Title: Evaluating predictive markers for viral rebound and safety assessment in blood and lumbar fluid during HIV-1 treatment interruption

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Abstract

BACKGROUND:

Validated biomarkers to evaluate HIV-1 cure strategies are currently lacking, therefore requiring analytical treatment interruption (ATI) in study participants. Little is known about the safety of ATI and its long-term impact on patient health.

OBJECTIVES:

ATI safety was assessed and potential biomarkers predicting viral rebound were evaluated.

METHODS:

PBMCs, plasma and CSF were collected from 11 HIV-1-positive individuals at four different timepoints during ATI (NCT02641756). Total and integrated HIV-1 DNA, cell-associated (CA) HIV-1 RNA transcripts and restriction factor (RF) expression were measured by PCR-based assays. Markers of neuroinflammation and neuronal injury [neurofilament light chain (NFL) and YKL-40 protein] were measured in CSF. Additionally, neopterin, tryptophan and kynurenine were measured, both in plasma and CSF, as markers of immune activation.

RESULTS:

Total HIV-1 DNA, integrated HIV-1 DNA and CA viral RNA transcripts did not differ pre- and post-ATI. Similarly, no significant NFL or YKL-40 increases in CSF were observed between baseline and viral rebound. Furthermore, markers of immune activation did not increase during ATI. Interestingly, the RFs SLFN11 and APOBEC3G increased after ATI before viral rebound. Similarly, Tat-Rev transcripts were increased preceding viral rebound after interruption.

CONCLUSIONS:

ATI did not increase viral reservoir size and it did not reveal signs of increased neuronal injury or inflammation, suggesting that these well-monitored ATIs are safe. Elevation of Tat-Rev transcription and induced expression of the RFs SLFN11 and APOBEC3G after ATI, prior to viral rebound, indicates that these factors could be used as potential biomarkers predicting viral rebound.

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Keywords

Restriction factors
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Analytical treatment interruption
Time to viral rebound
HIV reservoir
HIV Cure

Research in context

Evidence before this study

Analytical treatment interruption (ATI) trials are an essential tool to measure the impact of new HIV-1 cure strategies and to identify potential biomarkers predicting viral rebound post-ATI. Nevertheless, little is known about the safety of ATI and their long-term impact on patients' health. Only few studies have demonstrated that treatment interruption is safe by evaluating the stability of the HIV reservoir pre- and post-ATI. Levels of total HIV-1 DNA, cell-associated HIV-1 RNA, and viral production after stimulation) did not differ before and after ATI. Additional analyses evaluating virological and immunological characteristics pre- and post-ATI are crucial for further safety assessment. Chronic immune activation in the blood and central nervous system can occur despite virally-suppressive antiretroviral therapy (ART) and contributes to disease morbidity. Recently, neuro-inflammatory and neuronal injury markers have been evaluated after early treatment initiation, clearly showing a beneficial effect on the levels of brain-related chronic inflammation and therefore also potentially on the size of the central nervous system (CNS) reservoir. A better understanding of the CNS reservoir formation prior to treatment, and its preservation during ART, is important when exploring HIV eradication strategies, but also to avoid severe CNS adverse events during the course of HIV cure studies.

Recently, a new panel of reverse transcription droplet digital polymerase chain reaction (RT-ddPCR) assays specific for different HIV transcripts (TAR, long LTR, polyA, Pol and Tat-Rev) that define distinct blocks to transcription was introduced. Levels of HIV-1 multiply spliced RNA indicate the ability to overcome blocks to initiation, elongation and termination and were reported to increase at viral rebound. A wide range of host restriction factors (RF) are induced early upon HIV-1 infection to suppress viral infectivity. Limited data is available evaluating the role of RF in vivo. A recent report nicely showed that expression of several restriction factors (APOBEC3G, MX2 and TRIM5) is linked with HIV-1 viral load. Contrarily, SLFN11 and SAMHD1 expression was found to be negatively correlated with levels of total and integrated HIV-1 DNA and SLFN11, BST2, and SAMHD1 were furthermore highly expressed in LTNPs. These data indicate that RF SLFN11, BST2 and SAMHD1 could form a signature associated with protection from disease progression and that the induction of SLFN11 and SAMHD1 could limit viral load and viral reservoir size which could contribute to the design of novel antiviral therapeutics. Evaluating kinetics of restriction factors in ATI might reveal new insights on viral control and enlighten on the complexity of the innate immunity and its response against HIV, which might require individual treatment strategies.

Added value of this study

In the present study, we aimed to further explore safety of ATIs and their potential impact on patients health by evaluating virological and immunological factors, and neuronal inflammatory and -injury markers. Besides confirming a stable viral reservoir in the blood compartment after ATI, we were the first to show that short ATI do not give rise to signs of increased inflammatory activation in the brain, nor neuronal injury.

Furthermore, we were the first to assess potential promising biomarkers for viral rebound, like cell-associated HIV-1 RNA transcripts and HIV-1 specific RF at four different time points during ATI. Enabling researchers to predict viral rebound would be a massive asset in future CURE trials, assessing new therapeutic strategies without requiring participants to stop cART to evaluate their range of success. This is the first study demonstrating upregulated levels of RF SLFN11 and to a lesser extent also

APOBEC3G after ATI, but before HIV plasma viral load becomes detectable. Similarly, Tat-Rev transcription was increased before actual viral rebound, suggesting potential for these markers in prediction of viral rebound. Furthermore, levels of total and integrated HIV-1 DNA at baseline (before ATI) correlated with amount of plasma VL at rebound depicting that HIV-1-infected individuals with a higher viral reservoir have typically highest VL levels at rebound.

Implications of all the available evidence

This study, together with other ATIs confirm that short and well monitored ATIs are safe in terms of maintaining a stable HIV reservoir over time and having no impact on neuronal injury and/or inflammation.

Although we were not able to identify a biomarker that can predict viral rebound without cART interruption, we have found some potential interesting markers that increased very rapidly after ATI. Tat/Rev transcription was found to proceed viral rebound. RF SLFN11 and APOBEC3G elevation occurred also rapidly after ATI, suggesting that these factors are upregulated by other mechanisms than interferon alone and might predict viral rebound at an early stage upon ATI. Validation of these findings in larger cohorts is required to confirm these results.

Introduction

Combination antiretroviral therapy (cART) is able to suppress viral replication in HIV-1 infected individuals, but cannot completely eradicate HIV-1. Life-long cART remains mandatory as the latent viral reservoir refuels viral replication after treatment interruption, condemning HIV-1 to a chronic disease. This latent HIV-1 reservoir is the last hurdle towards an HIV-1 cure and consequently the target of cure strategies. Analytical treatment interruption (ATI) trials are becoming the gold standard to evaluate the potency of cure strategy interventions, in lack of biomarkers assessing reservoir reduction or virological control.[1,2] Although these intensively monitored ATIs are well tolerated by most of the participants, little is known about the safety of ATI and their long-term impact on patients' health.[3,4] Recent reports demonstrated no changes in viral reservoir size (total HIV-1 DNA, intact HIV-1 DNA

(near full-length), cell-associated (CA) HIV-1 RNA and infectious units per million cells (IUPM), measured by quantitative Viral Outgrowth Assay (qVOA)) and little diversity in composition of virus populations pre- and post-ATI.[5,6] This data is reassuring, but additional analyses evaluating virological and immunological characteristics in different body compartments pre- and post-ATI are crucial for further safety assessment. Neopterin, kynurenine and kynurenine/tryptophan ratio are regularly assessed immune activation markers in plasma and cerebrospinal fluid (CSF)[7,8] and further, YKL-40 and neurofilament light chain (NFL) are used as markers of neuro-inflammation and neuronal injury respectively. Increased levels of these markers have been detected in several diseases characterized by persistent inflammation (e.g. Alzheimer's disease, cancer and viral infections, including HIV-1).[8,9] Assessing neopterin, kynurenine, kynurenine/tryptophan ratio, YKL-40 and NFL in CSF pre- and post-ATI could inform on the potential consequences of ATI on the brain.

ATI combined with extensive patient sampling, could give broader insights on the origin of viral rebound and can help identify potential biomarkers to predict viral rebound post-treatment interruption. Because restriction factors (RF), specific HIV-1 antiviral factors, are induced early upon HIV-1 infection after IFN production,[10] these factors could be potential biomarkers for viral rebound. Mechanisms that drive RF expression are not fully elucidated and measurement of RF levels *in vivo* before, during and after ATI could contribute to understanding the timing of RF induction. The best characterized RFs include bone marrow stromal cell antigen 2 (*BST2*)/tetherin, apolipoprotein B mRNA editing enzyme catalytic subunit 3G (*APOBEC3G*), SAM domain and HD domain containing protein 1 (*SAMHD1*) and tripartite motif containing 5 (*TRIM5*), performing their antiviral activity at different stages of the viral replication cycle (inhibition of virion release, viral hypermutation at reverse transcription, depletion of dNTP pool and targeting the viral capsid, respectively).[11] Other RFs have been described recently, such as *MX2*, *SLFN11* and *PAF1*, interfering with nuclear import, translation of viral RNA to proteins and early events in replication cycle.[11-13] HIV-1 encodes accessory proteins able to counteract RF activity, such as viral infectivity factor (Vif), viral protein U (Vpu), negative regulatory factor (Nef), and viral protein R (Vpr),[14] allowing virus replication to continue.[15] *In vivo* RF data is limited but their expression was found to be positively correlated to interferon stimulated gene (ISG) levels and to viral load for some factors (*APOBEC3G*, *TRIM5* and *MX2*).[16,17] Additionally,

APOBEC3G, *TRIM5*, *BST2* and *MX2* expression levels are increased in seroconverters (SRCV),[18] ART-naïve acutely infected HIV-1 patients, confirming RF expression is induced as an early antiviral defense mechanism of the host immune system and suggesting that RF levels could be induced early post-ATI. Determination of different CA HIV-1 RNA transcripts during ATI could give insights in various blocks and peaks of viral transcription. TAR, long LTR, polyA, Pol, and Tat-Rev transcripts represent resp. transcription initiation, elongation, termination, CA unspliced HIV-1 RNA (usRNA) and multiply spliced HIV-1 RNA (msRNA). Levels of HIV-1 msRNA indicate the ability to overcome blocks to initiation, elongation and termination and were reported to predict viral rebound after ATI.[19,20]

By assessing HIV reservoir size, inflammation markers and RF expression levels at different time points during ATI (baseline on cART (T1; undetectable VL), post-ATI (T2; undetectable VL), at viral rebound (T3; detectable VL) and 3 months after restart cART (T4; undetectable VL)), we have comprehensively evaluated the safety of these trials and verified that some of these markers could potentially be used as biomarkers predicting viral rebound.

Materials and Methods

Patient cohort

The STAR-STUDY ATI trial was designed to identify and characterize the relevant anatomical compartment(s) of the replication competent HIV reservoir. The study design allowed us to further investigate virological and immunological characteristics and their relationship towards viral rebound. The study protocol, experimental design and recruitment strategy were approved by the Ethics Committee of the University Hospital of Ghent (Belgian registration number: B670201525474). Participants were recruited from the AIDS reference center at the Ghent University Hospital depending on strict inclusion criteria and after intensive counseling (NCT02641756). Written informed consent was obtained from all participants. Eleven HIV-1 infected, long-term treated participants under chronic cART were included. In a second phase of the study, treatment was interrupted and participants were monitored intensively upon viral rebound.

RF and viral reservoir markers (total HIV-1 DNA, integrated HIV-1 DNA and different transcripts of CA HIV-1 RNA indicating specific transcriptional blocks: TAR, long LTR, Pol, polyA and Tat-Rev)

were measured in PBMCs at T1 (on cART, undetectable VL), T2 (7-15 days after ATI, undetectable VL), T3 (viral rebound, detectable VL) and T4 (3 months after cART restart) (Figure 1). Additionally, inflammation markers were determined in plasma at these four different time points and from 10/11 participants at T1 and T4 in CSF.

Primers

APOBEC3G and *SAMHD1* assays were commercially derived (Bio-Rad, Belgium). Reference gene assays were described in RTprimerDB, a freely accessible database containing PCR primers and probes. RF primers were designed manually using PrimerXL, a primer design platform based on Primer3 using *in silico* assay validation. After *in silico* validation, primers were validated in the lab and selected based on adequate PCR efficiency. Supplementary Table 1 depicts used assays (*Table S1*).

Quantitative real-time PCR

RNA was extracted from 10^7 PBMCs (RNA innuprep mini kit, Analytik, Germany) and reverse transcribed into cDNA using the qScript cDNA SuperMix (Quantabio, MA, USA) according to the manufacturer's protocol. 20 ng cDNA was used in a 10 µl real-time PCR reaction with SYBR Green (LightCycler480 SYBR Green I Master, Roche Applied Science, Belgium). Per PCR reaction, 5 µl SYBR Green Master Mix, 2.5 µl H_2O and 250 nM of each primer were added and every reaction was performed in duplicate. Cycling conditions on LightCycler 480 (Roche Applied Science, Germany) were 95°C for 5 min, 45 amplification cycles of 95°C for 10s, 58°C for 30s and 72°C for 30s. A melting curve from 60°C to 95°C was included for subsequent melting curve analysis to determine assay specificity. Reference gene stability was assessed for actin beta (*ACTB*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), TATA-box binding protein (*TBP*), beta-2-microglobulin (*B2M*), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*), procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1 (*PLOD1*), hydroxymethylbilane synthase (*HMBS*) and ubiquitin C (*UBC*) on a subset of the samples. *ACTB*, *GAPDH* and *YWHAZ* were selected for normalization after analysis with the GeNorm algorithm.[21] Expression analysis was performed with qbasePLUS software (Biogazelle)[22] allowing automated normalization, calculation of PCR efficiency and adequate inter-run calibration. Relative gene expression values are expressed as normalized relative quantities (NRQ).[23]

Neuro-inflammation markers

NFL and YKL-40 protein were measured in CSF as markers of neuronal injury and neuroinflammation, respectively. In addition, neopterin, tryptophan and kynurenine were measured both in plasma and CSF as markers of immune activation. NFL concentration was measured using an in house enzyme-linked immunosorbent assay as previously described in detail (PMID: 29370869). YKL-40 concentration was measured using a commercially available ELISA according to instructions from the manufacturer (R&D System, Minneapolis, MN). NFL and YKL-40 measurements were performed by board-certified laboratory technicians in one round of experiments with baseline and follow-up samples side-by-side on the ELISA plates to minimize variation. Intra-assay coefficients of variation were below 8% for both NFL and YKL-40. Neopterin was measured by ELISA (BRAHMS, Hennigsdorf, Germany), and kynurenine calculating the kynurenine/tryptophan ratio were done by HPLC.[24]

Total and integrated HIV-1 DNA

Total genomic DNA was extracted from 10^7 PBMCs (DNeasy Blood & Tissue Kit, Qiagen, The Netherlands). Restriction and ddPCR reactions were performed as described previously.[25] Briefly, following primers were used to quantify total HIV-1 DNA (Forward: 5'-GCCTCAATAAAGCTTGCC-3'Reverse: 5'-GGCGCCACTGCTAGAGATTTT-3', Probe: 5'-AAGTRGTGTGTGCCC-3'). Reference gene ribonuclease P/MRP subunit p30 (RPP30) was used for normalization and quantification of total HIV-1 DNA was performed with ddpcRquant, an in-house developed software.[26] Integrated HIV-1 DNA was quantified using the repetitive sampling Alu-HIV PCR method involving a nested PCR approach.[27] This method was performed as described previously.[18] Briefly, in the first PCR, a forward and reverse primer targeting respectively a human Alu fragment and the HIV-1 g*ag* region were used to quantify 40 replicates. Additionally, 20 replicates of background quantification were performed using only the HIV-1 *gag* primer.[18]

Cell-associated HIV-1 RNA transcripts

RNA was extracted from 10^7 million PBMC (RNA innuprep mini kit, Analytik, Germany). TAR, long LTR, polyA, Pol and Tat-Rev CA HIV-1 RNA transcripts were quantified as described previously by Yukl et al.[19] Input for reverse transcription (RT) reactions was maximized based on RNA mass recovery:

median 0.385 µg and 3.286 µg for resp. 1) TAR and 2) long LTR, Pol, polyA and Tat-Rev RT was used. Normalization was performed depending on input RNA mass as previously described.[19]

Statistical analysis

Non-parametric Friedman test with post-hoc Dunn statistical analysis was performed. Holm p-value adjustment method was used for multiple comparisons. Spearman correlation analysis was performed to define significant correlations between RF, neuro-inflammation markers and immunological and virological reservoir parameters at single time points. Additionally, repeated measurements correlation analysis was performed to detect similar profiles for these markers over the different time points. Statistical analyses and graphing was performed with R software using the following packages: PMCMR, Hmisc, graphics, ggplot2, corrplot and rmcorr.

Results

Study participants

Eleven HIV-1-positive individuals were included in the study and sampled at four time points pre- and post-ATI (*Figure 1*). Participants were on stable cART for ≥ 3 years and did not have significant co-morbidities at time of inclusion. Participants received a standard cART regimen involving an integrase inhibitor for at least 3 months before study inclusion. The median age of the participants was 40 years (range 32-56) (*Table 1*). Median time to viral rebound (TTVR) was 21 days (range 15-36). Demographic variables are available in Table 1.

Quantification of viral reservoir change during ATI

Levels of total and integrated HIV-1 DNA were determined at the four time points during ATI to further assess the stability of the viral reservoir and the safety of ATI. No remarkable changes at the different time points were detected for both markers (Figure 2a and 2b). Spearman correlation analysis at T1, T2, T3 and T4 revealed a highly significant positive correlation between total and integrated HIV-1 DNA levels (Spearman R 0.75, 0.90, 0.90 and 0.90, respectively).

TAR, long LTR, polyA, Pol and Tat-Rev HIV-1 RNA transcripts were determined at the different time points and exhibited maximal levels at T3 (viral rebound; detectable plasma VL). Significant increases

were detected for long LTR, polyA, Pol and Tat-Rev at T3 as compared to T1 and T4 (T1: $p<0.0001$, $p<0.0001$, $p=0.0006$ and $p=0.0002$, respectively; T4: $p<0.0001$, $p=0.019$, $p=0.001$ and $p=0.0077$, respectively; Figure 2c). Repeated measurement correlations indeed indicated highly significant positive correlation between TAR, long LTR, polyA, Pol and Tat-Rev RNA transcription levels over the four time points, confirming similar profile for these transcripts (data not shown). Interestingly, Tat-Rev levels were also significantly elevated at T2, time point post-ATI before detectable VL, as compared to T1 ($p=0.016$) (Figure 2c).

No increased inflammation in plasma and CSF after ATI

No significant increase in NFL or YKL-40 in CSF was observed between baseline (T1) and viral rebound (T3) (*Figure 3a*). Furthermore, markers of immune activation, neopterin, kynurenine and kynurenine/tryptophan ratio, were also found to be stable in CSF (*Figure 3b*). Similarly, plasma levels of neuro-inflammatory markers did not increase during viral rebound (*Figure 3c*). Interestingly, Neopterin levels in CSF pre-ATI were negatively correlated with time on treatment ($p = 0.004$), suggesting that neuronal injury decreases with time on cART.

To further investigate viral replication in the central nervous system (CNS), we sequenced the virus derived from CSF at T1 and T3. The V1/V3 *env*-sequences detected in CSF demonstrated no genetic evidence for compartmentalization in the CNS responsible for viral rebound. In 3/5 patients where we sequenced free virus from CSF at T3, at least one of the sequences was identical to a rebound virus, most probably by crossing the blood-brain barrier (*Figure 3d*).

***SLFN11* and *APOBEC3G* expression elevation prior to viral rebound**

IFIT1 and *MX1*, well-characterized ISGs but not RF specifically interfering with HIV, were used as markers for IFN exposure. Significant *MX1* upregulation was detected at viral rebound (T3) as compared to time points with undetectable VL (T1, T2 and T4; $p=0.03$, 0.02 and 0.01, respectively, *Figure 4a*). Similarly, *IFIT1* levels demonstrated upregulation at viral rebound (T3) in comparison to T1 ($p=0.001$), suggesting that IFN induction is linked to VL (*Figure 4a*). Because most RF are also induced by IFN, we wanted to determine if *APOBEC3G*, *SAMHD1*, *BST2*, *TRIM5*, *MX2*, *SLFN11* and *PAF1* expression profile would follow *IFIT1* and *MX1* levels and therefore were as well upregulated at viral rebound (T3).

Additionally to RF, we hypothesized that expression levels of two HIV-1 dependency factors (*NLRX1* and *PSIP1*) could also be linked to VL levels.

RF *MX2*, *TRIM5* and *BST2* levels followed this assumption and demonstrated highest levels at T3 although not reaching statistical significance for *TRIM5* and *BST2*, suggesting a link between VL levels and *MX2*, *TRIM5* and *BST2* elevation (*Figure 4b*). *SAMHD1* and *PAF1* expression was not changed during ATI (*Figure 4c*). Similarly, HIV-1 dependency factors, *NLRX1* and *PSIP1*, involved in inhibition of IFN response and encoding an integrase cofactor LEDGF/75, demonstrated no differences in expression levels between the different time points during ATI (*Figure 4d*). Surprisingly, *SLFN11* demonstrated significant upregulation at T2, time point post-ATI but with still undetectable VL, as compared to T1 and T4 (p=0.002 and p=0.049, respectively). *APOBEC3G* followed similar pattern as *SLFN11* although upregulation at T2 was not significant (*Figure 4e*). Repeated measurements correlation analysis demonstrated a highly significant correlation between *SLFN11* and *APOBEC3G* over the four time points (R = 0.80 and p<0.0001), indeed indicating their similar expression profile (*Supplementary Figure 1*).

RF expression levels are not correlated with time to viral rebound

Because *SLFN11* and *APOBEC3G* levels are upregulated before plasma viral load becomes detectable, we were interested if their expression was linked to TTVR and therefore could be used as a marker to predict viral rebound. Unfortunately, no association could be detected for *SLFN11*, *APOBEC3G* or other antiviral factors with TTVR. Interestingly, levels of total and integrated HIV-1 DNA at T1 and T2 were positively correlated with viral load at rebound (T3) (T1: R= 0.52 and 0.70, respectively; T2: R=0.9 and 0.85, respectively; *Supplementary Figure 2*), suggesting that a larger viral reservoir gives rise to highest plasma VL levels.

Furthermore, we analyzed possible correlations between expression of RF, participant characteristics, virological and immunological parameters. Surprisingly, a strong negative correlation was detected between expression levels of antiviral factors (*MX1*, *IFIT1*, *APOBEC3G*, *PAF1*, *SAMHD1* and *SLFN11*) at T1 (cART; undetectable VL) and NK cell count.

Discussion

ATIs remain the gold standard to evaluate new possible HIV-1 cure strategies, as we lack surrogate biomarkers that are able to assess the efficiency of cure strategies and their impact on time to viral rebound after treatment interruption. However safety of these ATIs and the long-term effects on the HIV reservoir in the blood or other compartments is unclear. In the present study, the effect of ATI on the viral reservoir size and inflammation status was evaluated at different time points before, during and after ATI to further address these safety concerns. Additionally, our study investigated the expression levels of HIV-1 RF, dependency factors and the expression of different CA viral RNA transcripts defining distinct blocks to transcription at these time points to identify possible biomarkers for viral rebound.

ATI did not increase levels of total and integrated HIV-1 DNA over the four different time points, nor of the different CA HIV-1 RNA transcripts pre- and post-ATI (T1 vs T4). This data is consistent with previous findings and indicates a stable viral reservoir after a short period of treatment interruption.[5,6] Interestingly, levels of total and integrated HIV-1 DNA at T1 and T2 were positively correlated with viral load at rebound (T3) suggesting that HIV-1 positive individuals with a larger viral reservoir before ATI have highest plasma VL levels at viral rebound.

We further assessed the effect of ATI on the HIV brain compartment. No detectable signs of neuronal injury or major CNS inflammation during viral rebound in plasma after (short) ATI were determined, suggesting that it is very unlikely that replication in CNS has started to a large extent this early after treatment cessation. This is in line with the limited sequencing data we obtained from CSF giving no sign of compartmentalization, nor of early rebound in the brain compartment.

Expression of RF and ISGs at the different time points was evaluated to find possible biomarkers predicting viral rebound. ISGs and RF *MX2*, *TRIM5* and *BST2* followed similar profile and were induced at the time VL became detectable (T3), suggesting that VL drives ISG and RF elevation. These findings are in line with previous reports depicting a link between levels of several RF and VL in different cohorts of HIV-1 positive individuals.[16,18] Interestingly, we detected that *SLFN11* and *APOBEC3G* were induced prior to other RF and prior to time of viral rebound (T3) and could therefore serve as biomarkers for viral rebound, predicting viral rebound at an early stage after ATI. *SLFN11* elevation has been linked to LTNP status and reduced viral reservoir size,[16,18] suggesting a role for *SLFN11* in VL control. Therefore,

early induction of *SLFN11* could be a host defense mechanism attempting to control the virus. Unfortunately, we could not find a correlation between the upregulation of *SLFN11* and *APOBEC3G* and TTVR. We also did not identify significant links between TTVR and expression of other RF, participant characteristics (time since primary infection, time on cART, time before cART initiation), virological (total HIV-1 DNA, integrated HIV-1 DNA, VL zenith) and immunological parameters (CD4 nadir). Surprisingly, a strong negative correlation was detected between expression levels of antiviral factors (*MX1*, *IFIT1*, *APOBEC3G*, *PAF1*, *SAMHD1* and *SLFN11*) at T1 (cART; undetectable VL) and NK cell count. Lower NK count and higher RF expression levels in ART-treated individuals have been linked to lower immune activation and better preservation of immune responses, respectively. Therefore, this data suggests that ART-treated individuals displaying increased RF levels could be associated with reduced amount of immune activation.

Another interesting mechanism that should be further investigated is the expression of CA HIV-1 RNA transcripts defining distinct blocks to transcription during ATI. Transcription of TAR, long LTR, polyA, Pol and Tat-Rev fragments is highly induced at viral rebound (T3). Interestingly, Tat-Rev expression preceeds viral rebound, whereas the other transcripts only statistically increase at the time point of viral rebound (T3). Tat-Rev transcription indicates the ability to overcome blocks to initiation, elongation and termination and could therefore be used as a marker for viral rebound.[19,20] However, caution should be taken in interpreting these results, as Tat-Rev levels are mostly very low or undetectable in patients on cART[28] and the observed significant Tat-Rev increase at T2 could be related to the assays limit of detection.

The current study has several limitations, most importantly the limited amount of participants included in this trial, making it difficult to draw population based conclusions. The design of these ATIs makes it a real challenge to include more participants, both from an ethical as financial point of view. Furthermore, all participants rebounded between D15 and D36 after ATI, so the time between T2 and T3 is often very limited which might explain why we do not find significant differences for most of the markers analyzed. We only performed biomarker analysis in PBMCs and not in CD4+ T-cells or T-cell subsets which could also decrease the sensitivity of our results.

Overall, our data supports that ATI is safe and if combined with close monitoring and reinitiation of treatment, this intervention can be considered as the final and most comprehensive read-out of HIV cure trials. Furthermore ATIs give rise to unique opportunities to further investigate the viral reservoir both in blood and other anatomical compartments and should be used to investigate potential biomarkers for viral rebound.

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Declaration of Interests

HZ has served at scientific advisory boards for Roche Diagnostics, Wave, Samumed and CogRx, has given lectures in symposia sponsored by Biogen and Alzecure, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg (all outside the submitted work). The other authors declare that no competing interests exist.

Author Contributions

HZ generated the NFL and YKL-40 data, interpreted data and revised the manuscript for important intellectual content.

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