-1.03] in SMART, p=0.047). Among participants on cART, D-dimer levels were similar between those receiving protease inhibitors and non-nucleoside reverse transcriptase inhibitors.

Both hsCRP and IL-6 were independently associated with higher D-dimer (Figure 2). IL-6 was positively correlated with D-dimer levels (Figure 3). There was no evidence of interaction between age and hsCRP (p=0.33) or IL-6 (p=0.98).

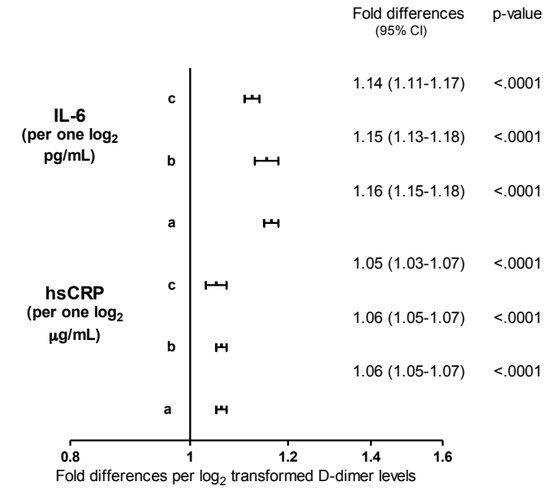

Figure 2: Biomarkers of Inflammation and D-dimer levels (a) SMART/ ESPRIT/ SILCAAT (N=9848); adjusted for demographics, HIV-specific variables and biomarkers of inflammation; (b) SMART/ ESPRIT (N=6928); as in (a) and also adjusted for co-morbidities (CVD, DM and hepatitis B/C) and eGFR and (c) SMART (N=4488); as in (b) and also adjusted for smoking, cystatin C and cholesterol levels .

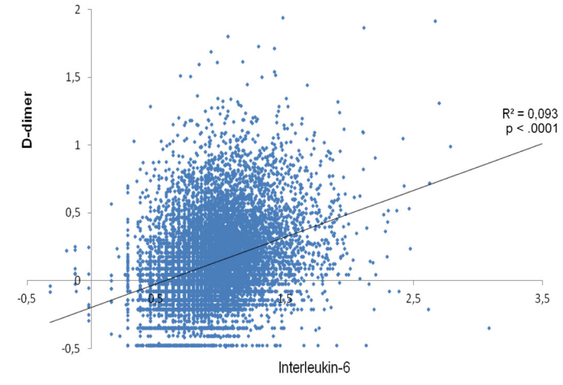

Figure 3: Correlation between D-dimer and IL-6 levels. Plotted values refer to log_{10} transformed levels of units of measurement based on the molecular masses of D-dimer and IL-6 (nmol/L for D-dimer and fmol/L for IL-6)

HBV and HCV were the only co-morbidities found to be associated with increased D-dimer levels. Smoking and other co-morbidities, such as cardiovascular disease and diabetes were related to higher D-

dimer in univariable analyses only, but did not have a significant association with D-dimer after adjustment. In SMART, adjusted FDs (95% CI) for HBV and HCV were 1.27 (1.07-1.50) and 1.19 (1.10-1.29), respectively. In HBV and HCV co-infected participants, the degree of liver fibrosis, as demonstrated by higher hyaluronic acid levels (1.05[1.01-1.09] per 1 log_2 ng/mL, p= 0.0078, for HBV and 1.05[1.00-1.09], p= 0.0315, for HCV), but not the viral load of hepatitis viruses (1.01[1.00-1.02] per 1 log_2 IU/mL, p= 0.17, for HBV and 0.99[0.97-1.00], p= 0.09, for HCV) was found to be positively correlated with D-dimer levels.

Poorer renal function, as demonstrated by lower eGFR or higher cystatin C, was associated with higher D-dimer. When eGFR and cystatin C were mutually adjusted for, this association was apparently stronger for cystatin C (adjusted FD [95% CI]: 1.37 [1.24-1.51] per 1 log_2 mg/dL in SMART). Higher total (0.97 [0.96-0.98] per 10 mg/dL, p< .0001) and HDL cholesterol (0.98 [0.96-1.00] per 10 mg/dL, p< .0151) were found to be associated with lower D-dimer levels.

Overall, goodness of fitness was not improved by adding more covariates to the models. The adjusted R^2 values ranged from 0.15 to 0.22

Paper II

Among the 1,410 SILCAAT participants with haemoglobin, MCV and biomarkers levels measured at study entry, 313 (22.2%) had anaemia, defined as a haemoglobin of 14 g/dl or less in men ad of 12 g/dl or less in women. Anaemic participants had significantly higher levels of IL-6, hsCRP and D-dimer. Moreover, they were more likely to be older, black, male and receive zidovudine, as well as have lower $CD4^+$ cell counts and lower Karnofsky scores at study entry.

Of all anaemic participants, 13 (4.1%), 85 (27.2%) and 215 (68.7%) had microcytic, normocytic and macrocytic anemia, respectively. Zidovudine use was significantly more common in participants with macrocytic versus normocytic anemia (78.6% versus 14.1%, p<.001).

Higher levels of IL-6 and D-dimer, but not of hsCRP, were found to be independently associated with significantly higher odds of anaemia at study entry (Figure 4). This association was observed consistently in those with normal and high MCV values. The presence of anaemia increased most dramatically in those with the highest quintiles of IL-6 and D-dimer (Figure 5).

Multivariable linear regression models using haemoglobin as a continuous variable showed similar association between biomarkers and lower haemoglobin. Adjusted regression coefficients (95% CI) for 1 log_2 higher IL-6, hsCRP and D-dimer were: -0.13 (-0.21 to -0.06), 0.00 (-0.04 to 0.04) and -0.25 (-0.34 to -0.16), respectively.

Secondary analyses excluding participants receiving zidovudine showed findings similar to the ones above described.

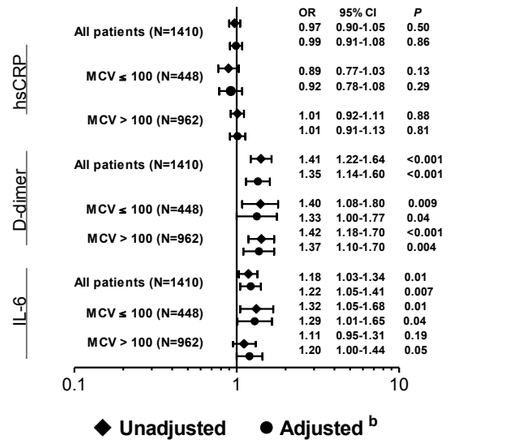

Figure 4: Associations between anaemia and biomarkers across MCV levels [a]

[a] The number of anaemic patients with MCV< 80 (N=11) was too low to calculate ORs. Adjusted ORs for patients with normocytic anaemia (MCV 80-100) were 1.27 (p= 0.09) for D-dimer and 1.18 (p= 0.17) for IL-6. [b] Adjusted for demographics (age, race, gender), BMI, HIV RNA levels, CD4+ cell counts (nadir and baseline), Karnofsky score, previous AIDS diagnosis, hepatitis B and C co-infection and use of zidovudine. Log_2-transformed levels of IL-6, hsCRP and D-dimer were adjusted for simultaneously.

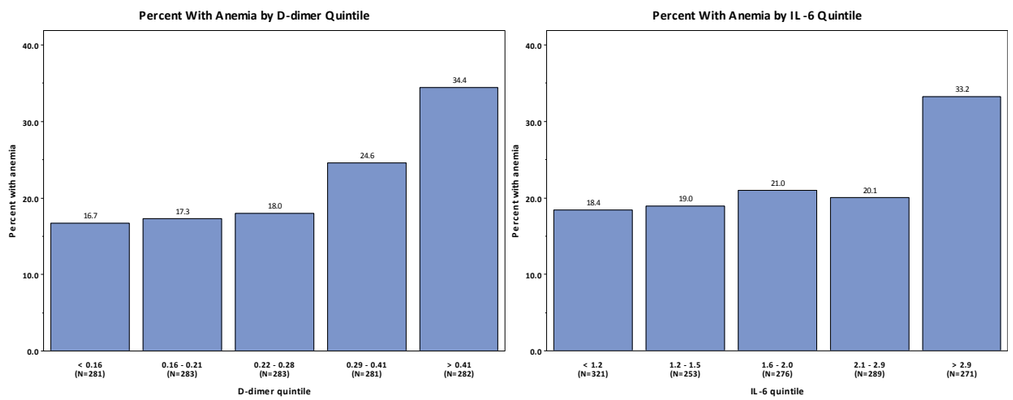

Figure 5: Percent of SILCAAT participants with anaemia by D-dimer and IL-6 quintile

Paper III

During 62,898 person-years of follow-up (PYFU) among 14,515 EuroSIDA participants with platelet data available after 1 January 2015, there were 1,168 non AIDS-defining events (crude incidence ratio [IR] 18.6/1,000 PYFU; 95% CI 17.5–19.6) and 735 AIDS-defining events (IR 11.7; 10.8–12.5). We found a 'reverse J' shaped relationship between current platelet count and AIDS and non-AIDS-defining events. Persons with thrombocytopenia, defined as platelet count of 100 x 10^9/L or less [79], had the highest incidence of both events, this declined as current platelet count went from 101-200 x10^9/L to 201-300 x10^9/L, but then increased again when platelet counts were >300 x10^9/L (Figure 6).

In multivariable Poisson models, thrombocytopenic patients had a slightly increased incidence of AIDS-defining events (adjusted IRR 1.42; 95% CI 1.07-1.86). However, the incidence of non AIDS-defining events was more than two-fold higher (aIRR 2.66; 2.17-3.26) in patients with thrombocytopenia. Those with the highest platelet counts have also a significantly higher risk of both AIDS and non AIDS-defining events (Figure 6). On the one hand, the association between thrombocytopenia and non-AIDS defining events (aIRR 1.43; 95% CI 1.20-1.71) remained significant after lagging platelets by 12 months prior to clinical events. On the other hand, the associations between thrombocytopenia and AIDS-defining-events (aIRR 1.04; 95% CI 0.84-1.28) and between higher platelet counts and AIDS-defining (aIRR 1.05; 95% CI 0.76-1.45) and non-AIDS-defining events (aIRR 1.10; 95% CI 0.87-1.41) were no longer significant in time-lagged analyses.

Among non AIDS-defining events, after adjustment, the incidence of non AIDS-defining cancer (aIRR 2.20, 95% CI 1.61-3.01), but not of cardiovascular disease (aIRR 0.66, 0.32-1.34) was significantly higher in patients with thrombocytopenia (Figure 7). The association between thrombocytopenia and cancer remained unaltered in time-lagged analyses and in secondary analyses that (1) required a confirmed platelet count to define thrombocytopenia, (2) included only virologically suppressed participants with $CD4^+$ counts at least 200 cells/mm^3 and (3) excluded HBV and HCV co-infected participants.

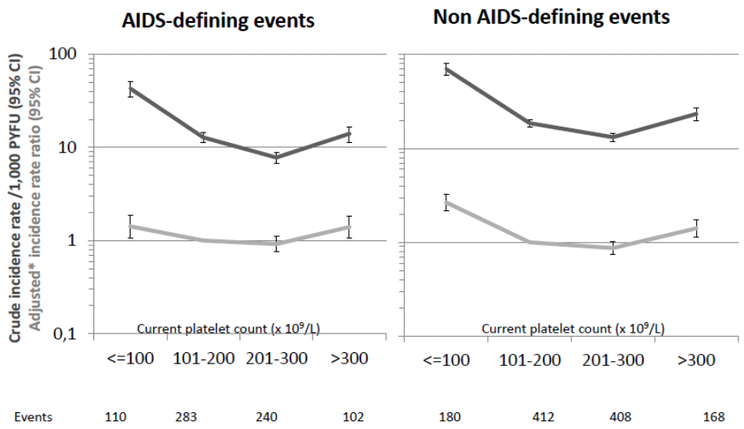

Figure 6: Platelet counts, AIDS-defining and non AIDS-defining events. *adjusted for gender, ethnicity, HIV exposure group, baseline date, CD4 nadir, *age, hepatitis B, hepatitis C, CD4 counts, viral load, diabetes, hypertension, smoking status and anaemia (time-updated variables)*. AIDS was adjusted for AIDS at baseline and non-AIDS as time-updated, non-AIDS was adjusted for non-AIDS at baseline and AIDS as time-updated.

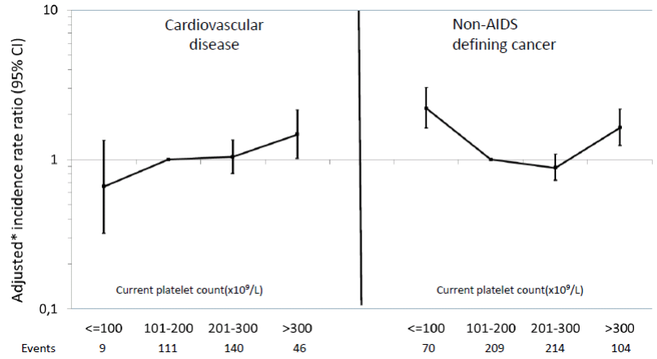

Figure 7: Association between cardiovascular disease, non-AIDS defining cancer and current platelet counts. *adjusted for gender, ethnicity, HIV exposure group, baseline date, CD4 nadir, *age, hepatitis B, hepatitis C, CD4 counts, viral load, diabetes, hypertension, smoking status and anaemia (time-updated variables).* AIDS was adjusted for AIDS at baseline and non-AIDS as time-updated, non-AIDS was adjusted for non-AIDS at baseline and AIDS as time-updated.

Table 2: ESPRIT, SMART, and SILCAAT
Hazard Ratios Associated with Baseline Biomarkers (per one log_2 (i.e. doubling) increment)
Cohort: All control Patients

	Any Cancer						Infection-related cancers#						Infection-unrelated cancers					
	hsCRP		D-dimer		IL-6		hsCRP		D-dimer		IL-6		hsCRP		D-dimer		IL-6	
	HR (95% CI)*	p-value	HR (95% CI)*	p-value	HR (95% CI)*	p-value	HR (95% CI)*	p-value	HR (95% CI)*	p-value	HR (95% CI)*	p-value	HR (95% CI)*	p-value	HR (95% CI)*	p-value	HR (95% CI)*	p-value
Model 1 [a]	1.21 (1.11;1.32)	< .001	1.25 (1.10;1.43)	< .001	1.48 (1.30;1.69)	< .001	1.22 (1.07;1.39)	.003	1.25 (1.01;1.54)	.04	1.49 (1.21;1.84)	< .001	1.20 (1.08;1.34)	< .001	1.26 (1.06;1.49)	.007	1.47 (1.24;1.74)	< .001
Model 2 [b]	1.16 (1.06;1.26)	.001	1.17 (1.01;1.35)	.03	1.38 (1.19;1.59)	< .001	1.18 (1.03;1.35)	.02	1.19 (0.95;1.50)	.13	1.42 (1.14;1.78)	.002	1.15 (1.02;1.28)	.02	1.15 (0.96;1.38)	.12	1.35 (1.12;1.63)	.002
Model 3 [c]	1.06 (0.96;1.17)	.22	1.06 (0.91;1.23)	.43	1.29 (1.09;1.52)	.003	1.08 (0.92;1.26)	.34	1.06 (0.83;1.36)	.64	1.32 (1.01;1.71)	.04	1.06 (0.93;1.20)	.40	1.06 (0.88;1.28)	.53	1.27 (1.02;1.58)	.03
No. patients[§]	*5022*		*5006*		*4994*		*5022*		*5006*		*4994*		*5022*		*5006*		*4994*	
No. events[§]	*172*		*171*		*171*		*70*		*70*		*70*		*102*		*101*		*101*	

All AIDS-defining-malignancies plus vagina, vulva, penis, anal and oral cavity/ pharynx cancers; Hodgkin lymphoma; liver and stomach cancer. All other malignancies were considered to be infection-unrelated cancers
[a] Stratified for study and unadjusted.
[b] Stratified for study and adjusted for demographics (age, race, gender, and continent), and time-updated $CD4^+$ cell counts.
[c] As in model 2 and also adjusted for all biomarkers.
* HR = hazard ratio for one log_2 (i.e. doubling) increase in indicated biomarker.
§ Due to missing data, numbers are reduced in model containing all biomarkers to 4982 participants with 171 any cancer, 70 infection-related cancer, and 101 infection-unrelated cancer events.

Paper IV

During approximately 24,000 PYFU among 5,023 participants in the control arms of SMART, ESPRIT and SILCAAT with biomarkers measured at study entry, there were 70 infection-related cancers and 102 infection unrelated cancers.

In Cox models with biomarkers modelled as continuous variables, IL-6, hsCRP and D-dimer demonstrated significant unadjusted (model 1) and adjusted (model 2) associations with increased risk of any type, infection-related and infection-unrelated cancers (Table 2). When all biomarkers were adjusted for mutually (model 3), only IL-6 remained independently associated with cancer risk (HR 1.29, 95% CI 1.09-1.52, p=0.003) (Table 2). Further adjustment for other traditional cancer risk factors, namely BMI, diabetes and smoking, which were available for SMART participants only, did not change our findings. The Kaplan-Meier curve for all cancers grouped for quartiles of IL-6 levels is shown in Figure 8.

When we tested the proportional hazards assumption, we found no evidence that HRs of cancer varied over follow-up for hsCRP (p=0.25) and D-dimer (p=0.92). For IL-6, however, the HRs varied across the time (p=0.04). The association between higher IL-6 and any type cancer was stronger during the first two years, but remained highly significant afterwards (Figure 9). Based on model (2), the HR for any type cancer in the first two years of follow-up (68 cancer events) associated with a doubling of IL-6 was 1.50, 1.21-1.85 (p<0.001); for follow-up after 2 years (103 cancer events), the HR was 1.31, 1.08-1.60 (p=0.007). The association between higher hsCRP levels and long-term cancer also remained significant after exclusion of early events, but D-dimer was no longer associated with cancer risk (Figure 9).

When using the Wei-Lin-Weissfeld test, we found no evidence that biomarker associations varied between infection-related and infection unrelated cancers. With the model 2 adjustment, p-values for differences between the associations with infection-related and infection-unrelated cancers were 0.90, 0.90, and 0.89, for hsCRP, D-dimer and IL-6, respectively. When we studied associations between biomarkers and type-specific cancers (non-Hodgkin lymphoma, Hodgkin lymphoma, HPV-related cancers, lung, prostate, colorectal and Kaposi sarcoma), there was no evidence that biomarker associations varied by type of cancer for D-dimer (p=0.12) or IL-6 (p=0.65); however, there was evidence for unequal effects across cancer types for hsCRP (p=0.04). Higher hsCRP levels were more strongly related to Hodgkin lymphoma (HR 1.72, 1.18-2.50, p=0.004) than to colorectal cancer (1.41, 1.02-1.92, p=0.04) or lung cancer (1.26, 1.00-1.58, p=0.05).

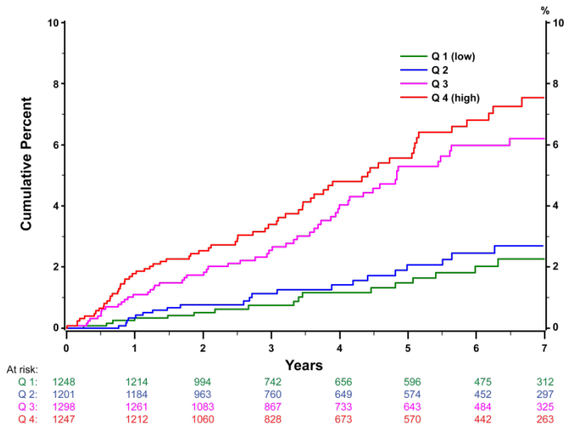

Figure 8: Kaplan-Meier curve: any type cancer by IL-6 quartile at study entry. Control arms of SMART, ESPRIT and SILCAAT.

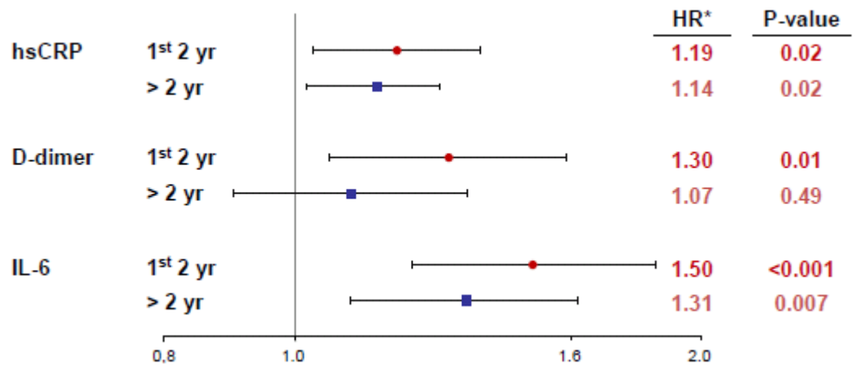

Figure 9: HRs and 95% CI for any type cancer by interval of follow-up. Control arms of SMART, ESPRIT and SILCAAT. *HR for one $\log_2$ higher biomarker level (i.e. a doubling)

Paper A

On the basis of our findings reported in paper *IV*, we decided to conduct a review to critically appraise the recent literature on factors associated with cancer risk during HIV infection. First, we attempted to provide a historical perspective on the epidemiological data linking cancer to HIV infection. Three cancer types, namely Kaposi sarcoma, non-Hodgkin lymphoma and invasive cervical cancer, were found to have a significantly higher incidence in the very beginning of the AIDS pandemics and have since then been referred to as AIDS-defining malignancies. The scope of cancer types associated with HIV infection was subsequently broadened by epidemiological surveillance. Several other cancer types, both infection-related and infection-unrelated, were found to be significantly more common among HIV+ persons when compared to the general population.

The reasons why HIV+ persons have an increased risk of cancer are not entirely understood. Persistent immunodeficiency seems to play a crucial role. Individuals with lower $CD4^+$ cells have higher cancer risk than those with immunological recovery, which is particularly true for infection-related cancers with a known viral aetiology. Enhanced inflammation and coagulation (paper *IV*), higher prevalence of traditional risk factors for cancer, such as smoking and alcohol use, and a potential direct oncogenic effect of HIV may also be important contributors.

With respect to cART exposure, there are very conflicting experimental and epidemiological data on how it affects cancer risk. Because cART restores immune function, controls HIV replication and reduces inflammation, some experts have recommended earlier initiation of cART as a means to reduce cancer risk during HIV infection. However, many studies have linked longer cART exposure to higher risk for cancer. We tried to shed further light in this debate by analysing secondary data reported from randomised controlled trials that compared immediate versus delayed cART initiation in treatment naïve individuals. Only two such trials, SMART [80] and the HIV Prevention Trials Network 052 trial (HPTN 052) [81], have reported incidence of non-AIDS defining cancers as a secondary outcome. There were 3 non-AIDS-defining cancers among 1,017 individuals who started cART immediately and 3 non-AIDS-defining cancers among 993 individuals who deferred cART initiation until median $CD4^+$ counts dropped below 250 cells/mm^3 (risk ratio: 0.98, 95% CI 0.20-4.83 for immediate versus deferred cART). This lack of difference could be explained, among other factors, by the very small number of events and the relatively short follow up of the trials. Because a randomised trial aimed at specially comparing cancer events between early and deferred cART is unfeasible, a better understanding of the effect of cART on cancer will require continued epidemiological surveillance. In this respect, data from the ongoing START study [77,78], a large (N=4,685) randomized trial, will be of particular interest. This study, with a composite clinical endpoint including non-AIDS-defining cancers, is comparing immediate versus deferred (i.e., when CD4+ counts drop below 350) cART initiation in HIV+ persons with $CD4^+$ counts higher than 500 cells/mm^3.

Discussion

Factors associated with D-dimer levels

The association between elevated D-dimer levels and mortality, which was consistently demonstrated by reports involving both HIV+ persons [36,82], as well as the general population [83,84], called for a better understanding of the factors associated with plasma D-dimer levels. In paper *I*, we report, among approximately 10,000 HIV+ individuals from across the globe, the largest investigation of determinants of D-dimer ever published.

The demographic factors that we found to be associated with higher D-dimer, namely older age [47,85,86], black race [47,87] and female sex, are all in accordance with previous reports. The increase of D-dimer levels with age was not attenuated by adjustment by comorbidities and biomarkers of inflammation and there was no significant interaction between age and these biomarkers. Therefore, the higher levels of D-dimer observed in older individuals, differently from what we had previously hypothesized, are most probably not attributable to a higher burden of comorbidities or an age-related pro-inflammatory state. As D-dimer is strongly correlated with arterial disease [85], it is possible that worsening subclinical atherosclerosis, among other factors, may play an important role in the increase of D-dimer with age. In the light of this hypothesis, however, the lack of an association between cardiovascular disease and D-dimer levels appears counter-intuitive. A possible explanation may reside with the fact that participants with prior cardiovascular disease may have been more likely to be on anti-coagulant therapy, which could have reduced plasma D-dimer levels.

In analyses stratified by gender, we found that while a steeper increase in D-dimer with age occurred in HIV+ men, HIV+ women had high D-dimer levels from an early age. Pre-menopausal women may have higher D-dimer levels possibly due to higher estrogen levels [88] and/ or an intrinsically exacerbated immune activation [89]. The independent association between D-dimer and inflammatory markers is consistent with a bi-directional interplay between inflammation and coagulation. Indeed, inflammation may trigger fibrin formation and lysis [90] and IL-6 has long been shown to directly activate the coagulation cascade [91].

In accordance with our hypothesis, uncontrolled HIV infection, as demonstrated by higher HIV RNA and lower $CD4^+$ cell count, was independently associated with higher D-dimer. On the other hand, the observation that higher nadir $CD4^+$ cell count was associated with higher D-dimer was counter intuitive and may have been just a chance finding. Alternatively, this may in part be explained by lower D-dimer levels in participants who had good response to cART and presented a large increase from nadir to study entry $CD4^+$ counts.

Among participants with HBV and HCV co-infection, we observed that hepatic fibrosis, as demonstrated by elevated hyaluronic acid levels, but not the viral load of hepatitis viruses, was independently associated with higher D-dimer. This suggests that the enhanced coagulation state observed in this subset of participants is mainly mediated by liver fibrosis. This is consistent with data from cirrhotic, HIV-uninfected individuals, in whom D-dimer levels increased with progressing hepatic impairment [92]. Therefore, the activated coagulation state seen during HIV infection is most probably determined by a complex and synergistically deleterious interaction of HIV-related and unrelated factors.

The R^2 adjustment coefficients showed that models with an increasing number of covariates were not better to predict the overall variance of D-dimer levels. However, the fact that covariates found to be significantly associated with D-dimer continued so after further adjustment proves the robustness of results.

Enhanced inflammation and coagulation and anaemia in SILCAAT

In the largest study to date (paper *II*) to investigate, in the setting of treated HIV infection, the relationship among inflammation, coagulation and anemia, we found that higher levels of IL-6 and D-dimer, but not hsCRP, are independently associated with the presence of anaemia. This association was not restricted to participants with normocytic anemia, as we hypothesized, but was observed across different ranges of MCV values. This suggests that enhanced inflammation and coagulation may be linked with the development of all types of anaemia. Questions remain as to whether anaemia is merely a bystander of underlying severe disease or is indeed causally related to morbidity and mortality in HIV+ individuals. Our findings suggest that the previously identified link between anemia and increased morbidity and mortality may have a component involving activated inflammation and coagulation. This is consistent with earlier studies reporting correlations between lower haemoglobin and other inflammatory biomarkers [93-95].

The haemoglobin thresholds of 14 g/dl or less in men and of 12 g/dl or less in women have been used in number of studies involving HIV+ persons [51,69,96]. However, because this threshold may be debatable, we sought to confirm our results modelling haemoglobin as a continuous variable in multivariable linear regression models. Here, again, higher IL-6 and D-dimer, but not hsCRP, were significantly associated with lower haemoglobin. Adjustment for a higher number of variables and for other biomarkers had already been shown to significantly reduce associations between hsCRP and clinical outcomes [97]. Among other factors, this may be due to the fact that the hepatic production of hsCRP is determined by IL-6 [38].

The overall prevalence of anemia in this study (22.2%) was similar to other reports using the same haemoglobin thresholds to define anemia and involving European and American HIV-treated participants [51,52,69]. Microcytic anemia was rare among our study participants. This is in sharp contrast with reports involving African HIV+ persons [98,99], in whom microcytic anemia is the most common type of anemia. These discrepant findings could be explained by a possibly lower prevalence of iron deficiency and inherited hemoglobinopathies in Europe and America. Moreover, studies in Africa have used more strict haemoglobin thresholds to define anemia and recruited treatment-naïve participants with more advanced disease. Macrocytosis is a well-known manifestation of zidovudine toxicity [100] and, not surprisingly, this drug was significantly associated with macrocytic anaemia in our study. This was not an important bias in our report because our main findings were confirmed after the exclusion of participants receiving zidovudine.

Thrombocytopenia and the risk of clinical events during HIV infection

In paper *III*, which is one of the first studies with enough power to explore associations between platelet counts and clinical event risk, we report that thrombocytopenia was more strongly associated with non-AIDS-defining events than with AIDS-defining events. On the basis of reported correlations between low platelet counts and higher D-dimer [57] and IL-6 levels [101], we believe that thrombocytopenia may be an epiphenomen as a consequence of activated inflammatory and coagulation pathways, which have the potential to be causally related to the development of end-stage organ disease and cancer.

High platelet counts of 300x 10^9/L or more were also associated with AIDS and non-AIDS-defining events in adjusted analyses, but this association disappeared after lagging platelet counts by one year prior to clinical events and is thus most probably attributable to reverse causality. It is possible that this association reflected reactive thrombocytosis, that is an unspecific response as a part of host's attempt to manage cancer and opportunistic infections [102].

The most striking finding of our report was the fact that incidence of cancer, but not of cardiovascular disease, was significantly higher among thrombocytopenic participants. The association between thrombocytopenia and cancer held in time lagged analyses, which reduced the possibility of confounding by reverse causality. There was no potential bias by laboratory errors, as the results in secondary analyses requiring two consecutive platelet counts to define thrombocytopenia rendered consistent results. Because the association between thrombocytopenia and cancer was also observed among virologically suppressed participants with high $CD4^+$ cell counts, it is very unlikely that this association was driven by the impact of untreated HIV disease. Finally, this association was not explained by hepatic impairment or hypersplenism because the results were consistent after the exclusion of HBV and HCV co-infected participants.

Activation of inflammation and coagulation and cancer risk during HIV infection

Paper *IV* is the largest study thus published to investigate the association between biomarkers and cancer risk in the setting of HIV infection. Higher levels of IL-6, hsCRP and D-dimer were found to be independently associated with a significantly higher risk for cancer. This association was strongest for IL-6, which was the only biomarker that remained significantly associated with any type, infection-related and infection-unrelated cancer when all three biomarkers were adjusted for simultaneously. It is true that risk gradients with IL-6, but not hsCRP, did vary over follow up, but the HRs associated with higher IL-6 levels also remained significant for all cancer endpoints after excluding early cancer events.

We found a stronger association between IL-6 and cancer than between hsCRP and cancer. This finding has biological plausibility as IL-6, an upstream inflammatory marker, regulates the hepatic downstream production of hsCRP [38]. Moreover, IL-6 has been shown to fuel tumour growth through autocrine [103] and paracrine [104] pathways. Experimental data indicates that IL-6 may play a role in several steps of cancer, including initiation, promotion, progression and dissemination [105]. The independent relation that we observed between higher D-dimer and cancer risk was most probably explained by reverse causality, because D-dimer was no longer associated with cancer after the exclusion of early cancer events.

On the one hand, The Wei-Lin-Weissfeld test demonstrated that associations between hsCRP and cancer risk differed according to cancer type, which is consistent with data from the general population. For instance, elevated plasma levels of hsCRP were found to be linked with lung [64,106] and colorectal cancer [64], but not with breast cancer [107]. It is possible that these differences in associations reflect the pathophysiological mechanisms underlying specific cancer types, which can be entirely distinct from each other. On the other hand, we found no evidence that the strength of associations varied by cancer type for IL-6. This is in contrast with data from HIV-uninfected individuals, in whom a significantly increased risk of lung and colorectal cancer [63], but not of prostate cancer [108], was observed with raised circulating levels of IL-6. In contrast with the few previous reports involving HIV+ persons [65,66], we did not find a significant association between IL-6 and non-Hodgkin lymphoma. However, our data should be interpreted with caution owing to our limited ability to examine type-specific associations given the small numbers of specific cancer types.

Strengths and limitations

Overall, the main strengths of the papers included in this thesis are: large sample size, heterogeneous HIV+ populations from across the globe, carefully collected clinical data, measurement of biomarkers by central laboratories (papers *I*, *II* and *IV*) and long follow up period (papers *III* and *IV*). A number of limitations of our work need to be acknowledged. The cross-sectional design and the lack of HIV-uninfected controls in paper *I* hampered our ability to study the temporal direction of associations between covariates and D-dimer levels. In paper *II*, besides the same afore mentioned cross-sectional limitation, we could not look into specific causes of anaemia. Further, SILCAAT recruited participants with persistently low $CD4^+$ counts despite suppressive cART, which raises questions over the generalizability of our results to HIV+ persons with complete immunological recovery. In paper *III*, the most important limitation was the lack of information on important causes of thrombocytopenia in the setting of HIV infection, such as alcohol abuse and immune thrombocytopenic purpura. In paper *IV*, owing to the small number of less common malignancies, we had to categorise them into two broad categories of infection-related and infection-unrelated cancer. As a result, widely heterogeneous and etiologically distinct malignancies were grouped together. Finally, the papers presented in this thesis analysed observational data and, therefore, do not provide definitive evidence for a causal relationship between activated inflammation and coagulation and the development of AIDS-related and non-AIDS-related disease.

Conclusion and perspectives

SMART was the seminal study to link activated inflammation and coagulation, as demonstrated by elevated plasma levels of IL-6, hsCRP and D-dimer, to mortality risk among HIV+ persons [36]. cART interruptions led simultaneously to bursts of plasma levels of biomarkers [36], anaemia [52] and thrombocytopenia [57]. Subsequent studies, most of which were nested in SMART, linked higher levels of IL-6, hsCRP and D-dimer to other adverse clinical outcomes during HIV infection, including progression to AIDS [109,110], cardiovascular disease [72,111] and diabetes mellitus [112]. These ground breaking findings served as the basis of the work conducted in this thesis. Here, we report that:

(paper *I*) demographics, uncontrolled HIV infection, HBV and HCV co-infection, activated inflammation and poorer renal function are independently associated with higher plasma levels of D-dimer, the biomarker most strongly associated with death risk [36];

(paper *II*) higher plasma IL-6 and D-dimer levels, but not hsCRP levels, are independently associated with all types of anaemia during treated HIV disease;

(paper *III*) thrombocytopenia is associated with an increased risk of cancer during HIV infection;

(paper *IV*) higher plasma levels of IL-6, hsCRP and D-dimer are independently associated with subsequent risk of developing infection-related and infection-unrelated cancer in the setting of HIV infection; this association is stronger for IL-6 than for the other biomarkers.

One important finding of paper *I* was that hepatic fibrosis, but not the viral load of hepatitis viruses, affects D-dimer levels. Because HIV infection has been shown to impair the production of anti-coagulant factors leading to a net-pro-coagulant state [113], additional research is required to understand the impact of hepatic dysfunction on coagulation abnormalities, in particular in the subset of HCV and HBV co-infected persons.

With respect to paper *II*, confirmation of our findings linking anaemia to enhanced inflammation/coagulation is warranted among HIV+ persons with high $CD4^+$ cell counts. Further, information on reticulocyte counts, ferritin, transferrin saturation, folic acid, cobalamin and hepcidin levels would have been helpful to assess specific causes of anaemia and better understand the clinical relevance of our findings.

Despite the large number of previous reports on biomarkers that have investigated associations with all-cause death and specific clinical outcomes, information regarding the strength of associations between biomarker levels at study entry and future risk of multiple pathologies is still lacking. This poses an important research question as to whether activated inflammation and hypercoagulation contribute in a general way to the development of multiple diseases or are instead more strongly associated with the development of specific pathologies. Further, it remains to be determined which biomarker best predicts AIDS-related and non-AIDS-related morbidity among persons receiving standard-of-care. Addressing this research question should be a natural continuation of this thesis. Indeed, we have already carried out preliminary analyses deploying the Wei-Lin-Weissfeld test to assess the strength of associations between plasma levels of IL-6, hsCRP and D-dimer and the risk of different clinical outcomes in the control arms of SMART and ESPRIT. Initial results pointing out a potential superiority of IL-6 have recently been presented at an international conference [114].

Our findings in paper *III* poses an important research question as to whether the pathophysiological mechanisms underlying thrombocytopenia contribute to cancer development during treated HIV disease. Thrombocytopenia may be an epiphenomenon as a consequence of activated inflammatory and coagulation pathways [57,101]. Alternatively, it is also possible that thrombocytopenia may reflect the removal from circulation of activated platelets that bind to leukocytes and endothelial cells [115]. Activated platelets release a variety of inflammatory mediators, such as chemokines and molecules from the TNF-α ligand superfamily, into the circulation [116,117]. The plasma levels of platelet-derived inflammatory mediators have been shown to persist raised or even increase despite successful cART [116] and may contribute to chronic immune activation [118]. It is thus enticing to hypothesise that thrombocytopenia is the tip of the iceberg of a feed-forward loop, in which immune activated platelets would release inflammatory mediators that contribute to a chronic immune activation and hypercoagulation status that is not entirely down-regulated by cART. This feed forward loop may have important clinical implications and may explain the epidemiological association between thrombocytopenia and development of cancer that we described in paper *III*. Future studies assessing the relationship between platelet-derived cytokines, biomarkers of platelet activation and cancer risk should be performed to further understand this relationship.

No study has thus far provided irrefutable evidence for a causal relationship between activated inflammation/coagulation and AIDS-related and non-AIDS-related morbidity among HIV+ persons. In the general population, gene-association studies using principles of Mendelian randomisation support a causal role of IL-6 in cardiovascular disease [119] and cancer [120-122]. With respect to hsCRP, polymorphisms in genes that are associated with higher levels of hsCRP were not associated with increased risk of cardiovascular disease [123]. hsCRP seems, therefore, to be more of a epiphenom of underlying pathophysiological processes than a biomarker causally linked to adverse outcomes. Prospective gene-associations studies involving HIV+ persons would be helpful to further clarify this.

A number of concurrent pathophysiological processes, such as HIV persistence in latent cells [124], low level HIV viraemia [125,126], microbial translocation across the gastrointestinal tract [127], persistent damage to lymphoid tissues [128,129] and other chronic viral co-infections, such as cytomegalovirus [130,131] and hepatitis C [132], have been postulated as underlying causes of enhanced inflammation and coagulation and may be causally linked to morbidity during HIV disease. Several therapeutic strategies to reduce immune activation and inflammation have already been tested or are being considered for clinical trials among HIV+ persons [133,134]. Overall, these very small proof-of-concept studies with surrogate endpoints have produced conflicting results. Furthermore, owing to the small sample size and short follow up period, none of these studies have had enough power to simultaneously evaluate the impact of interventions on both biomarker levels and clinical outcomes [133].

Because timing of cART initiation is an important parameter that influences inflammation and coagulation [70,135] by suppressing HIV replication, the results from the ongoing START study [77,78] will be helpful to further elucidate this. START recruited HIV+ persons with early HIV infection and high $CD4^+$ cell counts. The risk of AIDS-related morbidity is, therefore, expected to be low in this population. In case

early treatment turns out to be superior to treatment deferral reducing the risk of clinical endpoints (presumably mainly consisting of cardiovascular disease and cancer events as in SMART [31]), the hypothesis of persistent inflammation as a cause of non-AIDS morbidity during treated HIV disease will be strengthened, although not fully proved because the potential benefits of early cART may also be mediated by mechanisms other than reduction of inflammation. START will also provide an excellent opportunity to assess the surrogacy of inflammatory and coagulation biomarkers for clinical outcomes. Indeed, if biomarkers are good surrogates for clinical outcomes, a dose response relationship should be expected: the larger the reduction in biomarker levels, the larger the reduction in clinical event risk.

The Evaluating the Use of Pitavastatin to Reduce the Risk of Cardiovascular Disease in HIV-Infected Adults (REPRIEVE) trial (NCT02344290) is another ongoing large and clinical endpoint-driven study whose results will be relevant to the debate on whether inflammation is causally related or not to non-AIDS morbidity. This study will randomise 6,500 HIV+ individuals with low cardiovascular risk receiving cART to start statin or placebo. In the Justification for the Use of Statins in Primary Prevention: An Intervention Trial Evaluating Rosuvastatin (JUPITER) (NCT00239681), which recruited HIV-uninfected individuals with low LDL cholesterol and elevated hsCRP levels at baseline, statins were found to reduce risk of cardiovascular disease by 50% [136]. However, because REPRIEVE and JUPITER have cardiovascular disease as the main study endpoint, it is impossible to determine whether the clinical benefit of statin therapy is mediated by a reduction of LDL cholesterol, a reduction of inflammation or a combination of both [137].

Two additional placebo-controlled trials of anti-inflammatory therapies to prevent secondary cardiovascular events in patients with prior coronary disease have started enrolling participants: the Canakinumab Anti-Inflammatory Thrombosis Outcomes Study (CANTOS) (NCT01327846), which is assessing a monoclonal antibody against interleukin-1β [138], and the Cardiovascular Inflammation Reduction Trial (CIRT) (NCT01594333), which is assessing low dose methotrexate [139]. By deploying therapies that directly target inflammation, CANTOS and CIRT will be important to test the inflammatory hypothesis of cardiovascular disease. However, their results will not address the question as to whether inflammation is causally linked to cancer and other non-AIDS-defining morbidities.

To conclude, our findings presented in this thesis highlight the need for clinical endpoint-driven trials to determine whether anti-thrombotic and anti-inflammatory therapies to lower levels of IL-6, hsCRP and D-dimer can reduce morbidity and mortality in treated HIV infection. Such trials would not only determine the potential role of adjuvant anti-inflammatory and anti-thrombotic therapies in the management of HIV infection, but also elucidate whether enhanced inflammation and coagulation are causally linked to the development of AIDS-related and non-AIDS-related morbidity. The inflammatory hypothesis will be strengthened if an intervention that directly target inflammation and coagulation leads to a reduced risk of clinical events.

Danske resume

I de fem artikler, sammenfattet i denne PhD afhandling, har vi søgt at udvide den nuværende viden om den kliniske betydning af aktiveret inflammations- og koagulationssystem, i form a lavere trombocyttal og forhøjede biomarkører for inflammation (IL-6 og hsCRP) or koagulation (D-dimer), ved HIV infektion. Ved brug af data fra HIV positive personer inkluderet i tre internationale randomiserede studier, og i en stor heterogen HIV kohorte, identificerede vi faktorer som er associeret med koncentrationen af D-dimer, og undersøgte associationerne mellem trombocyt kinetikken, biomarkører og risikoen for alvorlige kliniske manifestationer. Vi påviste at demografiske faktorer, ukontrolleret HIV infektion, hepatitis B og hepatitis C ko-infektion, aktiveret inflammation og nedsat nyrefunktion alle er uafhængigt associeret med forhøjede plasma koncentrationer af D-dimer. Forhøjede plasma koncentrationer af IL-6 og D-dimer, men ikke hsCRP, var uafhængigt associeret med alle former for anæmi ved behandlet HIV infektion. Vi rapporterer også at trombocytopeni i forbindelse med HIV infektion var uafhængigt associeret med risikoen for at udvikle cancer. Endeligt, viste vi at forhøjede IL-6, hsCRP og D-dimer koncentrationer hos HIV positive, var uafhængigt associeret med risikoen for udvikling af både infektions-relaterede cancere og cancere som ikke er relateret til infektion. Samlet set, understreger vores fund behovet for studier som undersøger om adjuverende anti-trombotisk og anti-inflammatorisk behandling kan nedsætte morbiditet og mortalitet ved behandlet HIV infektion.

English summary

With the work reported in the five papers summarised in this PhD thesis, we sought to extend the current knowledge on the clinical significance of activated inflammatory and coagulation pathways, as demonstrated by lower platelet counts and elevated levels of inflammatory (IL-6 and hsCRP) and coagulation biomarkers (D-dimer), in the setting of HIV infection. By using data from HIV+ persons participating in three international randomized trials and in one large and heterogeneous HIV cohort, we identified factors associated with circulating D-dimer levels and explored the associations between platelet kinetics, biomarkers and the risk of adverse clinical outcomes. We found that demographics, uncontrolled HIV infection, hepatitis B and hepatitis C co-infection, activated inflammation and poorer renal function are independently associated with higher plasma levels of D-dimer. Higher plasma IL-6 and D-dimer levels, but not hsCRP levels, were found to be independently associated with all types of anaemia during treated HIV disease. We also report that thrombocytopenia was independently associated with the development of cancer during HIV infection. Finally, higher IL-6, hsCRP and D-dimer levels were found to be independently associated with the development of infection-related and infection-unrelated cancers during HIV infection. Taking together, our findings highlight the need for trials to determine whether adjuvant anti-thrombotic and anti-inflammatory therapies can reduce morbidity and mortality in treated HIV disease.

Abstract

Background

In the SMART study, cART interruptions led simultaneously to anaemia, thrombocytopenia and elevations in plasma levels of biomarkers of inflammation (IL-6 and hsCRP) and coagulation (D-dimer). These biomarkers were subsequently associated with excess mortality risk. The majority of deaths were attributable to non-AIDS-defining conditions and a strikingly higher mortality risk was seen in persons with elevated D-dimer. Here, we sought to: (1) identify factors independently associated with D-dimer levels; (2) assess the relationship between biomarkers and presence/ type of anaemia at study entry; (3) study the relationship between platelet kinetics and risk for AIDS-defining and non-AIDS-defining conditions and (4) between plasma biomarker levels and risk of developing cancer during HIV infection

Methods

For objective 1, a cross-sectional study combining baseline data of all participants in three randomised trials (SMART, ESPRIT and SILCAAT) was done. Determinants of elevated D-dimer were identified by multivariable linear regression. For objective 2, the association between IL-6, hsCRP and D-dimer and anaemia was cross-sectionally assessed in SILCAAT by multivariable logistic regression. For objective 3, multivariable Poisson regression quantified the associations between platelet counts and AIDS and non-AIDS-defining conditions in EuroSIDA, a large international HIV cohort. For objective 4, a prospective cohort involving the control arms of SMART, ESPRIT and SILCAAT was used. Cox regression quantified the associations between hsCRP, IL-6 and D-dimer levels measured at study entry and the risk of infection-related and -unrelated cancer.

Results

Demographics, uncontrolled HIV infection, HBV and HCV co-infection, activated inflammation and poorer renal function are independently related to significantly higher plasma levels of D-dimer. Higher plasma IL-6 and D-dimer levels, but not hsCRP, are independently associated with all types of anaemia during treated HIV disease. Thrombocytopenia was independently associated with the development of cancer during HIV infection. The association between thrombocytopenia and cancer was robust in several sensitivity analyses. This finding was consistent after adjustment for confounders, in analyses restricted to virologically suppressed participants with immune recovery and in analyses when the time between platelet counts and cancer events was lagged by 12 months, reducing the possibility of reverse causality. Higher IL-6, hsCRP and D-dimer levels were independently associated with the development of cancer during HIV infection. This association was strongest for IL-6 and was present for both infection-related and infection-unrelated malignancies after excluding early events and adjusting for confounders.

Conclusions

Elevated plasma D-dimer levels are most probably determined by a complex and synergistically deleterious interaction of HIV-related and unrelated factors. Lower haemoglobin levels may provide indirect evidence of activation of coagulation/inflammation during treated HIV disease. Activated inflammation and coagulation, as demonstrated by lower platelet counts and higher IL-6, hsCRP and D-dimer levels, are associated with the development of cancer during HIV infection. Trials to determine whether pharmacological interventions to lower IL-6, hsCRP and D-dimer can reduce morbidity and mortality risk in the setting of HIV infection are warranted.

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Factors Associated with D-Dimer Levels in HIV-Infected Individuals

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Abstract

Background: Higher plasma D-dimer levels are strong predictors of mortality in HIV+ individuals. The factors associated with D-dimer levels during HIV infection, however, remain poorly understood.

Methods: In this cross-sectional study, participants in three randomized controlled trials with measured D-dimer levels were included (N = 9,848). Factors associated with D-dimer were identified by linear regression. Covariates investigated were: age, gender, race, body mass index, nadir and baseline $CD4^+$ count, plasma HIV RNA levels, markers of inflammation (C-reactive protein [CRP], interleukin-6 [IL-6]), antiretroviral therapy (ART) use, ART regimens, co-morbidities (hepatitis B/C, diabetes mellitus, prior cardiovascular disease), smoking, renal function (estimated glomerular filtration rate [eGFR] and cystatin C) and cholesterol.

Results: Women from all age groups had higher D-dimer levels than men, though a steeper increase of D-dimer with age occurred in men. Hepatitis B/C co-infection was the only co-morbidity associated with higher D-dimer levels. In this subgroup, the degree of hepatic fibrosis, as demonstrated by higher hyaluronic acid levels, but not viral load of hepatitis viruses, was positively correlated with D-dimer. Other factors independently associated with higher D-dimer levels were black race, higher plasma HIV RNA levels, being off ART at baseline, and increased levels of CRP, IL-6 and cystatin C. In contrast, higher baseline $CD4^+$ counts and higher high-density lipoprotein cholesterol were negatively correlated with D-dimer levels.

Conclusions: D-dimer levels increase with age in HIV+ men, but are already elevated in women at an early age due to reasons other than a higher burden of concomitant diseases. In hepatitis B/C co-infected individuals, hepatic fibrosis, but not hepatitis viral load, was associated with higher D-dimer levels.

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Introduction

Chronic inflammation and activated coagulation are well-known features of HIV infection [1,2] and evidence has accrued indicating that both processes contribute to an increased risk of death. Out of a panel of inflammatory and coagulation biomarkers tested in participants of the Strategies for Management of Antiretroviral Therapy (SMART) study [3], D-dimer, a fibrin degradation product, was the most predictive biomarker of overall mortality [4]. Furthermore, elevated D-dimer levels were found to be strongly associated with early mortality following ART initiation among severely immunosuppressed South-African patients [5].

A strong association between HIV replication and raised D-dimer levels has been demonstrated. D-dimer levels decline following antiretroviral therapy (ART) initiation [1,5,6] and

increase after stopping ART in treatment experienced patients [1,4]. Correlations of D-dimer with HIV viremia and markers of endothelial dysfunction and microbial translocation [1,4,7,8] have also been reported. This favors the hypothesis that HIV replication and microbial translocation are among the main determinants of the hypercoagulable state seen in HIV-infected persons.

On the other hand, correlations with other biomarkers may also indicate that elevations of D-dimer levels are not mainly determined by HIV infection, but just reflect the presence of co-morbidities or unmeasured confounders that are truly associated with activated coagulation. Indeed, an increase of D-dimer levels with age has been reported in both HIV+ and HIV- individuals [9,10] and it has been hypothesized that a higher burden of co-morbidities and an age-related pro-inflammatory state could explain this [11,12]. Given the complex interaction of multiple factors leading to inflammation, endothelial dysfunction and activated coagulation in persons aging with HIV [13], questions remain as to what is the individual contribution of HIV-specific factors, demographics, co-infections and co-morbidities to the variance in D-dimer levels.

The purpose of this study is to identify factors independently associated with D-dimer levels in a large group of HIV+ individuals. Our main *a priori* hypotheses were that (1) the higher levels of D-dimer seen in older individuals are mainly attributable to a higher burden of co-morbidities and enhanced inflammation, and (2) that HIV-specific variables (HIV viremia, $CD4^+$ cell count and ART use) are independently associated with higher D-dimer levels and that this association remains strong after adjustment for demographics, co-morbidities, smoking, and biomarkers of inflammation and renal function.

Materials and Methods

The present study used baseline data from participants in three randomized controlled trials: (1) SMART (ClinicalTrials.gov number, NCT00027352) [3]; (2) Evaluation of Subcutaneous Proleukin in a Randomized International Trial (ESPRIT) (ClinicalTrials.gov number, NCT00004978); and (3) Subcutaneous Recombinant, Human Interleukin-2 in HIV-Infected Patients with Low $CD4^+$ Counts under Active Antiretroviral Therapy (SILCAAT) (ClinicalTrials.gov number, NCT00013611) [14], whose methods have been described in detail elsewhere. Briefly, the SMART trial compared, in 5,472 individuals with $CD4^+ > 350$ cells/mm^3 at baseline, continuous use of ART with structured treatment interruption guided by $CD4^+$ cell count, involving stopping ART when $CD4^+$ was >350 cells/mm^3 and re-initiating ART when $CD4^+$ was <250 cells/mm^3**.** The ESPRIT and SILCAAT trials compared IL-2 plus ART with ART alone in 4,111 individuals with $CD4^+ >300$ cells/mm^3 and 1,695 individuals with $CD4^+$ between 50 and 299 cells/mm^3, respectively. Participants from all three trials who had consented to storing blood for future research and whose serum D-dimer levels were measured at baseline (N = 9,848) were included in this study.

The SMART, ESPRIT and SILCAAT studies, including the consent for stored specimens, was approved by the institutional review board or ethics committee of each clinical site and of the University of Minnesota. A written informed consent was obtained from all participants involved in the three trials.

Biomarker Measurements

In SMART participants, D-dimer, CRP, IL-6 and cystatin C were measured at the Laboratory for Clinical Biochemistry Research at the University of Vermont (Burlington). D-dimer levels were measured with immunoturbidometric methods on the Sta-R analyzer, Liatest D-DI (Diagnostic Stago, Parsippany, New Jersey, USA). IL-6 was measured with Chemiluminescent Sandwich ELISA (R&D Systems, Minneapolis, Minnesota, USA), CRP with a NBTMII nephelometer, N Antiserum to Human CRP (Siemens Diagnostics, Deerfield, Illinois, USA) and cystatin C with a BNII nephelometer (Dade Behring Inc., Deerfield, Illinois, USA). In the ESPRIT and SILCAAT trials, laboratory measurements were performed by SAIC-Frederick (Frederick, Maryland, USA). D-dimer was measured using an enzyme-linked fluorescent assay (ELISA) on a VIDAS instrument (bioMerieux Inc., Durham, North Carolina, USA), and CRP and IL-6 were measured using ELISA (R&D Systems, Minneapolis, Minnesota, USA). In SMART, lower limits of detection for IL-6, CRP, D-dimer and cystatin C were 0.16 pg/mL, 0.16 μg/mL, 0.01 μg/mL and 0.195 mg/dL, respectively. In ESPRIT and SILCAAT, lower limits of detection for IL-6, CRP and D-dimer were 0.156 pg/mL, 0.078 μg/mL and 0.045 μg/mL. The assays used to measure D-dimer and CRP, while different, compared very well on 20 duplicate samples. Estimated glomerular filtration rate was calculated using the Cockcroft-Gault formula [15] in ESPRIT and SMART participants. Total cholesterol, low-density lipoprotein cholesterol (LDLc) and high-density lipoprotein cholesterol (HDLc) were measured in SMART by Quest Diagnostics, Inc. (Madison, NJ) using standard enzymatic methods. LDLc was directly measured. Samples were not required to be fasting and were analyzed blinded to treatment arm.

The screening of SMART and ESPRIT participants for co-infection with hepatitis B (HBV) or hepatitis C (HCV) has been reported elsewhere [16]. Baseline plasma obtained from individuals with antibody tests positive for HBV and HCV was analyzed for levels of HCV RNA and HBV DNA using branched DNA assays (Versant HCV RNA 3.0 and Versant HBV DNA 3.0, respectively; Bayer Diagnostics), whose lower limits of detection were 615 and 357 IU/mL, respectively. Participants with a positive HBV/HCV antibody and/or viral load test were considered to have hepatitis co-infection. Baseline hyaluronic acid levels were measured in co-infected patients using an enzyme-linked binding protein assay (Corgenix, Colorado, USA) with a lower limit of detection of 10 ng/mL [17].

Statistical Analyses

Factors independently associated with elevated D-dimer levels were identified by multivariable linear regression models. The distributions of D-dimer, CRP and IL-6 were right-skewed; thus a logarithmic transformation was used in the analyses. Log_2-transformed D-dimer levels were modeled as the outcome. PROC REG was used in SAS (version 9.3; SAS Institute, Cary, NC, USA) to produce estimates with 95% confidence intervals (CI) to assess the contribution of covariates. Estimates were then exponentiated in order to correspond to fold differences in D-dimer levels per unit or category difference in the covariates included in the linear regression models. The impact of inter-study, inter-laboratory and inter-method variability was minimized by entering terms for each study in all models. The goodness of fit of the models was assessed using the adjusted R^2 coefficient. A two-sided *P*-value of <0.05 was used as the threshold of statistical significance.

As data on some variables of interest were not collected in all three trials, the regression models were fitted to three different datasets:

(1) The largest dataset combining SMART, ESPRIT and SILCAAT participants (N = 9,848) included: age, gender, race, body mass index (BMI), $CD4^+$ cell counts (nadir and baseline), markers of inflammation (CRP and IL-6), ART use and ART regimens;

(2) A smaller dataset consisting of SMART and ESPRIT participants (N = 6,928) included: co-morbidities (HBV and HCV, diabetes mellitus, prior cardiovascular disease; defined as prior myocardial infarction, stroke or coronary artery disease requiring surgical procedure) and renal function (eGFR);

(3) The smallest dataset consisting only of SMART participants (N = 4,488), included: smoking, cholesterol levels (LDLc and HDLc) and additional information on renal function (cystatin C).

Given the significantly higher levels of D-dimer seen in women, we found it helpful to investigate if determinants of D-dimer levels could differ in analyses stratified by gender. Since the three trials involved participants with different baseline characteristics, we also investigated interactions between D-dimer levels, study (SMART, ESPRIT and SILCAAT), plasma HIV RNA levels, inflammatory biomarkers (IL-6 and CRP) and demographic covariates found to be correlated with D-dimer levels (age, race and gender).

Table 1. Baseline Characteristics by Dataset ESPRIT, SILCAAT and SMART Patients.

	SMART, ESPRIT & SILCAAT (N = 9,848)	SMART & ESPRIT (N = 6,928)	SMART (N = 4,488)
D-dimer (median, IQR) (μg/mL)	**0.24 (0.15–0.38)**	**0.22 (0.15–0.37)**	**0.20 (0.13–0.36)**
Demographics			
Age in Years (median, IQR)	42 (36–49)	42 (36–49)	44 (38–50)
Female Gender (%)	21.8	23.2	25.5
Black Race (%)	19.4	21.3	27.8
BMI (median, IQR)	24.34 (22.12–27.00)	24.45 (22.15–27.30)	24.99 (22.50–28.09)
HIV-specific variables			
Baseline CD4+ cell count (median, IQR) (cells/mm^3)	490 (368–671)	540 (422–722)	601 (470–799)
Nadir CD4+ cell count (median, IQR) (cells/mm^3)	200 (84–316)	229 (121–335)	250 (154–358)
Plasma HIV RNA ≤500 copies/mL (%)	76.4	76.3	73.3
ART regimen			
Off ART (%)	8.3	10.1	15.2
PI-based (%)	37.5	33.7	31.8
NNRTI-based (%)	36.5	38.9	37.8
Other (%)	17.7	17.3	15.2
Biomarkers of Inflammation			
IL-6 (median, IQR) (pg/mL)	1.80 (1.20–2.89)	1.81 (1.17–2.90)	1.72 (1.07–2.93)
CRP (median, IQR) (μg/mL)	1.59 (0.70–3.67)	1.61 (0.71–3.76)	1.70 (0.71–4.07)
Co-morbidities			
Cardiovascular disease (%)*	n/a	2.7	3.6
Diabetes Mellitus (%)*	n/a	5.3	6.7
Hepatitis B (%)*	n/a	3.7	2.2
Hyaluronic Acid (median, IQR) (ng/mL)***	n/a	23.80 (14.63–43.69)	n/a
HBV DNA (median, IQR) (IU/mL)***	n/a	71,704 (2,000–100,000,000)	n/a
Hepatitis C (%)*	n/a	14.4	13.4
Hyaluronic Acid (median, IQR) (ng/mL)****	n/a	33.17 (18.75–59.82)	n/a
HCV RNA (median, IQR) (IU/mL)****	n/a	2,576,804 (583,936–7,610,964)	n/a
Smoking**	n/a	n/a	40.5
Renal Function			
eGFR (median, IQR) (mL/min per 1.73 m^2)*	n/a	111.56 (100.66–121.03)	110.82 (100.25–120.56)
Cystatin C (median, IQR) (mg/dL)**	n/a	n/a	0.81(0.71–0.92)
Cholesterol Levels			
Total Cholesterol (median,IQR) (mg/dL)**	n/a	n/a	192 (164–222)
LDL-c (median,IQR) (mg/dL)**	n/a	n/a	112 (90–137)
HDL-c (median,IQR) (mg/dL)**	n/a	n/a	40 (33–51)

*Not ascertained for patients in SILCAAT.
**Not ascertained for patients in SILCAAT or ESPRIT.
***Data available for n = 245 participants.
****Data available for n = 860 participants.
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In the subset of hepatitis co-infected individuals, we sought to investigate the contribution of liver fibrosis and replication of hepatitis viruses to the variance of D-dimer levels. We then entered hyaluronic acid levels (a validated marker of hepatic fibrosis, which was measured at baseline in 245 study participants co-infected with HBV and in 860 co-infected with HCV), as well as HBV and HCV viral load, into models adjusted for demographics (age, gender and race) and restricted to HBV- and HCV- co-infected participants, respectively.

Sensitivity Analyses

Correlates of D-dimer levels were also investigated by using multivariable logistic regression models. Participants were dichotomized into two groups: low and elevated D-dimer levels; the latter defined as levels greater than 0.377 μg/mL (4^{th} quartile for the participants in all three trials). Odds ratio (OR) with 95% CI were calculated to assess the contribution of correlates.

We also carried out two additional sensitivity analyses using linear regression models: (a) stratified by current ART use (i.e., yes versus no) and (b) stratified by plasma HIV RNA levels (i.e., plasma HIV RNA ≤500 versus >500 copies/mL). Analysis (a) was performed in order to investigate the effect of plasma HIV RNA on D-dimer levels, since plasma HIV RNA was not included in the primary analyses because of the possibility that the colinearity between ART use and plasma HIV RNA levels could affect valid interpretation of our findings. Analysis (b) was performed to determine whether the suppression of viral replication would change the predictors of D-dimer levels.

Results

Baseline demographic, clinical and laboratory characteristics are summarized separately for each of the three datasets included in the analyses and are presented in Table 1. In analyses investigating possible interactions between study and demographic covariates, the following interactions were found to be significant: study and gender ($p = 0.0005$), and study and race ($p<.0001$). There was, however, no evidence of an interaction between study and age ($p = 0.20$). Because the interactions suggested only moderate differences in effect and the biomarker assays compared well on duplicates, we found it appropriate to fit models to datasets pooling the three trials. Moreover, given that our main results are fairly consistent between datasets which used the two different D-dimer assays we believe that the associations presented in this study are not artificially influenced by the use of different assays.

Demographics

The demographic factors found to be positively and independently correlated with D-dimer levels were older age, black race and female sex. The results were robust, with similar fold differences seen consistently across multiple models using different datasets and after adjustment for an increasing range of covariates (Figure 1). BMI, on the other hand, was not found to be associated with D-dimer levels.

In analyses stratified by gender, older age was found to be independently associated with higher D-dimer levels; the effect of increasing age on D-dimer was, however, much stronger in men than in women (Figure 2). Women in all age groups were found to have significantly higher D-dimer levels when compared to males aged 25–34 years (data not shown). The interaction between age and gender was found to be significant ($p<.001$), but there was no evidence of an interaction between age and plasma HIV RNA ($p = 0.40$) and between age and biomarkers of inflammation ($p = 0.98$ for IL-6 and $p = 0.33$ for CRP).

The addition of an increasing number of covariates in multiple regression models consisting of SMART and ESPRIT datasets did not substantially change the power to predict D-dimer levels (adjusted R^2 values ranged from 0.15 to 0.22).

HIV-specific Variables

Uncontrolled HIV infection, as demonstrated by lower baseline $CD4^+$ cell counts and higher plasma HIV RNA, was found to be positively correlated with higher D-dimer levels. This could well explain why being off ART at baseline was also independently associated with elevated D-dimer. We also found a positive and independent correlation between nadir $CD4^+$ cell counts and D-dimer levels. Among those on ART, protease inhibitor (PI)-based and non-nucleoside reverse transcriptase inhibitor (NNRTI)-based regimens were associated with similar D-dimer levels and no significant differences were noted (Figure 3).

Biomarkers of Inflammation

Both CRP and IL-6 were independently and positively correlated with D-dimer. Once again, the results were robust and observed consistently in all linear regression models (Figure 4). The linear positive relationship between IL-6 and D-dimer levels is graphically illustrated in Figure 5.

Co-morbidities, Renal Function and Cholesterol Levels

Prior cardiovascular disease (Fold Difference 0.90, 95% CI [0.79–1.03], $p = 0.14$), smoking (0.98, [0.93–1.03], $p = 0.45$) and diabetes mellitus (0.94, [0.85–1.04], $p = 0.23$) did not have a significant association with D-dimer levels in adjusted models. On the other hand, HBV (1.27, [1.07–1.50], $p = 0.0061$) and HCV (1.19, [1.10–1.29], $p<.0001$) co-infection were independently associated with raised D-dimer levels. In co-infected participants, the degree of liver damage, as demonstrated by higher hyaluronic acid levels (1.05[1.01–1.09] per 1 $\log_2$ ng/mL, $p = 0.0078$, for HBV and 1.05[1.00–1.09], $p = 0.0315$, for HCV), but not the viral load of hepatitis viruses (1.01[1.00–1.02] per 1 $\log_2$ IU/mL, $p = 0.17$, for HBV and 0.99[0.97–1.00], $p = 0.09$, for HCV) was found to be positively correlated with D-dimer levels.

Higher eGFR levels at baseline were found to be significantly associated with lower D-dimer in the dataset consisting of SMART and ESPRIT participants (0.99 [0.98–1.00] per 10 mL/min per 1.73 m^2, $p = 0.023$). However, in SMART participants, after further adjustment for cystatin C, as well as for smoking and cholesterol levels, the association between higher eGFR count and lower D-dimer was no longer significant (1.02, [1.00–1.04], $p = 0.06$). Higher cystatin C levels were strongly associated with elevated D-dimer (1.37 [1.24–1.51] per 1 $\log_2$ mg/dL, $p<.0001$). In contrast, higher total (0.97 [0.96–0.98] per 10 mg/dL, $p<.0001$) and HDL cholesterol (0.98 [0.96–1.00] per 10 mg/dL, $p<.0151$) were found to be associated with lower D-dimer levels.

Sensitivity Analyses

Logistic regression models yielded results highly consistent with linear models (data not shown). The factors associated with D-dimer levels did not differ between study participants off and on ART and between those with and without virological suppression (data not shown).

Discussion

A better understanding of predictors of plasma D-dimer levels became particularly relevant in the light of new evidence indicating that both HIV+ [4,5] and HIV- individuals [18,19] with higher D-dimer levels are at a significantly increased risk of

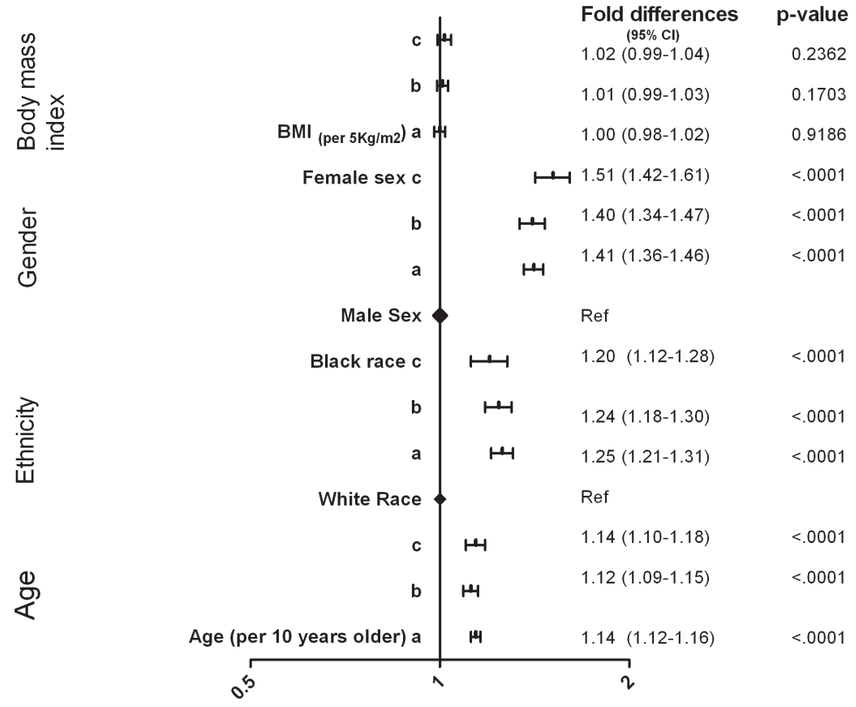

Figure 1. Demographics and D-dimer levels. (a) SMART/ESPRIT/SILCAAT; adjusted for demographics, HIV-specific variables and biomarkers of inflammation. (b) SMART/ESPRIT; as in (a) and also adjusted for co-morbidities (CVD, DM and hepatitis B/C) and eGFR. (c) SMART only; as in (b) and also adjusted for smoking, cystatin C and cholesterol levels.
doi:10.1371/journal.pone.0090978.g001

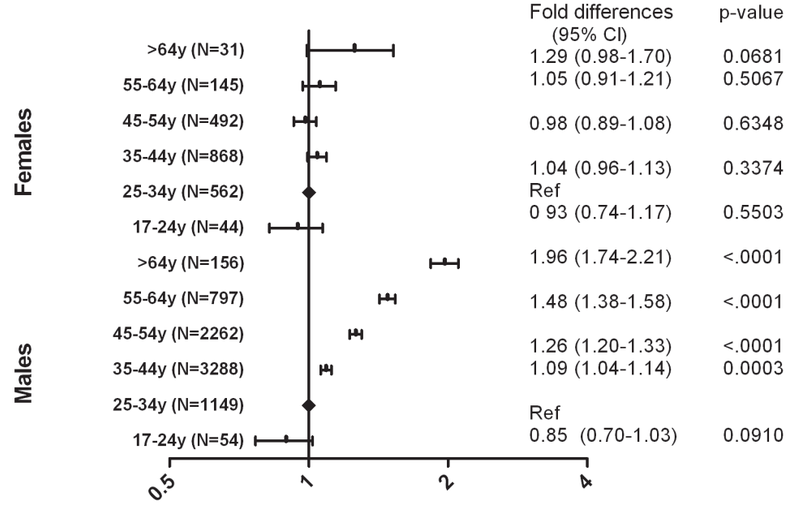

Figure 2. D-dimer levels across age groups stratified by gender (a). (a) SMART/ESPRIT/SILCAAT; adjusted for demographics, HIV-specific variables and biomarkers of inflammation.
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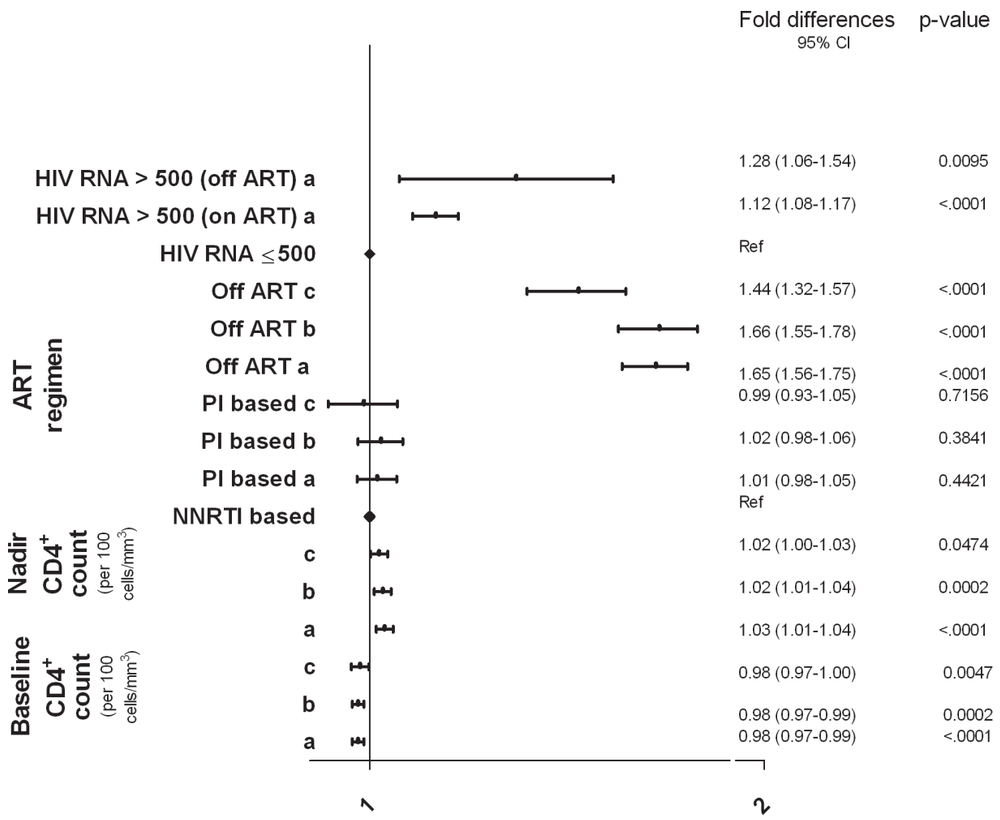

Figure 3. HIV-specific variables and D-dimer levels. (a) SMART/ESPRIT/SILCAAT (N = 9848; 821 of whom were off ART at baseline); adjusted for demographics, HIV-specific variables and biomarkers of inflammation. (b) SMART/ESPRIT (N = 6928); as in (a) and also adjusted for co-morbidities (CVD, DM and hepatitis B/C) and eGFR. (c) SMART (N = 4488); as in (b) and also adjusted for smoking, cystatin C and cholesterol levels.
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death. To our knowledge, this is the largest study investigating determinants of D-dimer published thus far. We have found that while a significant increase in D-dimer with age occurs in HIV+ men, HIV+ women have high D-dimer levels from an early age. These findings cannot be explained by an increased burden of co-morbidities or enhanced inflammation, as previously hypothesized. In those co-infected with HBV/HCV, hepatic fibrosis, but not hepatitis virus load, is independently associated with higher D-dimer. We also observed that HIV-specific variables, other demographic factors, biomarkers of inflammation, renal function and cholesterol levels are independently associated with higher D-dimer levels.

We found that black race, female sex and older age were demographic factors independently associated with higher D-dimer levels. African-American ethnicity was also found to be associated with higher plasma levels of D-dimer in HIV+ participants in the Veterans Aging Cohort Study (VACS) [10] and HIV- hypertensive adults [20]. Not surprisingly, the inter-racial variability in circulating D-dimer levels was shown to be, in part, genetically determined [21].

Increases in D-dimer levels with age have been previously reported [9,10,20,22] and deleterious interactions between vascular damage, co-morbidities, inactivity and activated inflammation have been postulated as possible mechanisms [11,12]. However, we did not find, except for hepatitis, significant associations between co-morbidities and D-dimer levels. Moreover, we found no significant interaction between age and inflammatory biomarkers. Taken together, our findings suggest that the increase of D-dimer with age is primarily attributable to causes other than a higher burden of concomitant diseases or an age-related pro-inflammatory state. Since elevated D-dimer was found to be correlated with arterial disease severity [23], worsening subclinical atherosclerosis may play an important role.

We found a significant interaction between age and gender and demonstrated that older age was more strongly associated with higher D-dimer in men than in women. We hypothesize that the significantly higher D-dimer levels observed in younger women may be due to higher estrogen levels and higher immune activation. Pregnancy, hormone replacement therapy and estrogen-containing contraceptive pills increase plasma levels of procoagulant factors and are well-known risk factors for thromboembolism [24,25], which suggests a potential interplay between estrogen and D-dimer. After estrogen levels fall (among post-menopausal women), the gender difference is then attenuated as

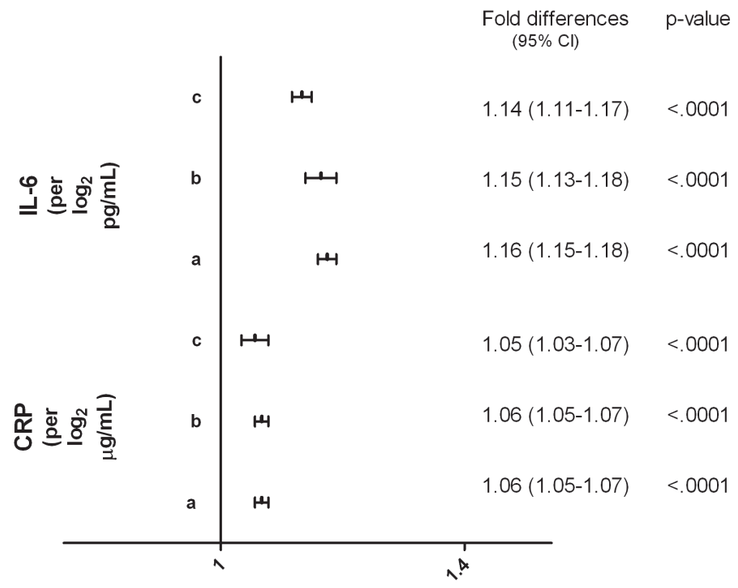

Figure 4. Biomarkers of Inflammation and D-dimer levels. (a) SMART/ESPRIT/SILCAAT (N = 9848); adjusted for demographics, HIV-specific variables and biomarkers of inflammation. (b) SMART/ESPRIT (N = 6928); as in (a) and also adjusted for co-morbidities (CVD, DM and hepatitis B/C) and eGFR. (c) SMART (N = 4488); as in (b) and also adjusted for smoking, cystatin C and cholesterol levels.
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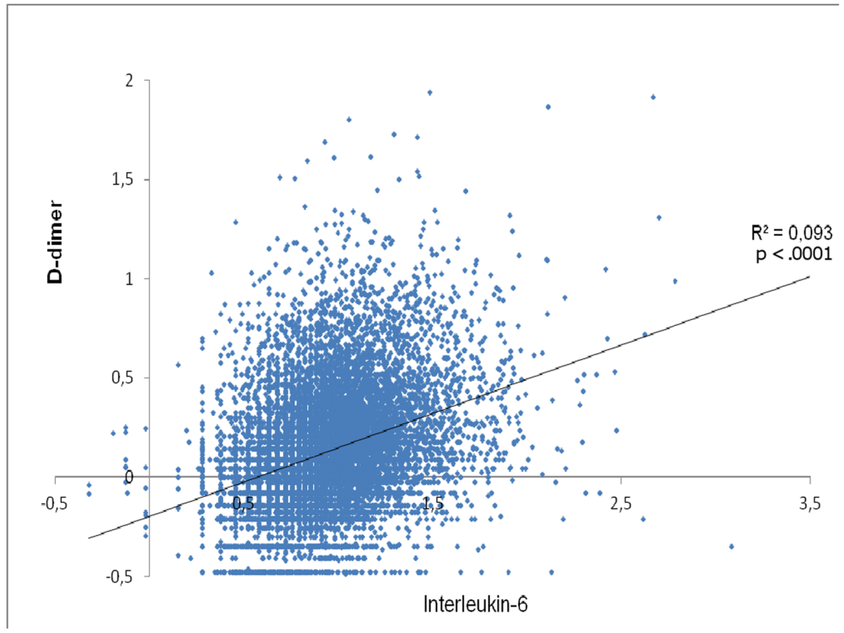

Figure 5. Correlation between D-dimer and IL-6 levels*. * Plotted values refer to $\log_{10}$ transformed levels of units of measurement based on the molecular masses of D-dimer and IL-6 (nmol/L for D-dimer and fmol/L for IL-6).
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determinants of D-dimer levels for both genders may be more related to similar clinical and environmental factors. HIV+ women have also been found to have higher activation of $CD8^+$ T cells than HIV+ men with comparable HIV plasma levels [26] and this exacerbated immune activation may have contributed to the higher D-dimer levels observed primarily in pre-menopausal women.

Significantly higher D-dimer levels have been seen in HIV-infected patients with ongoing viral replication and lower $CD4^+$ cell counts [4,6,10]. Individuals receiving ART had significantly lower D-dimer levels than those off ART at baseline, but no remarkable differences between PI- and NNRTI-based regimens were noted. The factors independently associated with elevated D-dimer levels, however, did not differ considerably between individuals with suppressed or unsuppressed plasma HIV RNA levels. The control of HIV viral replication, therefore, did not substantially affect the main factors driving coagulation, a finding that suggests the potential benefit of adjunctive anti-thrombotic therapies during HIV infection, even in those with HIV viral suppression.

The positive correlation between nadir $CD4^+$ cell counts and D-dimer levels that we observed was surprising and counter-intuitive. Nadir $CD4^+$ counts were significantly associated with D-dimer levels only after adjustment for baseline $CD4^+$ counts, but not in univariable analysis. The interplay between D-dimer levels, $CD4^+$ cell counts and plasma HIV RNA is complex with dynamic changes after ART initiation [1,5,6] and this may in part be explained by lower D-dimer levels in participants who had good response to ART and presented a large increase from nadir to baseline $CD4^+$ counts. However, this observation may have been a chance finding and further investigation is required.

Congruent with previous reports [4,21], D-dimer and biomarkers of inflammation were positively correlated. Inflammatory responses promote fibrin formation and lysis, resulting in elevated D-dimer levels [27], with IL-6 being shown to directly activate the coagulation cascade [28]. Furthermore, D-dimer and other fibrin degradation products have been found to modulate the production of IL-6 and other inflammatory mediators [29]. This is consistent with a bi-directional interplay between inflammation and coagulation. We have also confirmed a previously reported negative correlation between HDLc and D-dimer [30]. Indeed, HDLc has been shown to down-regulate thrombotic pathways by multiple mechanisms, including inhibition of endothelial and platelet activation, promotion of endothelium-dependent vasodilatation and attenuation of thrombin generation [31].

We demonstrated for the first time that the degree of hepatic fibrosis, as demonstrated by higher hyaluronic acid levels, but not the replication of hepatitis viruses, was associated with higher D-dimer levels in co-infected patients. This is consistent with data from cirrhotic, HIV-uninfected individuals, in whom D-dimer levels were found to increase as hepatic impairment progresses [32]. However, similarly to other fibrosis markers, HA is not liver specific and may reflect other pathologies. Therefore, information on other measures of hepatic fibrosis, such as Fibroscan, APRI and FIB-4, would have been helpful. Of interest, recent data demonstrated that HIV replication, in part through associated reductions in levels of hepatocyte-dependent anti-coagulant factors, leads to a net-procoagulant state [33]. Additional research is needed to better understand the potential consequences of hepatic function for coagulation abnormalities, and clinical risk, among HIV positive patients.

Decreased renal function, as demonstrated by lower eGFR, was associated with elevated D-dimer in partially adjusted models in this study. We observed, however, that this association became no longer significant after adjustment for cystatin C, which, in turn, was found to be positively correlated with D-dimer levels. This finding indicates that cystatin C, as a surrogate measure of renal impairment, is a better predictor of D-dimer levels than eGFR. Higher D-dimer levels in renal failure may reflect both decreased D-dimer clearance and increased fibrin turnover [34], as well as an inherent pro-inflammatory state [35].

A number of caveats need to be noted regarding the present study. First, its cross-sectional design hampered our ability to infer causality and to characterize associations over time. Second, as data on variables of interest were not uniformly collected in the three trials, adjustment for important co-variates had to be done in smaller datasets. Conversely, given the large sample size of this study, some of the statistically significant associations we have found may not be clinically relevant. Finally, we have not investigated factors found to be associated with higher D-dimer levels in the general population, such as blood pressure, alcohol intake and physical activity [36].

In conclusion, D-dimer levels increase with age in HIV+ men, but are already high in women at an early age. This seems to be primarily attributable to causes other than a higher burden of concomitant diseases or an age-related pro-inflammatory state. In those with HBV/HCV co-infection, the only co-morbidity found to be associated with raised D-dimer, hepatic fibrosis, but not hepatitis virus replication, seems to influence D-dimer levels. The control of viral replication did not substantially affect the main factors driving coagulation in HIV+ persons and the role of adjunctive anti-thrombotic therapies should be investigated in this population. As only 20% of D-dimer variance could be explained by the factors we investigated, further studies on genetic, socio-economic and clinical correlates of D-dimer in HIV+ individuals are warranted. Prospective studies and randomized trials are also needed to determine whether pharmacologic interventions to lower elevated D-dimer levels can reduce morbidity and all-cause mortality during HIV infection. We believe that our findings can be instrumental in building the basic knowledge and in selecting suitable candidates for such studies.

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Determinants of D-Dimer during HIV Infection

CONCISE COMMUNICATION

Markers of inflammation and activation of coagulation are associated with anaemia in antiretroviral-treated HIV disease

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Objective: The objective of this study is to determine the relationship between inflammatory interleukin-6 (IL-6) and high-sensitivity C-reactive protein (hsCRP)] and coagulation (D-dimer) biomarkers and the presence and type of anaemia among HIV-positive individuals.

Design: A cross-sectional study.

Methods: Combination antiretroviral therapy (cART)-treated adults participating in an international HIV trial with haemoglobin and mean corpuscular volume (MCV) measurements at entry were categorized by presence of anaemia (haemoglobin ≤14 g/dl in men and ≤12 g/dl in women) and, for those with anaemia, by type [microcytic (MCV < 80 fl), normocytic (80–100 fl), macrocytic (>100 fl)]. We analysed the association between inflammation (IL-6 and hsCRP) and coagulation (D-dimer) and haemoglobin, controlling for demographics (age, race and sex), BMI, HIV plasma RNA levels, $CD4^+$ T-cell counts (nadir and baseline), Karnofsky score, previous AIDS diagnosis, hepatitis B/C coinfection and use of zidovudine.

Results: Among 1410 participants, 313 (22.2%) had anaemia. Of these, 4.1, 27.2 and 68.7% had microcytic, normocytic and macrocytic anaemia, respectively. When compared with participants with normal haemoglobin values, those with anaemia were more likely to be older, black, male and on zidovudine. They also had lower baseline $CD4^+$ T-cell counts and lower Karnofsky scores. Adjusted relative odds of anaemia per two-fold higher biomarker levels were 1.22 ($P = 0.007$) for IL-6, 0.99 for hsCRP ($P = 0.86$) and 1.35 ($P < 0.001$) for D-dimer. Similar associations were seen in those with normal and high MCV values.

Conclusion: Persistent inflammation and hypercoagulation appear to be associated with anaemia. Routine measurements of haemoglobin might provide insights into the inflammatory state of treated HIV infection.

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Keywords: anaemia, coagulation, C-reactive protein, D-dimer, HIV, interleukin-6, inflammation

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Introduction

Anaemia is the most common haematological abnormality in HIV disease [1,2] and is associated with increased morbidity and mortality [3–8]. Even minor decreases in haemoglobin levels are clinically relevant: a 1 g/dl decrease in haemoglobin was found to be independently correlated with a 57% higher risk of death in those with HIV [3].

The morphological assessment of red cell size based on mean corpuscular volume (MCV) is helpful for assessment of the cause of anaemia. Whereas microcytic (MCV < 80 fl) and macrocytic (MCV > 100 fl) anaemias are often caused by deficiencies of iron and folic acid/cobalamin, respectively, normocytic anaemia (MCV 80–100 fl) is predominately seen in patients with chronic disease [9]. In the general population, there is mounting evidence that enhanced inflammation contributes to the development of normocytic anaemia via interleukin-6 (IL-6) dependent pathways [10]. IL-6 induces the formation of hepcidin, a hepatic hormone that interferes with iron absorption and promotes iron uptake by the reticuloendothelial system, ultimately leading to anaemia [11,12].

In this study, we set out to determine the relationship between inflammatory biomarkers [IL-6 and high-sensitivity C-reactive protein (hsCRP)] and the presence and type of baseline anaemia among HIV-positive individuals participating in an international HIV treatment trial. Given the close link between inflammation and coagulation in HIV disease, we also determined the association between D-dimer levels and anaemia.

Materials and methods

The design and results of the Subcutaneous Recombinant, Human Interleukin-2 in HIV-Infected Patients with Low $CD4^+$ Counts under Active Antiretroviral Therapy (SILCAAT) trial have been described elsewhere [13]. Briefly, the SILCAAT trial compared IL-2 as well as combination antiretroviral therapy (cART) with cART alone in 1695 individuals with entry $CD4^+$ T-cell counts between 50 and 299 cells/μl. All participants with biomarker levels, haemoglobin and MCV measurements at entry were included in this study.

We defined anaemia as a haemoglobin of 14 g/dl or less in men and of 12 g/dl or less in women [3,5,8]. Anaemia was classified as microcytic, normocytic or macrocytic on the basis of MCV values below 80, between 80 and 100, and over 100 fl, respectively. On the basis of strong associations of IL-6, hsCRP and D-dimer with all-cause mortality [14] and the observation that these biomarkers were elevated in treated HIV-positive individuals compared with the general population [15], IL-6, hsCRP and D-dimer were measured on stored plasma at baseline for all consenting SILCAAT participants. IL-6 and hsCRP were measured using the Chemiluminescent Sandwich immunoassay (R&D Systems, Minneapolis, Minnesota, USA) and D-dimer was measured using the VIDAS immunoassay (BioMerieux Inc., Durham, North Carolina, USA). Measurements were performed by SAIC-Frederick and lower limits of detection for IL-6, hsCRP and D-dimer were 0.156 pg/ml, 0.078 and 0.045 μg/ml, respectively.

Demographics and clinical data from participants with and without anaemia and those with normocytic and macrocytic anaemia were compared. The association between inflammatory (IL-6 and hsCRP) and coagulation (D-dimer) biomarkers and anaemia was assessed by multivariable logistic regression. IL-6, hsCRP and D-dimer levels were $\log_2$-transformed because their distributions were right-skewed. With this approach, a one $\log_2$ higher level of a biomarker corresponds to a two-fold higher level of the marker. Variables adjusted for were chosen on the basis of their epidemiological importance and biological plausibility and included demographics (age, race and sex), BMI, HIV plasma RNA levels, $CD4^+$ T-cell counts (nadir and baseline), Karnofsky score, previous AIDS diagnosis, hepatitis B [defined as hepatitis B surface antigen (HBsAg) seropositivity by ELISA] and hepatitis C (defined as seropositivity for antihepatitis-C antibodies by enzyme immunoassay) coinfection and use of zidovudine. Log_2-transformed levels of IL-6, hsCRP and D-dimer were also entered into the logistic regression models and were thus adjusted for simultaneously.

Table 1. Demographic and clinical variables by presence of anaemia at baseline.

Baseline variable	Anaemia at baseline[a] Yes ($N=313$) Median (IQR)	No ($N=1097$) Median (IQR)	P[b]
Haemoglobin (g/dl)	13.3 (12.4–13.7)	15.2 (14.5–15.9)	0.001
Age (years)	43 (38–51)	40 (36–47)	0.001
Black race (%)	15.7	6.9	0.001
Female (%)	10.2	17.4	0.002
BMI (kg/m^2)	23.5 (21.3–25.8)	23.9 (21.8–26.1)	0.09
HIV RNA ≤500 copies/ml (%)	80.8	82.0	0.62
Baseline CD4$^+$ T cell count (cells/μl)	192 (136–241)	205 (153–257)	0.001
Nadir CD4$^+$ T cell count (cells/μl)	50 (24–101)	60 (27–104)	0.13
Prior AIDS diagnosis (%)	33.5	30.9	0.38
Hepatitis B coinfection (%)	9.0	8.1	0.82
Hepatitis C coinfection (%)	25.1	25.7	0.62
Karnofsky score	100 (90–100)	100 (100)	0.008
On PI (%)	72.8	66.3	0.03
On NNRTI (%)	41.5	45.6	0.20
On zidovudine (%)	60.1	38.3	0.001
On stavudine (%)	58.1	38.8	0.001
D-dimer (μg/ml)	0.3 (0.2–0.5)	0.2 (0.2–0.4)	0.001
IL-6 (pg/ml)	2.0 (1.3–3.3)	1.7 (1.2–2.6)	0.001
hsCRP (μg/ml)	1.7 (0.7–4.6)	1.4 (0.6–3.1)	0.02
MCV (fl)	107 (98–114)	105 (99–111)	0.37
MCV < 80 (%)	4.2	0.4	0.001
80 ≤ MCV ≤ 100 (%)	27.2	31.5	
MCV > 100 (%)	68.7	68.1	

hsCRP, high-sensitivity C-reactive protein; IL-6, interleukin-6; MCV, mean corpuscular volume; NNRTI, nonnucleoside reverse-transcriptase inhibitors; PI, protease inhibitors. P value for comparing MCV categories (<80, 80–100, >100) are from chi-square test.
[a]Anaemia is defined as haemoglobin ≤14 mg/dl for men and ≤12 mg/dl for women.
[b]P values are from t-test. For D-dimer, hsCRP and IL-6, $\log_2$ transformed values were used.

As the best haemoglobin cut-off to define anaemia may be debatable, a multivariable linear regression model, which was adjusted for the aforementioned covariates, was deployed to further explore associations between biomarkers and haemoglobin. Continuous haemoglobin levels were modelled as the outcome. Regression coefficients with 95% confidence intervals (95% CIs) were calculated to assess the independent contribution of IL-6, hsCRP and D-dimer to the variance of haemoglobin levels.

Because macrocytic anaemia can be a manifestation of zidovudine toxicity [16,17], analyses were repeated excluding participants receiving zidovudine. Statistical analyses were performed using SAS (version 9.2; SAS Institute Inc., Cary, North Carolina, USA).

Results

Baseline haemoglobin, MCV and biomarker levels were available in 1410 out of 1695 individuals enrolled in SILCAAT. Among the 1410 participants in the present study, the median (interquartile range, IQR) age was 41 (36–48) years; 84.2% were men, 81.1% were white, 8.3% had hepatitis B and 25.6% had hepatitis C coinfection. Study participants received cART for a median (IQR) of 4.4 (2.0, 7.8) years. The median (IQR) baseline CD4$^+$ T-cell count was 202 (149–255) cells/μl. Most (81.8%) had an HIV RNA lower than 500 copies/ml plasma. A total of 285 participants lacked biomarker measurements at baseline and were excluded. Data were missing mainly among participants from Argentine, Brazilian and Belgian study sites, wherein blood samples were not routinely collected. Relative to the studied cohort, these individuals had received cART for a shorter time, were younger, were more likely be on zidovudine and less likely to be white, but had otherwise similar demographic and clinical characteristics (data not shown).

A total of 313 (22.2%) participants had anaemia. When compared with participants with normal haemoglobin levels, those with anaemia were more likely to be older, black, male and on zidovudine. They also had lower baseline CD4$^+$ T-cell counts and lower Karnofsky scores (Table 1). D-dimer, hsCRP and IL-6 levels were significantly higher in those with anaemia than in those without anaemia (Table 1).

Of the 313 anaemic patients, 13 (4.1%), 85 (27.2%) and 215 (68.7%) had microcytic, normocytic and macrocytic anaemia, respectively (supplementary Table 1, http://links.lww.com/QAD/A533). As expected, zidovudine use was significantly more common in participants with macrocytic anaemia than in those with normocytic anaemia (78.6 versus 14.1%, respectively; $P<0.001$).

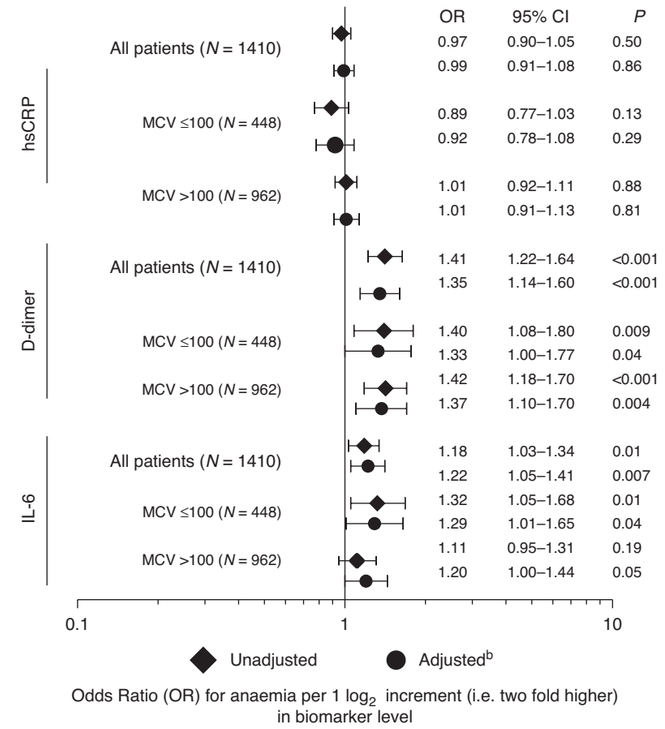

Fig. 1. Associations between anaemia and biomarkers across mean corpuscular volume levels[a]. [a]The number of anaemic patients with MCV < 80 ($N=11$) was too low to calculate ORs. Adjusted ORs for patients with normocytic anaemia (MCV 80–100) were 1.27 ($P=0.09$) for D-dimer and 1.18 ($P=0.17$) for IL-6. [b]Adjusted for demographics (age, race, sex), BMI, HIV RNA levels, CD4$^+$ cell counts (nadir and baseline), Karnofsky score, previous AIDS diagnosis, hepatitis B and C coinfection and use of zidovudine. Log_2-transformed levels of IL-6, hsCRP and D-dimer were adjusted for simultaneously.

Furthermore, those with macrocytic anaemia were more likely to be black, male and to be virologically suppressed than those with normocytic anaemia. The levels of IL-6, hsCRP and D-dimer did not differ significantly between participants with normocytic and macrocytic anaemia (Supplementary Table 1, http://links.lww.com/QAD/A533).

Considering all participants, higher levels of IL-6 and D-dimer, but not hsCRP, were found to be associated with anaemia in univariate and adjusted analyses (Fig. 1). Adjusted relative odds of anaemia per two-fold higher biomarker levels were 1.35 ($P<0.001$) for D-dimer, 0.99 for hsCRP ($P=0.86$) and 1.22 ($P=0.007$) for IL-6. Similar associations were seen in those with normal and high MCV values (Fig. 1). The percentage of patients with anaemia increased with higher quintiles of D-dimer and IL-6 levels (supplementary Figures 1 and 2, http://links.lww.com/QAD/A533). The presence of anaemia did not appear to be linearly associated with activated coagulation, as it was much higher in the top two D-dimer quintiles and similar in the bottom three quintiles (supplementary Figure 1, http://links.lww.com/QAD/A533).

In multivariable liner regression models, higher IL-6 and D-dimer levels were found to be independently correlated with lower haemoglobin. Adjusted regression coefficients (95% CI) were −0.13 (−0.21 to −0.06), $P<0.001$, for IL-6 (i.e. haemoglobin levels were 0.13 g/dl lower on average per two-fold higher IL-6 level) and −0.25 (−0.34 to −0.16), $P<0.001$, for D-dimer. No significant association was observed between hsCRP and haemoglobin [regression coefficient (95%CI) 0.00 (−0.04 to 0.04), $P=0.98$] in adjusted analyses.

Analyses excluding individuals using zidovudine rendered consistent results (data not shown).

Discussion

In the largest study to date investigating the relationship among inflammation, coagulation and anaemia in the

setting of antiretroviral-treated HIV infection, we found that, among antiretroviral-treated adults with moderate $CD4^+$ T cells counts, higher levels of both IL-6 and D-dimer are associated with the presence of anaemia after adjustment for demographic and clinical variables. The risk for anaemia appeared to increase most dramatically in those with the highest levels of IL-6 and D-dimer. This association was not restricted to normocytic anaemia, as we initially hypothesized, but was consistently seen across all MCV levels, although it is possible that zidovudine, which was used by the majority of participants with macrocytosis (supplementary Table 1, http://links.lww.com/QAD/A533), could have caused macrocytic anaemia in patients who otherwise would have had normocytic anaemia.

The pathogenesis of anaemia in HIV infection is complex and multifactorial. Possible causes include blood loss (i.e. neoplastic disease or gastrointestinal opportunistic infection), bone marrow infiltration or suppression, drug-related or infection-related haemolysis, nutritional deficiencies (iron, folic acid or cobalamin), hypogonadism and myelo-suppressive drugs [18]. It is clear that anaemia is associated with decreased survival in several other chronic conditions [19,20]. With HIV infection, however, questions remain as to whether anaemia is an epiphenomenon as a consequence of the underlying severe disease or is causally related to morbidity and mortality. Our findings suggest that the previously identified link between anaemia and increased morbidity and mortality in HIV-positive individuals may reflect in part elevated inflammation and coagulation.

Our data are broadly consistent with the results of the Veterans Aging Cohort Study, which also found higher D-dimer and IL-6 levels in those with lower haemoglobin values [21], although in that study, the correlation between haemoglobin and biomarkers was not adjusted for confounding. Furthermore, an earlier study found an association between increasing haemoglobin levels after cART initiation and decreased plasma levels of the inflammatory biomarkers neopterin and tumour necrosis factor-alpha (TNF-α) [22]. Finally, structured cART interruptions resulted in increased levels of inflammatory and coagulation markers [14] and an increased risk of new and worsening anaemia [8]. In multivariable models adjusted for all three biomarkers simultaneously, we did not find significant associations between hsCRP and anaemia or between hsCRP and haemoglobin levels. Adjustment for a higher number of variables and for other biomarkers had already been shown to significantly reduce associations between hsCRP and clinical outcomes [23]. Among other factors, this may be due to the fact that the hepatic production of hsCRP is determined by IL-6 [24].

Several issues need to be considered when interpreting our results. First, because this is a cross-sectional study, we could not establish temporal relationship, and no definitive inference of causality between enhanced inflammation/coagulation and anaemia can be made. Second, we could not adjust for environmental factors or for socio-economic status. Third, we have not explored specific causes of anaemia, and information on reticulocyte counts, ferritin, folic acid, cobalamin and hepcidin levels was not routinely collected. Fourth, the number of participants with microcytic anaemia was too small to perform statistical analyses. Fifth, we had no data on hepatitis C virus (HCV) RNA and hepatitis B virus (HBV) DNA plasma levels. As participants with detectable or higher HCV-RNA/HBV-DNA may have had more active hepatic disease, examining differences between them and coinfected participants with undetectable HCV-RNA/HBV-DNA would have been helpful. Finally, SILCAAT recruited participants who did not achieve immunological reconstitution despite cART use for several years. As the bone marrow from immunological nonresponders have impaired clonogenic capability and altered cytokine production [25], which might have contributed to the development of anaemia, confirmation of our findings by studies involving cART-treated participants with high $CD4^+$ T-cells counts is warranted. As the occurrence of prior AIDS-defining events was comparable in those with and without anaemia, we do not believe that our findings can be explained by concurrent opportunistic infections resulting in anaemia and raised levels of inflammatory and coagulation biomarkers.

In conclusion, we have shown that higher levels of IL-6 and D-dimer are associated with anaemia in HIV-treated adults. These associations remained strong after adjustment for clinical and demographic covariates and are seen across all MCV levels, a finding that suggests that activation of coagulation and inflammation may contribute to the development of all types of anaemia in HIV-infected individuals. Lower haemoglobin levels may provide indirect evidence of activation of coagulation/inflammation among HIV-infected adults. Further studies are needed to explore whether the established link between anaemia and increased morbidity and mortality has a causal component related to enhanced inflammation and coagulation.

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A.H.B., J.D.N., J.D.L. and S.G.D. contributed to the conception and design of the study. G.C. and J.D.N. performed all statistical analyses. A.H.B. drafted the manuscript. All authors contributed to data analysis and interpretation and reviewed the manuscript.

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Conflicts of interest

The authors declare no conflicts of interest.

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CONCISE COMMUNICATION

Thrombocytopenia is associated with an increased risk of cancer during treated HIV disease

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Objective: To assess the relationship between platelet counts and risk of AIDS and non-AIDS-defining events.

Design: Prospective cohort.

Methods: EuroSIDA patients with at least one platelet count were followed from baseline (first platelet $\geq$ 1 January 2005) until last visit or death. Multivariate Poisson regression was used to assess the relationship between current platelet counts and the incidence of non-AIDS-defining (pancreatitis, end-stage liver/renal disease, cancer, cardiovascular disease) and AIDS-defining events.

Results: There were 62 898 person-years of follow-up (PYFU) among 12 279 patients, including 1168 non-AIDS-defining events [crude incidence 18.6/1000 PYFU, 95% confidence interval (CI) 17.5–19.6] and 735 AIDS-defining events (crude incidence 11.7/1000 PYFU, 95% CI 10.8–12.5). Patients with thrombocytopenia (platelet count $\leq 100 \times 10^9$/l) had a slightly increased incidence of AIDS-defining events [adjusted incidence rate ratio (aIRR) 1.42, 95% CI 1.07–1.86], when compared to those with platelet counts $101–200 \times 10^9$/l, whereas the incidence of non-AIDS-defining events was more than two-fold higher (aIRR 2.66, 95% CI 2.17–3.26). Among non-AIDS-defining events, the adjusted incidence of cancer (aIRR 2.20, 95% CI 1.61–3.01), but not cardiovascular disease (aIRR 0.66, 95% CI 0.32–1.34), was significantly higher in patients with thrombocytopenia. The association between thrombocytopenia and cancer remained unaltered in sensitivity analyses requiring repeated platelet counts to confirm thrombocytopenia and lagging platelets by 1 year prior to clinical events.

Conclusion: Patients with thrombocytopenia had increased incidence of AIDS-defining and non-AIDS-defining events, but the association with the latter, in particular cancer, was stronger. Future studies should investigate whether the pathophysiological processes underlying thrombocytopenia are associated with the development of cancer during treated HIV disease.

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Introduction

Thrombocytopenia, a haematological abnormality frequently observed during HIV infection [1], has been linked to adverse clinical outcomes, including progression to AIDS [2], neurological impairment [3,4] and death [5–9]. Platelet decline may reflect activated coagulation and inflammation. In the Strategies for Management of Antiretroviral Therapy (SMART) study [10], antiretroviral therapy interruption resulted in lower platelet counts [11] and bursts of plasma levels of inflammatory and coagulation biomarkers [12]. Platelet decline was inversely correlated with plasma levels of D-dimer, a fibrin degradation product indicative of hypercoagulation [11]. Thrombocytopenic HIV-infected persons have also higher levels of interleukin (IL)-6, a pro-inflammatory cytokine [13]. Indeed, there has been mounting evidence that platelets exert a pivotal role not only in homeostasis, but also in inflammation [14].

The effect of platelet kinetics on clinical events remains poorly understood during treated HIV disease. Previous attempts to investigate this have not been appropriately powered [11]. Here, we sought to assess the relationship between platelet counts and the risk of developing AIDS and non-AIDS-defining events among a heterogeneous patient population from across Europe.

Methods

The EuroSIDA study, a multinational prospective cohort of 18 786 HIV-infected individuals, has been documented elsewhere [15]. In this study, we included patients aged at least 16 years with at least one platelet count after 1 January 2005 (from when information on platelets has been routinely collected) with prospective follow-up data and $CD4^+$ cell count or HIV RNA measured within 6 months of baseline, defined as the first platelet count during prospective follow-up after 1 January 2005. Fatal and nonfatal events were included; causes of death were determined using the Coding Causes of Death in HIV (CoDe) [16].

Multivariate Poisson regression quantified the association of the latest, or current, platelet count, categorized as 100 or less, 101–200, 201–300 and above 300×10^9/l, with the incidence of AIDS-defining events [17] and non-AIDS-defining events (cardiovascular disease, end-stage hepatic/renal disease, non-AIDS-defining cancers and pancreatitis [18]). Thrombocytopenia was defined as a single platelet count 100×10^9/l or less [19]. Factors adjusted for were chosen on the basis of their epidemiological and biological importance and included baseline (sex, ethnicity, HIV exposure group, baseline date, $CD4^+$ nadir) and time-updated [age, hepatitis B and hepatitis C co-infection (defined as seropositivity for hepatitis B surface antigen and anti-HCV antibody), $CD4^+$ cell counts, HIV RNA, diabetes, hypertension, smoking and anaemia] variables [18]. Anaemia was defined as haemoglobin 14 g/dl or less in men and 12 g/dl or less in women [20]. We repeated analyses using non-AIDS-defining cancer and cardiovascular disease, the most commonly occurring non-AIDS-defining events, as separate endpoints.

We carried out sensitivity analyses requiring a repeated platelet count 100×10^9/l or less (at least 3 months apart) to confirm thrombocytopenia (analysis 1), re-calculating incidence rates for AIDS-defining and non-AIDS-defining events in analysis restricted to treated and virologically suppressed persons (HIV RNA <400 copies/ml) with $CD4^+$ at least 200 cells/μl (analysis 2) and lagging platelets by 12 months prior to clinical events (analysis 3). Analysis 1 was done to account for laboratory errors and spontaneous thrombocytopenia remission [21]. Analysis 2 was done to exclude the impact of untreated HIV disease and immunological non-response to therapy. Analysis 3 was done to assess if thrombocytopenia reflected the subclinical disease rather than truly preceding the event (reverse causality). The above analyses were performed using SAS (version 9.3; SAS Institute, Cary, North Carolina, USA).

Results

Of the 18 786 participants enrolled in EuroSIDA, 14 515 persons had follow-up after 1 January 2005 and 2236 were excluded (Supplementary Fig. 1, http://links.lww.com/QAD/A568). Among the 12 279 eligible persons, there

was a median of nine platelet measures per person [interquartile range (IQR) 4–14], measured a median time apart of 6.2 months (IQR 4.7–8.0). The majority of persons were men (73.6%), MSM (41.4%), white (87.3%) and on antiretroviral therapy (88.5%). Median age at baseline was 42 years (IQR 36–49) and baseline CD4$^+$ cell count was 452 (IQR 308–640/μl).

During 62 898 person-years of follow-up (PYFU), there were 1168 non-AIDS-defining events [crude incidence 18.6/1000 PYFU, 95% confidence interval (CI) 17.5–19.6] and 735 AIDS-defining events (crude incidence 11.7/1000 PYFU, 95% CI 10.8–12.5). The three most common non-AIDS-defining events were non-AIDS-defining cancer ($n = 597$), cardiovascular disease ($n = 306$) and end-stage liver disease ($n = 118$); and the three most common AIDS-defining events were oesophageal candidiasis ($n = 93$), pulmonary tuberculosis ($n = 65$) and non-Hodgkin lymphoma ($n = 56$). Crude incidence of AIDS and non-AIDS-defining events increased as current platelet decreased (Fig. 1). There was a 'reverse' J-shaped relationship between current platelet count and AIDS and non-AIDS-defining events. Persons with thrombocytopenia had the highest incidence of both events; this declined as current platelet count went from 101–200 to 201–300 $\times 10^9$/l, but then increased again when platelet counts were above 300×10^9/l.

In multivariate analysis, the adjusted incidence of either AIDS or non-AIDS-defining events was higher in patients with thrombocytopenia, but the association between thrombocytopenia and non-AIDS-defining events was much stronger (Fig. 1). Thrombocytopenic patients had a slightly increased incidence of AIDS-defining events [adjusted incidence rate ratio (aIRR) 1.42, 95% CI 1.07–1.86]. However, the incidence of non-AIDS-defining events was more than two-fold higher (aIRR 2.66, 95% CI 2.17–3.26) in patients with thrombocytopenia. Those with the highest platelet counts have also a significantly higher risk of both AIDS and non-AIDS-defining events (Fig. 1).

Sensitivity analyses requiring a repeated platelet count 100×10^9/l or less to confirm thrombocytopenia and restricted to treated participants with HIV RNA below 400 copies/ml and CD4$^+$ cell counts at least 200/μl showed consistent results (data not shown). In adjusted analyses with platelet counts lagged by 12 months, persons with thrombocytopenia had a similar incidence of AIDS-defining events (aIRR 1.04, 95% CI 0.84–1.28) when compared to those with 101–200 $\times 10^9$ platelets/l, as did those with platelet counts above 300×10^9/l (aIRR 1.05, 95% CI 0.76–1.45). After adjustment and lagging by 12 months, persons with thrombocytopenia remained with a significantly higher incidence rate of non-AIDS-defining events (aIRR 1.43, 95% CI 1.20–1.71). In contrast, those with platelet counts above 300×10^9/l had similar incidence of non-AIDS-defining events after adjustment (aIRR 1.10, 95% CI 0.87–1.41) in time-lagged analysis.

Among non-AIDS-defining events, after adjustment, the aIRR of non-AIDS-defining cancer (aIRR 2.20, 95% CI 1.61–3.01), but not of cardiovascular disease (aIRR 0.66, 95% CI 0.32–1.34), was significantly higher in patients

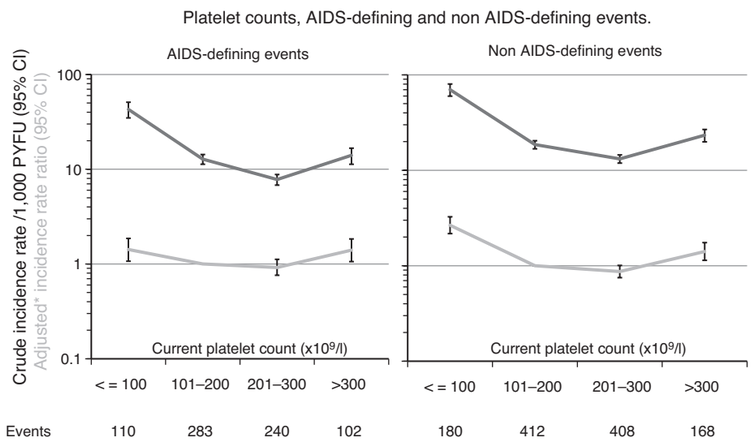

Fig. 1. Platelet counts, AIDS-defining and non-AIDS-defining events. *Adjusted for sex, ethnicity, HIV-exposure group, baseline date, CD4$^+$ nadir, *age, hepatitis B, hepatitis C, CD4$^+$ cell counts, HIV RNA, diabetes, hypertension, smoking status and anaemia (time-updated variables)*. AIDS was adjusted for AIDS at baseline and non-AIDS as time-updated; non-AIDS was adjusted for non-AIDS at baseline and AIDS as time-updated. CI, confidence interval; PYFU, person-years of follow-up.

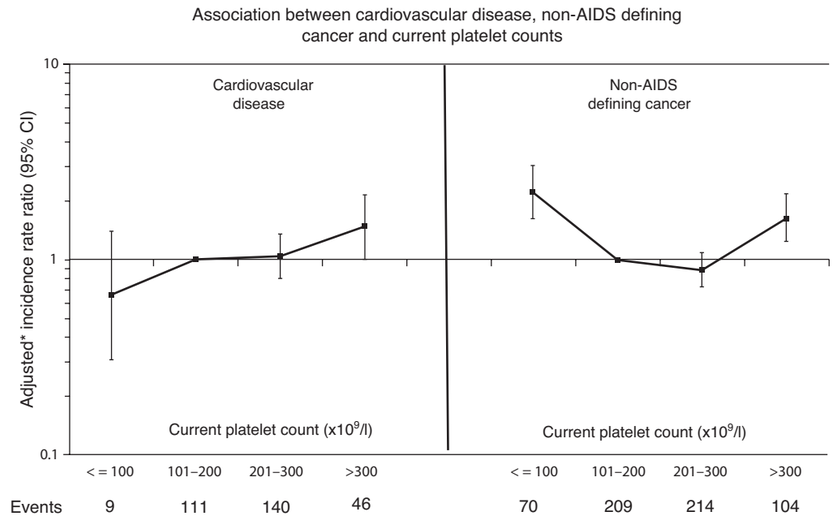

Fig. 2. Association between cardiovascular disease, non-AIDS-defining cancer and current platelet counts. *Adjusted for sex, ethnicity, HIV-exposure group, baseline date, CD4$^+$ nadir, *age, hepatitis B, hepatitis C, CD4$^+$ cell counts, HIV RNA, diabetes, hypertension, smoking status and anaemia (time-updated variables)*. AIDS was adjusted for AIDS at baseline and non-AIDS as time-updated; non-AIDS was adjusted for non-AIDS at baseline and AIDS as time-updated. CI, confidence interval.

with thrombocytopenia (Fig. 2). The increased incidence of non-AIDS-defining cancer in those with thrombocytopenia remained unaltered in fully adjusted time-lagged analysis (aIRR 2.26, 95% CI 1.34–3.33). Hepatocellular carcinoma was the most frequently observed cancer in thrombocytopenic persons (Supplementary Fig. 2, http://links.lww.com/QAD/A568).

Discussion

In a large, contemporary cohort of HIV-infected persons receiving standard of care, thrombocytopenia was more strongly related to non-AIDS-defining events than to AIDS-defining events. Among non-AIDS-defining events, the incidence rate of cancer, but not cardiovascular disease, was higher in persons with thrombocytopenia. The association between thrombocytopenia and cancer was robust in several sensitivity analyses. This finding was consistent after adjustment for confounders, in analysis restricted to virologically suppressed participants with CD4$^+$ at least 200/μl and in analysis when the time between platelet counts and cancer events was lagged by 12 months, reducing the possibility of reverse causality.

To our knowledge, this is one of the first studies adequately powered to assess the effect of platelet kinetics on clinical event risk in HIV-positive persons. The strong association between thrombocytopenia and non-AIDS-defining events can be explained by many possible mechanisms. Lower levels of circulating platelets may reflect a state of chronic immune activation and hypercoagulability, which is a hallmark of HIV infection [22]. Thrombocytopenic individuals have higher plasma levels of IL-6 [13] and D-dimer [11]. Both biomarkers have been linked to subsequent risk of non-AIDS-defining events, such as cancer [23] and cardiovascular disease [24].

We found that hepatocellular carcinoma was more frequently seen in thrombocytopenic patients, which possibly reflects liver impairment/fibrosis or hyperesplenism as contributing causes of thrombocytopenia in patients with hepatocellular carcinoma. The association between thrombocytopenia and non-AIDS-defining cancers, however, was not entirely explained by hepatic impairment. In analyses excluding hepatitis B and hepatitis C co-infected participants – those at greatest risk of liver cancer – there was still a significantly increased incidence of non-AIDS-defining cancer in those with thrombocytopenia when compared to those with 101–200 $\times 10^9$ platelets/l (data not shown). Patients with activated inflammation and hypercoagulability may have a higher risk of cancer [23] than cardiovascular disease [24]. This could explain why we found no association between platelet counts and cardiovascular disease, although other factors may play a role.

We hypothesize that thrombocytopenia may be an epiphenomen as a consequence of activated inflammatory and coagulation pathways, which have the potential to be causally related to the development of end-stage organ disease and cancer. It is also possible that activated platelets, via production of soluble CD40 ligand and pro-inflammatory cytokines [25–27], may contribute to enhanced immune activation during treated HIV disease [28], indirectly leading to an increased risk of non-AIDS-defining events. In this case, thrombocytopenia could reflect the rapid removal of activated platelets from the circulation [29].

We also found an increased incidence of AIDS and non-AIDS-defining events in patients with platelet counts above 300×10^9/l. An independent association between higher platelet counts and progression to AIDS and death had been reported previously [30]. With respect to non-AIDS-defining events, the association between higher platelet counts and clinical events was driven mainly by cancer (data not shown). IL-6 may induce hepatic synthesis of thrombopoietin, thereby causing thrombocytosis [31], and growth factors secreted by platelets can increase proliferation of cancer cells [32]. However, the association between higher platelet counts and AIDS and non-AIDS-defining events was considerably weaker in time-lagged analyses and may be thus attributable to reverse causality. It is possible that this association reflected reactive thrombocytosis, that is an unspecific response as a part of the host's attempt to manage cancer and opportunistic infections [30].

The large sample size, long follow-up, quality assurance of data collection, time-updated information on demographic factors, comorbidities and viro-immunological parameters are the main strengths of this study. There are some issues to consider when interpreting our results. First, our analyses were unadjusted for alcohol use, an important cause of thrombocytopenia in HIV-infected persons [33]. Second, we did not look into specific causes of thrombocytopenia. Data on bone marrow aspirates and on plasma levels of IL-6, D-dimer, thrombopoietin and platelet-derived inflammatory mediators were not available, but would be helpful to interpret our findings. Finally, we did not have data on hepatitis B virus DNA or hepatitis C virus RNA among all persons with serological evidence of hepatitis co-infection.

To conclude, thrombocytopenia was independently associated with the incidence of both AIDS and non-AIDS-defining events during HIV disease. It was associated with the development of non-AIDS-defining cancer in a greater degree than to the development of AIDS-defining events or other non-AIDS-defining events, such as cardiovascular disease. Future studies should investigate if the pathophysiological processes underlying thrombocytopenia are associated the development of cancer during treated HIV infection. Studies assessing the relationship between platelet-derived cytokines, biomarkers of platelet activation and cancer risk may be helpful to further understand this relationship. The association between thrombocytopenia and subsequent risk of cancer should be confirmed by studies involving the general population.

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A.H.B., J.D.L. and A.M. conceived the study. A.M. performed all statistical analyses. A.H.B. drafted the manuscript. All authors contributed to data interpretation, critically revised the manuscript and approved the final version.

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Conflicts of interest

P.G. reports honoraria and/or travel support from Gilead, ViiV, Astellas, Novartis, BMS, AstraZeneca, Pfizer, Abbvie, Janssen and Merck. A.M. reports consultancy, honoraria, and/or travel support from BMS, BI, Gilead, Pfizer and Merck.

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Predicting risk of cancer during HIV infection: the role of inflammatory and coagulation biomarkers

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Objective: To investigate the relationship between inflammatory [interleukin-6 (IL-6) and C-reactive protein (CRP)] and coagulation (D-dimer) biomarkers and cancer risk during HIV infection.

Design: A prospective cohort.

Methods: HIV-infected patients on continuous antiretroviral therapy (ART) in the control arms of three randomized trials ($N = 5023$) were included in an analysis of predictors of cancer (any type, infection-related or infection-unrelated). Hazard ratios for IL-6, CRP and D-dimer levels ($\log_2$-transformed) were calculated using Cox models stratified by trial and adjusted for demographics and $CD4^+$ cell counts and adjusted also for all biomarkers simultaneously. To assess the possibility that biomarker levels were elevated at entry due to undiagnosed cancer, analyses were repeated excluding early cancer events (i.e. diagnosed during first 2 years of follow-up).

Results: During approximately 24 000 person-years of follow-up (PYFU), 172 patients developed cancer (70 infection-related; 102 infection-unrelated). The risk of developing cancer was associated with higher levels (per doubling) of IL-6 (hazard ratio 1.38, $P < 0.001$), CRP (hazard ratio 1.16, $P = 0.001$) and D-dimer (hazard ratio 1.17, $P = 0.03$). However, only IL-6 (hazard ratio 1.29, $P = 0.003$) remained associated with cancer risk when all biomarkers were considered simultaneously. Results for infection-related and infection-unrelated cancers were similar to results for any cancer. Hazard ratios excluding 69 early cancer events were 1.31 ($P = 0.007$), 1.14 ($P = 0.02$) and 1.07 ($P = 0.49$) for IL-6, CRP and D-dimer, respectively.

Conclusion: Activated inflammation and coagulation pathways are associated with increased cancer risk during HIV infection. This association was stronger for IL-6 and persisted after excluding early cancer. Trials of interventions may be warranted to assess whether cancer risk can be reduced by lowering IL-6 levels in HIV-positive individuals.

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Keywords: biomarkers, cancer, C-reactive protein, D-dimer, HIV, interleukin-6

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Introduction

Since the beginning of the HIV pandemic, an increased risk of cancer has been observed in HIV-infected individuals. Some infection-related cancers, namely Kaposi sarcoma, non-Hodgkin lymphoma (NHL) and invasive cervical cancer (ICC), were found to have a particularly high incidence in patients with advanced HIV infection. With the aim of tracking the emerging epidemic, these cancers were included in the case definition of AIDS and, since then, have been classically referred to as AIDS-defining malignancies (ADMs) [1]. Epidemiological surveillance, however, subsequently broadened the spectrum of malignancies associated with HIV infection. HIV-infected individuals have been found to be at a higher risk of an ever-increasing range of both infection-related and infection-unrelated non-AIDS defining malignancies (NADMs) [2–5].

Although many reasons have been postulated, the mechanisms by which HIV infection increases the risk of cancer remain poorly understood. A higher prevalence of traditional cancer risk factors (e.g. smoking, alcohol use, oncogenic virus coinfection) in HIV-positive persons seems to play an important role [6]. Furthermore, clinical data suggest that HIV-associated immunodeficiency may lead to accelerated viral oncogenesis and reduced immune surveillance of malignant cells. Not only infection-related ADM but also infection-unrelated NADM have been shown to occur more frequently in HIV-infected persons, particularly in individuals with lower $CD4^+$ cell counts [4,7–10]. Finally, the scale up of antiretroviral therapy (ART) has greatly improved survival outcomes, and as HIV-infected individuals age, the incidence of cancer is expected to increase [11], as it does in the general population.

Evidence has recently accrued suggesting that activated inflammatory and coagulation pathways may also contribute to cancer risk in HIV-infected individuals. In the Strategies for Management of Antiretroviral Therapy (SMART) study [12], structured interruptions of ART were associated with a significantly higher incidence of cancer [13] and a rise in plasma levels of D-dimer, a fibrin degradation product, and of interlukin-6 (IL-6), an inflammatory cytokine [14]. IL-6 production has been shown to be an important component of autocrine [15] and paracrine [16] circuits that fuel the growth of solid tumours. In the general population, elevated plasma levels of IL-6 and C-reactive protein (CRP), a marker of inflammation whose production by hepatocytes is driven by IL-6 [17], are associated with an increased risk of developing cancer [18–20]. Except for a few reports linking elevated IL-6 levels with the risk of future NHL [21,22], no systematic studies examining the interplay between inflammation, coagulation and cancer in the setting of HIV infection have been carried out.

The purpose of this study is to investigate the relationship between inflammatory (IL-6 and CRP) and coagulation (D-dimer) biomarkers and the risk of cancer during HIV infection. Our main *a priori* hypothesis was that activated inflammation and coagulation, as demonstrated by elevated plasma levels of IL-6, CRP and D-dimer, contribute to the risk of developing both infection-related and infection-unrelated cancer in HIV-infected individuals.

Materials and methods

Study design and study population

This is a cohort study involving participants in the control arms of three randomized controlled trials, who had consented to storing blood for future research and whose plasma levels of IL-6, CRP and D-dimer were measured at study entry ($N = 5023$). The methodology of SMART [12], the Evaluation of Subcutaneous Proleukin in a Randomized International Trial (ESPRIT) and the Subcutaneous Recombinant, Human Interleukin-2 in HIV-Infected Patients with Low $CD4^+$ Counts under Active Antiretroviral Therapy (SILCAAT) [23] trial has been described in detail elsewhere. Briefly, the SMART trial compared, in 5472 individuals with $CD4^+$ cell count more than 350 cells/μl at baseline, continuous use of ART versus structured treatment interruption guided by $CD4^+$ cell count. The ESPRIT and SILCAAT trials compared IL-2 and ART with ART alone in 4111 individuals with $CD4^+$ cell count more than 300 cells/μl and 1695 individuals with $CD4^+$ cell count between 50 and 299 cells/μl, respectively. In the three trials, individuals in the control arms received standard of care according to HIV guidelines and were to be continuously maintained on ART.

Inflammatory and coagulation biomarkers

On the basis of strong associations of CRP, IL-6 and D-dimer with all-cause mortality in a nested case–control study [14] and the observation that these biomarkers were elevated in HIV-infected individuals compared with the general population [24], IL-6, CRP and D-dimer were measured on stored plasma at baseline for all consenting participants in ESPRIT, SILCAAT and SMART. For SMART participants, biomarkers were measured at the Laboratory for Clinical Biochemistry Research at the University of Vermont (Burlington). In the ESPRIT and SILCAAT trials, laboratory measurements were performed by SAIC-Frederick (Frederick, Maryland, USA).

IL-6 was measured by the same method at each laboratory (Chemiluminescent Sandwich ELISA; R&D Systems, Minneapolis, Minnesota, USA). D-dimer levels were measured by ELISA on the Sta-R analyser, Liatest D-DI (Diagnostic Stago, Parsippany, New Jersey, USA) for SMART participants and on a VIDAS instrument (BioMerieux Inc., Durham, North Carolina, USA) for ESPRIT and SILCAAT participants. These assays, while different, compared very well on 20 duplicate samples. CRP was measured by ELISA by both laboratories. For SMART participants, an NBTMII nephelometer, N Antiserum to Human CRP (Siemens Diagnostics, Deerfield, Illinois, USA) was used. For ESPRIT and SILCAAT participants, an R&D Systems ELISA assay was used. The assays used were different, but as for D-dimer, they compared very well on duplicate samples. Lower limits of detection for IL-6, CRP and D-dimer were 0.16 pg/ml, 0.16 and 0.01 μg/ml for SMART. In ESPRIT and SILCAAT, lower limits of detection were 0.156 pg/ml, 0.078 and 0.045 μg/ml. All samples were analysed blinded to the treatment group and cancer event status.

Follow-up and cancer ascertainment

Patients were followed from study entry until first cancer event, death, loss to follow-up or the closing date of each study, whichever occurred first. Clinical assessment intervals and total follow-up time varied by study. Median follow-up time was 29 months in SMART, 81 months in ESPRIT and 91 months in SILCAAT; overall, median follow-up was 59 months for the entire cohort. In SMART and ESPRIT, all malignancies were systematically reported to and centrally adjudicated by the Endpoint Review Committee (ERC). In SILCAAT, only ADM and Hodgkin lymphoma were centrally adjudicated, whereas other NADMs were identified from the adverse event reporting system. In sensitivity analyses, the exclusion of SILCAAT had no major impact on findings (data not shown).

The following malignancies were considered to be infection-related: ADM, which have viral causes [Kaposi sarcoma, NHL and ICC are related to human herpes virus 8 (HHV-8), Epstein–Barr virus (EBV) and human papillomavirus (HPV), respectively); vagina, vulva, penis, anal and oral cavity/pharynx cancers (HPV); Hodgkin lymphoma (EBV); liver cancer (hepatitis B and C viruses) and stomach cancer (*Helicobacter pylori*) [25]. No pharyngeal lymphoepitheliomas were identified in this study. All other malignancies were considered to be infection-unrelated cancers.

Statistical analyses

All analyses were restricted to patients in the control arms of each study for whom baseline biomarker data were available. Other than the events identified through adverse event reporting, cancer events were included if they were considered 'confirmed or 'probable' by the ERC, or if the patient died and the ERC attributed the underlying cause of death to cancer. Cancers were characterized by type on the basis of the Medical Dictionary for Regulatory Activities (MedDRA) High Level Term (HLT) assigned.

Kaplan–Meier curves giving the cumulative percentage of participants with cancer are shown for biomarker quartiles. To control for any differences in biomarker distributions among studies, quartiles were defined separately for participants in each study. Associations between IL-6, CRP and D-dimer levels at study entry and the risk of the first incident cancer event were estimated using proportional hazards (Cox) models from which hazard ratios corresponding to one $\log_2$ increase in biomarker (i.e. a doubling) and 95% confidence intervals (CIs) were estimated. Biomarkers were $\log_2$-transformed because their distributions were right-skewed. Cox models for each biomarker were stratified by study to account for differences in underlying risk for the different cohorts. This stratification also served to stratify on the two laboratories that measured the biomarkers. Three models were considered: (1) unadjusted; (2) adjusting for age, sex, race, continent of enrolment, study entry and time-updated $CD4^+$ counts; and (3) adjusting for the same covariates and all biomarkers simultaneously.

In order to compare the association of each biomarker with different types of cancer, a competing risk model for multiple unordered events of different types was fit [26]. With this approach, the fit of a model that assumed a common association for each biomarker with infection-related and infection-nonrelated cancers was compared with the fit of a model that allowed the association to vary. A similar approach was taken for assessing the association of the biomarkers with type-specific cancers of interest.

With the proportional hazards model, we assume that the risk of cancer associated with higher versus lower biomarker levels is constant over the follow-up period. This assumption was tested in a model that included an interaction term between each $\log_2$-transformed biomarker and log follow-up time along with the baseline covariates in model (2) above. In addition, two other analyses were carried out: first, we produced graphic displays of log (−log) of the adjusted survival curves for participants with biomarker levels above versus below the median (departure from parallelism of these graphs indicates that the risk of cancer for those with higher versus lower levels is not constant over follow-up); and second, we performed analyses excluding cancer events diagnosed in the first 2 years of follow-up. These analyses were performed because clinically undetected malignancies may lead to raised plasma levels of inflammatory and coagulation biomarkers. In that case, one would expect that risk ratios for cancer events closer to the time of measurement of the biomarkers would differ from those events more remote from the measurement.

In other sensitivity analyses, additional important traditional cancer risk factors were adjusted for obesity/BMI

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(data available for participants in the three trials); diabetes (SMART and ESPRIT participants) and smoking (SMART only). All statistical analyses were performed using SAS software (version 9.2; SAS Institute, Cary, North Carolina, USA)

Results

A total of 5023 HIV-infected individuals were included. Six hundred and fifteen individuals without biomarker measurements were excluded. Excluded individuals were more likely to be younger, black and female and have lower nadir $CD4^+$ cell counts (data not shown). During approximately 24 000 persons-years of follow-up, 172 patients developed cancer (70 infection-related; 102 infection-unrelated). The most common infection-unrelated cancers were lung, prostate and colorectal; the most common infection-related cancers were NHL, anal and Hodgkin lymphoma (Table 1). Patients who developed cancer were more likely to be older males, have hepatitis B/C coinfection and diabetes, as well as to smoke. Patients who developed cancer were also found to have higher baseline levels of CRP, D-dimer and IL-6, and lower nadir $CD4^+$ cell counts (Table 2).

Kaplan–Meier curves for any type, infection-related and infection-unrelated cancers are shown for quartiles of each biomarker in Supplementary Figure 1, http://links.lww.com/QAD/A313. When biomarkers were modelled as continuous variables, all three biomarkers demonstrated significant unadjusted associations (model 1) with increased risk of any type, infection-related and infection-unrelated cancers (see Table 3). After adjustment for demographics (age, race, sex and continent) and for study entry and time-updated $CD4^+$ cell counts (model 2), each biomarker remained significantly associated with any type of cancer. When all biomarkers were adjusted for (model 3), only IL-6 remained independently associated with cancer risk (hazard ratio 1.29, 95% CI 1.09–1.52, $P=0.003$). There was no evidence that biomarker associations varied between infection-related and infection-unrelated cancers. With the model 2 adjustment, P values for differences between the associations with infection-related and infection-unrelated cancers were 0.90, 0.90 and 0.89, for CRP, D-dimer and IL-6, respectively.

Table 1. Frequency distribution of cancers ESPRIT, SILCAAT and SMART control patients.

	Number	Frequency (%)
Infection-unrelated cancers	102	100
Lung	26	26
Prostate	14	14
Colorectal	12	12
Breast	8	8
Melanoma	7	7
Urinary tract	6	6
Pancreas	3	3
Oesophagus	2	2
Leukemia	2	2
Unknown primary site	9	9
Other[a]	13	13
Infection-related cancers	70	100
Non-Hodgkin lymphoma	21	30
Anus	13	19
Hodgkin lymphoma	10	14
Kaposi sarcoma	10	14
Liver	5	7
Oral cavity/pharynx	4	6
Cervix	4	6
Other[b]	3	4

[a]Female reproductive tract, mesotheliomas, larynx, small intestine, thyroid, testicle, adrenal gland.
[b]Penis, vagina, stomach.

Table 4 summarizes the association between inflammatory and coagulation biomarkers and specific types of cancer that occurred in at least 10 patients. On the basis of the adjusted competing risk model summarizing cancers by type (NHL, Hodgkin lymphoma, HPV-related cancers, lung, prostate, colorectal, Kaposi sarcoma and all other types), there was no evidence that biomarker associations varied by type of cancer for D-dimer ($P=0.12$) or IL-6 ($P=0.65$); however, there was evidence for unequal effects across cancer types for CRP ($P=0.04$). Higher baseline plasma levels of CRP were independently associated with risk of developing any type of lymphoma (hazard ratio 1.35, $P=0.005$), Hodgkin lymphoma (HR = 1.72, $P=0.004$) and colorectal cancer (hazard ratio 1.41, $P=0.04$). Elevated IL-6, on the contrary, was found to be statistically associated with risk of lung cancer (hazard ratio 1.62, $P=0.01$). However, owing to the limited number of cancer events and the consequent wider CI, our estimates are imprecise and need to be interpreted with caution.

Sensitivity analyses

For all cancers and for infection-related and infection-unrelated cancers, there was no evidence that hazard ratios for higher versus lower levels of CRP and D-dimer varied over follow-up; P values testing the proportionality assumption ranged between 0.25 and 0.92. For IL-6, however, the hazard ratios varied across time for all cancers ($P=0.04$) and infection-unrelated cancers ($P=0.03$). On the basis of model (2), the hazard ratio for any type of cancer in the first 2 years of follow-up (68 cancer events) associated with a doubling of IL-6 was 1.50 (95% CI 1.21–1.85; $P<0.001$); for follow-up after 2 years (103 cancer events), the hazard ratio was 1.31 (95% CI 1.08–1.60; $P=0.007$). For infection-unrelated cancers, the hazard ratios were 1.52 (95% CI 1.17–1.97, $P=0.002$) and 1.24 (95% CI 0.95–1.62, $P=0.12$) for the two time periods. For IL-6, this is graphically illustrated in Supplementary Figure 2, http://links.lww.com/QAD/A313. During the early follow-up period, differences between the quartiles were greater, whereas after 1–2 years, the curves were more parallel.

Table 2. Baseline characteristics ESPRIT, SILCAAT and SMART control patients.

	Developed cancer		
	Yes	No	P*
Age in years (median, IQR)	48 (41, 56)	42 (36, 49)	<0.001
Female sex (%)	12.2	22.7	0.005
Race (%)			0.05
Black	16.3	19.6	
White	76.7	67.2	
Other	7.0	13.1	
BMI (median, IQR)	24 (22, 26)	24 (22, 27)	0.67
AIDS (%)	25.0	26.1	0.67
Hepatitis B/C coinfection (%)[a]	27.9	18.6	0.04
Diabetes[a]	8.6	4.9	0.02
Smoking[b]	60.3	40.5	0.003
$CD4^+$ cell count (cells/μl) (median, IQR)	421 (305, 585)	488 (370, 672)	0.27
$CD4^+$ nadir (cells/μl)(median, IQR)	143 (44, 262)	200 (87, 319)	0.01
HIV RNA ≤500 copies/ml (%)	72.7	76.7	0.15
CRP (μg/ml) (median, IQR)	2.51 (1.25, 5.24)	1.54 (0.67, 3.54)	<0.001
D-dimer (μg/ml) (median, IQR)	0.28 (0.22, 0.47)	0.24 (0.15, 0.38)	<0.001
IL-6 (pg/ml) (median, IQR)	2.40 (1.80, 3.58)	1.80 (1.17, 2.80)	<0.001
Study			0.03
ESPRIT participant	40.7	35.2	
SILCAAT participant	25.6	13.6	
SMART participant	33.7	51.2	
No. of patients	172	4851	

CRP, C-reactive protein; IQR, interquartile range.
[a]Not ascertained for patients in SILCAAT.
[b]Not ascertained for patients in SILCAAT or ESPRIT.
*P value from a univariate Cox regression model stratified by study.

Data on some important traditional cancer risk factors, namely obesity/BMI, diabetes and smoking, were not uniformly collected in the three trials. Consequently, these factors had to be adjusted for in smaller datasets (see Table 5). Their inclusion in multivariable models did not alter the associations between the biomarkers and risk of any type, infection-related and infection-unrelated cancer.

Discussion

This is the largest prospective study to investigate, in the setting of HIV infection, the relationship between plasma levels of coagulation and inflammatory biomarkers and cancer risk. Here, we report that activated inflammation and coagulation pathways, as measured by higher IL-6, CRP and D-dimer levels, are associated with an increased risk of cancer. This association was strongest for IL-6 and evident for both infection-related and infection-unrelated cancer after excluding early events and adjusting for demographics, $CD4^+$ cell counts and traditional risk factors. During the entirety of follow-up, IL-6 had the steepest risk gradients for all cancer endpoints and, when the three biomarkers were entered into a multivariable Cox regression model, only IL-6 remained significantly associated with cancer risk.

The strong association between elevated plasma levels of IL-6 and cancer risk that we observed is corroborated by a body of evidence suggesting that IL-6 is a tumourigenic cytokine that influences, through autocrine [15] and paracrine [16] pathways, all stages of cancer development, including initiation, promotion, progression and dissemination [27]. Gene association studies using principles of Mendelian randomization have provided further evidence to support a role of IL-6 in cancer aetiogenesis, with IL-6 gene polymorphisms associated with colorectal [28], cervical [29] and oral cancer [30].

In the present study, associations between CRP and cancer risk differed according to cancer type. Site-specific associations between inflammatory markers and cancer have also been described in the general population. Elevated plasma levels of CRP were found to be linked with lung [31,32] and colorectal cancer [31], but not with breast cancer [33]. On the contrary, a significantly increased risk of lung and colorectal cancer [18], but not of prostate cancer [34], was observed in HIV-uninfected individuals with raised circulating levels of IL-6. In contrast with the few previous reports in HIV-infected populations [21,22], we did not find a significant association between IL-6 and NHL. Instead, we did observe an association between CRP and Hodgkin lymphoma and all lymphomas grouped. Nevertheless, we had limited ability to examine site-specific associations, which should be confirmed in larger studies.

The possibility that raised plasma levels of biomarkers, rather than predicting clinical endpoints, just reflect the presence of subclinical disease (i.e. reverse causality) is

Table 3. ESPRIT, SMART and SILCAAT hazard ratios associated with baseline biomarkers [per one log_2 (i.e. doubling) increment] cohort: all control patients.

	Any cancer						Infection-related cancers[a]						Infection-unrelated cancers					
	CRP		D-dimer		IL-6		CRP		D-dimer		IL-6		CRP		D-dimer		IL-6	
	HR (95% CI)*	*P*	HR (95% CI)*	*P*	HR (95% CI)*	*P*	HR (95% CI)*	*P*	HR (95% CI)*	*P*	HR (95% CI)*	*P*	HR (95% CI)*	*P*	HR (95% CI)*	*P*	HR (95% CI)*	*Pe*
Model 1[b]	1.21 (1.11–1.32)	<0.001	1.25 (1.10–1.43)	<0.001	1.48 (1.30–1.69)	<0.001	1.22 (1.07–1.39)	0.003	1.25 (1.01–1.54)	0.04	1.49 (1.21–1.84)	<0.001	1.20 (1.08–1.34)	<0.001	1.26 (1.06; 1.49)	0.007	1.47 (1.24; 1.74)	<0.001
Model 2[c]	1.16 (1.06–1.26)	0.001	1.17 (1.01–1.35)	0.03	1.38 (1.19–1.59)	<0.001	1.18 (1.03–1.35)	0.02	1.19 (0.95–1.50)	0.13	1.42 (1.14–1.78)	0.002	1.15 (1.02–1.28)	0.02	1.15 (0.96; 1.38)	0.12	1.35 (1.12; 1.63)	0.002
Model 3[d]	1.06 (0.96–1.17)	0.22	1.06 (0.91–1.23)	0.43	1.29 (1.09–1.52)	0.003	1.08 (0.92–1.26)	0.34	1.06 (0.83–1.36)	0.64	1.32 (1.01–1.71)	0.04	1.06 (0.93–1.20)	0.40	1.06 (0.88; 1.28)	0.53	1.27 (1.02; 1.58)	0.03
No. of patients[e]	5022		5006		4994		5022		5006		4994		5022		5006		4994	
No. of events[e]	172		171		171		70		70		70		102		101		101	

CI, confidence interval; CRP, C-reactive protein; HR, hazard ratio; IL-6, interleukin-6.
[a]All AIDS-defining-malignancies along with vagina, vulva, penis, anal and oral cavity/pharynx cancers; Hodgkin lymphoma; liver and stomach cancer. All other malignancies were considered to be infection-unrelated cancers.
[b]Stratified for study and unadjusted.
[c]Stratified for study and adjusted for demographics (age, race, sex and continent), and study entry and time-updated $CD4^+$ cell counts.
[d]As in model 2 and also adjusted for all biomarkers.
[e]Due to missing data, numbers are reduced in model containing all biomarkers to 4982 participants with 171 any cancer, 70 infection-related cancer and 101 infection-unrelated cancer events.
*HR, hazard ratio for one log_2 (i.e. doubling) increase in indicated biomarker.

difficult to exclude in cancer or other diseases with long latency. In this study, risk gradients with CRP did not vary over follow-up and although risk gradients with IL-6 did vary over follow-up, hazard ratios associated with higher IL-6 levels remained significant for all cancers after exclusion of early cancer events. Furthermore, HIV-positive patients frequently have more aggressive cancer and poorer clinical outcomes [35]. Therefore, we consider it unlikely that our results can be explained by occult cancer.

The main strengths of our study are its large sample size, long follow-up period and carefully collected clinical data. A number of limitations need to be considered. First, the measurement of a more extensive panel of inflammatory and coagulation biomarkers would have been helpful to further clarify the relationship between activated inflammatory and coagulation pathways and the risk of cancer in HIV-infected individuals. Second, our analyses were not adjusted for some important traditional cancer risk factors, namely alcohol use, diet and sun exposure. Third, given the small numbers of cases, we were unable to carry out separate analyses for less common cancers and had no alternative but to use, as endpoints, broader cancer categories involving widely heterogeneous and etiologically distinct malignancies. However, this implies that the strong associations observed for IL-6 are conservative and may be stronger for individual cancers within broad categories. Finally, this study does not provide definitive evidence for a causal relationship between activated coagulation/inflammation and cancer during HIV infection; prospective gene association studies in HIV-infected individuals or a protective clinical effect on risk of cancer development from medical interventions able to specifically reduce the production or effect of IL-6 may be useful to elucidate this.

Our results indicate that activated inflammation and coagulation, as demonstrated by higher IL-6, CRP and D-dimer levels, are associated with the development of cancer during HIV infection. This association was strongest for IL-6 and was present for both infection-related and infection-unrelated malignancies after the exclusion of early events and after adjustment for demographics, $CD4^+$ cell counts and traditional cancer risk factors. Trials of interventions may be warranted to assess whether cancer risk can be reduced by lowering IL-6 levels in HIV-positive individuals.

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Table 4. ESPRIT, SMART and SILCAAT hazard ratios for one log_2 (i.e. doubling) increment in biomarkers for cancer subsets.

	CRP			D-dimer			IL-6		
	Adj HR[a]	95% CI	*P*	Adj HR[a]	95% CI	*P*	Adj HR[a]	95% CI	*P*
Any cancer	1.16	1.06–1.26	0.001	1.17	1.01–1.35	0.03	1.38	1.19–1.59	<0.001
No. of patients (events)		5022 (172)			5006 (171)			4994 (171)	
Lymphoma – any	1.35	1.09–1.66	0.005	1.31	0.93–1.85	0.12	1.34	0.95–1.88	0.10
No. of patients (events)		5022 (31)			5006 (31)			4994 (31)	
Non-Hodgkin lymphoma	1.21	0.94–1.56	0.13	1.49	1.00–2.21	0.05	1.27	0.83–1.93	0.27
No. of patients (events)		5022 (21)			5006 (21)			4994 (21)	
Hodgkin lymphoma	1.72	1.18–2.50	0.004	0.98	0.51–1.91	0.96	1.52	0.85–2.72	0.16
No. of patients (events)		5022 (10)			5006 (10)			4994 (10)	
HPV-related cancers[b]	1.06	0.84–1.35	0.61	0.99	0.65–1.50	0.96	1.47	0.98–2.19	0.06
No. of patients (events)		5022 (23)			5006 (23)			4994 (23)	
Lung cancer	1.26	1.00–1.58	0.05	0.84	0.58–1.20	0.34	1.62	1.12–2.35	0.01
No. of patients (events)		5022 (26)			5006 (25)			4994 (25)	
Prostate cancer	0.84	0.62–1.15	0.28	0.91	0.59–1.42	0.68	0.62	0.33–1.17	0.14
No. of patients (events)		*5022 (14)*			*5006 (14)*			*4994 (14)*	
Colorectal cancer	1.41	1.02–1.94	0.04	0.95	0.56–1.59	0.84	1.32	0.77–2.26	0.31
No. of patients (events)		5022 (12)			5006 (12)			4994 (12)	
Kaposi sarcoma	1.05	0.72–1.51	0.81	1.55	0.93–2.57	0.09	1.58	0.92–2.69	0.09
No. of patients (events)		5022 (10)			5006 (10)			4994 (10)	

CI, confidence interval; CRP, C-reactive protein; HPV, human papillomavirus.
[a]HR, hazard ratio for one log_2 increase in indicated biomarker. Model is stratified by study and adjusted for demographics (age, race, sex and continent), and study entry and time-updated $CD4^+$ cell counts.
[b]Invasive cervical cancer, anal, vagina, vulva, penis and oral cavity/pharynx.

Table 5. Sensitivity analysis – adjustment for BMI, diabetes and smoking hazard ratios for one log_2 (i.e. doubling) increment in biomarkers ESPRIT, SMART and SILCAAT.

	CRP		D-dimer		IL-6	
	HR[b]	*P*	HR[b]	*P*	HR[b]	*P*
Any cancer						
Full adjustment[a]	1.16	0.001	1.17	0.03	1.38	<0.001
No. of patients (events)	5022 (172)		5006 (171)		4994 (171)	
Full adjustment + BMI	1.16	<0.001	1.16	0.04	1.37	<0.001
No. of patients (events)	4948 (170)		4932 (169)		4920 (169)	
Full adjustment + smoking (SMART)	1.10	0.20	1.12	0.28	1.37	0.006
No. of patients (events)	2541 (58)		2526 (57)		2514 (57)	
Full adjustment + diabetes (SMART and ESPRIT)	1.15	0.008	1.14	0.10	1.42	<0.001
No. of patients (events)	4307 (128)		4291 (127)		4279 (127)	
Infection-related cancer						
Full adjustment[a]	1.18	0.02	1.19	0.13	1.42	0.002
No. of patients (events)	5022 (70)		5006 (70)		4994 (70)	
Full adjustment + BMI	1.18	0.02	1.19	0.15	1.40	0.004
No. of patients (events)	4948 (69)		4932 (69)		4920 (69)	
Full adjustment + smoking (SMART)	1.15	0.30	1.25	0.22	1.45	0.07
No. of patients (events)	2541 (18)		2526 (18)		2514 (18)	
Full adjustment + diabetes (SMART & ESPRIT)	1.20	0.03	1.25	0.08	1.56	<0.001
No. of patients (events)	4307 (51)		4291 (51)		4279 (51)	
Infection-unrelated cancer						
Full adjustment[a]	1.15	0.02	1.15	0.12	1.35	0.002
No. of patients (events)	5022 (102)		5006 (101)		4994 (101)	
Full adjustment + BMI	1.15	0.02	1.15	0.13	1.35	0.002
No. of patients (events)	4948 (101)		4932 (100)		4920 (100)	
Full adjustment + smoking (SMART)	10.08	0.39	1.06	0.65	1.35	0.04
No. of patients (events)	2541 (40)		2526 (39)		2514 (39)	
Full adjustment + diabetes (SMART & ESPRIT)	1.12	0.10	1.08	0.45	1.32	0.02
No. of patients (events)	4307 (77)		4291 (76)		4279 (76)	

CRP, C-reactive protein.
[a]Stratified by study and adjusted for demographics (age, race, sex, continent), and study entry and time-updated $CD4^+$ cell counts.
[b]HR, hazard ratio for one log_2 increase in indicated biomarker.

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Conflicts of interest

There are no conflicts of interest.

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Factors contributing to risk for cancer among HIV-infected individuals, and evidence that earlier combination antiretroviral therapy will alter this risk

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Purpose of review
To critically appraise recent published literature about factors associated with cancer risk likely to be influenced by combination antiretroviral therapy (cART) in HIV-infected individuals, and the potential of earlier cART initiation to reduce this risk.

Recent findings
Factors leading to increased risk of non-AIDS-defining malignancies (NADMs) in particular remain poorly understood. Immunodeficiency appears to be key, whereas evidence is emerging that a direct pro-oncogenic effect of HIV, activated inflammatory and coagulation pathways, and cART toxicity may also contribute. By reducing HIV replication, improving immune function, and limiting chronic inflammation, cART initiation at higher $CD4^+$ cell counts may, therefore, reduce NADM risk. However, cART only partly normalizes enhanced inflammation and coagulation seen during HIV infection and conflicting laboratory and epidemiological data have been reported as to whether (and how) cART affects NADM risk. Furthermore, secondary analyses of randomized controlled trials comparing early versus delayed cART initiation were inconclusive.

Summary
Continuous epidemiological surveillance is warranted to monitor trends in cancer incidence among HIV-infected individuals and to better understand the impact of earlier cART on NADM risk. The role of adjuvant anti-inflammatory or antithrombotic therapies to reduce cancer risk deserves further investigation.

Keywords
antiretroviral therapy, cancer, HIV, inflammation

INTRODUCTION

Cancer and HIV infection have been inextricably intertwined since the beginning of the AIDS pandemic [1,2]. Three cancer types, namely Kaposi sarcoma, non-Hodgkin lymphoma (NHL), and invasive cervical cancer (ICC), were soon found to have a particularly higher incidence in HIV-infected individuals and, for epidemiological surveillance purposes, have classically been referred to as AIDS-defining malignancies (ADMs) [3]. Including ICC in the list of ADM also served the purpose of emphasizing the importance of integrating gynecologic care into medical services for HIV-infected women [3].

However, the spectrum of cancer types observed in excess in HIV-infected individuals was subsequently broadened to encompass a number of non-AIDS defining malignancies (NADMs). With the advent of combination antiretroviral therapy (cART) resulting in prolonged life expectancy, the incidence of NADM rose more than three-fold [4] and its burden has now surpassed the burden of ADM [4,5,6▪▪]. Moreover, when compared with

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KEY POINTS

- Cancer, in particular NADMs, imposes a growing burden on HIV-infected individuals. Immunodeficiency appears to be key to the increased cancer risk observed in this population, whereas evidence is emerging that a direct pro-oncogenic effect of HIV, activated inflammatory and coagulation pathways, and cART toxicity may also contribute.
- Because cART improves immune function, lowers HIV viral load, and reduces inflammation, cART initiation at higher $CD4^+$ cell counts has been proposed as a potentially effective approach to reducing NADM risk.
- Nevertheless, cART only partly normalizes the enhanced inflammation associated with HIV infection and conflicting laboratory and epidemiological data have been reported as to whether (and how) cART affects cancer risk.
- Continuous epidemiological surveillance is warranted to better understand the impact of earlier cART on NADM risk. The role of adjuvant anti-inflammatory or antithrombotic therapies to reduce cancer risk deserves further investigation.

the general population, HIV-infected individuals have been found to be at higher risk of NADM [7–9], with a standardized incidence ratio of approximately 2.0 [10]. Risk estimates differ considerably for individual NADM types. For instance, HIV-infected individuals have been shown to have, in large cohort studies, a 55-fold higher risk of anal cancer, 19-fold higher risk of Hodgkin lymphoma, 2.1-fold higher risk of nonmelanoma skin cancer, and 1.8-fold higher risk of melanoma and liver cancer [9,11▪▪]. Of interest, the risk seems to be distinctly higher for those cancer types associated with viral infection and smoking [5,10,12,13], factors that are particularly prevalent in those with HIV. As a result, NADMs are now a leading cause of death in the setting of HIV infection [14▪].

The changing epidemiology of cancer during HIV infection has rendered the categorization of malignancies into ADM and NADM out-of-date. Anal cancer, a human papillomavirus (HPV)-related NADM, is more strongly associated with HIV infection than ICC [15], with incidence rates 80–110 times as high for HIV-infected MSM compared with the general population [16▪▪,17▪▪]. Moreover, the incidence of anal cancer was found to be five-fold higher in HIV-positive than HIV-negative MSM [18]. Hodgkin lymphoma, an Epstein–Barr virus (EBV)-related NADM [19], is about five to 20 times more common in HIV-infected than in HIV-uninfected individuals [11▪▪,20]. Hence, an emerging trend in recent studies is to categorize cancer into infection-related and infection-unrelated [5,7,9,10,12,21,22▪▪].

The factors leading to an increased cancer risk among HIV-infected individuals remain poorly understood. Immunodeficiency and high prevalence of traditional cancer risk factors (e.g., smoking, oncogenic virus infection) [23–26] appear to be key, whereas evidence is emerging that direct pro-oncogenic effects of HIV, activated inflammatory and coagulation pathways, and cART toxicity may also contribute [6▪▪,22▪▪,25,27]. It remains elusive, however, whether these factors act independently or synergistically. By reducing HIV replication, improving immune function, and reducing inflammation at earlier stages of HIV infection, cART initiation at higher $CD4^+$ cell counts has been proposed as a potentially effective approach for reducing NADM risk [6▪▪,9,28,29]. The purpose of this review is to critically appraise recent evidence regarding established and suspected factors associated with cancer risk likely to be influenced by cART, including immunodeficiency, HIV viral load, enhanced inflammation and coagulation, cART toxicity, and the potential of earlier cART initiation to reduce this risk. We focused on Medline-indexed English literature published from June 2012 to June 2013 that evaluated cART effects, including immunodeficiency and viral replication, on NADM risk. In this review, we will not address traditional cancer risk factors.

IMMUNODEFICIENCY

The strong relationship between lower $CD4^+$ cell count (i.e., immunodeficiency) and increased ADM risk is well established [6▪▪]. Furthermore, there is mounting evidence for an inverse relationship between $CD4^+$ cell count and NADM risk as well [6▪▪]. Although earlier studies that used static (i.e., time-fixed) $CD4^+$ cell measures were inconsistent regarding this relationship, more recent studies that used time-updated measures of $CD4^+$ cell count have observed associations between lower recent $CD4^+$ cell count and increased risk of NADM (grouped) and of a range of specific cancer types. These reports have been reviewed in detail elsewhere [6▪▪]. As mentioned above, HIV-infected individuals have been found to be at a particularly higher risk of infection-related NADM [5,10,12,13]. Moreover, infection-related NADM may be diagnosed at later stages and may be associated with elevated morbidity and mortality in people with HIV infection [30–32]. Furthermore, the augmented risk of infection-unrelated NADM observed among individuals with lower $CD4^+$ cell counts [9,28,29,33–36] is

consistent with a possibly impaired surveillance of premalignant and malignant cells (or with an unknown viral component to cancer types that are currently considered infection-unrelated). Thus, HIV-associated immunodeficiency appears to exert its cancer-predisposing effects through two main mechanisms: reduced clearance and control of oncogenic virus infection and reduced immune surveillance of malignant cells.

HIV VIRAL LOAD AND DIRECT ONCOGENIC EFFECTS OF HIV

Some studies have reported an association between ongoing viral replication and cancer risk. In one report, both cumulative and current HIV RNA levels more than 500 copies/ml were independently associated with increased risk of ADM [37]. In another study, a direct relationship was found between current HIV RNA level and risk of Kaposi sarcoma and NHL, and between duration of time with HIV RNA more than 100 000 copies/ml and anal cancer risk [28]. Evidence has accrued indicating that HIV itself, via tat and Vpr proteins, may have direct pro-oncogenic effects. The potential mechanisms are multiple and complex, involving synergism with other pro-oncogenic viruses [38], disruption of cell cycle regulation [39], blockage of tumor suppressor gene function [40], promotion of chromosome instability through the inhibition of telomerase activity [41], impairment of DNA repair function [42], induction of tumor angiogenesis [38,43], and enhancement of the effects of exogenous carcinogens [44,45].

ENHANCED INFLAMMATION AND COAGULATION

More recently, evidence has emerged linking activated inflammatory and coagulation pathways, as demonstrated by higher plasma levels of biomarkers, to cancer risk. In the Strategies for Management of Antiretroviral Therapy (SMART) study [46], structured cART interruptions were simultaneously associated with higher levels of coagulation and inflammatory biomarkers [47] and increased risk of cancer [48]. In a recent study of ours, we investigated the relationship between plasma levels of interleukin-6 (IL-6), a pro-inflammatory cytokine, C-reactive protein (CRP), an inflammatory marker whose hepatic production is stimulated by IL-6, and D-dimer, a fibrin-degradation product and marker of enhanced coagulation, and the risk of cancer among 5000 HIV-infected individuals enrolled in the control arms (i.e., standard of care) of three randomized trials [22▪▪]. Increasing baseline biomarker plasma levels were independently associated with higher cancer risk; the hazard ratio per doubling in biomarker level was 1.38 ($P<0.001$) for IL-6, 1.16 ($P=0.001$) for CRP, and 1.17 ($P=0.03$) for D-dimer. Results were similar for infection-related and infection-unrelated cancers. This association was strongest for IL-6, the only biomarker that remained significantly associated with cancer risk with simultaneous adjustment for all three markers. Although not providing definitive evidence for a causal link between enhanced inflammation/coagulation and cancer risk during HIV infection, these findings do indicate that trials of interventions that reduce inflammatory and coagulation biomarker levels, in particular IL-6, may be warranted.

COMBINATION ANTIRETROVIRAL THERAPY TOXICITY

With regard to cART toxicity as a risk factor for cancer, a number of studies have failed to detect positive associations between cART use and cancer risk [49–52]. Furthermore, the beneficial effects of cART on HIV replication, immune function, and inflammation suggest that cART use would lead to a reduction in overall cancer risk [6▪▪,9,28,29]. Nevertheless, potential carcinogenic effects of specific cART agents and drug classes may result in increased risk of cancer. This outcome is the case not only for toxic, older drugs, such as zidovudine [53,54], but also for antiretrovirals currently recommended as first-line therapy for treatment-naive patients. Protease inhibitors have been linked to a higher risk of anal cancer in observational studies after adjustment for important confounders [55▪▪,56,57] and efavirenz, a nonnucleoside reverse transcriptase inhibitor, was associated with increased risk of Hodgkin lymphoma in one study [58]. In a recent report, raltegravir, an integrase inhibitor, was found to induce host DNA rearrangements, which, from a theoretical point of view, may have unforeseen consequences including an increased risk of cancer [59]. It is also biologically plausible that, by reducing immunological surveillance of malignant cells, CCR5 inhibitors, a drug class increasingly used in treatment-experienced individuals who failed previous cART regimens, may also lead to an increased incidence of NADM [60]. However, in the absence of any epidemiologic evidence, the clinical relevance of the potential carcinogenic effects of integrase and CCR5 inhibitors remains to be determined.

WOULD EARLIER ANTIRETROVIRAL THERAPY INITIATION REDUCE THE RISK OF NON-AIDS-DEFINING MALIGNANCIES?

There is global consensus that the overall risk:benefit ratio of cART initiation at $CD4^+$ cell counts below 350 cells/μl is favorable. However, given the lack of

randomized trial evidence and inconsistent results from observational studies [61,62], a debate on whether and when to initiate cART at higher $CD4^+$ cell count thresholds is still unfolding [63▪,64▪]. This has resulted in inconsistencies among treatment guidelines. The US Department of Health and Human Services guidelines [65] recommend cART for all HIV-infected persons, regardless of $CD4^+$ cell count (i.e., no threshold), whereas the British HIV Association guidelines only recommend cART initiation in asymptomatic persons with $CD4^+$ cell counts below 350 cells/μl, an exception being serodiscordant couples, wherein cART can be initiated in asymptomatic HIV-positive individuals with higher $CD4^+$ cell counts to reduce the risk of transmission to the HIV-negative partner [66]. The WHO, in its newest guidelines, recommends cART initiation when $CD4^+$ cell counts drop below 500 cells/μl [67]. Earlier cART initiation has clear benefits in terms of reduced HIV transmission at the population level [68], but is not without potential drawbacks in individuals with early HIV infection and thus low risk of disease progression, including cART toxicity, risk of drug resistance, and required commitment to life-long therapy.

There is evidence that earlier cART initiation, by preventing immune deterioration associated with the decline in $CD4^+$ cell counts, reduces Kaposi sarcoma and NHL risk [69–71]; indeed, cART initiation results in regression of early stage Kaposi sarcoma [72]. Furthermore, because cART improves immune function, lowers HIV viral load, and reduces inflammation, earlier cART initiation has been suggested as a potential approach for reducing NADM risk as well, among HIV-infected individuals [6▪▪,9,28,29]. Thus, reduced incidence [73▪,74▪] and even regression of HPV-related premalignant squamous intraepithelial lesions [75▪] following cART initiation have been reported. Alongside their potential benefit in terms of cancer prevention through immune reconstitution and reduced inflammation and viral suppression, some drugs used in cART regimens have been found to have a direct antineoplasic effect. In in-vitro and in-vivo experiments, protease inhibitors were shown to block angiogenesis [76,77] and inhibit tumor growth and invasion [77]. Similarly, efavirenz was found to have selective antitumor cytoxic effects [78] and to inhibit proliferation and differentiation of neoplastic cells [79]. However, the clinical relevance of these findings is yet to be determined and, as discussed above, conflicting laboratory and epidemiological evidence suggests that some cART agents or classes may be associated with increased NADM risk.

The definitive way to determine the effect of earlier cART initiation on risk of NADM is to conduct a large, cancer endpoint-driven randomized controlled trial. However, such a trial would require a very large sample size with extended follow-up. Currently, additional randomized evidence to inform this debate can be obtained only from secondary analyses of trials in which cancer events were not primary endpoints. For two [80,81] of three contemporary randomized trials comparing immediate versus delayed cART initiation in treatment-naive patients [68,80,82], data on NADM outcomes have been reported (Table 1), with no difference noted between the two strategies. The number of NADM events was, however, too small and much longer follow-up will be required to demonstrate differences (if any) between early versus deferred cART initiation. The deferred strategy in these trials allowed $CD4^+$ cell counts to drop far below the thresholds currently recommended for cART initiation by the majority of treatment guidelines [65,66,83]. In this respect, data from the ongoing Strategic Timing of AntiRetroviral Treatment (START) study [84], a large ($N=4600$) randomized trial, will be of particular interest. This study, with a composite clinical endpoint including NADM, is comparing immediate versus deferred (i.e., when $CD4^+$ cell counts drop below 350) cART initiation in HIV-positive persons with $CD4^+$ cell counts higher than 500 cells/μl.

Finally, virological suppression induced by cART only partly normalizes the activated inflammatory and coagulation pathways observed in persons with HIV [85,86]: the reduction of T-cell activation as a result of effective therapy does not reach the level of HIV-uninfected controls [87]. Should activated inflammatory or coagulation pathways be demonstrated definitively to play a causal role in carcinogenesis among HIV-infected individuals, adjunctive anti-inflammatory or antithrombotic therapies may be required to further reduce cancer risk in this population [22▪▪]. In an AIDS Clinical Trial Group observational study investigating whether statin use is associated with decreased risk of serious non-AIDS-defining events [88▪▪], statin use was found to be associated with a 57% reduction in NADM risk. As no significant benefits were observed for cardiovascular events, it was hypothesized that the reduction in cancer risk was driven by cholesterol-independent, anti-inflammatory properties of statins. The lack of an inverse association between statin use and cardiovascular events may also be explained by unknown biases (as such an association would be expected) or by low statistical power for cardiovascular events (adjusted and weighted hazard ratio = 0.89; 95% confidence

Table 1. Impact of immediate versus deferred initiation of combination antiretroviral therapy on non-AIDS-defining malignancy incidence: data from randomized controlled trials involving combination antiretroviral therapy-naive HIV-positive persons

Study	Sample size	Median follow-up time (years)	Median baseline $CD4^+$ cell count (cells/μl)	Deferral strategy	Median $CD4^+$ cell count (cells/μl) at cART initiation in the deferred arm	NADM in immediate cART initiation arm	NADM in deferred cART initiation arm	Relative risk (95% CI) for NADM (immediate versus deferred cART)
SMART[a] [80]	249	2.6	437	cART deferred until:				
				1. $CD4^+$ cell declined to < 250 cells/μl	245	0/131	0/118	n/a
				2. $CD4^+$ cell percentage declined to <15%				
				3. Symptoms of HIV disease developed				
HPTN 052 [81]	1761	2.1	428	cART deferred until:	229	3/886	3/875	0.99 (0.20–4.88)
				1. $CD4^+$ cell declined to ≤250 cells/μl				
				2. AIDS-defining illness developed				
Pooled data from the two trials						3/1017	3/993	0.98 (0.20–4.83)

cART, combination antiretroviral therapy; CI, confidence interval; NADM, non-AIDS-defining malignancy; SMART, Strategies for Management of Antiretroviral Therapy.
[a]Only includes the subset of patients who were treatment-naive at study entry.

interval = 0.32–2.44). However, the report findings are consistent with a case–control study nested within an HIV cohort in which statin exposure, but not use of other lipid-lowering drugs, was found to be associated with a significantly decreased risk of NHL [89], a cancer type whose development was shown to be preceded by chronic immune activation [90,91].

CONCLUSION

Cancer, in particular NADM, imposes a growing burden on the aging population of HIV-infected individuals. Immunodeficiency and the high prevalence of traditional cancer risk factors (e.g., smoking, oncogenic virus infection) appear to be key to the increased cancer risk observed in this population, whereas evidence is emerging that a direct pro-oncogenic effect of HIV, activated inflammatory pathways, and cART toxicity may also contribute to the higher risk. Because cART improves immune function, lowers HIV viral load, and reduces inflammation, cART initiation at higher $CD4^+$ cell counts has been proposed as a potentially effective approach to reducing NADM risk. cART is, however, not without risks and there is no conclusive laboratory or epidemiological evidence regarding the association of cART and NADM risk. Therefore, continuous epidemiological surveillance is warranted to monitor trends in cancer incidence among HIV-infected individuals and to better understand the impact of earlier cART on NADM risk. As cART alone only partly normalizes the enhanced inflammation and coagulation associated with HIV infection, the role of adjuvant anti-inflammatory or antithrombotic therapies to reduce NADM risk deserves further investigation.

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Conflicts of interest

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