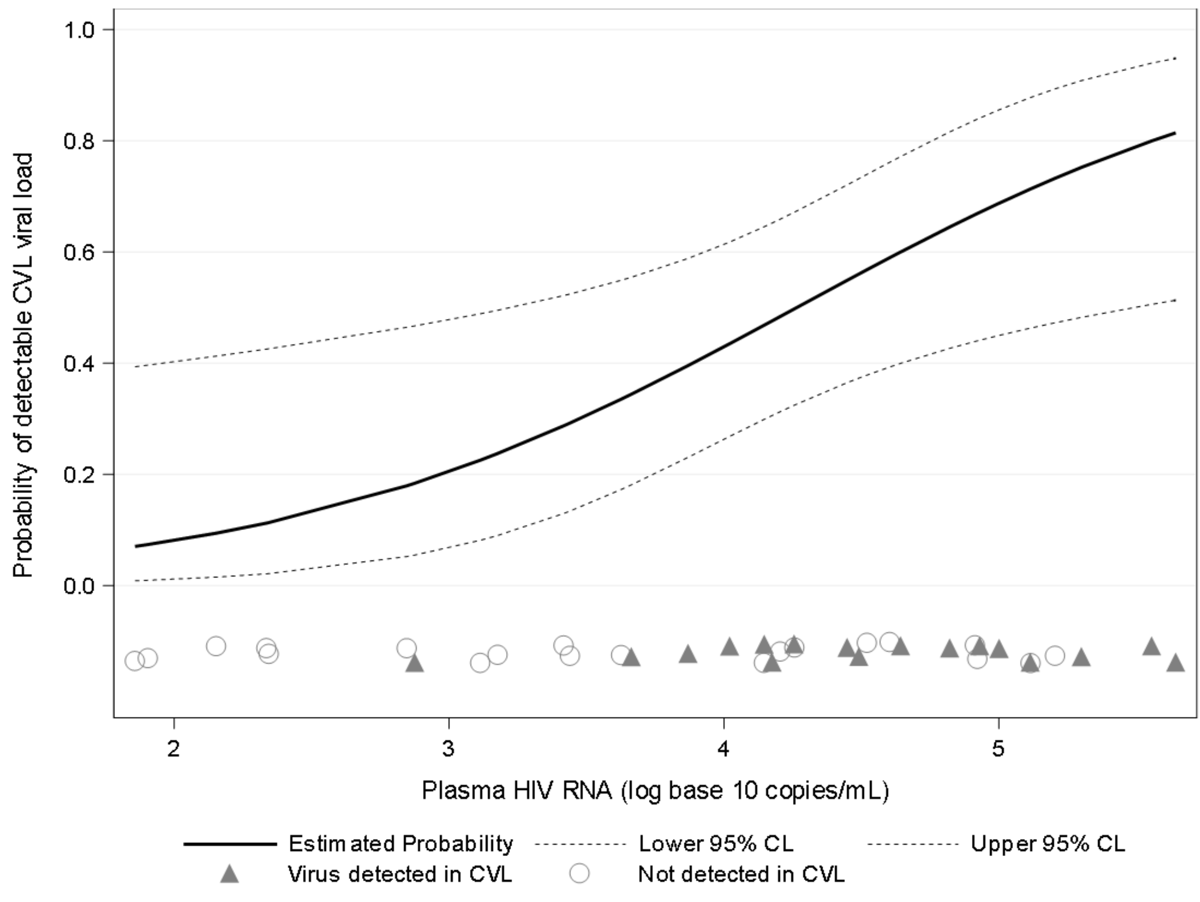
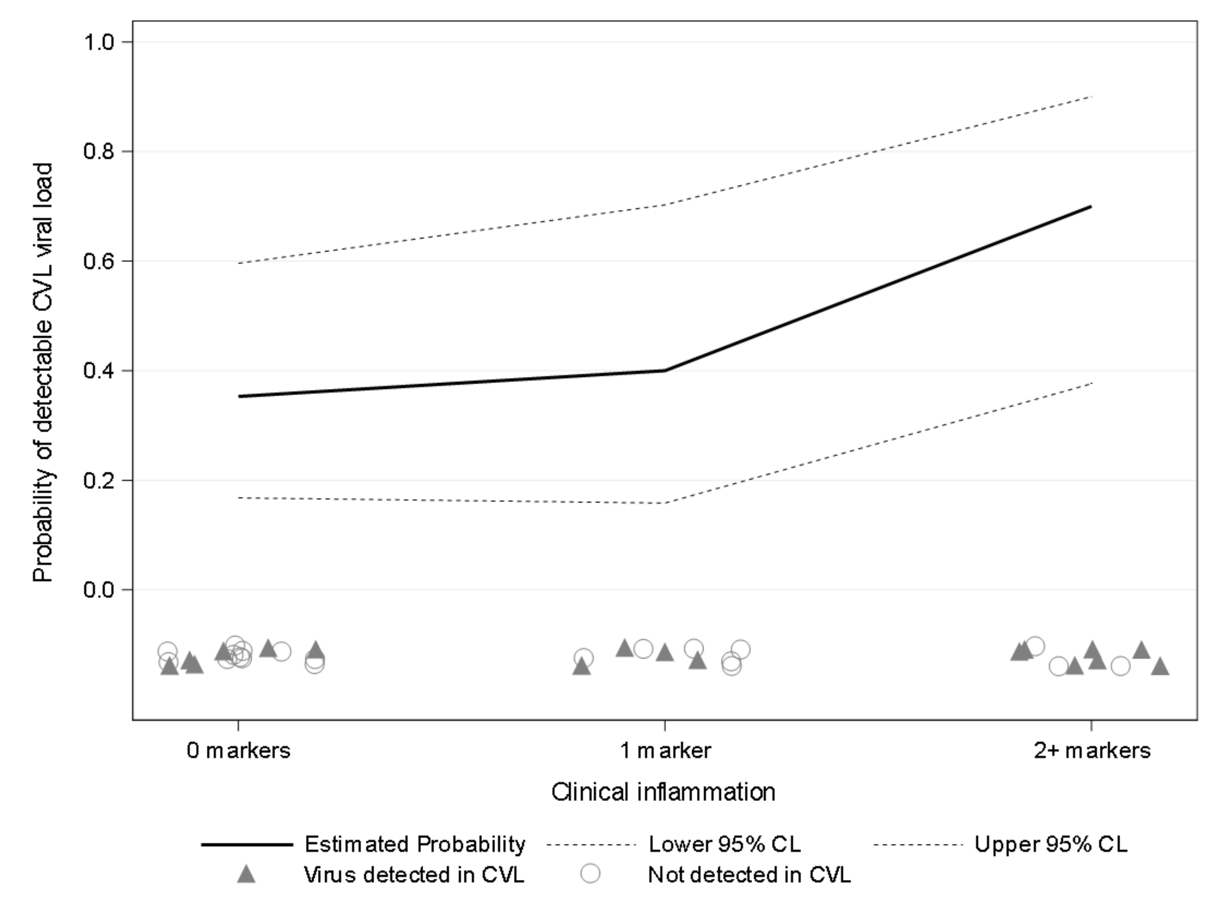
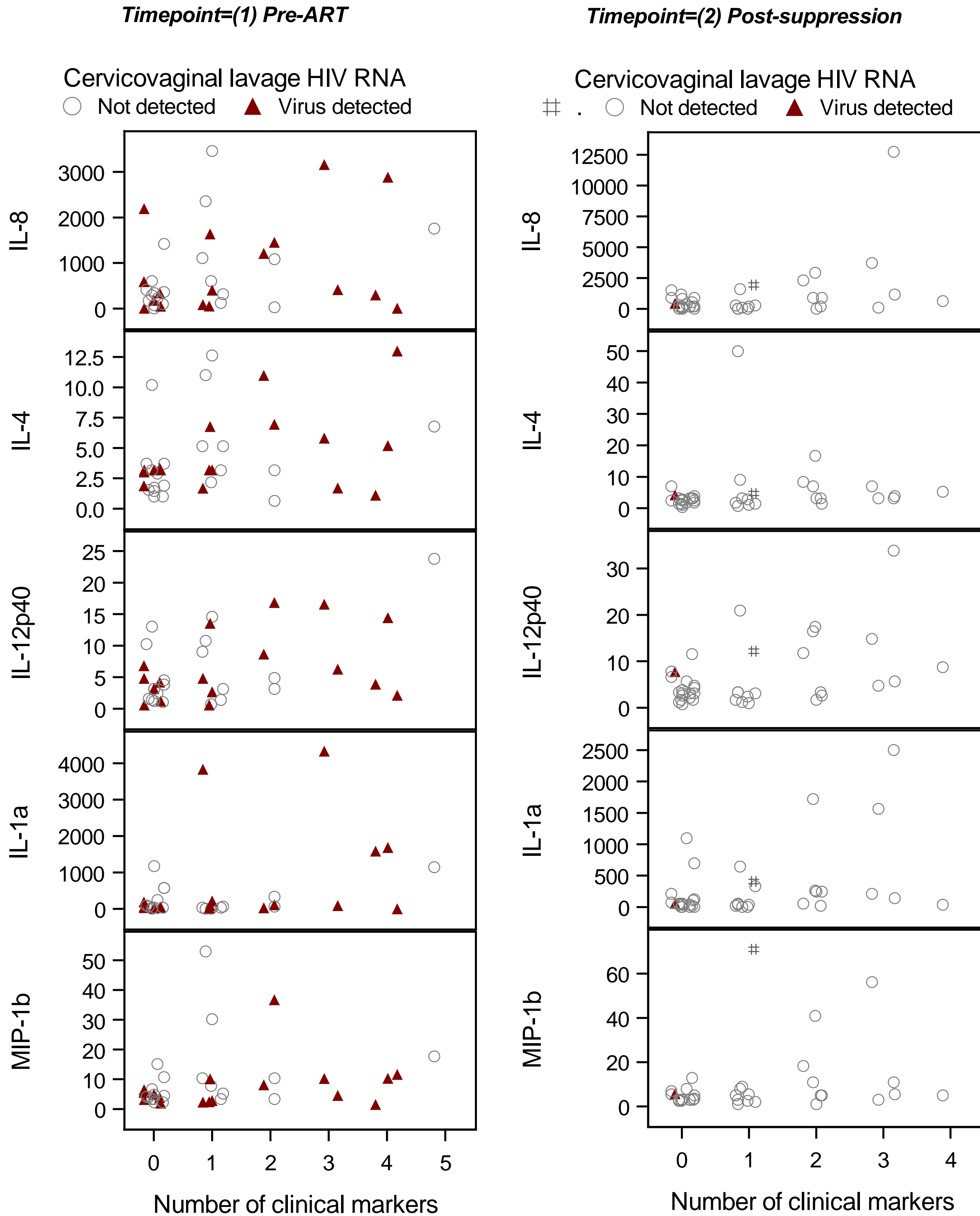
**Supplemental Figure S1. Estimated probability of detectable viral load in cervicovaginal lavage before ART initiation among WIHS participants: relationship to plasma viral load.** Model-based predicted probability of detectable viral load in CVL (solid line) and a 95% CI (dashed lines) are displayed. Plasma viral load ($\log_{10}$ cp/mL) on the x-axis was fit as linear in the logit. The observed data distribution of plasma viral load is plotted at the bottom with no associated y values.



**Supplemental Figure S2. Probability of detecting HIV-1 in cervicovaginal lavage (CVL) among HIV-1 seropositive WIHS participants with 0, 1, or multiple clinical inflammation markers.** Model-based predicted probability of detectable viral load in CVL (solid line) and a 95% CI (dashed lines) are displayed. Plasma viral load ($\log_{10}$ cp/mL) on the x-axis is shown clustered to indicate the number of observations in each group.



**Supplemental Figure S3. Number of clinical inflammation markers vs. 9 biomarker levels in 38 WIHS participants at pre-ART and post-suppression visits.** Clinical inflammation markers included vaginal pH>5.5, visible cervical lesions, cervical ectopy, cervical friability, cervical exudate, trichomoniasis, and inflammation noted on Pap smear. Each biomarker scale is in pg/mL.



***Timepoint=(1) Pre-ART***

Cervicovaginal lavage HIV RNA
○ Not detected ▲ Virus detected

GM-CSF
6
5
4
3
2
1
0

IL-10
8
6
4
2
0

RANTES
1500
1000
500
0

CD163
125000
100000
75000
50000
25000
0

0 1 2 3 4 5

Number of clinical markers

***Timepoint=(2) Post-suppression***

Cervicovaginal lavage HIV RNA
# . ○ Not detected ▲ Virus detected

GM-CSF
5
4
3
2
1
0

IL-10
40
30
20
10
0

RANTES
400
300
200
100
0

CD163
12500
10000
7500
5000
2500

0 1 2 3 4

Number of clinical markers

**Supplemental Table S1. Viral load testing of unfractionated cervicovaginal lavage (CVL) spiked with HIV-1 using different pre-treatments of the CVL samples.**

| | | CVL VL | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Nominal spiked value** | **PBS VL** | **Pre-treatment*** | **Donor 1** | **Donor 2** | **Donor 3** | **Donor 4** | **Donor 5** | **Donor 6** |
| 500 | 653 | None | 124 | 231 | 326 | 151 | | |
| 500 | 355 | Pellet cells/debris | 85 | 225 | 240 | 314 | | |
| 100 | 70 | Lysis buffer + Prot K | ND[a] | 240 | ND | 178 | | |
| 100 | 198 | Lysis buffer + Prot K | ND | 117 | ND | 249 | | |
| 250 | 302 | Lysis buffer + Prot K | 298 | 254 | 272 | 307 | | |
| 100 | 198 | Dilute with plasma | ND | | ND | | | |
| 100 | 198[b] | Add different virus | | | ND | | | |
| 100 | 198[b] | Lysis/Prot K + 95° | ND | | | | | |
| 100 | 198[b] | Lysis buffer + 2X PK | | | ND | | | |
| 100 | 138 | PBS + Prot K | 226 | | 131 | | | |
| 100 | 185 | PBS + Prot K | ND | 229 | ND | 311 | 111 | 77 |
| 100 | 204 | PBS + Prot K | 186 | | 150 | | | |
| 100 | 166 | Prot K | | | 160 | | | |
| 100 | 189 | Prot K | 174 | 188 | 214 | 144 | 283 | 261 |
| 100 | 149 | Prot K | | | 171 | 256 | 209 | 240 |
| 50 | 53 | Prot K | | | ND | 103 | 83 | 76 |
| 25 | 42 | Prot K | | | ND | ND | ND | 50 |
| 12.5 | ND | Prot K | | | ND | <42 det[c] | ND | 45 |

*See Methods for details on pre-treatments.
[a]ND = Not detected.
[b]Same comparator PBS for each of these pre-treatments.
[c]Detected but under the lower limit of quantitation.

**Supplemental Materials**

Optimization of HIV-1 RNA quantitation in CVL: Assay optimization and validation were performed using unfractionated CVL collected from HIV-negative donors using a protocol and informed consent approved by the UNC-CH Institutional Review Board. Frozen aliquots of the CVL were thawed, spiked with a known amount of HIV-1, and then treated with a specific pre-treatment (detailed below) before testing with the Abbott RealTime HIV-1 assay. A 0.6 mL aliquot of phosphate-buffered saline (PBS) was always spiked with the same amount of HIV-1 at the same time the CVL was spiked as a control and was tested with the CVL, albeit without any pre-treatment. Briefly, the different pre-treatment procedures were: (i) CVL was pelleted after thawing (1000g for 5 min); (ii) CVL was treated as described for breastmilk [1] with the addition of RNA lysis buffer (Promega) and 60 ul proteinase K (Abbott), followed by incubation at 53°C for 20 min; (iii) CVL was diluted 1:2 with normal plasma; (iv) addition of 600 cp of the RCAS virus [2] to increase the amount of (non-HIV) viral RNA as a carrier; (v) treatment of CVL with lysis buffer and proteinase K as above plus 5 min of heating at 95°C to inactivate the proteinase K; (vi) treatment of CVL with lysis buffer as above with double the proteinase K amount; (vii) treatment of CVL with PBS and proteinase K; (viii) or addition of only proteinase K to the CVL. This last pre-treatment procedure with only proteinase K was used for the rest of the testing as follows: 0.06 mL proteinase K (Abbott) was added to 0.74 mL CVL and manually mixed by pipetting, incubated at 53°C for 20 min, vortexed briefly, and spun at 3200g for 5 min. The mixture was run on the Abbott m2000sp with the 0.6 mL plasma program. The small amount of dilution resulted in LLQ of 42 cp/mL.

**Validation of HIV-1 quantification in unfractionated CVL.** To ensure that the VL testing could be done successfully on unfractionated CVL from WIHS participants, unfractionated CVL from multiple uninfected donors was spiked with known levels of HIV-1 and tested on both a laboratory developed Droplet Digital PCR (ddPCR) HIV-1 assay and the Abbott RealTime HIV-1 assay. Both assays yielded less viral amplification in CVL than in plasma spiked with the same quantity of HIV-1 (data not shown). We tested the performance of the Abbott assay using pre-treatment procedures, which have been previously used successfully with other specimen types such as breastmilk and semen, to compare recovery of spiked HIV-1 in CVL to recovery of HIV-1 in PBS (all samples spiked with the same amount of HIV-1 at the same time and tested in parallel). As detailed in Supplemental Table S1, CVL was collected from 6 donors and spiked with HIV-1 at different levels from 100–500 cp/mL. CVL VL was always compared to the PBS VL obtained in the same run. HIV-1 recovery on the Abbott assay was poor under conditions of no pre-treatment and pelleting of the CVL cells and debris. Pre-treatment with Abbott Proteinase K and RNA lysis buffer with heating, a method previously successfully applied to breastmilk specimens [34] resulted in better, but inconsistent, recovery among donors. Other pre-treatments with poor and variable results included diluting with plasma, spiking with 6-fold more of a different retrovirus (RCAS) to increase the amount of viral RNA as carrier, heating to 95°C after proteinase K treatment, and doubling the amount of proteinase K. After replacing the lysis buffer with PBS during the proteinase K treatment we obtained consistent results with the 5 donors tested. We validated pre-treatment with and without PBS using a sample that previously gave inconsistent results (donor 1), and a set of samples spiked with 12.5 to 100 cp/mL HIV-1

(Supplemental Table S1). This method of proteinase K treatment without any other additions was applied to all of the WIHS CVL samples tested in this study.

References

1. Farr SL, Nelson JAE, Ng'ombe TJ, Kourtis AP, Chasela C, Johnson JA, *et al.* **Addition of 7 days of zidovudine plus lamivudine to peripartum single-dose nevirapine effectively reduces nevirapine resistance postpartum in HIV-infected mothers in Malawi.** *J Acquir Immune Defic Syndr* 2010; **54**:515–523.
2. Nasioulas G, Hughes SH, Felber BK, Whitcomb JM. **Production of avian leukosis virus particles in mammalian cells can be mediated by the interaction of the human immunodeficiency virus protein Rev.** *Proc Natl Acad Sci U S A* 1995; **92**:11940–4.