The Impact of a Therapeutic HIV-1 Vaccine on HIV-1 Proviral DNA and RNA Transcription in a Phase I Clinical Trial

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Human immunodeficiency virus (HIV) is an ongoing public health issue. Currently, antiretroviral therapy (ART) is used to treat people living with HIV (PLWH), but the virus persists in latent memory T cells despite viral suppression on ART. Recent therapeutic advances are aimed at finding functional cures of HIV. Alongside therapeutic research, there is a need for the development of quantitative assays to accurately measure the viral reservoir within individuals and evaluate the impact of interventions on reducing the size of the reservoir. In this thesis, I implement an automated nucleic acid extraction method followed by quantitative molecular assays to quantify longitudinal changes in Cell-Associated HIV RNA and DNA (CARD) using samples from a Phase I clinical trial. The results will help determine if study participants responded to vaccination with autologous dendritic cells (DC) that were loaded with or without HIV peptides, directly or through natural processing, compared to unloaded DC. As this is the first usage of the automated CARD system on clinical trial samples, we are also further evaluating its performance characteristics compared to the existing manual CARD assay.

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Preface

I would like to acknowledge my colleagues and mentors in the Mellors Lab. I am thankful to Asma Naqvi and Jana Jacobs for providing me with mentorship and guidance throughout the research process. I would also like to express my thanks to Leah Brandt who has been a great mentor and listener. Leah made herself available to talk through concepts or to read over working drafts and helped me to turn in a final product that I am proud of. I am thankful to those in the Mellors Lab that provided me with support throughout the project: Lou Halvas, Joshua Cyktor, Erin Salese, and Nathan McKenna. I would also like to express my thanks to John Mellors for giving me the opportunity to do research in the lab and supporting my growth as a scientist.

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I would like to acknowledge those involved in DC04, the Phase I autologous dendritic cell-based therapeutic vaccine clinical trial. The protocol chairs, Sharon Riddler, Bernard Macatangay, and John Mellors allowed me to use the data generated for the clinical trial in this thesis. Members of the Macatangay Lab in the School of Medicine received and processed samples from participants: Lori Caruso and Jenny Roscher. I would also like to acknowledge and thank the individuals that volunteered to participate in this study.

1.0 Introduction

Human immunodeficiency virus type-1 (HIV-1) is the etiologic agent of acquired immunodeficiency syndrome (AIDS), which began spreading rapidly worldwide in the 1980s. AIDS severely weakens the immune system and leads to other opportunistic infections, including tuberculosis, pneumonia, and Kaposi's syndrome (Greene, 2007). In 1983, HIV-1 was isolated and determined to be the retrovirus causing AIDS (Barré-Sinoussi et al., 1983; Gallo et al., 1984; Levy et al., 1984). Researchers around the world were focused on understanding the newly identified virus and its impact on human health. The first discovered antiretroviral was a repurposed cancer drug, azidothymidine (AZT), which was approved by the FDA for the treatment of HIV/AIDS in 1987. The application of AZT as AIDS treatment was a significant milestone in HIV/AIDS research; but the drug itself had harsh side effects, limited effectiveness, and could lead to drug resistant HIV. Research showed HIV-1 plasma RNA levels were indicative of disease progression, and reductions in HIV-1 plasma RNA resulted in delayed disease progression (Mellors et al., 1996; O'Brien et al., 1996). Combination therapies, highly active antiretroviral therapy (HAART), were developed to inhibit HIV-1 replication through several mechanisms, compared to AZT treatment that attacked HIV-1 through only inhibiting viral reverse transcriptase activity. Targeting multiple stages of the viral life cycle through HAART led to transformed HIV/AIDS treatment, combination therapy significantly reduced HIV-1 replication and improved immune function. Clinical responses to HAART were dramatic, and deaths from AIDS began to decrease in 1997. Although many clinical outcomes were positive, overall, they were still somewhat variable. Scientists began to understand HIV-1 persists in a latent reservoir of infected resting memory $CD4^+$ T cells (Blankson et al., 2002). Around this time, it was also discovered that HIV-1

continued to proliferate in individuals on HAART, even if those individuals had undetectable levels of viremia (Dornadula et al., 1999; Furtado et al., 1999). Since the initial discovery of HIV-1, scientists have made significant strides. With the introduction and continued development of antiretroviral therapy (ART) early treatment of HIV infection and viral suppression provides people living with HIV a more typical life expectancy (Ford et al., 2018). However, latent HIV persists in memory T cells in the form of integrated proviral DNA that prevents an individual from being completely cleared of the virus. Interruptions in ART regimes can result in viral rebound; thus, adherence to ART is critical in preventing new HIV infection of cells within individuals (Leal et al., 2021).

The HIV cure research field has undertaken various strategies to develop functional cures: latency reversal agents, CRISPR/Cas9 strategies, broadly neutralizing antibodies (bnAbs), and therapeutic vaccines (Deeks et al., 2016). Latency reversal agents reactivate viral transcription in an effort to allow ART and the host's immune system to attack and kill HIV-1 infected cells (Laird et al., 2015). CRISPR/Cas9 is a gene therapy method with the goal of directly removing HIV-1-infected cells by selectively deleting proviral DNA that is integrated into the host cell's genome (Hu et al., 2014). HIV-1 bnAbs are capable of neutralizing a virus by targeting highly conserved regions of the HIV envelope (Caskey et al., 2015). Different bnAbs target sites on the viral envelope, and humanized mice and macaque studies have shown these antibodies can suppress active HIV-1 infection (Barouch et al., 2013; Klein et al., 2012; Shingai et al., 2013). Therapeutic vaccines are used to strengthen the host's immune response to HIV-1. As previously learned with AZT and combination therapies, multiple targeted approaches are needed to target HIV-1; therefore, these therapies are being used concurrently in an effort to achieve HIV-1 remission. For

example, latency reversal agents reactivate HIV-1-infected cells in order for other therapy methods, like ART or a therapeutic vaccine, to kill infected HIV-1 cells.

One important and universal aspect of HIV-1 cure research is the need to accurately measure how a therapeutic intervention affects HIV-1 infected cells in the body. Baseline measurements of the HIV-1 reservoir can serve as a pre-intervention parameter to monitor responses to therapy. Cell-associated HIV-1 DNA and RNA are often measured as virological markers before, during, and after interventions. Cell-associated (CA) HIV-1 RNA originates from spliced transcripts that the cellular machinery is actively transcribing, which is obtained from the RNA fraction of nucleic extract. CA HIV-1 RNA is reverse transcribed and followed by qPCR for analysis (Pasternak & Berkhout, 2018). HIV-1 DNA is proviral DNA that has been integrated into genomic DNA, unintegrated DNA, and 2-LTR circles. HIV-1 DNA is used as a measure of total infected cells, and is directly analyzed from the genomic DNA in infected cells (Hong et al., 2016). CA HIV-1 RNA and DNA serve as markers used to measure the viral reservoir, which would provide value in determining changes from clinical interventions (Besson et al., 2014).

In this thesis, the author utilizes an adaptation from Hong et al. to measure CA HIV-1 RNA and DNA on a subset of samples from an ongoing clinical trial (Hong et al., 2016). The assay used in this trial differs from the assay utilized in Hong et al. because an automated extraction method is used to extract nucleic acid, compared to a manual extraction method employed previously. This thesis will assess differences in the automated extraction method compared to the manual extraction method by analyzing differences in performances characteristics between the two assays. Furthermore, this assay was implemented for the first time in a Phase 1 clinical trial of a therapeutic dendritic-cell based vaccine to gauge changes to HIV-1 reservoir size and transcriptional activity after therapeutic intervention. Specifically, CA HIV-1 RNA and DNA is

used to assess differences between vaccine and placebo arms. One of the secondary objectives of the Phase I clinical trial, and the objective driving this thesis, is to assess potential changes in CA HIV-1 RNA or DNA over 42 weeks. Given the clinical trial design uses participant-specific antigen-presenting cells combined with highly conserved regions in *gag* and *pol* with epitopes associated with viremia control, our team expects to observe a decrease in CA HIV-1 DNA and RNA from baseline to the end of the study, indicative of a cellular response to eliminating HIV-1-infected cells. However, any trends observed are speculative since the clinical trial is ongoing and all samples and corresponding treatment arms are masked. This paper will assess longitudinal changes in proviral DNA and RNA transcription through measuring CA HIV-1 RNA and DNA from 10 participants from the Phase I clinical trial.

1.1 Previous use of CARD assay

The published CARD assay was described by Hong et al., along with inter-assay variability and intra-assay variability (Hong et al., 2016). In the original study, PBMC from 5 viremic people living with HIV-1 (PLWH) and 20 PLWH receiving ART were used in the development and validation of a sensitive and specific cell-associated assay. CA HIV-1 RNA and DNA were quantified using qPCR and probes and primers that target a highly conserved region of the 3' end of *pol* on the HIV-1 genome (Li et al., 2016; Williams et al., 2014).

Results from Hong et al. showed CA HIV-1 RNA and DNA levels were strongly correlated, with an r value of 0.77 to 1. The longitudinally obtained PBMC samples, separated by at least 10 days, showed little overall inter-assay variation in both groups. The study also measured inter-assay and intra-assay variability. Inter-assay variability was measured by five replicates of

samples from the same 12 virally suppressed individuals on different assay runs. The standard deviation for CA HIV-1 DNA and CA HIV-1 RNA was 0.15 $\log_{10}$ copies/10^6 PBMCs and 0.4 $\log_{10}$ copies/10^6 PBMC respectively. The estimated standard deviation in intra-assay variability was measured to be 0.1 $\log_{10}$ copies/10^6 PBMCs and 0.19 $\log_{10}$ copies/10^6 PBMC for CA HIV-1 DNA and RNA, respectively. The sensitivity of the assay was measured to be between 3 to 5 copies per 1 million cells. Overall, the modest variability among assays along with the high specificity and sensitivity shows that CARD offers reliable and quantifiable biomarkers within the scope of clinical interventions.

The automated CARD assay is a useful tool to apply to large scale clinical trials because of its high throughput, efficient, and consistent abilities to assay a large number of samples in parallel. For an ongoing clinical trial, the automated CARD assay could detect changes in the HIV-1 reservoir related to the total number of HIV-1 infected calls in the body. For example, an increase in HIV-1 DNA levels would imply that cells are proliferating following the intervention. A decrease in HIV-1 DNA would imply a decrease in the number of infected cells. An increase in CA HIV-1 RNA implies an increase in transcriptional expression of proviruses, and a decrease in CA HIV-1 RNA implies transcriptional activity has decreased. When a therapeutic treatment such as an autologous dendritic cell vaccine is given to a participant, CA HIV-1 RNA and DNA can be an initial screening tool used to measure changes in proviral DNA and RNA transcription in response to the therapeutic intervention.

1.2 Previous dendritic cell vaccines

HIV-1 cure researchers are evaluating a number of diverse therapies for their abilities to clear the HIV-1 reservoir and provide a functional cure. Of particular interest are therapeutic dendritic cell (DC) vaccines. DCs are potent antigen presenting cells, and therapeutic DC vaccines enhance the immune system's capacity to recognize and clear HIV-1 infection through antigen specific immune responses (deLuca & Gommerman, 2012; Lu et al., 2004; Mohty & Gaugler, 2003). The clinical trial being presented in this thesis is measuring two treatment arms: autologous DCs loaded with peptides covering conserved and immunogenic HIV-1 epitopes or autologous DC being loaded with autologous heat-inactivated virus (Ondondo et al., 2016). Either treatment is given as 6 monthly doses to prime the immune system to induce an effective $CD8^+$ T cell response. DCs are a strong candidate for therapeutic vaccines due to their effectiveness in antigen-presentation, and have been shown *in vitro* to enhance HIV-1 specific T cell responses, which has been demonstrated in previous studies through elevated frequency of IFNγ-producing cells and correlated lower levels of plasma HIV-1 RNA (Smith et al., 2014). Furthermore, previously established HIV-1 and cancer DC-based vaccines have shown strong safety profiles while eliciting measurable immune responses (Connolly et al., 2008; Ide et al., 2006; Lévy et al., 2014; Lu et al., 2004; Macatangay et al., 2016).

Several previous studies have examined the efficacy of autologous DC vaccines (García, Climent, et al., 2013; García, Plana, et al., 2013; Ide et al., 2006; Lu et al., 2003, 2004). Initially, non-human primate models showed aldrithiol-2 (AT-2) inactivated HIV-1 loaded DC vaccines to elicit immune responses against simian immunodeficiency virus (SIV), measured by an increase in $CD4^+$ T cell counts (Lu et al., 2003). Non-human primates vaccinated with autologous DC loaded with inactivated virus had a decrease in SIV cellular DNA and plasma RNA levels 10 days

after vaccination, and a 1,000 fold decrease in plasma RNA measured after 6 weeks (Lu et al., 2003). Although 30% of the vaccinated non-human primates experienced a rebound in viral load after 8-17 weeks, the remaining 70% of vaccinated non-human primates retained a low, controlled viral load (<1,000 copies/mL) until the end of the study (42 weeks).

In subsequent human clinical trials, Garcia et al. found that myeloid derived DC vaccines designed for HIV-1 in humans had measurable decreases in plasma HIV-1 RNA copies/mL; however, the effects were not long lasting, which was thought to be due to persistent viral replication (García, Plana, et al., 2013). Similarly, Lu et al. showed HIV-1 infected individuals immunized with AT-2 activated HIV-1 loaded DCs experienced an 80% decrease in plasma viral RNA load, which remained stable throughout the year-long study. CA DNA concentrations decreased by 50% in the first 112 days of the study, but eventually showed fluctuating concentrations two-thirds of the way into the study, which was thought to imply a disconnect between virus specific $CD4^+$ T cells in promoting virus specific $CD8^+$ T cells, as well as a lack of a humoral response (Lu et al., 2004). More recently, an autologous DC vaccine combined with interferon alpha-2a showed no significant change in total HIV-1 DNA and no changes in T-cell responses between vaccine and placebo groups. However, the study did not measure changes in CA HIV-1 RNA (Leal et al., 2021).

These examples of therapeutic DC vaccines have all demonstrated a commonality of decreases in plasma HIV-1 RNA and cellular HIV-1 DNA over the short term, which suggests that HIV-1 plasma RNA, CA RNA as well as DNA assays are sensitive enough to measure immediate changes in immune responses, although the effects of the interventions may not be long lasting. The temporary control of the reservoir evidenced by autologous DC vaccines should continue to be an avenue in combination with additional interventions in the field of HIV-1 cure. Furthermore,

rapid, high-throughput screening tools (i.e. CA HIV-1 RNA and DNA), shown to be useful and sensitive in such large-scale intervention studies, are capable of quantifying trends in CA HIV-1 DNA and RNA.

2.0 Methods

2.1 Study design and clinical specimen

The automated CA HIV-1 RNA and DNA assay is being used in a Phase I, randomized, double-masked, clinical trial to quantify longitudinal changes in proviral HIV-1 DNA and cell-associated HIV-1 RNA being transcribed from these proviruses. Eligible participants included men and women between the ages of 18 and 65 that have maintained suppressive antiretroviral therapy for a minimum of 24 months with $CD4^+$ T cell counts of ≥350 cells/mm^3. This study was approved by the University of Pittsburgh Institutional Review Board (20110377). Written informed consent was obtained from all participants. Study participants were recruited from two sites, the University of Pittsburgh and The Ohio State University. Upon the completion of this trial (anticipated at study week 42), we will be able to examine the impact of DC-HIV-1 vaccination on CA HIV-1 RNA and DNA and to assess if there was a difference between vaccine arms.

Participants were enrolled in this study if HIV-1 virus was successfully isolated using a variation of the quantitative viral outgrowth assay into a fully autologous system (Finzi et al., 1997; Laird et al., 2013). For participants with successful isolation, leukapheresis was planned at Week 4 in the clinical trial. These participants were then randomly assigned into one of six arms of treatment (Table 1). Participants were scheduled to receive 6 doses of GMP-manufactured DC product every 4 weeks and were followed prospectively for an additional 48 weeks for a total study duration of 80 weeks. Participants underwent blood sample collections that were subsequently isolated into PBMC fractions through centrifugation (Figure 1). The cells were collected in 5 million cell aliquots and cryopreserved upon collection. Timepoints at which PBMCs were

collected to quantify CA HIV-1 RNA and DNA levels were weeks 4, 12, 16, 22, 29, 34, and 42 (Figure 1).

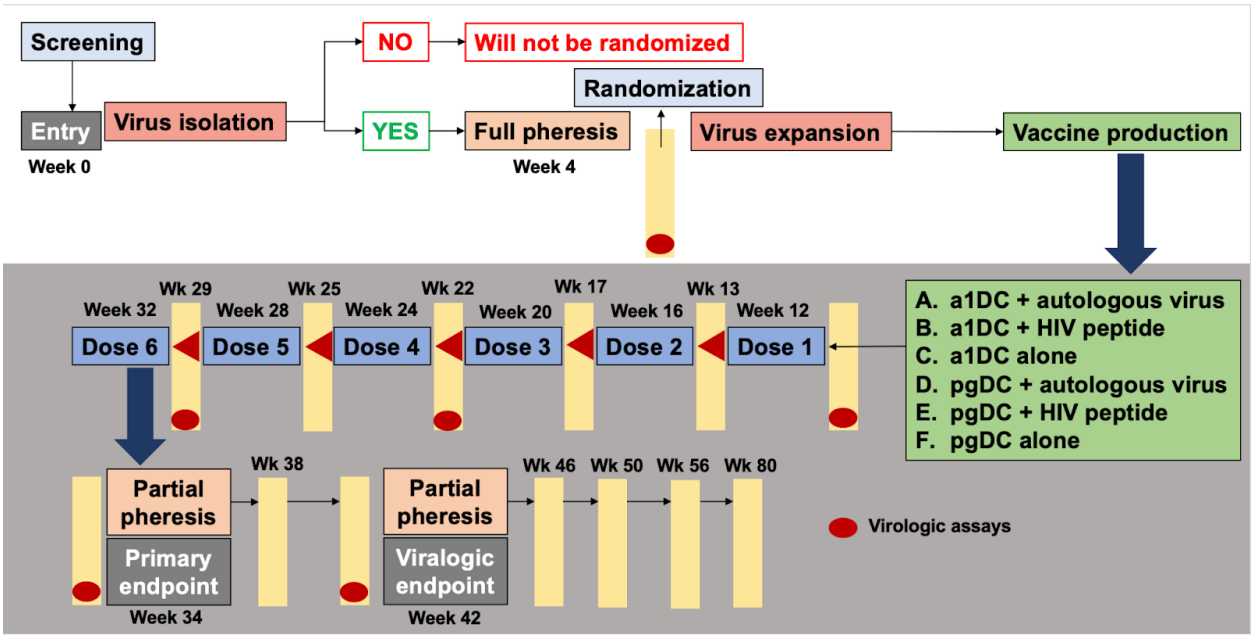
Table 1: Description of study groups

Arm	N	DC	Antigen	Dose	Frequency
A	8	a1DC[1]	Autologous HIV-1	10^7 DC	Q 4 week x 6
B	8	a1DC	Conserved peptides	10^7 DC	Q 4 week x 6
C	4	a1DC	None	10^7 DC	Q 4 week x 6
D	8	pgDC[2]	Autologous HIV-1	10^7 DC	Q 4 week x 6
E	8	pgDC	Conserved peptides	10^7 DC	Q 4 week x 6
F	4	pgDC	None	10^7 DC	Q 4 week x 6

[1] a1DC: optimized cocktail for alpha-1 DC

[2] pgDC: prostaglandin E2 cocktail

Figure 1: Study design relative CA HIV-1 RNA and DNA assay



2.2 CA HIV-1 RNA and DNA assay protocol and controls

The CA HIV-1 RNA and DNA assay used cell associated HIV-1 RNA and DNA as a measure of longitudinal change in reservoir size. In this study we utilized the KingFisher Flex Purification System (KingFisher Flex System, ThermoFisher Scientific, Waltham, MA, USA) because of its high throughput efficiency to extract both RNA and DNA in independent fractions. Our team has adapted the published CARD assay to incorporate an automated extraction of nucleic acids that allows for a higher throughput of samples to be processed at one time (Jacobs et al., 2019). Previous unpublished in-house experiments showed comparable nucleic acid recovery when compared with the manual nucleic acid extraction cited in Hong et al. In summary, the KingFisher Flex System uses magnetic beads to bind to either RNA or DNA with an external magnetic block to separate the nucleic acid-bound beads from the remaining sample components.

The KingFisher then washes the nucleic acid-bound beads to remove any impurities. Finally, the nucleic acid is eluted from magnetic beads and released into an elution buffer. Separate kits are used to extract either RNA or DNA (MagMAX mirVana Total RNA Isolation Kit or MagMAX DNA Multi-Sample Ultra 2.0 Kit).

The positive controls used in the CA HIV-1 RNA and DNA assay come from aliquots of PBMC from an individual with HIV-1 that has been previously analyzed in house with consistent CAD values that range from 1198-4311 copies/10^6 PBMC and CAR values between 47-636 copies/10^6 PBMC (Table 5). The KingFisher can accommodate up to 10 participant samples for each extraction, of which we include that positive extraction control as well as a negative control, which is PBMC from an individual without HIV-1. Cells from the control samples were incorporated in each sample plate for nucleic acid extractions and subsequently analyzed in the same downstream CA HIV-1 RNA and DNA protocols. Positive controls were also taken through a manual extraction in order to compare the recovery and concentrations of the target HIV-1 markers between manual and automated extractions. Positive control values from manual extractions were previously run by Asma Naqvi in the Mellors Lab and were included in the results with her permission.

2.3 CA HIV-1 DNA extraction

We examined CA HIV-1 RNA and DNA from 10 participant samples through quantification using an automated extraction, to quantify changes in $\log_{10}$ copies/10^6 PBMC. PBMC were thawed at 37 °C and split into 1.25 million cell aliquots, designated for RNA and DNA analyses. This cell number was previously found optimal in nucleic acid recovery. Equal

amounts of RPMI solution was added to the cells and centrifuged at 0.5 g to pellet the cells and remove the storage buffers. After removal of the supernatant, 100 µl of lysis buffer (GuHCl + ProK) was added and sonicated according to previously published protocol (Hong et al., 2016). Samples were then incubated in a bead bath at 42°C for an hour to lyse the cells. To prepare the King Fisher automated extraction, 380 µl of PBS and 440 µl DNA binding bead mixture (MagMAX DNA Multi-Sample Ultra 2.0 Kit) were added to the sample in a 96 deep well plate. We prepared all remaining reagents according to manufacturer instructions for the KingFisher Flex System (ThermoFisher Scientific, Waltham, MA, USA). The "MagMAX_Ultra2_Buccal_FLEX" system parameters were run on the KingFisher Flex Purification System (Thermo Fisher Scientific) for the extraction of DNA. Nucleic acids were eluted in 80 µl of elution buffer (MagMAX DNA Multi-Sample Ultra 2.0 Kit). After extraction, CA HIV-1 DNA was quantified using OD_{260} values (DeNovix DS-11 Spectrophotometer). The elution buffer is used downstream for qPCR.

2.4 CA HIV-1 RNA extraction

The second 1.25 million cell aliquot for RNA analysis was extracted on the KingFisher Flex Purification System (Thermo Fisher Scientific) using the MagMAX mirVana Total RNA Isolation Kit. To the washed cell pellet, as previously described, 200 µl of lysis binding mix was added and subsequently shaken for 5 minutes, per the manufacturer instructions. The lysate was used for extraction according to the manufacturer's instructions with the following modification: samples were eluted in 80 µl and a 15-minute heated, fast-bottom mix was added to the "AM1830DW" protocol. The final RNA eluent was quantified using OD_{260} values (DeNovix DS-

11 Spectrophotometer) and carried through immediately to RT-qPCR for the analysis of HIV-1 integrase.

2.5 Quantification of total CA HIV-1 DNA

CA HIV-1 DNA was quantified with qPCR using the Roche LightCyler 480 II in triplicate wells, 10 µl of DNA extract was added to 25 µl of qPCR master mix (LightCycler 480 Master ready-to-use reaction mixture, 400 nM forward and reverse primers, 200 nM probes designed to target *int* region [bases 4186 to 5913 of the HIV-1 reference sequence HXB2]) (Table 2) (Cillo et al., 2014). Sample DNA integrase concentrations were derived from within-run standard curves created by a serial 3.16-fold dilution of a purified in-house 1,000 copy/ul integrase standard template, ranging from 3,000 to 3 copies per PCR well (Hong et al., 2016). To assess the number of cell equivalents assayed by PCR, a 1:30 dilution of the nucleic acid extract was quantified in duplicate wells by PCR for the CCR5 gene (Malnati et al., 2008). A CCR5 DNA standard curve was derived from a 1:1 dilution of control genomic DNA (Invitrogen TaqMan Control Genomic DNA, Thermo Fisher Scientific, USA) to generate a standard curve, CCR5 copy numbers were used to calculate the number of integrase copies per 10^6 cell equivalents.

2.6 Quantification of total CA HIV-1 RNA

The CA HIV-1 RNA eluent was quantified for HIV-1 integrase bases 4186 to 5913 of the HIV-1 reference sequence HXB2) using a two-step RT-qPCR assay on the Roche LightCyler 480

II. A 10 µl volume of the RNA extract was used to generate cDNA in five separate reactions with the AffinityScript multiple temperature reverse transcriptase (Aligent, USA) on a thermocycler (Bio-Rad S1000 Thermal Cycler) [25 °C for 15 minutes, 42 °C for 40 minutes, 90 °C for 30 minutes, 25 °C for 10 minutes]. A no-reverse transcriptase control reaction was also run on the plate to ensure that no HIV-1 DNA was carried over into the RNA eluent.

Three of the cDNA reactions from the CA-RNA fraction were analyzed for the same HIV-1 integrase region, as described above (Table 2). Two of the five cDNA reaction wells were used to assay a cellular housekeeping gene, IPO8, which serves as an internal control for RNA recovery, as it is expressed in resting and activated lymphocytes (Cillo et al., 2014).

Table 2: Probes and primer sequences

Probe/Primer	**Sequence**
INT Fw	**5'-TTTGGAAAGGACCAGCAAA-3'**
INT Rv	**5'-CCTGCCATCTGTTTTCCA-3'**
INT probe	**5'-[6FAM]AAAGGTGAAGGGGCAGTAGTAATACA[TAMRA]-3'**
CCR5 Fw	**5'-ATGATTCCTGGGAGAGACGC-3'**
CCR5 Rv	**5'- AGCCAGGACGGTCACCTT-3'**
CCR5 Probe	**5'-[6FAM]AACACAGCCACCACCCAAGTGATCA[TAMRA]-3'**
IPO8 Fw	**5'-GCTCTGATAACTGTGCAG-3'**
IPO8 Rv	**5'-CAGTGTGTACACCTCCTG-3'**
IPO8 Probe	**5'-[6FAM]TGCTGTCCTCTGATCCTCGC[TAMRA]-3'**

2.7 Statistical analysis

Within-assay variation was determined using the coefficient of variation and reported in Table 5. The mean, range, standard deviation, and coefficient of variation of the positive controls from this automated assay was compared to the manual version of this assay using the same controls. A t test of the means was performed to determine if there is a significant difference in $\log_{10}$ copies/10^6 PBMC between the automated assay and the manual assay.

The LightCycler 480 software (Version 1.5.1) was used to determine cycle thresholds and to quantify the copy numbers based on previously described standard serial dilutions. Levels of CA HIV-1 RNA that were reported as less than the limit of detection were analyzed as one-half of the corresponding limit of detection. The fold change between baseline, the third dose, and the final dose were determined and compared. A paired t test was used to determine significant change over time for an individual. R and R-Studio (version 12.0) and Prism (version 10.2.1) were used to perform statistical analysis.

3.0 Results

The purpose of this thesis was to measure and analyze the levels of CA HIV-1 RNA and DNA in trial participants and to test performance characteristics of the automated CA HIV-1 RNA and DNA assay compared to the manual CA HIV-1 RNA and DNA assay. As this is an ongoing trial, data from 10 participants are analyzed without unmasking of treatment arm participants were randomly assigned to. Rather, changes in CA HIV-1 RNA and DNA across the group and individually will be presented. Therefore, the research objective is to characterize any general changes associated with vaccine dosing and length of study in CA HIV-1 RNA and DNA through the course of the clinical study. Specifically, the analysis will focus on whether there are significant fold changes in CA HIV-1 RNA or DNA from baseline to the third dose, from the third dose to the final dose, and from baseline to the final dose across all 10 participants.

3.1 Performance characteristics

The positive controls used for this assay are aliquots from one donor at the same time point with 5 million PBMC per aliquot. Therefore, these values provide a reliable measure of performance characteristics. For the automated CAD assay performed in this study, the mean was 3.33 $\log_{10}$ copies/10^6 PBMC with an estimated standard deviation of 0.22 $\log_{10}$ copies/10^6 PBMC (Table 3). Seven runs of manual CAD using the same positive control were used as a comparison. The mean for the manual CAD assay was 3.19 $\log_{10}$ copies/10^6 PBMC with a standard deviation of 0.26 $\log_{10}$ copies/10^6 PBMC (Table 3). A t test of means generated a test statistic of -1.2 (p-

value = 0.25). The small test statistic and large p-value implies the null hypothesis cannot be rejected and there is no significant change in $\log_{10}$ copies/10^6 PBMC between the automated and manual CAD assay. This is also shown in Figure 14, the median and interquartile ranges are similar across assays, the auto CAD assay produced a median of 3.32 $\log_{10}$ copies/10^6 PBMC with an interquartile range of 3.13 to 3.5 $\log_{10}$ copies/10^6 PBMC and the manual CAD assay has a median of 3.19 $\log_{10}$ copies/10^6 PBMC with an interquartile range of 3.04 to 3.5 $\log_{10}$ copies/10^6 PBMC.

The mean $\log_{10}$ copies/10^6 PBMC from the auto CAR assay measured in this study is 2.41 $\log_{10}$ copies/10^6 PBMC, with a standard deviation of 0.37 $\log_{10}$ copies/10^6 PBMC. The manual CAR assay produced a mean of 1.88 $\log_{10}$ copies/10^6 PBMC with a standard deviation of 0.72 $\log_{10}$ copies/10^6 PBMC (Table 3). A t test of means generated a test statistic of -1.8 (p-value = 0.11), therefore the difference in RNA $\log_{10}$ copies/10^6 PBMC between the two assays is not significant. However, Figure 2 shows the automated CAR assay produced more consistent results, which is in line with the smaller standard deviation of 0.37 $\log_{10}$ copies/10^6 PBMC compared to 0.72 $\log_{10}$ copies/10^6 PBMC. Similarly, the interquartile range from the automated CAR assay is 2.17 to 2.67 $\log_{10}$ copies/10^6 PBMC compared to 1.56 to 2.33 $\log_{10}$ copies/10^6 PBMC from the manual CAR assay (Table 3).

Figure 2: Positive control log_{10} copies/10^6 PBMC from the automated CAD assay, automated CAR assay, manual CAD assay, and manual CAR assay. Median and interquartile range shown

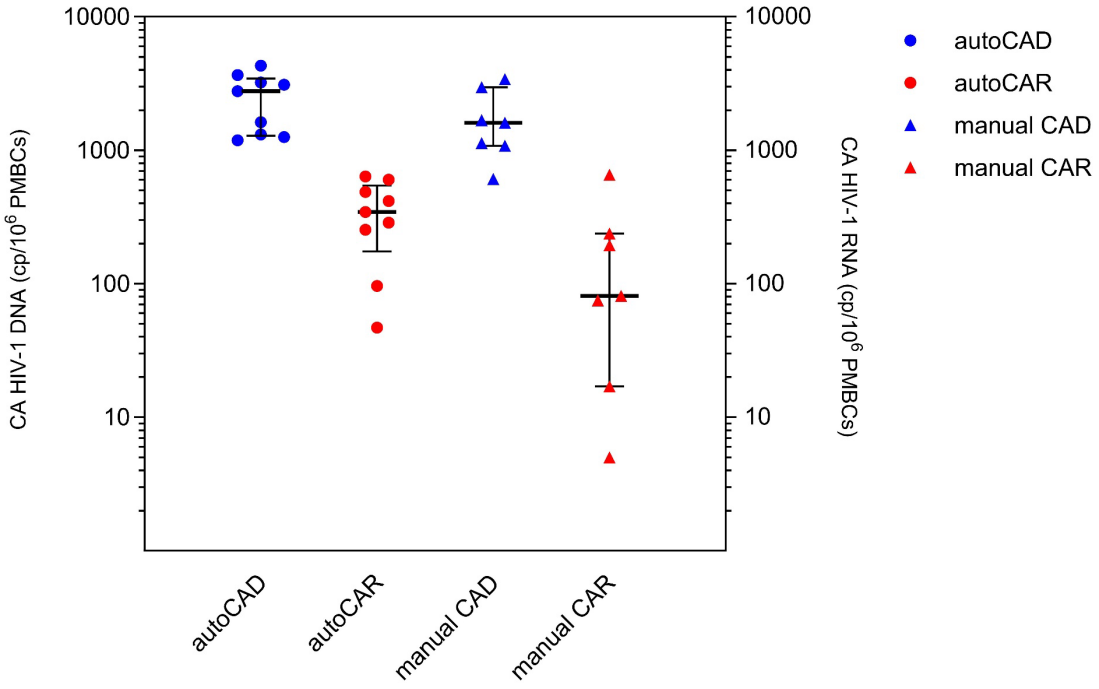


Table 3: Manual and auto comparison for CA HIV-1 DNA and RNA

CAD Assay			
Parameter	Manual	Automated	Paired t test (p-value)
Mean ± SD (log_{10} cps/10^6 PMBC)	3.19 ± 0.26	3.33 ± 0.22	-1.2 (0.25)
Median (IQR) (log_{10} cps/10^6 PMBC)	3.21 (3.04 - 3.5)	3.32 (3.13 - 3.5)	
Coefficient of Variation	8.2	6.5	
CAR Assay			
Mean ± SD (log_{10} cps/10^6 PMBC)	1.88 ± 0.72	2.41 ± 0.37	-1.8 (0.11)
Median (IQR) (log_{10} cps/10^6 PMBC)	1.91 (1.56 - 2.33)	2.5 (2.17 - 2.67)	
Coefficient of Variation	38.2	15.5	

3.2 Overall change in CA HIV-1 RNA and DNA

Table 4 shows the fold change in CA HIV-1 DNA across participants at baseline, post-third dose, and after the final dose of a vaccine or placebo. The mean fold change in CA HIV-1 DNA from baseline to the third dose was 1.11 ± 0.69, from the third dose to the sixth dose was 1.08 ± 0.38, and from baseline to the final dose was 1.09 ± 0.56. These fold changes were not significant (paired t test): from baseline to the third dose and third dose to final dose (p-value = 0.90), baseline to third dose and baseline to final dose (p-value = 0.74), or third dose to final dose and baseline to final dose (p-value = 0.97). Figure 3 shows the median fold change for CA HIV-1

DNA among all participants from baseline to the end of the study (Week 42). The median fold change was slightly above or below zero for all timepoints, with overlapping interquartile ranges (Figure 3). The data show that there were not significant differences in CA HIV-1 DNA fold change at the time points measured.

Table 5 shows the fold change of CA HIV-1 RNA across participants at baseline, post-third dose, and after the final dose of a vaccine or placebo. The mean fold change in CA HIV-1 RNA from baseline to the third dose was 1.05 ± 0.55, from the third dose to the sixth dose was 1.12 ± 0.5, and from baseline to the final dose was 1.06 ± 0.67. These fold changes were not significant (paired t test): from baseline to the third dose and third dose to final dose (p-value = 0.82), baseline to third dose and baseline to final dose (p-value = 0.73), or third dose to final dose and baseline to final dose (p-value = 0.99) (Table 5). The median CA HIV-1 RNA fold change was around 1 through Weeks 12 to 34, there was a slightly bigger fold change observed at Week 42, however interquartile ranges are overlapping (Figure 4). The data for CA HIV-1 RNA changes among the group of participants shows slight variation in fold changes across time points, but no significant changes.

Figure 3: CA HIV-1 DNA fold change in log_{10} cps/10^6 PBMC

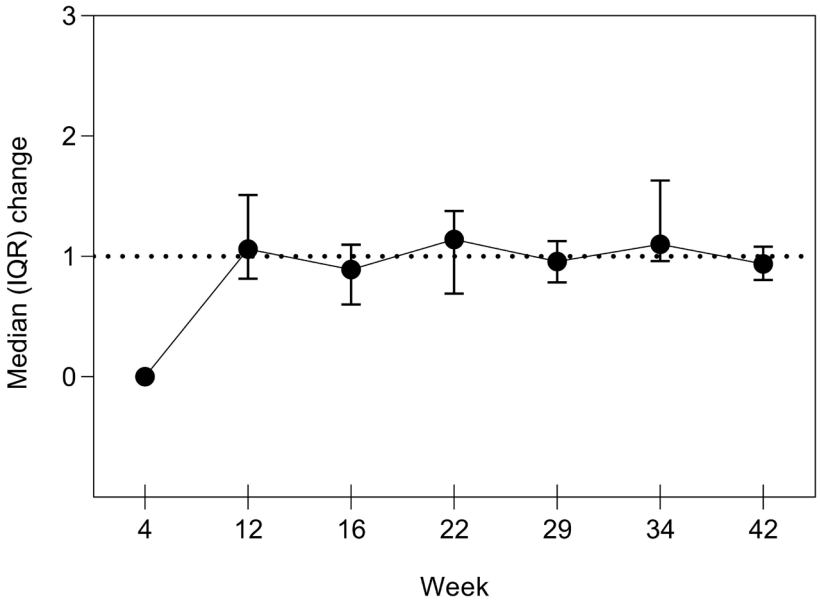


Figure 4: CA HIV-1 RNA fold change in log_{10} cps/10^6 PMBC

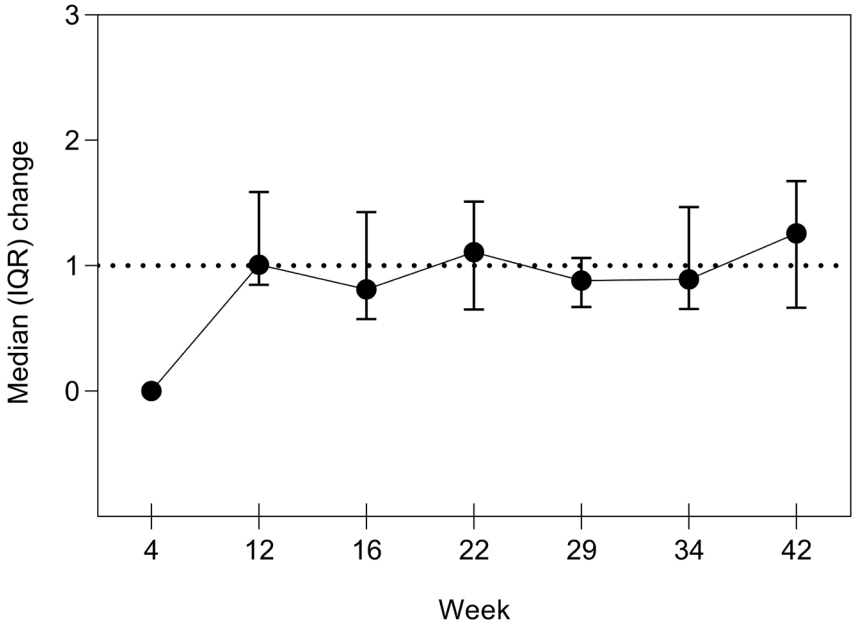


Table 4: Summary of fold change for CAD levels in PBMC (log_{10} cps/10^6 cells)

Participant	Fold change between Baseline and Dose 3 CA HIV-1 DNA	Fold change between Dose 3 and Dose 6 CA HIV-1 DNA	Fold change between Baseline and Dose 6 CA HIV-1 DNA
1	0.46	1.17	0.54
2	0.52	1.81	0.94
3	1.02	1.01	1.03
4	0.63	1.70	1.07
5	1.25	0.75	0.94
6	2.89	0.89	2.57
7	0.91	0.94	0.85
8	1.05	0.69	0.73
9	1.48	0.85	1.26
10	0.95	0.97	0.92
Mean	1.11 ± 0.69	1.07 ± 0.38	1.09 ± 0.56

Table 5: Summary of fold change for CAR levels in PBMC (log_{10} cps/10^6 cells)

Participant	Fold change between Baseline and Dose 3 CA HIV-1 RNA	Fold change between Dose 3 and Dose 6 CA HIV-1 RNA	Fold change between Baseline and Dose 6 CA HIV-1 RNA
1	0.29	1.42	0.41
2	0.85	0.8	0.68
3	1.19	1.62	1.37
4	0.59	0.84	0.50
5	1.92	0.22	0.42
6	0.88	1.25	1.10
7	1.0	1.92	1.92
8	2.01	1.08	2.18
9	1.14	1.37	1.57
10	0.67	0.65	0.44
Mean	1.05 ± 0.55	1.12 ± 0.5	1.06 ± 0.67

3.3 Individual changes in CA HIV-1 RNA and DNA

When looking at individual participants, there are trends in CA HIV-1 RNA and DNA across the time points measured. All participants had higher levels of CA HIV-1 DNA compared to CA HIV-1 RNA. Participant 1 showed a steady decrease in CA HIV-1 DNA (3.0 to 2.1 log_{10} copies/10^6 PBMC) and an increase in CA HIV-1 RNA at Week 16 (2.5 log_{10} copies/10^6 PBMC) that decreased again at Week 22 (0.6 log_{10} copies/10^6 PBM) (Figure 5). Participant 2 showed a decrease in CA HIV-1 DNA log_{10} copies/10^6 PBMC between Weeks 16 and 29 (3.0 to 2.6 log_{10} copies/10^6 PBMC) and then a return to baseline at Week 34 (3.3 log_{10} copies/10^6 PBMC) (Figure

6). CA HIV-1 RNA levels showed a modest change for Participant 2, ranging from 1.7 to 2.2 $\log_{10}$ copies/10^6 PBMC from Week 4 to Week 42. Participant 3 had steady levels of CA HIV-1 RNA and DNA throughout the trial, with a modest increase in RNA at Week 34 that decreased again at Week 42 (2.3 to 1.9 $\log_{10}$ copies/10^6 PBMC) (Figure 7). Other participants had more fluctuations in CA HIV-1 RNA levels, 4 participants experienced a spike in RNA levels, followed by a decrease and return to baseline (Figure 5, 12, 13, 14). Participant 7 had no CA HIV-1 RNA detected on this assay at Weeks 4, 22 and 34, but maintained consistent levels of CA HIV-1 DNA throughout the study (mean = 2.4 $\log_{10}$ copies/10^6 PBMC) (Figure 11).

Figure 5: Participant 1. Total CA HIV-1 DNA $\log_{10}$ copies/10^6 PBMC in blue on the left axis in log scale. Total CA HIV-1 RNA $\log_{10}$ copies/10^6 PBMC in red on the right axis in log scale. The dotted vertical lines represent injection timepoints.

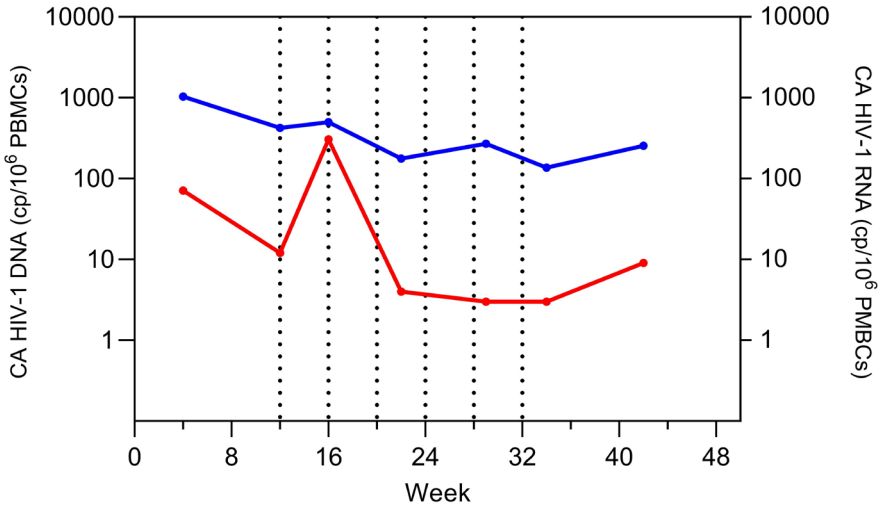


Figure 6: Participant 2. Total CA HIV-1 DNA log_{10} copies/10^6 PBMC in blue on the left axis in log scale. Total CA HIV-1 RNA log_{10} copies/10^6 PBMC in red on the right axis in log scale. The dotted vertical lines represent injection timepoints.

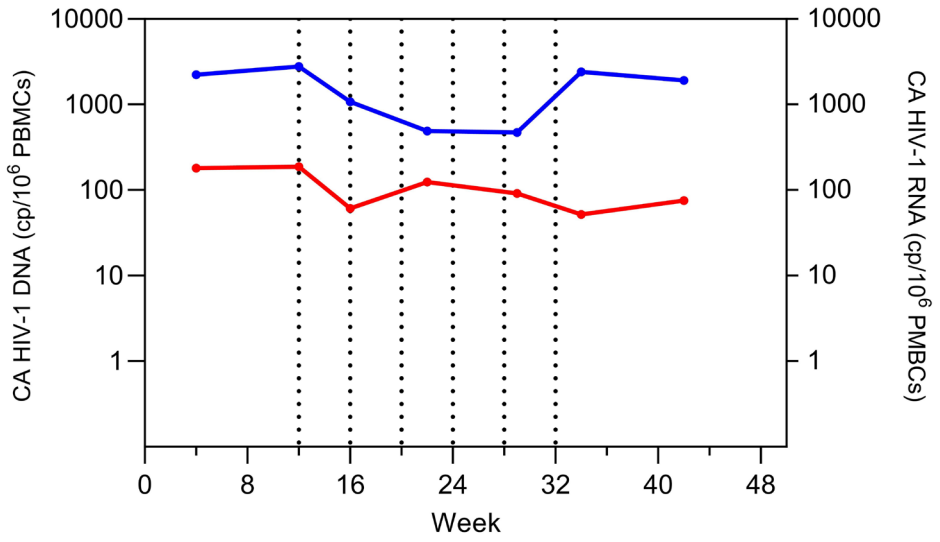


Figure 7: Participant 3. Total CA HIV-1 DNA log_{10} copies/10^6 PBMC in blue on the left axis in log scale. Total CA HIV-1 RNA log_{10} copies/10^6 PBMC in red on the right axis in log scale. The dotted vertical lines represent injection timepoints.

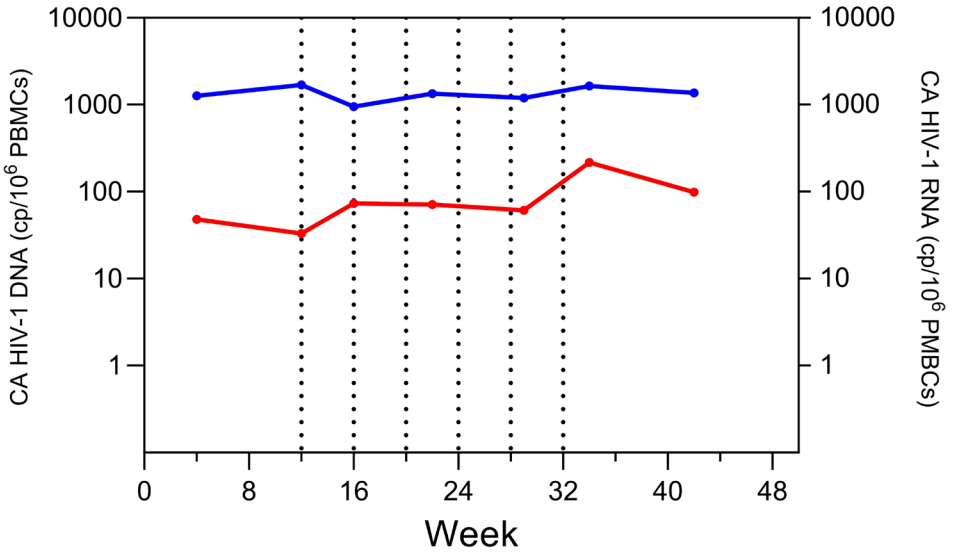


Figure 8: Participant 4. Total CA HIV-1 DNA log_{10} copies/10^6 PBMC in blue on the left axis in log scale. Total CA HIV-1 RNA log_{10} copies/10^6 PBMC in red on the right axis in log scale. The dotted vertical lines represent injection timepoints.

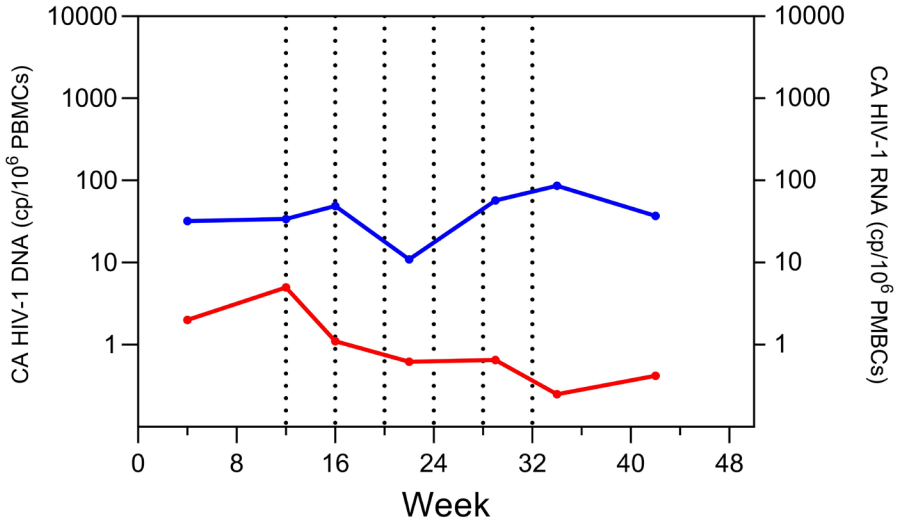


Figure 9: Participant 5. Total CA HIV-1 DNA log_{10} copies/10^6 PBMC in blue on the left axis in log scale. Total CA HIV-1 RNA log_{10} copies/10^6 PBMC in red on the right axis in log scale. The dotted vertical lines represent injection timepoints.

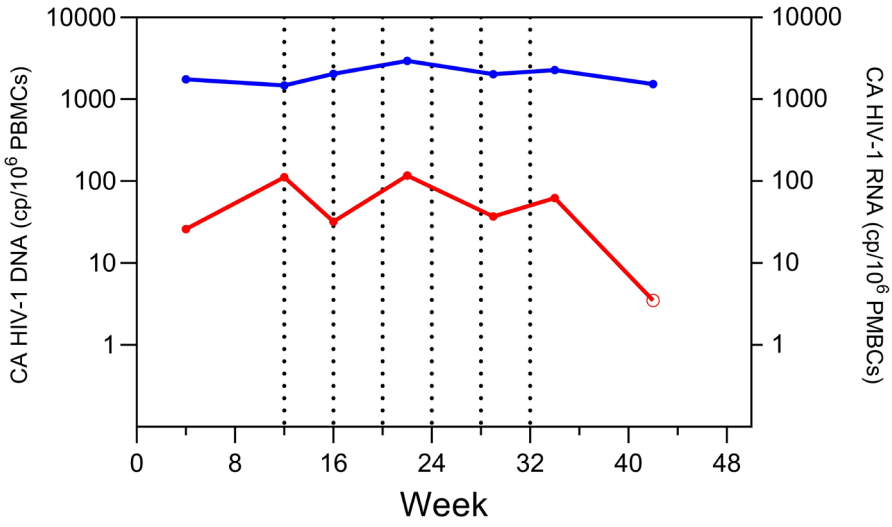


Figure 10: Participant 6. Total CA HIV-1 DNA log_{10} copies/10^6 PBMC in blue on the left axis in log scale. Total CA HIV-1 RNA log_{10} copies/10^6 PBMC in red on the right axis in log scale. The dotted vertical lines represent injection timepoints.

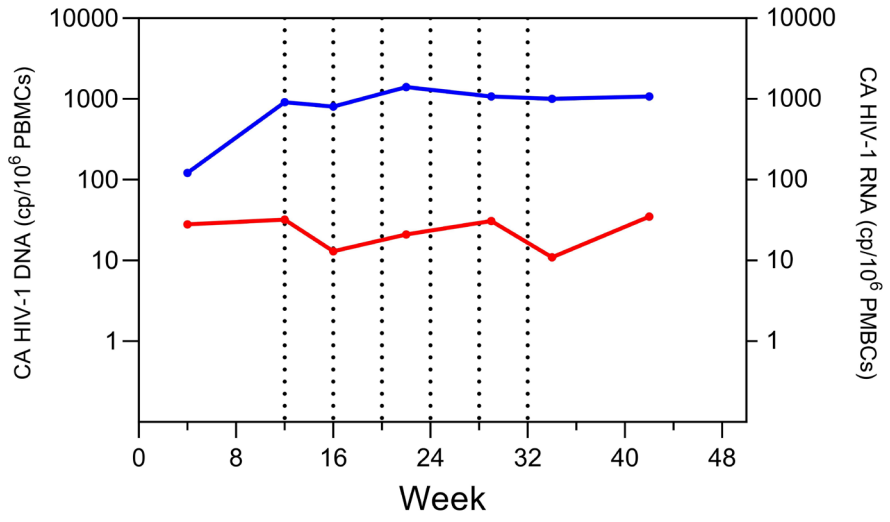


Figure 11: Participant 7. Total CA HIV-1 DNA log_{10} copies/10^6 PBMC in blue on the left axis in log scale. Total CA HIV-1 RNA log_{10} copies/10^6 PBMC in red on the right axis in log scale. The dotted vertical lines represent injection timepoints.

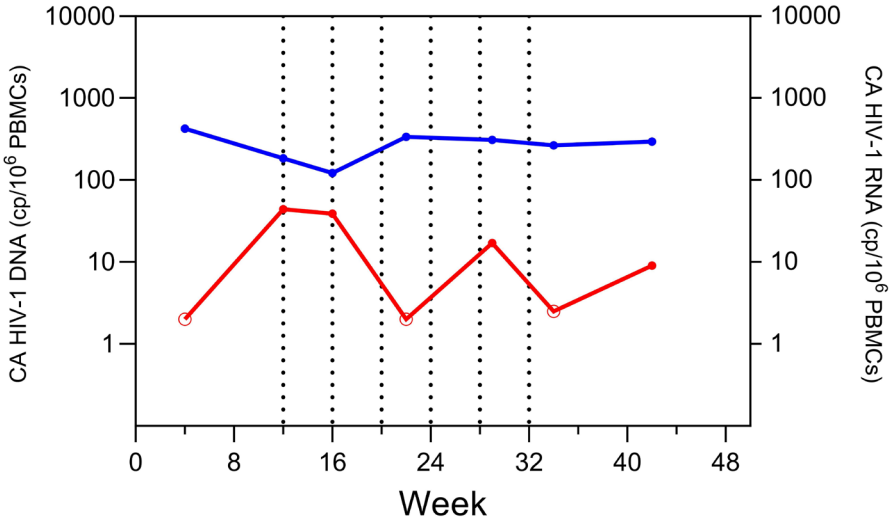


Figure 12: Participant 8. Total CA HIV-1 DNA log_{10} copies/10^6 PBMC in blue on the left axis in log scale. Total CA HIV-1 RNA log_{10} copies/10^6 PBMC in red on the right axis in log scale. The dotted vertical lines represent injection timepoints.

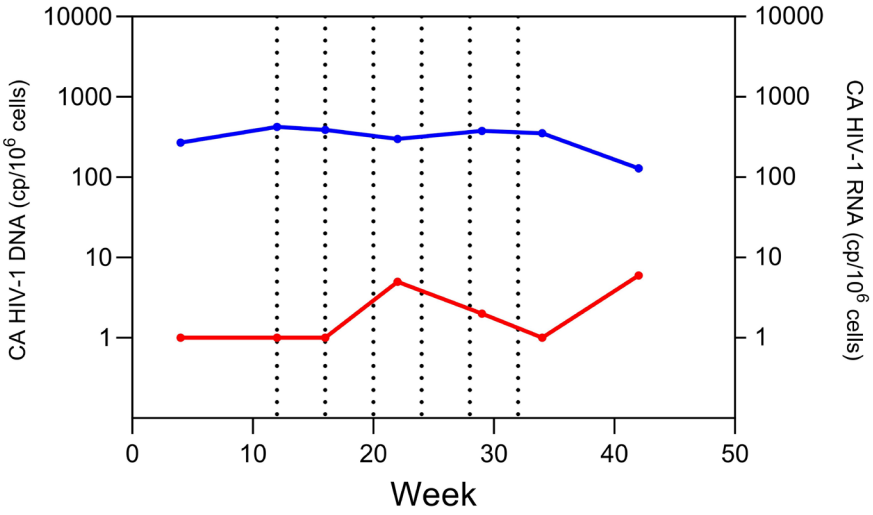


Figure 13: Participant 9. Total CA HIV-1 DNA log_{10} copies/10^6 PBMC in blue on the left axis in log scale. Total CA HIV-1 RNA log_{10} copies/10^6 PBMC in red on the right axis in log scale. The dotted vertical lines represent injection timepoints.

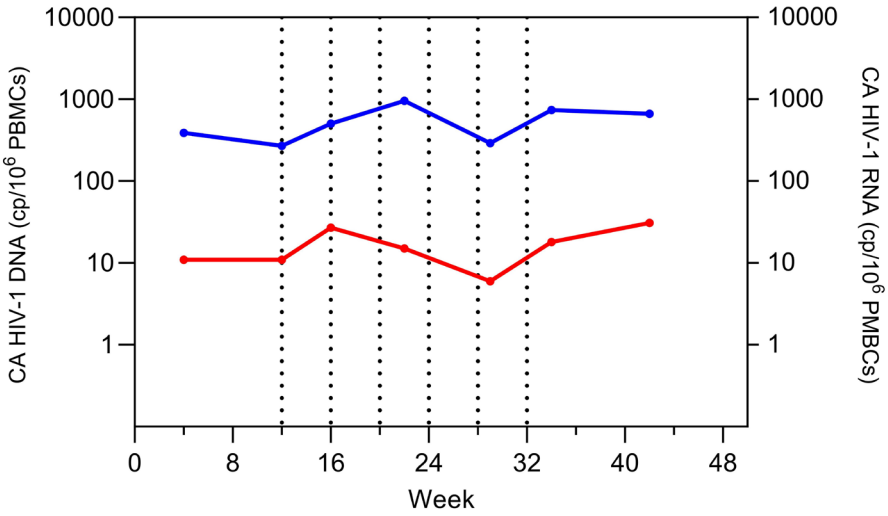
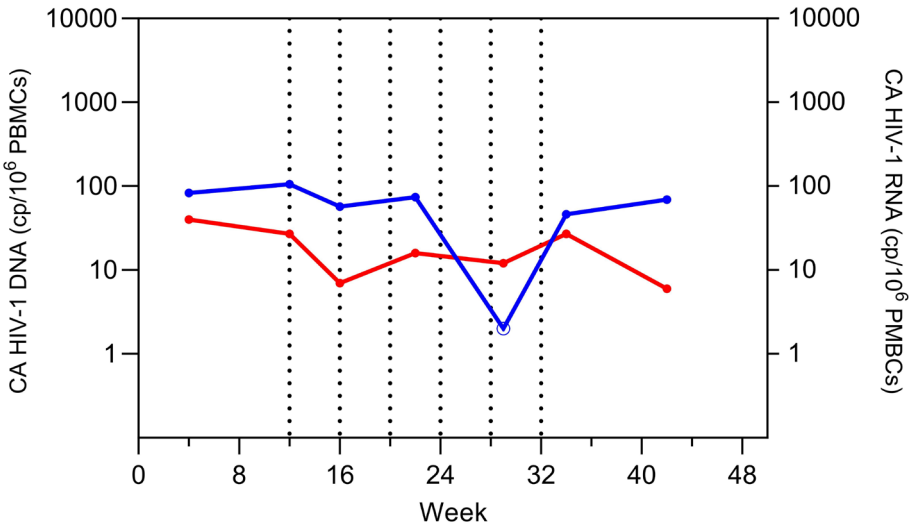


Figure 14: Particpant 10. Total CA HIV-1 DNA log_{10} copies/10^6 PBMC in blue on the left axis in log scale. Total CA HIV-1 RNA log_{10} copies/10^6 PBMC in red on the right axis in log scale. The dotted vertical lines represent injection timepoints.



4.0 Discussion and conclusions

In summary, the automated DNA extraction method showed a modest difference in variation compared to the manual DNA extraction method, which was insignificant. The CAR extraction method showed less inter-assay variation compared to manual CAR, although a paired t test found the difference in means to be insignificant (Table 3). When evaluating changes in CA HIV-1 RNA or DNA among the group of 10 participants, there were no significant changes. In individual circumstances, CAR levels did fluctuate; however, there was no clear pattern to discern among the group. CAD levels were more stable among individuals, with some temporary decreases that resulted in a return to levels similar to baseline.

Although results comparing the automated and manual extraction methods were not significant, the automated extraction method did have slightly less variation among repeated samples for both RNA and DNA, with standard deviations of 0.37 and 0.22, respectively. The automated extraction has qualitative benefits as well; it is faster, high-throughput, and decreases the chances of human error. On the King Fisher Flex System, the DNA extraction runs for 30 minutes, and the RNA extraction runs for 40 minutes. In total, the automated extraction method takes about 2.5 hours to complete, compared to the manual extraction method that takes at least double the amount of time. The automated extraction method utilizes the King Fished Flex System, which can handle up to 96 samples during a single extraction. The manual extraction method is done by hand, which limits the number of samples capable of being extracted at once. Because the manual extraction method takes longer and is done by hand, there is a higher chance of human error playing a role. RNA is unstable and degrades easily, therefore a longer extraction process could lead to degradation of the nucleic acid. The streamlined and quicker automated extraction

protocol limits the amount of time RNA is being handled and as a result can control for potential degradation of the nucleotides.

The ongoing nature of this trial means participants' group allocation cannot yet be unmasked. There could be a change in CA HIV-1 RNA or DNA copies that is occurring among vaccine groups that was not measured in this analysis. Because there was no dependent variable to anchor the independent variable to, we were limited in the statistical tests that could be utilized. A paired t test was used to determine if there was a difference between mean fold changes measures, however with more information a repeated measures ANOVA test could provide a stronger conclusion. Another limitation of the CARD assay is that it does not distinguish whether the provirus is intact and capable of producing virions or just merely defective. The assay measures a highly conserved region on the 3' end of *pol*, but does not discern whether the genome is intact or defective. Therefore, we cannot make any conclusions about the impact of this increase on the rest of the viral reservoir based on this assay. However, there are assays that use multiplex digital droplet PCR to distinguish and quantify intact proviruses. Bruner et al. has reported on an intact proviral DNA assay that quantifies intact HIV-1 provirus, and this assay can used to distinguish provirus that is implied to be replication competent from defective virus (Bruner et al., 2019). The Mellors lab is developing a similar assay that includes more targets to better model the intact provirus. Also, sequencing could be done on these samples to determine if the genome is inferred intact, which would help to determine if active transcription of HIV-1 mRNA implies replication competent virus is being made (Wright et al., 2021). A third limitation is the small sample size, which limits the strength of the analysis. As this cohort was taken from an ongoing clinical trial, the ability to measure all timepoints of a participant relied on completion of the study.

The CARD assay was included in this Phase I clinical trial with the purpose of measuring longitudinal changes in CA HIV-1 RNA and DNA. In a clinical trial setting, the automated nature of this assay provides a reliable method of measuring changes in the reservoir. In this autologous DC vaccine trial, the CA HIV-RNA and DNA assay is being used alongside single copy assay, $CD8^+$ T cell immune response responses, intracellular cytokine staining, and measurements of immune activation and systemic inflammation. Together, these measurements will provide insight that helps understand how the therapeutic vaccine is impacting the intact viral reservoir in a participant living with HIV-1.

Furthermore, the assay measured in this study is part of a larger goal to develop a functional cure for HIV-1. A functional cure for HIV-1 is possible when the immune system can control an HIV-1 infection without the assistance of combination ART with the virus is still present but remaining latent (Bailon et al., 2020). A functional cure is different from a sterilizing cure, in which there is no replication competent provirus in the body. Although combination, life-long ART has changed the HIV-1 landscape for the better, access and adherence to treatment are key factors in a favorable prognosis (Espinar-Buitrago & Muñoz-Fernández, 2022). Both access and adherence can be impacted by outside variables, including availability of ART, cost, social stigma, or drug toxicities (Espinar-Buitrago & Muñoz-Fernández, 2022; Leal et al., 2021). Therefore, a more sustainable solution is needed for people on life-long ART. The purpose of functional cure research is to explore therapies that can lead to HIV remission. To reach this goal, precise, quantitative assessments of an individual's viral reservoir are necessary to measure changes after a therapeutic intervention. This thesis evaluated the performance characteristics, throughput, and capabilities of an automated assay to quantify HIV-1 proviral DNA and RNA transcription in samples from a Phase I clinical trial for the first time. The formal results from our assay will be

analyzed alongside other measurements to fully characterize the effect of this unique intervention on the HIV-1 reservoir and determine if vaccination with specialized DC could lead to a functional cure of HIV-1.

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