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In silico thrombin generation: Plasma composition imbalance and mortality in human immunodeficiency virus

Kathleen E. Brummel-Ziedins PhD[1] | Matthew Gissel MS[1] | Jacqueline Neuhaus MS[2] |
Álvaro H. Borges MD, MSc, PhD[3,4] | David R. Chadwick MRCP, PhD[5] |
Sean Emery PhD[6,7] | James D. Neaton PhD[2] | Russell P. Tracy PhD[1,8] |
Jason V. Baker MD, MS[9,10] | for the INSIGHT SMART, ESPRIT Study Groups

[1]Department of Biochemistry, University of Vermont, Colchester, Vermont, USA

[2]Department of Biostatistics, University of Minnesota, Minneapolis, Minnesota, USA

[3]Centre of Excellence for Health, Immunity, and Infections (CHIP), Department of Infectious Diseases, Rigshospitalet, University of Copenhagen, Copenhagen, Denmark

[4]Institute for Clinical Medicine, University of Copenhagen, Copenhagen, Denmark

[5]James Cook University Hospital, Middlesbrough, UK

[6]Faculty of Medicine, University of Queensland, Brisbane, Australia

[7]The Kirby Institute, University of New South Wales, Sydney, Australia

[8]Department of Pathology and Laboratory Medicine, University of Vermont, Burlington, Vermont, USA

[9]Department of Medicine, University of Minnesota, Minneapolis, Minnesota, USA

[10]Department of Infectious Diseases, Hennepin County Medical Center, Minneapolis, Minnesota, USA

Correspondence
Dr. Kathleen E. Brummel-Ziedins, University of Vermont, Department of Biochemistry, 360 South Park Drive, Colchester, VT 05446, USA.
Email: kathleen.brummel@uvm.edu

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Abstract

Background: Effective HIV treatment with antiretroviral therapy has prolonged survival and shifted causes of death to non-AIDS illnesses such as cardiovascular disease. We have shown that inflammation and HIV viral load associate with pro- and anticoagulant factor imbalances resulting in increased thrombin generation when mathematically modeled. We explore the hypothesis that factor compositional imbalance, corresponding to increased in silico thrombin generation, predicts mortality among HIV+ persons.

Methods: In a nested case-control study of HIV+ individuals on continuous antiretroviral therapy in two large trials, we evaluated cases (any non-violent mortality, n = 114) and matched controls (n = 318). Thrombin generation in response to a tissue-factor initiator for each individual was calculated by a mathematical model incorporating levels of factors (F)II, V, VII, VIII, IX, X, antithrombin, tissue factor pathway inhibitor, and protein C (PC) measured at study entry to the trials. In silico thrombin generation metrics included clot time, maximum rate (MaxR), maximum level (MaxL), and area under the curve (AUC).

Results: Levels of antithrombin and PC decreased, while FV and FVIII were higher in cases vs controls. This resulted in a more procoagulant phenotype with increased MaxR, MaxL, and AUC in cases compared to controls ($P < 0.05$ for all).

Conclusions: Antithrombin, FV, FVIII, and PC were the major contributors to the increased thrombin generation associated with mortality risk. Our results suggest that mortality in HIV is associated with an increase in in silico thrombin generation via altered balance of pro- and anticoagulant factors, likely due to an inflammatory response signal, and resulting coagulopathy.

KEYWORDS

blood coagulation factors, HIV, in silico, inflammation, mathematical model, mortality, thrombin generation

Essentials

- Individuals with HIV frequently present with a procoagulant phenotype.
- Thrombin generation was simulated in death cases and matched controls in a HIV cohort.
- Although clot time was unaffected, the cases did produce more thrombin than controls.
- Differences in factors V and VIII, antithrombin, and protein C accounted for this imbalance.

1 | INTRODUCTION

Aberrant coagulation is a well-described consequence of HIV infection.[1] Deficiencies in key anticoagulants such as protein C (PC)[2,3] and antithrombin (AT)[4] have been detected in HIV-infected patients, and risk for thromboembolic disease may increase at lower CD4+ counts.[4] With the development and use of effective antiretroviral therapy (ART), morbidity and mortality have been reduced among HIV-infected populations. However, rates of non-AIDS defining conditions, such as cardiovascular disease, have been increasing.[5] Increases in many chronic diseases would be expected with prolonged life expectancy,[6] but a key factor contributing to excess rates of non-AIDS events among treated HIV+ patients includes persistent abnormalities in systemic inflammation and coagulation activity.[7-9] Higher levels of biomarkers such as D-dimer and interleukin-6 (IL-6)[8-10] among HIV+ patients have been shown to be positively associated with anemia,[11] cancer,[12] cardiovascular disease,[10] type 2 diabetes,[13] progression to AIDS,[14] and composite non-AIDS related clinical events and all-cause mortality.[7,8,15] However, a mechanistic basis for the connection between HIV-associated alterations in coagulation activity and risk for a broad spectrum of non-AIDS defining diseases has yet to be elucidated.

Qualitative or quantitative alterations in the proteins and cells responsible for maintaining the hemostatic balance can result in morbidity and mortality.[16,17] At the center of many hemostatic processes that regulate bleeding and clotting is the generation of thrombin.[18,19] Thrombin generation is the central event in hemostasis[20] and a culprit in thrombosis, whereby the suppression of thrombin production and/or function has been proven to be an effective anticoagulant therapy.[21-23] Thrombin generation assays can be used to define an individual's phenotype through the use of the phases of thrombin generation (initiation, propagation, termination) which are governed by the underlying kinetic reactions and can provide descriptors of the coagulation process (eg, rates, levels, and timing of thrombin formation and inhibition).[24] Studies in the healthy population have shown that thrombin generation dynamics differ largely between individuals, but are relatively stable over time in a given individual, supporting the concept that they may have utility as a phenotypic marker.[25-27] The mechanism-based bridge between thrombin generation assays and individualized risk prediction is plasma composition–based computational modeling.[28,29] Recently, computational models that kinetically describe the tissue-factor initiated coagulation process (extrinsic pathway) have been utilized to define an individual's thrombin phenotype based upon their individual plasma composition of pro- and anticoagulant factors.[30]

The central idea with this approach is that in any individual, the balance of the procoagulant and anticoagulant factor levels together act to generate a unique coagulation or thrombin phenotype upon a tissue-factor stimulus. We believe that the sum of developmental, environmental, genetic, nutritional, and pharmacological influences are reflected in the protein levels at any given time that specifically alter the synthesis and/or presentation of the proteins and active enzymes. We have described changes in thrombin generation and related these changes to plasma coagulation factor composition in the following groups: genetic bleeding tendencies[30-33] and clotting tendencies[34,35]; women undergoing in vitro fertilization[36]; chronic obstructive pulmonary disease[37]; stroke[38]; rheumatoid arthritis[39]; and cardiovascular disease.[40] In all cases, thrombin generation was more pronounced in individuals with procoagulant vs bleeding tendencies.[30-32] Individuals with acute conditions appeared to have

more pronounced thrombin generation than individuals with chronic conditions.[38,40] Individuals with inflammatory diseases displayed a wide range of thrombin generation.[37,39]

We previously used this approach to study the consequences of HIV viral replication.[41] We found short-term increases in factor (F) VIII and decreases in both AT and PC when HIV replication increased after stopping ART; reciprocal changes occurred after initiating ART. The protein concentrations for both groups (treated and untreated HIV infection) were within the normal ranges for healthy individuals, however, these differences resulted in significantly increased thrombin generation during untreated HIV infection, which was shown to be reversible with ART treatment. Whether this increased procoagulant potential translates into a higher risk of adverse clinical endpoints is unknown. In this nested case-control study, we report associations between mortality risk and computationally generated thrombin generation among HIV+ patients treated with ART.

2 | PATIENTS AND METHODS

2.1 | Study population

The study design was a nested case-control study, sampling patients from the control arms (continuous use of ART) of two large, international HIV clinical outcome trials, Strategies for Management of Antiretroviral Therapy (SMART)[42,43] and Evaluation of Subcutaneous Proleukin in a Randomized International Trial (ESPRIT).[44] In SMART, 5472 HIV-positive adults with CD4+ cell count >350 cells/mm^{3} in 33 countries were randomized to receive continuous ART with the goal of viral suppression (control group) or episodic ART guided by the CD4+ count. In ESPRIT, 4111 HIV-positive adults with CD4+ cell counts ≥300 cells/mm^{3} in 25 countries were randomized to receive continuous ART alone (control group) or continuous ART in combination with subcutaneous IL-2. Therefore, participants in the control arms of SMART and ESPRIT received continuous ART along with standard of care according to HIV treatment guidelines.

Cases were defined as death from any non-violent cause (n = 114) during follow-up (median 3 years), and were matched with controls based on age, enrollment date, study, and continent (three controls for every one case). Twenty-four controls were omitted due to an insufficient blood sample to measure factor levels; the remainder (n = 318) were included in our analyses. All participants provided written informed consent.

2.2 | Measurement of factor levels and other biomarkers

Blood was collected using EDTA (ethylenediaminetetraacetic acid) tubes and plasma was processed, frozen at −70°C, and stored at a central repository until analysis. Procoagulants FII, FV, FVII, FVIII, FIX, and FX were measured using an ELISA (Enzyme Research Laboratories, South Bend, IN); analytical inter-assay coefficients of variation (CV) range for these measures were 6.4%-11.1%,

9.6%-10.8%, 6.7%-12.2%, 3.8%-5.2%, 7.4%-7.8% and 5.8%-8.0%, respectively. AT was measured on a Stago STA-R analyzer (Stago Diagnostics, Parsippany, NJ); with a CV range of 2.6%-10.2%. PC and tissue factor pathway inhibitor (TFPI) were measured using ELISAs (Stago Diagnostics and R&D Systems, Minneapolis, MN, respectively). Corresponding CV ranges were 5.5%-6.2% and 8.8%-11.3%. The assays were standardized with EDTA plasma. All measurements used the sample collected at trial entry. Factors are expressed as percentages of the mean values in the healthy population.

IL-6 was measured by ELISA (R&D Systems). D-dimer was measured using the Liatest D-DI (Stago Diagnostics) for SMART or on a VIDAS instrument (BioMerieux Inc., Durham, NC) for ESPRIT. High-sensitivity C-reactive protein (hsCRP) was measured by ELISA (Siemens Diagnostics, Munich, Germany for SMART and R&D Systems for ESPRIT).

2.3 | Computational model of thrombin generation

Mathematical simulations of tissue factor–initiated thrombin generation were produced as previously described.[45] For each individual, concentrations of procoagulants FII, FV, FVII(a), FVIII, FIX, and FX and anticoagulants TFPI, AT, and PC are electronically "exposed" to 5 pM tissue factor (TF) in the presence of 1 nmol/L thrombomodulin. Simulated reactions were solved for thrombin over a 1200 seconds time frame. Individual profiles can be summarized by parameters describing the initiation, propagation, and termination phases of in silico thrombin generation: maximum level of thrombin generation (MaxL), maximum rate of thrombin generated (MaxR), time to 10 nmol/L thrombin (clot time), and total thrombin generated (area under the curve [AUC]).[46] Results for a particular group of individuals are graphically presented as a curve, which represents the mean thrombin concentration at each second of the reaction (± standard error of the mean). In silico thrombin generation from a hypothetical "control" is defined as all factors set at 100% of the mean physiologic concentration for each factor.

To determine which factor(s) were responsible for differences between cases and controls, we used a previously described procedure[39,40] in which each of the individuals in the mortality group had one of the factor levels sequentially adjusted to the mean of the control group and the reaction rerun with a 5 pmol/L TF stimulus and in silico thrombin outputs recalculated. This process was repeated for each factor (and expanded to multiple factors simultaneously). The impact of a given factor was evaluated by quantifying the differences between various thrombin generation parameters pre- and post-adjustment.

2.4 | Statistical methods

We evaluated the effect of variables used to describe the initiation, propagation, and termination phases of thrombin generation, baseline characteristics, and initial protein concentrations on the risk of mortality with conditional logistic regression analyses for matched case-control studies. Odds ratios (OR) for mortality per 1 $\log_2$ increase in factor levels and modeling parameters are cited. A 1 $\log_2$

increase corresponds to a two times higher factor level or modeling parameter. To assess whether associations between maximum thrombin level and mortality varied by subgroups, an interaction term (product of $\log_2$ level and subgroup indicator) was included in the logistic models. The effect of adjusting for individual factor levels on the maximum level OR for mortality was assessed.

3 | RESULTS

3.1 | Participant characteristics

Table 1 presents the participant demographic and clinical characteristics at trial entry. Distribution of underlying causes of death are included in Table 2, with the most frequent causes being non-AIDS defining cancer (n = 27; 23.7%), unknown (n = 16; 14.0%), cardiovascular disease (CVD) (n = 15; 13.2%), unwitnessed death (n = 12; 10.5%), liver disease (n = 10; 8.8%) and AIDS (n = 10; 8.8%). The median age was 46, and 81% were male. When compared to controls, cases had higher prevalence of viral hepatitis co-infection, prior CVD, smoking, and use of blood pressure–lowering therapy. Baseline use of non-nucleoside reverse transcriptase inhibitors (48.2%), protease inhibitors (44.7%), abacavir (24.8%), and tenofovir (10.2%) was similar between cases and controls. Cases were less likely to be virologically suppressed and had significantly higher levels of high-sensitivity C-reactive protein (hsCRP), IL-6, and D-dimer.

TABLE 1 Participant demographic and clinical characteristics

	Cases	Controls	P-value[a]
Number	114	318	
Age (median, IQR)	47 (40-54)	46 (40-53)	N/A
Female (%)	19.3	19.2	0.98
Black race (%)	20.2	17.3	0.36
White race (%)	73.7	72.3	
Other race (%)	6.1	10.4	
BMI (median, IQR)	23.4 (21.2-27.1)	24.5 (22.6-26.9)	0.18
CD4+ (cells/mm^3) (median, IQR)	460 (371-630)	499 (405-660)	0.15
Nadir CD4+ (cells/mm^3) (median, IQR)	194 (84-297)	201 (98-320)	0.16
HIV RNA (% ≤500 copies/mL)	66.7	76.1	0.036
Hepatitis B or C co-infection (%)	33.7	18.6	0.002
BP-lowering treatment (%)	23.7	15.4	0.025
Lipid lowering treatment (%)	19.3	15.7	0.40
Diabetes (%)	8.8	7.2	0.65
Prior CVD (%)	9.6	3.1	0.005
Current smoker[b] (%)	59.3	37.9	0.009
Total cholesterol[b] (mg/dL) (median, IQR)	190 (165-238)	202 (176-234)	0.39
HDL cholesterol[b] (mg/dL) (median, IQR)	40 (31-53)	41 (33-50)	0.68
LDL cholesterol[b] (mg/dL) (median, IQR)	111 (84-134)	111 (88-133)	0.64
Triglycerides[b] (mg/dL) (median, IQR)	168 (110-251)	219 (149-324)	0.32
Total/HDL ratio[b] (median, IQR)	4.5 (3.6-6.4)	5.1 (3.8-6.5)	0.62
hsCRP (µg/mL) (median, IQR)	3.10 (1.59-7.61)	1.77 (0.79-3.84)	0.005
Interleukin-6 (pg/mL) (median, IQR)	3.10 (2.10-4.48)	1.90 (1.30-3.00)	<0.001
D-dimer (µg/mL) (median, IQR)	0.34 (0.23-0.54)	0.26 (0.15-0.39)	0.008

BMI, body mass index; BP, blood pressure; CVD, cardiovascular disease; HDL, high density lipoprotein; hsCRP: high-sensitivity C-reactive protein; IQR, interquartile range; LDL, low density lipoprotein.
[a]From univariate conditional logistic regression model.
[b]Available only for SMART participants (n = 54 cases and 153 controls).

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Cause of death	ESPRIT, n (%)	SMART, n (%)	Total, n (%)
AIDS	7 (11.7)	3 (5.6)	10 (8.8)
Non-AIDS cancer	14 (23.3)	13 (24.1)	27 (23.7)
CVD	8 (13.3)	7 (13)	15 (13.2)
Unwitnessed death	7 (11.7)	5 (9.3)	12 (10.5)
Hepatic	7 (11.7)	3 (5.6)	10 (8.8)
Infection	3 (5)	4 (7.4)	7 (6.1)
CNS disease	3 (5)	0 (0)	3 (2.6)
Renal	0 (0)	2 (3.7)	2 (1.8)
Digestive system disease	1 (1.7)	1 (1.9)	2 (1.8)
Respiratory disease	0 (0)	1 (1.9)	1 (0.9)
Substance abuse	3 (5)	6 (11.1)	9 (7.9)
Unknown	7 (11.7)	9 (16.7)	16 (14)
Total	60 (100)	54 (100)	114 (100)

CNS, central nervous system; CVD, cardiovascular disease.

3.2 | The pro- and anticoagulant balance in HIV mortality

Concentrations of plasma factors (expressed as a percentage of mean physiologic values in the healthy population) are shown in Table 3. In the unadjusted comparisons, the cases had higher procoagulants FVIII ($P = 0.003$) and FV ($P = 0.018$), but lower anticoagulants PC ($P = 0.002$) and AT ($P = 0.034$). After adjustment for age, sex, and race, FVIII (OR 1.80; 95% CI 1.16-2.80; $P = 0.009$), FV (OR 1.73; 95% CI 1.10-2.71; $P = 0.017$), and PC (OR 0.45; 95% CI 0.26-0.77; $P = 0.003$) remained significantly associated with mortality risk.

3.3 | Thrombin generation

The mean thrombin generation (±SEM) for cases and HIV+ controls are shown in Figure 1 along with a dashed line representing the 100% mean physiologic curve. Thrombin generation was increased,

representing a more procoagulant potential, in both groups of HIV+ individuals (cases and controls) over a mean physiologic thrombin profile, with mortality cases having the higher thrombin generating procoagulant potential when compared to controls. From the individual profiles, parameters that objectively characterize the kinetic reaction of thrombin generation (MaxR, MaxL, AUC, and clot time) were extracted and are presented by group in Table 4. The OR for mortality risk per $\log_2$ higher level for each thrombin generation parameter is also presented in Table 3. Although clot time was similar between the two groups, the thrombin generation parameters of MaxR ($P = 0.020$), MaxL ($P = 0.012$), and AUC ($P = 0.006$) were significantly associated with increased mortality risk.

The association of thrombin generation was further evaluated by subgroups defined by traditional risk factors, with data for mortality risk by maximum thrombin levels presented in Table 5. There was no evidence of a significant interaction of subgroup by higher maximum thrombin level for mortality risk.

TABLE 3 Factor levels and odds ratios for mortality. Median (IQR) factor levels for cases and controls and odds ratios for mortality associated with a doubling of the factor

Factor	Cases median (IQR)	Controls median (IQR)	OR (95% CI)[a]	*P*-value	Adjust. OR (95% CI)[b]	*P*-value
II (%)	101 (84-117)	105 (91-121)	0.56 (0.32-1)	0.049	0.63 (0.35-1.13)	0.12
V (%)	111 (87-135)	98 (85-120)	1.72 (1.1-2.69)	0.018	1.73 (1.1-2.71)	0.017
VII (%)	94 (77-112)	103 (84-124)	0.65 (0.41-1.02)	0.062	0.67 (0.41-1.09)	0.10
VIII (%)	147 (110-189)	130 (100-163)	1.91 (1.25-2.92)	0.003	1.80 (1.16-2.8)	0.009
IX (%)	96 (82-113)	97 (86-113)	0.83 (0.56-1.23)	0.36	0.84 (0.56-1.26)	0.40
X (%)	90 (78-105)	93 (81-108)	0.76 (0.42-1.39)	0.38	0.74 (0.4-1.38)	0.35
PC (%)	109 (91-126)	117 (100-135)	0.44 (0.27-0.74)	0.002	0.45 (0.26-.77)	0.003
TFPI (ng/mL)	27 (21-35)	27 (22-32)	1.39 (0.91-2.12)	0.13	1.40 (0.9-2.17)	0.14
AT (%)	127 (110-140)	130 (114-143)	0.47 (0.23-0.95)	0.034	0.51 (0.25-1.05)	0.068

AT, antithrombin; IQR, interquartile range; PC, protein C; TFPI, tissue factor pathway inhibitor.

[a]Odds ratio for mortality per 1 $\log_2$ higher factor level from unadjusted conditional logistic regression model.

[b]OR for mortality per 1 log2 higher factor level, adjusted for age, sex, and race.
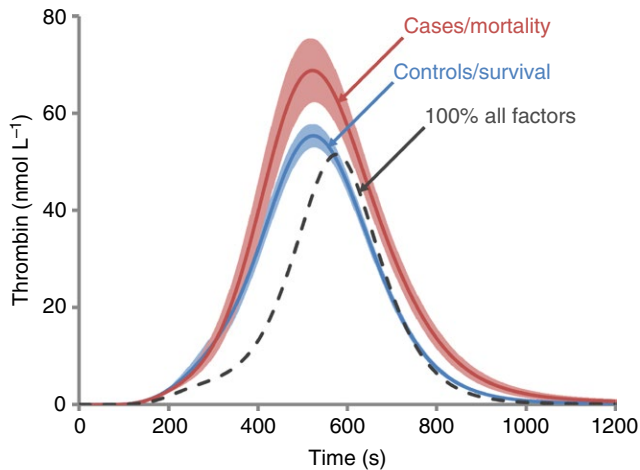
FIGURE 1 Computational model of all patients by survival status. Cases (in red) and controls (in blue) are displayed as the mean thrombin concentration at each second. The shaded areas represent the SEM at those points. Also shown (as a dashed gray line) is the curve generated when all coagulation factors are set to the mean physiologic value for the healthy population

3.4 | Factor contribution to thrombin generation

In order to determine which factors were responsible for the altered thrombin generation between cases and controls, each of the cases had their thrombin generation curves re-simulated with each factor level set to the median value of the corresponding factor concentration observed in the HIV control cohort. The protein with the largest impact using this methodology was AT. Figure 2A shows the disappearance of differences in simulated thrombin generation between the groups when the cases had their AT set to the median value for controls of 130% (with all other factor levels for cases left unchanged). Figure 2B shows a similar diminishing of thrombin generation differences was seen when a combination of both FV and FVIII in cases were altered to the median values in the controls (111% and 147%, respectively).

This modeling approach was recapitulated with conditional logistic regression. The association between mortality and MaxL of thrombin generation was adjusted for each of the individual factors separately, and the OR for mortality associated with a doubling of

MaxL was determined (Table 6). Individual adjustment for FV, FVIII, AT, or PC demonstrated the largest attenuation in the association between mortality and MaxL. In addition, adjustment of FV and FVIII together had the most substantial impact on attenuating the association with mortality.

4 | DISCUSSION

We present data demonstrating for the first time that risk for all-cause mortality among HIV+ patients receiving continuous ART is associated with a factor compositional imbalance that corresponds to an increase in simulated thrombin generation. Mortality was associated with increased inflammation, D-dimer, and an altered pro- and anticoagulant balance with higher levels of FV and FVIII and lower levels of PC and AT, yielding a higher procoagulant state among cases of non-violent mortality when compared with controls. These findings expand our understanding of HIV-associated abnormalities in coagulation biology and the consequences for mortality from a broad spectrum of disease, both CVD and non-CVD related.

We have shown in previous analyses of different pathologic states that normal range variation in coagulation factor composition can modulate the relative severity of the thrombin generation defect.[30,47] The balance between these procoagulant and anticoagulant processes provides a defense against excessive fluid loss (hemorrhage) and against inappropriate vascular occlusion (thrombosis). Previous studies have shown that in HIV+ patients, there exists lower PC and protein S and elevated D-dimer and von Willebrand factor compared to HIV- controls.[48] Higher fibrinogen has also been identified in HIV+ patients.[49] We have also shown from the SMART study that increased HIV replication after stopping ART correlated with increased levels of FVIII and decreased levels of AT and PC, resulting in increased thrombin generation in this computational model.[41] In this current study, both groups of ART-treated HIV+ patients had higher average AT than the mean physiologic population. This finding is in agreement with Hsue et al,[50] who also found higher AT levels in HIV patients (both untreated and those receiving ART) compared to uninfected controls. That study found the higher AT levels contributing to a relatively decreased thrombotic state. In contrast, our HIV+ cohort had a significantly higher prothrombotic

TABLE 4 Thrombin modeling parameters and odds ratios for mortality

	Cases median (IQR) n = 114	Controls median (IQR) n = 318	OR (95% CI)[a]	P-value
Maximum rate (nmol/L per s)	0.44 (0.28-0.62)	0.35 (0.2-0.56)	1.25 (1.04-1.51)	0.02
Maximum level (nmol/L)	68 (45-97)	54 (33-87)	1.31 (1.06-1.63)	0.012
Clot time (s)	348 (284-420)	359 (298-425)	1.04 (0.84-1.27)	0.75
AUC (μmol/L × s)	18 (11.2-26)	13.5 (8.7-22.1)	1.35 (1.09-1.67)	0.006

AUC, area under curve; IQR, interquartile range.
[a]Odds ratio for mortality per 1 $\log_2$ higher parameter level from unadjusted conditional logistic regression model.

TABLE 5 Mortality risk by maximum thrombin level (MaxL) for key subgroups

	Cases		Controls				
	Number	Med (IQR)	Number	Med (IQR)	OR (95% CI)	P-value[a]	P-value[b]
Age							
≤50 years	69	72 (49-98)	217	53 (31-87)	1.49 (1.14-1.96)	0.004	0.15
>50 years	45	67 (38-96)	101	55 (36-87)	1.08 (0.77-1.52)	0.65	
Sex							
Male	92	62 (42-95)	257	51 (33-81)	1.32 (1.04-1.67)	0.022	0.78
Female	22	84 (74-108)	61	77 (41-108)	1.43 (0.85-2.4)	0.18	
Race							
Black	23	72 (49-117)	55	75 (54-113)	1.04 (0.67-1.62)	0.87	0.25
Other	91	67 (42-93)	263	50 (30-83)	1.39 (1.1-1.76)	0.006	
Hepatitis coinfection							
Yes	36	80 (57-116)	50	71 (57-97)	1.46 (0.87-2.43)	0.15	0.52
No	78	65 (38-91)	268	50 (31-82)	1.21 (0.95-1.53)	0.12	
HIV RNA							
≤500 copies/mL	76	67 (43-88)	242	51 (31-81)	1.34 (1.04-1.74)	0.026	0.59
>500 copies/mL	38	84 (48-115)	76	69 (44-100)	1.18 (0.81-1.74)	0.39	
IL-6							
<2.11 pg/mL	30	53 (42-76)	185	52 (34-81)	1.08 (0.74-1.59)	0.69	0.33
≥2.11 pg/mL	80	74 (48-112)	133	59 (33-89)	1.36 (1.04-1.78)	0.023	

CI, confidence interval; IQR, interquartile range; OR, odds ratio.
[a]Conditional logistic regression OR for mortality per 1 $\log_2$ higher level.
[b]P-value for interaction.

potential than the hypothetical control with all factors at mean physiologic levels despite the increase in AT. This increased AT assisted in mediating the combined effects of higher prothrombotic factors and decreased TFPI, when compared to physiologic means, which were found in both groups of this HIV+ population. As seen in Figure 2B, an alteration of the concentrations of FV and FVIII in the cases to the level seen in the controls diminished the observed differences in thrombin generation between the two groups. This particular combination is notable because they are the two procofactors of the coagulation system, converted by thrombin to the active forms FVa and FVIIIa.[51–53] These proteins are the respective cofactors of the prothrombinase (FVa–FXa) and intrinsic tenase (FVIIIa–FIXa) complex, with the intrinsic tenase being the principal generator of FXa and subsequent thrombin generation.

Thrombin's procoagulant activity is neutralized by the plasma serine proteinase inhibitor AT and through the PC anticoagulant pathway. The PC anticoagulant pathway plays a major role in the balance of procoagulation and anticoagulation, by providing a dynamic inhibitory system to regulate thrombin production. Activated protein C, produced by the thrombin–thrombomodulin complex, inactivates the cofactors FVa and FVIIIa,[54] thereby downregulating further generation of thrombin and stopping clot propagation. In this study, it is the suppression of the anticoagulants PC and AT that allows for the continued formation of the procoagulant complexes by not inactivating FVa and FVIIIa, which converts FX to FXa and allows

for the continuation of thrombin generation. The increase in these critical enzymes (FXa and thrombin) also further promotes the observed hypercoagulation in part through the activation of protease-activated receptors 1 and 2,[55–57] which is seen in this population by the increased proinflammatory cytokines such as IL-6. This combined effect may be driving the excess thrombin generation that is seen among the mortality cases.

We have previously reported that risk for all-cause mortality, as well as a composite of serious non-AIDS defining conditions, is associated with increased levels of D-dimer, a known marker for thrombosis, as well as the inflammatory cytokine IL-6.[7] D-dimer has also been shown to be a predictor of mortality in cancer,[58,59] acute pulmonary embolism,[60] and (along with CRP) in acute aortic dissection.[61] In this study, mortality risk was associated with parameters of thrombin generation along with differences in the individual factors FV, FVIIII, AT, and PC. Our findings build on our previous work[41] demonstrating that decreased AT and increased FVIII were the primary proteins responsible for the increased thrombin generation associated with HIV viral replication. Here, we show associations between mortality and estimated thrombin generation among ART-treated HIV+ patients that is, in part, accounted for by this same pattern of lower AT and higher FVIII levels. Cumulatively, these data are consistent with our overall hypothesis that HIV-related coagulation abnormalities improve but may persist while on ART, have consequences for a broad spectrum of end-organ disease risk, and may be mediated, at least
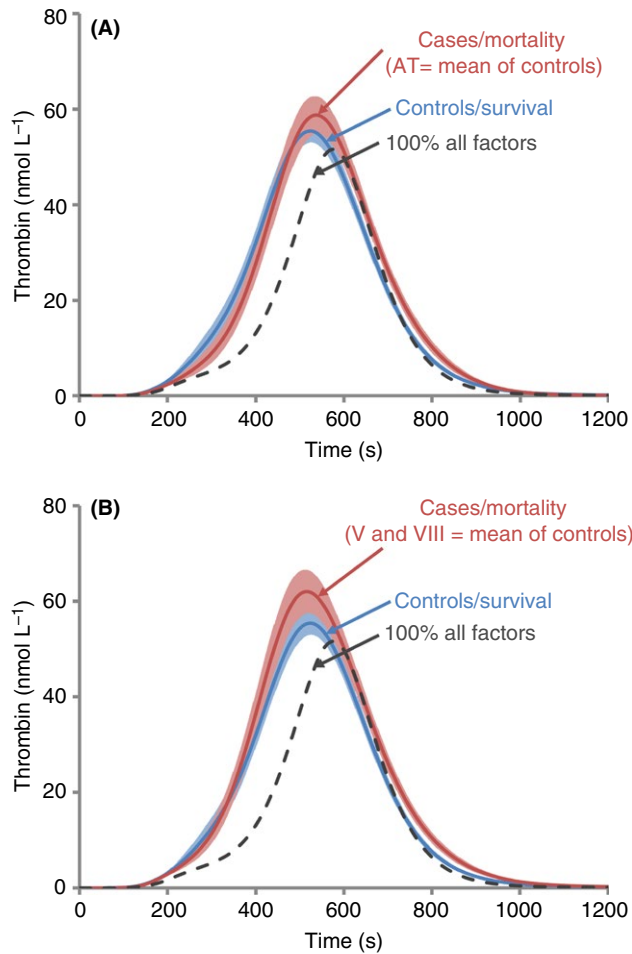
FIGURE 2 Individual factor level impact on computational thrombin generation. Left: For the cases (in red), the antithrombin levels were adjusted to the mean levels of those in the control group (127.9%). Right: For the cases (in red), the factor V and factor VIII levels were adjusted to the mean levels of those in the control group (105.5% and 135.3%, respectively). The control group (in blue) is displayed as in Figure 1 in both panels. Also shown (as a dashed gray line) is the curve generated when all coagulation factors are set to the mean physiologic value for the healthy population

TABLE 6 Maximum level (MaxL) and odds ratios (OR) for mortality after adjustment for individual factor levels

Adjustment	OR (95% CI)[a]	P-value[a]
Age, sex, and race only	1.32 (1.06-1.64)	0.014
Factor II	1.29 (1.03-1.61)	0.026
Factor V	1.23 (0.98-1.56)	0.079
Factor VII	1.28 (1.02-1.6)	0.03
Factor VIII	1.21 (0.96-1.53)	0.11
Factor IX	1.32 (1.06-1.64)	0.012
TFPI	1.52 (1.18-1.97)	0.001
AT	1.27 (0.98-1.65)	0.071
Protein C	1.20 (0.95-1.51)	0.13
Factors V and VIII	1.13 (0.88-1.46)	0.32

CI, confidence interval.

[a]Conditional logistic regression OR for mortality per 1 $\log_2$ higher level of maximum level. All models are adjusted for age, sex and race.

events are a precious resource in this context, and we did not have any additional plasma available to perform in vitro thrombin generation assays. As computational modeling has made advancements into the area of predictive modeling of hemostatic status, ultimately having input concentrations of both antigen and functional activity would be optimal, and (iv) Any coagulation related genetic mutations such as prothrombin G20210A or FV Leiden, were not accounted for, which would also influence the individual's prothrombotic state.

Determining who is at risk for mortality from thrombotic disorders is difficult because the pathogenesis is complicated and multifactorial. Risk predictions for thrombotic-related outcomes are mostly based upon genetic factors (AT,[62] PC,[63] and protein S[64] deficiencies; FV Leiden[65] and prothrombin G20210A[66] mutations), increases in coagulation factors (notably FVIII,[67] FIX,[68] FXI[69]) and environmental factors (obesity,[70] oral contraceptive use,[71] hormone replacement therapy,[71] age,[72] alcohol use,[73] and potentially smoking[74]). The heterologous presence of any of these circumstances in any individual may still be asymptomatic and does not ordinarily necessitate clinical intervention. In HIV+ patients, ART reduces but does not appear to fully normalize abnormalities in coagulation activation. In this study, all HIV+ patients were on ART therapy, and we show that that residual abnormalities in coagulation factor composition (FV, FVIII, PC, AT), in part, resulting from ongoing inflammation (eg, reflected in IL-6 levels), promote FXa activity which can become an important contributor to the excess thrombin generation observed among mortality cases. Having predictive models that evaluate each individual's specific blood coagulation profile may better inform mortality risk, and help identify potential treatment interventions, among ART-treated HIV+ persons.

in part, by changes in coagulation factors as a result of hepatocyte dysfunction in the context of systemic inflammation.

This study has several limitations: (i) There are too few events to examine associations with specific causes of death or any individual end-organ disease condition, and we have limited power for assessing subgroup interactions, (ii) We estimate the kinetics of thrombin generation through an empirically validated computational model, however, the incompleteness of any coagulation model is a potential confounder.[47] Here, in this focused case-control study, we ask the question of whether our previously validated model can identify HIV+ patients who die vs those who do not, (iii) Specimens used for this study were from adjudicated cases, and controls, enrolled in a large clinical outcome trial. The stored plasma allocated for this study was collected as EDTA specimens, which precluded functional factor level measurements. As well, specimens associated with clinical

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DC received grants and personal fees from Pfizer. RPT is one of the owners of Haematologic Technologies, Inc. All other authors claim no conflicts of interest.

AUTHOR CONTRIBUTIONS

KBZ designed and performed the research, analyzed data, and wrote the manuscript. MG performed the research, analyzed data, and wrote the manuscript. JN analyzed data and wrote the manuscript. AHB, DC, and SE critically evaluated the manuscript and provided edits. JDN, RPT, and JVB designed, analyzed data, and wrote the manuscript.

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