nature portfolio

Peer Review File

Longitudinal Plasma Proteomic Signatures of Elite and Viremic Spontaneous HIV Controllers

Corresponding Author: Dr Nadira Vadaq

This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Nature Communications.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The authors have provided thorough and thoughtful rebuttals to all the points raised previously. I do not have any further concerns at this point.

Reviewer #3

(Remarks to the Author)
Vadaq and colleagues explore in this study the plasma levels of more than 2000 proteins in a large cohort of PLHIV with different levels of control of viral replication: elite controllers (EC, <50copies/mL) and viremic controllers (VC, <10000copies/mL). There are two clear strengths in the study: 1)the number of individuals (n=183 individuals for the discovery cohort, n=19 individuals for the validation cohort) and 2)the longitudinal analysis, up to 17 years, that allowed the authors to identify participants that lost their control of the viral replication (transient controllers). Three proteins were increased in VC (CRTAM, LY9, CD6) and one in EC (VAT1). With retrospective analysis, they checked whether differences in the levels of plasma protein could predict loss of viral control or CD4 decay years after the first sampling (in the so-named transient controllers). They developed an accurate model for prediction of the loss of control.
Overall, the study is descriptive and lacks depth in the mechanisms underlying the control of HIV replication. It is ambitious and involves high dimensional data, but some concerns regarding the methodology and interpretation should be addressed to strengthen the conclusions of the study.
Comment 1. There are significant age differences between the groups. Age has been compared for the first approach (EC vs VC). However, in the second step of the analysis (EC, permanent VC and transient VC) this difference should be addressed as well. Even though age is included in the analysis as a covariate, it may not fully be controlled and should be taken carefully for the interpretations as residual confounding effect could remain. At the time of the second sampling, EC are ~60 y.o. while transient VC are ~50 y.o. and this difference might be relevant in terms of inflammation and aging.
Comment 2. There is also a difference between pre and post ART and the duration of ART before the second sample is different between EC (~3 years) and VC (>5years). Are all the individuals virologically suppressed at the time of the post ART sampling?
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Comment 5. It seems that the pathway analysis was only performed in persistent VC since few proteins were significantly downregulated post ART in transient VC and none in the EC. However, it might be relevant to understand why the pathway analysis has been restricted to persistent VC. Moreover, in the manuscript it is indicated that this analysis was performed in 47/57 VC. Is there any specific reason to use a subset of the persistent VC group?
Comment 6. Figure 3 is the most relevant of the study and it might help to identify potential biomarkers for loss of viral

control. In figure 3C and 3D it is not indicated whether EC and VC were included or only one of the groups (as indicated in figure 3E and 3F). Proper labeling might help to interpretate the figure.
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Comment 8. The relevance of the sequencing of HIV RNA in four samples of VC is not clear. Sequences are from “chronic phase”, without a clear identification of this timing. The study opens the possibility of viral evolution as a driver of control loss. However, several aspects make this statement too speculative. First, diversity has been only addressed at one timepoint in a small subsets of VC. It would have been interesting to compare viral diversity with the reservoir landscape of EC, transient VC and normal progressors. They acknowledge they cannot confirm whether this plasma represents replication-competent virus (line 843), which weakens even more possible conclusions from the virological aspect of the study. These findings do not seem solid enough and they do not complement the rest of the analysis.
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Minor comments:
• The study design is complex. Indicating the sample size in each analysis might help to follow the study.
• Reference 7 is contextually unrelated (it is an ATI study), reference 8 does not demonstrate any genetic aspect of the loss of control (as mentioned in the introduction), reference 20 seems irrelevant.
• Mislabeling: line 455 (should be Figure 1E), line 477 (should be Figure 1D).

Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
The authors have addressed all my comments very well. the reasoning is sound and the added analyses improve the manuscript. Manuscript has improved substantially following revision.
Comments 1–3. The additional analysis shows that the reported differences are not age-dependent, which is helpful. The authors should consider adding the relevant panels to the supplementary material, as age is in general a very relevant factor for plasma proteomics.
Comment 4. The new analysis is very good and provides deeper mechanistic insight into viral escape. I highly appreciate that the authors have included this figure in the supplementary figures.
Comment 8. In line with my previous remark and the authors’ reply, this specific analysis does not add mechanistic insight into loss of control or identify clear biomarker candidates. If the authors want to keep the analysis, I highly recommend moving the whole figure to the Supplementary Figures and briefly describing it as an exploratory analysis in the Discussion (as proposed by them).
Comment 9. The authors substantially improved the discussion paragraph. The revised text plausibly explains the counterintuitive findings and is well reasoned.

Dear Reviewers,

In response to the constructive evaluation of our manuscript, “Longitudinal Plasma Proteomic Signatures of Elite and Viremic Spontaneous HIV Controllers”, we appreciate the detailed comments , which hopefully led to improved clarity and robustness of our work.

In response, we have carefully revised the manuscript to improve clarity, scientific rigor, and interpretability. In particular, we expanded the cellular analyses, enhanced methodological descriptions, and refined the interpretation and discussion of our findings. In addition, we corrected mislabeling errors, provided more descriptive and informative figure legends, and performed additional sub-analyses to evaluate potential confounders. Collectively, these revisions substantially strengthen the robustness and transparency of the study.

We hope that the changes meet your expectations and that the revised manuscript now conveys our findings with greater precision and impact. We are grateful for your guidance and for the opportunity to further improve our study.

Thank you for your time and feedback.

Reviewers' Comments:

Reviewer #2 (Remarks to the Author):

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Reviewer #3 (Remarks to the Author):

Vadaq and colleagues explore in this study the plasma levels of more than 2000 proteins in a large cohort of PLHIV with different levels of control of viral replication: elite controllers (EC, <50copies/mL) and viremic controllers (VC, <10000copies/mL). There are two clear strengths in the study: 1)the number of individuals (n=183 individuals for the discovery cohort, n=19 individuals for the validation cohort) and 2)the longitudinal analysis, up to 17 years, that allowed the authors to identify participants that lost their control of the viral replication (transient controllers). Three proteins were increased in VC (CRTAM, LY9, CD6) and one in EC (VAT1). With retrospective analysis, they checked whether differences in the levels of plasma protein could predict loss of viral control or CD4 decay years after the first sampling (in the so-named transient controllers). They developed an accurate model for prediction of the loss of control.
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Comment 1. There are significant age differences between the groups. Age has been compared for the first approach (EC vs VC). However, in the second step of the analysis (EC, permanent VC and transient VC) this difference should be addressed as well. Even though age is included in the analysis as a covariate, it may not fully be controlled and should be taken carefully for the interpretations as residual confounding effect could remain. At the time of the second sampling, EC are ~60 y.o. while transient VC are ~50 y.o. and this difference might be relevant in terms of inflammation and aging.

Answer: We acknowledge the age differences between EC and VC subgroups at the time of the second sampling, particularly between EC (median age ~60 years) and transient VC (median age ~50 years). While age was included as a covariate in the linear modeling, we agree that residual confounding may persist and thus interpret our findings with caution.

Fortunately, there are no direct comparisons between EC and VC in the second step of the analysis. All comparisons are exclusively within pairs (EC, permanent VC and transient VC, respectively). Within pairs, the age difference between samples was 10.5 years for transient VC, 9.4 years for persistent VC, and 8.1 years for persistent EC. To further address the potential impact of age on the results, we correlated the differentially expressed proteins with time (Figure 2B for Persistent VC,

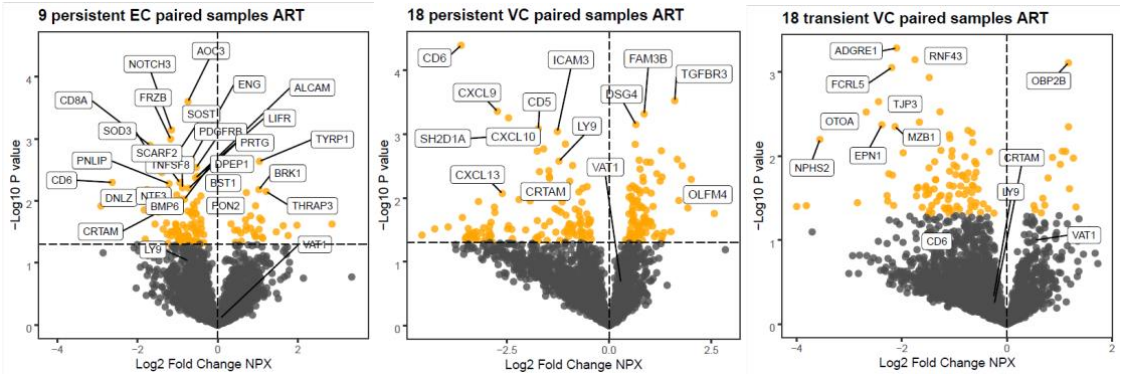
Concerning the absolute difference in age between EC, permanent VC and transient VC (~60, ~47, ~52 years at second sampling), it is possible that this age difference partly explains why the differential proteins do not fully overlap across the paired analyses (i.e. the analysis of EC pairs [Fig 2D] had a slightly different set of differential proteins than the analysis of permanent-VC pairs [Fig 2A] and likewise for transient VC [Fig 2C]). Notwithstanding, a number of top differential proteins in permanent VC (such as CD6, CD48, FCRL1, FCRL2, SEMA7A, TNFRSF13B) are also top differential proteins in transient VC. Of these, EC have only CD6 and CRTAM among their top differential proteins. In fact, EC do not exhibit the predominant downregulation that we see in the VC groups. Although one explanation could be the minor absolute age difference of 10 years pointed out by the reviewer, the analyses in Figure 1 and Figure 3 underline the differential impact of plasma viral load of the plasma proteome.

To explore the effect of age further, here presented is an additional set of paired analyses before and after ART, but for age-matched groups, ensuring similar age across groups. Median age is 60 at the time of second sampling for all three groups.

After 1:2 Age-matching EC:Persistent VC and EC:TransientVC, these are the ages:

stat	EC_preART	EC_onART	VCpersistent_preART	VCpersistent_onART	VCtransient_preART	VCtransient_onART
median age (IQR)	49.5 (19.1)	60.2 (16.8)	46.1 (21.4)	60.1 (15.8)	48.4 (18.4)	59.6 (15.1)

We reperformed the within-pair comparisons for these age-matched groups and the variation in top differential proteins across paired comparisons persisted (see the figures below). This stresses the importance of other factors than age, of which our main findings highlight plasma viral load as the main contributor.



Nonetheless, we recognize that age remains a potential confounder, and we have added this as a limitation (line 839-842) in the revised manuscript:
"Third, we acknowledge age differences when comparing pre- and post-ART samples in persistent and transient EC and VC. To address this, we again adjusted our analyses for age, sex, recruitment center, and storage time. Consistently, within-pair age-matched comparisons confirmed similar results."

Comment 2. There is also a difference between pre and post ART and the duration of ART before the second sample is different between EC (~3 years) and VC (>5years). Are all the individuals virologically suppressed at the time of the post ART sampling?

Answer: Indeed, there was a difference in ART duration between the groups at the time of post-ART sampling. Specifically (Based on Table 2):

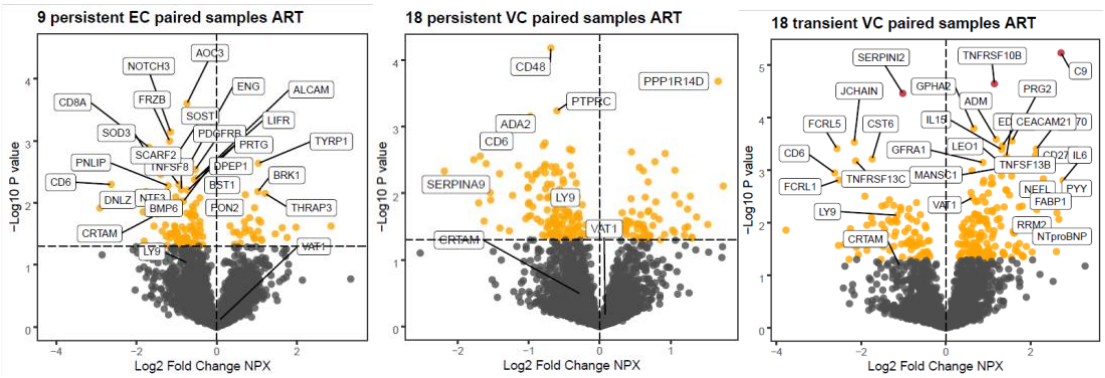
- Elite Controllers (EC) received ART for a median of 3.07 years [IQR: 2.55–5.24],
- Persistent Viremic Controllers (VC) for 5.34 years [IQR: 2.96–8.05],
- Transient VC for 5.40 years [IQR: 3.37–7.98].

Fortunately, no direct comparisons are done between EC and VC groups in the paired analyses. Moreover, stratified analyses based on ART duration (<1 year, 1–5 years, 5–10 years, ≥10 years) showed no major impact on the overall proteomic profile, as demonstrated in Principal Component Analysis Plot (Supplementary Figure 3A).

To further accommodate, we performed a sub-analysis comparing pre- and post-ART samples within VC and EC groups that were more closely matched in ART duration (median ~3 years in all three groups).

stat	EC_preART	EC_onART	VCpersistent_preART	VCpersistent_onART	VCtransient_preART	VCtransient_onART
median ART duration in years (IQR)	NA (NA)	3.1 (2.7)	NA (NA)	3.4 (3)	NA (NA)	4.3 (2.6)

Top differential proteins overlapped with the analyses with the full groups. It should be noted that the absence of complete overlap with the proteins main analyses is most likely an effect of extreme substratification and, consequentially, very limited sample size. Notwithstanding, this subgroup analysis underscores our other analyses that ART duration does not explain the differences across the paired comparisons of EC, persistent VC and transient VC, respectively.



Additionally, we confirm that all individuals included in the post-ART sampling were virologically suppressed at the time of sampling.

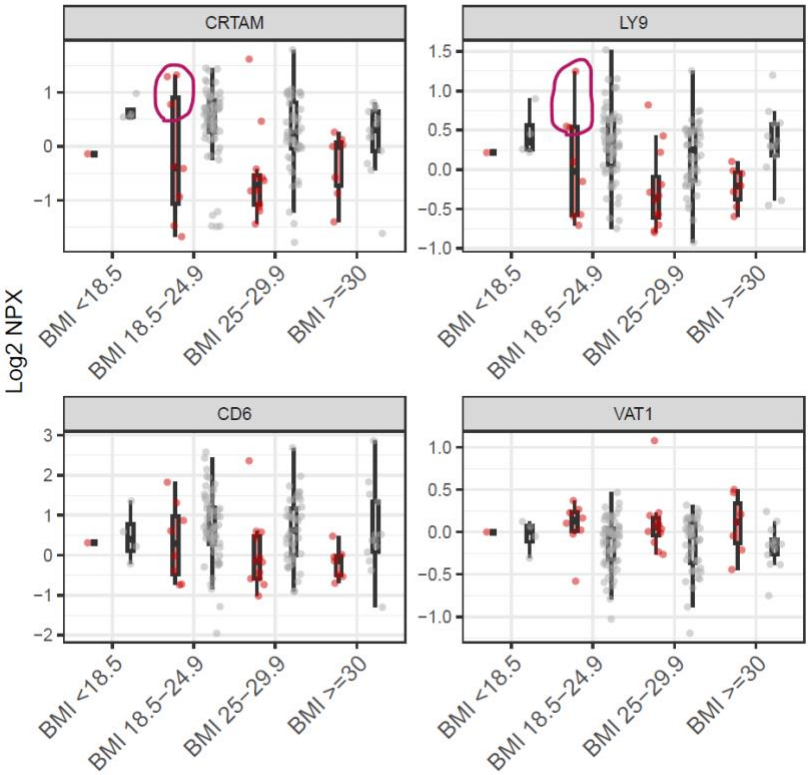
Comment 3. It is interesting to observe the difference in the levels of CRTAM, LY9, CD6 and VAT1 are maintained independently of smoking status or ethnicity; but regarding BMI they only appear in those >25. Do the authors have any hypothesis in this regard? Is it related to metabolic or inflammatory pathways?

Answer: The observation that differences became statistically significant only in the BMI >25 group is influenced by limited sample sizes after the stratification. BMI was missing in N=31 individuals (22% [8/36] of EC and 16% [23/147] of VC).

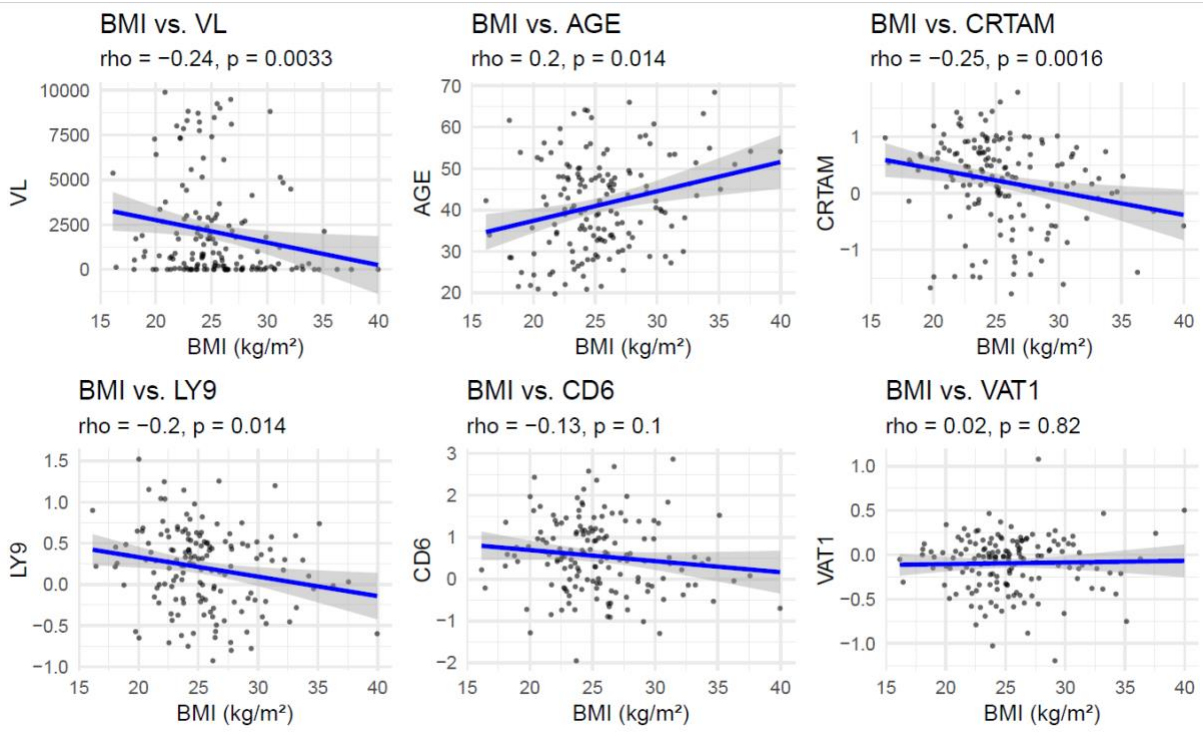
Across BMI categories the sample sizes after stratification were as follows:

Group	BMI <18.5 (N)	BMI 18.5-24.9 (N)	BMI >=25 (N)
EC	1	8	19
VC	4	63	57

Importantly, the EC BMI 18.5-24.9 group is small and strongly influenced by 3/8 EC having high levels of CRTAM and LY9, skewing the data for this stratum. In fact, the two EC with the highest levels of CRTAM and LY9 in the BMI 18.5-24.9 were blipping at the time of sampling (VL 857 and 61, respectively). This hints that viral load may be the main determinant here, not BMI. See below the figure with these three individuals encircled: (and also stratified between BMI 25-30 and >30)



Upon further inspection, we confirmed that BMI and viral load are indeed well associated. The pattern for BMI~VL represents the associations BMI~CRTAM and BMI~LY9. Correlations with BMI are shown here for the 28 EC and 124 VC who had available BMI data:



Indeed, EC had slightly higher BMI than VC (median [IQR] 27.7 [24.3, 30.0] and 24.5 [22.7, 26.4] for EC and VC, respectively). The associative nature of our study prohibits differentiating cause from consequence here (i.e. higher BMI might alter immune regulation <vs> better immune control could

result in less inflammation-associated wasting). Interestingly, across the various analyses in this study, metabolic status- or adiposity-related proteins in plasma were not associated with controller status, loss or viral load.

In conclusion, although our study suggests an association between BMI and viral control, the differences in CRTAM/LY9/CD6 between EC and VC were consistent across BMI categories. The subgroups where this did not reach statistical significance were either too small (BMI <18.5) or had blipping EC (BMI 18.5-24.9).

Comment 4. The 21-color flow cytometry panel is not described in the methods. The only result that appears is the level of CD8+ HLA-DR+ CD38+ cells as it shows positive correlation with levels of CRTAM and SH2D1A. However, it is unclear whether there are not significant correlations with the activation of other populations or whether this aspect has not been addressed. A lack of correlation with other cell populations might be biologically relevant.

Answer: We have now added a detailed description of the 21-color flow cytometry panel and gating strategy to the Methods section (line 207-211) of the revised manuscript:
"Data were collected in CytExpert v2.3 and analyzed in Kaluza v2.1.2 via sequential gating to resolve 355 distinct populations. From these, we selected 58 populations co-expressing HLA-DR and/or CD38, including CD4⁺ and CD8⁺ T-cell subsets, NK cells, γδ-T cells, and regulatory T cells, to quantify activation states. Antibody selection has been described previously in detail [22]"

Initially, we focused on CD8⁺ HLA-DR⁺ CD38⁺ T cells as a proxy for cytotoxic T cell activation, given their established role in chronic HIV infection and immune surveillance. However, as suggested, we expanded the analysis to include all HLA-DR⁺ and/or CD38⁺ immune cell subsets.

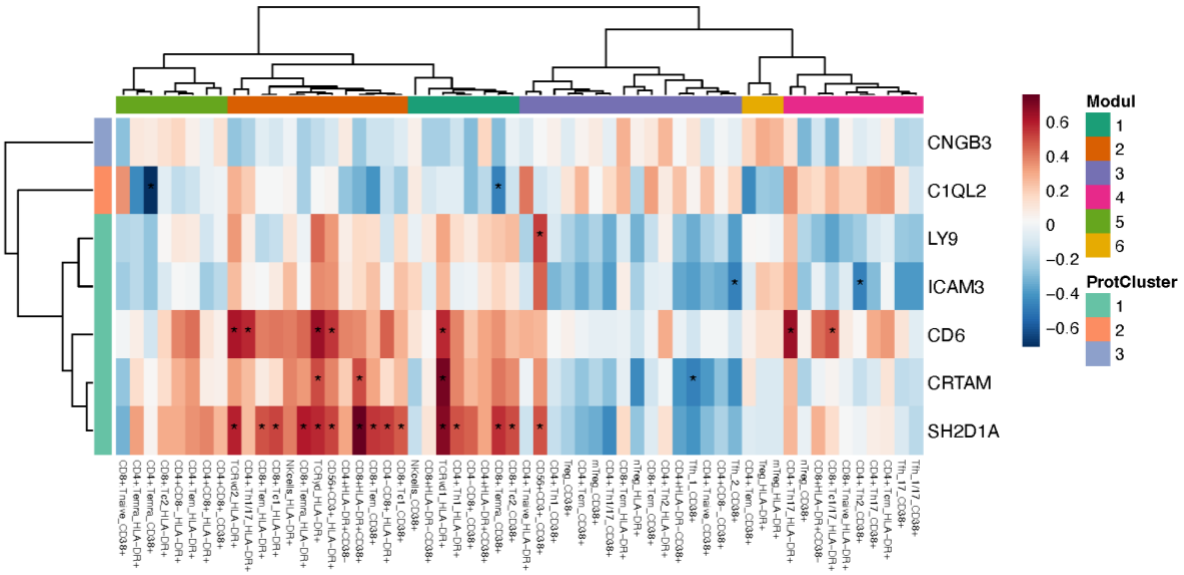


Figure 7B. Heatmap illustrates the Spearman correlations between seven proteins (CRTAM, LY9, CD6, ICAM3, SH2D1A, C1QL2, and CNGB3) and the absolute number of HLA-DR⁺CD38⁺ T-cell subsets. Data derive from ART-naive HIV controllers (HIC) in the 2000HIV cohort, including elite controllers (EC, n = 14) and viremic controllers (VC, n = 5). Tiles are colored by rho value (red = positive, blue = negative), with hierarchical clustering (Ward's method on 1−|rho| distances) grouping proteins and cell subsets by similarity of their correlation profiles. Each tile displays an asterisk (*) if the correlation is significant (P < 0.05). Each rho and P value reflects one protein–subset pairing across individual participants.

As shown in the heatmap, we found significant correlations between SH2D1A, CRTAM, and CD6 and the cell subsets in activation Modules 2 and 3. These modules are dominated by cytotoxic and

memory compartments, including HLA-DR$^+$CD38$^+$ CD8$^+$ central/effector-memory T cells, and HLA-DR$^+$ γδ T cells, defining a focused cytotoxic/memory activation axis. Among the top associations were SH2D1A with CD8$^+$HLA-DR$^+$CD38$^+$ (rho = 0.75, P value = 0.0002), CRTAM with TCRγδ HLA-DR$^+$ (rho = 0.71, P value = 0.001), and SH2D1A with TCRγδ HLA-DR$^+$ (rho = 0.69, P value = 0.001). These results might indicate antigen-driven CD8$^+$ and TCRγδ activation as the main drift toward viral escape, likely driving immune exhaustion and eventual loss of viral control. In contrast, the regulatory Module 6, which comprises mTreg HLA-DR$^+$, nTreg HLA-DR$^+$, and CD4$^+$ Tem CD38$^+$ T cells showed no significant correlations with any of the seven biomarkers (CRTAM, LY9, CD6, ICAM3, SH2D1A, C1QL2, CNGB3), suggesting that escalating activation, rather than failed regulation, may be the early indicator of impending loss of control.

These additional results are included in the:
- Results section line 639-647:
"Additionally, we found significant correlations between SH2D1A, CRTAM, and CD6 and the cell subsets in activation Modules 2 and 3. These modules are dominated by cytotoxic and memory compartments, including HLA-DR$^+$CD38$^+$ CD8$^+$ central/effector-memory T cells and HLA-DR$^+$ γδ T cells, defining a focused cytotoxic/memory activation axis. Among the top associations were SH2D1A with CD8$^+$HLA-DR$^+$CD38$^+$ (rho = 0.75, P value = 0.0002), CRTAM with TCRγδ HLA-DR$^+$ (rho = 0.71, P value = 0.001), and SH2D1A with TCRγδ HLA-DR$^+$ (rho = 0.69, P value = 0.001). These results might indicate antigen-driven CD8$^+$ and TCRγδ activation as the main drift toward viral escape, likely driving immune exhaustion and eventual loss of viral control (Supplementary Figure 7B, Supplementary Table 9)."
- Discussion section line 787-791:
"Flow-cytometry integration revealed that SH2D1A, CRTAM, and CD6 correlated with cytotoxic/memory activation modules dominated by HLA-DR$^+$CD38$^+$ CD8$^+$ T cells and HLA-DR$^+$ γδ T cells, suggesting that escalating antigen-driven cytotoxic activation, rather than failed regulatory control, may precede loss of viral control."
- Supplementary figure 7B, and
- Supplementary Table 9.

Comment 5. It seems that the pathway analysis was only performed in persistent VC since few proteins were significantly downregulated post ART in transient VC and none in the EC. However, it might be relevant to understand why the pathway analysis has been restricted to persistent VC. Moreover, in the manuscript it is indicated that this analysis was performed in 47/57 VC. Is there any specific reason to use a subset of the persistent VC group?

Answer: The pathway analysis was indeed limited to the persistent VC group, as this was the only subgroup in which a sufficient number of proteins (47) were significantly downregulated after ART initiation, passing FDR correction. In contrast, the pre-post ART comparisons in transient VC (n=36) and EC (n=9) yielded only 5 and 0 proteins, respectively, that met the threshold for multiple testing correction. As such, we were unable to perform a meaningful pathway analysis in these two groups due to the limited number of significant findings.

We have updated the relevant sections of the manuscript to clarify this point in the result section line 526-527: "Pathway analysis for both comparisons was not done due to the limited number of significant proteins."

Additionally, we would like to clarify that the pathway analysis was performed on all 57 persistent VC participants with paired pre- and post-ART samples, as stated in the main text (line 503-505) and in the legend of Figure 2A. The reference to "47" pertains to the number of proteins that passed FDR correction, not to a subset of individuals analyzed.

Comment 6. Figure 3 is the most relevant of the study and it might help to identify potential biomarkers for loss of viral control. In figure 3C and 3D it is not indicated whether EC and VC were included or only one of the groups (as indicated in figure 3E and 3F). Proper labeling might help to interpretate the figure.
In this figure, ICAM3 emerges as a potential biomarker, but not when comparing VC and EC in figure 1. Was it analyzed the same way as the other proteins in Figure 1?

Answer: We sincerely appreciate the reviewer's thoughtful and detailed comments. The analyses presented in Figure 3C and D included all 181 ART-naive HIV controllers (145 VC and 36 EC). We have now added the number of individuals included in the figure 3 and figure 3 legends to improve clarity and interpretation.

Regarding ICAM3, this protein emerged as significant in both longitudinal prediction models: one predicting a substantial increase in viral load, and the other predicting loss of controller status. Both models were based on Cox regression, adjusted for age, sex, study center, and sample storage duration. ICAM3 also appeared among the top six proteins in the cross-sectional comparison between EC (n=36) and VC (n=145), with higher levels in VC ($\beta = 0.39$, raw $P = 0.00022$). However, this difference narrowly missed significance after multiple testing corrections (FDR = 0.08). This analysis used a linear regression model, adjusted for the same covariates: age, sex, storage time, and center of inclusion.

The discrepancy between these findings likely reflects both biological and analytical aspects. The cross-sectional analysis (Figure 1) compares groups at a single timepoint and may not capture markers that reflect risk of future loss of control. In contrast, the longitudinal Cox models (Figure 3) assess time-to-event outcomes and may be more sensitive to early changes associated with virologic instability. ICAM3, involved in immune cell adhesion and trafficking, could be an early marker of immune activation that precedes viral rebound, making it more detectable in the longitudinal setting.

Comment 7. The supplementary figure 7 is unclear and there is no labeling for the different graphs that are depicted per row. The measure of inflammatory markers is not defined in the methods and the graph suggests some normalization issues, with values of <0 (e.g. TNF). Please, clarify whether this data is normalized. In figure B, the manuscript refers to% of CD8+ HLA-DR+ CD38+ but the measure in the graph is more likely the absolute number of activated CD8 T cells.

Answer: The associations shown in Supplementary Figure 7A were based on Olink data and involved correlations between selected proteins of interest (CRTAM, LY9, CD6, VAT1, ICAM3, SH2D1A, C1QL2, and CNGB3) and established inflammatory markers (TNF, IL-6, IL-1β, and sCD14).

Both the X- and Y-axes represent NPX values (Normalized Protein eXpression), which are Olink's arbitrary units reported on a log2 scale. NPX values are derived from Ct values and undergo a normalization process to reduce intra- and inter-assay variation (as detailed on the Olink website: https://olink.com/knowledge/documents). Due to the log transformation and normalization steps, NPX values can occasionally be negative, which typically reflects very low levels of the corresponding protein. We have now clarified this in the legend of Supplementary Figure 7A and updated the figure labels for clarity.

Regarding Supplementary Figure 7B, the reviewer is indeed correct, the values shown reflect the absolute number of $CD8^+$ $HLA\text{-}DR^+$ $CD38^+$ T cells, not percentages or frequencies. We have revised the figure label and legend accordingly to ensure accuracy and avoid confusion.

Comment 8. The relevance of the sequencing of HIV RNA in four samples of VC is not clear. Sequences are from "chronic phase", without a clear identification of this timing. The study opens the possibility of viral evolution as a driver of control loss. However, several aspects make this statement too speculative. First, diversity has been only addressed at one timepoint in a small subsets of VC. It would have been interesting to compare viral diversity with the reservoir landscape of EC, transient VC and normal progressors. They acknowledge they cannot confirm whether this plasma represents replication-competent virus (line 843), which weakens even more possible conclusions from the virological aspect of the study. These findings do not seem solid enough and they do not complement the rest of the analysis.

Answer: We thank the reviewer for this important comment. Indeed, the plasma viral RNA sequencing was performed on stored samples from four viremic controllers (VC) during the chronic phase of infection. As indicated in Supplementary Table 10, these samples were collected at a median of 4.97 years after the date of infection (range: 3.42–6.27 years).

Participant ID	PVL (copies/ml)	Sampling year	Loss of control (Y/N), If yes loss date	End of follow-up	HIV-1 duration (till plasma collection) in years	HIV-1 subtype	Co-receptor tropism
VC01	8711	Mid 2007	End 2007	2021	6.27	CRF_07 BC	CCR5
VC02	6119	Early 2007	Early 2008	2021	5.74	B	Mixed tropic*
VC03	5370	Early 2004	No	2019	3.42	B	CCR5
VC04	5570	Mid 2012	No	2022	4.20	B	CCR5

Snippet of Supplementary Table 10

We have now included this information in the main Methods section (line 214-216) to clarify the timing of sample collection:
"Stored plasma from four viremic controllers in the elite-ATHENA cohort was sequenced. Viral loads ranged from 5,370 to 8,711 copies/mL, and all samples were collected during chronic infection at a median of 4.97 years post-infection (range 3.42–6.27 years).".

The aim of this exploratory analysis was to assess the genetic composition of circulating viruses in VC and explore whether viral diversity or integrity might contribute to the observed loss of viral control. Our sequencing results showed a mixture of genetically intact and defective genomes, and a high degree of viral diversity was observed across all four VC, regardless of whether they maintained or lost control shortly after sampling. This suggests that despite harboring genetically diverse and intact virus populations, some individuals are still able to sustain viral control, highlighting the likely importance of host immune factors.

That said, we fully acknowledge the limitations of this analysis, particularly the small sample size, lack of longitudinal data, and absence of comparative sequences from EC or normal progressors.

Furthermore, as noted in the limitation of the manuscript, we cannot confirm whether the plasma RNA sequences reflect replication-competent virus, which further limits the value of this paragraph. Should the editor find it appropriate, we are open to downscale the section, move this exploratory paragraph to the supplements, or remove it altogether from the manuscript.

Comment 9. There are two paragraphs in the discussion that appear to be counterintuitive. The three proteins upregulated in VCs (CRTAM, LY9 and CD6) are associated with immune control (respectively: improved cytotoxicity, proviral silencing and inhibition of viral entry). However, in this study they are more likely linked to reduced control of viral replication. This "paradox" is repeated in lines 725 and 747, but without a clear biological interpretation why those molecules might be increased in VC compared to EC.

Answer:
We adapted these paragraphs in the discussion to better reflect the overall message of the paper:

- We removed the ATI study on CRTAM (Corley et al., AIDS 2021) investigated CpG islands but did not measure RNA transcripts or plasma proteins. As such, this study was not able to prove the direction of the association. We removed the line below:
  "In a previous analytical treatment interruption study in 13 normal progressors PLHIV on ART, it was observed that the DNA methylation levels of the CRTAM gene prior to intervention correlated with the timing of viral rebound"
- We added the line (704-706): "In PLHIV, CRTAM levels are higher in untreated normal progressors than PLHIV on ART, suggesting that increased CRTAM levels are part of an activated T cell response that aims to curb the uncontrolled viral replication. (Sperk et al. Medicine (Baltimore), 2018)"
- We added the line at the end of LY9 paragraph (720-722):
  "Our findings demonstrate, however, that this virus-inhibitory effect is insufficient to prevent loss of HIV control and that, paradoxically, increased plasma LY9 are indicative of deteriorating viral replication."
- We conclude the CD6 paragraph with (line 733-737):
  "Collectively, the upregulation of these three proteins in VC may be a "last-stand" attempt to maintain control status play a role in maintenance of HIV control status in the presence of low-level viral replication by improving cytotoxicity (CRTAM), silencing proviral DNA (LY9), and by inhibiting viral entry (CD6). Nonetheless, the need for such upregulation is a herald of imminent loss of HIV control and these biomarkers may therefore be utilized for their high predictive potential."

Minor comments:
• The study design is complex. Indicating the sample size in each analysis might help to follow the study.

Answer: We have now added the number of individuals included in the figure and/or figure legends to improve clarity and interpretation.

• Reference 7 is contextually unrelated (it is an ATI study), reference 8 does not demonstrate any genetic aspect of the loss of control (as mentioned in the introduction), reference 20 seems irrelevant.

Answer: We have now excluded reference 7 (previous version of the manuscript) as reviewer's suggested. As to reference 8 (factors predictive of loss of control), the word "genetics" has been removed from the text (line 56-58). Instead, we added key reference Gasca-Capote et al. JCI 2024 to

support the notion that viral factors contribute to loss of viral control. Other studies on factors of control loss are left out but mentioned here for transparency: Noel et al PLoS One 2015, Pernas et al J Virol. 2018, Rodriguez-Gallego et al JID 2019, Tarancon-Diaz et al eBioMedicine 2019, Masip et al Front Imm 2022. In addition, we have also excluded reference 20 along with the phrase in line 75-77 (previous version of the manuscript) that mentioned it.

• *Mislabeling: line 455 (should be Figure 1E), line 477 (should be Figure 1D).*

Answer: We thank the reviewer for the detailed observation. We have corrected the labels in the figure 1 accordingly.

Dear Reviewer,

In response to the rebuttal of our manuscript "**Longitudinal Plasma Proteomic Signatures of Elite and Viremic Spontaneous HIV Controllers**", we have carefully revised the manuscript and incorporated the requested additions/changes. We hope that these revisions fully meet your expectations.

Thank you very much for your time and thoughtful feedback.

Reviewers' Comments:

Reviewer #3 (Remarks to the Author):
The authors have addressed all my comments very well. the reasoning is sound and the added analyses improve the manuscript. Manuscript has improved substantially following revision.

Comments 1–3. The additional analysis shows that the reported differences are not age-dependent, which is helpful. The authors should consider adding the relevant panels to the supplementary material, as age is in general a very relevant factor for plasma proteomics.

Response: We have now added the additional age-matched analyses to the supplementary materials and referenced them in the main manuscript (lines 227:238), as follows:

"Given the observed age differences across subgroups at the time of the second sampling, with persistent EC having a median age of 60.2 years, persistent VC 46.9 years, and transient VC 52.1 years (Table 2), we further assessed the extent to which age could contribute to the variation in proteomic changes. We repeated the paired analyses pre- and post- ART in age-matched subgroups. After 1:2 matching, all three groups reached a comparable age distribution at the second sampling, with median ages of 60.2 years (IQR 16.8) in persistent EC, 60.1 years (IQR 15.8) in persistent VC, and 59.6 years (IQR 15.1) in transient VC. Correspondingly, their pre-ART ages were also aligned, with median ages of 49.5 years (IQR 19.1), 46.1 years (IQR 21.4), and 48.4 years (IQR 18.4), respectively. The variation in top differentially expressed proteins across the age-matched paired comparisons persisted, indicating that the observed proteomic differences are unlikely to be attributable to age alone (Supplementary Figure 3D)."

Comment 4. The new analysis is very good and provides deeper mechanistic insight into viral escape. I highly appreciate that the authors have included this figure in the supplementary figures.

Response: We thank the reviewer for the positive evaluation.

Comment 8. In line with my previous remark and the authors' reply, this specific analysis does not add mechanistic insight into loss of control or identify clear biomarker candidates. If the authors want to keep the analysis, I highly recommend moving the whole figure to the Supplementary Figures and briefly describing it as an exploratory analysis in the Discussion (as proposed by them).

Response: We have now moved the full figure to the supplementary section and describe it as an exploratory analysis in the Discussion (lines 498:508):

“As an exploratory extension of these findings, we examined plasma RNA genomes in viremic controllers, which provides insights into the intactness and diversity of viral sequences in this group.”

“This exploratory analysis also revealed a high level of genetic diversity among plasma RNA sequences in all four VC (Supplementary Figure 8B; Supplementary Table 10).”

Comment 9. The authors substantially improved the discussion paragraph. The revised text plausibly explains the counterintuitive findings and is well reasoned.

Response: We thank the reviewer for the positive evaluation.