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## Laidlaw Scholar Report

# Characterisation of the Cellular HIV Reservoir in Differently Treated Children Using PCR Methods

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# Introduction

Human Immunodeficiency Virus (HIV), first identified in the 1980s, continues to be an ongoing pandemic (25). As a retrovirus, it uses ribonucleic acid (RNA) as its genetic material, using reverse transcriptase to reverse transcribe the RNA into deoxyribonucleic acid (DNA) which gets integrated into the human host's DNA (15). Ongoing development of antiretroviral therapy (ART) which targets this process has transformed the infection into a manageable chronic condition for adults, but treatment for paediatric HIV remains challenging. An estimated 2.38 million children worldwide are living with HIV, a vulnerable population who face unique barriers to accessing and adhering to ART (12). These barriers include poor palatability, unsuitable formulations—which do not consider the differences between adults and children—or significant drug interactions, for example between HIV and tuberculosis (TB) medications, which can affect children more severely (6, 13). In addition, despite effective suppression of the virus with ART, HIV persists in the body as a latent reservoir in infected cells. This means that HIV treatment must be taken lifelong, as the presence of a latent reservoir can lead to viral rebound if treatment is discontinued. For children, the reliance on caregivers for medication adherence makes this harder, and for adolescents, psychosocial struggles can lead to intentional non-adherence. Poor adherence increases the risk of viral rebound and the development of drug-resistances, a growing global concern (16, 19).

The ODYSSEY trial, conducted between 2016 and 2018, was a pivotal randomized controlled trial (RCT) that compared the efficacy and safety of dolutegravir (DTG), a second-generation integrase strand transfer inhibitor (INSTI), to standard of care (SOC) regimens in children and adolescents. This study enrolled over 700 participants from diverse geographical regions, including low- and middle-income countries, where the burden of paediatric HIV is highest. Participants were divided into two main cohorts: ODYSSEY A, children starting first-line ART, and ODYSSEY B, children starting second-line ART, switching from a failing regimen to a regimen with or without dolutegravir. Within each cohort, patients either received a DTG-based treatment, which included dolutegravir plus two nucleoside reverse transcriptase inhibitors (NRTIs), or SOC, which included a protease inhibitor (PI) or non-nucleoside reverse transcriptase inhibitor (NNRTI) plus two or three NRTIs. Results demonstrated that DTG-based regimens are non-inferior to SOC in preventing treatment failure among children, consistent across different weight bands, age groups, and geographic regions. The findings highlighted dolutegravir's potential to address key challenges in paediatric HIV treatment, offering advantages such as lower cost, once-daily dosing, and fewer drug-drug interactions. The development of child-friendly dolutegravir formulations, like dispersible tablets, as well as its ability to be continued seamlessly into adulthood, has further improved accessibility in resource-limited settings (7, 8, 11, 18, 26).

So far, the ODYSSEY trial measured differences in the viremia—copy numbers of the virus in the plasma. As part of a sub study furthering results from this trial, current research at University College London (UCL) Great Ormond Street Institute of Child Health (GOS ICH) is investigating the virological characteristics of the reservoir, looking at the proviral load within peripheral blood mononuclear cells (PBMCs), comparing the effects observed of DTG against SOC. This report will reflect on the work I did in assisting this research.

# Background

HIV primarily targets CD4+ helper T-cells but also macrophages and dendritic cells, using CD4 as a main receptor and CCR5 or CXCR4 as co-receptors. The virus consists of two single strands of positive-sense ribonucleic acid (RNA). The genome comprises of three main genes: gag, pol, and env, which encode structural proteins, enzymes essential for viral replication, and surface proteins, respectively. These genes are flanked by long terminal repeats (LTRs), critical for regulating viral replication. In the viral particle, the RNA is enclosed by a nucleocapsid protein (p24) and matrix proteins (p17), all surrounded by a viral envelope containing embedded transmembrane proteins (gp41) and surface glycoproteins (gp120) (15).

The HIV replication cycle includes two main phases: early replication, involving viral entry and integration into the human host genome, and late replication, which relies on the host's cellular machinery to produce new viral particles. During early replication, HIV binds to the CD4 receptor and co-receptors on target cells, leading to the fusion of the viral envelope with the host cell membrane. The viral RNA is then reverse transcribed into DNA, which is transported to the nucleus and integrated into the host's chromosomal DNA by the viral integrase enzyme. Once integrated, the virus enters the late replication phase. From this point the host's enzymes transcribe and translate the HIV DNA, producing new viral proteins and particles at the ribosomes. The virus is then assembled and released from the cell via budding, and after maturation is ready to infect other cells. The process can be seen in Figure 1 (15).

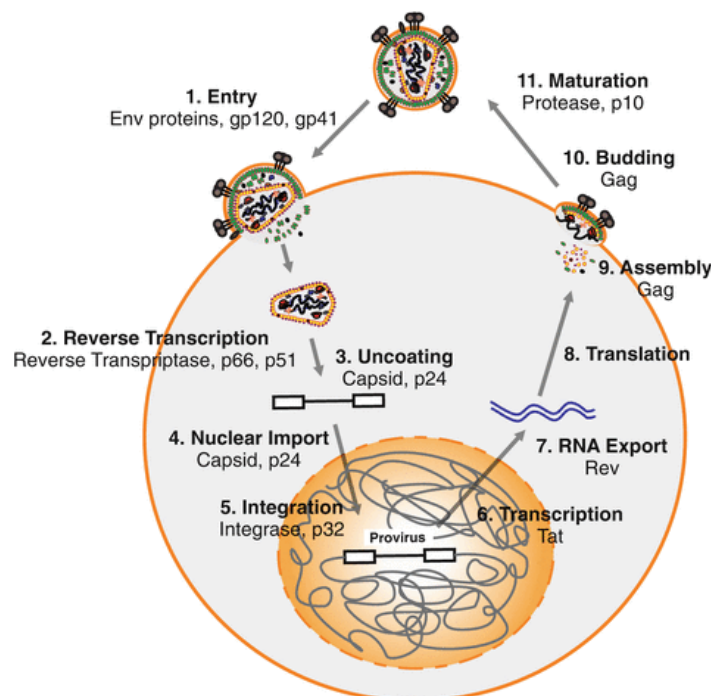


*Fig. 1 The HIV replication cycle (15)*

Dolutegravir is an integrase inhibitor, meaning that it specifically targets the integration step of the HIV replication cycle. It does this by inhibiting the integrase enzyme, which is essential for the integration of viral DNA into the host genome, a process that involves two key catalytic reactions: 3' processing and strand transfer, which can be seen in Figure 2 (22-23)

1. **3’ Processing**: This step involves the removal of two nucleotides from each 3’ end of the viral DNA by the integrase enzyme, which exposes reactive hydroxyl (-OH) groups essential for the subsequent integration process.
2. **Strand Transfer**: The strand transfer reaction is the crucial step where integrase facilitates the covalent linkage of viral DNA to the host DNA, integrating the viral genome into the host’s chromosomal DNA.

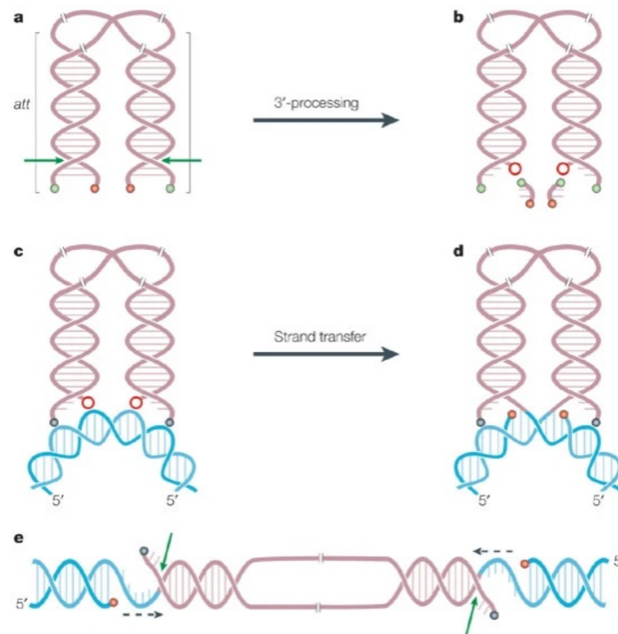


*Fig. 2 An illustration of the two key steps in integration of HIV to the host’s DNA (17)*

Dolutegravir targets the strand transfer step and specifically, it binds to two divalent metal cations ($Mg^{2+}$) located at the integrase enzyme’s active site. Through its chelating groups, dolutegravir forms coordinate bonds with these cations, effectively interfering with the enzyme’s ability to insert viral DNA into the host genome (Figure 3). By stabilizing the integrase-DNA complex in a non-productive form, dolutegravir blocks viral integration, thereby halting further replication of the virus. Dolutegravir also has a slow dissociation rate from the integrase enzyme, contributing to its higher genetic barrier to resistance than other antiretrovirals (21).

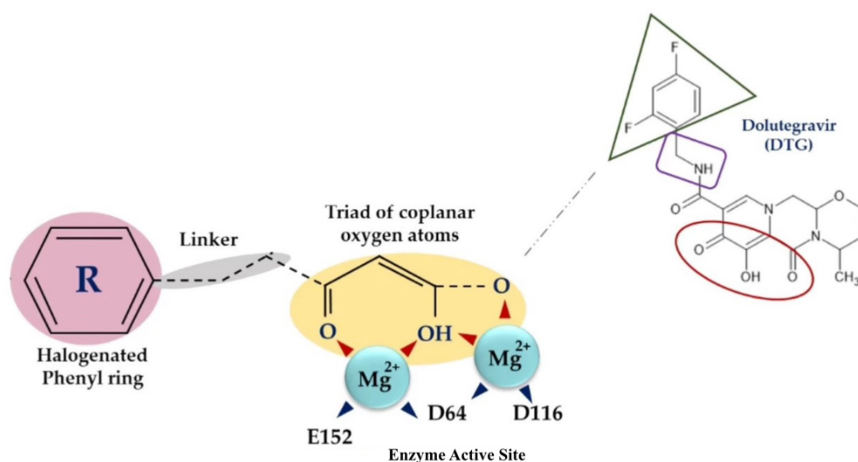


*Fig. 3 Showing where dolutegravir binds to the cations on the integrase active site (21)*

While ART limits the virus spreading throughout the body via the blood, HIV latently persists within cells. The formation of these cellular reservoirs, known as "seeding," occurs early in infection when the virus integrates its genetic material into the DNA of cells that take on a "resting state", as seen in Figure 4. These latently infected cells can proliferate, usually after activation, maintaining the integrated HIV genome as they divide. Although ART can suppress viral replication, it does not eliminate the latent reservoir, necessitating lifelong treatment to prevent viral rebound. Understanding the factors that influence the size of this reservoir is crucial for developing strategies aimed at eradicating HIV (1-5, 19-20, 24).

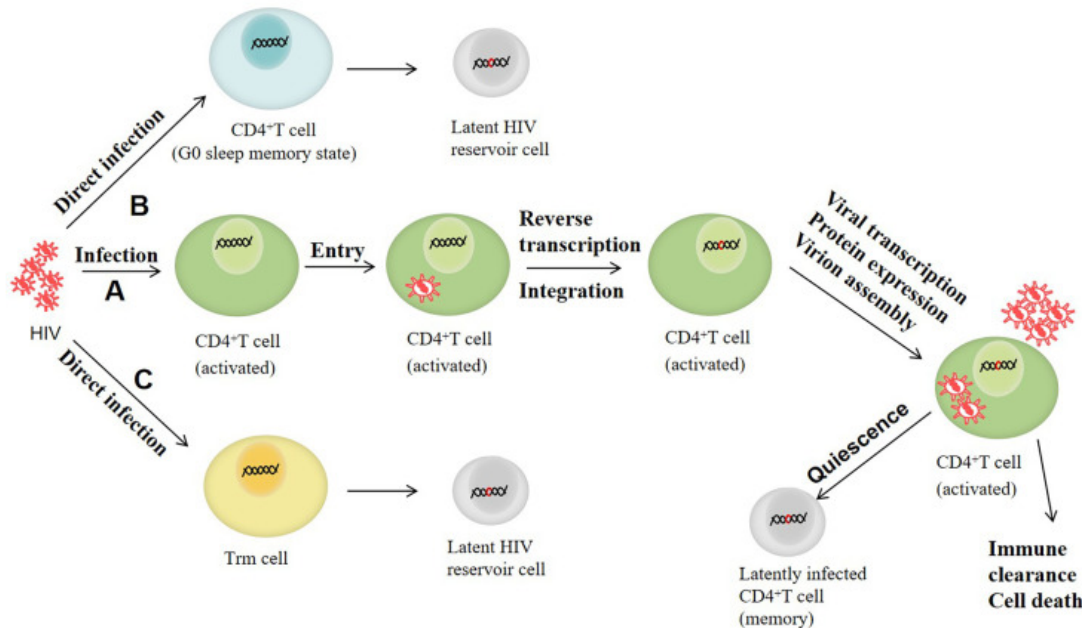


*Fig. 4 The formation of the latent reservoir. (A) shows the classical pathway of HIV infecting a human CD4+ T cell, however some of these cells can be converted into a resting state, called "quiescence". (B) shows HIV infecting a CD4+ T cell that immediately reverts to a dormant state. (C) shows HIV infecting a resting memory CD4+ T cell (5)*

This begs the question that given the mechanism of dolutegravir, could it potentially be affecting the size of the HIV latent reservoir? As it targets the integration phase, with maintaining long-term viral suppression, it may reduce the likelihood of new integration events, thereby limiting the size of the latent reservoir. This hypothesis is what forms the basis of this project. Current research at GOS ICH aims to shed light on dolutegravir's potential role in affecting the size of the reservoir and enhancing the effectiveness of future HIV cure strategies.

# Materials and Methodology

**Ethics Statement:**

The trial was approved by local ethics committees and participants gave consent to participate in the study.

**Nucleic Acid Extraction from PBMCs:**

To extract HIV DNA and RNA from the cellular samples, we followed a modified version of the Qiagen DNeasy Blood and Tissue Kit protocol. Selected subpopulations of CD3-, CD3+ CD4-, and CD3+ CD4+ HIV+ cells from PBMCs were obtained in a Containment Level 3 (CL3) Laboratory. They were inactivated and lysed in a saline solution. The DNA was then extracted according to protocol with some changes made: After adding proteinase K and Buffer AL for lysis, the mixture was pulse vortexed for at least 15 seconds and incubated at 56°C for 30 minutes. Ethanol was added to the sample, which was placed on a column and washed with A1 and A2 Buffers. Then, instead of eluting in 100µl AE Buffer, we eluted in 60µl of water, after which the column was placed on a hot block at 56°C for 5 minutes before being centrifuged. We then added a last step where the 60µl were aspirated from the bottom of the Eppendorf and pipetted back on the column (the Eppendorf was re-used) to be centrifuged again for 1 minute at 13,000 rpm. This is to maximise the amount of nucleic acid obtained. The column was discarded and the extracted nucleic acid, left in the Eppendorf, was stored at +4°C before use in Polymerase Chain Reaction (PCR) and -80°C after for long-term storage.

**To measure the copy numbers of HIV DNA in the sample, we followed a Quantitative PCR (qPCR) protocol:**

To measure the HIV copy numbers, 0.25µl of HIV Long Terminal Repeat (HIV-1-LTR) Taq forward primer (20µM), 0.25µl of HIV-1-LTR Taq reverse primer (20µM), and 0.25µl of HIV-1 Taq PR probe (FAM/ZEN) (10µM) were added. The assay used the human Pyruvate Dehydrogenase (PDH) gene as a reference gene to measure the total number of cells. Hence, 0.25µl of PDH forward primer (10µM), 0.25µl of PDH reverse primer (10µM), and 0.25µl of PDH probe (HEX/ZEN) (10µM) were added. 12.5µl Qiagen multiplex mastermix, containing the polymerase, and 6µl water ($H_2O$) were added. 5µl of sample DNA was then added, making the mixture a total of 25µl.

The programme was 95°C for 15 minutes, followed by 45 cycles of 94°C for 60 seconds, followed by 60°C for 60 seconds, and reading of fluorescence in the plate.

**To amplify the whole HIV genome, we used a nested PCR protocol and compared 2 different polymerases:**

In the first round, we conducted a whole genome PCR, using 0.75µl of the HIV Whole forward primer and 0.75µl of the HIV Whole reverse primer, depicted in red in Figure 5. We used 25µl of RepliQa HiFi Mix. We calculated the amount of DNA sample needed to get the number of copies wanted and added the water volume accordingly to get a total of 50µl for the mixture. The second polymerase used was HiFid Platinum Taq Polymerase using a mixture of 1µl of 10x Taq buffer, 0.4µl of 50mM $MgSO_4$, 0.2µl of 10mM dNTPs, 0.2µl of each primer, 0.05µl of the polymerase (5U/µl), 5.95µl of water, and 2µl of cell extract to make up a 10µl total reaction.

For the RepliQa polymerase, initial denaturation was at 98°C for 30 seconds. Then for 10 cycles: denaturation at 98°C for 10 seconds, annealing at 68-58°C (goes down by 1°C after each cycle) for 5 seconds, extension at 68°C for 1.5 minutes. Then for 15 cycles: denaturation at 98°C for 10 seconds, annealing at 58°C for 5 seconds, extension at 68°C for 1.5 minutes. The final extension was 68°C for 2

minutes followed by a cooldown hold at 12˚C. For the HiFid Platinum polymerase, extension times were increased to 7.5 minutes.

For the second round, we did a four fragment PCR, which you can see in blue in Figure 5. For each primer pair we used 1.5µl each, with 25µl of the RepliQa HiFi Mix. We took 2.5µl of sample from the first PCR and made up the mixture to 50µl with 19.5µl of water. For the HiFid Platinum polymerase, we used 0.4µl each for the primers with 0.1µl of the polymerase. We took 2µl of sample, diluted 1:2, from the first PCR and made up the mixture to 20µl with 0.8µl of 50mM $MgSO_4$, 0.4µl of 10mM dNTPs, and 13.9µl of water.

The temperature regimen for the second round of PCR was as follows: an initial step at 98˚C for 30 seconds, followed by 10 cycles of 98˚C for 10 seconds, 68-58˚C for 5 seconds, and 68˚C for 1.5 minutes, with the annealing temperature decreasing by 1˚C each cycle. This was followed by 35 cycles of 98˚C for 10 seconds, 58˚C for 5 seconds, and 68˚C for 1.5 minutes. The final extension was at 68˚C for 2 minutes, and then the reaction was held at 12˚C. Extension time for the HiFid Platinum polymerase was 4.5 minutes.

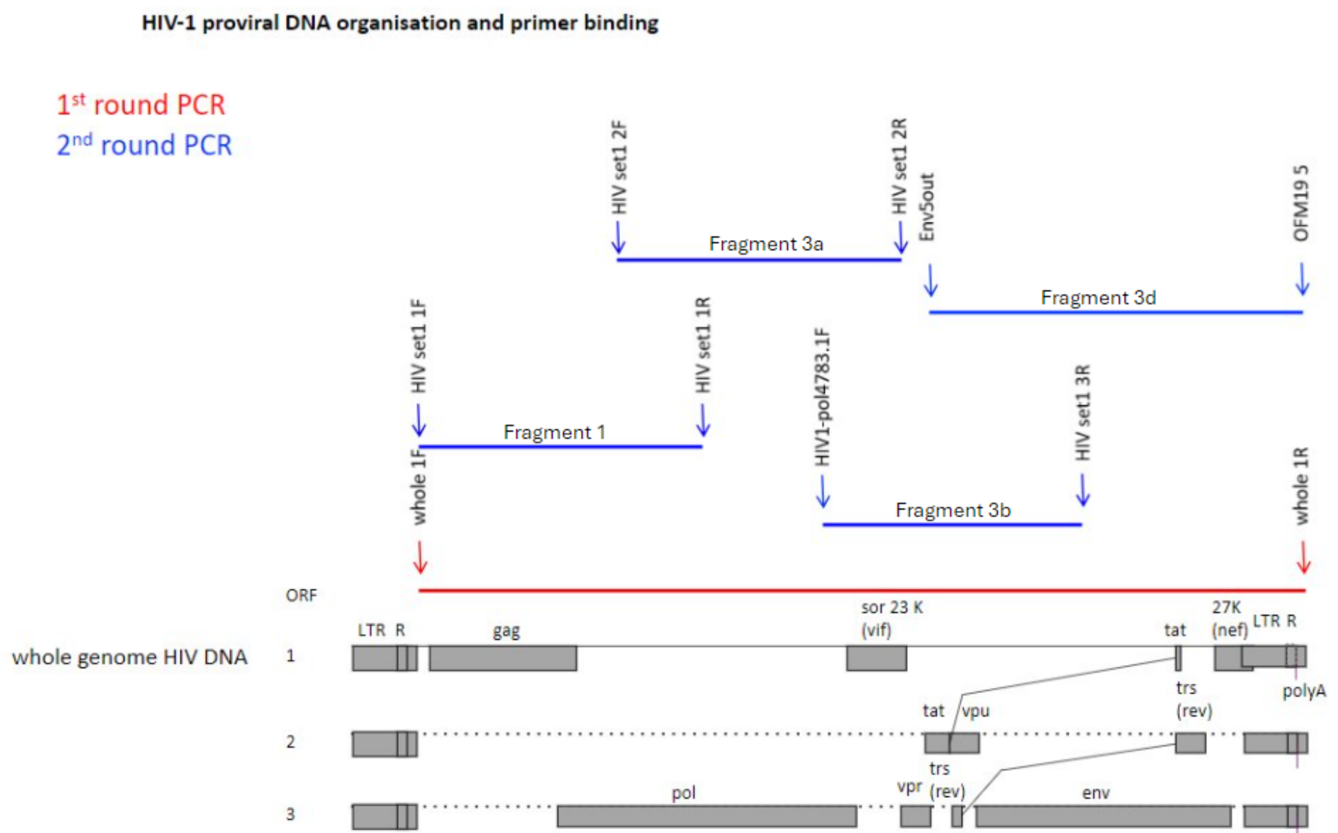


*Fig. 5 Showing where different primers cut the genome (From Kathleen Gärtner. Personal Communication)*

The four fragment PCR can then be used for sequencing and can be pieced together to give a picture of the whole genome using bioinformatics (9-10, 14, 27-28).

# Results and Discussion

From the qPCRs we received copy numbers of PDH and HIV in all subpopulations (data not shown). Copy numbers per $10^6$ cells were calculated as:

$$x = \frac{y}{z} \cdot 10^6$$

Where $x =$ copy numbers per $10^6$ cells, $y =$ raw HIV copy numbers, $z =$ half the PDH copy numbers

An example for 7 patients can be seen in Figure 6 below. As can be observed, the population of cells containing HIV DNA was mostly highest in the CD4+ populations, which was to be expected, since HIV primarily targets CD4+ cells. For patient 1, this trend did not follow, which may have to do with the levels of HIV DNA being low. For patient 5, the DNA concentration in the CD3+ CD4+ population was too low to detect any HIV copies. For patient 3, a large amount of HIV DNA can be seen.

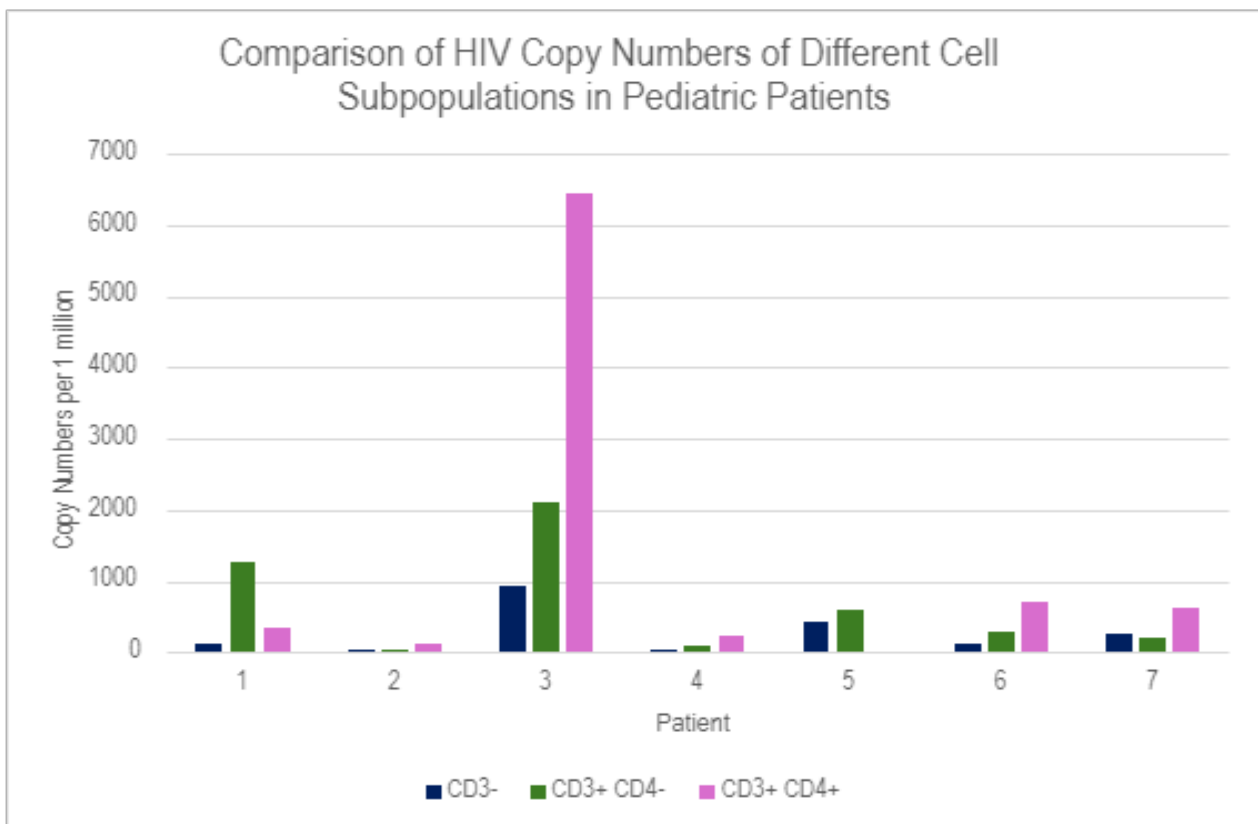


*Fig. 6 Comparison of HIV Copy Numbers of Different Cell Subpopulations in Paediatric Patients.*

In an initial comparison of the DTG versus SOC arms, which can be seen in Figures 7-9, one can see that unexpectedly, patients receiving DTG seem on average to have higher copy numbers of HIV present within their cells. However, no conclusion can be drawn from this data analysis alone, as the number of patients is too small, reflected in the error bars. Additionally, these initial results may be explained by one patient, patient 3 in Figure 6, having much higher levels of HIV copies present than the other patients. While this patient was in the DTG arm, many other factors could contribute to the high HIV copy numbers, for example, poor adherence to treatment.

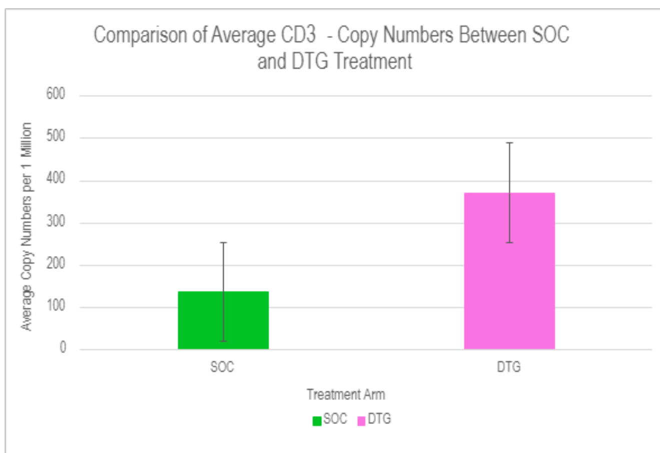


*Fig. 7 Comparison of Average CD3- Copy Numbers Between SOC and DTG Treatment*

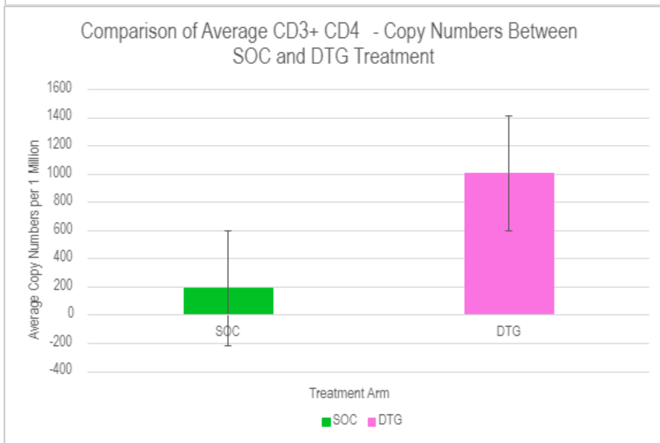


*Fig. 8 Comparison of Average CD3+ CD4- Copy Numbers Between SOC and DTG Treatment*

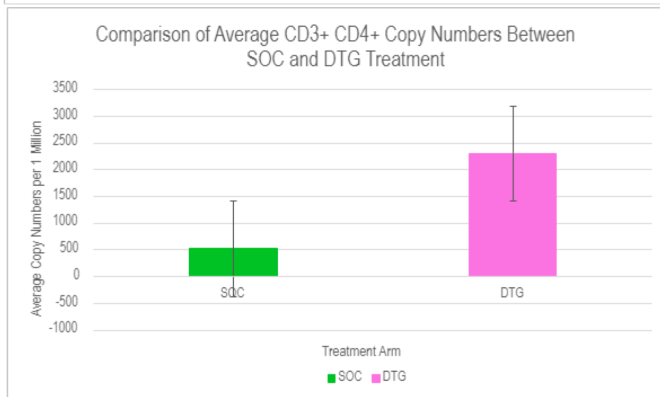


*Fig. 9 Comparison of Average CD3+ CD4+ Copy Numbers Between SOC and DTG Treatment*

As the project continues and more data is collected, these results may change. The initial analysis above begins to address the key question of "How many HIV DNA genomes are integrated per million PBMCs?" Another key question to keep in mind is "How many intact and/or defective genomes can we detect in sequencing?" This could give more detail about the characteristics of the latent reservoir and whether they may be different between the two groups. While high numbers of HIV DNA genomes may be detected, whether they are intact or defective can bring about different effects clinically. Other questions guiding this research are "How much cell-associated-ribonucleic acid (CA-RNA) is expressed in treated children?" and the overarching question, "Is there a difference between the two treatment arms (DTG vs. SOC) regarding these markers?"

Additionally, we worked on optimizing the PCR assay, which can be seen in the gel results. Figure 10 is a nested PCR of an HIV sample using the HiFid Platinum polymerase, the four fragments labelled along with a positive and negative control for each. It can be noted that the positive controls for Fragments 1 and 3a appropriately came up positive.

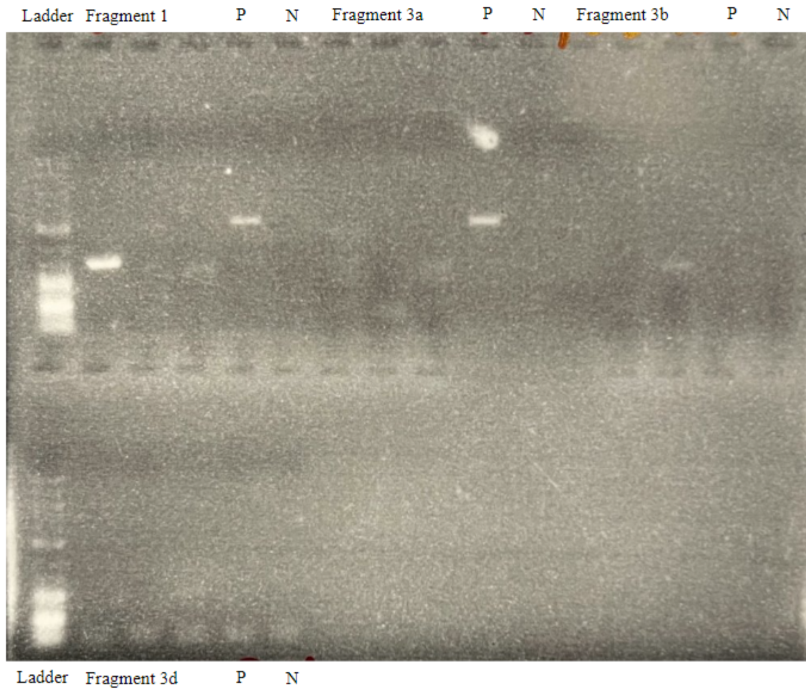


*Fig. 10 Gel image of 4-fragments nested PCR on positive samples from a first round PCR*

Figure 11 shows the results when we switched to using the High Fidelity RepliQa QuantaBio polymerase. It allowed our PCR process to be much faster, from 2 days with the HiFid Platinum polymerase to 3 hours, and, as can be seen in the gels, improved accuracy and imaging.

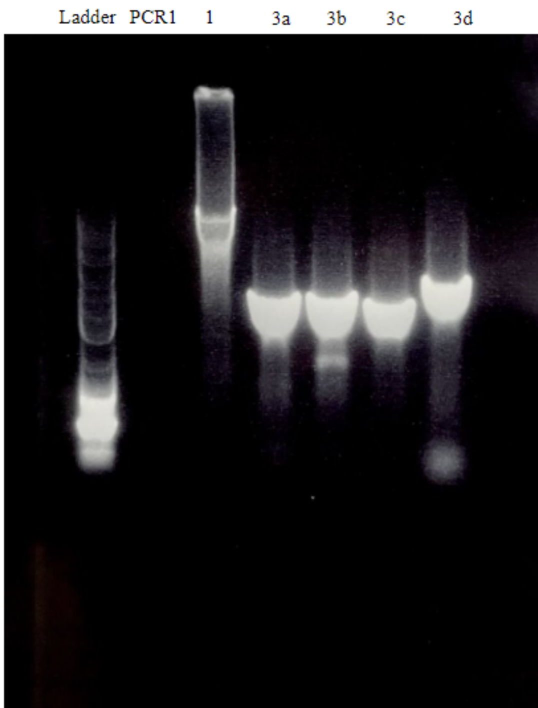


*Fig. 11 Gel image of 4-fragments nested PCR on a positive control*

# Reflection and Conclusion

This six-week research project was completed under the guidance of my supervisor, Kathleen Gärtner. Although the project remains ongoing, it has been a privilege to witness the trajectory of the research, and I hope to continue a lasting relationship with everyone who taught me at GOS ICH.

Initially, I felt a sense of disorientation, recognizing terms and techniques I had learned about in lectures but realizing I understood very little in practice. The transition from theoretical knowledge to hands-on application was both exhilarating and humbling. It made me reflect on why certain practices were in place. For example, on a very basic level, when should I use which pipette to improve accuracy? The process of calibrating instruments and preparing samples demands a meticulous attention to detail that I did not fully appreciate before.

Additionally, I was not in the regular habit of recording down what I was learning to do until having to write down everything in my lab book. When having to write things out and especially in writing this report, I realized how much of what I had learned to do in practice, watching and then repeating in the lab, I still did not fully understand. For instance, I would add primers mindlessly without thinking about why those primers were chosen and what they would help us target. When the realization hit me, it encouraged me to be better about reading more literature around the project and to be more organized and thoughtful in my notes. It made me see more clearly that to truly learn something, I must actively do it with my hands and then sit down by myself and really devote time to thinking or talking it through. Often, I rush through life hoping to passively absorb whatever is thrown at me and for that to be sufficient, but it is not, at least, not in my case. I also shy away from asking too many questions out of fear, which only makes things more difficult when the content I am meant to understand progresses.

Working alongside experienced researchers provided invaluable insights into the subtleties of experimental design and data analysis. Observing them perform procedures and discuss their findings in lab meetings highlighted to me the importance of collaborative, critical thinking and problem-solving in research. Their feedback and guidance were instrumental in refining my techniques and building my confidence. They also were very kind and let me help with multiple projects to learn more lab techniques beyond the ODYSSEY project.

As I became more familiar with the lab environment, I became more organized and paying attention to details became more habitual. Reflecting on myself now, I realize I actively ask myself more questions to ensure my understanding. Before starting any work, I think, “Why am I doing this? What is this experiment meant to achieve in the larger scheme of the project?” This was a huge change from the beginning of my internship. The challenges I faced have been crucial in shaping my approach to scientific research, and I look forward to applying these lessons in future projects.

Most importantly, I am grateful for the opportunity to immerse myself in such a dynamic and supportive research setting. It has been a profound learning journey, and I would like to express my deepest gratitude to Kathleen and her colleagues, particularly Kumu Azad, Dagmar Alber, and Ian Botha.

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