Title

Semen HIV-1 Concentration is Strongly Associated with Altered Levels of Semen Interferon-Gamma, Interleukin-17, and Interleukin-5

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Semen HIV-1 Concentration is Strongly Associated with Altered Levels of Semen Interferon-Gamma, Interleukin-17, and Interleukin-5

A thesis submitted in partial satisfaction

of the requirements for the degree Master of Science

in Clinical Research

by

Jennifer Claire Hoffman

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ABSTRACT OF THE THESIS

Semen HIV-1 Concentration is Strongly Associated with Altered Levels of Semen Interferon-Gamma, Interleukin-17, and Interleukin-5

by

Jennifer Claire Hoffman

Masters of Science in Clinical Research

University of California, Los Angeles, 2013

Professor Robert M. Elashoff, Chair

Semen HIV-1 level is an important determinant of the risk of HIV-1 sexual transmission. In this prospective observational study, we investigated potential associations between seminal cytokine levels and HIV-1 concentration in the seminal plasma of HIV-infected men, using paired blood and semen samples from 18 HIV-1 chronically-infected men off antiretroviral therapy. HIV-1 RNA levels and cytokine levels in seminal plasma and blood were measured and analyzed using simple linear regressions to screen for an effect of cytokines on seminal plasma HIV-1. Forwards stepwise regression was performed to construct the final multivariate model. The median HIV-1 concentration was 4.42 $\log_{10}$ RNA copies/ml (IQR 2.98, 4.70) and 2.96 $\log_{10}$ RNA copies/ml (IQR 2, 4.18) in blood and semen plasma, respectively. In stepwise multivariate linear regression analysis, blood HIV-1 level ($p<0.00005$), seminal plasma IFN-γ level ($p=0.03$),

and seminal plasma IL-17 level ($p=0.03$) were positively associated with seminal plasma HIV-1 level; seminal plasma IL-5 was negatively associated with seminal HIV-1 level ($p=0.0007$). These data indicate that, in addition to plasma levels of HIV-1, cytokine profiles in the male genital tract are an important determinant of HIV-1 levels in semen. Th1 and Th17 cell profiles are associated with increased levels of virus while the Th2 cellular profile is associated with decreased levels of virus. These results support the importance of genital tract immunomodulation in HIV-1 transmission.

The thesis of Jennifer Claire Hoffman is approved.

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Janet S. Sinsheimer

Robert M. Elashoff, Committee Chair

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2013

DEDICATION

To my family, without whose support this would not have been possible, and to my mentors, who have taught me so much and encouraged me over the years.

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ACKNOWLEDGEMENTS

Chapter 1 is a version of a manuscript by Hoffman J, Anton P, Cocita Baldwin G, Eliott J, Anisman-Posner D, Tanner K, Grogan T, Elashoff D, Sugar C, Yang O, and Hoffman R. "Semen HIV-1 Concentration is Strongly Associated with Altered Levels of Semen Interferon-Gamma, Interleukin-17, and Interleukin-5" which has been submitted to AIDS Research and Human Retroviruses for consideration of publication. J. Hoffman performed the data and statistical analyses and wrote the primary manuscript; P.Anton participated in study design, subject recruitment/sample collection, participated in data analysis, and revised the manuscript; G. Cocita Baldwin participated in study design, performed laboratory experiments, and revised the manuscript; J. Eliott participated in study design, optimization/performance of laboratory experiments; D. Anisman-Posner participated in study design and optimization/performance of laboratory experiments; K. Tanner participated in study design and optimization/performance of laboratory experiments; D. Elashoff performed statistical analyses and revised the manuscript; C. Sugar performed statistical analyses; O. Yang participated in study design, performed data analysis, and revised the manuscript; R. Hoffman was principal investigator for the study and designed the study, enrolled participants, performed laboratory experiments, participated in data analysis, and revised the manuscript.

Introduction

As approximately 85% of new HIV-1 infections worldwide are acquired through sexual transmission [1] it is of paramount importance to understand factors associated with that risk. It is known that the concentration of virus in semen is an important determinant for sexual transmission.[2,3] Although antiretroviral therapy (ART) reduces blood plasma HIV-1 levels and concurrently semen HIV-1 RNA levels in HIV-infected men, 6-48% of treated men with undetectable plasma viremia continue to exhibit intermittently detectable virus in semen.[4-6].

While plasma viremia has been demonstrated in multiple studies to be strongly correlated with semen HIV-1 level,[2,7] other factors influence semen HIV-1 level as well. Sexually transmitted infections such as genital herpes,[3,8] gonorrhea,[9,10] and non-gonococcal urethritis,[10-12] as well as genital tract CMV reactivation are associated with increased genital tract inflammation and increased semen HIV-1.[13,14]

Consistent with a role for local inflammation driving increased HIV-1 in semen, several recent studies have shown correlations between increased levels of various pro-inflammatory cytokines in seminal plasma and seminal plasma HIV-1 levels. Berlier *et al* described a positive correlation with IL-1β,[15] while Storey *et al* described a positive correlation with RANTES,[16] and Sheth *et al* showed a positive correlation with IL-6, IL-8, IL-12, and IFN-γ.[17] Furthermore, Lisco *et al* recently suggested that HIV-1 infection causes a "reprogrammed cytokine network" in the semen with elevated levels of several pro-inflammatory cytokines including IL-1α, IL-1β, IL-6, and IL-8, which do not correlate to blood plasma cytokine levels.[18] This suggests that cytokine

expression is compartmentalized and that semen cytokines are likely produced within the male genital tract.

In this pilot study, we examine the relationship between seminal plasma HIV-1 and a panel of 17 blood and seminal plasma cytokines in a group of 18 HIV-1-infected men with viremia >200 RNA copies/ml. We hypothesized that higher levels of pro-inflammatory cytokines in seminal plasma would be independently associated with higher seminal plasma HIV-1 concentrations.

Methods

Study subjects and specimens:

All participants provided written informed consent under a UCLA Institutional Review Board-approved protocol. The study population was comprised of 18 HIV-1-infected adult men from the Los Angeles area, who were not on ART with blood plasma HIV-1 level ≥ 5,000 copies/ml within the past 3-6 months by self-report, and who were willing to provide blood, urine, and semen samples. These samples were collected during a single study visit. Due to our intention to study men with detectable HIV-1 viremia, men with viremia <200 RNA copies/ml at the time of the study visit were excluded.

Clinical screening tests:

Blood and urine samples were transported to Foundation Labs and processed within 24 hours for routine clinical testing for blood $CD4^+$ T cell counts and urine for *Neisseria gonorrhea* and *Chlamydia trachomatis* (APTIMA Combo 2®, Hologic Gen-Probe, San Diego, CA).

HIV-1 quantification in blood and seminal plasma:

Plasma was isolated from blood and semen samples by centrifugation and aliquots were stored at -80°C prior to nucleic acid extraction. The Biomerieux NucliSENS Easy Q HIV-1 v1.1 and v2 (Durham, NC) assay systems were used for this study. This assay is designed for Nucleic Acid Sequence-Based Amplification and real-time detection of isolated HIV-1 RNA using proprietary instrumentation. Nucleic acid extractions were performed using the NucliSENS miniMAG extraction system as per manufacturer's protocol. Using the manufacturer's recommendations as a framework, the final protocol for nucleic acid isolation and subsequent real-time detection of

HIV-1 RNA for both blood plasma and seminal plasma was derived following optimization of the assay. Briefly, assay optimization was performed as follows: using matched blood and semen samples from HIV-infected (n=11) and uninfected donors provided on two separate visits, we found a high degree of reproducibility for serial HIV-1 RNA measurements in both blood (R^2=0.9754) and semen (R^2=0.7878) as well as a significant correlation between blood and semen plasma HIV-1 RNA concentrations (R^2=0.4343; p=0.027) (see **Figure 1**). During the process of assay optimization we also noted that, in contrast to the manufacturer's protocol, in order to achieve reproducible and valid results from seminal plasma, the silica/nucleic acid mixtures needed to be resuspended at each extraction washing step to prevent clumping.

Cytokine quantification:

Blood and seminal plasma were analyzed for concentrations of IL-1α, IL-1Rα, IL1-β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 P70, IL-17, IFN-γ, MIP-1β, RANTES, and TGF-β using Luminex® assays as per manufacturer's protocol (Millipore). As done in previous studies,[19,20] raw fluorescence intensities were used as the final readout because many of the observed values were outside the range of the standard curve.

Statistical analyses:

To achieve symmetric distributions, blood and seminal plasma HIV-1 RNA and cytokine values were $\log_{10}$ transformed. Initial screening of all cytokines for correlations with seminal plasma viral levels was performed using linear regression models. The Pearson correlation was used to compare each cytokine level with seminal plasma virus level; additionally, the partial correlation of each cytokine, when viremia was included, was also calculated. If the partial p-value for a

given cytokine was <0.20, the cytokine was included as a candidate in the multivariate model. Forwards stepwise regression was used to construct the final model with semen cytokines and blood virus included as candidate covariates. JMP10.0 software (SAS Institute, Cary, NC) was used with minimum AICc (Akaike Information Criterion, corrected) used as a stopping rule.

To assess for model overfitting due to the relatively large number of variables for the small number of observations, we performed a modeling simulation to examine the likelihood that the high R^2 observed for our model was due to chance. Each iteration of the simulation randomly permuted the semen cytokines while maintaining linked blood and seminal plasma viral level for each subject, permitting an assessment of the probability of selection bias in this model. Once the seminal plasma cytokines were randomly permuted, the same procedure as above was followed for model construction.

A sub-analysis was performed on the group of men with blood/semen viral discordance, defined as seminal plasma HIV-1/blood HIV-1 ≥0.5, given that HIV-1 in semen is typically ~10% of that in blood.[17,21] Median levels of blood and seminal plasma cytokines were compared between "discordant" and "non-discordant" men. Seminal plasma cytokine levels from men with semen HIV-1 RNA >100 copies/ml versus <100 copies/ml were associated with their respective blood levels creating a seminal:blood plasma cytokine ratio, which was compared between groups using the Wilcoxon test. Significance was defined as $p \leq 0.05$.

Results

Characteristics of study subjects.

Of 31 men screened, 18 who self-reported not to be taking ART were enrolled. Thirteen were excluded for the following reasons: 11 with viremia <200 HIV-1 RNA copies/ml, one with active chlamydia infection and one not providing semen were excluded. The median age was 45 years (IQR 35.5, 50.5), with racial distribution of 14 African-American, 3 Caucasian, and 1 undeclared. The median blood $CD4^+$ T cell count was 478 cells/mm^3 (IQR 285, 603) and median blood plasma viremia was 4.4 $\log_{10}$ HIV-1 RNA copies/ml (IQR 2.9, 4.7). The median seminal plasma HIV-1 concentration was 2.97 $\log_{10}$ HIV-1 RNA copies/ml (IQR 2, 4.2) with 12/18 subjects (66.7%) having detectable seminal plasma HIV-1. The cytokine profile of seminal plasma is shown in Table 1.

Relationship between blood and seminal plasma HIV-1 concentration. Consistent with prior reports [2,7], comparison of viremia levels to seminal plasma HIV-1 levels showed a significant correlation, with $R^2 = 0.44$ (**Figure 1)**. While there was a linear relationship between $\log_{10}$ HIV-1 RNA levels in these two compartments, the concentration in blood was approximately 30-fold higher than in seminal plasma, at 4.42 $\log_{10}$ RNA copies/ml (IQR 2.98, 4.70) versus 2.96 $\log_{10}$ RNA copies/ml (IQR 2, 4.18) respectively.

Multiple regression modeling to evaluate associations between seminal plasma and blood plasma cytokines and HIV-1 levels.

Initial comparisons between seminal plasma levels of individual cytokines with seminal plasma HIV-1 levels demonstrated positive correlations only for IL-1α (R=0.56, p=0.016) and IL-1ra

(R=0.65, p=0.004). There were no statistically significant correlations between levels of specific cytokines in seminal plasma and the corresponding cytokine in blood (not shown). However, linear regression analysis including blood plasma HIV-1 level as a covariate (**Figure 3**) revealed that seminal plasma IFN-γ, IL-1α, IL-1RA, and IL-17 showed positive correlative trends ($p < 0.20$) to seminal plasma HIV-1, while IL-6, IL-8, MIP-1β, and IL-5 showed negative correlative trends ($p < 0.20$).

Blood plasma HIV-1 level, as well as seminal plasma IFN- γ, IL-5, and IL-17 were included in a multiple regression model of associations between seminal plasma cytokines and HIV-1 concentrations. Blood plasma HIV-1 concentration ($p < 0.0001$), as well as higher seminal plasma IFN-γ ($p=0.03$) and IL-17 ($p=0.03$) levels were positively associated with seminal plasma HIV-1 concentrations, while seminal plasma IL-5 was negatively associated ($p=0.0007$). This model demonstrated a strong fit between the covariates and seminal plasma HIV-1 (**Figure 4,** $R^2=0.83$).

Testing of the model for variable selection bias.

To differentiate true predictive value of the above associations versus selection bias ("overfitting"), simulation modeling with 10,000 iterations was performed. Of the 10,000 iterations, only 340 (3.4%) had a higher R^2 than the one observed for our final model (**Figure 5**, $R^2 = 0.826$), suggesting less than 3% likelihood that fitting of our model is due to chance or selection bias. Note that because the relationship between blood and seminal plasma viral level was maintained in this analysis, the lowest observed R^2 in this simulation was 0.438, which is the correlation between blood and seminal plasma viral level in our data set.

Analysis of men with disproportionately high seminal plasma HIV-1 levels compared to blood plasma HIV-1 levels.

Five of the 18 men (28%) had discordance of HIV-1 in seminal plasma versus blood, with the ratio of seminal plasma $\log_{10}$ HIV-1 RNA versus blood plasma $\log_{10}$ HIV-1 RNA $\geq$0.5. In the seminal plasma compartment, men with blood/semen HIV-1 discordance demonstrated a trend towards higher levels of IL-2 (p=0.06), while men without blood/semen HIV-1 discordance demonstrated higher levels of IL-5, IL-8, and IL-10 (p=0.02 for all), and a trend towards higher levels of MIP-1β (p=0.07) (**Table 2 and Table 4**). In the blood compartment, men with blood/semen HIV-1 discordance showed higher levels of blood plasma IL-12 (p=0.02) and IFN-γ (p=0.005), with trends towards higher levels of blood IL-1β, IL-2, and IL-17 (**Table 2 and Table 4**). Men without discordance showed a trend towards lower levels of blood RANTES (p=0.06).

The ratio of seminal plasma to blood plasma IL-10 was significantly lower in men with detectable HIV-1 in seminal plasma (p=0.03), compared to men without detectable seminal plasma HIV-1, and there was a trend towards an association with a higher ratio of seminal plasma to blood IL-1α (p=0.06) in these individuals. Several other cytokines demonstrated markedly different seminal to blood plasma ratios in persons with detectable seminal plasma HIV-1, but these differences did not reach statistical significance (**Table 3**).

Discussion

Our study adds to the growing evidence that pro-inflammatory cytokines in the seminal compartment are associated with higher semen HIV-1 levels. Compared to prior reported values for healthy HIV-1-uninfected men,[22] several pro-inflammatory cytokines appeared to be elevated, including IL-1α, IL-1β, IL-6, and RANTES, although IL-5, MIP-1 β, and TNF-α appeared to be near normal, and IL-17 levels were lower (**Table 1** and **Figure 2**). The immunosuppressive cytokine TGF-β appeared markedly depressed with HIV-1 infection. The identified seminal plasma cytokine profile is consistent with increased inflammation in the semen of men with untreated HIV-1-infection.

We found the concentration of virus in blood plasma was approximately 30-fold higher than in seminal plasma. In the context of reports of genetic compartmentalization of HIV-1 in semen,[5, 23] this suggests that local factors could influence HIV-1 levels in semen. Additionally, we observed altered seminal plasma to blood plasma ratios of IL-1α and IL-10 in subjects with detectable HIV-1 in seminal plasma (**Table 3**); these findings are consistent with previous work by Lisco et. al reporting increased compartmentalization of many pro-inflammatory cytokines in the setting of HIV-1 infection.[18]

In a group of chronically-HIV-1 infected men not on ART, we demonstrate significant associations between seminal plasma IFN-γ, IL-17 and IL-5 with seminal plasma HIV-1 concentration. In contrast to other studies, we did not see an association between seminal plasma RANTES or IL-1β and seminal plasma HIV-1 concentration, but we do replicate the previously reported association between seminal plasma IFN-γ and seminal plasma HIV-1 levels.[15-17] Our

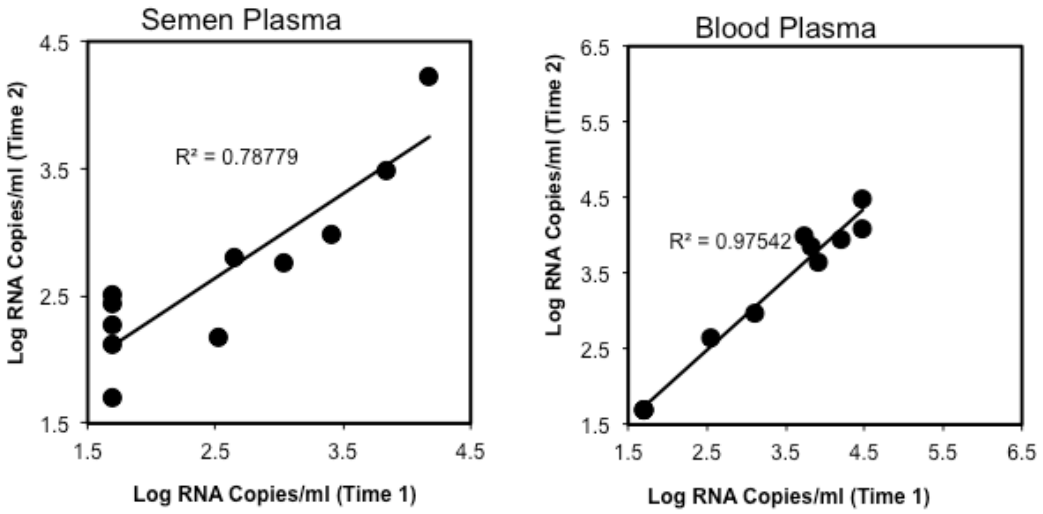
finding of a positive correlation between IL-17 and HIV-1 levels in seminal plasma directs further attention to the potential role of Th17 cells and/or NK cells in driving inflammation that increases HIV-1 levels.[24]

We have expanded upon previous studies examining the relationship between seminal plasma HIV-1 level and seminal cytokines by controlling for blood plasma HIV-1 level in our model. Blood HIV-1 level is known to be the strongest single predictor of semen HIV-1 concentration, with reported Pearson R^2 correlations in the range of 0.4-0.5.[2,7] This strong correlation may have obscured the effects of various cytokines on semen HIV-1 concentration in prior studies. However, when we performed analyses for associations between seminal plasma cytokines and HIV-1 levels *without* including blood plasma HIV-1 concentration as a model covariate, we still did not observe the previously-reported associations between semen HIV-1 and semen IL-1β or RANTES levels (data not shown). This may have been due to technical differences in laboratory technique; standardizing these techniques has been the focus of working groups such as the Semen Best Practices Working Group at the NIH). Moreover, we analyzed the Luminex® data using units of fluorescence intensity, rather than calculated concentrations from standard curves, which allowed analysis of low cytokine levels that were clearly detectable but below the lowest standard. This methodology has been used by some investigators in other fields,[19,20] but most prior HIV-1 studies have used calculated concentrations.

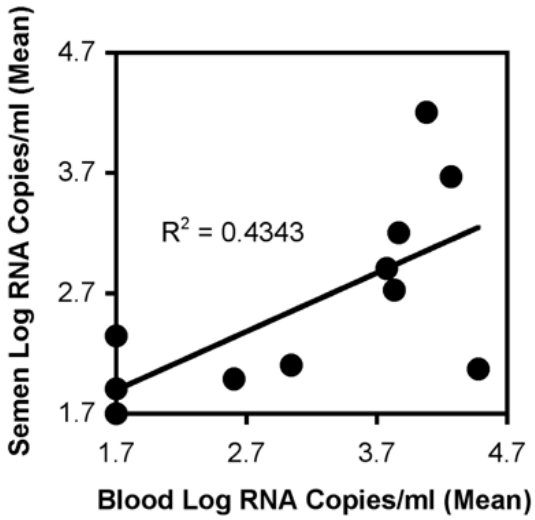
Our study has several limitations. Our sample size was small due to the exclusion of 11 subjects (35% of the sample) with viremia <200 copies/ml. Also, we had limited information on other factors that can influence genital tract inflammation, such as CMV serostatus.[14,18] Additionally,

our measurements of semen HIV-1 only assessed seminal plasma, and thus would not quantify cell-associated virus. Our model is exploratory, and did not control for multiple testing. Finally, the performance of the model may be overly optimistic due to variable selection bias (large number of candidate cytokines compared to the number of samples).

In conclusion, this study adds to the growing knowledge about determinants of semen HIV-1 levels. Although semen HIV-1 and thus the risk of sexual transmission of HIV-1 is greatly decreased by ART, a substantial subset of patients continue to have intermittently elevated semen HIV-1 and remain at risk for transmitting HIV-1 to their sexual partners. More comprehensive studies will be required to explore the mechanisms behind genital tract inflammation that drive HIV-1 production in semen.



A.



B.

Figure 1. Reproducibility of serial HIV-1 RNA measurements in blood and semen plasma and relationship of blood and semen plasma HIV-1 RNA concentrations. A. Concentrations of HIV-1 RNA from serial measurements are plotted. Undetectable values (<1.7 $\log_{10}$ RNA copies/ml) were assigned a value of 1.7 $\log_{10}$ RNA copies/ml.B. The mean values for blood and semen plasma HIV-1 RNA concentrations ($\log_{10}$ RNA copies/ml) across two visits for each person are plotted. The correlation between blood and semen HIV-1 RNA levels was significant (p = 0.027).

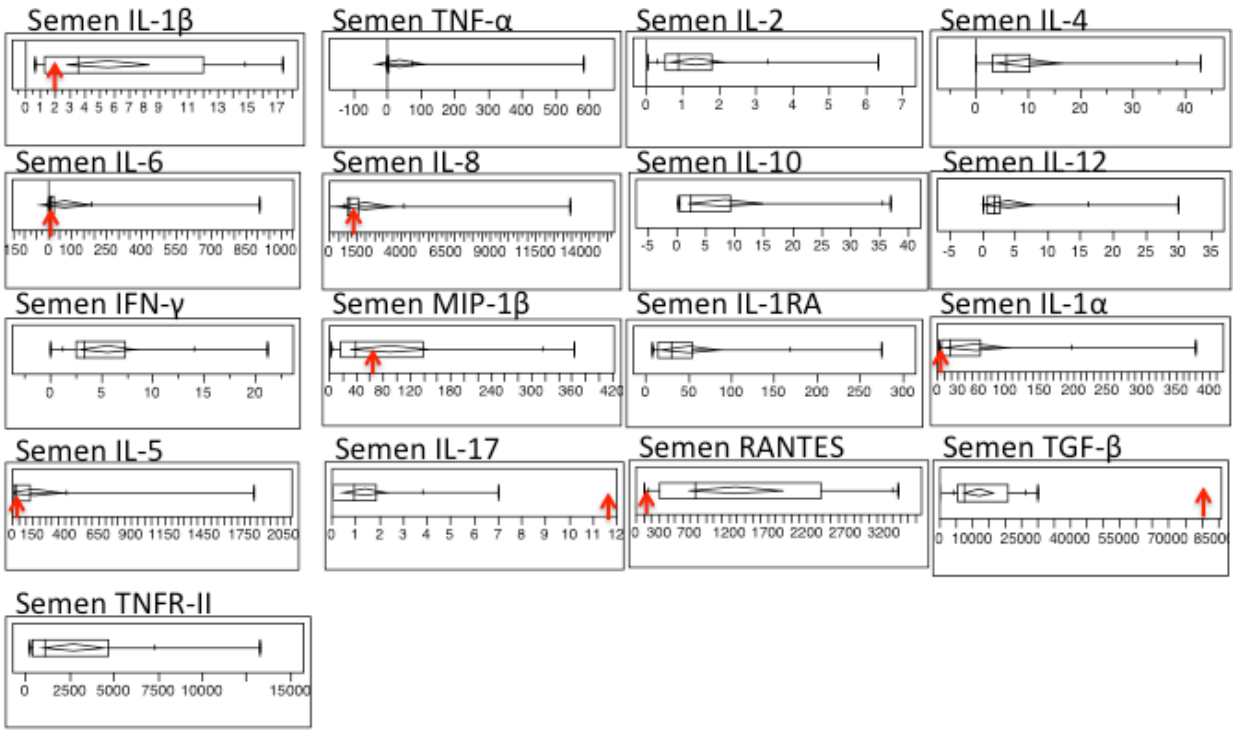


Figure 2. Boxplots of median semen cytokine concentrations in pg/ml for the HIV-1-infected study subjects. Previously published median values for healthy volunteers, where available, are indicated by the red arrow (values taken from Politch et al., 2007).

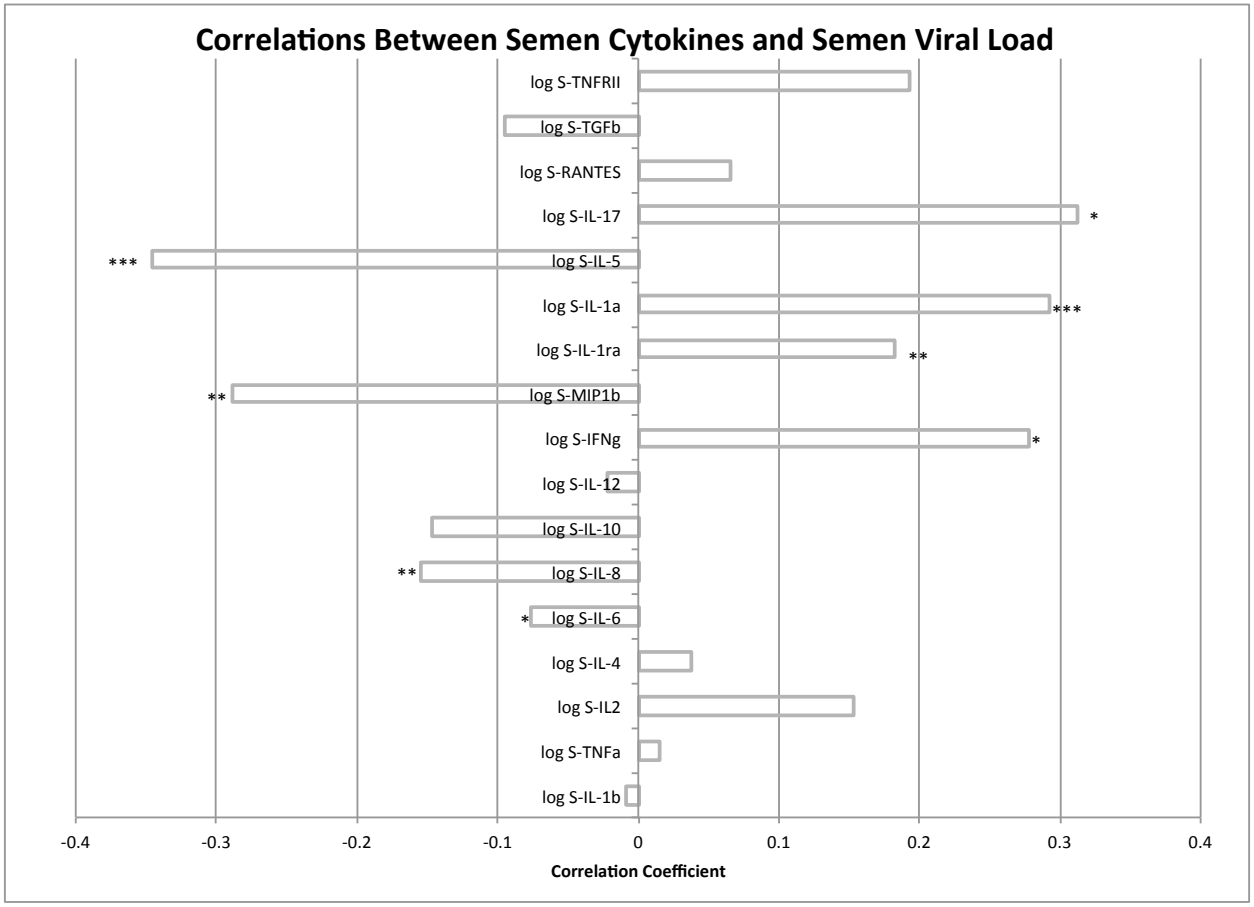


Figure 3: Graph of the relationship between seminal plasma cytokines and seminal HIV-1 concentrations in the simplified linear model (log semen VL=log blood VL + log semen cytokine). Negative numbers indicate an inverse correlation; positive numbers indicate a positive correlation.
*indicates p-value ≤0.05
**indicates p-value ≤0.10
***indicates p-value ≤0.20

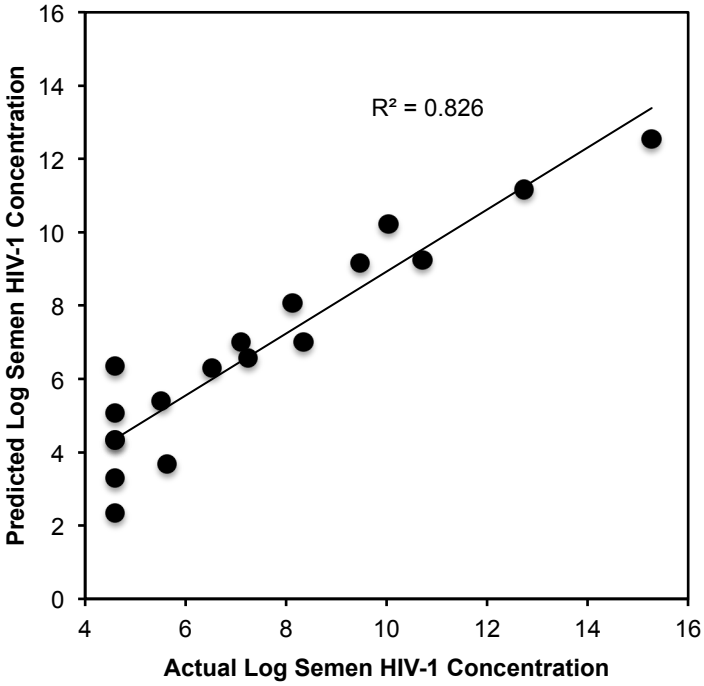


Figure 4: **Correlation of actual versus predicted HIV semen viral load.** Actual log semen HIV-1 concentration plotted against log semen HIV-1 concentration predicted by the multivariate regression model, showed high correlation ($R^2 = 0.826$).

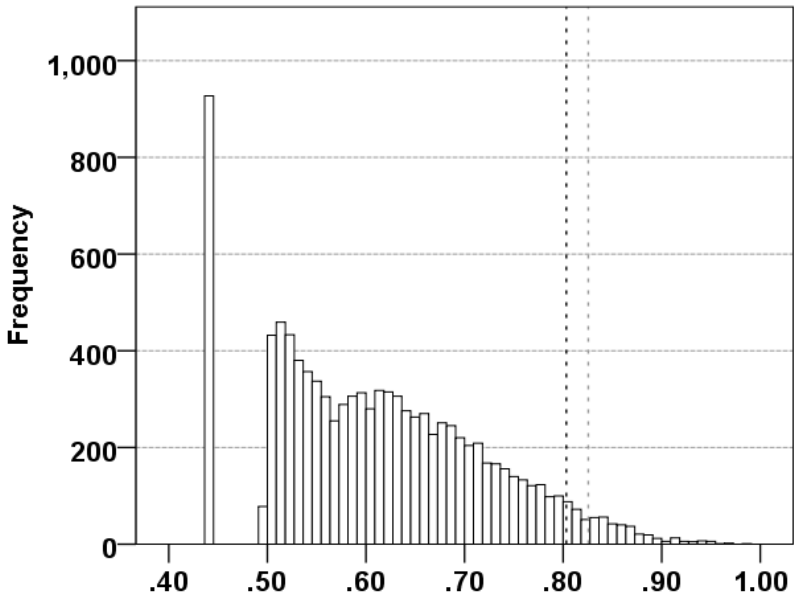


Figure 5. Simulation to examine for variable selection bias: A simulation was conducted with log semen HIV-1 as the response variable and log blood HIV-1 plus a subset of 17 randomly permuted semen cytokines with partial p-values <0.20 included as possible predictors for a potential multivariate model. For the variables meeting the p<0.20 threshold, stepwise reduced models (using lowest AICc criteria) were constructed. Of the 10,000 simulations performed, the mean R2 was 0.604, with the 95th percentile of 0.805 (indicated by black dotted line). The R2 for the actual model was 0.826, placing the model's performance at about the 97th percentile of the distribution (indicated by grey dotted line).

Semen Cytokine	Median Concentration (pg/ml)	IQR	***Published:*** **Median Concentration and IQR-HIV-negative men** ***(Politch JA 2007; pg/ml)***	Median Flourescence Intensity (FI)	Percent out-of-range*
IL-1RA	29.1	14.06-53.81	Not assessed	105.5	0
IL-1α	18.3	6.47-62.62	6.0	720.3	0
IL-1β	3.54	1.35-11.99	2.0	103.3	0
IL-2	0.87	0.54-1.81	***Not detected*** (Lower limit of detection, 7.0 pg/ml)	54	0
IL-4	5.69	3.05-10.33	*Not assessed*	161	11%
IL-5	33.63	8.34-128.50	31.3	1256.3	0
IL-6	11.30	6.95-26.23	6.0	328	0
IL-8	1120.78	970.75-1644.49	1305	25,159	5.5%
IL-10	2.17	0.40-9.17	***Not detected*** (Lower limit of detection, 3.9 pg/ml)	75	0
IL-12 P70	1.71	0.66-2.48	***Not detected*** (lower limit of detection, 5.0 pg/ml)	58.5	11%
IL-17	0.94	0-1.84	11.6	202.5	38.9%
IFN-γ	3.26	2.52-7.23	***Not detected*** (lower limit of detection, 3.0 pg/ml)	89	5.5%
MIP-1β	37.76	15.46-138.21	66.0	690	0
TNF-α	1.57	0.9-4.49	***Not detected*** (lower limit of detection, 1.95 pg/ml)	136.8	0
RANTES	758.95	300.05-2385.92	126.0	4964.3	0
TGF-β	7577.57	5311.66-20919.80	85,120.0	25278.5	38.9%
TNF-RII	1075.03	374.25-4709.51	*Not assessed*.	5616.3	0

Table 1: Median concentrations and IQR of semen cytokines *(compared to published values)*

*Out of range specimens had an FI below the lowest value on the standard curve for all cytokines except TGF-β, where 6/18 semen specimens had an FI above the highest value on the standard curve, and 1/18 had an FI below the lowest value on the standard curve.

Cytokine	Median Concentration in Men with Blood/Semen HIV-1 RNA Discordance (pg/ml) (IQR)	Median Concentration in Men with Blood/Semen HIV-1 RNA Non-Discordance (pg/ml) (IQR)	p-value (Wilcoxon test)
Blood IL-1β	0.80 (0, 1.97)	0 (0, 0.51)	0.09
Blood TNF-α	4.44 (2.49, 10.96)	5.46 (4.28, 6.55)	0.59
Blood IL-2	0.01 (0, 11.07)	0 (0, 0)	0.09
Blood IL-4	2.25 (1.09, 9.78)	0.69 (0, 2.58)	0.14
Blood IL-6	0.42 (0.21, 0.87)	0.33 (0.13, 0.56)	0.43
Blood IL-8	1.33 (0.87, 2.83)	2.09 (0.84, 3.66)	0.35
Blood IL-10	5.63 (3.66, 132.93)	4.99 (3.08, 7.38)	0.73
Blood IL-12 P70	0.99 (0.27, 100.81)	0 (0, 0.43)	0.02
Blood IFN-γ	1.68 (1.15, 78.04)	0 (0, 0.7)	0.005
Blood MIP-1β	2.38 (0, 3.56)	0 (0, 4.64)	0.66
Blood IL-1ra	14.54 (7.73, 87.36)	10.51 (9.2, 14.54)	0.28
Blood IL-1α	0 (0, 11.10)	0 (0, 0)	0.81
Blood IL-5	0.37 (0.17, 1.0)	0.19 (0.14, 0.30)	0.17
Blood IL-17	0 (0, 2.07)	0 (0, 0)	0.09
Blood RANTES	126,031 (57,026, 178,632)	103284 (52428, 193511)	0.07
Blood TGF-β	3,727 (3112, 6827)	5915.79 (4600, 5915)	0.18
Blood TNF-RII	6397.17 (3300, 10949)	6676.01 (4834, 13933)	0.59
Semen IL-1β	1.52 (0.75, 9.03)	3.73 (1.57, 12.30)	0.26
Semen TNF-α	0.94 (0.69, 2.77)	1.79 (0.99, 7.44)	0.13
Semen IL-2	0.32 (0.18, 3.44)	1.16 (0.82, 1.83)	0.07
Semen IL-4	3.54 (2.88, 11.89)	6.26 (2.98, 12.29)	0.73
Semen IL-6	8.25 (6.41, 10.04)	18.66 (7.02, 68.01)	0.13
Semen IL-8	1013 (953, 1069)	1294 (1013, 2707)	0.03
Semen IL-10	0.44 (0.17, 1.34)	3.72 (1.44, 23.57)	0.03
Semen IL-12 P70	0.86 (0.30, 3.45)	1.75 (0.95, 3.03)	0.46
Semen IFN-γ	2.56 (2.08, 7.88)	3.26 (2.63, 9.0)	0.52
Semen MIP-1β	20.16 (13.10, 30.95)	58.04 (17.51, 233.36)	0.07
Semen IL-1ra	30.36 (12.83, 97.54)	27.84 (16.20, 60.15)	0.96
Semen IL-1α	58.39 (6.41, 239.45)	15.73 (6.04, 46.07)	0.35
Semen IL-5	3.26 (1.99, 28.31)	79.58 (13.20, 209.69)	0.02
Semen IL-17	1.47 (0, 4.33)	0.85 (0, 1.9)	0.96
Semen RANTES	589 (149, 2154)	794 (370, 2646)	0.59
Semen TGF-β	7494 (6165, 19406)	7661 (4971, 21914)	0.59
Semen TNF-RII	554 (183, 9965)	1316 (592, 3977)	0.88

Table 2: Median blood and semen cytokine concentrations for men with blood/semen HIV-1 discordance (n=5) (defined as semen HIV RNA/blood HIV RNA >0.5) vs. non-discordance (n=13). The Wilcoxon test was used to determine the p-value for the difference in median concentration between men with discordance and men without discordance.

Cytokine	Overall Median Semen/Blood Cytokine Ratio (n=18)	Men with Detectable Semen HIV-1--Median Semen/Blood Cytokine ratio	Men with Undetectable Semen HIV-1--Median Semen/Blood Cytokine ratio
IL-1β	1.44	1.49(0.97, 3.92)	1.35 (1.14, 6.73)
TNF-α	0.40	0.34 (0.22, 0.69)	0.73 (0.26, 1.27)
IL-2	2.30	2.62 (1.11, 3.72)	2.22(1.43, 2.37)
IL-4	1.35	1.15 (1.01, 2.10)	1.37 (1.21, 2.16)
IL-6	3.30	3.22 (2.17, 11.24)	4.29 (2.96, 89.19)
IL-8	90.48	88.59 (66.80, 125.60)	90.48 (61.37, 98.19)
IL-10**	0.68	0.55 (0.40, 1.11)	1.25 (0.71, 7.03)
IL-12	1.13	1.09 (0.82, 1.62)	1.25 (1.09, 1.77)
IFN-γ	1.80	1.72 (1.02, 2.80)	2.61 (1.59, 4.07)
MIP-1β	2.08	1.58 (1.30, 5.95)	2.34 (1.63, 7.60)
IL-1ra	1.82	2.16 (1.13, 5.02)	1.49 (0.73, 2.15)
IL-1α *	3.73	6.24 (3.20, 18.54)	2.86 (1.93, 5.28)
IL-5	21.48	20.26 (3.82, 98.15)	59.97 (12.60, 262.85)
IL-17	1.56	1.39 (0.96, 2.31)	1.93 (1.14, 2.29)
RANTES	0.64	0.86 (0.07, 0.95)	0.39 (0.10, 0.85)
TGF-β	64.89	66.43 (40.62, 187.75)	43.67 (1.92, 188.48)
TNFR2	0.37	0.37 (0.10, 0.96)	0.36 (0.13, 0.84)

* indicates $p < 0.10$ and ** indicates $p < 0.05$ by Wilcoxon test for comparison of cytokine level in men with detectable versus undetectable semen HIV-1 level.

Table 3. Seminal plasma to blood plasma cytokine ratios, men with detectable seminal plasma HIV-1 vs. undetectable seminal plasma HIV-1.

Blood

Higher in Discordant Men	Lower in Discordant Men
IL-12**	RANTES*
IFN-γ**	
IL-1β*	
IL-2*	
IL-17*	

Semen

Higher in Discordant Men	Lower in Discordant Men
IL-2*	IL-5**
	IL-8**
	IL-10**
	MIP-1β*

Table 4. Cytokines in blood and seminal plasma of men with blood/semen HIV-1 discordance vs. non-discordance.*indicates $p \leq 0.10$; ** indicates $p \leq 0.05$.

Statistical Addendum

Simple Correlations Between Semen Cytokines and Semen Viral Level

Simple correlations (the Pearson R) were calculated for the relationship between each $\log_{10}$ semen cytokine and $\log_{10}$ semen HIV-1 concentration, without any adjustment for blood HIV-1 concentration (see **Table 5**). Increased levels of semen IL-1α and semen IL-1RA were associated with increased semen HIV-1 concentration ($p \leq 0.05$).

Multivariate Regression Model

The multivariate regression model was constructed using a forward stepwise procedure as described previously. In the final model, in addition to blood HIV-1 concentration, increased levels of semen IFN-γ and semen IL-17 were both associated with increased semen HIV-1 concentration ($p=0.03$ and $p<0.001$, respectively). Increased levels of semen IL-5 were associated with decreased semen HIV-1 concentration ($p<0.001$). The effect size of each log-transformed variable in the final model is shown in **Table 6.**

Assessment for Multicollinearity

The variables included in the final mulitivariate model were assessed for multicollinearity. Correlations between each of the predictor variables included in the final model are shown in **Table 7**. None of the predictor variables included in the final model were highly correlated with each other; the highest spearman ρ value was 0.38. This suggests that multicollinearity is not a serious problem in our model.

Leave-One-Out Crossvalidation

A leave-one-out crossvalidation was performed to examine the model for overfitting. When the predicted values for log semen VL using the leave-one-out crossvalidation were plotted against actual values for log semen VL, the standard deviation of the residuals (RMSE) was 1.77, compared to a standard deviation of residuals (RMSE) of 1.50 for the actual multivariate model (data not shown). The fact that the RMSE for the crossvalidation is only 18% greater than for the model suggests that the model is reasonable and that overfitting is not a serious problem.

Semen Cytokine	Correlation with Semen HIV-1 Concentration (R)	P-value
IL-1β	0.017	0.95
TNF-α	-0.063	0.79
IL-2	0.385	0.12
IL-4	0.214	0.40
IL-6	-0.122	0.62
IL-8	-0.197	0.43
IL-10	-0.283	0.26
IL-12 P70	0.032	0.90
IFN-γ	0.329	0.18
MIP-1β	-0.288	0.26
IL-1ra	0.645	0.004*
IL-1α	0.557	0.016*
IL-5	-0.217	0.39
IL-17	0.219	0.38
RANTES	0.126	0.61
TGF-β	0.228	0.36
TNFRII	0.315	0.20

Table 5: Simple correlations (Pearson R) between each log transformed semen cytokine and log-transformed semen viral levels.

Variable	Coefficient (95% CI)	P-value
Log blood HIV-1 RNA	1.174 (0.806, 1.542)	0.00005
Log semen IFN-γ	1.656 (0.198, 3.114)	0.03
Log semen IL-5	-1.067 (-1.572, -0.562)	0.0007
Log semen IL-17	3.569 (0.421, 6.717)	0.03

Table 6: Multivariable Linear Regression Model: Forwards stepwise regression was performed using all semen cytokines with $p<0.20$ in the above simplified linear regression model, as well as blood viral load as candidate predictors. Log semen viral load was the outcome variable. Log blood viral load, log semen IFN-gamma, and log semen IL-17 were all strongly associated with increased log semen viral load in the final parsimonious model; log semen IL-5 was strongly associated with decreased log semen viral load.

Variable	Variable	Spearman ρ	p-value
Log blood VL	Log S-IL-5	0.35	0.15
Log blood VL	Log S-IL-17	-0.23	0.36
Log blood VL	Log S-IFN-γ	0.20	0.43
Log S-IL-5	Log S-IL-17	0.16	0.54
Log S-IL-5	Log S-IFN-γ	0.38	0.12
Log S-IFN-γ	Log S-IL-17	0.29	0.25

Table 7: Correlations between the variables used in the multivariate regression model.

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