

Effects of HIV integrase inhibitors on cardiovascular components

Renos Keniyopoullos

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Faculty of Medicine of Imperial College London

National Heart & Lung Institute
Faculty of Medicine
Imperial College London
ICTEM Building, Du Cane Road
London
W120NN
United Kingdom

STATEMENT OF ORIGINALITY

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PUBLICATIONS, CONFERENCE PRESENTATIONS AND AWARDS

PUBLICATIONS

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"In vitro and patient studies with platelets, to explore off-target effects of antiretroviral drugs"

R. Keniyopoullos, Dr A. A. Khawaja, Prof M. Boffito, Dr M. Emerson.

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2024 The Platelet Society Meeting (Aberdeen):

"Differential effects of HIV integrase inhibitors on platelets"

2023 EACS (European AIDS Clinical Society) (Warsaw):

"Dolutegravir but not bictegravir exposure increases *in vitro* platelet aggregation"

2023 NHLI (National Heart & Lung Institute) Away Day (London):

"Effects of integrase inhibitors on platelets: towards personalisation of HIV treatment according to cardiovascular risk"

2023 International Workshop on Clinical Pharmacology of HIV, Hepatitis & Other Antiretrovirals (Rome):

"Dolutegravir but not bictegravir exposure increases *in vitro* platelet aggregation"

2023 CROI (Conference for Retroviruses & Opportunistic Infections) (Seattle):

"Dolutegravir exposure, but not bictegravir, increases *in vitro* platelet aggregation"

2022 EUPLAN (European Platelet Network conference) (Milan):

"Exploring the cardiovascular risk in people living with HIV by determining the platelet and endothelial effects of integrase inhibitors used in current anti-retroviral therapies"

2022 NHLI (National Heart & Lung Institute) Away Day (London):

"Do integrase inhibitors protect people living with HIV from an increased cardiovascular disease risk?"

2022 The Platelet Society Meeting (Hull):

"Exploring the endothelial & platelet effects of integrase inhibitors in people living with HIV"

AWARDS

2021-2024 Received the A.G. Leventis Foundation scholarship for 3 consecutive years

2023 Received the Young Investigators Scholarship award at CROI (Seattle)

COVID-19 STATEMENT

This project commenced in July 2021. Due to college-specific Covid-19 regulations, the original plan to perform platelet experiments, by recruiting healthy volunteers from Imperial College, had to be postponed. For this reason, the first year of the project was re-directed into endothelial cell experiments, until a higher number of college personnel (including students and staff) was available to perform donor-dependent research.

LIST OF ABBREVIATIONS

3TC	Lamivudine
Ab	Antibody
ABC	Abacavir
ABP	Actin Binding Protein
ADP	Adenosine Diphosphate
AIDS	Acquired Immunodeficiency Syndrome
ART	Antiretroviral Therapy
ARV	Antiretroviral
ATP	Adenosine Triphosphate
AZT	Azidothymidine
BBB	Blood Brain Barrier
BIC	Bictegravir
BIK	Biktarvy
BMD	Bone Mineral Density
C/EBP- α	CCAAT / Enhancer Binding Protein α
CAB	Cabotegravir
cAMP	Cyclic Adenylyl Monophosphate
cAMP-PK	Cyclic Adenylyl Monophosphate Protein Kinase
cART	Combination Antiretroviral Therapy
CCR5	C-C Chemokine Receptor type 5
CD	Cluster of Differentiation
CD4	Cluster of Differentiation
CDC	Centres for Disease control and Prevention
cGMP	Cyclic Guanylyl Monophosphate
cGMP-PK	Cyclic Guanylyl Monophosphate Protein Kinase
CHB	Chronic Hepatitis B
Cldn	Claudin
CLEC-2	C-type Lectin-like Receptor 2
COX-1	Cyclooxygenase 1
CROI	Conference for Retroviruses and Opportunistic Infections
CRP	C Reactive Protein

CVD	Cardiovascular Disease
CXC4	C-X-C Chemokine Receptor type 4
D:A:D	Data on Adverse effects of anti-HIV Drugs
DAG	Diacylglycerol
DC-SIGN	Dendritic Cell-Specific Intercellular Adhesion Molecule-3-Grabbing Non-Integrin
DNA	Deoxyribonucleic Acid
DOV	Dovato
DRV	Darunavir
dSTORM	Direct Stochastic Optical Reconstruction Microscopy
DTG	Dolutegravir
DTS	Dense Tubular System
EC	Endothelial Cell
EFV	Efavirenz
ELISA	Enzyme-Linked Immunosorbent Assay
eNOS	Endothelial Nitric Oxide Synthase
ER	Endoplasmic Reticulum
ERK	Extracellular Regulated Protein Kinase
ESAM	Endothelial cell Selective Adhesion Molecules
ESL-1	E-Selectin Ligand 1
F-actin	Filamentous Actin
FCR γ	Fc Receptor γ Chain
FDA	Food and Drug Administration
FI	Fibrin
Fla	Fibrinogen
FIIa	Thrombin
FTC	Emtricitabine
GALT	Gut Associated Lymphoid Tissue
GPCR	G Protein Coupled Receptor
GPIb-IX-V	Glycoprotein Ib-IX-V
GPIb β	Glycoprotein Ib β
GTP	Guanylyl Triphosphate
HAART	Highly Active Antiretroviral Therapy
HCAEC	Human Coronary Artery Endothelial Cells

hCMEC	Human Cerebral Microvessel Endothelial Cells
HDL	High Density Lipoprotein
HF	Heart Failure
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HUVEC	Human Umbilical Vein Endothelial Cells
ICAM-1	Intracellular Adhesion Molecule – 1
ICAM-3	Intracellular Adhesion Molecule – 3
IL-1	Interleukin 1
IL-11	Interleukin 11
IL-2	Interleukin 2
IL-6	Interleukin 6
IL-8	Interleukin 8
INF- γ	Interferon γ
INSTI	Integrase Strand Inhibitor
IP ₃	Inositol-1, 4, 5-triphosphate
IR	Incidence Rate
ITAM	Immunoreceptor Tyrosine-based Activation Motif
JAK	Janus Kinase
JAM	Junctional Adhesion Molecules
KS	Kaposi Sarcoma
L-Arg	L-Arginine
LAT	Linker for Activation of T cells
LDL	Low Density Lipoprotein
LFA-1	Leukocyte Function Associated Antigen 1
MAPK	Mitogen Activated Protein Kinase
MI	Myocardial Infarction
MLC	Myosin Light Chain
MRV	Maraviroc
mtDNA	Mitochondrial DNA
NADC	Non-AIDS Defining Cancers
NET	Neutrophil Extracellular Traps
NF- κ B	Necrosis Factor κ B

NHL	Non-Hodgkin Lymphoma
NHS	National Health System
NK	Natural Killer
NNRTI	Non-Nucleotide Reverse Transcriptase Inhibitor
NRTI	Nucleotide Reverse Transcriptase Inhibitor
Ocln	Occludin
oxLDL	Oxidised LDL
PAR-1	Protease-Activated Receptor 1
PAR-4	Protease-Activated Receptor 4
PECAM-1	Platelet Endothelial Cell Adhesion Molecule 1
PG-I ₂	Prostacyclin
PI	Protease Inhibitor
PI3K	Phosphoinositide 3 Kinase
PKA	Protein Kinase A
PKB	Protein Kinase B
PL	Phospholipids
PLC	Phospholipase C
PLWH	People Living With HIV
PrEP	Pre-Exposure Prophylaxis
PS	Protein S
PSGL-1	P-Selectin Glycoprotein Ligand 1
PtdIns(4,5)P ₂	Phosphatidyl Inositol-4,5-bisphosphate
RAL	Raltegravir
Rap1b	Ras-associated protein 1b
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RPV	Rilpivarin
RR	Relative Risk
RT-qPCR	Real time - quantitative Polymerase Chain Reaction
TAF	Tenofovir Alafenamide
TDF	Tenofovir Disoproxil Fumarate
TEM	Trans Endothelial Migration
TF	Tissue Factor

TFPI	Tissue Factor Pathway Inhibitor
TGF- β	Tissue Growth Factor β
Tjp1	Tight Junction Protein 1
TNF- α	Tumour Necrosis Factor - alpha
TPO	Thrombopoietin
TRAP-6	Thrombin Receptor Activating Peptide - 6
TXA2	Thromboxane A2
UNAIDS	Joined United Nations Programme on HIV/AIDS
VASP	Vasodilator-Stimulated Phosphoprotein
VCAM-1	Vascular Cell Adhesion Molecule - 1
VEGF	Vascular Endothelial Growth Factor
VEH	Vehicle
VT	Venous Thrombosis
VTE	Venous Thromboembolism
vWF	Von Willebrand Factor
WHO	World Health Organization

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ABSTRACT

Approximately 39 million people live with HIV (PLWH) today. With combination antiretroviral therapy (cART), PLWH can no longer develop AIDS and maintain an undetectable viral load, but they have been shown to acquire double the risk in developing a cardiovascular disease (CVD) compared to the general population. Several antiretrovirals have been associated with off target effects on the endothelium and platelets. In particular, integrase strand inhibitors (INSTIs), including bictegavir (BIC) and dolutegravir (DTG), are largely used in clinics today, and recent studies associated DTG with an enhanced platelet activity, highlighting the need to explore any possible impact DTG, or similar INSTIs may have on CVD risk. Here, I hypothesize that DTG affects endothelial and platelet activation more than BIC and I aimed to explore and compare the effects of the two, post *in vitro* treatment with clinically relevant concentrations of the drugs, alone or in therapeutical combinations and conduct some *ex vivo* platelet assays from PLWH to create a pilot study. Adhesion and permeability markers of Human Umbilical Vein Endothelial Cells (HUVEC) and Human Coronary Endothelial Cells (HCAEC) were measured post stimulation in flow cytometry or confocal microscopy. Platelet aggregation and surface activation markers were measured in light transmission aggregometry and flow cytometry, respectively. Additionally, platelet adhesion to collagen was measured under physiological conditions upon *in vitro* treatment with INSTIs. Major findings show no effects of DTG or BIC on HUVEC or HCAEC. A higher platelet aggregation profile is displayed in samples treated with INSTIs (especially DTG) versus vehicle control, however this was not extended in *ex vivo* assays from patients. A neutral effect of INSTIs on the endothelium was demonstrated, though clinical relevance of this yet to be determined. This pilot study offers a template for future patient and pathophysiological studies to allow personalized treatments with lower CVD risk.

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CHAPTER 1

Introduction

1.1 HUMAN IMMUNODEFICIENCY VIRUS (HIV)

1.1.1 HISTORY OF HIV/AIDS

“HIV does not make people dangerous to know, so you can shake their hands and give them a hug, Heaven knows they need it” Princess Diana said in April 1991, a decade after AIDS (Acquired Immunodeficiency Syndrome) was officially announced as a new disease by the Centre for Disease Control and Prevention (CDC) [1]. AIDS, initially called “the gay plague”, was first reported in 1981, in the United States, affecting mostly the homosexual community and in 1982 the first cases were reported in Europe and Asia [2], [3].

In the UK, AIDS cases were initially reported in 1982 and within 3 years the number rose to approximately 260 [4]. From those, an estimated 90% were gay or bisexual and were living in London. By the end of 1980s, 7000 individuals died from AIDS in the UK alone [4].

In 1984, a virus was identified by Robert Gallo as the causative agent of the disease [2], [3] and blood tests to detect it were officially licensed by the Food Drug Administration (FDA) a year later. This virus was later named Human Immunodeficiency Virus (HIV) in 1986, a year before the first anti-HIV drug azidothymidine (AZT) was introduced. By the end of 1980s, approximately 9 million people were infected with the virus [2], [3].

HIV is thought to originate from the Democratic Republic of Congo [1], [2] and is believed to be passed on from chimpanzees to humans, decades before the first documentation of HIV or AIDS cases were reported [3], but the exact timeline or how this occurred, still remains a question.

In 1990s, a dual therapy to suppress the virus was introduced, two years before documenting transmission of AZT-resistant HIV [3], with a big rise in cases across Asia. Highly active anti-retroviral therapy (HAART) was introduced mid-90s, around the same time as another drug from the same class, Nucleotide Reverse Transcriptase Inhibitors (NRTIs), was introduced [1], [3]. Towards the end of 1990s, serious adverse effects of HAART have been reported, like lipodystrophy and fat redistribution syndrome [3].

In 2000, more than 26 million people were thought to be living with HIV and around 14,000 new cases were reported daily [5]. In 2006, the first oral once-a-day medication got approved and a year later the first HIV Integrase Strand Inhibitor (INSTI) was approved by the FDA. A huge milestone in the timeline of HIV and AIDS was the development of PrEP or pre-exposure prophylaxis, after clinical trials showing the promising efficacy of Truvada, a combination of two antiretroviral (ARV) drugs in preventing the transmission of HIV [6], [7].

In 2014, a goal named 90-90-90 was set by UNAIDS (the Joint United Nations Programme on HIV & AIDS) where they expected that by 2020, 90% of people with HIV would know their status, 90% of them would access antiretroviral therapy (ART), and 90% of those on ART would reach viral suppression [8], [9]. Some countries managed to accomplish this but some others not, therefore the goal was re-set for 2025 and the target changed to 95-95-95 [5], [8] as a more reasonable date, hoping to end the AIDS pandemic by 2030.

The latest report released by UNAIDS in 2023, shows that on average 86% of people living with HIV (PLWH) know their status, 89% of them are on treatment, and 93% of PLWH on ART are virally suppressed [5]. Poor education, stigma and healthcare access remain the biggest challenges in achieving this.

Today, 40 years post beginning of the pandemic, more than 39 million people worldwide live with HIV and a total of 40 million people have died so far from AIDS or AIDS-related illnesses [5]. From 2010 to 2022, lives lost due to HIV have been reduced by half [5] and great progress has been made in understanding and treating HIV, yet no successful vaccine or cure has been identified.

1.1.2 LIFE CYCLE & PATHOGENESIS OF HIV

Two types of HIV have been discovered so far, HIV-1 and HIV-2, with similar transmission routes, life cycle and pathogenesis [10], [11] although HIV-2 has been associated with decreased transmissibility and progression to AIDS [10]. HIV-2 predominantly affects West Africa, with only a small proportion of PLWH being infected with HIV-2 [10], [11]. This thesis will focus on HIV-1.

The route of HIV transmission from human to human is through sexual intercourse, exchange of body fluids, including semen and vaginal secretions, blood transfusion, use of needles, and in rarer occasions through childbirth, breast feeding and tissue or organ transplantation [12]. Once in the bloodstream, the virus interacts with and attacks CD4 (Cluster of differentiation 4) positive cells as shown in **Figure 1.1.** resulting in the production of new virions [12], [13]

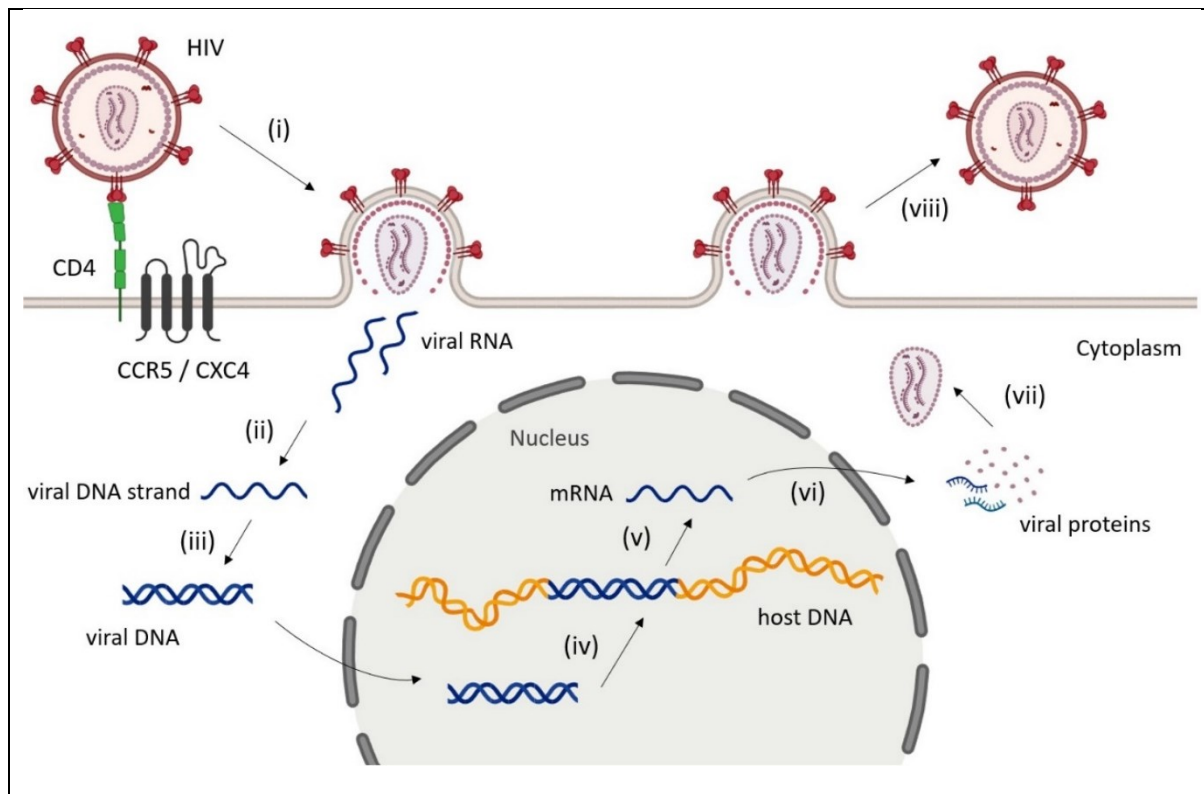


Figure 1.1. Life cycle of HIV within host T cell. The figure shows the attachment, entry and replication cycle of HIV within a human CD4+ (Cluster of differentiation 4 positive) cell. Viral surface proteins interact with surface receptors of the host cell. Fusion of the two takes place (i) with the virus releasing its content within the cytoplasm of the host cell. In (ii) the viral RNA strand is used by the HIV Reverse Transcriptase to produce a DNA strand which is in turn used to make the complementary strand by the DNA polymerase of the host (iii). Within the cytoplasm, viral DNA is then integrated into host DNA by HIV integrase enzyme (iv) and gets transcribed into mRNA together with the host DNA (v) before being translated into a single large polypeptide. This is then cleaved by the HIV protease enzyme into smaller functional proteins (vi) which later assemble into a new virion (vii) and buddies off from the host cell (viii). Figure made on Biorender using template “HIV Sites for Therapeutic Intervention” from <https://app.biorender.com/biorender-templates>.

As demonstrated in **Figure 1.1**, the virus fuses its membrane with that of the host cell, after interacting its surface proteins gp120 and gp41 with the host receptor CD4 and co-receptor CCR5 (C-C Chemokine

Receptor type 5) or CXCR4 (C-X-C Chemokine Receptor type 4) [11], [12]. During fusion, the HIV genome encoded in a single strand of RNA, together with some essential proteins are released into the cytoplasm of the host. These proteins include the reverse transcriptase enzyme, the integrase enzyme and the protease enzyme. The RNA strand is used to form the DNA single strand by the HIV reverse transcriptase, and the product is then used by the host DNA polymerase to form the complementary DNA strand, resulting in a double stranded DNA of the virus which makes it to the nucleus of the host.

Within the nucleus, the HIV integrase enzyme then inserts the newly formed viral DNA into the host DNA [11], [12]. The viral genes, now part of the host's genome, undergo transcription followed by translation, using the host machinery producing a single polypeptide, later cleaved by the HIV protease inhibitor into smaller functional viral proteins [12], [13]. Those proteins then assemble into a new virion that fuses to and gets released from the cell membrane, budding off to attack a new host cell. As newly matured viruses leave host cells, they attack neighboring CD4⁺ T cells in a process known as virological synapse [15]. This process was found to be dependent on adhesion molecule expression clustering and interaction of donor cells (ones from which HIV budded off) and recipient cells (ones of close proximity being attacked by HIV) [15].

If left untreated, HIV infection can progress to different stages, potentially leading to AIDS [16], [17], [18]. There are 4 phases of the HIV pathogenesis in ART-naïve individuals, starting with the first one called primary or acute infection [16], [17]. During this phase, which can last from just a few days post acquiring HIV until a few months later, the individual might face typical symptoms of a viral infection including fever, fatigue, lymphadenopathy or weight loss. At this stage about 100 million to 1 billion new virions can be produced daily and seroconversion takes place, where the body starts producing HIV-specific antibodies which can be detected in HIV testing.

The following 3 phases are considered as the clinical stages, where stage 1 involves the long or chronic early-stage HIV infection which can last for decades and can be asymptomatic making it harder to detect with individuals having a CD4⁺ count of more than 500 cells per μL of blood [16], [17]. Stage 2, or mid-stage HIV infection, is characterized by a CD4⁺ cell count between 200 and 500 per μL of blood and

mild symptoms start being noticeable, including development or worsening of skin conditions, further weight loss and recurrent herpes [16], [17].

HIV infection at the advanced (final) stage, or AIDS, is characterized by a CD4⁺ cell count of less than 200 per μL of blood [16], [17]. An individual with AIDS is considered immunocompromised and Pneumocystis pneumonia, encephalitis or other opportunistic infections are usually observed at this stage and can result in death.

1.1.2.1 HIV Controllers

A proportion of PLWH has been reported to control the HIV from progressing without the use of ARV medication [18], [19]. This group is divided into 2 sub-groups, the viraemic controllers and the elite controllers, where the first is characterised by 3 consecutive tests confirming HIV RNA levels of less than 2000 copies per mL, and elite controllers by less than 50 copies per mL [18].

Controllers can control or limit the viral replication by presenting an enhanced immunological response to HIV [18] with elite controllers demonstrating a greater production of INF- γ (Interferon- γ) and IL-2 (Interleukin-2) by HIV specific CD4 and CD8 cells [19]. The same study demonstrated an association of controllers and enhanced levels of human leukocyte antigen (HLA) alleles (HLA-B*57 or HLA-B*27). Genetic factors are associated with the clinical outcomes between controllers and non-controllers [19] and elite as well as viraemic controllers can be studied more to provide better insights and knowledge on how HIV and T lymphocytes interact, offering possible therapeutical ideas.

Controllers were also shown to have a smaller reservoir relative to non-controllers with HIV [18]. Reservoirs are groups of cells that the virus can persist in an inactive state and during ART interruption can result in viral rebound [20]. It was initially believed that CD4⁺ cells were the main type of cells that could act as HIV reservoirs but was later shown that more immune cell types like macrophages or cells of the CNS, including astrocytes and microglia. Additionally, tissues and organs like lymph nodes or components of the genitourinary system were shown to potentially act as viral reservoirs [20].

Being able to identify and target HIV reservoirs is still a big challenge, if not the biggest, and it is a critical step for the development of a cure for HIV. Today, acquiring HIV is still considered incurable, but

medical advances in drug development allow PLWH to live to a near-normal life expectancy, given they adhere to their recommended ART consistently and continuously.

1.1.3 DRUG CLASSES OF ANTIRETROVIRAL THERAPY

1.1.3.1 Nucleoside (or Nucleotide) Reverse Transcriptase Inhibitors – NRTIs

The first drug developed in late 1980s to treat PLWH was an NRTI, which as the name suggests, its structure resembles that of a nucleoside and works by fitting in the active site of the HIV enzyme reverse transcriptase and blocking it from carrying out its function [21]. NRTIs are administered in the form of a prodrug and require metabolic processing, mainly a cascade of phosphorylation steps by the host enzymes to result in the active drug form [22].

A few years later, NRTI-related toxicity started being noticeable and studies associated the drug class with diseases like neuropathy, lactic acidosis, cardiomyopathy and other mitochondrial dysfunction related illnesses [23]. This was because the structure of NRTIs made it possible to also inhibit human DNA polymerase γ , the enzyme responsible for replication of mitochondrial DNA. This was depleting mitochondrial DNA including genes for mitochondrial enzymes resulting in mitochondrial dysfunction [23].

At present, this drug class still exists, but mostly comprises of a newer generation NRTI drugs which form the basis or backbone of many antiretroviral combinations prescribed in clinics today. Their chemical structures differ slightly from first generation NRTIs to minimize toxicity and adverse side effects [22]. Examples of NRTIs, in order of approval year, include lamivudine (3TC) (1995), abacavir sulphate (ABC) (1998), tenofovir disoproxil fumarate (TDF) (2001), emtricitabine (FTC) (2003) and tenofovir alafenamide (TAF) (2016) [22], [24] [25].

Development of genetic resistance from the virus towards NRTIs was observed in the active site of the HIV RT enzyme, not long after establishing the ARV class [22], [26] [27] Residues within the protein structure of the HIV reverse transcriptase have undergone favorable mutations allowing the viral enzyme to discriminate in favor of nucleosides rather than NRTIs, including amino acids like A62, Q151, V75 [26]. In addition, residues like M41, D67, K70 have been shown to mutate into ones that allow an

ATP-driven removal of integrated NRTIs within the enzyme. A combination of those has led to multidrug resistance strains of HIV [27].

TDF/FTC – PrEP

A combination of 2 NRTIs TDF and FTC, commercially known as Truvada, was approved in 2012 by many countries globally to be used as a prophylactic means of protection from the virus prior to possible exposure. Truvada, also known as PrEP (pre-exposure prophylaxis), is a single-tablet administered orally daily or on-demand before a potential high-risk event [7], [24], [28]. Recent studies suggest replacing TDF with TAF as TDF has been associated with kidney and bone toxicity, despite TAF being associated with dyslipidemia and increased weight gain [24], [28].

1.1.3.2 Non-Nucleoside Reverse Transcriptase Inhibitors – NNRTIs

Similar to NRTIs, are the NNRTIs which also function by inhibiting the reverse transcriptase enzyme of the virus but not by being nucleotide analogues. NNRTIs can target the enzyme either at its active site or at different parts of its structure [22]. As a result, drugs from either of the two classes prevent the enzyme from producing the single stranded viral DNA which is essential in the life cycle and replication of HIV.

An example of an NNRTI currently in use is the second-generation drug, rilpivirine (RPV) [22], [29]. First-generation NNRTIs including efavirenz (EFV) have been associated with a number of adverse side effects including possible association with teratogenicity thus alternative ones like RPV are preferred [29]. In fact, RPV has been proven to be very efficient as an ARV drug and confer less off target effects, including neuropsychiatric effects and plasma lipid levels compared to EFV, though mutations on the HIV transcriptase were reported, increasing the genetic resistance against this too [29], [30]

1.1.3.3 Protease Inhibitors - PIs

Protease inhibitors were the class of anti-HIV drugs developed after (N)NRTIs and this allowed the introduction of combination therapy, where 2 or more drugs were used simultaneously to treat PLWH, reducing the chances for genetic resistance and increasing the viral suppression [25]. First PI developed and approved was saquinavir, which acts by binding to the active site of the protease responsible for

cleaving the newly-formed polypeptide chain of HIV into smaller functional proteins that later assemble into new virions [13], [25][31]. Similar to (N)NRTIs, off target effects and genetic resistance were observed soon after global use, at multiple residues of the protease structure, and second-generation PIs had to be developed [31], [32].

Although this class of drugs is not as popular as other drug classes due to a several links with lipodystrophy, hyperlipidemia and possible resistance to insulin [32], second-generation PIs with improved safety and efficacy are still in use, with darunavir (DRV) being the most recent and commonly used PI today [25].

1.1.3.4 Capsid Inhibitors

Capsid inhibitors or molecules that bind to the capsid hexameric structure surrounding the genetic material of HIV have been under development with most candidates failing before approval. Inhibiting the HIV capsid would be a great target as it has essential functions in both protecting and transferring the viral genome into the host nucleus [25].

The first capsid inhibitor approved and used today is lenacapavir, which was only approved in 2022, and has been proven to inhibit viral replication in two ways. By binding to the capsid subunits, it obstructs the transportation of viral DNA into the nucleus but also inhibits the assembly of new virions, targeting the HIV life cycle at both an early and a late phase of viral replication [33], [34]. Moreover, lenacapavir or GS-6207, is shown to be effective in multidrug resistant HIV strains, with slow-release kinetics making possible a subcutaneous administration once every few months [33], [34] becoming a great ARV candidate.

1.1.3.5 Entry Inhibitors

The first HIV entry inhibitor to be FDA-approved in 2003 and is still used today, is enfuvirtide [35]. This drug suppresses the viral load by inhibiting the fusion process that occurs between the viral membrane and the host cell membrane, a step critical for the entry of HIV and its genome into the human target cell [36]. It does so by binding the fusion-driving glycoprotein 41 (gp41) which makes up the envelope or membrane structure around HIV preventing membrane-to-membrane interactions to occur [35]

Overall, enfuvirtide was well-tolerated in clinical trials and can be used for multidrug resistant HIV strains as well [36]. Unfortunately, it was associated with injection-site reactions following its subcutaneous administration, making its use less frequent [35]

Another type of drugs that belongs to this group of ARVs is the CCR5 inhibitors [35]. They prevent the entry of HIV into host cells by binding the CCR5 co-receptor on human cells, which mediates viral recognition and interaction prior to the fusion of the viral and human cell membranes. An example of an FDA-approved CCR5 inhibitor used today is maraviroc (MRV) [35], [37]. In addition to its successful efficiency in viral suppression, by binding to the human CCR5 chemokine receptor, it initiates a number of intracellular signalling that also aids in fighting latent HIV and other diseases, by enhancing the T cell response of the immune system [37]

1.1.3.6 Integrase Strand Inhibitors - INSTIs

The last class of ARV drugs I will discuss here is the integrase inhibitors, including bictegravir (BIC) and dolutegravir (DTG), the 2 drugs this thesis will focus on. The first integrase inhibitor to be approved and used in clinics to treat PLWH in 2007 was raltegravir (RAL), which established the class in the world of HIV medicine and allowed the development of some of the main protagonists of current ART [38].

INSTIs inhibit the viral replication within host by binding onto the integrase enzyme of HIV. The role of the enzyme is to cleave the viral double stranded DNA at its 3' (3-prime) ends, followed by the removal of 2 nucleotides at each 3' end, making it subject to integration within the human DNA, also mediated by the integrase[38]. By blocking the active site of the enzyme, INSTIs can prevent this viral-to-human genome integration and stall the viral replication at a very critical step of the HIV life cycle. Popular second-generation INSTIs globally used today include dolutegravir, bictegravir and more recently cabotegravir [38] with their chemical structures shown in **Figure 1.2**.

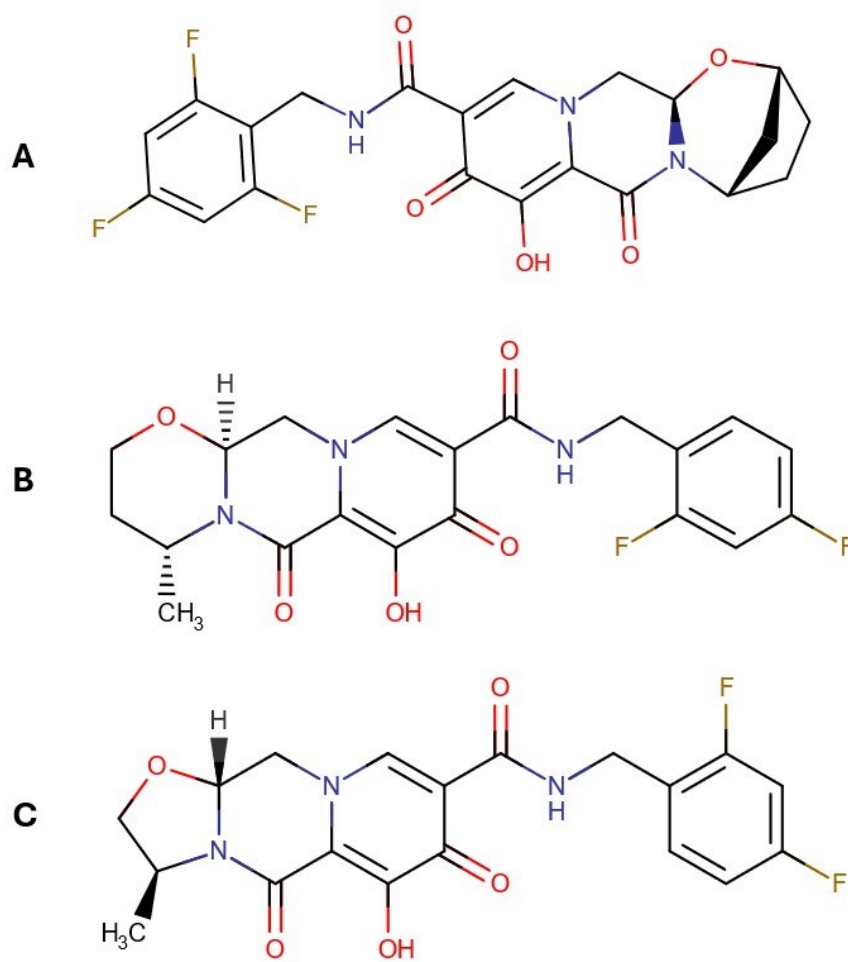


Figure 1.2. Chemical structures of currently popular integrase strand inhibitors (INSTIs). The figure shows in **A** the chemical structure of dolutegravir, in **B** the structure of bictegravir and in **C** the structure of cabotegravir. Compound structures of the drugs were obtained from DrugBank website (go.drugbank.com).

Dolutegravir

Dolutegravir (DTG), manufactured and released by ViiV Healthcare (GlaxoSmithKline), appeared to be a better candidate than raltegravir soon after its release, by demonstrating a more effective viral suppression and a higher threshold for genetic resistance [39]. It showed great effectiveness in ART-naïve PLWH as well as in PLWH with multi-class resistant strains [40]. Approved by FDA in 2013, DTG also known as Tivicay, was shown to dissociate from the HIV integrase in a gradual manner and maintain a plasma half-life of 14h, allowing just a single oral administration daily, as opposed to raltegravir that required two [39], [41].

DTG with ABC and 3TC was a potent combination that showed great effectiveness relative to alternative cART [42]. One of the first trials to examine this combination (the SINGLE trial) showed that a significantly higher number of ART-naïve volunteers with HIV receiving DTG/ABC/3TC had less than 50 copies/mL viral RNA, compared to the alternative treatment group receiving EFV/TDF/FTC [42]. Additionally, participants in the DTG/ABC/3TC arm reached viral suppression earlier than those in the EFV/TDF/FTC arm. More participants discontinued due to adverse effects in the EFV/TDF/FTC treatment group relative to DTG/ABC/3TC group.

This study only examined the efficacy and effects of short-term treatments with the two cART groups, therefore another trial was conducted looking at longer treatments outcomes of DTG/ABC/3TC versus EFV/TDF/FTC, including cost effectiveness and quality of life [43]. Despite showing a better QALY (quality-adjusted life in years) in patients receiving DTG-containing cART, they concluded that the higher cost of the regimen might not be worth it compared to cheaper EFV/TDF/FTC.

Patel et al., conducted a meta-analysis of 31 studies comparing DTG versus different ARV agents, called third agents, the third drug (often an integrase or protease inhibitor) given in combination with a backbone – usually 1 or 2 (N)NRTIs [44]. Their results showed an enhanced viral suppression and a higher number of CD4+ cells in participants receiving DTG + NRTI backbone, compared to a different third agent with a backbone. They also reported a lower number of adverse effects in people receiving DTG than those receiving an alternative ARV.

More recent studies explored the efficacy of DTG/3TC, commercially known as Dovato, as a two-drug therapy, without abacavir [45]. This trial included 3616 patients, including treatment-naïve and treatment-experienced individuals switching to Dovato. Results showed viral suppression from early weeks in ART-naïve patients as well as in patients receiving three-drug regimens beforehand. Moreover, they demonstrated good tolerability and no superior adverse effects compared to alternative three-drug treatments examined. In the group of participants switching to Dovato from a three-drug regimen, viral suppression was maintained in 97.4% of them at the week 24 post-switch.

Bictegravir

Bictegravir (BIC), another popular integrase inhibitor was released by Gilead Sciences a few years after DTG, as a co-formulated tablet with 2 NRTIs, FTC and TAF. Commercially known as Biktarvy, the cART tablet was globally approved in February 2018 [38] ,[46] to be used as a single oral administration once daily and contains 50mg BIC, 200mg FTC and 25mg TAF. It was approved for both treatment naïve and experienced HIV patients [46].

Gilead Sciences has extended licensing arrangements with several pharmaceutical companies to produce BIC alone or in co-formulations for distribution in 116 developing (low- and medium-income) countries, allowing Biktarvy to rapidly become one of the most used cART worldwide [46].

In a phase Ib double-blinded trial, patients receiving BIC alone had a significant reduction in HIV viral load compared to placebo, in a dose-dependent manner, with some of them reaching undetectable viral load from as early as 10 days of receiving treatment [47].

A phase III trial compared the effectiveness of Biktarvy with a well-established ARV combination, DTG/ABC/3TC in 631 patients, and an undetectable viral load was achieved in 92.4% and 93% of patients respectively, by week 48 [48]. No treatment was found to be superior to the other and no INSTI-related genetic resistance was observed in any of the groups with the only difference reported being in the adverse effects, with patients in DTG/ABC/3TC arm demonstrating a higher incident of drug-related nausea compared to those in the BIC/FTC/TAF arm.

A similar phase III trial by Sax et. al [49] compared BIC and DTG, both given in combination with the same backbone, FTC and TAF in ART-naïve patients from countries in Europe, Australia and North and Latin America. 330 participants were given the DTG-containing regimen and 327 the BIC-containing regimen. By week 48, 93% of the patients in the DTG group were virally suppressed and 89% in BIC group, a non-significant difference. No HIV genetic resistance was observed in any group and adverse effects reported were similar in both arms [49].

Pharmacokinetics as well as tolerability and safety of Biktarvy were measured in children as young as 2 years old in another study [50], where a lower dose of 30mg BIC, 120mg FTC and 15mg TAF was

administered in children of a weight between 14 and 25kg. Viral suppression together with good tolerability and adherence were reported by the end of the study confirming effectiveness of the lower dosage Biktarvy in younger patients.

Although many drugs of the same class share a similar chemical structure (**Figure 1.2**) given the extreme specificity of the HIV enzymes and their active site ARV drugs are targeting, a big financial battle commenced in 2018 soon after Bictegravir (or Biktarvy) was released. ViiV, part of GlaxoSmithKline, alleged that the chemical formula of bictegravir was too similar to that of dolutegravir and sued Gilead Sciences [51], [52]. A trial took place in June 2022 post Covid-19-related delays, and the court decided that Gilead Sciences should compensate GlaxoSmithKline with the huge sum of \$1.25 billion, and on top of that pay an annual 3% of Biktarvy sales (in the U.S.A) until 2027, when the dolutegravir patent expires [51], [52].

Cabotegravir

Early in 2021, cabotegravir (CAB) another INSTI released by ViiV Healthcare was FDA-approved to be used as the first long-acting injectable treatment with rilpivavine, a combination commercially known as Cabenuva [53]. Administration involves a single subcutaneous injection intramuscularly of the dual treatment once every 1-2 months allowing a slow release of the ARV drugs in patients. In addition to treatment, long-acting injectable cabotegravir (also known as Apretude) was approved by FDA at the end of 2021 to be used as PrEP [53].

The LATTE-2 trial examined patient tolerability and satisfaction of long-acting Cabenuva injected intramuscularly once every 4 or 8 weeks in comparison to patients receiving CAB+(N)NRTIs orally once daily [54]. Outcomes showed a higher satisfaction in patients receiving the injectable treatments as opposed to the oral regimen. Additionally, in the ATLAS-2M phase III trial, PLWH receiving once monthly or bimonthly an injectable administration of cabenuva had similar outcomes in viral suppression and pharmacokinetics confirming treatment potency and patient tolerability throughout a 152-week trial period [55]. This proved the effectiveness of a single injection bimonthly in maintaining viral suppression.

Several clinical trials, including ECLAIR [56] and HPTN 077 [57], confirmed that long-acting CAB was safe and well-tolerated in HIV-negative participants proving it could be a good alternative to daily Truvada tablets, potentially improving adherence as it would only require single injections once monthly or bimonthly [58]. Cabenuva is the most recently approved combination therapy for HIV. A full list of FDA-approved treatment combinations for HIV is shown in **Table 1**, starting from the first one approved in 1997 until the most recent to date, Cabenuva [59].

	List of drug combinations	
FDA approval year	Commercial name	Anti-retroviral (ARV) drugs
1997	Combivir	Lamivudine, zidovudine
2000	Kaletra	Lopinavir, ritonavir
2000	Trizivir	Abacavir, lamivudine, zidovudine
2004	Truvada	Emtricitabine, Tenofovir Disoproxil Fumarate
2004	Epzicom	Abacavir, lamivudine
2006	Atripla	Efavirenz, emtricitabine, tenofovir disoproxil fumarate
2011	Complera	Emtricitabine, rilpivirine, tenofovir disoproxil fumarate
2012	Stribild	Elvitegravir, cobicistat, emtricitabine, tenofovir disoproxil fumarate
2014	Triumeq	Abacavir, dolutegravir, lamivudine
2015	Prezcobix	Darunavir, cobicistat
2015	Evotaz	Atazanavir, cobicistat
2015	Genvoya	Elvitegravir, cobicistat, emtricitabine, tenofovir alafenamide
2016	Odefsey	Emtricitabine, rilpivirine, tenofovir alafenamide
2016	Descovy	Emtricitabine, tenofovir alafenamide
2017	Juluca	Dolutegravir, rilpivirine
2018	Symfi Lo	Efavirenz, lamivudine, tenofovir disoproxil fumarate
2018	Biktarvy	Bictegravir, emtricitabine, tenofovir alafenamide
2018	Cimduo	Lamivudine, zidovudine
2018	Symtuza	Darunavir, cobicistat, emtricitabine, tenofovir alafenamide
2018	Delstrigo	Darunavir, lamivudine, tenofovir disoproxil fumarate
2019	Dovato	Dolutegravir, lamivudine
2021	Cabenuva	Cabotegravir, rilpivirine

Table 1. List of FDA-approved combination treatments to date for HIV, listed in ascending order from the first combination approved to the most recent.

1.1.4 CURRENT COMPLICATIONS OF HIV TREATMENTS

Despite the recent advances in HIV medicine which undeniably saved millions of people from death or other AIDS-related conditions, cART has been associated with a number of complications affecting patients' quality of life. Is cART a double-edged sword?

1.1.4.1 cART and cancer

It was observed by many that years after introducing cART and being able to suppress the viral replication in PLWH, cancer cases including AIDS-related Kaposi Sarcoma (KS) and Non-Hodgkin lymphoma (NHL) were gradually decreasing [60], [61]. Nasti et al., reported that PLWH with KS receiving cART had a slower and less severe progression of KS compared to ART-naïve PLWH [60]. Risk of NHL in HIV patients was also reduced post cART initiation even in severely immunocompromised patients with less than 50 CD4+ cells/ μ L [61].

NNRTIs were associated with a lower risk of developing KS or NHL, whereas PIs were associated with a lower risk of KS but a significantly higher risk of non-AIDS defining cancers (NADC), specifically anal cancer [62]. Powles et al., also reported a higher incidence of NADC with patients on recent exposure to or established ARV therapy [63] including anal, liver and lung cancer compared to the general population.

More recently, a higher number of NHL and KS cases has been observed [64] but it is unclear whether the increased risk in NADC in PLWH is due to cART or age, as there is a higher ageing population with HIV now compared to pre-cART era.

1.1.4.2 cART and bone deficits

Effects on bone density were observed by a number of ARVs [65], [66]. In a study including young HIV-negative men on different levels of TDF/FTC (PrEP) exposure, a decrease in bone mineral density (BMD) was reported in participants with higher levels of TDF. An increase of parathyroid hormone and decrease in fibroblast growth factor 23 (both of which affect bone density) was also reported in participants with higher exposure to TDF in the same study [65]. In another study by Mulligan et al.,

HIV-negative volunteers were randomized into receiving TDF/FTC or placebo and the PrEP group showed a significantly lower BMD [66].

Brown et al., reported bigger loss on BMD in PLWH on PIs than INSTIs (both co-administered with TDF/FTC) [67]. Interestingly, in the Phase III trial by Sax et al., TDF was associated with worse bone and renal effects when compared to TAF. Both tenofovir derivatives were co-administered with the same combination of ARV regimens [68]. In chronic Hepatitis B (CHB) patients receiving TDF, an improved bone and kidney activity was observed in those switching to TAF compared to those remaining in TDF for a 96-week period [69].

1.1.4.3 cART, increase in weight and diabetes

Significant reduction in weight once defined PLWH pre-cART era, a trend which now transitioned into the other extreme, with many studies reporting overweight and obese patients on effective ART [70]. Increase in weight was shown to be higher in individuals receiving TAF relative to those receiving TDF (both in combination with FTC) and greater weight gain was also observed on INSTI (dolutegravir or raltegravir) recipients when compared to NNRTI (efavirenz) recipients [71]. Additionally, DTG was associated with the higher weight gain in PLWH compared to alternative ARVs like darunavir, rilpivavine or efavirenz. Ayissi et al., demonstrated that the currently popular INSTIs BIC and DTG were reported to cause adipocyte hypertrophy and fibrosis as well as insulin resistance [72].

In a large-cohort study involving PLWH in US and Canada, patients on INSTI-based treatments exhibited an increased risk on insulin resistance possibly due to off target effects on adipocytes resulting in lower adiponectin levels [73]. In the same study, the incidence rate (IR) for developing diabetes mellitus was found to be double than the one in the general US population [73]. Specifically, INSTI- or PI-based cART was associated with an increased risk for diabetes when compared to NNRTI-based cART.

1.1.4.4 cART and cardiovascular disease (CVD)

Amongst other factors heightening the risk of cardiovascular disease (CVD), including HIV-associated chronic inflammation, the continuous exposure to ARV regimens is largely affecting the CVD development and progression in both older and young HIV patients [74], [75], [76], [77]. In fact, CVD

was recently reported to be the leading non-AIDS related cause of death in PLWH [76] mirroring the global population. What is noteworthy is that PLWH face more than a 60% higher risk of developing a CVD than HIV-negative individuals and PLWH on cART double the risk than ART-naïve PLWH[78].

From early 2000s, just a few years after cART was established, an increase in myocardial infarction (MI) was noticed [75] where a 26% increased risk for MI was reported in PLWH per year (during the first 4-6 years) of receiving cART. Factors reported to affect this increased risk include heightened total cholesterol and triglyceride levels, as well as diabetes. Triant et al., reported an increased rate of acute MI in PLWH when compared to an HIV-negative cohort [79] in addition to increased rates of hypertension, dyslipidemia and diabetes, all of which enhance MI risk.

From a worldwide (still ongoing) multi-cohort study D:A:D (Data on Adverse effects of anti-HIV Drugs) in 2008, NNRTIs have been linked with an increased risk of MI. Specifically they reported a 90% higher risk in PLWH on abacavir than those who never used the drug or used it more than 6 months ago prior to the study [75].

A few years later, a meta-analysis by Islam et al. explored and evaluated the relative risk (RR) of CVD in PLWH on different drug classes [78]. They calculated the RR in developing a CVD to be 1.65, 1.59, and 1.18 in PLWH on PIs, NRTIs and NNRTIs respectively, compared to ART-naïve individuals and suggested that PI-containing cART overall increases the CVD risk more than a non-PI containing cART. A similar report was published in 2019, where Alonso et al., conducted a study looking at risk ratios and incidence of various types of CVD in PLWH relative to HIV-negative population, and showed a significantly higher ratio of heart failure (HF) and stroke incidents in PLWH [80].

Incidence and risk of atherosclerosis is reportedly higher in both children and adults with HIV when compared to general population [77], [81] and ART is shown to be an independent factor in heightening this [74]. Over the last few years, there was a rise in the use of statins for PLWH mainly to cope with this.

Use of Statins

PLWH with heightened levels of low-density lipoprotein (LDL) cholesterol and dyslipidemia should be treated with statins as a first-line therapy, as recommended by EACS in their 2019 report [82], to help reduce their CVD risk.

A meta-analysis published earlier this year collectively measured the impact of statins versus placebo in PLWH from a number of studies and trials. Despite not showing a significant decrease in CVD risk in participants taking statins, they reported a significant decrease in LDL-cholesterol, total cholesterol, and in triglycerides [83]. They also reported possible drug interactions between statins, PIs and NNRTIs.

A phase III trial, including HIV patients at 12 different countries with a low to moderate risk profile of developing a CVD, with no previous CVD incident, showed great statin (specifically pitavastatin) effectiveness in significantly reducing the CVD risk in PLWH on cART [84]. Participants taking 4mg/day of the statin had a statistically significant decrease in CVD incidents versus those taking the placebo. Noteworthy is that pitavastatin was specifically chosen for the trial, as other statins were shown to be involved in drug interactions with cART components.

All of the above mainly focus on older HIV ARVs and medication, though not much is known about the cardiovascular impact of newer INSTIs used in clinics today which appear to be less metabolically toxic than alternative third agents like PIs [76].

1.1.5 CHRONIC INFLAMMATION

Consistent low-grade immune activation leading to chronic inflammation is observed in PLWH even on effective cART, due to a number of reasons [85], [86], [87]. Firstly, the presence of HIV proteins that circulate even in patients with undetectable viral load can stimulate production of pro-inflammatory cytokines [85], [86], [87].

Secondly, a destruction in the abundance of CD4+ cells found mainly in the gut associated lymphoid tissues (GALT) from the early stages of HIV infection is shown to not be restored even after initiation of effective cART, leading to the disruption of tight cell-to-cell junctions in the epithelium layer of the gut

[87], [88]. This allows leakage of bacterial products (e.g. lipopolysaccharides) in the bloodstream [85], [89] in the process known as microbial translocation that further activates immune response [87], [88].

The continuous immune activation leads to T cell exhaustion, which in turn results in the cells' decreased ability to produce essential cytokines, upregulation of their inhibitory receptors further reducing their activity and show metabolic dysregulation [85], [86].

The upregulation of pro-inflammatory cytokines in PLWH, including IL-6 (Interleukin 6), TNF- α (Tumour Necrosis Factor - α), INF- γ (Interferon - γ) and CRP (C-reactive Protein) has been reported [85], [89]. In a study by Claudio et al., several biomarkers of chronic inflammation were measured in HIV patients and were correlated with a higher cardiovascular risk e.g. levels of TNF- α with a higher risk for atherosclerotic CVD [90].

In another study, levels of IL-6 and TNF- α were quantified in PLWH pre- and post-cART as well as in HIV-negative individuals [89]. Results showed a decrease in cytokine levels post treatment initiation and upon viral suppression but TNF- α levels remained significantly higher in virally suppressed patients when compared to HIV-negative participants even at undetectable HIV.

Similarly, another study measured the soluble levels of several pro-inflammatory cytokines in HIV patients (before and after cART initiation) and in age-matched HIV-negative individuals [91]. Despite seeing most of the cytokine levels dropping and normalizing weeks after cART, TNF- α levels were reported to remain high even after almost 2 years of cART-mediated viral suppression. In fact, TNF- α levels in PLWH on effective cART were as high as those in the elderly control group 'non-survivors' who passed away during follow-up. Bernal et al., suggested an increased monocyte activation from circulating lipopolysaccharides or other cytokines as the main drive for the persistent TNF- α [91].

1.2 CARDIOVASCULAR SYSTEM & DISEASE

ARV medication that PLWH are exposed to daily, together with the HIV-associated chronic inflammation present even in virally suppressed individuals, are two major factors in compromising the cardiovascular system and increasing the risk for developing a CVD [78], [90].

This section will focus on the cardiovascular system and will include an overview of recent studies exploring at a molecular level, the effects of popular ARVs on key components of the cardiovascular system, endothelial cells and platelets. This will allow a summary of what is known in the context of INSTIs and CVD, but most importantly what are the existing gaps, in an effort to link cART with the increased CVD risk reported in PLWH today.

1.2.1 OVERVIEW OF THE CARDIOVASCULAR SYSTEM

The cardiovascular system comprises of the ‘cardio’, the Greek name for heart, and ‘vascular’ which refers to an extensive network of blood vessels in which blood circulates and delivers oxygen and essential nutrients in tissues, as well as removing waste products. The heart and blood vessels work together to ensure efficient circulation, adapting to the body's changing needs [92].

1.2.1.1 The heart and blood vessels

The heart is a muscular organ located in the thoracic cavity between the lungs and its function is to pump blood throughout the body. It has four chambers, two atria at the top and two ventricles below [92]. The right atrium receives deoxygenated blood from the body, which is then pumped to the lungs by the right ventricle, whereas oxygenated blood returns to the left atrium and is pumped out to the body by the left ventricle. The walls of the heart are composed of three layers, the epicardium, the endocardium and the myocardium, with the last one being the middle muscular layer responsible for the heart's contractile force aiding in blood flow [92].

Blood vessels include arteries, veins, and capillaries, each serving distinct roles in the cardiovascular system [93]. Arteries carry oxygen-rich blood away from the heart to the tissues and have thicker walls to withstand the higher pressure, while veins return deoxygenated blood back to the heart and have

thinner walls as blood flows at a decreased pressure. Capillaries, the smallest blood vessels of the cardiovascular, facilitate the exchange of gases, nutrients, and waste between blood and tissues. Endothelial cells line the blood vessels and play an essential role in regulating blood flow, vascular resistance, and maintaining vascular health and homeostasis [93].

1.2.2 ENDOTHELIAL CELLS

Endothelial cells (ECs) vary genetically and functionally depending on which type of blood vessel they align but all have the same general role, to maintain tissue homeostasis by responding to stimuli. Arterial and venous endothelial cells come from mesodermal progenitor cells followed by a series of differentiation steps and expression of E-phrinB2 ligand or Ephrin type B Receptor 4 is the critical step in an endothelial cell to develop into an arterial or venous EC [94], [95].

ECs line the vessel with an additional layer of smooth muscle cells as sheath (thicker in arteries due to higher pressure) and they adjust their permeability depending on location, type and stimuli [94], [95]. Permeability and stability of endothelial cells are maintained by a number of junction proteins that interact in neighboring cells to regulate the integrity of the endothelial cell monolayer, as illustrated in **Figure 1.3** [96], [97].

One protein type found between ECs is the tight junction proteins which consist of the Claudin (Cldn) and the Occludin (Ocln), and they are essential for excluding passage of molecules bigger than 800 Da [96], [97]. Both Cldn and Ocln span the cell membrane 4 times with their transmembrane domains, maintain reduced endothelial permeability and they are essential in blood-brain and blood-retina barriers [97].

Junctional Adhesion Molecules, or JAMs, is another type of proteins within the tight junction class with 4 specific ones found in endothelial cells, JAM-A, JAM-B, JAM-C and ESAM (Endothelial cell Selective Adhesion Molecules) [96]. Communication between tight junction proteins was observed with absence of JAM-A shown to downregulate the expression of Cldn5 through C/EBP- α (CCAAT / Enhancer Binding Protein- α) transcription factor mediated pathway [97].

Next are the adherens junctions, also illustrated in **Figure 1.3**, that nectins, Vascular Endothelial (VE)-Cadherin, and Platelet Endothelial Cell Adhesion Molecule 1 (PECAM-1) belong to. Nectins, despite being studied more extensively on epithelial cells, have an extracellular structure of 3 immunoglobulin-like domains and can interact in cis or trans conformations with themselves or other nectins, as well as with other growth factor receptors, and can regulate activity of immune cells including CD8+ and natural killer (NK) cells [98].

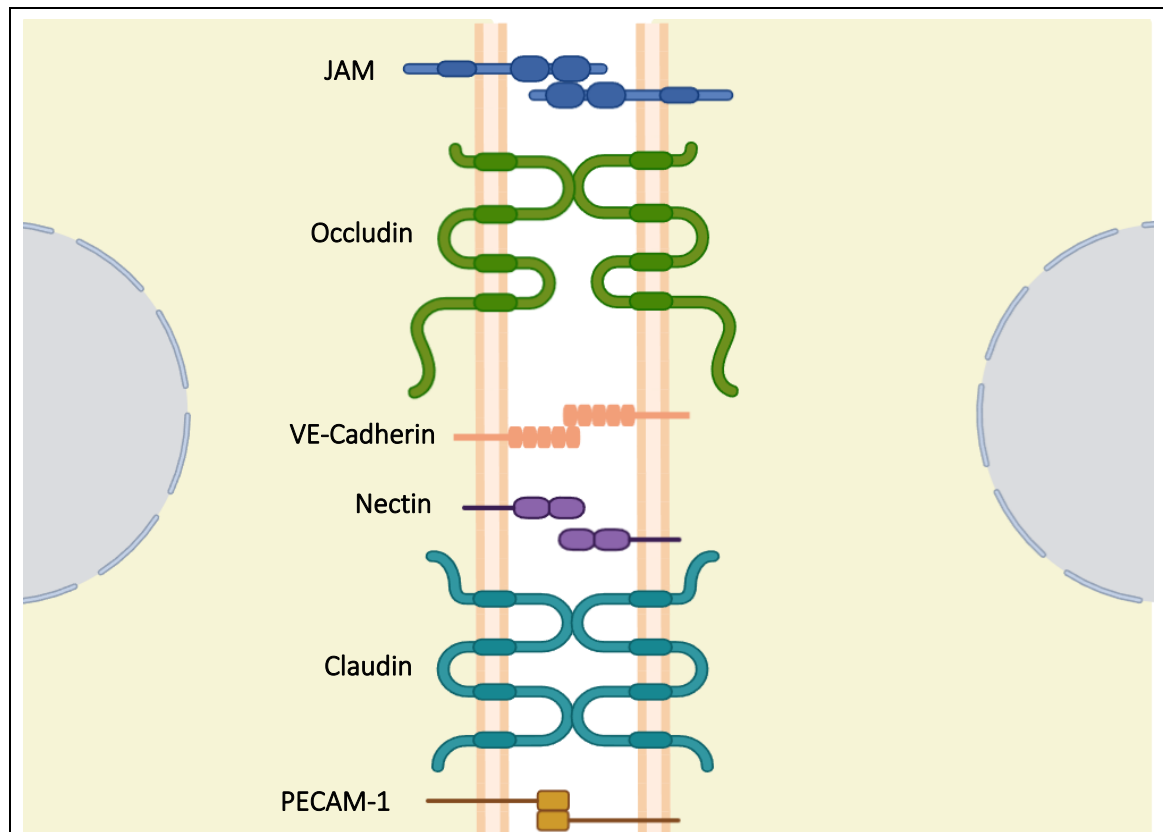


Figure 1.3. Endothelial cell-to-cell junction proteins. This figure summarizes the transmembrane proteins involved in stabilizing cell-to-cell adhesion in the endothelium lining. Junctional Adhesion Molecule (JAM) is shown in blue, Occludin in green, VE-Cadherin in pink, Nectin in purple, Claudin in light blue and PECAM-1 in orange. All proteins shown here have an intracellular, a transmembrane and an extracellular domain and they adhere to the cytoplasm of each endothelial cell by stable interactions with the actin cytoskeleton. JAM, Occludin and Claudin belong to the tight junctions, whereas VE-Cadherin, PECAM-1 and nectins belong to the adherens junctions, with both groups involved in the endothelium stability and integrity. Figure was produced in BioRender Software.

VE-Cadherin

VE-Cadherin has an essential role in endothelial cell-to-cell junction and stabilization, angiogenesis and development of new blood vessels [99]. Its carboxyl terminal domain is well anchored in the cytoplasm of the endothelial cells by strong interactions with other catenin proteins (α -, β - and γ -catenin) in the actin cytoskeleton, whereas its extracellular N-terminal domain maintains calcium-dependent homophilic interactions with adjacent VE-cadherin N-terminal domains of neighboring ECs [99], [100].

Another protein p120 is associated with the cytoplasmic domain of VE-Cadherin and regulates its expression levels as well as preventing its internalization and degradation at resting conditions [99]. Trio is a protein recruited intracellularly at the junction site upon VE-cadherin - VE-Cadherin interactions and aids in stabilizing the contact between the proteins [100].

PECAM-1

Similar to VE-Cadherin, the transmembrane protein PECAM-1 maintains strong endothelial junctions by homophilic interactions with other PECAM-1 proteins of adjacent cells [101], [102]. It provides an endothelial barrier at resting conditions, and PECAM-1 - PECAM-1 links are essential for ECs to recover post-stimulation [101]. Mutations on the extracellular domain of PECAM-1 can compromise self-interactions and reduce cell-cell barrier integrity more than PECAM-1 variants with mutations on the intracellular or transmembrane domain, confirming the significance of the homophilic interactions in vascular integrity [101].

In addition to ECs, PECAM-1 is also expressed on the surface of platelets and leukocytes and has a major role in transendothelial migration (TEM) or extravasation [96], [102]. Interactions of PECAM-1 proteins on ECs and leukocytes drive leukocyte transmigration through endothelial cells during an inflammatory state where leukocytes are required to migrate from the vessel to damaged tissue [102].

1.2.2.1 Leukocyte extravasation and adhesion molecules

During extravasation, membrane protein interactions involved in tight and adherens junctions between ECs loosen up, protein complexes dissociate, and often get internalized and degraded, to allow the passage of leukocytes across the endothelium [102]. This process takes place in smaller vessels like

capillaries or venous vessels adjacent to capillaries, during inflammation or upon extravascular damage [102], [103].

Leukocytes, like neutrophils or monocytes are recruited on the endothelium [103], [104]. This recruitment occurs by the first contact of leukocyte surface proteins and EC adhesion molecules allowing leukocyte rolling on the endothelium. Expression of the transmembrane P-Selectin on ECs is critical for the initial capture of leukocytes, as it interacts with the PSGL-1 (P-Selectin glycoprotein ligand-1) found on the surface of neutrophils, monocytes and other cells involved in dealing with inflammation or tissue damage [102], [104].

Additionally, E-Selectin and VCAM-1 (Vascular Cell Adhesion Molecule-1) are two more essential adhesion molecules expressed on ECs. They aid in tethering and rolling of neutrophils and monocytes respectively by interacting with ESL-1 (E-Selectin Ligand-1) and CD44 on neutrophils, or $\alpha 4\beta 1$ surface integrin on monocytes [103], [104].

To stabilize adhesion and lock leukocyte-EC interactions, upregulation and expression of ICAM-1 (Intercellular Adhesion Molecule-1) occurs in endothelial cells and is essential in all types of leukocyte migration [104]. ICAM-1 interacts with the leukocyte integrin LFA-1 resulting in the redistribution of integrins and adhesion molecules forming a ring-like structure, enabling leukocyte transmigration [105].

1.2.2.2 The role of TNF- α in leukocyte extravasation

Levels of TNF- α , one of the most extensively studied pro-inflammatory cytokines, are elevated during acute or chronic inflammation, especially in PLWH [89], [91]. TNF- α can be released by a range of cells, including endothelial or immune cells like macrophages and NK cells and is shown to significantly upregulate ICAM-1, VCAM-1 and E-Selectin expression on endothelial cells [104], [106], [107].

Chandrasekharan et al., showed that TNF- α binding to its receptors p55 (TNF- α Receptor I) or p75 (TNF- α Receptor II) is critical for downstream signalling, leading to the expression of adhesion molecules [107]. Phosphorylation of the p65 subunit of the transcription factor Nuclear Factor κ B (NF- κ B) is

followed by translocation in the nucleus, altering the gene expression of adhesion and junction proteins involved in EC permeability and extravasation [102], [106].

Aveleira et al., demonstrated a TNF- α -mediated reduction in both mRNA and protein levels of tight junctions like Cldn5 in ECs [108]. Tyrosine phosphorylation at the intracellular domain of VE-Cadherin was also shown to be mediated by the TNF- α signalling pathway followed by internalization of the protein subsequently increasing vascular permeability [109]. Similarly, Romer et al., demonstrated that PECAM-1 was also shown to be affected by the presence of TNF- α and INF- γ (Interferon- γ), another pro-inflammatory cytokine, by redistribution on the EC surface, weakening the PECAM-1 homophilic interactions [110].

The aforementioned confirms that the increase in TNF- α levels during inflammation result in a reduction of tight and adheren junctions between ECs compromising integrity and increasing permeability of the endothelium.

1.2.2.3 Endothelial activation & dysfunction

In addition to the pro-adhesive phenotype described above, during activation ECs also exhibit pro-inflammatory and pro-coagulant properties [111], [112]. TNF- α as well as other cytokines like INF- γ can bind to EC receptors and induce a series of signalling events further enhancing inflammation and coagulation processes by upregulation of adhesion molecules and other cytokines like IL-1 (Interleukin-1), Tissue Factor (TF) or von Willebrand Factor (vWF). This is considered the slow or late Type II EC activation [111].

Endothelial cells can also respond rapidly upon stimulation, releasing pre-made proteins like P-Selectin, or vWF from Weibel bodies, and this is known as the Type I EC stimulation [111]. It usually occurs upon ligand binding to the G-Protein Coupled Receptors (GPCRs) on ECs, and involve a conformational change of the receptor, activating the α subunit of the intracellular G-protein complex, to switch bound GDP with GTP[113]. This enables protein α to phosphorylate the enzyme Phospholipase C β (PLC β), which in turn cleaves the membrane bound PtdIns(4,5)P $_2$ (Phosphatidylinositol-4,5-bisphosphate) into 2 parts,

DAG (Diacylglycerol) which remains membrane bound, and IP₃ (Inositol-1, 4, 5-triphosphate) which in turn moves to the endoplasmic reticulum (ER) and induces the release of stored Ca²⁺ ions [113].

1.2.2.4 Nitric oxide (NO) in endothelial dysfunction

All these responses, pro-adhesive, pro-inflammatory and increasing vascular permeability, persist during endothelial dysfunction when ECs remain activated over an excessive amount of time [114]. Endothelial dysfunction is the term used to characterize changes in the NO availability or synthesis by the endothelium, with subsequent deleterious effects in the vascular responsiveness [114].

At physiological resting conditions, the endothelial-specific enzyme called Nitric Oxide Synthase (eNOS) utilizes L-arginine (L-Arg) to produce NO and L-citrulline as a by-product [115], [116]. This takes place upon ligand-receptor binding on the EC membrane (where ligands include acetylcholine or bradykinin) [117], [118] inducing mobilization of Ca²⁺ ions which results in calmodulin (a Ca²⁺-regulatory protein) binding and phosphorylation of eNOS [119], [120]. Low calcium levels allow phosphorylation of calmodulin which in turn dissociates from eNOS, reducing NO production [119], [120]. Shear stress also maintains an active mode of eNOS producing NO at physiological conditions [115], [116].

A baseline amount of NO is essential for homeo- and haemostasis as it controls expansion or diameter of blood vessels and maintains an anti-thrombotic activity of endothelial cells as well as other cells in the bloodstream like leukocytes and platelets [114], [118]. During endothelial dysfunction, this inhibitory effect of endothelial-derived NO in platelets is lost or reduced, resulting in a higher activity of platelets which in turn results in a pro-coagulant activity [114]. Platelet activation and coagulation will be reviewed in the following section of this chapter.

1.2.3 PLATELETS

In adults, approximately 1×10^{12} platelets flow in circulation with an average lifespan of 8-10 days, before being phagocytosed in the spleen or liver [121], [122]. Platelets are tiny, anucleated cells and one of the most abundant cell types in circulation [123], [124], [125]. They originate from megakaryocytes in a process stimulated by a combination of cytokines, like interleukins (IL-3, IL-6, IL-11) and thrombopoietin [124], [125].

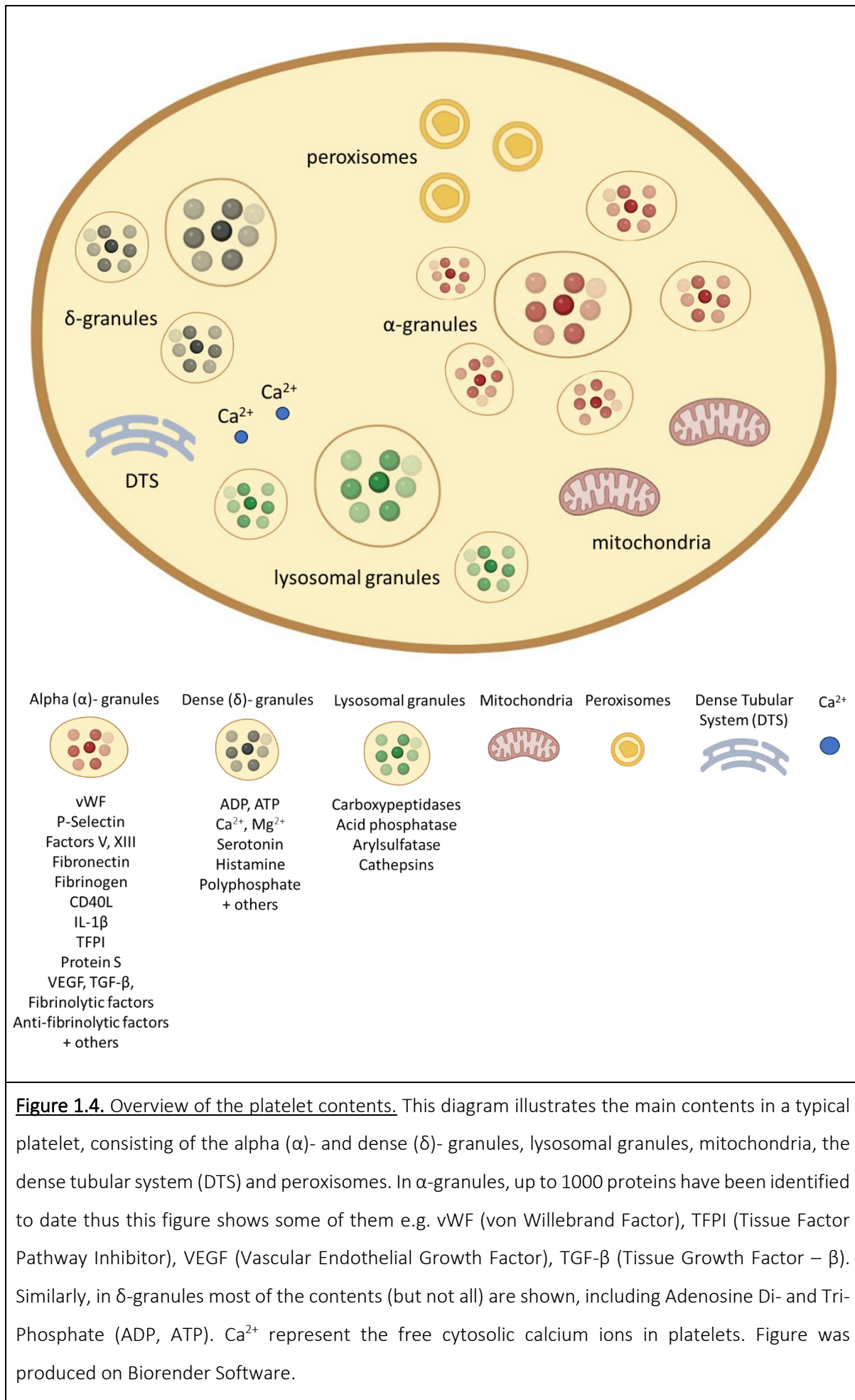
1.2.3.1 Platelet production form megakaryocytes

The liver hormone thrombopoietin (TPO) binds to its receptor (TPO-R or Mpl) on megakaryocyte cells in the bone marrow, a critical stage for initiating platelet production [125]. This binding promotes receptor dimerization, which in turn allows phosphorylation of intracellularly associated JAKs (Janus Kinases) on tyrosine residues, inducing downstream signalling [125]. This results in megakaryocyte maturation followed by expression of platelet integrins like $\alpha_{IIb}\beta_3$ on the megakaryocyte surface [125], [126].

Megakaryocytes then align on sinusoidal bone marrow endothelial cell [127]. Through the endothelial pores, megakaryocytes (being too massive to migrate through) extend some pseudopodia-like protrusions known as proplatelets in the bloodstream [128], [129]. Those projections are dependent on F-actin reorganization and the dynamic arrangement of microtubules [124], [130]. Proplatelets are then fragmented into platelets, by shear force exerted by circulating blood [122], [124].

1.2.3.2 The structure of platelets

Platelets are disc-like cells, with an average diameter ranging from 2 to 5 μm and a thickness of 0.5 μm [121], [122]. They do not contain a nucleus but carry other organelles in their cytoplasm, like mitochondria, alpha (α)-granules, dense (δ)-granules, peroxisomes and lysozymes, as shown in **Figure 1.4**, all of which have crucial roles in haemostasis [122], [123]. Moreover, they contain the platelet-specific endoplasmic reticulum known as dense tubular system (DTS), the open canalicular system (with a possible role as a store for cytoplasmic glycoproteins) and free Ca^{2+} ions [122].



Mitochondria are crucial in platelet function and survival as they are the only source of DNA in platelets, encoding more than 35 genes [131]. There are around 5-8 mitochondria in each platelet and in addition to hosting oxidative phosphorylation and producing energy in the form of ATP, they regulate apoptosis and stress responses as well as maintaining calcium ion homeostasis in platelets [131].

The dense tubular system (DTS) of platelets is a canalicular system which resembles the structure and function of the smooth endoplasmic reticulum, present in other nucleated cells, and is also known as the platelet store for calcium ions [123]. Moreover, it is involved in thromboxane A₂ (TXA₂) production and upon platelet activation releases high amounts of Ca²⁺ [123].

Platelets usually contain a maximum of 2 lysosomal granules each, which are responsible for endosomal digestion by degrading contents in their acidic environment using cathepsins and glycohydrolases and other proteases [121], [123], [131]. Peroxisomes break down reactive oxygen species (ROS) like hydrogen peroxide using the enzyme catalase promoting platelet survival [122].

Alpha-granules

As shown in **Figure 1.4**, there is a diverse range of proteins stored in α -granules, either coming directly from the megakaryocyte, or by platelets taking up proteins from the plasma and storing it in α -granules [123]. They are the largest and most abundant type of granules in platelets, with as many as 80 of them (typically 50 to 80) in each platelet and contain proteins involved in critical stages of haemostasis, coagulation, immunity, chemotaxis and inflammation [123].

Additionally, they express membrane-bound receptors like P-selectin (or CD62P) that upon platelet activation they fuse with the platelet membrane to induce extracellular signalling or get released into the bloodstream and communicate with other cells of the vasculature [132]. This process occurs upon platelet activation, where intracellular signalling allows rearrangement of the actin cytoskeleton and microtubules, fusing the membrane of α -granules with the platelet one [121], [123]. Platelet activation will be covered in more detail at the next section.

P-selectin, despite having important roles in endothelial adhesion and leukocyte rolling as mentioned in **Section 1.2.2**, it plays a crucial role in platelet function, either membrane-bound or at its soluble form

[132]. It binds its ligand PSGL-1 on cells of the immune system like neutrophils, monocytes and T cells, enhancing inflammation, or aiding in thrombus formation by recruiting more monocytes on site [132].

P-selectin also binds other surface molecules like the glycoprotein Ib-IX-V (GPIb-IX-V) specifically at its Ib α domain [133] found on endothelium. This results in platelet rolling on the endothelium at resting or activated conditions, which in turn brings platelets in close proximity, enhancing platelet aggregation when activated [133].

Dense-granules

Dense (δ)-granules in platelets share a few similarities with α -granules yet are quite different [134]. Both types of granules contain key proteins or mediators for processes like inflammation or coagulation and upon activation, fuse with the platelet membrane releasing their contents in circulation. In contrast to α -granules, δ -granules are smaller in size, with approximately only 3 to 8 of them found in each platelet [134]. Releasing their contents, δ -granules further augment platelet activation as ADP is one of the main platelet agonists studied to date [123], [134] initiating a positive feedback loop.

Amongst other ions and proteins, δ -granules are rich in Ca^{2+} which also have a critical role in platelet activation signalling and Trinh et al., imaged the granules using scanning transmission X-ray microscopy where calcium ions were used to identify them from other granule types [135]. Not only the characterized δ -granule structures but offered a new way of estimating their calcium content which is associated with δ -granule deficiencies leading to bleeding.

Actin cytoskeleton of platelets

The most abundant protein found in platelets is actin, mainly the filamentous form (F-actin), which covers the cytoskeleton and moves towards the cell protrusions upon activation [136]. Specifically, in response to stimuli, actin assembles into irregular networks of filaments adjacent to the microtubule bundles surrounding the platelet circumference, creating pseudopodia-like projections [137], [138].

Those filopodia expand into a spindle-like structure, allowing the platelet to spread increasing surface area and sensing as Mayr et al. demonstrated using a high-resolution microscopy technique called dSTORM (direct stochastic optical reconstruction microscopy) [137]. Moreover, they obtained a ‘fried

egg' like structure of platelets where the area between the filopodia was slowly being filled allowing platelet motility during an activated state.

1.2.3.3 Inhibition of platelet activation by EC-derived NO

As briefly mentioned at the end of **Section 1.2.2**, EC-derived NO maintains a resting phase in platelets by inhibiting activation. In more detail, NO diffuses through the platelet surface and activates soluble guanylyl cyclase, an enzyme responsible for catalysing the conversion of guanylyl tri-phosphate (GTP) into cyclic guanylyl mono-phosphate (cGMP) [139], [140]. This in turn activates the cGMP-dependent Protein Kinase (cGMP-PK) which phosphorylates key mediators involved in the inhibition of platelet activation signalling [139], [140].

Two of these targets are PLC and IP₃, which similar to endothelial cells described in **Section 1.2.2**, are responsible for increasing the levels of intracellular Ca²⁺ either by allowing them to enter the cell from plasma / circulation, or by being released from the platelet-specific DTS [139]. Re-organization of the platelet cytoskeleton is also inhibited by cGMP-PK phosphorylating proteins like VASP (Vasodilator-Stimulated Phosphoprotein) involved in actin arrangement [141].

Re-organization of actin cytoskeleton allows activation of the platelet surface integrins $\alpha_{IIb}\beta_3$ which are the main drivers of platelet aggregation, thus NO / cGMP-PK indirectly downregulates that as well [139]. Moreover, Roberts et al., published a NO-mediated inhibition of platelet spreading and adhesion on collagen by targeting the $\alpha_2\beta_1$ -mediated pathway on already activated platelets, possibly by decreasing the platelet-derived ADP release [140].

Another route through which NO / cGMP-PK maintain inhibition of platelet activation is by targeting the RhoA-mediated pathway, a GTPase allowing platelets to change shape and spread upon activation [142]. This is done by inhibiting the activation of RhoA therefore downstream signalling doesn't take place, preventing cytoskeletal changes or granule release [142]. Moreover, cyclic nucleotides like cGMP and cAMP (cyclic adenosine monophosphate) can block the activation of p38 and p42 MAPKs (Mitogen Activated Protein Kinases), also involved in platelet activation through calcium ion-elevating mechanisms [139].

Similar to cGMP and cGMP-PK are the cAMP and cAMP-PK (cAMP-Protein Kinase), a signalling pathway activated from prostacyclin (PG-I₂) instead of NO and shares common targets as the cGMP pathway during platelet inhibition, with some additional ones [139]. Those extra targets include the actin binding protein (ABP) and caldesmon, for stabilizing actin cytoskeleton in resting platelets, the glycoprotein Ib β (GPIb β) subunit for inhibiting polymerization of actin upon collagen-evoked platelet activation and the G-protein subunit α 13 for inhibiting the Rho kinase signalling pathway.

1.2.3.4 Platelet Activation

Platelets circulate at a resting state and upon activation, have several essential roles in haemostasis and immunity [121]. After looking at platelet contents and EC-mediated platelet inhibition through NO pathways, this subsection will focus on membrane receptors and signalling pathways involved in platelet activation followed by aggregation and adhesion. Several molecules were shown to induce platelet activation, referred to as platelet agonists [134] and here I will review the most popular ones, ADP, collagen, thrombin and TXA₂.

Platelet activation by ADP

Extracellular ADP, released by damaged or activated cells (including platelets) binds to its heptahelical transmembrane receptors P2Y₁ and P2Y₁₂ on the platelet surface, two GPCRs (G-protein-coupled receptors) that have a synergetic action in activating platelets [143]. Upon binding to ADP, the G protein associated with P2Y₁ activates Phospholipase C (PLC) which increases production of IP₃ from PIP₂. IP₃ then binds to its receptor on the DTS, releasing Ca²⁺ in platelets [144]. This results in cytoskeletal changes followed by degranulation and activation of the integrin composition which mediates platelet aggregation [144], [145].

Intracellular ADP/P2Y₁₂ downstream signalling is mediated by the G protein subunits, G α i and G β γ , where the first dissociates upon ADP binding, and inhibits the activity of AC, reducing the levels of cAMP, resulting in reduced activation of PKA, thus reduced phosphorylation of VASP [145]. This in turn decreases the regulation of actin cytoskeleton, increasing adhering properties of platelets,

degranulation of α -granules, and despite that ADP-activation is not as strong to release δ -granules, signal can be amplified by other platelet agonists [143].

G $\beta\gamma$ activates PI3K (Phosphoinositide 3-Kinase) which in turn activates the Ras-associated protein (Rap1b) signalling as well as phosphorylating and activating Protein Kinase B (PKB) which activates p38 and ERK (extracellular-regulated protein Kinase) and this results in TXA₂ production [145]. Both Rap1b and PKB pathways result in the activation of $\alpha_{IIb}\beta_3$.

Similar to ADP, ATP can also activate P2Y₁ or P2Y₁₂ but less strongly [144]. Moreover, ATP can bind another platelet surface receptor P2X₁, which is a ligand-gated ion channel, and upon activation it allows an influx of extracellular calcium ions in the platelets, magnifying the activation process and response [144].

Thrombin-mediated activation of platelets

Thrombin, a strong platelet agonist, is a serine protease that is mostly released during coagulation. There are two membrane receptors for thrombin on human platelets PAR-1 and PAR-4 (protease-activated receptors 1 and 4) [123]. PAR-1 is a high-affinity receptor and low levels of thrombin can activate it, whereas PAR-4 is a low-affinity receptor and high levels of thrombin are required to activate it [123], [146].

Thrombin cleaves the extracellular N-terminal domain of PAR-1 between residues 41 and 42, and between residues 47 and 48 of PAR-4, exposing a new N-terminal domain in both cases, which acts as a ligand and self-activates the receptor by binding to itself and allowing intracellular signalling [123], [146], [147]. Both PAR-1 and PAR-4 are GPCRs, and their effects are dependent on the G protein subunits they associate with. Common events between the two receptor pathways involve G_q and G_{12/13}, where the first activates PLC β , increasing IP₃ levels and subsequently releasing Ca²⁺ ions from DTS, and the latter one activates the GTPase RhoA resulting in cytoskeletal changes and degranulation [123], [146], [147].

Another PAR-1 pathway that takes place upon thrombin activation, is mediated by the G_i subunit [147]. Similar to the ADP/P2Y₁₂ pathway, the G subunit inhibits the AC, reducing cAMP levels which in turn

reduces PKA activity and results in less inhibition of platelet activation [147]. Overall, PAR-1 is quicker and has a short-term effect, whereas PAR-4 takes slightly longer to activate but has a long-term effect.

Thromboxane A₂ (TXA₂) activation

TXA₂ is another platelet agonist produced by already activated platelets from the cyclooxygenase-1 (COX-1) enzyme, utilizing arachidonic acid, and just like ADP, TXA₂ amplifies the activation signal in neighboring platelets through a membrane bound GPCR [123], [146]. Specifically, TXA₂ receptor can be also activated by prostaglandins, hence it's called TXA₂/prostaglandin receptor or TP [146].

Similar to thrombin/PAR signalling, upon binding of TXA₂ to TP, G protein subunits Gq and G_{12/13} dissociate from the receptor and initiate an intraplatelet cascade of events [148]. This results in elevated levels of IP₃ which enhance calcium ion mobilization from DTS and activation of RhoA which further removes the inhibition pathways of platelet activation, enabling actin reorganization, shape change and degranulation [148].

Collagen-induced platelet activation

Another strong platelet agonist is collagen. Collagen is exposed in the subendothelium layer of injured tissue and amongst other roles in coagulation, it binds platelets at two different receptors, the $\alpha_2\beta_1$ integrin composition that allows firm adhesion of platelets on the endothelium, and glycoprotein receptor VI, or GPVI [149].

GPVI, unlike the previous agonist receptors discussed, is not coupled to a G protein, but to FcR γ (Fc receptor γ chain) [150]. Moreover, it is coupled to two important kinase proteins, Fyn and Lyn, through its proline-rich domain and upon collagen binding to the receptor, Fyn and Lyn activate by phosphorylation and in turn phosphorylate the FcR γ on its ITAM (immunoreceptor tyrosine-based activation motif) domain [150].

Downstream signalling largely depends on LAT (linker for activation of T cells), a transmembrane protein with multiple phosphorylation sites, that allows a cascade of phosphorylation events to occur resulting in the activation of Phospholipase C γ (PLC γ) increasing IP₃ levels, hence elevating intraplatelet calcium

levels [149]. Subsequently, this results in cytoskeletal reorganization and shape change, degranulation and activation of the $\alpha_{IIb}\beta_3$ integrin composition on the platelet membrane driving platelet aggregation.

1.2.3.5 Role of calcium in platelet activation

A common stage in platelets activation, induced by any of the agonists described above, is the increase of intracellular Ca^{2+} , either by release from the DTS or from a calcium ion influx from the plasma. Gelsolin and cofilin are two calcium-dependent proteins which regulate the actin cytoskeleton [151]. Gelsolin is not only responsible for cleaving covalent bonds between actin subunits, but also capping them to prevent formation of new ones and cofilin binds to and disassembles filaments of actin allowing reorganization [151].

A rise in intraplatelet Ca^{2+} ions enables gelsolin and cofilin to promote cytoskeletal changes resulting in an activated state of platelets, disturbing F-actin and allowing extension of protrusions or lamellipodia, a conformational change that activates the $\alpha_{IIb}\beta_3$ complex [151]. Upon ligand binding, the $\alpha_{IIb}\beta_3$ downstream signalling (known as outside-in) together with high levels of calcium, enable another calcium-dependent protease calpain to cleave other cytoskeleton-regulating proteins making the change in platelet shape and activation easier.

Additionally, phosphorylation of the myosin light chain (MLC) by kinase enzymes is calcium-dependent as well [146]. This involves a RhoA-dependent pathway, where phosphorylation and inactivation of an MLC phosphatase occurs, allowing actin-myosin contractions to occur. All of the above cytoskeletal changes induce platelet restructuring, from a round to a star-like shape with lamellipodia, increasing surface area to volume ratio, degranulating and releasing contents of α - and δ -granules [144], [149], [151]. This enhances platelet aggregation and platelet adhesion.

1.2.3.6 Platelet aggregation mediated by $\alpha_{IIb}\beta_3$

Upon activation, platelets can interact with each other through CD62P (or P-Selectin) which moves to the cell membrane during a granule release, though this is a weak interaction and does not hold platelets firmly together [123]. The membrane-bound integrin complex $\alpha_{IIb}\beta_3$, that restructures during

cytoskeletal organization into its active form, is the main mediator of effective platelet aggregation [123], [152].

At resting conditions $\alpha_{IIb}\beta_3$ maintains a bent, inactivated shape and during platelet activation actin remodelling causes $\alpha_{IIb}\beta_3$ to elongate and acquire its activated form, through an intermediate state, exposing the fibrinogen binding site at the top of the integrins [152]. Neighbouring platelets are linked together by fibrinogen, which binds the activated $\alpha_{IIb}\beta_3$ on the surface of platelets at high affinity, resulting in firm aggregation and stabilization of thrombus formation [152].

Inhibiting $\alpha_{IIb}\beta_3$ destabilizes platelet aggregation and reduces the stability of thrombi [153]. This is considered a therapeutical target for people with a high risk in developing a CVD and eptifibatide (or integrilin) is a popular inhibitor used in clinical settings as well as in research to examine inhibition of platelet aggregation [152], [153], [154]. Eptifibatide mimics a toxic bleeding-causing peptide from rattlesnake venom, and as a typical competitive inhibitor, it binds $\alpha_{IIb}\beta_3$ at high affinity preventing platelet/fibrinogen interactions and subsequently effective aggregation [153].

1.2.3.7 Platelet adhesion to collagen

Exposed collagen on the subendothelium matrix of a damaged tissue interacts with its receptor GPVI on platelets directly, recruiting and activating platelets at the site of injury [149], [150]. In addition to that, collagen can also interact with platelets through von Willebrand factor (vWF) further enhancing platelet recruitment and adhesion to the vascular wall [155].

vWF is a large glycoprotein stored in various elements of the cardiovascular system, including platelets (in α -granules) or in vesicles called Weibel-Palade bodies in ECs. vWF has a globular structure and during activation it is released in plasma and binds to exposed collagen (through its A3 domain) on the subendothelium matrix at a site of injury [123], [155]. Shear stress from blood forces vWF to elongate into a tail-like structure exposing its A1 domain, the binding site for $\alpha_2\beta_1$ (or GPIb-V-IX), the integrin complex on the surface of platelets [155].

A few research groups developed flow models to study the dynamic of vWF and platelets during adhesion of the latter to collagen or endothelial cells [155]. This is a critical stage at the interface of haemostasis and thrombosis and makes a promising target for anti-CVD medication.

1.2.3.8 The process of coagulation

After reviewing different signalling pathways involved in endothelial cells and platelets, this section will focus on the coagulation pathway, a process which highly depends on both. Coagulation is a critical process that occurs in our body to stop bleeding, but if it occurs excessively or when not needed it can result in clots potentially blocking smaller arteries in the heart or brain resulting in myocardial infarction, stroke or death [156].

Coagulation can initiate from two separate pathways, the intrinsic or the extrinsic, both leading to the production of FXa from FX, followed by a common pathway [156], as shown in **Figure 1.5** below. The intrinsic pathway commences by contact of coagulation factors to damaged endothelium where negatively charged surfaces are exposed activating the enzyme responsible for cleaving FXII into FXIIa [157]. FXIIa catalyses the activation of FXI to FXIa, which in turn activates FIX into FIXa. FIXa together with FVIIIa and negatively charged phospholipids (PL) convert FX to FXa. Producing FIXa and FXa are two processes highly dependent on available Ca^{2+} ions [158].

The extrinsic pathway involves exposure of Tissue Factor (TF) on the subendothelium upon tissue disruption or insult [157], [158]. This activates FVII into FVIIa, and TF together with FVIIa, PL and Ca^{2+} ions catalyse the conversion of FX into FXa, as in the intrinsic pathway. Many molecules including Tissue Factor Pathway Inhibitor (TFPI) or Protein S (PS) are responsible for inhibiting this pathway under normal conditions or regulating the extent to which coagulation occurs [158].

The common pathway which follows either the intrinsic or extrinsic initiation pathway, involves the production of FXa (prothrombinase) from FX [156], [158]. This, together with Ca^{2+} and PLs, is responsible for converting prothrombin to thrombin, which in turn cleaves soluble fibrinogen to insoluble fibrin [156]. Fibrin is critical in cross-linking activated platelets together and stabilizing the thrombus, or a clot [153].

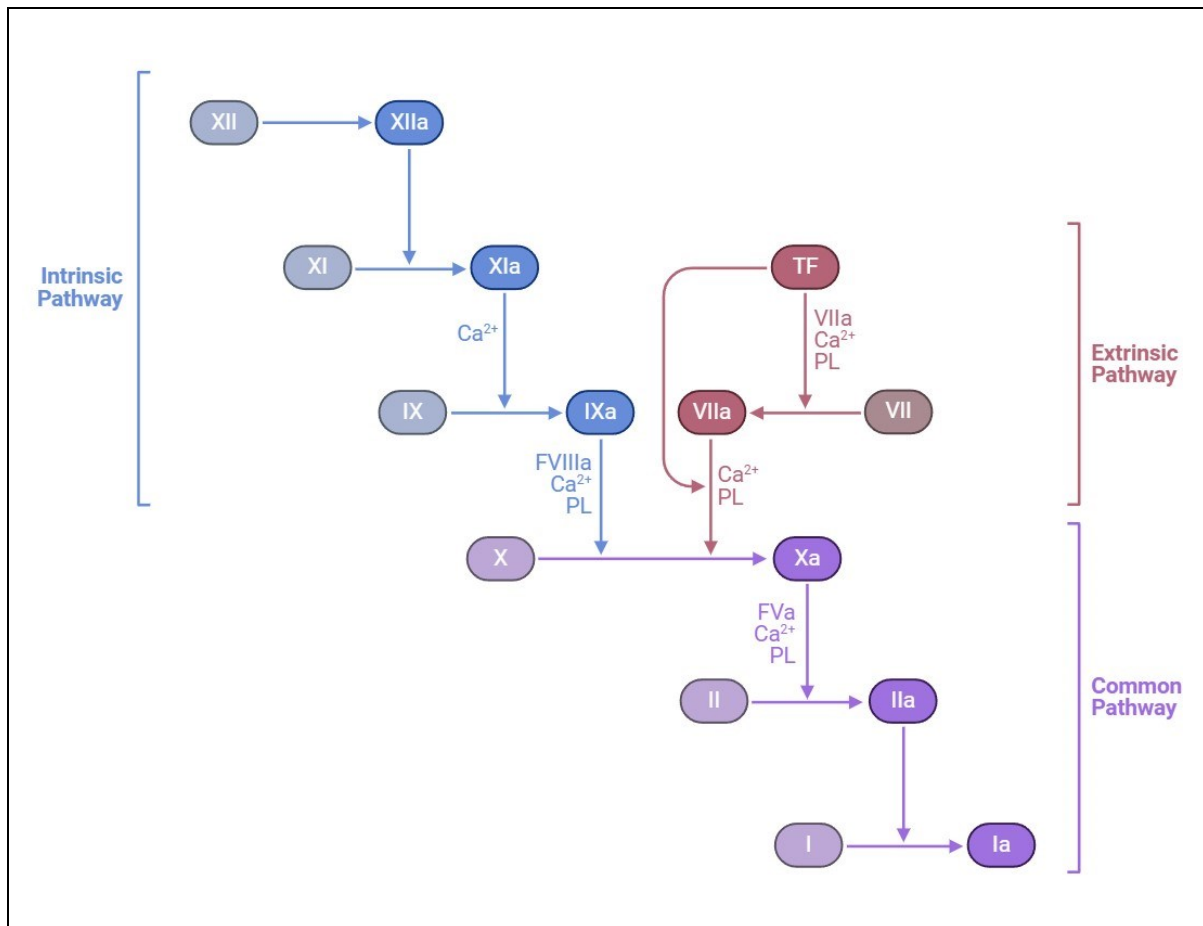


Figure 1.5. The coagulation pathway. The figure summarizes the cascade of events that occur in coagulation. Intrinsic pathway is shown in blue on the left and the extrinsic pathway is shown in pink on the right. Intrinsic pathway occurs from an activating surface cleaving FXII, whereas extrinsic pathway initiates from trauma, upon exposure of Tissue Factor (TF). Both pathways result in producing FXa from FX, and subsequently the common pathway takes place which results in thrombin (FIIa) formation, cleaving fibrinogen (FI) into fibrin (FIa). Figure is produced online, adapted from the 'Coagulation pathway' Biorender Software template.

1.2.4 CARDIOVASCULAR DISEASES (CVDs)

Balancing haemostasis and thrombosis requires excellent coordination to prevent unnecessary coagulation and clotting from occurring. Endothelial cells and platelets are in the interface of this balance and are linked to thrombosis-dependent obstructive CVDs. A common chronic state involved in CVD development and progression is atherosclerosis [159], [160].

1.2.4.1 Atherosclerosis

Initiation of atherosclerosis can result from different genetic or lifestyle factors that enhance endothelial dysfunction including smoking, diabetes or chronic inflammation. LDL molecules circulating in blood infiltrate the intima layer of the artery during endothelial dysfunction and get oxidized [114], [160]. This oxidation produces oxLDL which is highly pro-inflammatory and allows the entry of monocytes through ECs by an enhanced expression of adhesion molecules like ICAM-1 and VCAM-1, as well as by weakening junctions like VE-Cadherin or PECAM-1.

Monocytes entering the intima become tissue macrophages and engulf oxLDL through their scavenger receptors (e.g. CD36) resulting in lipid-loaded macrophages, also known as foam cells [160]. Foam cells in turn release a number of toxic atherogenic-mediators, like pro-inflammatory cytokines, reactive oxygen species (ROS), tissue factor and matrix metalloproteinases that degrade the matrix [160]. The recruitment and buildup of foam cells in the intima result in what is known fatty streak, the earliest lesion of atherosclerosis [114].

As this lesion develops, smooth muscle cells migrate from the media layer of the artery to the intima and proliferate, synthesizing collagen and other elements of the extracellular matrix, which stabilize the lesion or plaque [114], [160]. This fibromuscular plaque grows further as more oxLDL, foam cells, smooth muscle cells and collagen build up and accumulate forming a fibrous cap, mainly composed of smooth muscle cells and dense extracellular matrix and separates the lipid core of the plaque from the bloodstream.

Matrix metalloproteinases and other degrading enzymes produced from associated cells like macrophages, or other cells of the immune, weaken this fibrous cap making it more prone to rupture [114], [159], [160]. High shear from arterial blood can break this plaque exposing elements like vWF and collagen, who in turn activate platelets, which aggregate and form a thrombus [159]. This process can result in coronary artery disease and high blood pressure, and thrombi can occlude arteries leading to myocardial infarction or if in cerebral artery, ischaemic stroke [114], [160].

In addition to their role in thrombus formation after plaque rupture, platelets have a few other roles during atherosclerosis [159]. Firstly, they can adhere to the endothelium during endothelial dysfunction and enhance the recruitment and capture of monocytes at the site, by interactions between P-selectin on activated platelets and PSGL-1 on monocytes.

Additionally, platelets share the same oxLDL scavenger receptor as macrophages, CD36, suggesting a role in lipid metabolism [159]. Upon activation, platelets release a number of atherogenic mediators through their granules that enhance differentiation of monocytes to macrophages, subsequently foam cells, hence regulating the stability of the plaque core.

1.2.4.2 Venous thrombosis

Unlike arterial thrombosis, venous thrombosis (VT) does not depend on an insult on the endothelium [161], [162]. Reduced blood flow in veins (known as stasis) often due to the lack of mobility or pregnancy together with a pro-coagulant state are the main factors in the development of VT, where a thrombus in VT mainly consists of fibrin, erythrocytes, leukocytes and platelets, but in less amounts compared to arterial thrombi [161].

Immune response is highly associated with VT, as recruitment of neutrophils and monocytes was observed during blood stasis [161]. Moreover, Bruhl et al. showed that the release of neutrophil extracellular traps (NETs) as well as tissue factor expression by monocytes could initiate and magnify coagulation, resulting in high fibrin production. In VT, platelets also mediate recruitment of white blood cells and participate in coagulation [161], [162].

Venous thromboembolism (VTE) is the umbrella term to describe deep vein thrombosis that often occurs in legs, and the migration of the thrombus in pulmonary veins, known as embolism [162]. Unlike arterial thrombosis, VTE occurs in a low shear stress.

1.3 ARV EFFECTS IN ENDOTHELIAL CELLS AND PLATELETS

After discussing endothelial and platelet activation and their role in disease, the following section will focus on how different antiretroviral drugs discussed in **Section 1.1** were shown to affect those elements and bridge the sections together.

1.3.1 EFFECTS OF ARVS ON ENDOTHELIAL CELLS

Over the years, there is increasing evidence that despite the great efficacy of cART in suppressing the viral load, daily exposure of antiretrovirals can impact endothelial cell viability and function. In a review article by Marincowitz et al., vascular endothelial dysfunction was associated with ARV, through a rise in cellular levels of toxic ROS. This is due to several factors, including continuous ARV exposure and elevated levels of pro-inflammatory cytokines seen in chronic inflammation like TNF- α , IL-6 and IL-1 β [163].

Specifically, those cytokines together with viral proteins were linked to elevated levels of ICAM-1, VCAM-1 and reduced levels of eNOS and claudin proteins. Efavirenz (NNRTI) and lopinavir (PI) were linked to higher expression of adhesion molecules, apoptosis and oxidative stress in endothelial cells, and with decreased levels of junctional proteins like JAM or claudin [163].

NRTIs, especially older ones like AZT, were associated with other clinical manifestations including lipodystrophy and myopathies [164]. This is because they are nucleoside analogues and can get incorporated into the polymerase γ enzyme, responsible for replication of mitochondrial DNA (mtDNA), resulting in shorter mtDNA which in turn affects the process of oxidative phosphorylation, enhancing the production of toxic reactive oxygen species in the mitochondria and subsequently the cell [164].

Gomez-Garre et al. published a meta-analysis of ARV effects in 2012, especially (N)NRTIs and PIs, in atherosclerosis, by associating ARV exposure in years with increasing size of carotid intima-media thickness, mediated by vascular progenitor cells [165]. A study by Cohen et al., showed that two of the most popular NRTIs currently, TDF and TAF, increased oxidative stress and inflammation in HUVEC

(Human Umbilical Vein Endothelial Cells) promoting premature senescence, subsequently affecting adjacent astrocytes used in co-culture settings [166], [167].

Khawaja et al., explored the effects of TDF, TAF and ABC on two endothelial cell models, HUVEC and HCAEC (Human Coronary Artery Endothelial Cells) [167]. Cells were treated *in vitro* with clinically-relevant concentrations of the antiretroviral agents and levels of ICAM-1, VCAM-1 and tissue factor levels were measured. TNF- α -stimulated HUVEC following a 48h exposure of ARVs showed significantly higher levels of ICAM-1 and tissue factor in ABC-treated cells, compared to TDF- or TAF-treated cells but no significant difference was observed between those and the vehicle control.

Additionally, when HCAEC were used in a similar setting, Khawaja et al., observed a similar outcome, with ABC-treated cells showing higher levels of ICAM-1 and tissue factor levels and activity than TDF-, TAF-, or DMSO-treated (control) cells [167]. In fact, ABC was linked to endothelial dysfunction and indirectly to platelet dysfunction, through a NO-mediated pathway from endothelial cell toxicity [168]. This highlights the importance of comparing off target effects of drugs in the same drug class that are proven safe and efficient in suppressing HIV but may confer differential off-target effects.

1.3.1.1 Effects of INSTIs on endothelial cells

Currently, the effects of INSTIs on endothelial cells are being explored extensively with contradicting outcomes and conclusions [169], [170], [171]. Afonso et al., demonstrated a decrease in HCAEC-mediated inflammation in dolutegravir (DTG) treated cells compared to untreated, by interfering with the NF- κ B pathway [170].

Moreover, they showed a reduced senescence in DTG-treated compared to untreated HCAEC, as well as lower levels of soluble ICAM-1 and VCAM-1 [170]. In agreement to those outcomes were the results published by Auclair et al., which showed an NF- κ B mediated reduction in inflammation upon DTG treatment of HCAEC, resulting in lower levels of pro-inflammatory cytokine production like IL-6 and soluble ICAM-1 [169].

More recently, a contradictory study by Huang et al., revealed a possible cerebrovascular effect of INSTIs that could potentially explain the neuropsychiatric adverse effects reported by ARVs like DTG

[171], [172]. In particular, hCMEC (human cerebral microvessel endothelial cells) were treated *in vitro* with DMSO and DTG or BIC at a range of timepoints and DTG-treated cells (versus DMSO) exhibited a higher mRNA expression of IL-6 (at a 24h exposure) and IL-8 and IL-1 β (at a 48 exposure) [171]. BIC-treated cells (versus DMSO) only exhibited an increase in IL-6 mRNA levels 24h post exposure.

Similarly, they repeated the experiments using an *in vitro* mouse model of the blood-brain barrier (primary cultures of mouse brain microvascular ECs) [171]. They observed a significant reduction in the mRNA of tight junction proteins, including Tjp1 (Tight junction protein 1), occludins and claudins upon DTG versus DMSO treatment after a 6h drug exposure and enhanced IL-6 mRNA levels (post 24h exposure).

When BIC was compared to vehicle control in this cell model, Tjp1 levels were significantly higher than in the DMSO group, but no other significant effects were observed in other tight junction proteins, or pro-inflammatory cytokines [171]. These results suggest an INSTI-mediated effect on endothelial activation, more enhanced in DTG (versus DMSO) than BIC, but no direct comparison between the two INSTIs was conducted.

Similar *in vitro* experiments with human and mouse cell models of BBB, revealed an INSTI-mediated downregulation of several folate transporters linked with neuropsychiatric complications, where DTG effects were reportedly higher than the ones from BIC [173]. These results also agreed with experiments done in mouse *ex vivo* brain capillaries, confirming an INSTI effect on endothelial cells, potentially higher with DTG than BIC [173].

1.3.2 EFFECTS OF ARVs ON PLATELETS

Platelets are considered a double-edged sword in HIV pathogenesis. In particular, they express a number of CCR and CXCR receptors, as well as CLEC-2 (C-type lectin-like receptor 2) and DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin) which facilitate the internalization of HIV [174]. This can either result in the inhibition of viral replication by α -granules or macrophage phagocytosis, or platelets becoming a viral reservoir keeping the virus safe from the host defence mechanism.

In either case, direct interactions between platelets and HIV or HIV-associated proteins (e.g. in virologically suppressed patients), result in platelets circulating in an activated state [174], [175], enhancing processes like atherosclerosis and thrombosis by expressing surface integrins or releasing pro-coagulant mediators. This highlights the significance of understanding how other external factors influence platelet activity, for example the daily exposure of platelets to ARV agents.

A number of articles linked ART and platelet activity and summarized positive associations between PIs and NRTIs [175], [176]. Many PIs, especially ritonavir (RTV), were reported to elevate ROS and TGF- β 1 levels in platelets, leading to downstream pro-fibrotic signalling whereas abacavir was linked to higher platelet activity but no mechanism was yet identified [175].

Kann et al., reported differences in platelet activation in patients receiving cART, with the same NRTI backbone and a different third agent [177]. Specifically, those treated with a PI (e.g. DRV) as the additional agent showed higher levels of activated $\alpha_{IIb}\beta_3$ and CD62P, compared to patients receiving an NNRTI (EFV) or an INSTI (RAL). In a different study by Loelius et al., CD62P levels were unaffected post treatment of platelets (from healthy volunteers) with RTV but had altered levels of TXA₂ and prostaglandin E₂ [178]. Additionally, when compared to other ARV agents, RTV-treated platelets demonstrated enhanced platelet activation and aggregation *in vitro*.

Abacavir effects on platelets divided scientists until a clear mechanism was published [175], [179], [180]. Many reports linked ABC with higher risk of CVD like MI [175], [180] and proposed a platelet-mediated mechanism. In fact, Satchell et al., showed an enhanced agonist-induced platelet aggregation

from patients receiving ABC compared to non-ABC alternatives [180]. Falcinelli et al., previously showed that ABC enhances platelet activity through interfering with the NO-mediated platelet inhibition derived from ECs, showing that the drug had an indirect effect on platelets through the endothelium which was later confirmed by Alvarez et al. [168], [179].

In a model to measure platelet adhesion to HUVEC (from healthy volunteers), pre-treatment of both elements with ABC revealed an enhanced adhesion compared to treatment with other NRTIs like FTC, 3TC or TDF suggesting an ABC-specific and not a class-specific effect [168]. Kirk et al., confirmed this by demonstrating that an ABC metabolite abolished the NO-inhibition of platelets and platelet aggregation, an effect not seen in other NRTIs like TDF or TAF [181].

1.3.2.1 INSTIs and platelet activation

INSTIs are shown to be good candidates (in terms of safety and efficacy) to accompany NRTIs in current treatment combinations [177], [182], [183]. In a study by Tunjungputri et al., patients were divided into four groups, receiving either an INSTI-, a PI- or an NNRTI-based regimen, and the fourth group comprised of healthy controls [182]. After assessing platelet activity from all participants, it was shown that the INSTI group (receiving a RAL-based therapy) displayed a significantly lower expression of the degranulation marker CD62P and lower activation of $\alpha_{IIb}\beta_3$ integrins compared to NRTI- or PI-based regimens.

In a review article, a few studies showed that patients initiating or switching to RAL-based cART did not demonstrate improved platelet activity compared to non-INSTI-based cART, when CD62P or fibrinogen binding was assessed [183]. In the same review, it was also reported that patients switching from PIs to INSTIs had no subsequent improvement on arterial inflammation or platelet activity.

Dolutegravir, one of the newest INSTIs developed and currently used in cART today, has raised concerns in its ability to influence platelets [184], [185]. In 2019, an abstract was published and presented at CROI (Conference on Retroviruses and Opportunistic Infections) suggesting that DTG improves platelet activity by reducing aggregation and activation, relative to control or a PI alternative upon *in vitro* and *ex vivo* drug treatments [184].

Interestingly, in 2022, Madzime et al., published their work investigating potential effects of DTG on platelets and neutrophils post *in vitro* treatment [185]. Platelets from healthy volunteers were treated with vehicle (control), ADP or thrombin, and the agonist plus increasing concentrations of DTG. They demonstrated an increase in platelet degranulation (by measuring CD62P) and higher levels of platelet-neutrophil aggregates, post DTG-treatment compared to control.

In the same study, following experiments included blocking calcium channels on platelets and using calcium-depleted conditions [185]. They revealed that the DTG-mediated enhancement of platelet activity was potentially Ca^{2+} -dependent, and suggested an ionophore-like mechanism, where DTG is believed to increase the intraplatelet levels of calcium ions by delivering them through the platelet membrane, inducing downstream signalling which results in platelet activation. Important to note that DTG alone did not enhance platelet activity and drug effects were only observed in agonist-induced platelet activation.

To confirm this DTG ionophore-like mechanism, Throne et al., explored the DTG effects on neutrophils using a similar methodology, measuring neutrophil activation *in vitro* [186]. In fact, they demonstrated an increased neutrophil activation by measuring ROS production and enzyme release upon DTG treatment and confirmed that the presence of extracellular calcium ions was required for this.

SUMMARY

To summarize, PLWH today have a better quality and quantity of life than 40 years ago, with better understanding of the HIV pathogenesis and numerous treatment options. They still however exhibit double the risk in developing a CVD than the HIV negative population, that could be attributed to a combination of factors, mainly persistent chronic inflammation and continuous exposure to high amounts of ARV agents daily. After discussing the role and importance of cardiovascular elements like endothelial cells and platelets in haemostasis, thrombosis and disease, external factors that may influence their activity requires better understanding. Currently, INSTIs are largely used in combination with NRTIs as third agents, and the literature provides unclear or contradicting views of their impact on vasculature. This project aims to fill in the gaps and provide an unbiased view of their possible off-target

effects on endothelial cells and platelets, to better understand the CVD risk and potentially allow more targeted or personalized treatment options for patients.

1.4 HYPOTHESIS AND AIMS

Dolutegravir and bictegravir, two of the most popular INSTIs today, typically used in the clinical combinations Dovato or Biktarvy respectively, share a very similar chemical structure with just a few differences as shown in Table 1.2, and are commercially competing. Current literature suggests that ARV agents from the same drug class can have differential effects and that INSTIs, especially dolutegravir, might affect components of the cardiovascular system, including ECs and platelets, but not much is shown about bictegravir.

HYPOTHESIS

I hypothesize that dolutegravir enhances endothelial and platelet activation more than bictegravir, that may potentially lead to differences in CVD risk in PLWH.

AIMS AND OBJECTIVES

I divided my project into 3 major aims, with a few objectives in each, as shown in **Figure 1.6** below. Aim 1, covered in the first year of my PhD, focuses on measuring potential effects of INSTIs on endothelial activation using established endothelial models like HUVEC and HCAEC. Adhesion molecules and permeability markers were measured in flow cytometry and confocal microscopy, respectively, post *in vitro* treatment of cells with BIC and DTG.

Aim 2 involves extensive studies of platelets, from HIV negative volunteers, treated *in vitro* with BIC and DTG. Agonist-induced platelet aggregation was measured in light transmission aggregometry, and surface markers of platelet activation were measured in flow cytometry. Eptifibatide-mediated inhibition of platelet aggregation was also assessed in light transmission aggregometry. Lastly, platelet adhesion on a collagen surface was conducted in a flow model at our collaborator Dr Tom McKinnon's lab (Department of Immunology & Inflammation, Imperial College), where whole blood from HIV negative volunteers was exposed *in vitro* to the ARV agents.

Aim 3 in Year 3 includes *in vitro* treatment of platelets, from HIV negative volunteers, with clinically relevant combinations of BIC and DTG, commercially known as Biktarvy and Dovato respectively, and platelet aggregation and activation markers were measured similar to Aim 2. Additionally, samples from HIV patients receiving Biktarvy or Dovato were analysed in similar platelet experiments as well as in thrombin generation assays, at our collaborator Dr Josefin Ahnstroem's lab, to observe any effects in platelet-poor settings.

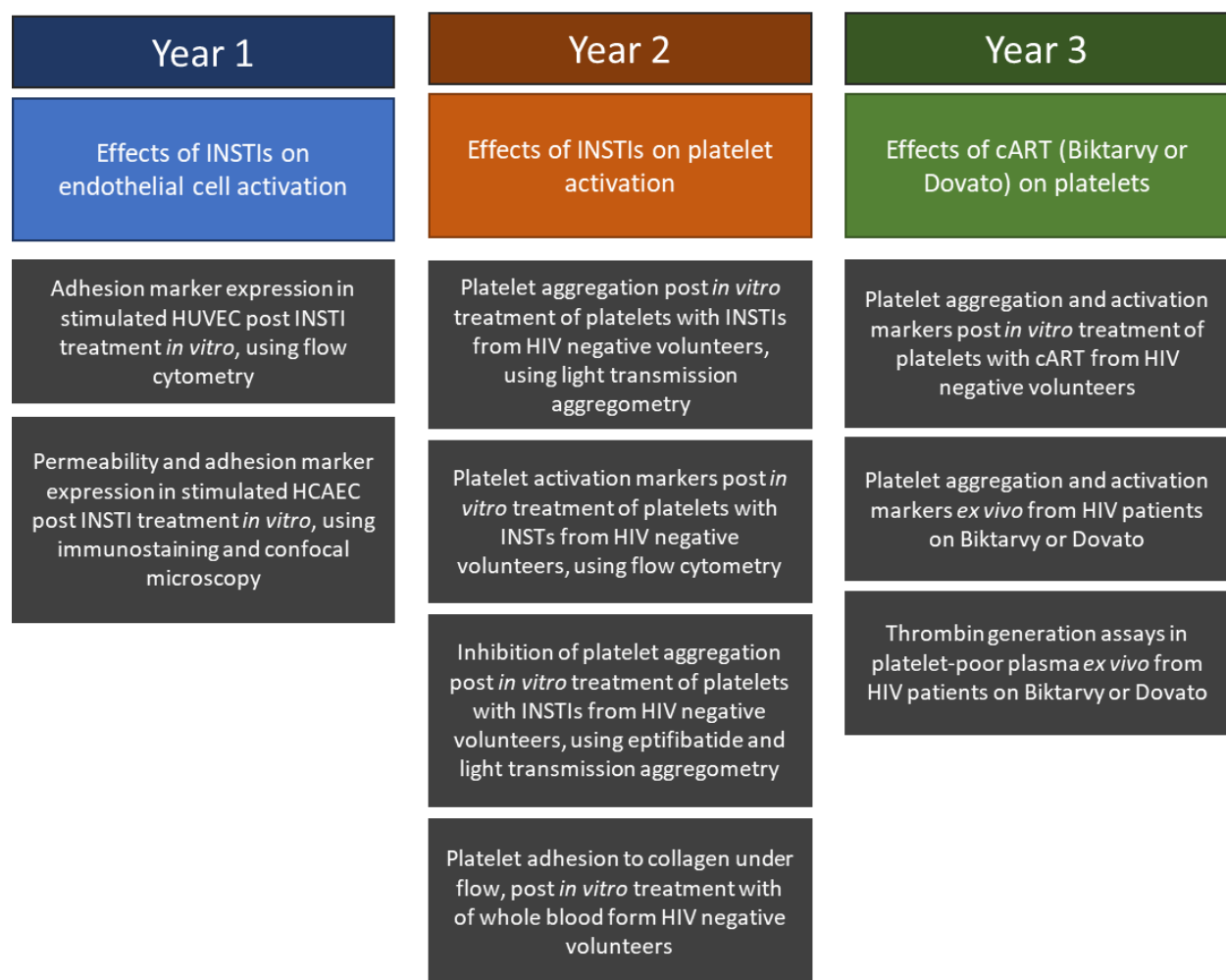


Figure 1.6. The project plan. The diagram breaks down my 3-year project plan into major aims and a list of objectives associated with each (where INSTIs, Integrase Strand Inhibitors, HUVEC, Human Umbilical Vein Endothelial Cells, HCAEC, Human Coronary Artery Endothelial Cells).

CHAPTER 2

Materials & Methods

2 MATERIALS & METHODS

2.1 ANTIRETROVIRAL (ARV) DRUGS

Bictegravir (BIC) and tenofovir alafenamide fumarate (TAF) were supplied by Gilead Sciences (CA, USA). Dolutegravir (DTG), emtricitabine (FTC) lamivudine (3TC) and abacavir sulphate (ABC) were supplied by Selleckchem (Texas, USA). All except ABC were diluted in DMSO to form 10mM aliquots and stored in -20°C until use whereas ABC was diluted in dH₂O. ARV concentrations were used as shown in **Table 2**.

	BIC	TAF	FTC	DTG	3TC	ABC
Plasma Cmax (μM)	13.7	0.2	8.6	8.8	6.1	4.5
Cell culture equivalent (μM)	0.3	0.2	8.6	0.3	6.1	4.5

Table 2. Antiretroviral concentrations used in this study. Top row shows the clinical plasma Cmax values for the antiretrovirals used for the platelet experiments. Bottom row shows the corresponding cell culture equivalent values of the drugs (usually lower due to less unspecific binding to plasma proteins) used for endothelial cell experiments. Cell culture equivalent values were determined upon equilibrium dialysis and mass spectrometry experiments by Gilead Sciences and kindly provided to us.

Details on the techniques used to calculate cell culture Cmax can be found on the poster by Mulato et al., from the 17th European Meeting on HIV & Hepatitis with title “Forgiveness of Antiretroviral regimens: *In vitro* HIV-1 viral breakthrough with 2-Drug versus 3-Drug Regimens Simulating Variable Adherence to Treatment”. Methodology performed by Gilead Sciences to deduce these values was confirmed to account for the 10% FBS used in our experiments.

2.2 VENOUS ENDOTHELIAL CELL CULTURE

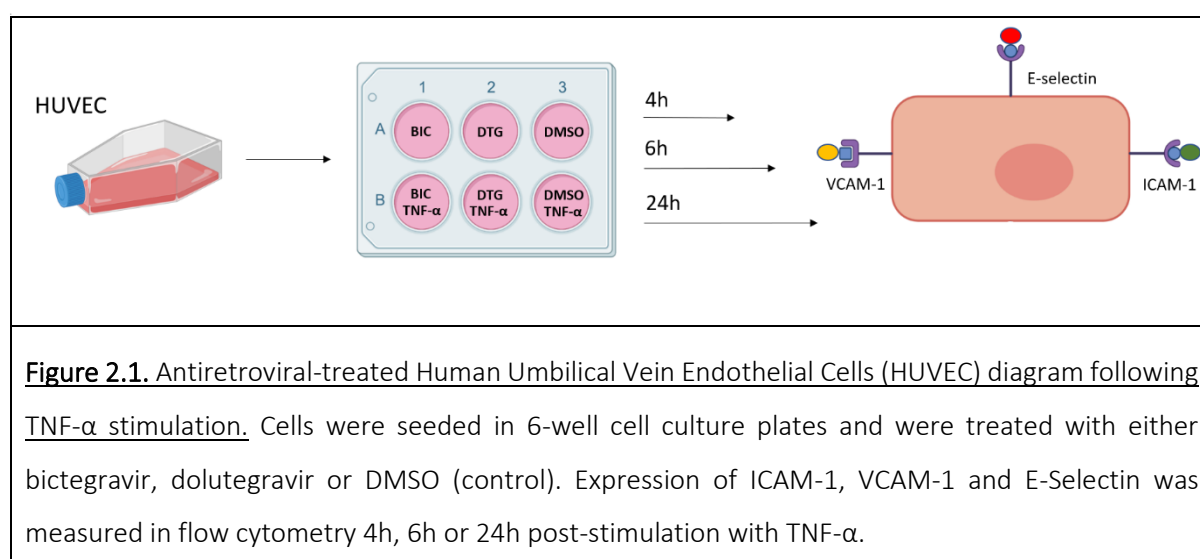
Pooled-donor Human Umbilical Cord Vein Endothelial Cells (HUVEC) (PromoCell, Germany), at passage 3, were grown to 90% confluency in T75 flasks in endothelial cell growth media-2 (PromoCell) supplemented with 10% FBS (Fetal Bovine Serum) (Life Tech), at 37°C and 5% CO₂. Cell splitting was performed upon 2X 5mL PBS (Cytiva, Utah) washes, following a 5min 4mL Trypsin (PromoCell) incubation at 37°C. 6mL of media was then added and the total cell solution was spun for 5mins at

350g. The pellet was resuspended in 5mL of media and cells were counted using Trypan Blue (ThermoFisher Scientific, UK) manually with a haemocytometer.

2.3 ENDOTHELIAL CELL ADHESION MOLECULES IN FLOW CYTOMETRY

Cells were seeded as illustrated below (**Figure 2.1**) and stimulated with 10ng/mL TNF- α (Peprotech, USA) for 4h, 6h or 24h followed by measurement of adhesion molecule expression in flow cytometry. Post-stimulation, media was discarded from the wells and cells were washed twice with 500 μ L of PBS before adding 500 μ L/well of Cell Dissociation buffer (Gibco, UK) for 5mins for cell detachment. Contents of every well were transferred in FACS glass tubes (a tube per well) and 500 μ L of PBS were also added in each well to obtain any remaining cells. Cells were centrifuged for 5mins at 350g, supernatant was then discarded, and pellet was resuspended in 500 μ L of HEPES buffer (HB) and centrifuged in the same way twice more.

A master mix of 3 antibodies, PE-Cy7 (CD54), APC (CD62E) and PE (CD106) (BD Biosciences), were diluted in HEPES buffer at 1:100, 1:20, 1:100 respectively, to measure adhesion molecule expression in HUVEC. 100 μ L of master mix were added in each tube for 30min in the dark, following 2X centrifuge cycles, with 2X washes between each step. Final resuspension was done in 4% PFA in dH₂O, and adhesion molecules expression was measured using Fortessa flow cytometer (BD Bioscience).



2.4 ARTERIAL ENDOTHELIAL CELL CULTURE

Human Coronary Artery Endothelial Cells (HCAEC) (PromoCell) were cultured at passage 5 similar to HUVEC (**Section 2.6**) but using endothelial cell growth MV2 media (PromoCell) plus 10% FBS, and cell counting prior to splitting was performed using an automated counter Cellometer T4 (Nexcelom Bioscience). Cells were seeded in 8-well chamber pre-treated μ -slides (IB-80806, Ibidi) with media added up to 300 μ L per well, after coating wells with 120 μ L Attachment Factor 1X (S006100, Gibco, NY, USA) and washed twice with PBS.

2.5 IMMUNOFLUORESCENCE STAINING

HCAEC were left to rest in the μ slide for 24h before treating with drugs, shown in **Figure 2.2**, for a 48h exposure prior to stimulation with 1ng/ μ L TNF- α for 4h, 6h and 24h. At the end of each stimulation timepoint, media was aspirated and wells were washed twice with 200 μ L PBS containing MgCl₂ and CaCl₂ (Sigma-Aldrich, GmbH, Germany). Cells were then treated with 100 μ L/well of 3.7% PFA in PBS for 15mins, followed by 2X PBS washes and 100 μ L/well of 0.1% Triton X-100 (Sigma) for 3min to permeabilise the cells. Wells were blocked with 3% BSA (in PBS) 150 μ L/well for 15min before adding 110 μ L/well of the primary antibody (Ab) master mix, including 1:100 of ICAM-1 (CD54), PECAM-1 (CD31) and VE-Cadherin (CD144) (Ab details in **Table 3**), diluted in 3% BSA in PBS and left overnight at 4°C. Cells were washed twice before adding 110 μ L/well of the secondary antibody master mix (1:250 each Ab), also made in 3% BSA in PBS, for 1h in the dark. Wells were washed twice and mounted using one drop of DAPI-containing mounting medium (Abcam).

Cells were imaged using confocal microscopy (Zeiss LSM-780 inverted). I utilized tailored Image J macros to automate the analysis of fluorescence microscopy images and obtain MFI values. The process involved setting measurement parameters and applying a series of image processing steps, as follows (in order of use): cell counting, background subtraction, Gaussian blurring and thresholding, to isolate nuclei and create cell masks. Subsequent analysis was performed on specified channels to quantify the expression of key markers PECAM-1, ICAM-1 and VE-Cadherin at 488 nm, 555 nm and 640 nm respectively. Exact macros developed for these experiments can be found in Appendix B.

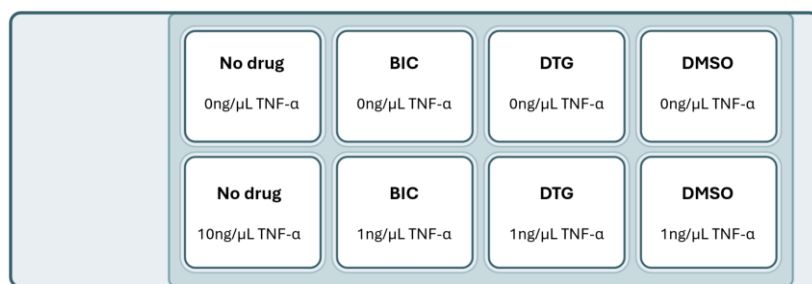


Figure 2.2. Illustration of antiretroviral-treated HCAEC in μ -slides. Top row in the schematic shows the unstimulated wells of the slide containing coronary artery endothelial cells (passage 6) exposed to cell culture equivalent Cmax values of bicitgravir, dolutegravir or DMSO. Bottom row shows the stimulated versions of the above, with 1ng/mL TNF- α , except the positive control well (bottom left) that was stimulated with 10ng/mL.

1° antibody master mix	2° antibody master mix
Goat anti-human ICAM-1 antibody (Bio-technie Ltd)	Donkey anti-goat 555 (ThermoFisher Scientific)
Rabbit anti-human CD31 antibody (Proteintech)	Donkey anti-rabbit 488 (FisherScientific)
Mouse anti-human VE-cadherin antibody (ThermoFisher)	Donkey anti-mouse 647 (FisherScientific)

Table 3. Master mix contents for 1° and 2° antibodies used in immunofluorescent staining. The table shows the 1° Ab master mix on the left including 26.4 μ L of each antibody plus 2560.8 μ L 3% BSA (in PBS) for all 3 slides (1 per timepoint). In the right column, the 2° Ab master mix was made by adding 10.5 μ L of each antibody plus 2608.5 μ L 3% BSA (in PBS).

2.6 BLOOD COLLECTION

Blood was collected by venipuncture in 3.2% sodium citrate-containing blood bottles (Greiner Bio-One) from HIV negative individuals (female : male ratio = 1, mean age 31 +/- 9 years old) by informed consent. Ethical approval was given by the Imperial College London Research Ethics Committee (REC reference: 15IC2615). Blood from PLWH was obtained by the same method from trained nurses in Kobler Clinic, Chelsea & Westminster hospital, in collaboration with Prof Marta Boffito under NHS Research Ethics Committee approval (REC reference: 21/NW/0148).

2.7 PLATELET-RICH PLASMA PREPARATION

Upon collection, blood was centrifuged at 175g for 15 mins (no break) to separate platelet-rich plasma (PRP) from whole blood and was transferred in a fresh 15mL centrifuge tube. 1mL of PRP was further centrifuged at 15,700g for 3min in a microcentrifuge to obtain platelet-poor plasma (PPP) to be used as blank for light transmission aggregometry. PRP was treated with plasma Cmax concentrations (Table 2) of antiretrovirals for a 25min incubation at room temperature prior to activation/ aggregation.

2.8 LIGHT TRANSMISSION AGGREGOMETRY

Agonist-evoked platelet aggregation was measured by light transmission using a plate reader (Sunrise TECAN) and Magellan software, using the following parameters: Absorbance measurement at 595nm every 20 seconds at 37°C (pre-heated). Shaking (at magnitude value 3) between every measurement, with a 7-second settle time between each cycle. 96-well flat bottom polystyrene plates (VWR, Leicestershire, UK) were used for these experiments, with a template layout shown below. Optical density (OD) values were converted into percentage aggregation using the following formula:

$$\left(1 - \frac{\text{final OD}}{\text{initial OD}} \right) * 100.$$

ADP (Sigma-Aldrich), TRAP-6 (Sigma-Aldrich) and collagen (Takeda) were used at various concentrations (1-30µM for ADP and TRAP-6, 1-30µg/mL for collagen) to activate platelets, where ADP and TRAP-6 were diluted in dPBS (Cytiva) and collagen in isotonic glucose (Takeda). Inhibition of platelet aggregation was later assessed in the presence of integrilin (GSK) used at 0.6µg/mL.

	1	2	3	4	5	6	7	8	9	10	11	12
A	PPP	PPP	PPP	PRP	PRP	PRP	PBS	PBS	PBS			
B	1	3	10	30	1	3	10	30	1	3	10	30
C	1	3	10	30	1	3	10	30	1	3	10	30
D	1	3	10	30	1	3	10	30	1	3	10	30
E	1	3	10	30	1	3	10	30	1	3	10	30
F	1	3	10	30	1	3	10	30	1	3	10	30
G	1	3	10	30	1	3	10	30	1	3	10	30
H												

Table 4. Plate layout for light transmission aggregometry upon ART exposure. Columns 1-4 (in orange) represent the bicitegravir-treated PRP, columns 5-8 (in green) represent the dolutegravir-treated PRP and columns 9-12 (in blue) the DMSO-treated PRP. Rows B-C were activated with ADP (1-30M), rows D-E with TRAP-6 (1-30 μ M) and rows F-G with collagen (1-30 μ g/mL). 10 μ L of agonist were pipetted first in each well, except row A, and 90 μ L of drug-treated PRP were added just before initiating measurement. Every sample was run in duplicate.

2.9 PLATELET ACTIVATION IN FLOW CYTOMETRY

ARV- or DMSO-treated PRP was diluted 1:10 in Tyrode's buffer (138mM NaCl, 2.6mM KCl, 12mM NaHCO₃, 0.2mM NaHPO₄) with 5.5mM glucose, added post-autoclaving. ADP and TRAP-6 were diluted in PBS to achieve 10 μ M concentration and collagen in isotonic glucose for 10 μ g/mL. PAC-1:FITC (BD), CD62P:APC (BD) and CD63:PE (BD) were used to measure α IIb β ₃ integrin activation, surface-bound P-selectin (marker for alpha-granule release) and dense granule release respectively. Antibodies were all used at a final dilution of 1:60 as shown in the table below. Each sample was measured for a total of 5min (30s baseline, 270s activation) in the flow cytometer (BD Accuri C6 Plus) using the 4 plots indicated in **Figure 2.3**. Average baseline MFI (mean fluorescence intensity) and the maximum value per measurement were used in the formula $\frac{\text{Maximum value}}{\text{average baseline}}$ to calculate the fold change in every activation marker post activation in the different ARV-treated plasma samples.

Component	Volume per sample (μL)
PAC-1: FITC	2
CD62P: APC	2
CD63: PE	2
Tyrodé's buffer	97
Platelets (1:10)	5
Agonist (ADP, TRAP-6, or collagen)	12

Table 5. Sample tube preparation for platelet activation markers in flow cytometry. The table shows the components added in each microcentrifuge tube to measure GPIIb/IIIa activation as well as alpha- and dense- granule release. Top 4 (antibodies and Tyrodé's) were prepared as a master mix, platelets were added just before recording, and the agonist 30s post initiation.

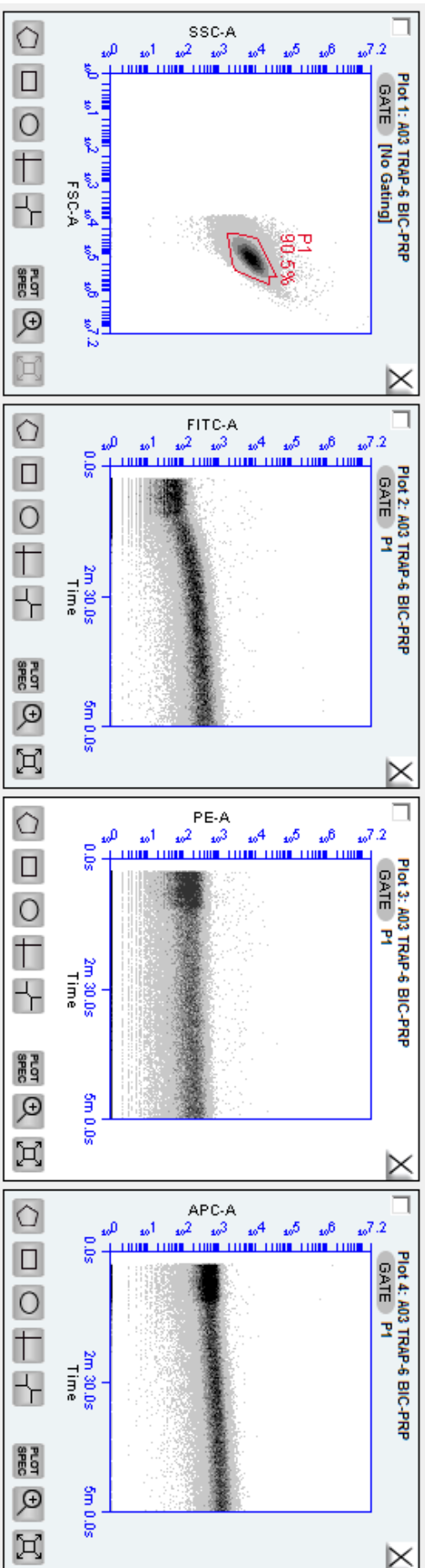


Figure 2.3. Example workspace in the BD Accuri C6 software during platelet activation. The diagram shows representative plots for platelet activation markers throughout a 5min timepoint. First row shows in Plot **1** the size separation of events and gating of platelets as population 1 (P1). Plot **2** shows FITC fluorescence of P1, as an indicator for PAC-1, Plot **3** the PE fluorescence indicating CD63, and Plot **4** the APC fluorescence for CD62P, all throughout a 5min timepoint.

2.10 PLATELET ADHESION UNDER FLOW

6-channel VI 0.4 μ -slides (Ibidi) were coated with 100 μ g/mL collagen (95% Type I; 5% Type III) (Chrono-log) overnight before being blocked with 2% BSA in PBS, with PBS washes in between every step, as reported in Riddell et al, 2018 [155]. Whole blood was collected as in **Section 2.2** and plasma concentrations of ARV drugs BIC, DTG, ABC or Vehicle control (**Table 2**) were added for 25min before starting the experiment. Platelets were fluorescently labelled with Calcein-AM (Sigma), at 4mM, and blood was pumped at $1500s^{-1}$ for 5min, using a perfusion pump (KDS250, KD Scientific). Schematic of the assay is shown in **Figure 2.4**. Platelet adhesion to collagen was visualised using an inverted epifluorescent microscope, CKX41 (Olympus) and real time recordings were taken via XR Camera (Q Imaging) using the Q-Capture Pro software. Images were then analysed in Fiji Software to obtain surface coverage values, following these steps: Image>Adjust>Threshold>Apply, Analyse>Measure. Blood from 3 healthy donors was used for these experiments and every ARV-treated sample was ran in duplicate.

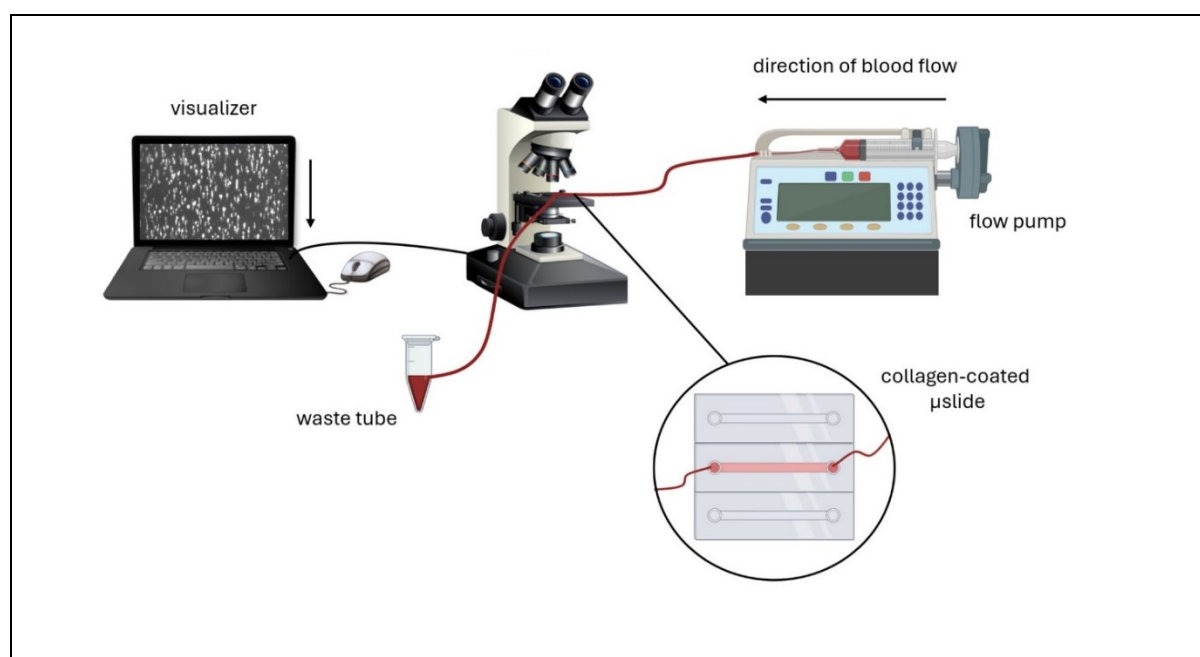


Figure 2.4. Schematic of flow assay measuring platelet adhesion to collagen. The diagram in **A** shows the flow assay used to record platelet adhesion on collagen-coated μ slides at a physiological arterial rate of $1500s^{-1}$. From right to left, whole blood from healthy volunteers, treated with clinical Cmax of antiretrovirals for 25min *in vitro*, is pumped into the μ slide and fluorescently labelled platelets are displayed in the laptop (visualizer) adhering to collagen surface and aggregating. Blood ends up in the waste tube from where it is appropriately discarded.

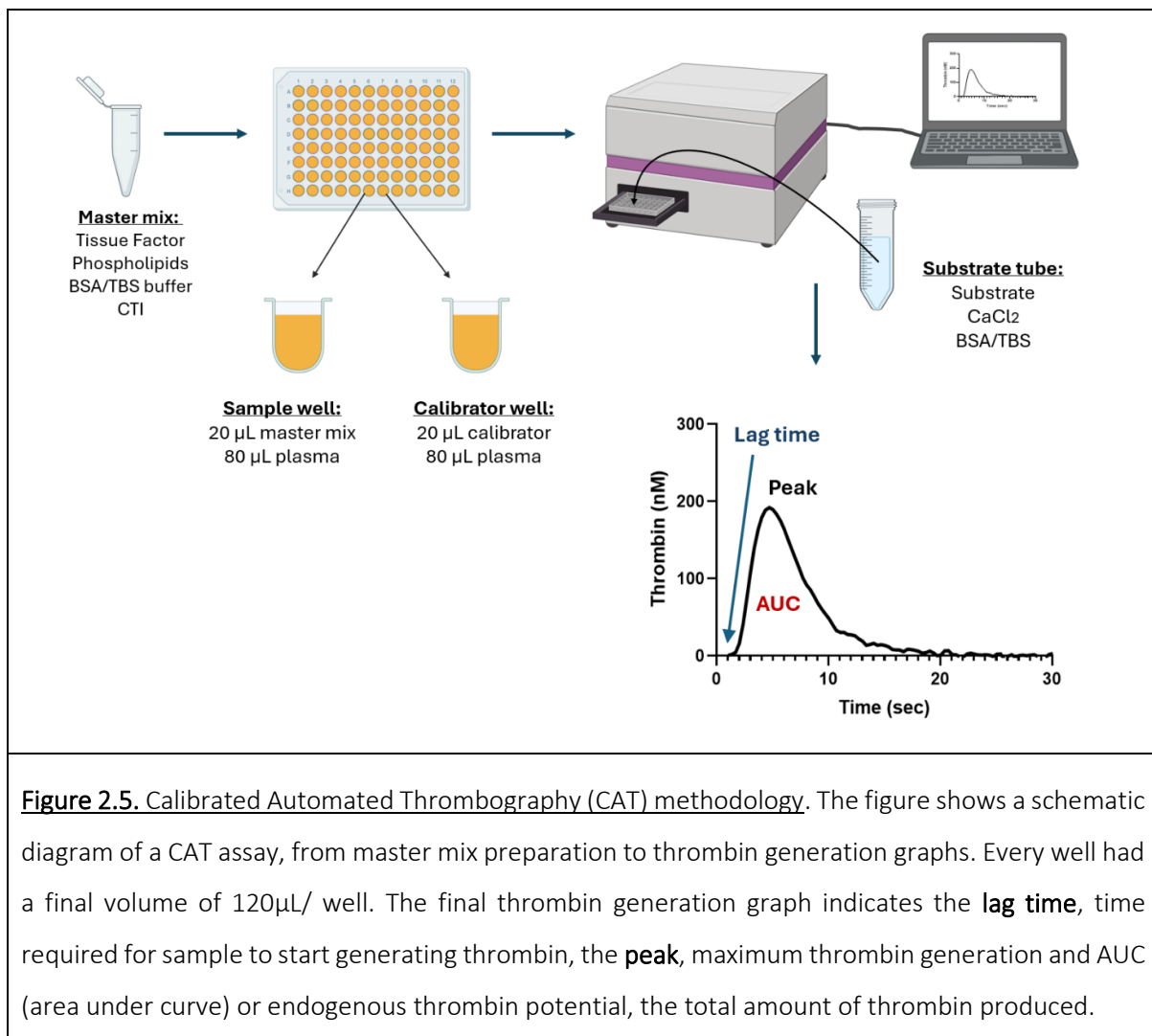
2.11 CALIBRATED AUTOMATED THROMBOGRAPHY (CAT)

CAT assays determine the procoagulant properties of a plasma sample by measuring thrombin generation over time. This occurs by activating the extrinsic pathway of coagulation, *in vitro*, with tissue factor (TF) in the presence of synthetic phospholipid membranes. Thrombin generation was assessed in platelet-poor plasma (PPP) from patients on Biktarvy (BIC/TAF/FTC) or Dovato (DTG/3TC). PRP obtained by centrifuging whole blood (blood collection and PRP preparation protocols in **Sections 2.2 & 2.3**) was further centrifuged at 3,500g for 15mins (no break) followed by 10,000g for 10mins (break) to obtain the PPP used in these assays. For every sample, 80µL/well of plasma was run in the presence of 4µM phospholipids (Avanti Polar Lipids), 65µg/mL of CTI (Prolytix) and 4pM TF (Innovin). Dilutions were made in 0.5% BSA (Sigma) in TBS (20mM Tris, pH 7.4, 150mM NaCl).

Phospholipids, DOPS (1,2-dioleoyl-sn-glycero-3-phospho-L-serine), DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine) and DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) were prepared by using 20µL for DOPS and DOPE and 60µL for DOPC. The phospholipid mixture was first dried using N₂ gas and TBS was used for resuspension making a final concentration of 1.25mM. Phospholipids were then passed through a 0.1µm-pore membrane (Millipore) 21X times using an extruder (Avanti Polar Lipids) and solution was kept in a foil-wrapped tube. Reaction was initiated by addition of 16.6mM CaCl₂ and 0.42mM Z-Gly-Gly-Arg-AMC (Bachem), a fluorogenic substrate of thrombin. Fluorescence was detected in a Fluoroscan Ascent microplate fluorometer (Thermo Lab System), and thrombin generation curves were simultaneously produced using the Thrombinoscope Software. The thrombin generation was quantified against a calibrator which is a thrombin-like molecule that can cleave the substrate with the same kinetics as thrombin but does not have any activity against coagulation factors. More details on protocol and reagents can be found at Andersson H. et al., 2010 [187].

A master mix for the sample wells shown in **Figure 2.5** was prepared including 24pM of TF (Innovin), 24µM of the phospholipid mixture including DOPS, DOPE and DOPC 260µg/mL Corn Trypsin Inhibitor (Enzyme Research Laboratories) to block the contact pathway of coagulation, and 0.5% BSA (Bovine Serum Albumin) in TBS (Tris-buffered saline). A 96-well round-bottom microplate (ThermoFischer Scientific, NY, USA) was used for this assay and samples were ran in triplicates, whilst a duplicate set of

calibrators was run per plasma sample. Assay was performed twice and results are presented as the average of the two assays.



2.12 DATA ANALYSIS & STATISTICS

Results are presented in histograms, produced in GraphPad PRISM software Version 9. Columns represent the mean average and error bars the standard error of the mean (SEM). 1-way ANOVA was used to analyze results in histograms with 1 variable, and 2-way ANOVA was used to analyze results with 2 variables. Tukey's multiple comparison was the statistical test used to compare the different groups, where *=p<0.05, **=p<0.01, ***=p<0.001 and ****=p<0.0001. No asterisks represent values of p>0.05, thus considered statistically insignificant.

CHAPTER 3

Effects of INSTIs on endothelial cells

3 EFFECTS OF INSTIs ON ENDOTHELIAL CELLS

Current literature covers the effects of different antiretroviral drugs or drug combinations on cardiovascular components including the endothelium, but no direct comparison of bictegravir and dolutegravir has been reported for possible differential effects on endothelial activation *in vitro*. For the following experiments, HUVEC have been chosen as the initial cell model for examining my first aim, to investigate the differences in adhesion molecule expression by TNF- α -stimulated endothelial cells, upon a 48h exposure to either BIC, DTG or the vehicle control.

HUVEC is a well-established endothelial cell (EC) model to study endothelial dysfunction and has been previously used to observe ARV effects on endothelial cells [181], [188]. TNF- α is a broad inflammatory stimulus relevant to many inflammatory conditions including vascular dysfunction [111], it enhances adhesion molecule expression, and is elevated in chronic inflammation and in PLWH despite viral suppression [85], [89].

3.1 TNF-A – INDUCED HUVEC STIMULATION

Firstly, without the presence of any ARVs, I tested 10ng/mL of TNF- α , a concentration previously shown to upregulate adhesion molecule expression in HUVEC at appropriate timepoints [188], [189]. HUVEC were cultured as explained in **Section 2.2** and prepared for flow cytometry as described in **Section 2.3**. **Figure 3.1** shows the median fluorescence intensity (MFI) measured for each of the adhesion molecules with or without TNF- α . Levels of E-Selectin were measured 4h post-stimulation, VCAM-1 6h post-stimulation and ICAM-1 24h post-stimulation.

TNF- α -stimulated HUVEC had a significantly higher expression of E-Selectin, 4h post-stimulation, compared to the unstimulated cells, by 9.8-fold. VCAM-1 expression was higher after a 6h stimulation by 210-fold, whereas ICAM-1 levels increased by 37.4 times 24h post-stimulation, relative to unstimulated HUVEC (**Figure 3.1**).

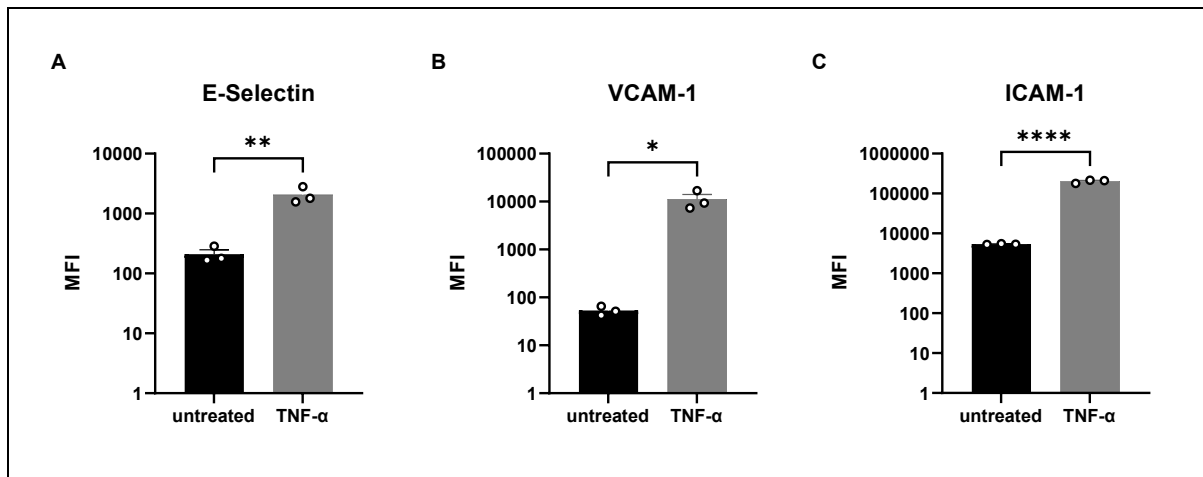


Figure 3.1. Adhesion molecule expression by Human Umbilical Vein Endothelial Cells (HUVEC) post-stimulation. Cells were stimulated with 10ng/mL of Tumour Necrosis Factor – alpha (TNF- α) for either 4h, 6h or 24h hours and levels of E-selectin, VCAM-1 and ICAM-1, respectively, were measured in flow cytometry. Graph **A** shows the difference in E-Selectin expression between untreated and TNF- α -treated HUVEC, and error bars represent the standard error of the mean from N=3. Graph **B** shows difference in VCAM-1 expression between unstimulated and stimulated cells and graph **C** in ICAM-1. A two-tailed t-test was performed to deduce significance in MFI differences observed for each endothelial marker between stimulated and unstimulated conditions. Asterisks represent * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, and **** = $p < 0.0001$.

3.2 RANGE OF TNF-A CONCENTRATIONS TO ACTIVATE HUVEC FOR 4H, 6H & 24H

My next step was to test a range of TNF- α concentrations, from 1 to 100ng/mL, and measure the expressions of ICAM-1, VCAM-1 and E-selectin but this time across all 3 different timepoints to confirm the optimal stimulation period for the highest expression of each marker. **Figure 3.2** shows the MFI obtained from every endothelial marker at 4h, 6h and 24h post-stimulation across a range of TNF- α concentrations. 10ng/mL of TNF- α resulted in the MFI values, of all 3 adhesion molecules, that lie best within the linear section of HUVEC activation, compared to alternative concentrations tested, for all 3 timepoints making it the optimal concentration to be used for the following experiments.

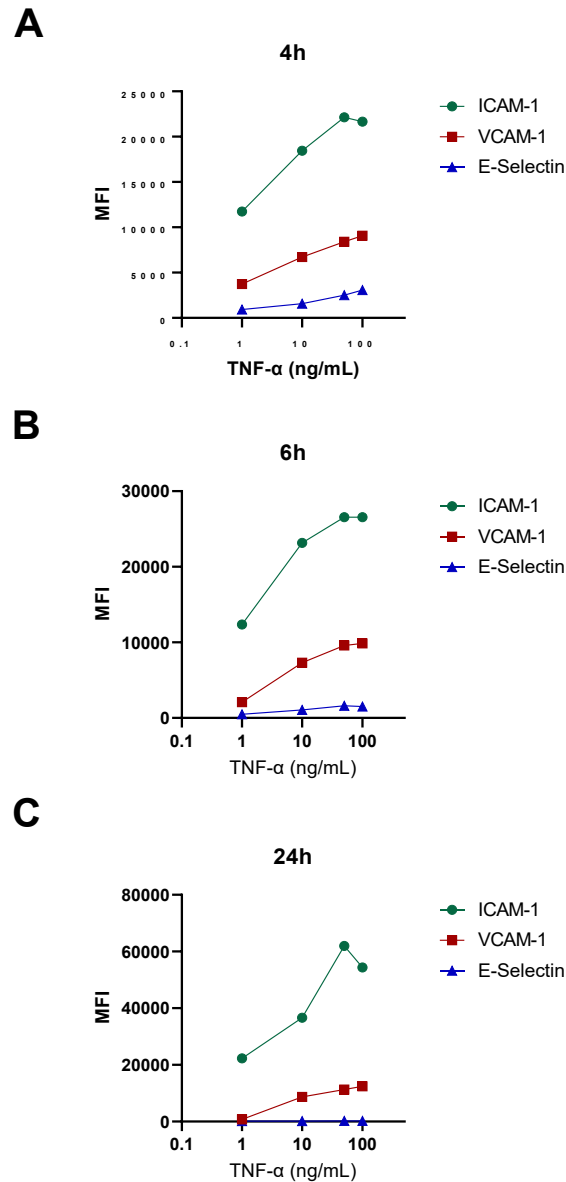
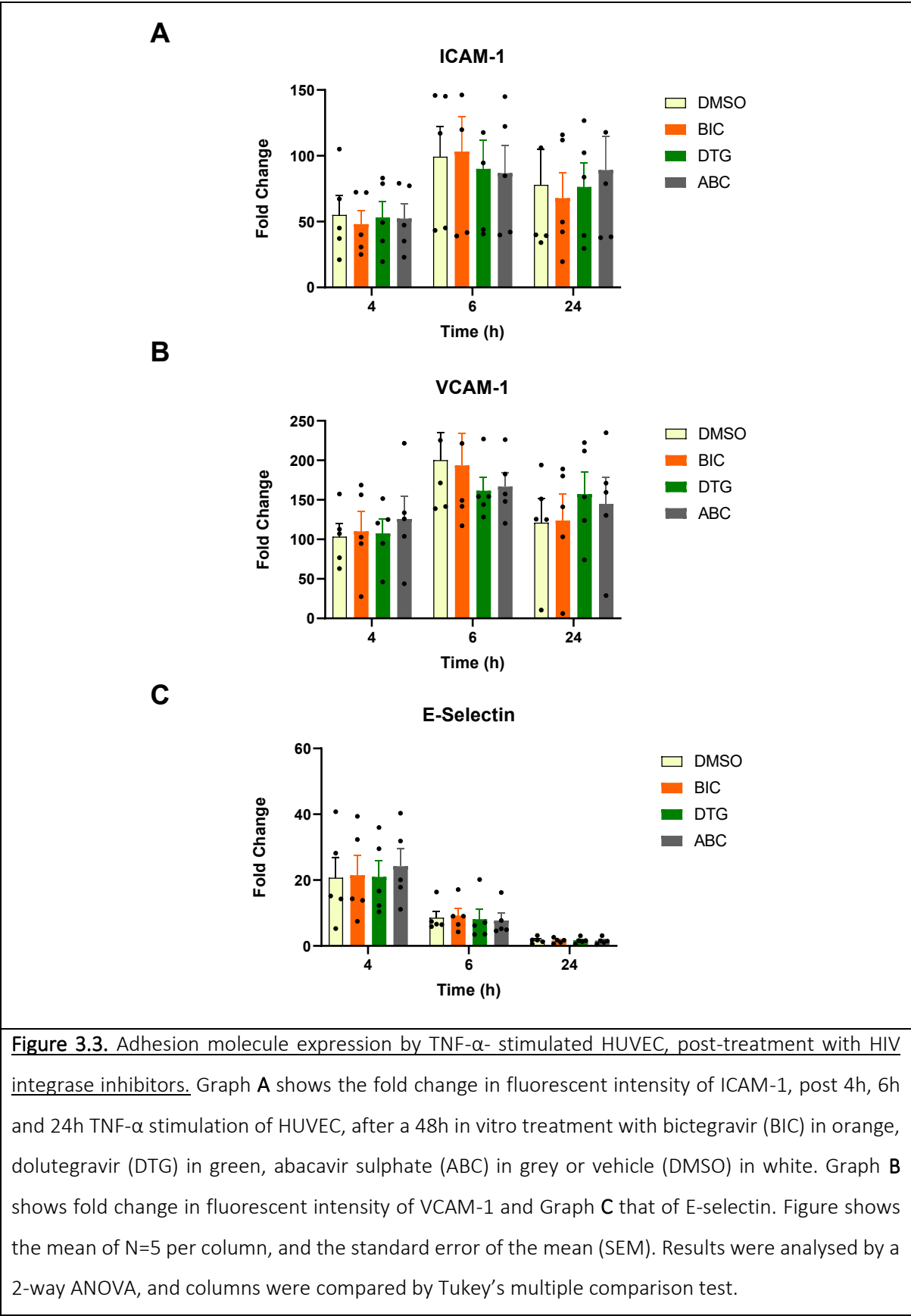


Figure 3.2. ICAM-1, VCAM-1 and E-Selectin expression post-TNF- α HUVEC stimulation. Graph **A** shows the median fluorescent intensity (MFI) of the 3 adhesion molecules, ICAM-1 (green), VCAM-1 (red) and E-Selectin (blue), 4h post stimulation of HUVEC with 1, 10, 50 or 100ng/mL of TNF- α . Similarly, Graph **B** shows the 6h stimulation and Graph **C** the 24h stimulation. The experiment was performed once thus no statistical tests were performed.

The next step was to treat the cells for 48h with bictegravir, dolutegravir, abacavir sulphate or vehicle (control), as described in **Section 2.3**, prior to stimulating with TNF- α and compare the differences in adhesion molecule expression. Results are shown in **Figure 3.3**. Abacavir Sulphate, an NRTI, was used

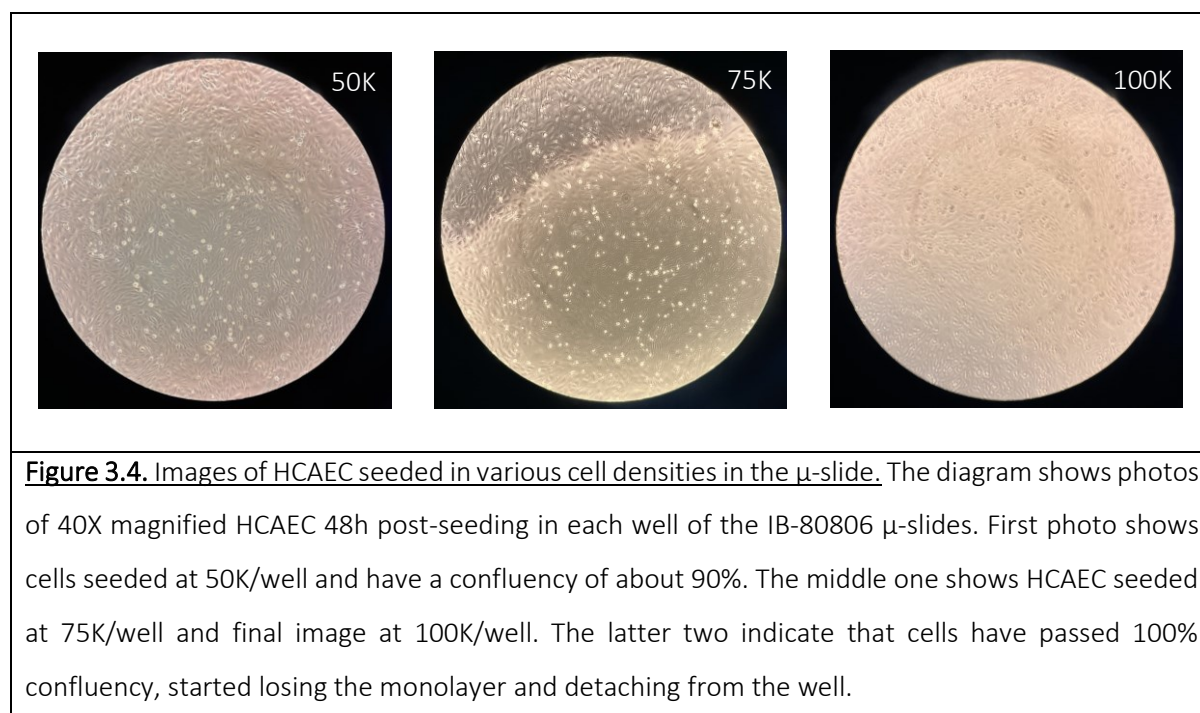
here as an investigational positive control after being shown previously to augment adhesion molecule expression in HUVEC compared to alternative NRTIs [188].



MFI measured in these experiments is a representation of cell surface expression of each adhesion molecule by HUVEC, after stimulating with TNF- α at various timepoints. **Figure 3.3** shows that there are no significant differences in fold change MFI of ICAM-1, VCAM-1 or E-Selectin between HUVEC treated with either integrase inhibitor, or between the control groups, DMSO- or ABC-treated HUVEC.

Upon reconsidering the cell model and technique, HCAEC from a single donor were used for the following experiments. As described in **Sections 2.4 - 2.5**, HCAEC were cultured and seeded in 8-well μ -slides where they were treated with clinically relevant concentrations of the drugs of interest, before being stimulated with TNF- α . The various activation markers were measured with immunostaining, in confocal microscopy, where actual images of the cells could be taken for each condition.

During culturing and seeding of HCAEC, there were a few technical complications that I had to solve by troubleshooting and altering various reagents before working out the optimal conditions to obtain a monolayer of cells, at about 90% confluency, before stimulation. Cells were dying and detaching from the wells before or just after stimulation, therefore I initially tested seeding different cell densities, as shown in **Figure 3.4**.



Moving forward with 50K/well as my chosen seeding density, cells would still detach and have a poor adherence to the slide, so I decided to try adding an attachment factor to wells prior to seeding, even

though the slides were purchased pre-treated for cell adhesion. I decided to use 120 μ L/well of the gelatin-containing Attachment Factor (**Section 2.4**), a volume enough to cover the bottom surface of each well and leave for 1h before removing and washing with PBS. Cells seemed to adhere to the slide surface better and longer than without the Attachment Factor and I could move to the next step, cell stimulation with TNF- α .

Upon stimulation, cells were rounding up, and once again detaching from the cells a few steps further down the immunostaining protocol described in **Section 2.4**. Few of these steps involved washing cells with PBS or diluting reagents in PBS so I thought I should try a different PBS to the one used for HUVEC experiments, this time containing MgCl₂ and CaCl₂ to enhance cell survival. This was the last stage of troubleshooting I performed to optimize the immunostaining protocol described in **Section 2.4**.

I carried out HCAEC activation with a range of TNF- α concentrations and measured ICAM-1 levels in confocal microscopy to determine a suitable concentration to prime the cells being treated with integrase inhibitors, but also a concentration to act as a positive control. **Figure 3.5** shows images of HCAEC, where ICAM-1 is shown in red, obtained by confocal imaging (described in **Sections 2.4 - 2.5**) from testing various TNF- α concentrations (0 to 50ng/mL) 24h post-stimulation. Staining levels of fluorescently labelled ICAM-1 were converted into MFI on Image J software after adjusting for background fluorescence.

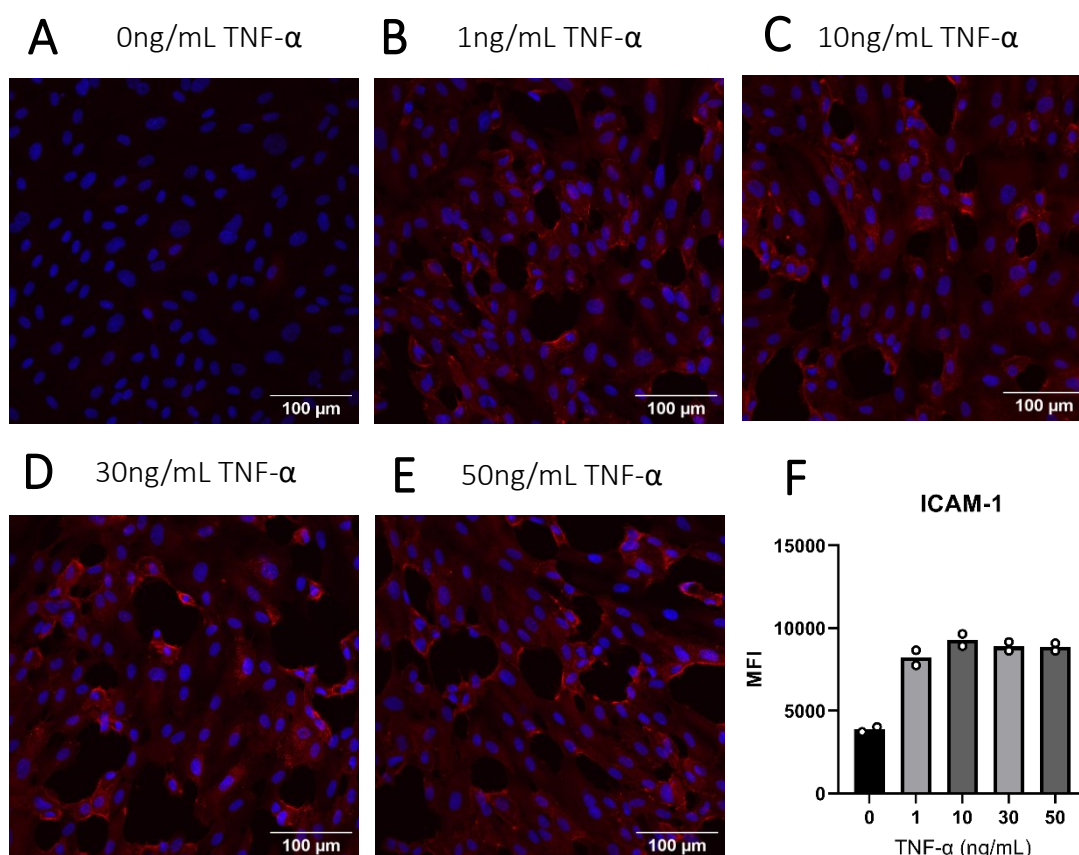


Figure 3.5. ICAM-1 levels upon a range of TNF- α -induced HCAEC stimulations. The figure shows 5 images (A-E) obtained in confocal microscopy where HCAEC were stimulated by 0, 1, 10, 30, or 50ng/mL of TNF- α respectively. Cell nuclei are shown in blue (DAPI) and ICAM-1 is shown in red (Alexa Fluor 555 staining). **Graph F** shows ICAM-1 levels obtained using the ImageJ software, by calculating MFI after adjusting for background and cell count. Bars are shown as a mean average of N=2.

Graph E in **Figure 3.5** shows numerical values of ICAM-1 MFI at each TNF- α concentration from an N=2. I decided to choose 1ng/mL as the concentration of TNF- α to activate HCAEC treated with BIC, DTG or DMSO for following experiments. Cells activated with more than 10ng/mL appeared to lose their monolayer dramatically and show a decrease in ICAM-1 MFI suggesting maxing out of the cell activation so decided to try 10ng/mL as an investigational positive control.

3.3 UNSTIMULATED HCAEC POST INSTI TREATMENT

For the following experiments I decided to measure ICAM-1, PECAM-1 and VE-cadherin instead of just adhesion molecules, in order to explore possible effects of drugs in vascular permeability and cell to cell adhesion. HCAEC were cultured and seeded in 3X 8-chamber μ -slides and stimulated for 4h, 6h and 24h with 1ng/mL of TNF- α following 48h treatment with BIC, DTG or vehicle control (**Figure 2**). Fluorescence microscopy images of the unstimulated HCAEC at the end of a 24h timepoint are shown in **Figure 3.6**, showing staining of ICAM-1, PECAM-1 and VE-Cadherin. Staining levels converted into MFI are shown in **Figure 3.7**.

As seen in **Figure 3.6**, the unstimulated DMSO-, BIC-, and DTG-treated cells formed a full monolayer with no gaps in between cells, and PECAM-1 and VE-cadherin are clearly defined surrounding cells, indicating a proper cell-to-cell adhesion, as expected. Moreover, no obvious staining of ICAM-1 is displayed in any of the three treatment groups.

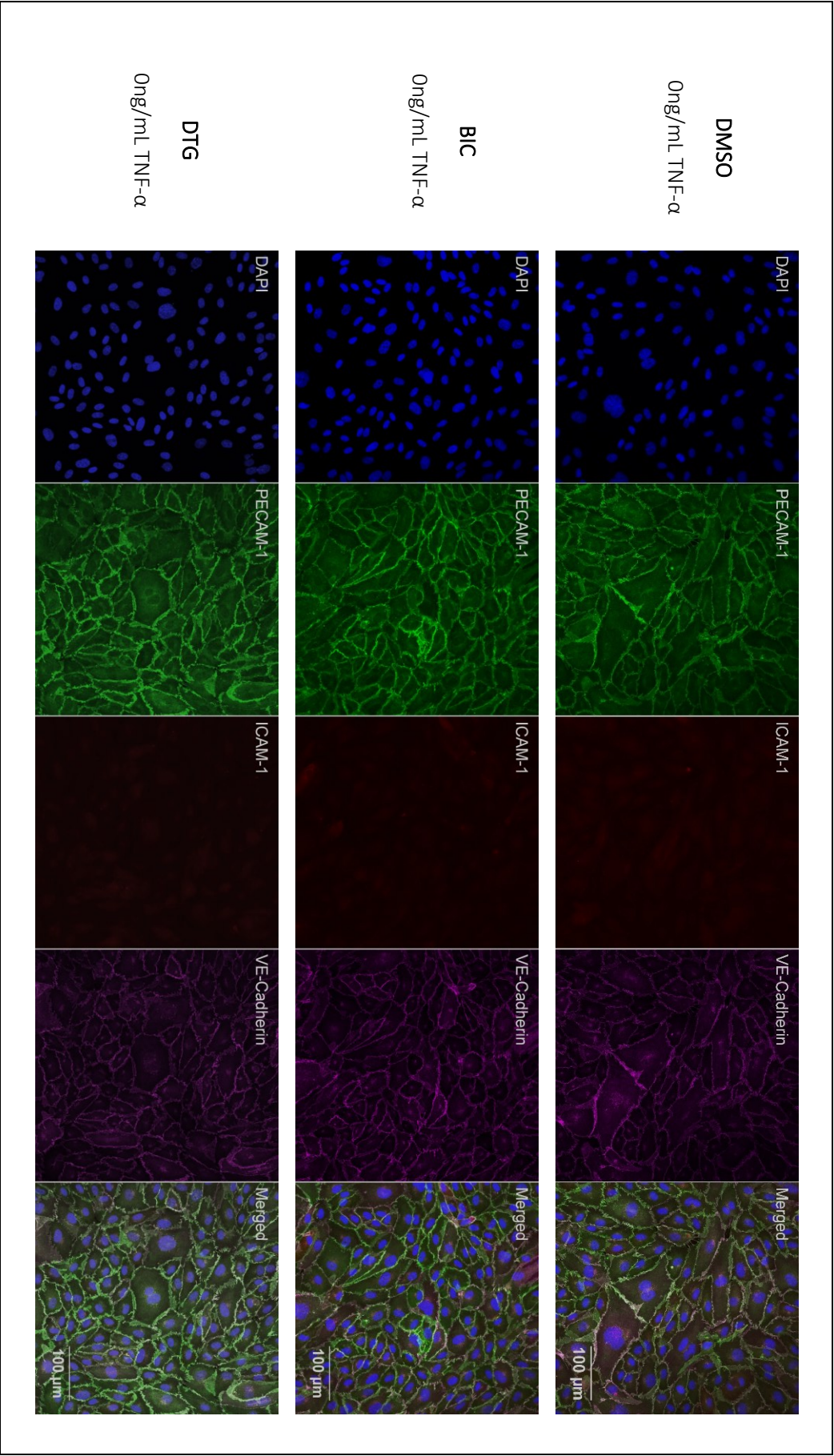


Figure 3.6. ICAM-1, PECAM-1 and VE-Cadherin levels in unstimulated HCAEC, at 24h timepoint, treated with integrase inhibitors. The figure shows confocal images obtained upon staining cell nuclei (DAPI) in blue, PECAM-1 (Alexa Fluor 488) in green, ICAM-1 (Alexa Fluor 555) in red and VE-Cadherin (Alexa Fluor 647) in pink. Top row shows the split channels of the well in which cells were treated with DMSO (vehicle), middle row shows the ones treated with bictegravir (BIC) and last row shows the cells treated with dolutegravir (DTG). Images were processed in Image J software.

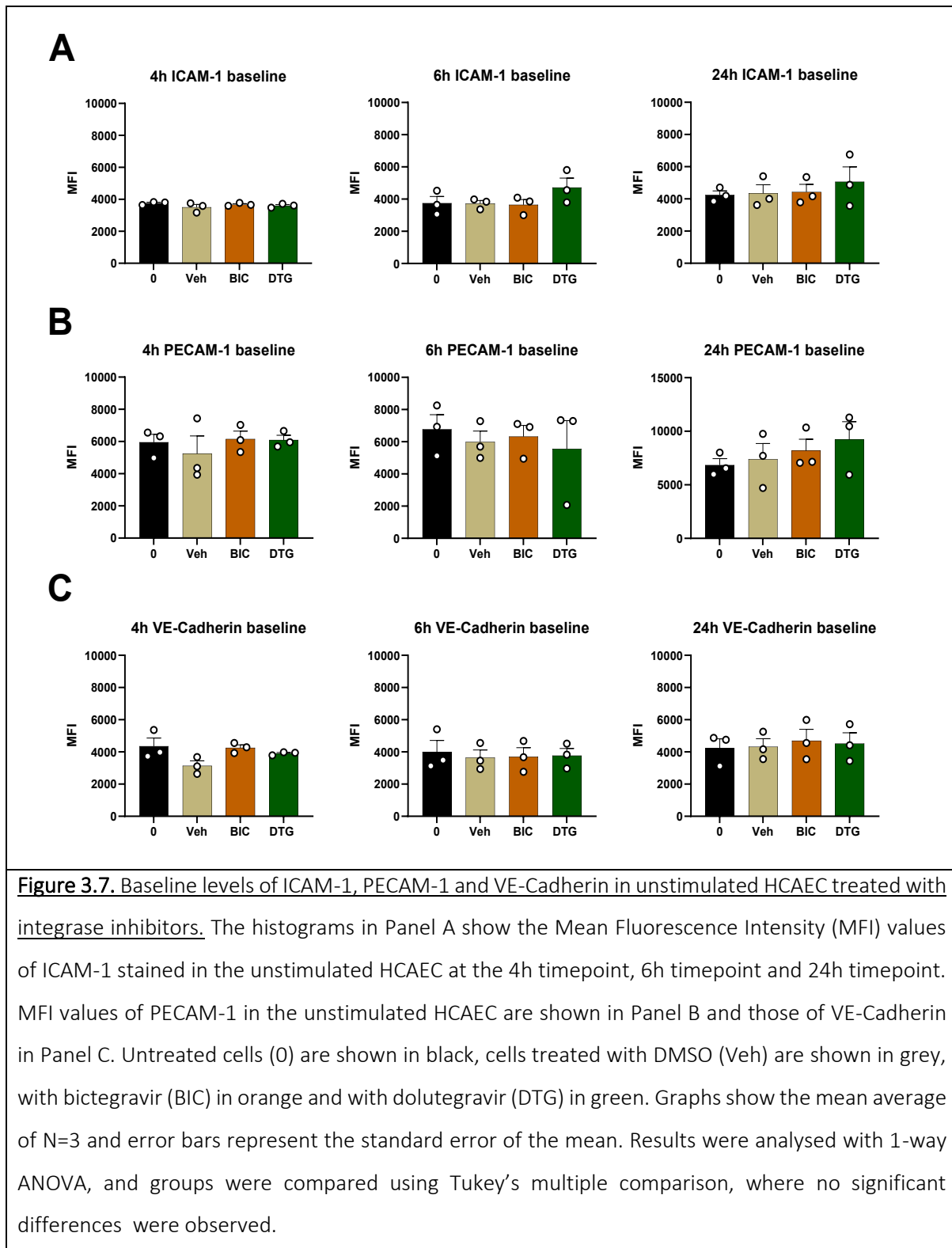


Figure 3.7 shows the baseline levels of ICAM-1, PECAM-1 and VE-Cadherin, in unstimulated HCAEC upon exposure to (cell culture equivalent) clinical values of BIC and DTG. No significant differences in ICAM-1 levels were observed between cells treated with BIC, DTG, DMSO or none. Additionally, PECAM-1 and VE-Cadherin levels, have no significant differences between unstimulated HCAEC treated with either integrase inhibitors or the control groups.

3.4 STIMULATED HCAEC POST INSTI TREATMENT

Figure 3.8 shows the confocal images of DMSO-, BIC- or DTG-treated HCAEC, stimulated with 1ng/mL of TNF- α for 24h, where ICAM-1, PECAM-1 and VE-Cadherin are stained. The MFI values of the three markers are shown in histograms in **Figure 3.9**. TNF- α stimulated HCAECs have gaps in the monolayer and cell-to-cell adhesion seems to be reduced. No obvious differences of ICAM-1, PECAM-1 or VE-Cadherin staining levels were observed between the different treatment groups (BIC, DTG or DMSO) from the confocal images which agrees with MFI values demonstrated in **Figure 3.9**.

As shown in **Figure 3.9**, ICAM-1 MFI levels in the TNF- α stimulated cells, where comparable between all treatment groups (DMSO 5454; BIC 5605; DTG 5332; positive control 5693) and all significantly higher compared to unstimulated cells (3743) at the 6h stimulation timepoint. Similarly, at 24h stimulation timepoints activated/treated cells had MFI values (DMSO 8424; BIC 8912; DTG 9053; positive control 8537) higher than the unstimulated group (4249). However, no differences were observed between stimulated groups treated with DMSO, BIC or DTG, suggesting that BIC and DTG have no effects on ICAM-1 levels.

Stimulation of untreated HCAEC with 10ng/mL of TNF- α , used as an investigational positive control, had no significant differences in ICAM-1 staining levels with 1ng/mL-stimulated HCAEC, treated with DMSO, BIC or DTG. This indicates that the 10ng/mL stimulation was not a suitable positive control for these experiments. In addition, vascular permeability of HCAEC measured in terms of PECAM-1 and VE-Cadherin levels had no significant changes between unstimulated and stimulated cells, with or without prior drug treatment, but also no difference with the investigational positive control.

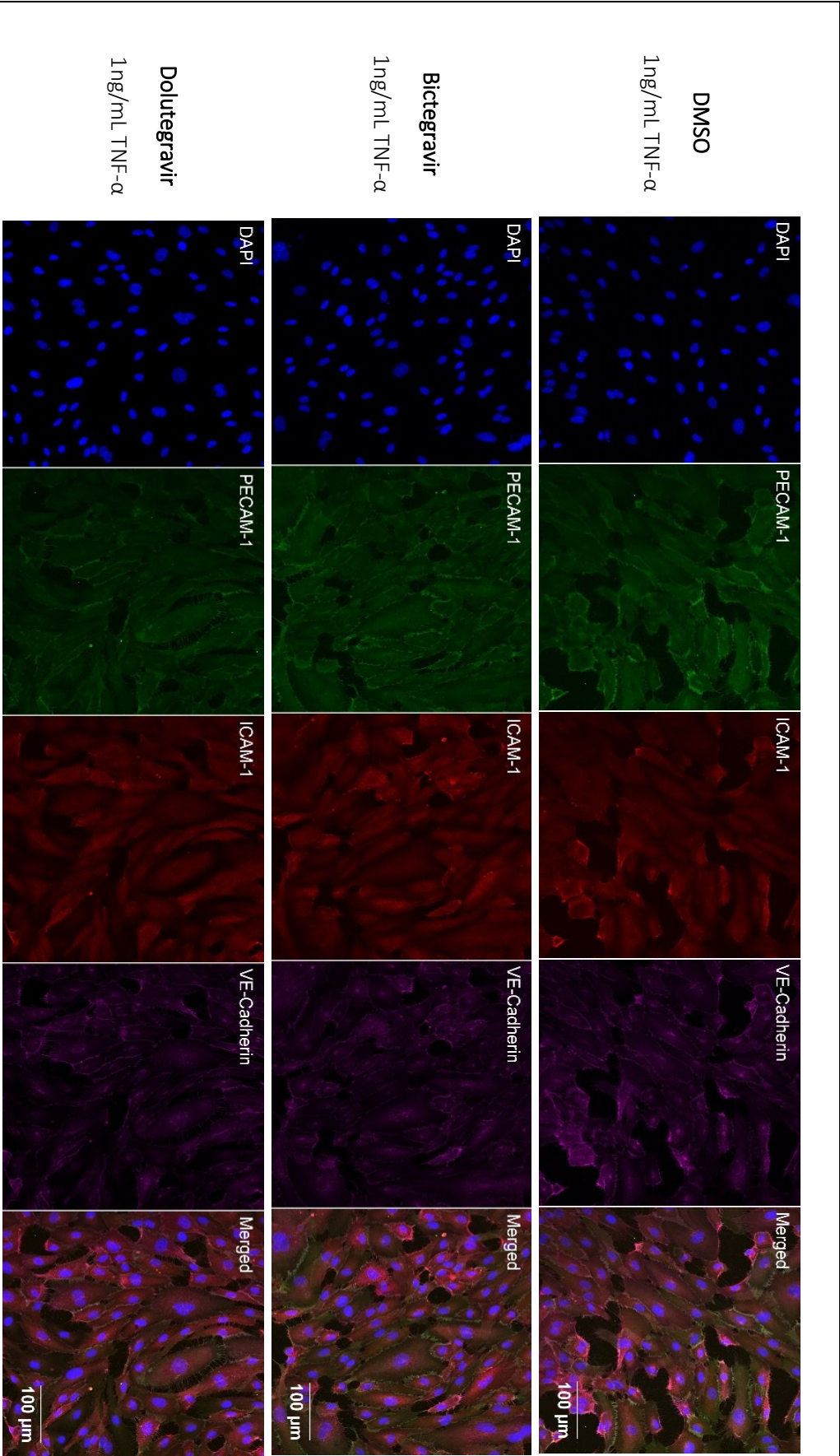


Figure 3.8. Levels of PECAM-1, ICAM-1 and VE-Cadherin post 48h treatment of HCAEC with integrase inhibitors followed by TNF- α stimulation. Figure shows confocal images of HCAEC upon immunostaining, where cell nuclei are shown in blue (DAPI), PECAM-1 in green (Alexa Fluor 488), ICAM-1 in red (Alexa Fluor 555) and VE-cadherin in pink (Alexa Fluor 647). Top row shows DMSO-treated HCAEC, second row bictegravir(BIC)-treated HCAEC, and bottom row dolutegravir(DTG)-treated HCAEC, followed by a 24h 1ng/mL TNF- α stimulation. Channels were split and merged in Image J software.

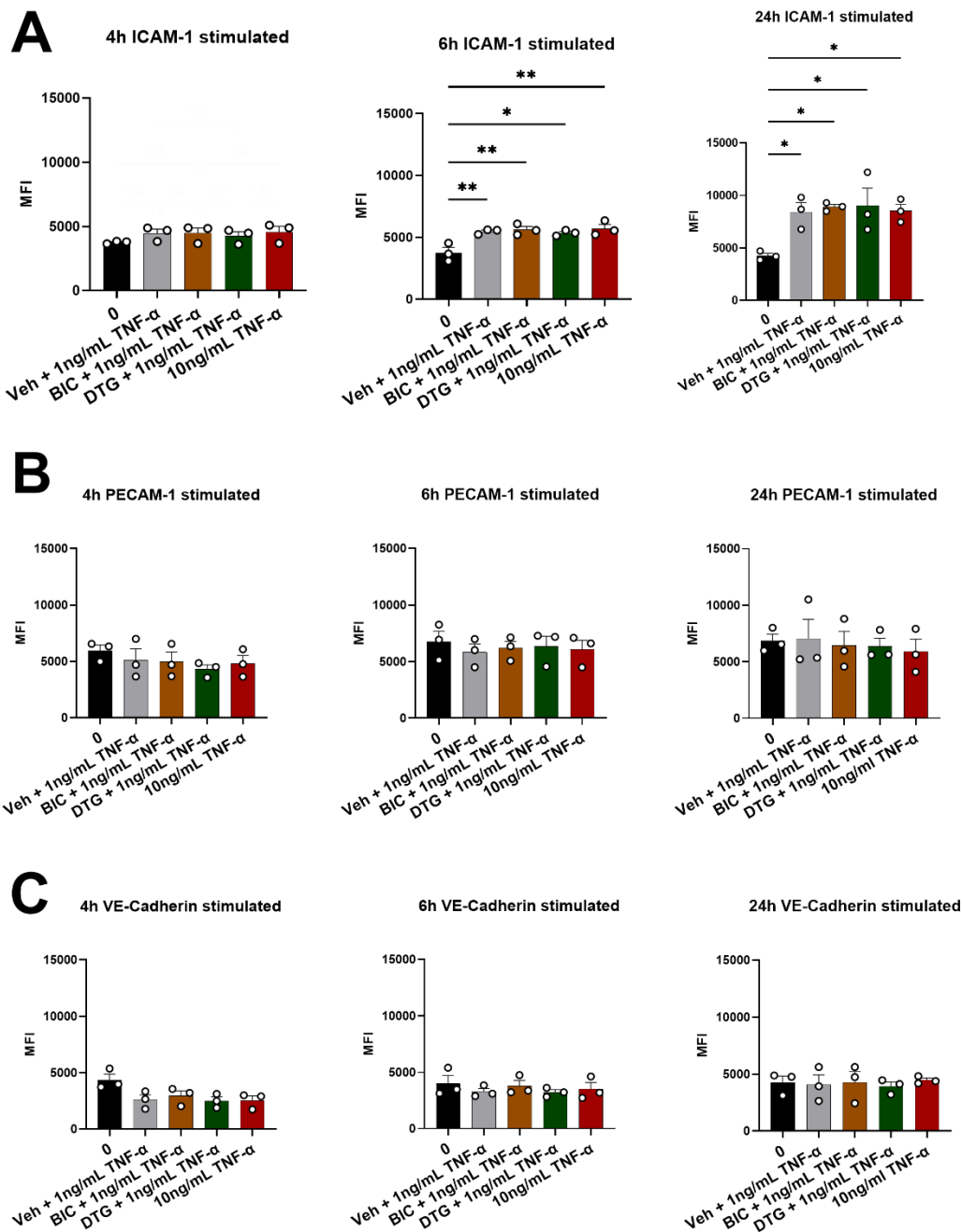


Figure 3.9. ICAM-1, PECAM-1 and VE-Cadherin staining levels in TNF- α -stimulated HCAEC. The figure shows the mean fluorescent intensity (MFI) values of ICAM-1, PECAM-1 and VE-Cadherin in HCAEC upon 1ng/mL TNF- α stimulation, following *in vitro* treatment of the cells with DMSO, bictegravir (BIC) or dolutegravir (DTG). Panel **A** shows the levels of ICAM-1 at 4h, 6h and 24h post stimulation, panel **B** the levels of PECAM-1 and panel **C** the ones of VE-Cadherin. Histograms also include the levels of the markers in the absence of TNF- α (labelled as 0) and the ones in cells stimulated with 10ng/mL TNF- α (labelled as 10ng/mL). Results were analyzed using 1-way ANOVA and groups were compared using Tukey's multiple comparison, where * = $p < 0.05$, ** = $p < 0.01$.

3.5 CHAPTER 3 - DISCUSSION

Augmented EC permeability and pro-adhesive properties are well-characterized in endothelial dysfunction, a process which contributes greatly to the development and progression of CVDs [99], [101], [104]. This makes PLWH, shown to acquire double the risk in developing a CVD, more susceptible to excessive endothelial activation.

Previous studies have explored drug effects on endothelial cell models following *in vitro* treatment with different ARV agents including INSTIs, providing conflicting or insufficient comparisons [166], [169], [188]. In this chapter, I explored and compared possible effects of two popular INSTI agents BIC and DTG, on HUVEC and HCAEC, by measuring changes in the levels of numerous adhesion and permeability markers post cytokine stimulations.

HUVEC experiments

Results from **Figures 3.1** and **3.2** confirm that 10ng/mL of TNF- α can upregulate the expression of ICAM-1, VCAM-1 and E-Selectin and agree with current literature [188], [189]. The statistical analysis for every data set is presented in each figure legend. Treating HUVEC with clinical concentrations of BIC, DTG, ABC or DMSO (vehicle control) *in vitro* prior to TNF- α stimulations (**Figure 3.3**) did not alter the surface expression levels of ICAM-1, VCAM-1 or E-Selectin implying that BIC and DTG share a similar neutral effect on endothelial activation. This finding disagrees with both Afonso et al., suggesting a DTG-mediated decrease in endothelial cell activation and Huang et al., suggesting an augmented endothelial activation in INSTI-treated cells, although different endothelial cell models were used in each study, therefore no direct comparison can be made.

Moreover, data from **Figure 3.3** showed that ABC cannot be used a positive control for this set of experiments. Khawaja et al., revealed that in similar settings, ABC treatment increases pro-adhesive properties of HUVEC, including ICAM-1 levels, when compared to alternative NRTIs like TDF or TAF [188]. However, they did not report differences in ICAM-1 expression between ABC and DMSO, hence the use of ABC here as an investigational positive control failed. A more suitable positive control could be a higher concentration of TNF- α to stimulate HUVEC, allowing an augmented increase in the surface

levels of the adhesion markers of interest. For that, data presented in **Figure 3.2** should be repeated to allow statistical analysis to be performed and deduce appropriate concentrations of TNF- α to prime and/ or fully activate endothelial cells.

In **Figure 3.3**, big discrepancies were observed between the data points of every repeat despite keeping every reagent and parameters consistent. HUVEC used for these experiments were obtained from pooled donors, potentially affecting the range of values in each repetition.

Reflecting on the technique, measuring cell surface markers from endothelial cells in flow cytometry was not ideal, as endothelial cells line blood vessels and therefore only part of their surface is exposed to the bloodstream, where they express adhesion molecules. Flow cytometry measures the whole cell surface in solution and one cell at a time rather than a monolayer of cells, as seen in real-life.

All the above imply that the technique chosen to measure changes in adhesion molecule expression might not be suitable and perhaps a different method that allows ECs exist in a more physiologically relevant setting should be investigated. In addition to that, although HUVEC are well-established in exploring drug effects and quantifying activation markers post-stimulation, they are umbilical cells thus not fully indicative for the context of CVD mostly observed in older people.

HCAEC experiments

HCAEC from a single adult donor were chosen for the following experiments, as arterial cells are more representative than umbilical venous cells for conditions like atherosclerosis, which occurs in mature arterial environments. Upon troubleshooting and tailoring the technique for my experimental objective, ICAM-1 and the permeability markers VE-Cadherin and PECAM-1 were measured post-stimulation in confocal microscopy, following a 48h drug treatment *in vitro*.

In the absence of TNF- α , no differences in the levels of ICAM-1 or permeability markers were observed between cells treated with DMSO, BIC or DTG, suggesting that the clinical concentrations of drugs in plasma cannot activate endothelial cells *in vitro*, and have a neutral effect on unstimulated HCAECs. Afonso et al., reported a decrease in soluble ICAM-1 levels from HCAEC treated with DTG but that was measured using ELISAs (Enzyme-Linked Immunosorbent Assays) and after 15 days of cell treatments *in*

vitro with the drug, which is more than seven times higher exposure time than the one in my experiments [170]. In the future, if those experiments were to be repeated, a higher treatment timepoint can allow better comparisons between the two studies.

To confirm the choice of 10ng/mL of TNF- α as an appropriate positive control, the experiments displayed in **Figure 3.5** should be repeated to allow statistical analysis and look for a significant increase in ICAM-1 levels. Moreover, a bigger range of TNF- α should be explored including concentrations between 1 and 10ng/mL to choose a more appropriate cytokine concentration for a positive control.

The confocal images from **Figure 3.6**, together with the associated MFI values displayed in **Figure 3.7** imply that BIC and DTG share a similar neutral effect on arterial endothelial cells and potentially do not influence endothelial permeability or adhesion at resting conditions. To confirm this, more adhesion molecules including VCAM-1 or E-Selectin, or permeability markers, like occludins and claudins, can be measured using immunostaining followed by confocal microscopy instead of flow cytometry used initially.

During stimulatory conditions, cells pre-treated with BIC or DTG displayed similar fluorescent intensities of PECAM-1, ICAM-1 and VE-Cadherin (**Figure 3.8**) between them and with the DMSO group, although gaps in the monolayer appeared to be larger in the DMSO group. MFI values presented at **Figure 3.9** confirm that INSTIs did not affect the expression levels of ICAM-1, VCAM-1 or E-Selectin at stimulated cells. As expected, ICAM-1 levels were higher in cells stimulated with 1ng/mL of TNF- α (+INSTI or Veh) at both 6h and 24h TNF- α induced stimulations versus the unstimulated cells. INSTI-treated cells had no differences with DMSO-treated cells, suggesting that differences in ICAM-1 were solely based on TNF- α stimulation, without any INSTI interference at MFI levels.

My results from this chapter suggest that BIC and DTG do not activate HUVEC or HCAEC at resting conditions, and do not enhance or reduce levels of adhesion or permeability markers in activated endothelial cells, thus share a similar potentially neutral effect on the endothelium. My hypothesis states that DTG affects endothelial activation more than BIC, but my findings do not support this. There were no significant differences between the two drug groups when various endothelial activation

markers were measured, suggesting that BIC and DTG, or potentially INSTIs as a drug class, may not affect endothelial dysfunction.

Endothelial dysfunction boosts CVD development due to excessive release of pro-inflammatory markers, enhanced endothelial permeability or adhesion to leukocytes and atherosclerosis development and progression. Demonstrating that certain INSTIs used in daily treatments could have a neutral effect in HUVEC or HCAEC activation, while other ARVs do not, has a significant value as it could contribute to allowing informed decisions by doctors and patients, shaping the way appropriate cART is chosen or tailored to each individual.

Limitations & Future work

The main limitations of these experiments include first and foremost the lack of an accurate positive control. The best would be an ARV agent, ideally an INSTI, with an established effect on vascular adhesion or permeability. Secondly, drug exposure for 48h *in vitro* is not representative to the daily, life-long treatment of PLWH with ARV drugs. In addition, those experiments only explored the individual effect of BIC or DTG, whereas in clinical settings these drugs are given in combination with NRTIs, thus cell treatments with drug combination could be investigated in the future.

In a real-life setting, during endothelial dysfunction a number of cytokines in addition to TNF- α circulate and further stimulate cells like INF- γ , or IL-6 [85], [89], [114]. Thus, additional stimuli could be assessed alone or in combination to explore drug effects in stimulated arterial cells. Moreover, given the role of other cell types in inflammation and atherosclerosis, co-culturing ECs with leukocytes or platelets could allow a more representative environment.

Another limitation of the study is that our experimental approaches were performed at static conditions whereas in real-life, shear stress can impact these processes especially at the arterial environment. This refers to gene expression and functionality changes dependent on shear flow, like NO production, anti-inflammatory responses and cytoskeletal organization.

For this, future flow studies with endothelial cells and blood from HIV negative volunteers treated with drugs *in vitro* or from PLWH (*ex vivo*) are recommended. HUVEC and HCAEC were derived from HIV-

negative donors, therefore future work can include cells from PLWH receiving treatments of interest, potentially allowing *ex vivo* experiments to support our *in vitro* work. Cell treatments *in vitro* using HUVEC and HCAEC can only partially provide a link between ARVs and CVD states like atherosclerosis or venous thrombosis, hence approaches where more cell types can be co-cultured in the presence of relevant inflammatory markers could offer a better association in a more pathophysiological environment, for example flow studies.

Immunostaining followed by confocal microscopy seemed to be a more reliable technique to measure the levels of adhesion and permeability markers in endothelial cells as variability between repeated experiments was improved. The use of HCAEC instead of HUVEC for similar experiments is recommended as they are mature arterial cells, thus more indicative than umbilical venous cells for mimicking atherogenic conditions.

An alternative approach to confocal microscopy that could be used in the future is the ELISA method, where EC-released pro-inflammatory cytokines or activation markers, like soluble vWF and IL-6, can be quantified from cell supernatants, upon stimulation and provide a better understanding on possible effects of INSTIs on endothelial activation. Another technique that could be used in the future, is Real-Time quantitative PCR (RT-qPCR) which measures gene expression during cell stimulation. This could provide numerical values of any up/downregulation of genes encoding markers of interest after drug treatments offering a more accurate comparison of drug effects at transcriptional level.

All the above include examples of how future changes to our approaches can represent a more physiologically relevant environment to that reported in chronic inflammation in PLWH. In the bigger picture, an established model of examining endothelial function could be assessed for measuring enhanced activity or dysfunction in the presence of older and currently popular ARV agents, individually or in clinically relevant combinations. Clinicians and researchers could collaborate and create a panel or a database for as many HIV drugs as possible, with possible off target effects in endothelial cells, in an effort to allow more targeted and personalized treatments for every individual living with HIV.

CHAPTER 4

Effects of INSTIs on platelets

4 EFFECTS OF INSTIs ON PLATELETS

This chapter includes a series of experiments with platelets that have been treated *in vitro* with clinically relevant Cmax values of antiretroviral drugs, in an effort to identify any possible effects of the drugs on platelet activation, aggregation or adhesion to collagen. Primary focus was given on bictegravir and dolutegravir before proceeding to combination treatments, relevant to what is commonly prescribed in clinics today.

Initially, I wanted to measure platelet aggregation in the absence of any agonists to observe any possible drugs effects on platelet aggregation in resting conditions. PRP was obtained from whole blood collected from healthy volunteers (Sections 2.6 - 2.7) and treated with ARV drugs (Section 2.1) or DMSO, the drug vehicle. Aggregation protocol using light transmission is described in Section 2.8. The results are plotted in the histogram shown in Figure 4.1.

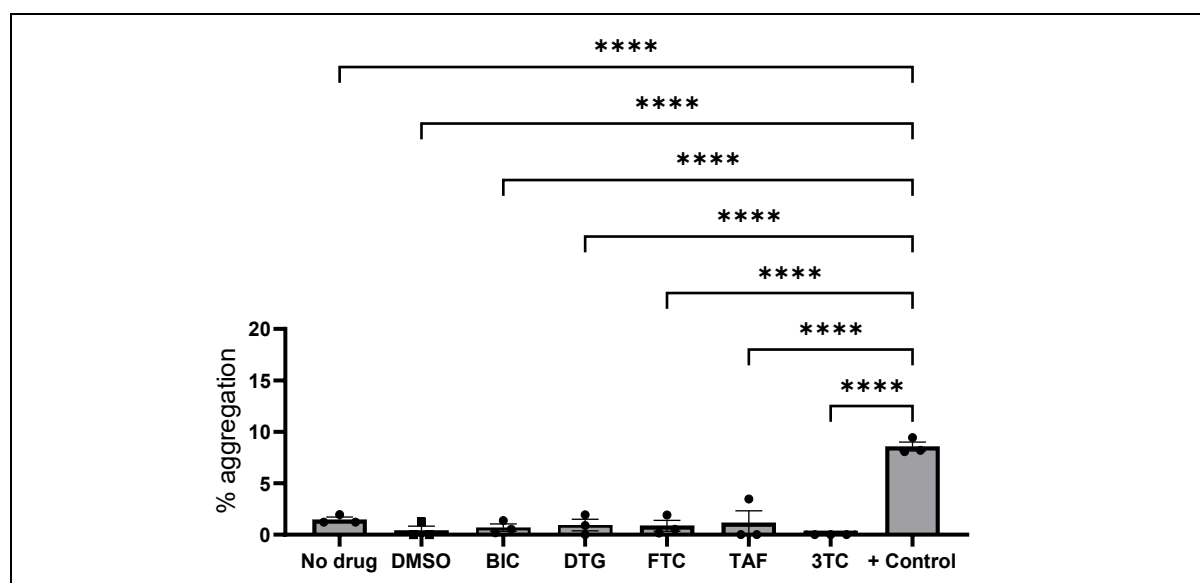
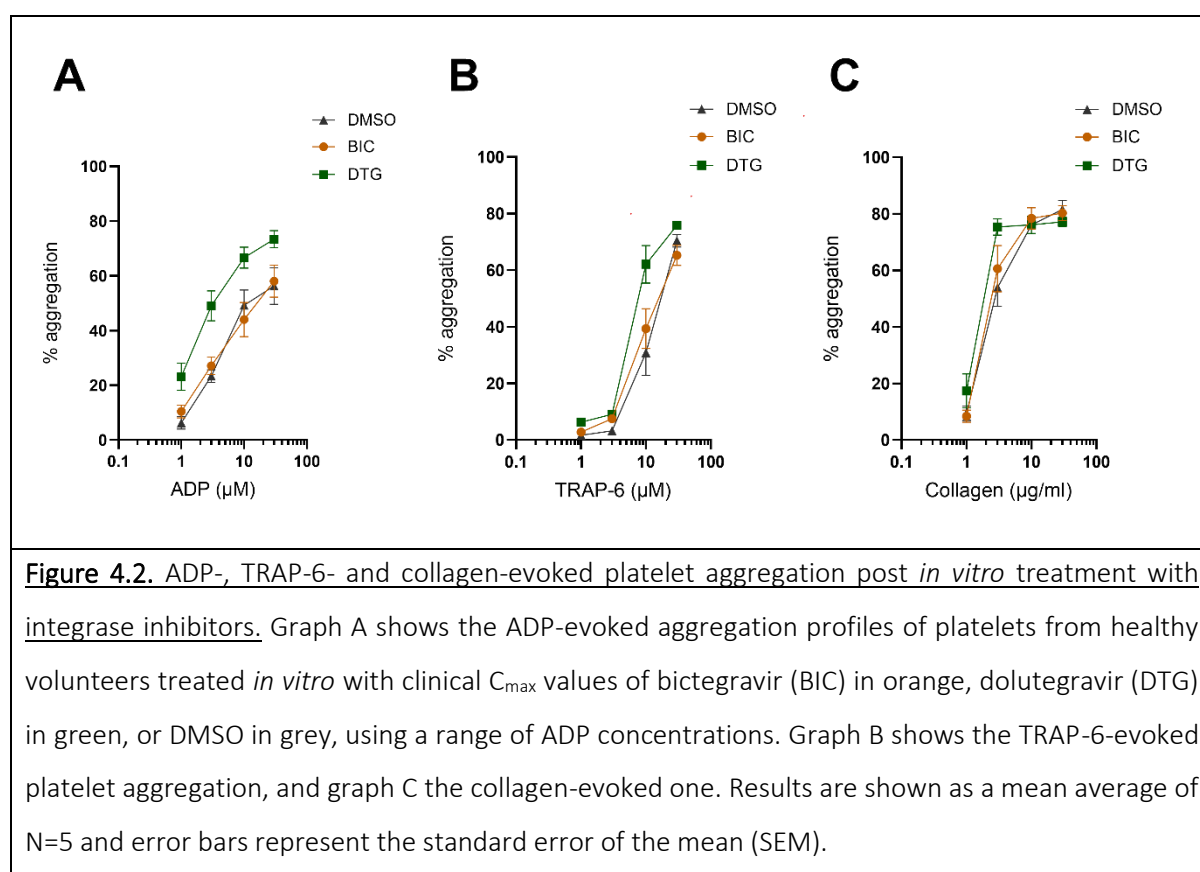


Figure 4.1 Antiretroviral (ARV)-treated platelet aggregation at resting conditions. The histogram shows the percentage platelet aggregation obtained upon treating platelet-rich plasma (PRP) from healthy volunteers with DMSO, bictegravir (BIC), dolutegravir (DTG), emtricitabine (FTC), tenofovir alafenamide (TAF), lamivudine (3TC), 1 μ g/mL collagen (positive control), or none (negative control). Bars show mean average from N=3, where error bars represent the standard error of the mean (SEM). Results were analysed with 1-way ANOVA and groups were compared using Tukey's multiple comparison, where *= p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001.

No significant differences were observed in platelet aggregation between samples treated with any ARV drug, DMSO, or with no treatment at all, but all of them were significantly lower ($p < 0.0001$) than PRP treated with 1 μ g/mL of collagen (the positive control).

4.1 EFFECTS OF BIC & DTG ON PLATELET AGGREGATION & ACTIVATION MARKERS

As illustrated in **Table 4 (Section 2.8)**, I carried out the aggregation assay 25min post treatment of PRP with DMSO, BIC or DTG, this time by activating platelets using a range of agonists including ADP, TRAP-6 and collagen. As no HIV integrase inhibitor has been previously reported to directly enhance platelet aggregation, no drug could act as an appropriate positive control. A range of agonist concentrations was therefore used, to demonstrate the aggregation potential of platelets at increasing agonist concentrations. **Figure 4.2** displays the platelet aggregation profiles post INSTI exposure, across the whole range of the agonist concentrations used.



An almost linear activation curve was observed in ADP-induced aggregation profiles, with a possible level-off after 10 μ M of the agonist. TRAP-6 activated platelets had a sudden increase in aggregation at

concentrations higher than 3 μ M and on the contrary, collagen aggregation profiles were more linear at lower concentrations, 1 and 3 μ g/mL, with a possible maxing out between 10 and 30 μ g/mL.

ADP, TRAP-6 and collagen induced aggregation profiles were divided into individual histograms, a graph per agonist concentration, to statistically compare treatment groups. Results from ADP-evoked platelet aggregation are shown in **Figure 4.3**.

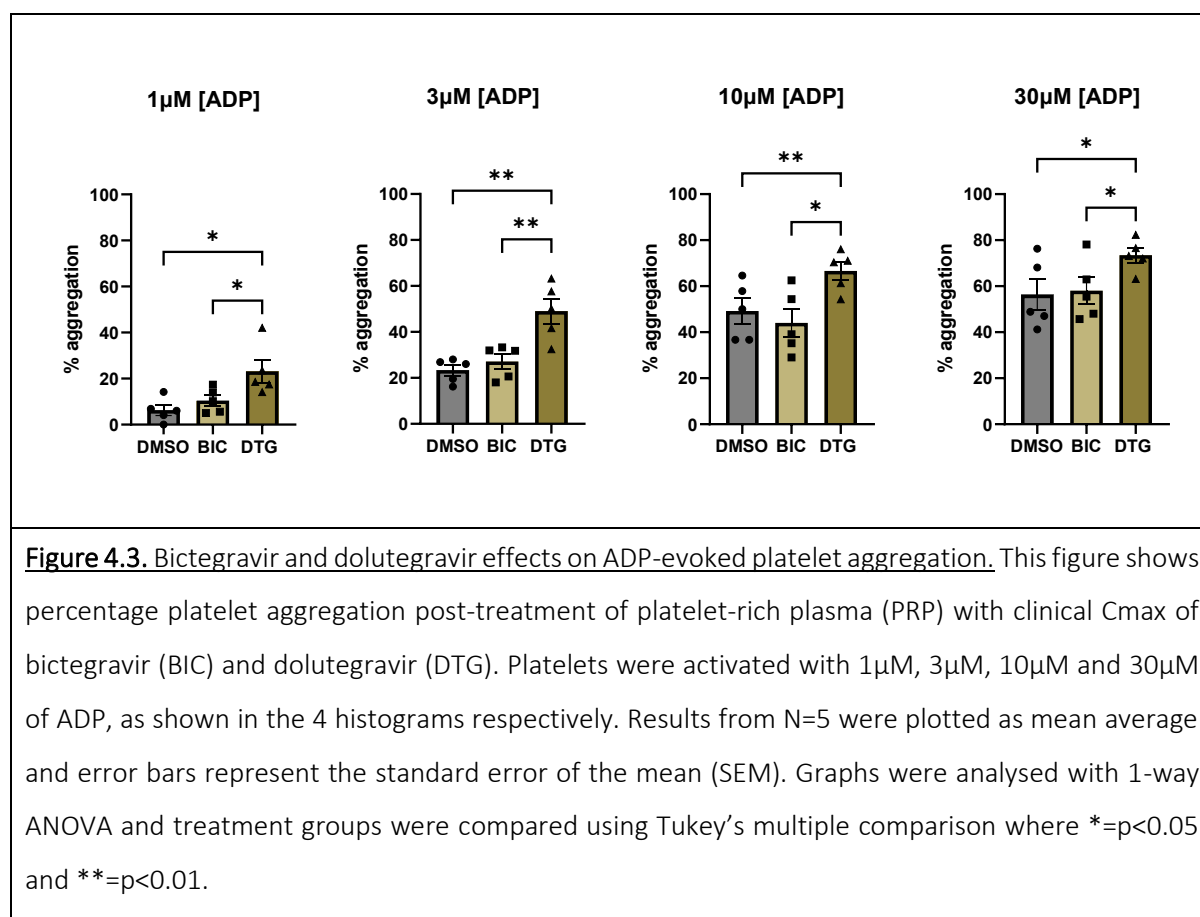
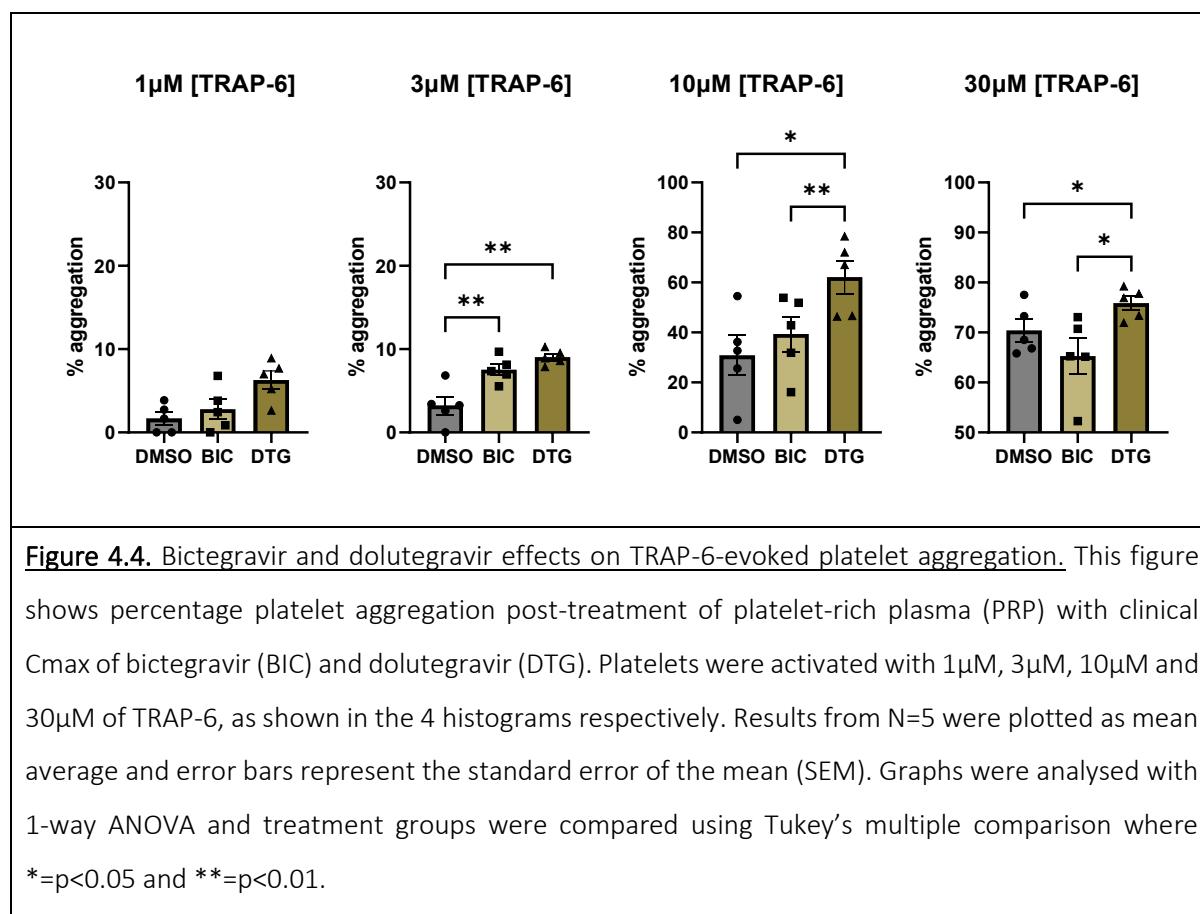


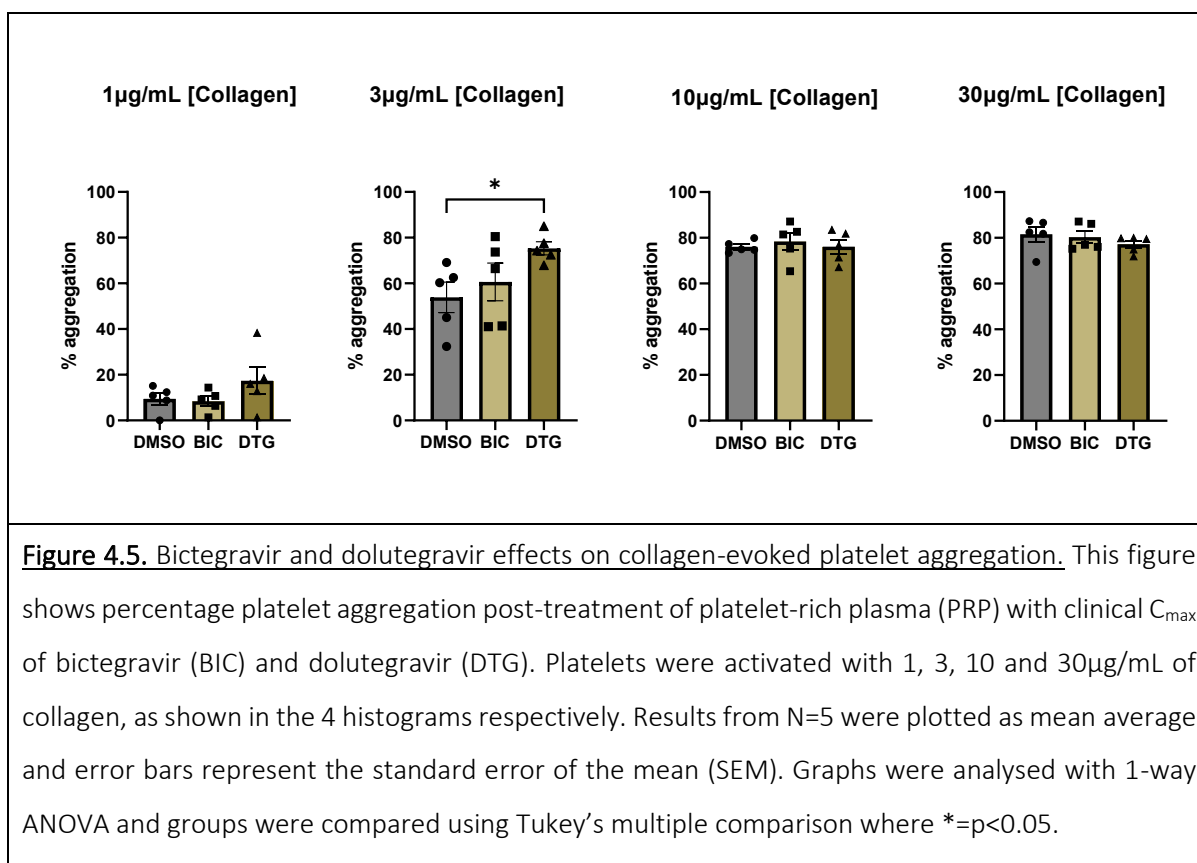
Figure 4.3 indicates that the percentage aggregation in DTG-treated platelets, evoked from 1, 3, 10 and 30 μ M of ADP, was significantly higher than that of DMSO (p values= 0.019, 0.0037, 0.0098, 0.031 respectively) and BIC (p values= 0.0303, 0.0037, 0.0246, 0.0226 respectively) treated groups. BIC on the other hand had no significant differences on ADP-evoked platelet aggregation compared to the vehicle control.

Similar to ADP, results from platelets activated using a range of TRAP-6 concentrations, are shown in **Figure 4.4**. At 1 μ M of TRAP-6, no significant differences were observed in aggregation of platelets treated with the vehicle or any of the INSTIs. However, at 3 μ M of the agonist, both BIC and DTG

enhanced aggregation relative to vehicle control by 2.3-fold and 2.8-fold respectively. The DTG group, at 10 and 30 μ M TRAP-6 concentrations, remained significantly higher than DMSO, with p values of 0.0107 and 0.0287 respectively, whereas BIC-treated platelets had no other significant changes from the DMSO group. Interestingly, DTG appeared to augment platelet aggregation notably more than BIC at 10 μ M ($p=0.0031$) and 30 μ M ($p=0.036$) of TRAP-6-activation.

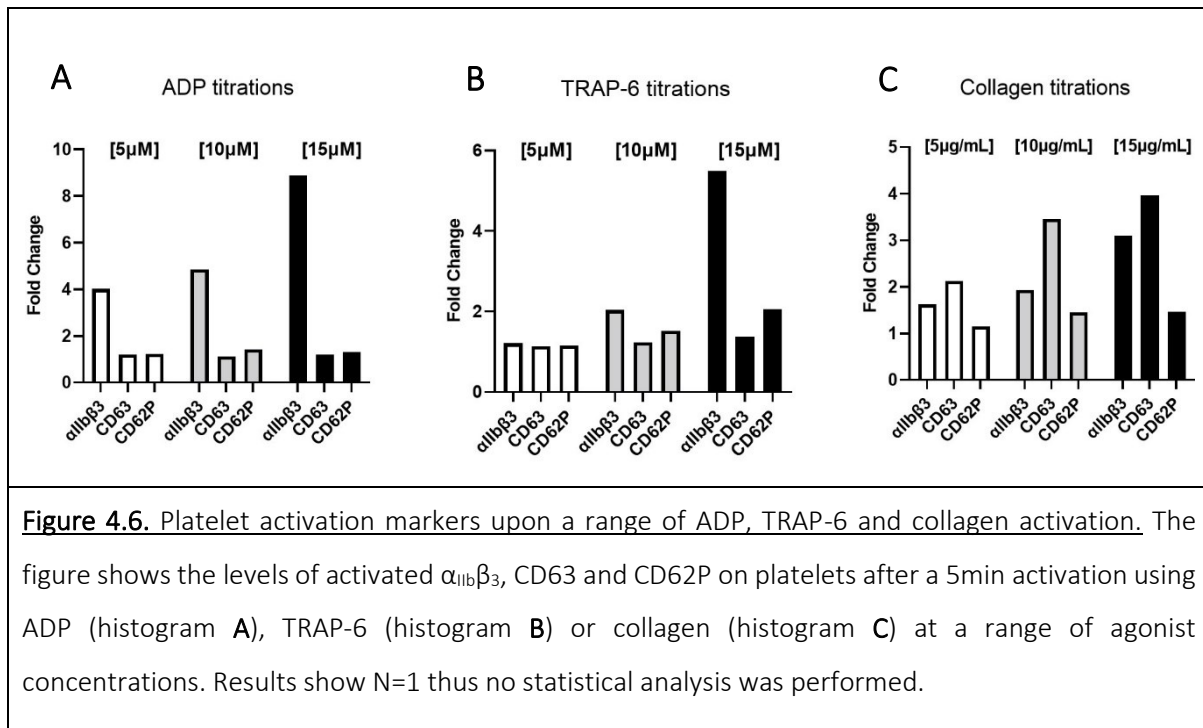


Results from collagen-evoked platelet aggregation, with agonist concentrations ranging from 1 to 30 μ g/mL, are displayed in **Figure 4.5**. BIC or DTG had no significant effects on platelet aggregation upon 1 μ g/mL collagen activation. At 3 μ g/mL however, platelets in PRP treated with DTG displayed a higher aggregation profile, approximately 1.4-fold, relative to the vehicle control group ($p= 0.0393$), but no differences were observed between the BIC group and the control or DTG group.



In the next set of experiments, the platelet activation markers, $\alpha_{IIb}\beta_3$, CD63 and CD62P, were labelled and quantified in flow cytometry (described in **Section 2.9**), during stimulation with the same agonists used for the aggregation experiments, ADP, TRAP-6 and collagen.

Before treating platelets with the drugs of interest, I carried out a single activation experiment using 3 different concentrations of each agonist to confirm an identifiable platelet activation without maxing out the response. For that, I stimulated platelets with 5, 10 or 15 µM of ADP, TRAP-6 and 5, 10 or 15 µg/mL of collagen, and results are shown in **Figure 4.6** below.



There is a trend of increasing levels of activated $\alpha_{IIb}\beta_3$ at increasing concentrations of ADP, TRAP-6 and collagen as shown in **Figure 4.6**. No obvious differences in degranulation markers CD63 and CD62P upon platelet activation with 5, 10 or 15 μ M of ADP in Graph A. On the contrary, in Graphs B and C there seems to be an increasing effect of TRAP-6 and collagen respectively in degranulation markers. Moving forward, I chose 10 μ M of ADP and TRAP-6 and 10 μ g/mL of collagen to activate platelets in the following experiments following *in vitro* treatment with INSTIs.

As described in **Sections 2.7 and 2.9**, PRP from healthy volunteers was treated with clinically relevant concentrations of BIC (13.7 μ M), DTG (8.8 μ M) or the vehicle control for 25min before fluorescently labelling and measuring platelet activation markers, similar to above. Results are shown in **Figure 4.7**. No significant changes were observed at activated $\alpha_{IIb}\beta_3$ levels between platelets treated with either drug or DMSO at any agonist activation. Similarly, no differences were shown in CD63 levels between the treatment groups at ADP-, TRAP-6- or collagen-induced activation, but CD62P (α -granule release marker) levels, were significantly higher (p value= 0.0414) in DTG-treated platelets compared to control group at ADP-evoked activation. No other notable CD62P differences were shown in TRAP-6 or collagen activation.

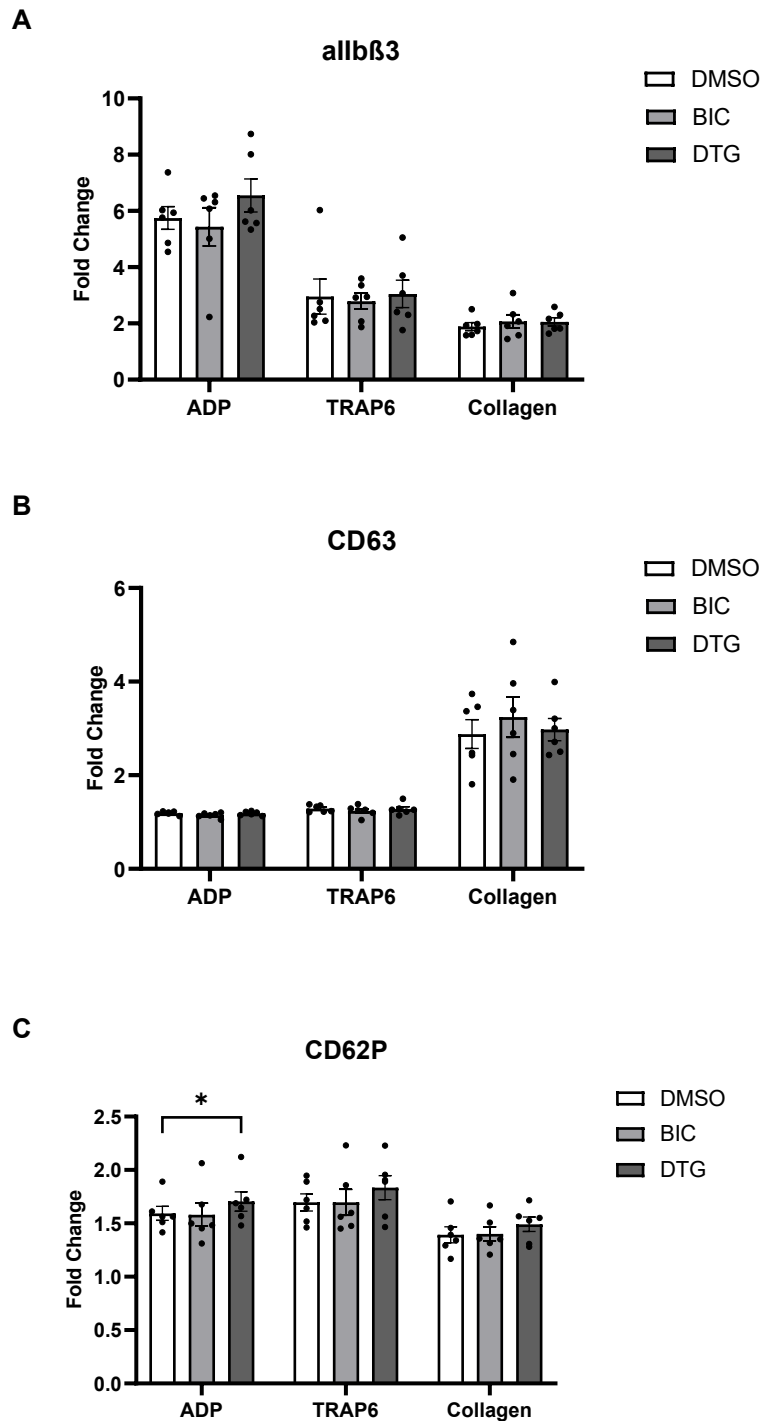
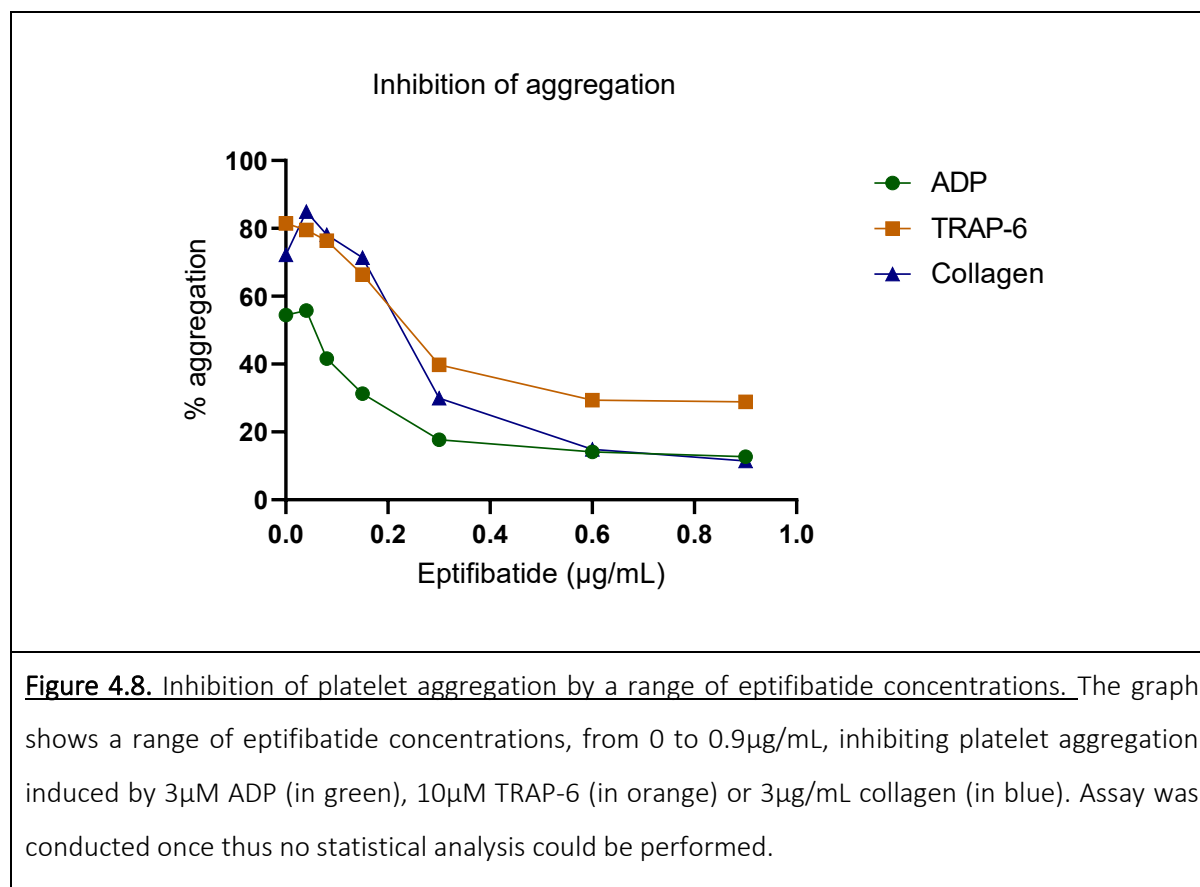


Figure 4.7. Change in $\alpha_{IIb}\beta_3$, CD63 and CD62P platelet levels post *in vitro* treatment with bicitegravir or dolutegravir. The graph shows in **A** the fold change in $\alpha_{IIb}\beta_3$ integrin activation in platelets treated with bicitegravir (BIC), dolutegravir (DTG) or vehicle control (DMSO) post ADP, TRAP-6 or collagen activation, in flow cytometry. Graph **B** shows the equivalent change in CD63 and graph **C** that of CD62P. Results from N=6 were analysed with 2-way ANOVA, and groups were compared using the Tukey's multiple comparison test, where *= p<0.05.

4.2 EPTIFIBATIDE INHIBITION OF PLATELET AGGREGATION

Results from the platelet aggregation assays suggest an enhanced platelet activity in samples treated with dolutegravir versus bicitegravir or DMSO and some effect of bicitegravir compared to control at low levels of TRAP-6. This effect was not clear from the fold change of $\alpha_{IIb}\beta_3$, the aggregation-mediating integrin, thus I decided to repeat a series of platelet aggregation assays but this time inhibiting aggregation as well. This was done using eptifibatide, an $\alpha_{IIb}\beta_3$ -inhibitor, to confirm that differences between initial findings resulted from true aggregation effects of the drugs.

I decided to use 3 μ M of ADP, 10 μ M of TRAP-6 and 3 μ g/mL of collagen to observe platelet aggregation, with or without inhibition, as those concentrations lie within the linear part of the activation curve for each agonist, shown in **Figure 4.2**. Inhibition of agonist-induced aggregation using 0, 0.04, 0.08, 0.15, 0.3, 0.6 and 0.9 μ g/mL of eptifibatide without any prior drug treatment is shown in **Figure 4.8** below. The eptifibatide concentration reached an almost maximum inhibition for all three agonist-induced aggregation profiles at 0.6 μ g/mL, thus this was the concentration used in the following experiments.



After choosing the appropriate concentrations, I repeated the platelet aggregation experiments post-treatment with BIC, DTG or DMSO with and without 0.6µg/mL. Results are shown in **Figure 4.9** below.

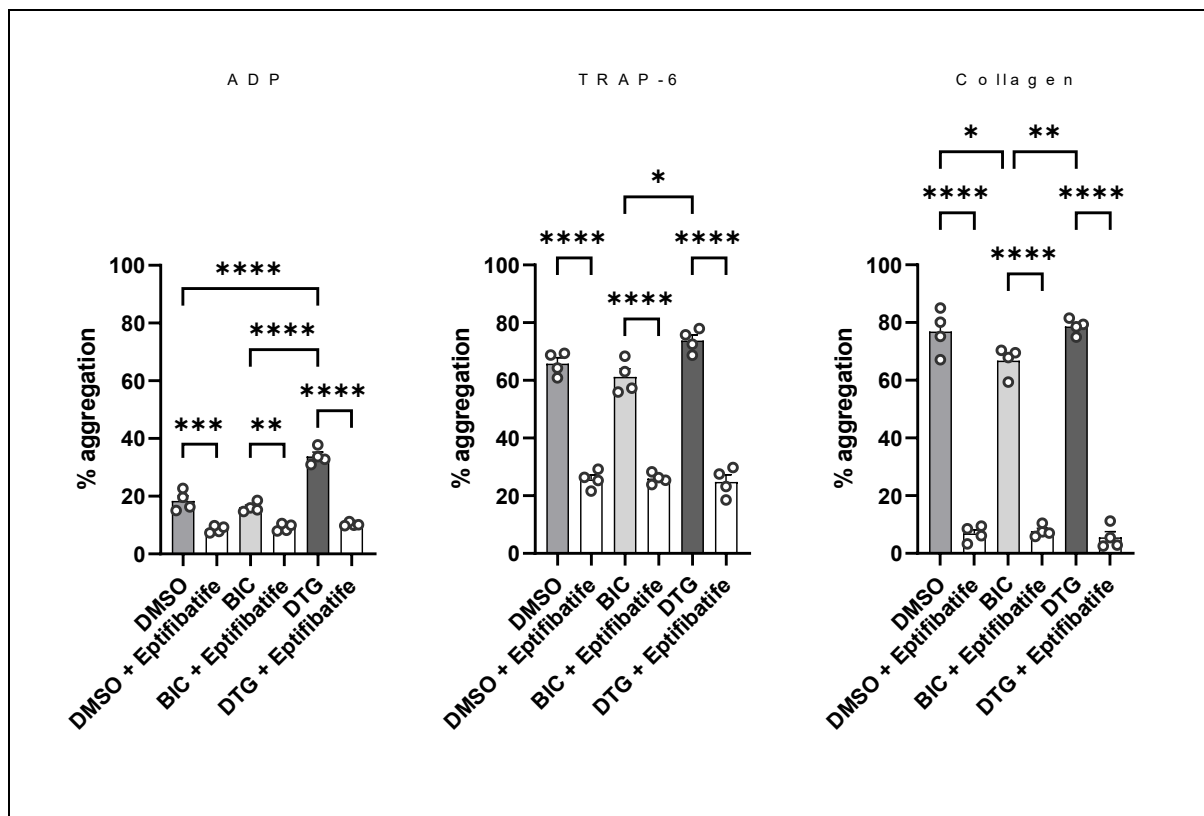


Figure 4.9. Eptifibatide inhibition in agonist-induced platelet aggregation post *in vitro* treatment with bicitegravir or dolutegravir. The figure shows ADP, TRAP-6 and collagen induced platelet aggregation in the three graphs respectively, with or without 0.6µg/mL of eptifibatide, in platelets treated with 13.7µM bicitegravir (BIC), 8.8µM dolutegravir (DTG) or DMSO vehicle control. Results from N=4 are shown as mean average and error bars represent the standard error of the mean (SEM). Groups were analysed using 2-way ANOVA and compared using Tukey's multiple comparison where *=p<0.05 **=p<0.01 ***=p<0.001 ****=p<0.0001.

A major finding from **Figure 4.9** is that the differences in aggregation from platelets treated with BIC, DTG or DMSO plus eptifibatide all have a p value > 0.05, in all three agonist activations. Moreover, DTG-treated platelets show an enhanced aggregation compared to BIC or DMSO, at comparable levels as already shown previously. BIC-treated platelets behave similar to control (p values > 0.05), except in collagen-induced aggregation where platelets seemed to have a lower aggregation profile in bicitegravir group compared to control.

4.3 EFFECTS OF BIC/TAF/FTC & DTG/3TC ON PLATELET AGGREGATION & ACTIVATION

MARKERS

After investigating the potential effects of the individual integrase inhibitors bicitegravir and dolutegravir, on platelet aggregation and activation markers, clinically relevant combinations of the drugs should be examined next. This includes the combination treatments commonly prescribed to PLWH, Biktarvy or Dovato. Biktarvy consists of bicitegravir (BIC), emtricitabine (FTC) and tenofovir alafenamide (TAF) and Dovato of dolutegravir (DTG) and lamivudine (3TC). Platelets were treated with the clinical Cmax values of each drug combination, and aggregation was initiated with ADP, TRAP-6 or collagen using a range of agonist concentrations. Results from ADP activation are shown in **Figure 4.10**.

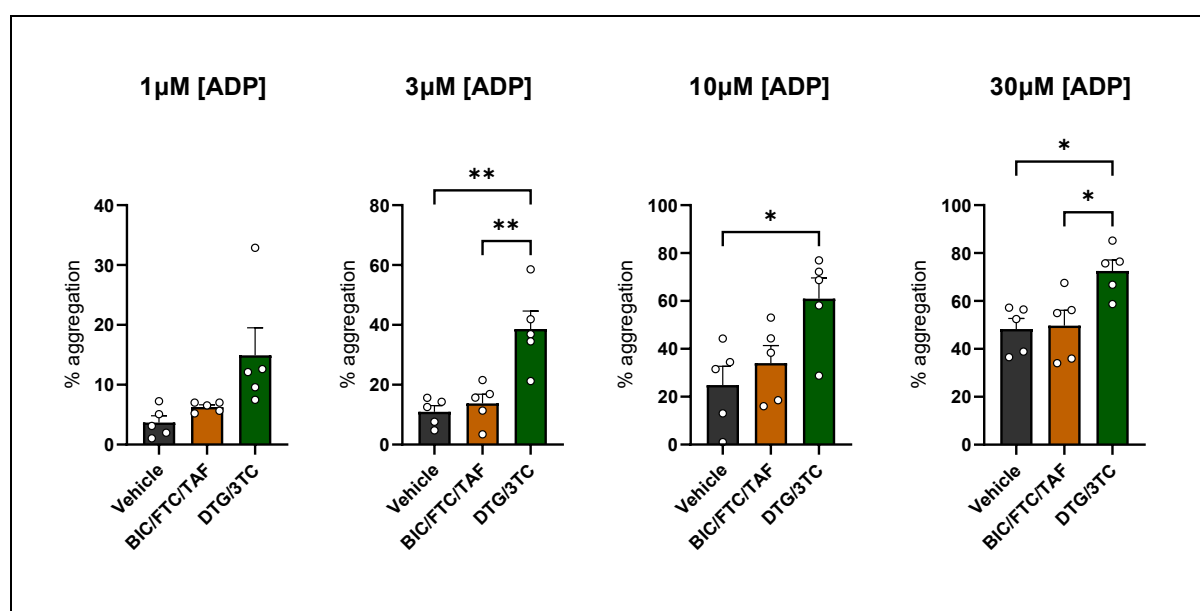
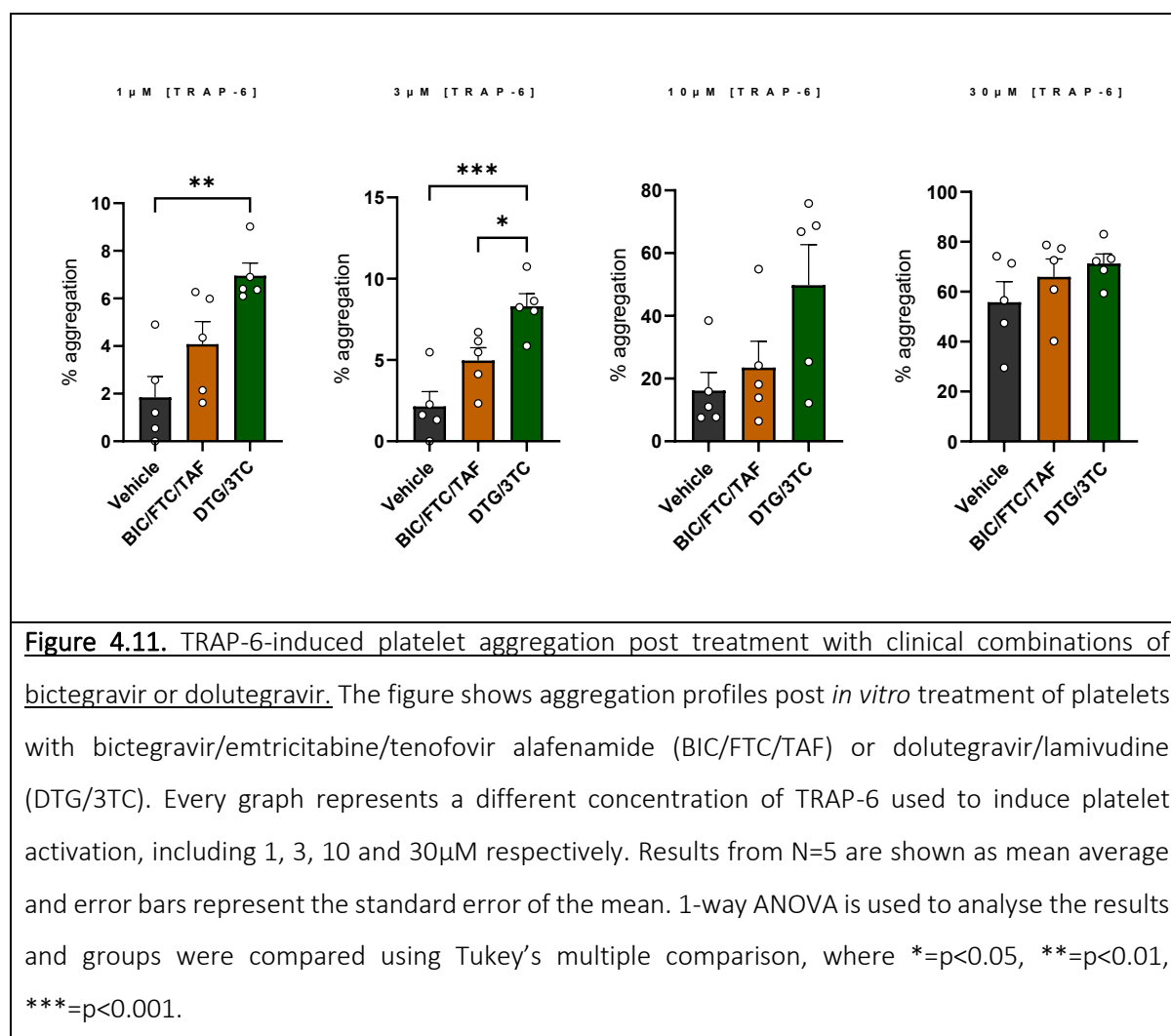


Figure 4.10. ADP-induced platelet aggregation post treatment with clinical combinations of bicitegravir or dolutegravir. The figure shows aggregation profiles post *in vitro* treatment of platelets with bicitegravir/emtricitabine/tenofovir alafenamide (BIC/FTC/TAF) or dolutegravir/lamivudine (DTG/3TC). Every graph represents a different concentration of ADP used to induce platelet activation, including 1, 3, 10 and 30μM respectively. Results from N=5 are shown as mean average and error bars represent the standard error of the mean. 1-way ANOVA was used to analyse the results and groups were compared with Tukey's multiple comparison, where *= $p < 0.05$, **= $p < 0.01$.

The dolutegravir-containing regimen, DTG/3TC, significantly altered ADP-induced platelet aggregation more than the bicitegravir-containing regimen, BIC/FTC/TAF ($p = 0.0028$ for 3μM ADP and $p = 0.0231$ for 30μM ADP), or the vehicle control ($p = 0.0012$ at 3μM ADP and $p = 0.0163$ at 30μM ADP). Additionally, at 10μM of ADP, percentage aggregation in DTG group was higher by 2.44-fold than the control group

($p=0.0185$). No significant changes were obtained between BIC/FTC/TAF and vehicle at any concentration.

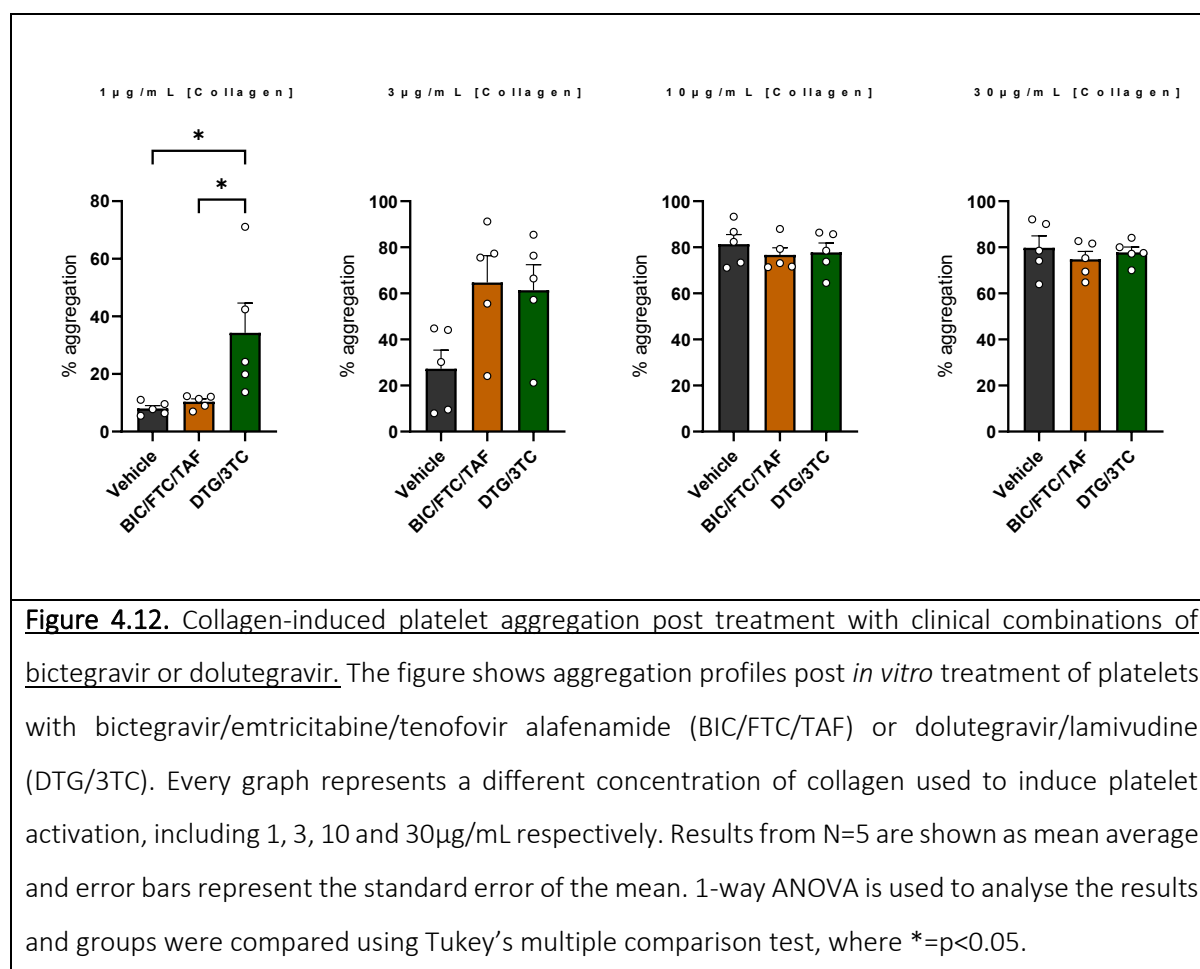
Results from platelets activated with a range of TRAP-6 concentrations after drug-combination treatments are shown in **Figure 4.11**.



DTG/3TC treatment enhanced platelet aggregation induced by TRAP-6 at low levels of the agonist compared to vehicle control at both 1 μM ($p=0.0021$) and 3 μM ($p=0.0005$) TRAP-6 activations, with the DTG/3TC group at the 3 μM exhibiting a significantly higher aggregation profile than BIC/FTC/TAF ($p=0.0364$) as well.

Finally, platelet aggregation induced by a range of collagen concentrations after BIC/FTC/TAF or DTG/3TC treatment *in vitro*, is shown below in **Figure 4.12**. The only significant differences obtained from these experiments were those between DTG/3TC and BIC/FTC/TAF ($p=0.0398$) or control group

($p=0.0246$) where DTG/3TC group exhibited more than 3-fold higher mean percentage aggregation than the other two groups.



Platelet activation markers in flow cytometry were also measured after incubating platelets with BIC/FTC/TAF, DTG/3TC or DMSO and results are illustrated in **Figure 4.13**. No significant differences were observed in activated $\alpha_{IIb}\beta_3$, CD63 or CD62P levels between platelets treated with either combination of antiretrovirals or DMSO. This was consistent at all three agonist-induced activations of platelets.

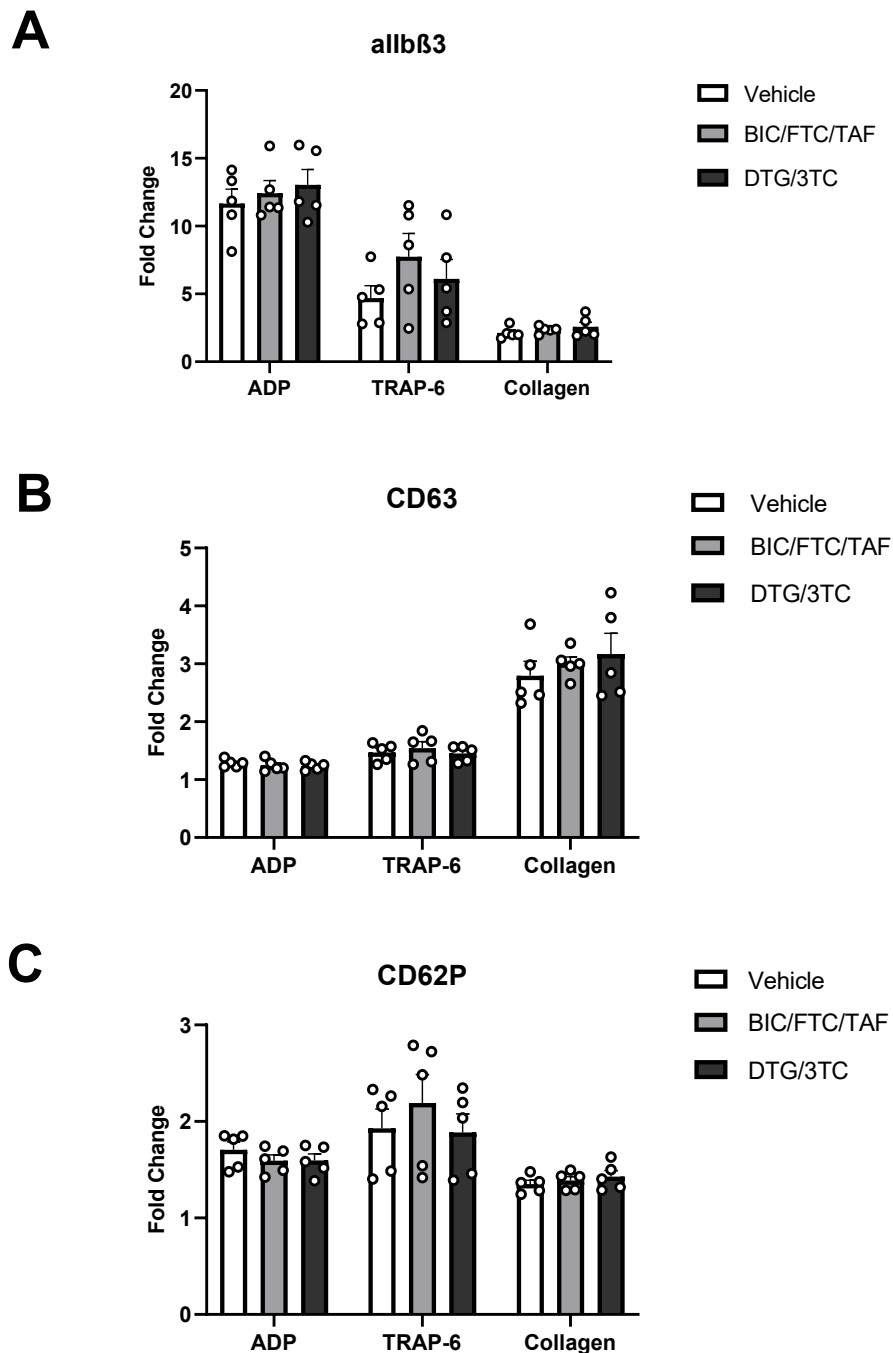


Figure 4.13. Change in $\alpha_{IIb}\beta_3$, CD63 and CD62P platelet levels in flow cytometry, post *in vitro* treatment with clinical combinations of bictegravir or dolutegravir. The graph shows in **A** the fold change in $\alpha_{IIb}\beta_3$ integrin activation in platelets treated with bictegravir/emtricitabine/tenofovir alafenamide (BIC/FTC/TAF), dolutegravir/lamivudine (DTG/3TC) or vehicle control (DMSO) post ADP, TRAP-6 or collagen activation. Graph **B** shows the fold change in CD63 and graph **C** that of CD62P. Results from N=5 were analysed with 2-way ANOVA, and groups were compared using the Tukey's multiple comparison test where no significant differences were observed.

4.4 EFFECTS OF FTC, TAF & 3TC ON PLATELET AGGREGATION & ACTIVATION MARKERS

The final step in this series of experiments was to investigate if the individual NRTI drugs in the combination treatments BIC/FTC/TAF and DTG/3TC, other than the integrase inhibitors, have any effects on platelets. For this, I measured agonist-induced platelet aggregation and platelet activation markers by flow cytometry, by treating platelets with clinically relevant concentrations of emtricitabine, tenofovir alafenamide, and lamivudine.

For the aggregation assays, I used a range of agonist concentrations that have been previously shown to adequately activate platelets without saturating the agonist effect. This includes 1-10 μ M of ADP, 3-10 μ M of TRAP-6 and 1-10 μ g/mL of collagen. Similar to **Sections 4.1** and **4.4** PRP from healthy volunteers was treated *in vitro* with 8.6 μ M FTC, 0.2 μ M TAF, 6.1 μ M 3TC or DMSO, the vehicle control, for 25min before initiating aggregation with agonists. Results are shown in **Figure 4.14**.

No significant differences in percentage aggregation were reported between platelets treated with FTC, TAF, 3TC or DMSO. This was consistent in all three agonist-induced aggregations, throughout the range of concentrations used for each agonist. This suggests that the NRTIs comprised in the combination treatments Biktarvy (BIC/FTC/TAF) and Dovato (DTG/3TC) have no differential effects on platelet aggregation.

Platelet activation markers post-NRTI treatment were measured in flow cytometry. Activation of platelets was done by 10 μ M ADP, 10 μ M TRAP-6 or 10 μ g/mL collagen and results are summarized in **Figure 4.15**. Platelets in PRP treated with the individual NRTIs of interest didn't show any significant differences between them or with vehicle control. This was consistent with every activation marker measured, including activated $\alpha_{IIb}\beta_3$ levels, or degranulation markers CD63 and CD62P. These findings agree with the ones from platelet aggregation, suggesting that emtricitabine, tenofovir alafenamide and lamivudine do not affect agonist-induced platelet activation or aggregation *in vitro*.

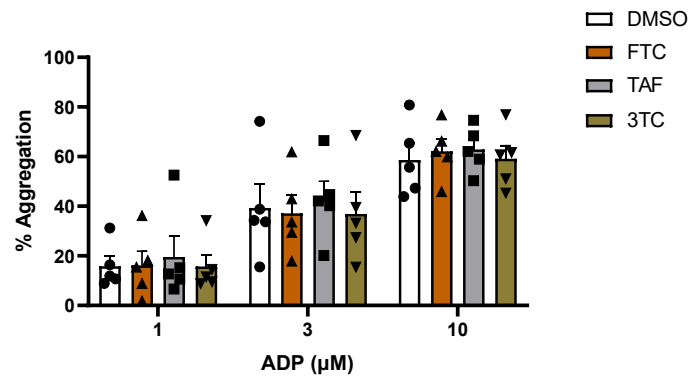
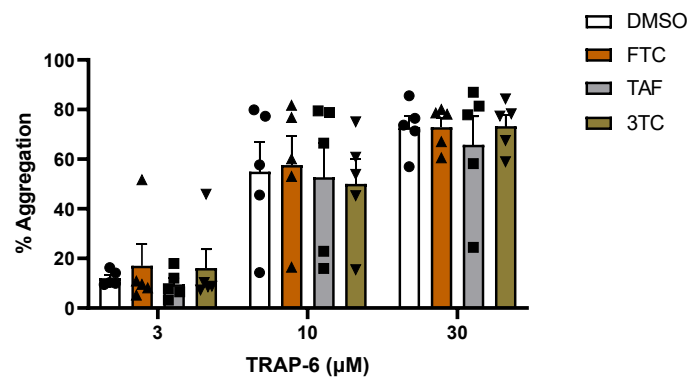
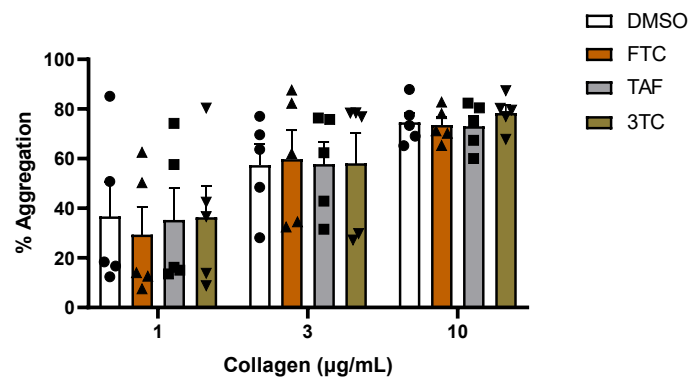
A**B****C**

Figure 4.14. Agonist-induced platelet aggregation post *in vitro* treatment with emtricitabine, tenofovir alafenamide and lamivudine. The figure shows in **A** the ADP-induced aggregation of platelets after a 25min treatment with emtricitabine (FTC), tenofovir alafenamide (TAF), lamivudine (3TC) or vehicle control (DMSO). Histogram **B** shows the TRAP-6-evoked platelet aggregation at agonist concentrations ranging from 3-30μM, and **C** that of collagen, at agonist concentrations ranging from 1-10μg/mL. Results are shown as mean average from N=5, and error bars represent the standard error of the mean. Results were analysed using 2-way ANOVA and groups were compared with Tukey's multiple comparison. No significant differences were observed between any of the drug treatment groups or the vehicle control.

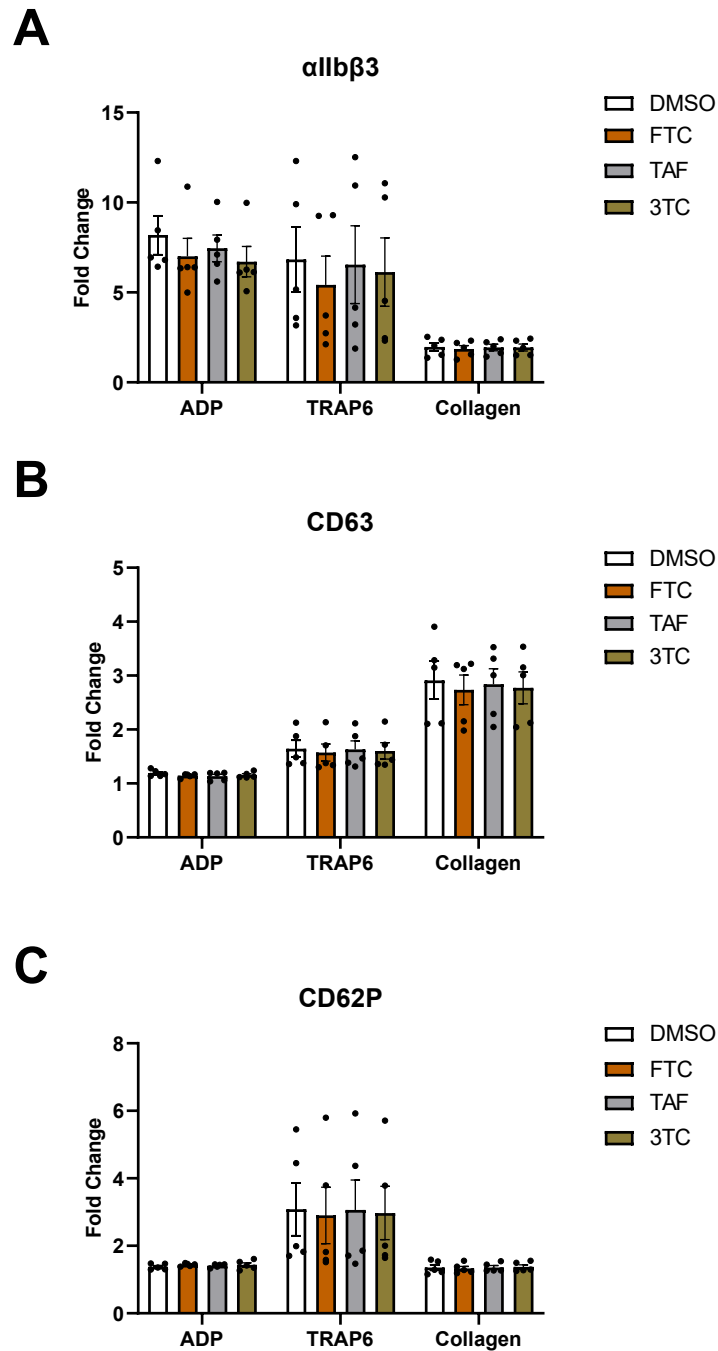


Figure 4.15. Change in α IIb β 3, CD63 and CD62P platelet levels in flow cytometry, post *in vitro* treatment with emtricitabine, tenofovir alafenamide or lamivudine. The graph shows in **A** the fold change in α IIb β 3 integrin activation in platelets treated with emtricitabine (FTC), tenofovir alafenamide (TAF) or lamivudine (3TC) or vehicle control (DMSO) post ADP, TRAP-6 or collagen activation. Graph **B** shows the fold change in CD63 and graph **C** that of CD62P. Results from N=5 were analysed with 2-way ANOVA, and groups were compared using the Tukey's multiple comparison test where no significant differences were observed.

4.5 PLATELET ADHESION TO COLLAGEN UNDER PHYSIOLOGICAL FLOW CONDITIONS

For the last section in this chapter, I will discuss the experiments I conducted in collaboration with Dr Tom McKinnon, at the Immunology & Inflammation department of Imperial College London. I used his well-established flow setup, where ART-treated whole blood was passed through a collagen-coated μ slide for 5min at $1500s^{-1}$, a physiological arterial flow rate. Fluorescently labelled platelets were recorded adhering to collagen and to each other. The technique protocol is described in **Section 2.10** and an illustrative diagram is shown in **Figure 3.5**.

Whole blood from 3 healthy volunteers was treated with clinical C_{max} values of BIC, DTG, ABC as an investigational positive control, or DMSO and platelet adhesion was recorded every minute. Percentage coverage of platelets on the collagen surface throughout the 5min period is shown in **Figure 4.16**.

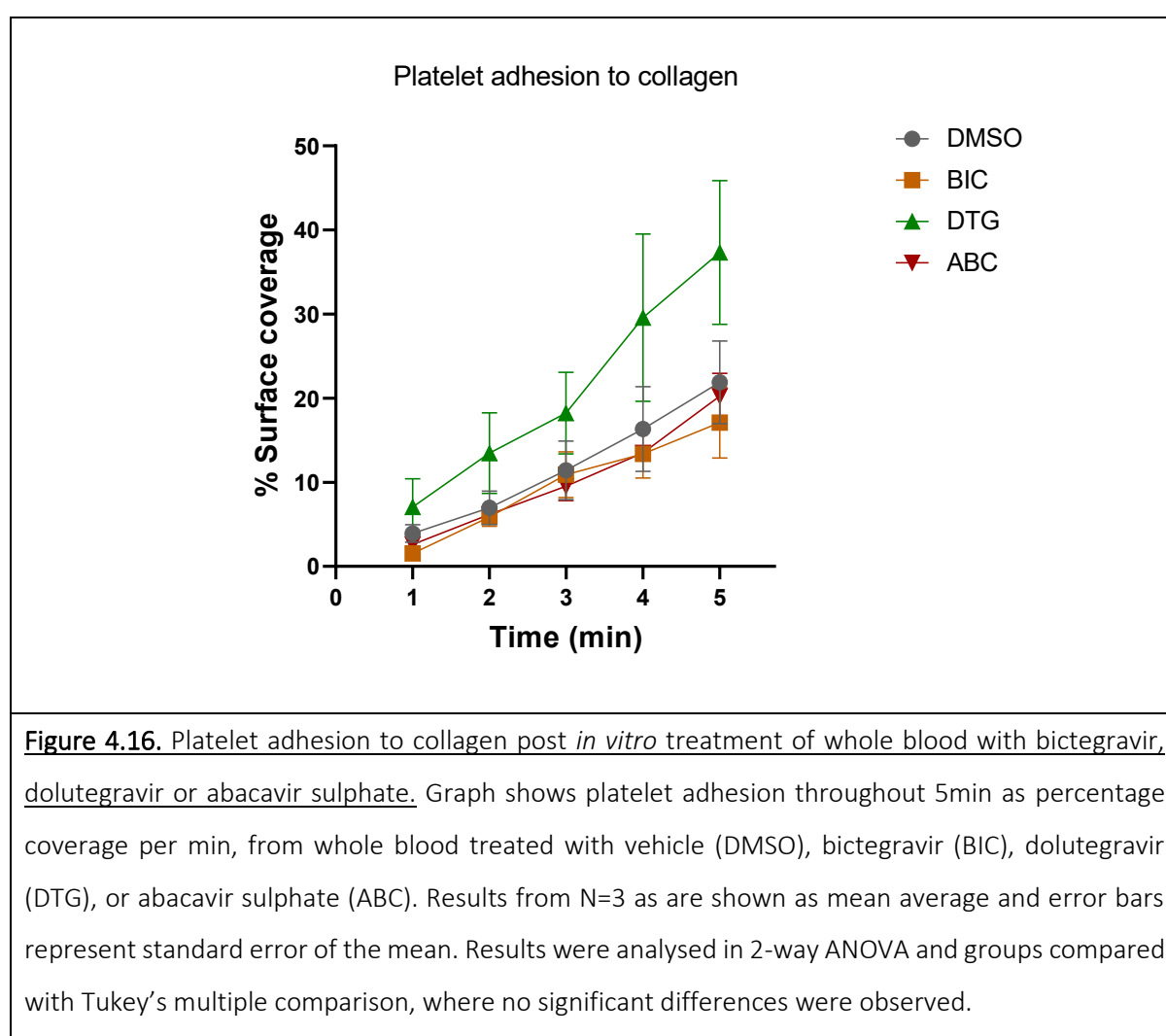
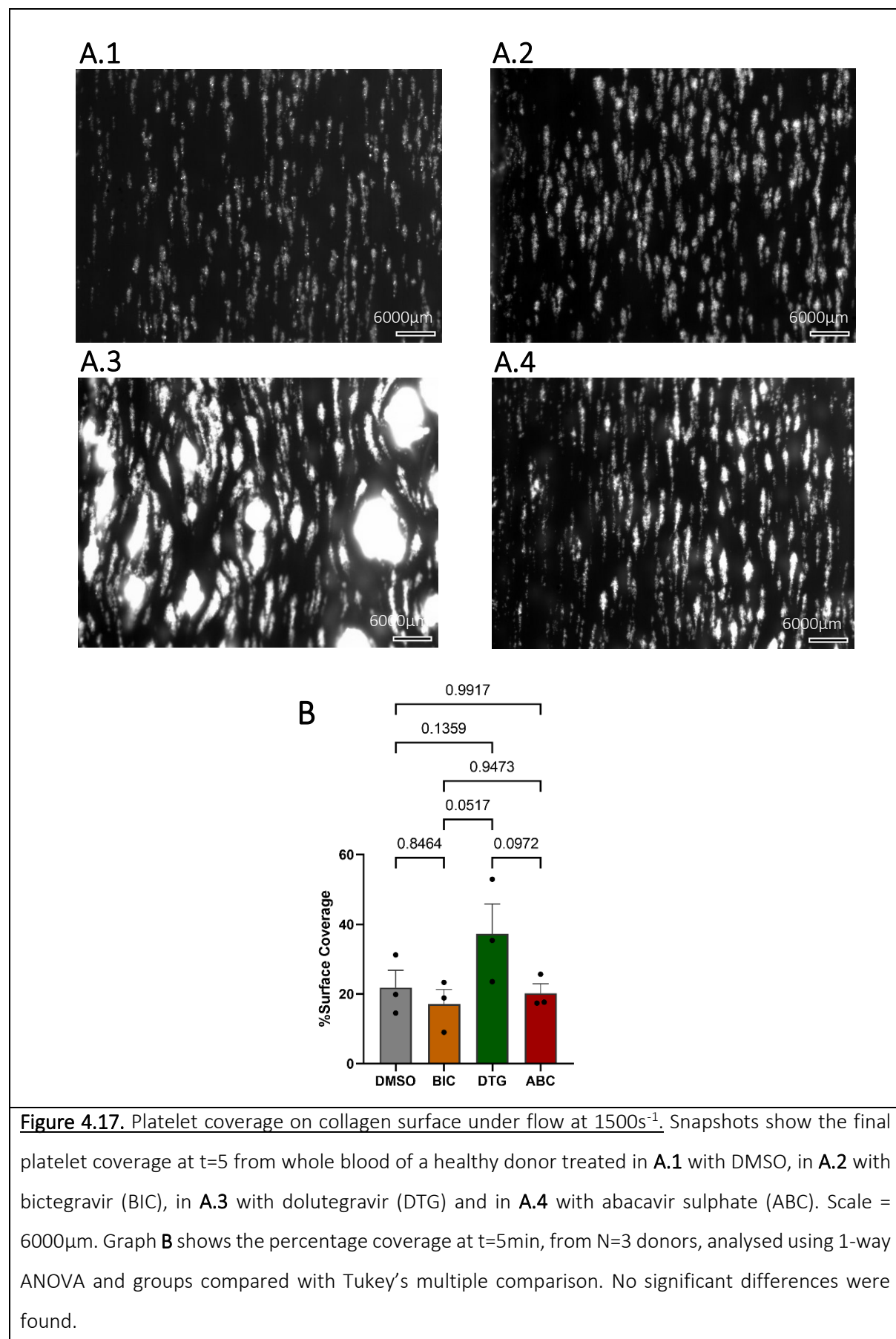


Figure 4.17 shows snapshots (A.1-A.4) taken at $t=5\text{min}$ from a single volunteer as well as the final percentage coverage (B) of platelets treated with DMSO, BIC, DTG or ABC from 3 healthy volunteers.



Data from **Figure 4.16** shows that ABC-treated blood had no differences in platelet coverage from the DMSO-treated group. Moreover, throughout the 5min passage of platelets on the collagen bed, those from DMSO-, BIC- and ABC-treated blood had a comparable trend in mean percentage coverage (21.88%, 17.09% and 20.24% respectively) unlike DTG group who appeared to exhibit an almost 2-fold higher percentage coverage (37.32%), though a higher N number is required to prove this.

Snapshots from the endpoint (t=5) of the first assay performed were included in **Figure 4.17** and it is obvious that there is a bigger recruitment of platelets in the DTG group compared to the rest, forming bigger thrombi and indicating a higher participation of platelets. However, endpoint percentage coverage from each group was included in graph **B** of **Figure 4.17**, and despite DTG exhibiting the bigger mean average compared to the rest of the groups, differences were not statistically significant.

4.6 CHAPTER 4 - DISCUSSION

PLWH present with a significantly higher incidence rate in developing arterial and venous thrombosis, myocardial infarction and ischemic stroke [80], [162]. The crucial role of platelets in thrombosis and CVD is well established [159], [174], [175], making them a critical component of the cardiovascular system and hence in PLWH. Previous studies linked ARV agents used in clinical settings with altered platelet function, potentially affecting the IR for CVD.

There was a big shift in integrase inhibitors over the last few years [38], [44], [177], [183] but not much is known about their possible off target effects on platelets. My study aims to offer a better understanding of this, particularly by assessing BIC and DTG *in vitro* effects in agonist evoked platelet activation in PRP obtained from HIV negative volunteers.

Initially, I confirmed in light-transmission aggregometry that DMSO, or the individual ARVs included in Biktarvy (BIC/FTC/TAF) and Dovato (DTG/3TC) used at clinically relevant concentrations, do not activate platelets at resting conditions. This agrees with current literature, as none of these ARVs were reported to affect resting platelets. Next, a range of agonists and agonist concentrations were used to explore the full aggregation potential of platelets in PRP treated with BIC, DTG or DMSO *in vitro*. ADP and collagen are naturally occurring platelet agonists [123], whereas TRAP-6 is an artificial agonist,

mimicking the ligand peptide of PAR-1 that thrombin naturally cleaves to allow PAR-1 mediated platelet activation and is used in research extensively [152], [181], [190]. Data from **Figure 4.2** were divided into three figures to display the aggregation profiles of platelets at different agonist concentrations and allow clear statistical comparisons.

Results in **Figure 4.3** indicate that DTG enhanced platelet aggregation, across the whole range of ADP concentrations used, more than BIC and/ or DMSO suggesting a potential DTG interference (or enhancement) of the ADP-induced activation pathway, making platelets more prone to aggregate. This data is supported by the work of Madzime et al., who revealed a DTG-mediated enhancement of ADP-evoked platelet activation, possibly through triggering a calcium influx [185]. Despite the similarities in chemical structure between BIC and DTG (**Figure 1.3**), not only no effects from BIC were observed in **Figure 4.3**, but DTG aggregation was approximately 2-fold higher than BIC at low agonist concentrations, suggesting a drug-specific effect rather than a drug class one.

Unlike ADP, at TRAP-6 activations in **Figure 4.4** both INSTIs augmented platelet aggregation compared to vehicle, at 3µM of the agonist suggesting a possible class specific effect. Interestingly, at higher agonist concentrations, this effect only persisted in the DTG group and was abolished in BIC with the former having significantly higher aggregation profiles than the latter. Madzime et al., reported a DTG-mediated enhancement of thrombin-evoked platelet activation [185] which agrees with my findings, given that TRAP-6 and thrombin share a similar activation mechanism in platelets [123], [190].

Aggregation profiles with collagen suggest a possible maximum aggregation capacity at approximately 10µg/mL of the agonist, as seen in **Figure 4.2** and **Figure 4.3**. At 3µg/mL of collagen activations, DTG-treated PRP exhibited higher aggregation than the control group, once again suggesting a DTG-specific augmentation of platelet aggregation at a low-grade agonist stimulation.

Overall, from the three agonists tested, my findings indicate a DTG-mediated enhancement of platelet aggregation post *in vitro* treatment with clinically relevant concentrations of the drug, an affect only seen in BIC-treated PRP at low-grade TRAP-6 activations, making it difficult to deduce if it is an INSTI-specific effect or not. Moreover, ADP and collagen are naturally occurring platelet agonists, whereas

TRAP-6 mimics thrombin activity, but does not occur naturally. For this, thrombin instead of TRAP-6 can be investigated in future studies and more INSTIs could be added in the experiments, like raltegravir or cabotegravir, to allow a better understanding of the overall effect of INSTIs on platelet activation.

These results agree with the findings of Madzime et al., who also reported a DTG specific effect in amplifying platelet activation [185] using a range of agonists. Every agonist/receptor signalling has a distinct pathway leading to platelet activation, with a common phase at increasing available intraplatelet calcium ions. The hypothesis of Madzime et al., suggesting a calcium influx in a DTG-mediated ionophore-like mechanism is a possible explanation for the differences they and I demonstrated. I would therefore recommend calcium studies similar to Madzime et al. or measuring intraplatelet Ca^{2+} levels in flow cytometry using calcium indicators post *in vitro* exposure to BIC or DTG to compare the effects of INSTIs on calcium levels and link to my original findings.

Platelet aggregation occurs after activation and light transmission aggregometry measures the cumulative effect of platelets in PRP. To confirm the effects of DTG or INSTIs as a class on platelet activation prior to aggregating, I decided to measure established surface activation markers on platelets [151], [152], [181] during real time activation. Fold change in the MFI of 1) the activated form of $\alpha_{\text{IIb}}\beta_3$ (aggregation-facilitating integrin complex), 2) CD62P (alpha granule release marker) and 3) CD63 (dense granule release marker) were measured using flow cytometry.

Agonist titrations were performed initially as shown in **Figure 4.6** to choose a concentration per agonist and 10 μM ADP, TRAP-6 and 10 $\mu\text{g}/\text{mL}$ of collagen were chosen for the following experiments as they elevated levels of markers of interest but did not reach maximum activation capacity of platelets. In the future, these experiments can be repeated to allow statistical testing, and another group (possibly PBS) with no agonist can be included as a negative control, in order to confirm the chosen concentrations.

Upon *in vitro* treatment with BIC, DTG or DMSO (control), surface activation markers were measured for 5min and fold change MFI was calculated for each agonist activation (**Figure 4.7**). No INSTI effects were reported in $\alpha_{\text{IIb}}\beta_3$, the integrin driving platelet aggregation, a result that does not agree with my findings from light transmission aggregometry. This might be due to different sensitivities of the two

assays, where light transmission aggregometry measures the collective effect of platelets together in PRP whereas flow cytometry measures a single platelet at a time. This incongruity might also be due to the time platelets are incubated with the agonists, 15min in aggregation assays and 5min in flow cytometry. Another possible reason to explain this, is the positive feedback observed in platelets during activation, where activated platelets further activate neighbouring ones increasing the cumulative effect, a setting not available in flow cytometry that only examines one platelet at a time. Another method to explore possible INSTI effects on $\alpha_{IIb}\beta_3$ activation could be conducted, to help deduce whether conclusions from light-transmission aggregometry or flow cytometry are more reliable.

No effects of BIC or DTG relative to control were observed in CD63, hence dense granule release, however a small but significant change was observed in CD62P between DTG-treated platelets versus control suggesting a possible enhancement of alpha-granule release. This difference though was only just significant and was only observed at ADP-induced activation, not with other agonists, thus no strong conclusions can be drawn from this. In order to confirm the validity of this, more ADP concentrations could be used in future assays, with a greater N size, and other techniques can be used as backup e.g. ELISA, to quantify levels of alpha granule release proteins, like soluble CD62P, released during ADP activation.

Tunjungputri et al., also reported higher MFI levels of CD62P (p value < 0.05) in platelets from patients receiving an INSTI-based regimen versus platelets from healthy volunteers [182]. My experiments did not involve patients so this study cannot support the DTG effect versus control demonstrated in **Figure 4.7** but it highlights the need to investigate DTG and other INSTI effects on platelet activation.

In order to better understand the INSTI effects observed in light transmission aggregometry but not in flow cytometry, I carried out the aggregation assays with a single concentration per agonist and measured percentage aggregation with or without eptifibatide, a popular $\alpha_{IIb}\beta_3$ -inhibitor used in similar assays previously [152], [153], [154]. After confirming the lowest concentration of the inhibitor (**Figure 4.8**) with the maximum reduction in platelet aggregation (in the absence of any ARVs) I repeated the experiments with pre-treatment with BIC, DTG or DMSO.

Results in **Figure 4.9** provide enough information to support previous outcomes. Initially, I confirmed that platelets in DTG-treated PRP displayed a significantly higher aggregation profile than BIC or DMSO groups in all three agonists tested, with different plasma donors, thus DTG-effect was reproducible. Additionally, for every drug (or DMSO) treated group, the corresponding group with eptifibatide was statistically significant, confirming the choice of inhibitor and inhibitor concentration used.

The most important finding from **Figure 4.9** is that significant differences observed between DTG and BIC or DMSO were all abolished in the presence of eptifibatide, confirming that changes observed are a true $\alpha_{IIb}\beta_3$ -mediated aggregation effect, hence supporting the evidence that DTG enhances platelet aggregation *in vitro* more than BIC or DMSO. Noteworthy is that even in the presence of eptifibatide, there was still a degree of platelet aggregation observed suggesting some possible platelet agglutination or platelet interactions not $\alpha_{IIb}\beta_3$ specific. In the absence of agonists, none of the drugs alone could trigger platelet aggregation as shown in **Figure 4.1** thus I cannot conclude that DTG enhances platelet interactions during resting conditions. At low grade collagen activation, BIC-treated platelets exhibited a lower percentage aggregation than DMSO but this effect was only observed in the eptifibatide assays and not previously, suggesting that this could be donor-specific effect.

Currently, PLWH receive a combination of ART so I wanted to explore the effect of BIC and DTG in the combinations they are usually prescribed in, BIC/FTC/TAF and DTG/3TC respectively [45], [46], where FTC, TAF and 3TC all belong in the same drug class, NRTIs. To do so, I repeated the same platelet assays, light transmission aggregometry and flow cytometry using PRP from HIV negative volunteers, this time treated *in vitro* with clinically relevant ARV concentrations and combinations. To my knowledge, this was never done before and can potentially offer a better understanding in the effects of INSTI-based regimens in a closer to real-life setting.

ADP-evoked aggregation profiles are shown in **Figure 4.10**, and similar to the individual INSTI exposures, platelets treated with the DTG containing regimen (DTG/3TC) had an overall augmented aggregation profile from both DMSO and BIC groups. This effect was maintained at low grade TRAP-6 activations (**Figure 4.11**) and 1 μ g/mL of collagen activation (**Figure 4.12**). This suggests that platelet aggregation can be potentially enhanced by DTG/3TC but further studies are required to prove this. No alterations

in platelet activation markers were observed in flow cytometry post cART exposure (**Figure 4.13**) implying that the possible effect of DTG (or DTG-containing regimen) is more evident during more physiological settings where platelets exist together (as in light transmission aggregometry) and not individually (as measured in flow cytometry). This can be due to the positive impact adjacent platelets have on each other, amplifying activation signals in the presence of platelet agonists.

Following experiments where NRTIs accompanying BIC and DTG in cART regimens were tested individually against each other in light transmission aggregometry (**Figure 4.14**) and flow cytometry (**Figure 4.15**) confirmed that none of them augment agonist-evoked platelet activity or aggregation. This can also validate that the platelet effects seen by DTG/3TC (versus BIC/FTC/TAF or DMSO) are mainly driven by DTG and not by 3TC.

To investigate the enhanced aggregation potential of platelets treated with DTG/3TC, a number of experimental approaches could be used. As this is expected to be Ca^{2+} -dependent, I would recommend calcium studies including intracellular calcium measurement, where washed platelets or platelets in PRP could be incubated with a calcium-sensitive fluorescent dye (e.g. Fura-2 AM), prior to INSTI or cART exposure. Calcium mobilization upon agonist-induced platelet activation could be measured in real-time flow cytometry, where addition of calcium ionophores or chelators could validate the involvement of Ca^{2+} ions in DTG/3TC – mediated enhanced platelet activity.

Another approach that could be used in the future is imaging washed platelets, treated with a calcium-sensitive dye (e.g. Fluo-4 AM), in fluorescence microscopy in a process known as Total Internal Reflection Fluorescence (TRIF) microscopy. INSTIs or drug combinations like DTG/3TC can be added, post-incubation with dye, on platelets resting on a collagen-coated surface. This could allow visualizing calcium-mobility as well as morphological platelet changes real-time in the presence of various drugs.

The last set of experiments presented in this chapter were conducted in collaboration with Dr McKinnon (Department of Immunology & Inflammation, Imperial College London) who established a flow assay allowing the measure of platelet adhesion on collagen under physiological conditions and shear stress [155]. Whole blood from HIV negative volunteers was treated *in vitro* for 25min with the

ARVs of interest and platelet capture images from the first run (shown in **Figure 4.17**) display noticeably bigger thrombi in the DTG-treated sample compared to the rest.

Statistical comparisons suggest that no significant differences in final percentage coverage were observed between platelets from different treatment groups, however a low p value of 0.05 between DTG and BIC groups was obtained, which requires a higher N number to clarify whether DTG enhances platelet adhesion more than BIC, as seen in the light transmission aggregometry data. Moreover, ABC appears to be an unsuitable positive control for this experiment since no endothelial cells were used for this assay, an outcome that supports previous findings showing that ABC indirectly enhances platelet activity through reducing the endothelium-mediated inhibition of platelets through NO [168], [179]. In future studies, in addition of surface coverage by platelets, the size of thrombi could be measured in different conditions as well as the rate by which thrombi are formed, to allow a range of numerical comparisons to be conducted between the different ARV-treated groups.

Relating back to my hypothesis, as expected, I saw an enhanced platelet activity upon *in vitro* treatment with DTG, throughout a number of light transmission aggregometry experiments which I confirmed to be a true DTG-mediated aggregation effect by inhibiting $\alpha_{IIb}\beta_3$. This was observed upon individual INSTI treatments and in clinical combinations, at a range of agonists and agonist concentrations used. Interestingly, BIC alone or in combination had an overall neutral effect in platelet activation or aggregation, except at low grade TRAP-6 platelet activation which appeared to enhance aggregation similar to DTG. My hypothesis is not proven despite that my findings lean towards it and I would like to highlight that further experimentations with fewer limitations can allow a stronger conclusion that could potentially clarify whether my hypothesis should be accepted or rejected.

Limitations & Future work

Whilst trying to mimic physiological exposure of platelets to ARVs *in vitro*, numerous limitations were encountered. Firstly, platelet exposure time to ARVs was 25min, whereas patient platelets are exposed to the cART regimens daily for the rest of their lives. Secondly, platelets were used in PRP for the light transmission aggregometry and flow cytometry assays, which limits the interaction of platelets to other

cells they would normally interact with, including leukocytes, and a number of platelet agonists that could exist in combination during an activated state. The lack of shear stress is also an important factor to consider when drawing conclusions from the aforementioned assays. To overcome this, studies including a physiological, arterial flow rate were performed but only 3 volunteers participated.

Overall limitations that involve all experimental approaches used in this chapter, are donor-based. The age of the HIV negative volunteers is much lower than the age of PLWH who are at risk with CVD. Moreover, 3-5 volunteers were included in each approach and a potentially higher number can allow stronger statistical analysis to be conducted. Consenting regulations for HIV negative volunteers did not allow reporting of demographics, lifestyle factors and other clinical conditions or co-medications further restricting me from linking my results with PLWH, in order to measure clinical significance.

Flow studies could be improved by measuring platelet adhesion on endothelial cells, like HCAEC to represent a more physiological setting of the arterial environment, and ABC could then be used as a suitable positive control, as its platelet effect is endothelium mediated. For light transmission aggregometry, activation capacity of platelets was assessed by using a range of agonist concentrations reaching maximum capacity. Flow cytometry and platelet adhesion assays however, lack a suitable positive control, ideally an INSTI with an established effect on platelet adhesion and activation markers.

Future studies could also include washed platelets instead of PRP for light transmission aggregometry assays to allow a better understanding of the platelet effects in the absence of any other plasma proteins. Moreover, to reflect a more physiological environment, combinations of agonists could be introduced in the assays and measure effects in the activation / aggregation capacity of platelets. In addition, more INSTIs can be included in the assays, individually or in clinical combination, including cabotegravir (with or without rilpivirine) as it raises a lot of attention lately for being the first ARV combination to be injected once or twice per month [53], [54], [58].

The chemical structure of BIC and DTG is so similar that the drug companies are still commercially competing, however the DTG demonstrated a higher effect in platelets than BIC. This could be attributed to the only differences of their chemical structure. A possible explanation might be due to

the additional fluoride ion in DTG stabilizing the negative charge in the ring domain, making the drug more prone to attract the positively charged Ca^{2+} ions across the platelet membrane, but experts in chemistry and drug design would be more appropriate to confirm or investigate this further.

The short lifespan and turnover of platelets allows any possible drug effects to be reversible, potentially by switching cART regimens in patients at risk. This makes platelets a key component of the cardiovascular system to be explored for possible drug effects not just in the context of HIV and antiretrovirals but potentially for other disease models and associated drug agents. However, platelets alone do not map exactly the cardiovascular risk in PLWH, thus more clinical or subclinical signs should be continuously monitored to better understand the CVD potential in these patient cohorts.

In my opinion, all ARV agents, used currently or in the future, should be assessed in regards to CVD as it makes the number one cause of death in PLWH other than AIDS-related illnesses, and platelets can be a suitable element to explore or to start with. A large database, accessible for scientists and clinicians, could be generated to display ARV effects individually or in clinical combinations on platelet aggregation and activation. This can allow more personalized choice or prescription of cART for PLWH.

CHAPTER 5

Patient study – Biktarvy vs Dovato

5 PATIENT STUDY – BIKTARVY VS DOVATO

In the previous chapter I examined potential effects of bicitgravir and dolutegravir on platelets obtained from healthy HIV negative volunteers treated with the drugs of interest *in vitro*. I have also investigated the effects of combination treatments containing these two integrase inhibitors, BIC/FTC/TAF and DTG/3TC which are currently used in clinics today. The next step was to move to a patient study, where samples from patients on Biktarvy (BIC/FTC/TAF) or Dovato (DTG/3TC) could be analyzed and look for possible patterns similar to the ones obtained from the *in vitro* experiments from healthy volunteers.

For this study to take place, I had to obtain a research passport in collaboration with Prof Marta Boffito, to gain access to the HIV clinic Kobler, part of Chelsea & Westminster Hospital NHS Foundation Trust. This passport allowed me to be in the clinic, recruit and consent patients for the study, as well as access the patient records of participants. Following consent, blood was collected by the clinical phlebotomy team and transported in a triple container, from the clinic to the laboratory, in a taxi.

PRP from blood samples was prepared as described in **Section 2.7**, from Biktarvy and Dovato patients. Platelet aggregation was assessed using light transmission aggregometry (**Section 2.8**) and platelet activation markers were measured in flow cytometry (**Section 2.9**). Experimental protocols were followed as previously mentioned minus the step involving the *in vitro* treatment of PRP with the drugs.

5.1 PATIENT DEMOGRAPHICS

In order to have a comparable list of patients in each treatment group, the appropriate inclusion criteria should be in place. For that, I decided to choose patients within the age range of 47 to 59 years old. **Table 6** displays the demographics of the patients included in this study. Demographic data was obtained from patient records.

Patients selected for the study had a viral load of less than 20 copies per ml of blood and have been receiving the current treatment daily for minimum 1 month from the day of recruitment. Priority was

given in the age factor when selecting patients from the two treatment groups Biktarvy or Dovato, to form age-matching cohorts. Ethnicity and gender were random and not selected for.

	BIC/FTC/TAF	DTG/3TC
N (sample size)	8	8
Age (+/- SD)	53.1 (+/- 5.9)	53.1 (+/- 5.8)
Ethnicity	4 White (English/Scottish/Welsh/ North.Irish/British) 2 White (other White background) 1 Black (African) 1 Not stated	2 White (English/Scottish/Welsh/ North.Irish/British) 3 White (other White background) 1 Multiple/Mixed Ethnicity 2 Not stated
Sex (at birth)	M	M
Viral load	< 20 copies	< 20 copies
Co-medications	P1. atorvastatin, perindopril, amlodipine P2. citalopram, acyclovir P3. none P4. atorvastatin P5. metformin, amlodipine, ramipril, glitazone P6. none P7. atorvastatin P8. finasteride, escitalopram	P9. propecia, minoxidil P10. atorvastatin, colecalciferol, doxycycline lansoprazole, zoledronic acid P11. none P12. sertraline P13. sertraline P14. none P15. atorvastatin, alendronic acid P16. vitamin D

Table 6. Patient demographics from BIC/FTC/TAF and DTG/3TC treatment groups. The table displays the demographics of the patients recruited in the study, including age, sex at birth, ethnicity and co-medications. All participants had an HIV viral load of less than 20 copies per mL. ‘P’ in co-medications stands for participant and ‘M’ for male.

5.2 PLATELET AGGREGATION - PATIENTS ON BIC/FTC/TAF vs DTG/3TC

Platelets from patients on Biktarvy or Dovato were activated *in vitro* using a range of ADP, TRAP-6 and collagen concentrations, and aggregation profiles were recorded. **Figure 5.1** shows the results of the agonist-evoked platelet aggregation from patients on the two treatment groups. No significant differences in platelet aggregation profiles were observed between patient samples from Biktarvy versus Dovato, at any agonist and agonist concentration used to induce aggregation.

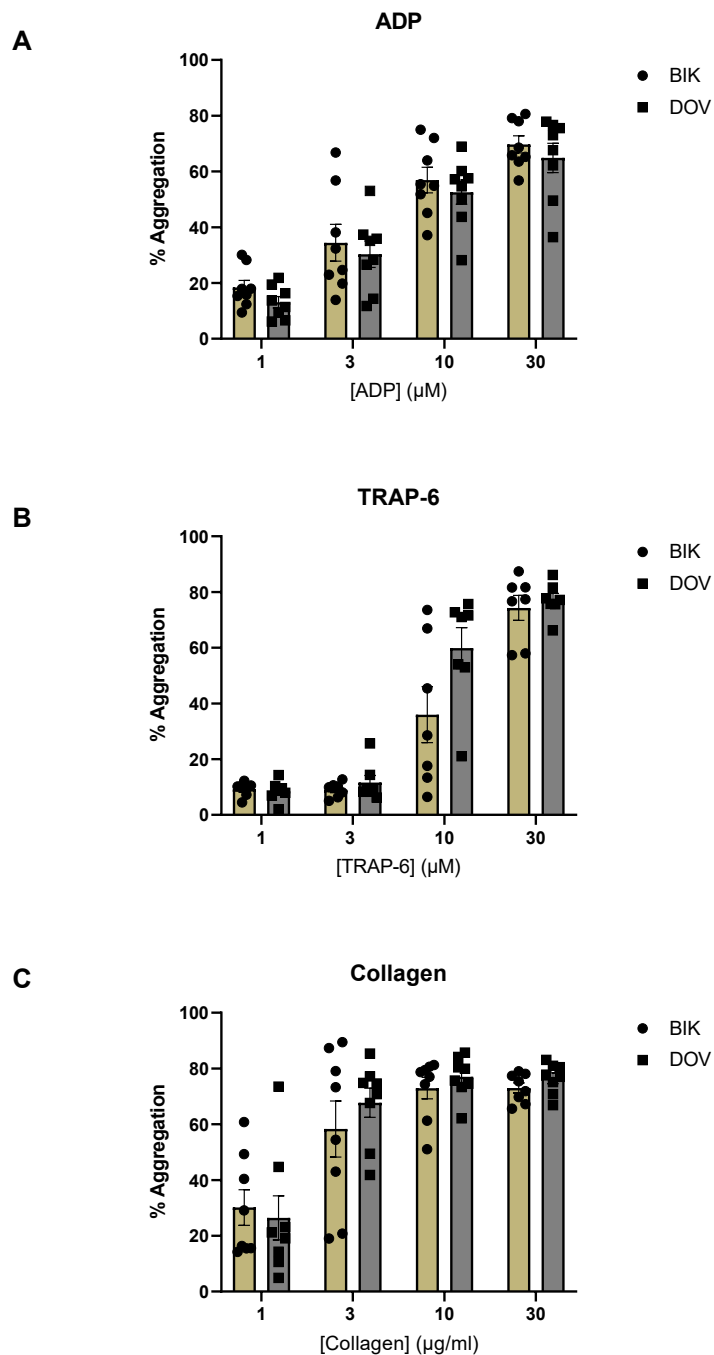


Figure 5.1. Platelet aggregation profiles from patients on Biktarvy or Dovato. The figure shows platelet aggregation from N=16 HIV patients, 8 on Biktarvy (BIK) and 8 on Dovato (DOV), upon *in vitro* activation with a range of ADP, TRAP-6 or collagen concentrations. Graph **A** shows the ADP-induced aggregation using agonist concentrations from 1-30μM, Graph **B** the TRAP-6-induced platelet aggregation with agonist concentrations ranging from 1-30μM, and Graph **C** collagen-induced aggregation with collagen ranging from 1-30μg/mL. Results were analysed using 2-way ANOVA and groups were compared using Tukey's multiple comparison test, where no significant differences were observed between the treatment groups.

5.3 PLATELET ACTIVATION MARKERS - PATIENTS ON BIC/FTC/TAF vs DTG/3TC

In addition to platelet aggregation displayed in **Section 5.2**, platelet activation markers from patients on Biktarvy or Dovato were also measured in flow cytometry, during activation with 10 μ M of ADP, 10 μ M of TRAP-6 or 10 μ g/mL of collagen. **Figure 5.2** displays the fold change in 1) $\alpha_{IIb}\beta_3$ integrin activation, 2) levels of CD63 and 3) levels of CD62P.

Results from **Figure 5.2** show that platelet activation markers from patients on Biktarvy did not vary with those from patients on Dovato: No significant differences were observed between the two groups in fold change of activated $\alpha_{IIb}\beta_3$ levels, or degranulation, measured in CD63 (dense granule) and CD62P (alpha granule) levels. This was consistent with platelets activated using ADP, TRAP-6 and collagen.

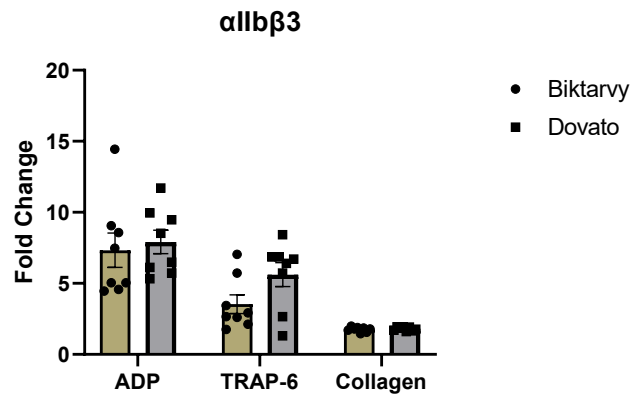
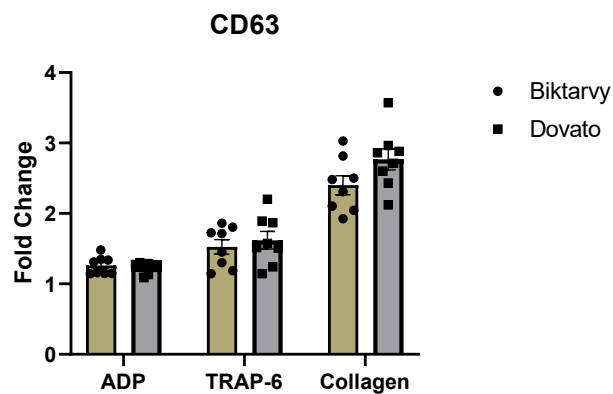
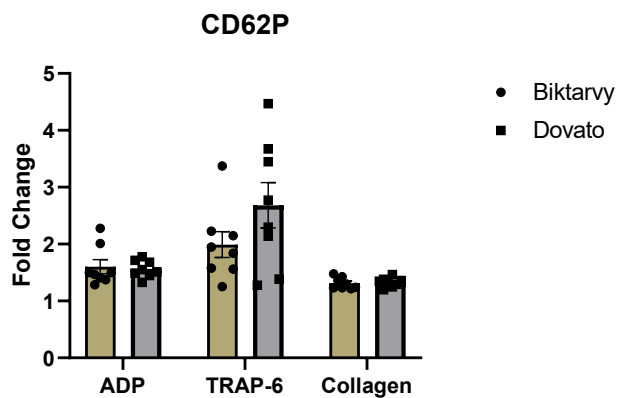
A**B****C**

Figure 5.2. Change in $\alpha_{IIb}\beta_3$, CD63 and CD62P platelet levels in flow cytometry from patients on Biktarvy or Dovato. Graph shows in **A** the fold change in activated $\alpha_{IIb}\beta_3$ levels after activating platelets from N=16 HIV patients (8 on Biktarvy and 8 Dovato) *in vitro*, using 10 μ M of ADP, 10 μ M TRAP-6 or 10 μ g/mL of collagen. Graph **B** shows the change in CD63 levels from activated platelets and Graph **C** that of CD62P. Results were analysed using 2-way ANOVA and treatment groups were compared using Tukey's multiple comparison, where no significant changes were observed.

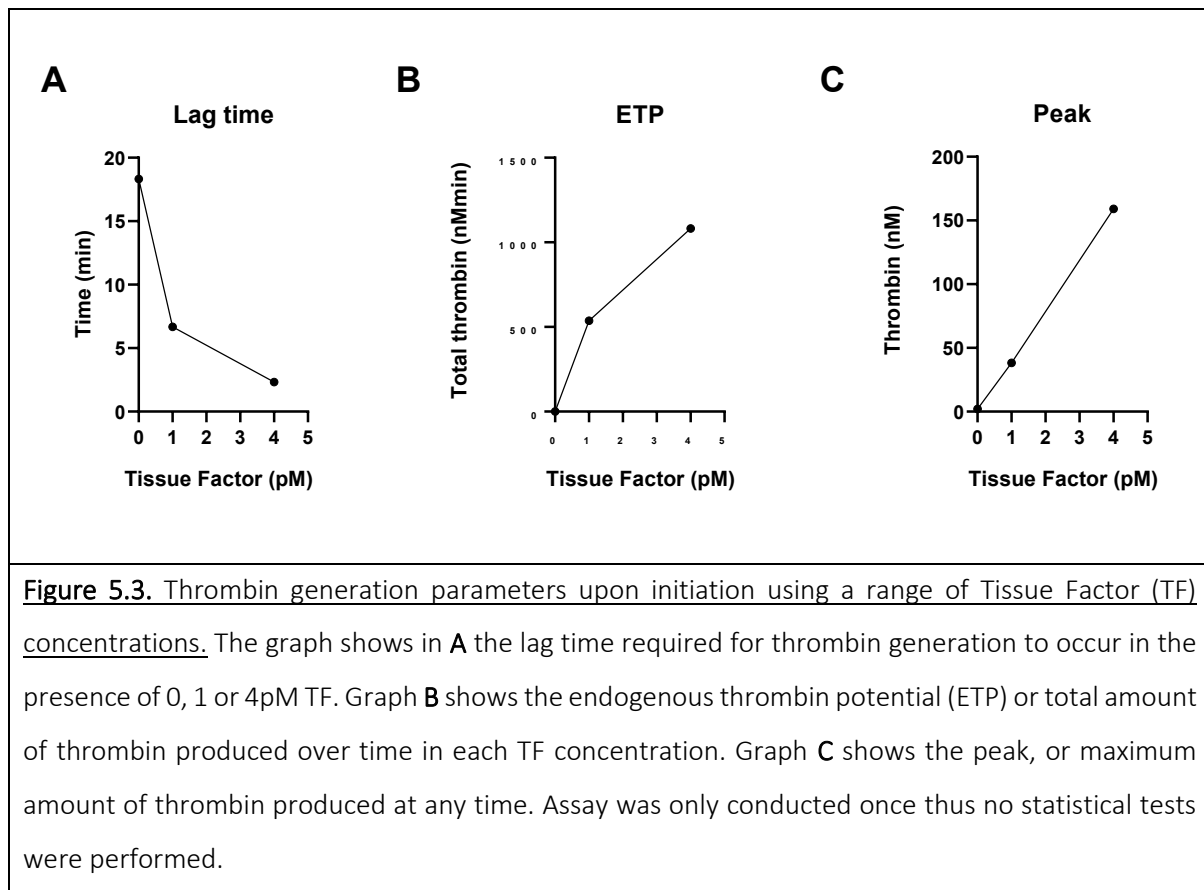
5.4 THROMBOTIC TENDENCY OF PLASMA FROM PATIENTS ON BIC/FTC/TAF vs DTG/3TC

Following the platelet experiments, I conducted a thrombin generation assay using platelet-poor plasma from patients on Biktarvy (BIC/FTC/TAF) or Dovato (DTG/3TC). This allowed me to compare the thrombotic tendency of patients from the two treatment groups in calibrated automated thrombography (CAT) and observe if the reduction of platelets affects the coagulant activity of the patient samples. CAT was run as described in **Section 2.11** for N=12 patients, 6X per treatment group, (shown in **Table 7**), as 2X patient samples in each treatment from original samples (**Table 6**) group did not yield enough PPP for CAT.

	BIC/FTC/TAF	DTG/3TC
N (sample size)	6	6
Age (+/- SD)	59.8 (+/- 5.7)	54.8 (+/- 6)
Ethnicity	2 White (English/Scottish/Welsh/ North.Irish/British) 2 White (other White background) 1 Black (African) 1 Not stated	1 White (English/Scottish/Welsh/ North.Irish/British) 3 White (other White background) 2 Not stated
Sex (at birth)	M	M
Viral load	< 20 copies	< 20 copies
Co-medications	P1. atorvastatin, perindopril, amlodipine P3. none P5. metformin, amlodipine, ramipril, glitazone P6. none P7. atorvastatin P8. finasteride, escitalopram	P9. propecia, minoxidil P10. atorvastatin, colecalciferol, doxycycline lansoprazole, zoledronic acid P11. none P14. none P15. atorvastatin, alendronic acid P16. vitamin D

Table 7. Patient demographics from BIC/FTC/TAF and DTG/3TC patient groups used in Calibrated Automated Thrombography (CAT) assays. The table displays the demographics of the 6X patients from each group that were included in the CAT assays. Samples were obtained from patients in **Table 6**.

Firstly, the CAT assay was only ran using reference plasma, pooled PPP samples from healthy volunteers, in the presence of 0, 1 or 4pM of Tissue Factor (TF). **Figure 5.3** shows the lag time, thrombin generation potential and total thrombin produced using the three TF concentrations and the reference plasma.



In **Figure 5.3**, 4pM of TF is shown to initiate thrombin generation quicker than 1pM. Moreover, 4pM of TF resulted in a higher yield of thrombin than 1pM thus 4pM was used in the following experiment to evoke thrombin production.

Next, I ran the CAT assay using PPP from 6 Biktarvy patients, 6 Dovato patients and the reference plasma. Results are shown in **Figure 5.4**. The average curve for thrombin generation in plasma from patients on BIK was almost identical to the one from patients on DOV, in Graph **A** of **Figure 5.4**. Both of those curves appear to have a higher peak compared to the reference plasma from healthy volunteers, but the difference was not significant when analyzed in the last graph of Panel **B** in the same figure. No significant differences were observed between the two treatment groups in lag time, as samples from

both groups started generating thrombin around the same time. The total amount of thrombin generated over time from the patient samples on Biktarvy was undistinguishable from that from patients on Dovato, as shown in the middle graph of Panel B (Figure 5.4).

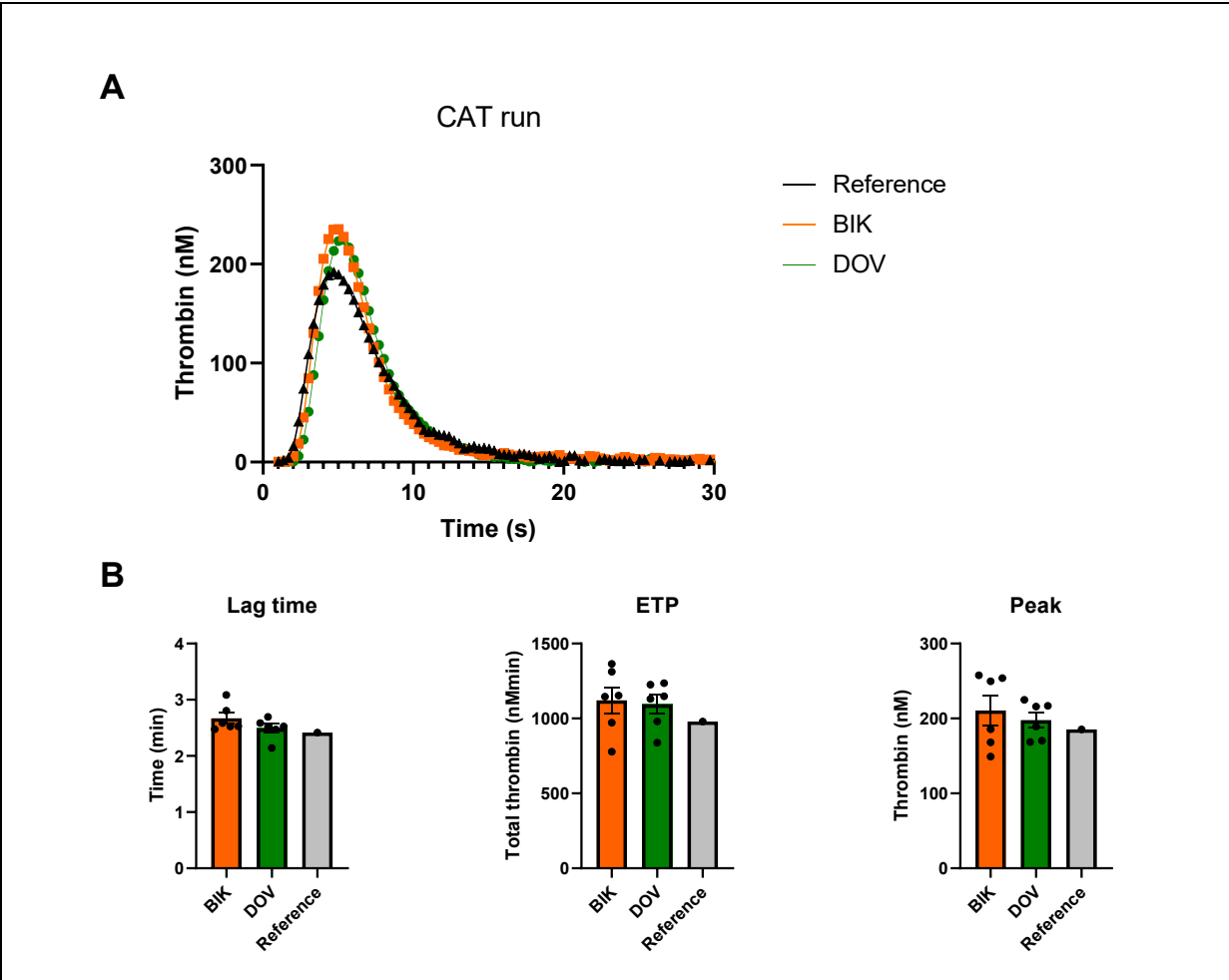


Figure 5.4. Thrombin generation *in vitro* from platelet-poor plasma (PPP) of patients on Biktarvy or Dovato. Graph A shows the thrombin generation curves from a 30min calibrated automated thrombography (CAT) run, conducted using plasma samples from N=6 patients on Biktarvy (BIK) in orange, N=6 patients on Dovato (DOV) in green and a reference plasma from pooled healthy volunteers, in grey. In panel B, graphs from left to right show the lag time, time required for thrombin generation to occur, the endogenous thrombin potential (ETP) or total amount of thrombin generated over time, and last the peak height, or maximum amount of thrombin generated at any time. Graphs in Panel B are statistically analyzed using 1-way ANOVA and groups were compared using Tukey’s multiple comparison test, where no statistical differences were obtained.

5.5 PILOT STUDY – POWER CALCULATIONS

In the patient study described in this chapter, I compare platelet activation and aggregation from patients on Biktarvy and Dovato. This was conducted in an effort to provide the medical field with preliminary data regarding the platelet profiles of PLWH on the two different INSTI-based treatments, but also to use the main findings as pilot data.

This pilot study enabled me to carry out power calculations and determine a proper sample size to be examined and analyzed in order to have a statistically appropriate number of patients in each treatment group. For this, I used the online tool OpenEpi, which can be found at the following link <https://www.openepi.com/SampleSize/SSMean.htm> and results are shown in **Figure 5.5**.

For these calculations, data from platelet aggregation shown in **Figure 5.1** were used to calculate the sample size for the two populations for an 80% power of the study, at a 95% confidence level. Specifically, I used the collagen-evoked platelet aggregation results using 3 µg/mL of agonist (Graph **C** in **Figure 5.1**) which is the concentration shown to induce platelet aggregation *in vitro* within the linear section of the activation curve (**Figure 4.1**, Chapter 4). The ratio for the sample size of each treatment group is 1. Results from **Figure 5.5** show that the sample size calculation recommends a total of 182 patients to be recruited for this study, N=91 from each treatment group.

Given the study is underpowered, there were some noticeable trends worth mentioning. Firstly, the 10µM TRAP-6 activation in platelet aggregation experiments, in **Section 5.2**, showing a 1.7-fold higher percentage aggregation in platelets obtained from PLWH on DTG/3TC compared to those on BIC/FTC/TAF. Similarly, there was a trend in higher platelet activity from patients on Dovato compared to those on Biktarvy at TRAP-6 activation, in the fold change of activated $\alpha_{IIb}\beta_3$ and CD62P MFI levels.

Sample Size For Comparing Two Means

Input Data

Confidence Interval (2-sided)	95%		
Power	80%		
Ratio of sample size (Group 2/Group 1)	1		
	Group 1	Group 2	Difference*
Mean	58.3	67.7	-9.4
Standard deviation	28.4	14.7	
Variance	806.56	216.09	
Sample size of Group 1	91		
Sample size of Group 2	91		
Total sample size	182		

*Difference between the means

Results from OpenEpi, Version 3, open source calculator--SSMean
 Print from the browser with ctrl-P
 or select text to copy and paste to other programs.

Figure 5.5. Sample size calculation for patient study. The figure shows a snapshot from the sample size calculation conducted at OpenEpi website, found at the following link: <https://www.openepi.com/SampleSize/SSMean.htm>. Based on the results shown, a total sample size of N=182 patients, 91X from each treatment group (Biktarvy or Dovato) are required to obtain a significant difference with 95% confidence interval for a power of 80%. This was based on pilot data from collagen-induced platelet aggregation, where mean and standard deviation of each group were obtained.

5.6 CHAPTER 5 – DISCUSSION

After reporting a significantly higher CVD risk in PLWH, many studies highlight the need for understanding the mechanisms behind this [78], [80]. The degree to which cART accounts for this increased risk is yet to be established, although many ARV agents have already been linked to an enhanced activity of cardiovascular components e.g. platelets, which have a critical role in atherosclerosis, thrombosis and subsequently CVD [174], [181], [188]. *In vitro* platelet studies shown in the previous chapter allow associating specific ARVs with possible effects in platelet activity, linked to CVD, but drug exposure times and volunteer profiles are not fully representative to HIV patient cohorts, hence a more real world setting was required.

In an effort to address this, I managed to recruit 16X HIV patients receiving either of the two popular INSTI-based regimens, Biktarvy or Dovato [45], [46] and conducted a series of platelet activation assays similar to Chapter 4, to compare and contrast the platelet activity from the two treatment groups. Aggregation potential of platelets from 8X patients on Biktarvy and 8X patients on Dovato (**Figure 5.1**) were comparable upon a number of agonist and agonist concentrations used to evoke platelet activation. These findings agreed with the statistically insignificant differences obtained in the average fold changes of platelet activation markers (degranulation markers and $\alpha_{IIb}\beta_3$ activation) measured in flow cytometry (**Figure 5.2**).

Cumulatively, these results suggest that HIV patients receiving Biktarvy or Dovato have no differences in platelet activity and potentially no platelet-mediated differences in CVD risk. In Chapter 4 though, platelets from HIV negative volunteers treated with clinically relevant ARV combinations and concentrations found in Biktarvy and Dovato, suggested that Dovato (specifically DTG) can largely impact platelet aggregation when compared to Biktarvy or vehicle control, therefore these two findings are contradicting. This could be due to the bigger variability of participants in the patient cohort versus the HIV negative cohort, where PRP from a single donor was exposed to all three treatments separately for each experiment. The main advantage from using samples from HIV negative cohorts for these experiments is that the same sample with the same platelet biology and activation potency (in the absence of HIV-related proteins) allows excluding other factors that could influence platelet activity

and makes the drug treatment the only different factor in every assay. On the other hand, recruiting patients for these studies represent the community more than HIV negative cohorts, therefore a combination of both makes this and future studies stronger, when exploring the overall effects of drugs on platelets.

I wanted to explore any possible pro- or anti-thrombotic tendencies in PLWH to confirm the shared neutral effect of Biktarvy and Dovato in the patient samples, in a platelet-poor environment as well. For this, in collaboration with Dr Josefin Ahnstroem (Department of Immunology & Inflammation, Imperial College London), I conducted a series of CAT assays to compare samples from Biktarvy patients against Dovato. The patient samples for this assay were chosen based on plasma volume availability and samples from 6X patients (in each group) were included, as shown in **Table 7**.

The results from CAT in **Figure 5.4** suggest that Biktarvy and Dovato share the same neutral effect in the coagulant properties of plasma samples tested in a platelet poor environment as well as in PRP reported previously. This was seen in all 3 parameters measured in CAT, 1) time to generate thrombin, 2) maximum thrombin produced over time and 3) total thrombin produced. Reference plasma used in the assays was a pooled PPP from healthy volunteers processed from PRP as previously indicated in **Section 2.11** and was just used as an indicator of the HIV negative population for the assay. No demographics are available for this hence cannot be used to compare the samples.

Reflecting on the technique, I would recommend carrying out CAT assays for HIV negative volunteers treated with the ARVs *in vitro*, as that is the cohort I observed the most differences between BIC and DTG (or Biktavry vs Dovato). This could also confirm if the differences observed in Chapter 3 between the two treatment groups are platelet specific and in platelet-poor environments the plasma alone does not have any altered procoagulant properties.

Limitations & Future work

Overall, this patient study is limited as confirmed by the power calculations demonstrated in **Figure 5.5**, where N=91 patients per treatment group is recommended for appropriate statistical calculations to measure and compare the differences in platelet activity from patients on Biktavry and Dovato. This

confirms that the experiments in this chapter are underpowered hence no conclusions can be made from these. A small N number is one of the limitations encountered in this set of experiments. Another one is the variability in the demographics used, where despite including participants of similar age groups and from various backgrounds to have an inclusive and representative study of the global population, all patients consented were male. For this, I suggest including more patients that are female at birth and trans men or women, to reflect the whole community. Another limitation in the patient demographics is the lack of lifestyle factors which could influence the results, including use of recreational or over-the-counter drugs, smoking, fitness levels and genetic predispositions to CVD.

A major challenge I faced during this study was patient recruitment at the clinics. There were numerous occasions when I travelled to the clinic aiming to recruit 3-4 patients at a time, that I selectively chose based on treatment regimens, age and viral load, and they would not show up or I would find out that they did a walk-in at a different time than planned for their blood tests, without anyone informing me. This could be attributed to the fact that HIV patients are no longer in pain and their hospital visits are more for check-up purposes instead of symptom relieving, potentially making them less sensible to adhere to specific dates and times provided. To address this issue, better communication is advised between Imperial College personnel and the NHS doctors or nurses at Kobler. For example a Kobler nurse could be appointed as a point of contact with our research group for future studies, and inform the group on any changes regarding patient appointments.

Another observation made from the overall demographics of the PLWH recruited for my study, is the noticeable use of statins in the patient cohort. Statins are known for reducing the CVD risk in PLWH [82], [84] and were recently reported to potentially reduce platelet activity and inhibit agonist evoked aggregation [191]. The effect of statins on vascular health, including reduction of vessel inflammation and improved endothelial function could potentially affect NO levels, hence platelet activity, and should be taken into consideration. Other co-medications reported in the patient cohort could also indirectly impact platelet activity including citalopram, escitalopram or sertraline that affect serotonin levels in blood, or zoledronic and alendronic acid, used for osteoporosis or to support bone tissue respectively, reducing inflammation. Thus, choosing selection criteria for participants in future studies is highly

recommended bearing in mind co-medications, especially statins, when designing platelet-based assays.

My results in this chapter cannot support or oppose my hypothesis. Considering the underpowered nature of the study I cannot draw conclusions on the effects of Biktarvy or Dovato on patients in regards to platelet activity or any potential differences in CVD risk.

Given more time, flow assays measuring platelet adhesion from PLWH on different treatments could be conducted. In addition to collagen, platelet adhesion to endothelial cells could also be investigated where cells could be obtained from patients to reflect physiological conditions as much as possible. Ideally, HCAECs from PLWH could be grown on microslides and allow passage of whole blood from PLWH at physiological or pathological arterial flow rates and record platelet adhesion and capture. This could be done for patients receiving Biktarvy, Dovato or Cabenuva, another popular INSTI-based regimen that has a different administration method.

Moreover, switching regimens could be a useful tool to investigate ARV-mediated effects on platelets in individual participants and this could allow quantifying how reversible the changes in platelets are. For example, patients on Biktarvy with recorded platelet activation/ aggregation profiles can switch regimens e.g. to Dovato and after a recommended period of receiving the new therapy quantify platelet potency again and compare it with previous data.

This chapter summarizes to a brief degree the clinical relevance of measuring platelet activation in light transmission aggregometry, and flow cytometry followed by the design of a pilot study which can be utilized for future studies in an effort to understand the heightened CVD risk in PLWH. My results may not offer enough information to draw significant conclusions for Biktarvy versus Dovato in regards to possible effects in platelet activity and thrombin generation, but provided a template for designing future projects with appropriate sample sizes and recommended points to look for when assessing patient demographics and selection criteria.

CHAPTER 6

Closing remarks

6 CLOSING REMARKS

Forty years ago, people, mainly gay men were overcrowding hospitals, dying in the streets in multiple continents and spreading this then-called “gay plague”. For many years, AIDS and death were inevitable for whoever contracted HIV leaving the gay community and in a greater extend the global population hopeless. Following effective cART, HIV patients are now referred to as people living with HIV, with an undetectable thus untransmissible viral load. Continuous research and medical innovations allow patients today to not only have a near-normal life expectancy but also the ability to choose, together with their clinicians, appropriate ART regimens from a pool of FDA approved ARVs. This choice involves considering parameters like administration method e.g. oral or injectable treatments and possible off-target effects.

Over the last few years, a number of agents like the NRTI ABC, were linked to direct and indirect effects on components of the cardiovascular system like endothelial cells and platelets. This raised a lot of concerns especially upon associating PLWH with double the risk in developing a CVD, including myocardial infarction or ischaemic stroke, compared to the global population. Development and progression of acute or chronic CVD conditions largely depend on endothelial cell and platelet activity, hence any possible off-target effects of ARVs on such elements of the cardiovascular system (exposed daily for a lifetime) require accurate monitoring.

INSTIs make up some of the most popular HIV medications used in clinics today, like BIC and DTG and are used globally as the first-line treatment in combination with 1 or 2 NRTIs, in people testing positive for, or living with HIV. Off-target effects of INSTIs on endothelial cells or platelets are not established but current literature may suggest a possible enhancement of platelet activity in platelets by allowing an intraplatelet flow of Ca^{2+} ions in an ionophore-like mechanism. This has not been explored for structurally similar INSTIs like BIC and my work can potentially contribute to establishing this DTG effect, as well as providing direct comparisons between the two drugs. For this, I performed numerous experimental approaches, laboratory techniques and models of endothelial cells and platelets from HIV negative people treated with the drugs *in vitro*, or by assessing samples *ex vivo* from PLWH on the

treatments of interest. The main findings from this study support the hypothesis suggesting a DTG enhancement of platelet aggregation, which I observed in light transmission aggregometry with or without the $\alpha_{IIb}\beta_3$ inhibitor, confirming a true aggregation effect specific to DTG but not BIC. There was only a minor enhancement of aggregation in BIC-treated platelets at a low TRAP-6 evoked activation, but the overall outcome suggests that DTG amplifies platelet aggregation more than BIC or vehicle control. These results can contribute in HIV clinics globally, when choosing a cART tailored to individual patients, considering their risk factors and possible off-target effects of drugs.

Results from my endothelial cell experiments in flow cytometry and immunostaining, as well as quantifying platelet activation markers and adhesion to collagen assays, showed an overall neutral effect of both INSTIs on the activation potency of endothelial cells and platelets, which agreed with the pilot study data obtained from PLWH. This pilot study was shown to be underpowered, however it provides templates for future studies with HIV patients that can allow establishment of the differential INSTI-mediated effects proposed, subsequently improving the CVD risk in PLWH.

Some challenges encountered during the study include inadequate sample sizes or experimental approaches that lack elements seen in physiological conditions to properly mimic the patient environment on a lab bench. Another limitation is the lack of an INSTI as a suitable positive control when assessing platelet or endothelial cell effects following *in vitro* exposure. My results provide the possibility of using DTG as a potential positive control for light transmission aggregometry in future experiments. Extending my research, performing more physiological experimental approaches, with more ARVs or ARV combinations could have a true global impact in tailoring ART regimens based on individual needs, potentially lowering their CVD risk.

To conclude, I feel privileged to be able to contribute to HIV medicine and accelerate the process in syncing PLWH with the global population, potentially offering ways to reduce their heightened CVD risk and improve their quality and quantity of lives. In the future, I hope PLWH have equal opportunities in medicine, health and life in general, reducing the inequality and stigma associated not just with the HIV community but also with the LGBTQ+ community, where I belong.

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Appendix

Appendix A

TITLE

In vitro and patient studies with platelets to explore off-target effects of integrase inhibitors

SHORTENED TITLE

Integrase inhibitors and platelets

AUTHORS & AFFILIATIONS

R. Keniyopoullos¹ (renos.keniyopoullos19@imperial.ac.uk)

Dr A. A. Khawaja¹ (akif.khawaja10@imperial.ac.uk)

Prof M. Boffito^{2,3} (marta.boffito@nhs.net)

Dr M. Emerson¹ (m.emerson@imperial.ac.uk)

¹ National Heart and Lung Institute, Imperial College, London, UK

² Department of Infectious Disease, Imperial College, London, UK

³ Chelsea and Westminster Hospital, NHS Foundation Trust, London, UK

CORRESPONDING AUTHOR

Name: Dr Michael Emerson

Email: m.emerson@imperial.ac.uk

Tel: +44 7941828744

Address: 537, 5th Floor, ICTEM building, Hammersmith Campus, W12 0NN, London, UK

KEYWORDS

Cardiovascular, platelets, integrase inhibitors, pharmacology, antiretroviral

ETHICS APPROVAL STATEMENT / VOLUNTEER CONSENT

People without HIV were bled following consent under Imperial College Research Ethics Committee approval, and people with HIV were recruited and consented at Chelsea & Westminster Hospital NHS Foundation Trust under NHS Research Ethics Committee approval.

CONFLICT OF INTEREST DECLARATION

This study was partly funded by an investigator-lead research grant by Gilead Sciences.

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ABSTRACT

Introduction: People with HIV currently face a 2-fold higher risk of developing cardiovascular disease (CVD) compared to HIV-negative individuals. Previous studies have shown various off-target effects of antiretroviral agents on the cardiovascular system, but little is known about the effects of currently used integrase strand inhibitors (INSTIs) on platelets. Platelet activation is associated with increased CVD, thrombus formation and release of pro-inflammatory mediators thus exploring platelet effects from currently prescribed antiretroviral therapies (ARTs) may contribute to the understanding of CVD etiopathogenesis in people with HIV. **Methods:** We aimed to identify potential effects of INSTIs on platelet aggregation and activation markers from individuals without HIV post *in vitro* treatment with clinically relevant drug concentrations. Bictegravir (BIC) and dolutegravir (DTG) were used individually or in therapeutic drug combinations, BIC/FTC/TAF or DTG/3TC. Additionally, we conducted a pilot study to compare platelet activity profiles from people with HIV on BIC/FTC/TAF and DTG/3TC. **Results:** Changes in *in vitro* platelet aggregation responses upon exposure to different INSTs were observed both upon individual drug application, or when using therapeutic combinations. These effects were not, however, reflected in flow cytometric evaluation of platelet degranulation. A pilot study in 8 people with HIV and 8 without HIV revealed no significant effects but established protocols for future patient studies. **Conclusion:** There is currently no consistent evidence of an effect of INSTIs on platelet activation and further study is warranted, focusing on models with more pathophysiological relevance including extensive studies in people with HIV.

INTRODUCTION

Effective antiretroviral treatment (ART) means that people with HIV are expected to live into older age and acquire a range of co-morbidities associated with aging. Cardiovascular disease (CVD) is the most prevalent co-morbidity observed despite the efficacy of ART in controlling HIV [1] and both HIV and certain ART have been shown to be independent risk factors for CVD [2, 3]. Both clinical cardiovascular events, including myocardial infarction (MI) [4], and subclinical cardiovascular damage are elevated in people with HIV [5]. Platelets interact directly with HIV and HIV-encoded proteins leading to abnormal function [6] that persists during effective therapy [7]. Furthermore, there are established links between inflammation and platelets despite control of HIV replication [8] and multiple reports of persistently heightened platelet function despite effective therapy [7, 9, 10]. There is a consensus,

therefore, that platelet function is modified in people with HIV although much remains unknown about exactly how platelet function is different and the causes of altered platelet function in people with HIV. It is also not known if or how altered platelet function in the context of HIV links mechanistically with CVD.

There is substantial evidence that certain antiretroviral drugs associate with increased MI in people with HIV [11] and that they affect platelet function [9, 10]. Many protease inhibitors activate platelets [12] and we have shown that elevated platelet responses *in vivo* result from exposure to the nucleotide reverse transcriptase inhibitor (NRTI) abacavir sulphate interrupting endothelial-dependent inhibition of platelet activation in human platelets and a mouse model of platelet thromboembolism [13]. Emerging evidence suggests that modern drugs outside of the NRTI class also affect platelet function [14]. Detailed comparisons of how antiretrovirals in common clinical use, as well as those emerging into clinical practice, affect platelet function will provide better understanding of the role of natural history *versus* drug effects in driving CVD in people with HIV and will identify potentially avoidable drivers of CVD in people with HIV. Platelet studies in the HIV field are therefore important and must be conducted carefully and with considered interpretation to avoid premature and unsubstantiated links between antiretroviral exposure and CVD being presented.

Modern ART regimens frequently include integrase strand transfer inhibitors (INSTIs) given in combination with other classes of drugs such as NRTIs. INSTI-based therapies are proven to be effective in both treatment-naïve and treatment experienced individuals with HIV [15]. The effects of INSTIs on platelet function and therefore CVD risk is incompletely understood. There are reports of possible associations between INSTI exposure and factors associated with CVD such as metabolic syndrome and weight gain [16, 17]. These studies are far from conclusive and inconsistencies in findings as well as confounding factors such as the effects of previous ART exposure have been highlighted [18]. INSTIs are an important cornerstone of modern, effective HIV control and therefore detailed understanding of their off-target effects is warranted alongside further studies to optimize their therapeutic value.

In this study, we have assessed the effects of two commonly prescribed INSTIs, dolutegravir (DTG) and bictegravir (BIC), on platelet function using well established platelet activation assays. We considered drugs given individually and in combinations used in treatment regimens. We compared *in vitro* studies with patient-derived data. Although no evidence of an effect of INSTIs on platelets in people with HIV was observed, our study provides justification for further research on this topic as well as insight into the design and interpretation of platelet and other cell-based pharmacological studies in the context of HIV and ART.

MATERIALS AND METHODS

Antiretrovirals & other reagents

BIC & tenofovir alafenamide fumarate (TAF) were supplied by Gilead Sciences (Ca, USA), DTG, emtricitibine (FTC) & lamuvidine (3TC) were purchased from Selleckchem (Texas, USA) and were used at clinical C_{max} values of 13.7 μ M, 0.2 μ M, 8.8 μ M, 8.6 μ M and 6.1 μ M respectively. ADP (Sigma-Aldrich) was used at 1-30 μ M, thrombin receptor activator for peptide 6 (TRAP-6) (Sigma-Aldrich) at 1-30 μ M and collagen (Takeda) 1-30 μ g/mL. ADP and TRAP-6 were diluted in PBS (Cytiva), collagen in isotonic glucose (Takeda). Eptifibatide (GSK) was used at 0.6 μ g/mL. CD62P:APC and CD63:PE were purchased from BD Biosciences.

Platelet-rich plasma (PRP) processing

Whole blood was centrifuged for 15min at 175g (no break) to separate platelet-rich plasma (PRP). 1mL of PRP was further centrifuged at 15,800g for 3min to obtain a platelet-poor plasma (PPP) to be used as blank for light transmission aggregometry. In experiments using samples from people without HIV, PRP was incubated with the drug or drug combinations for 25min before use.

Light transmission aggregometry

Platelets in PRP, previously treated with drugs of interest, were activated using ADP, TRAP-6 or collagen in a 96-well flat-bottom plate (VWR) where absorbance was measured for 15min, at 595nm. A measurement was made at 37°C every 20s in a plate reader (Sunrise TECAN), with a shaking step between every recording. PPP was used as blank and absorbance was used to calculate percentage aggregation. Inhibition of platelet aggregation was achieved by adding 0.6 μ g/mL of eptifibatide (an $\alpha_{IIb}\beta_3$ -inhibitor) per well.

Platelet activation markers by flow cytometry

Drug-treated PRP was diluted 10-fold in Tyrode's buffer (138mM NaCl, 2.6mM KCl, 12mM NaHCO₃, 0.2mM NaHPO₄, 5.5mM glucose). CD62P:APC (α -granule release) and CD63:PE (δ -granule release) antibodies were used at 1:60 each for every sample including PRP, Tyrode's buffer and agonist which was added last to activate platelets 30sec post initiation of measurement. ADP and TRAP-6 were used at final concentration of 10 μ M and collagen at 10 μ g/mL. Mean fluorescence intensity (MFI) was measured in Accuri C6 Plus (BD Biosciences) and fold change was calculated for every marker in every sample.

Statistical analysis

Results have passed the Shapiro-Wilko normality test and were analysed using 1- or 2-way ANOVA as indicated in figure legends. Groups were compared using Tukey's multiple comparison in GraphPad (V9). The histograms show the mean average in columns and error bars indicate the standard error of the mean (SEM). Statistical significance is shown as $*=p<0.05$, $**p<0.01$, $***=p<0.001$ and $****=p<0.0001$.

Recruitment of people with HIV

Sixteen people with HIV (mean age 53.1 $\pm$ 5.9 years) on stable and effective ART with a viral load < 50 copies/mL, were recruited and consented (Chelsea and Westminster Hospital NHS Foundation Trust) under NHS Research Ethics Committee approval. Whole blood was transported to Imperial College London for PRP processing and light transmission aggregometry.

RESULTS

Platelet aggregation *in vitro* shows comparative differences.

Platelets in PRP from N=5 people without HIV were activated using a range of agonist concentrations as shown in Figure 1, post 25min *in vitro* treatment with 13.7 μ M BIC or 8.8 μ M DTG. At 1, 3 and 10 μ M ADP, DMSO-treated platelets displayed a 6.3%, 23.3% and 49.2% platelet aggregation, BIC-treated platelets 10.4%, 27.1% and 44%, and DTG-treated platelets 23.2%, 49% and 66.6% respectively. At 3, 10 and 30 μ M TRAP-6, DMSO-treated platelets displayed a 3.23%, 30.8% and 70.4% platelet aggregation, BIC-treated platelets 7.53%, 39.3% and 65.3%, and DTG-treated platelets 9%, 62.1% and 75.8% respectively. Lastly at 1, 3 and 10 μ g/mL of collagen, DMSO-treated platelets displayed a 9.4%, 53.9% and 76% platelet aggregation, BIC-treated platelets 8.4%, 60.6% and 78.4%, and DTG-treated platelets 17.4%, 75.3% and 76.1% respectively. Comparisons between different treatment groups are displayed in Figure 1.

To confirm a true aggregation effect mediated by established platelet signalling events, rather than a non-specific effect on light transmission, 0.6 μ g/mL of eptifibatide, an $\alpha_{IIb}\beta_3$ inhibitor, was added to block integrin-mediated aggregation. 3 μ M of ADP, 10 μ M TRAP-6 and 10 μ g/mL of collagen were added in INSTI-treated PRP from N=5 volunteers without HIV, with and without eptifibatide, to assess any differences in the aggregation profiles. Figure 2 shows that the differences in percentage aggregation between the drug groups (as reported in Figure 1) were reproducible and in the presence of eptifibatide the differences were completely abolished confirming an $\alpha_{IIb}\beta_3$ -mediated effect in every agonist-mediated activation examined.

In vitro aggregation effects are sustained when modelling therapeutic drug combinations.

To examine a more clinically relevant effect of INSTIs on platelet aggregation, PRP from volunteers without HIV was treated *in vitro* with common drug combinations DTG and BIC are usually prescribed in, DTG/3TC and BIC/FTC/TAF. Figure 3 shows the ADP, TRAP-6 and collagen-induced platelet aggregation post combination ART treatment. The aggregation profiles of platelets treated with DTG/3TC, BIC/FTC/TAF or DMSO in ADP-evoked aggregation at 3 μ M of the agonist exhibited 38.6%, 13.8% and 11% respectively and at 10 μ M of the agonist, 61%, 34% and 24.9% respectively. At 3 μ M TRAP-6-evoked platelet activation, the DTG/3TC group demonstrated an 8.3% aggregation, the BIC/FTC/TAF group a 5% aggregation and the DMSO group 2.13%. Lastly, at 1 μ g/mL collagen-induced activation, platelets treated with DTG/3TC, BIC/FTC/TAF or vehicle displayed a 34.3%, 10.3% and 8% aggregation respectively. Comparisons between therapeutic drug combinations are displayed in Figure 3.

Enhanced platelet aggregation was not associated with changes in established markers of platelet activation.

Degranulation of platelets is an established component of the platelet activation process leading ultimately to platelet aggregation. Platelet degranulation was examined post INSTI-treatment. CD62P (or P-selectin) and CD63 are alpha-granule and dense-granule markers respectively and were measured by flow cytometry during real-time platelet activation with 10 μ M ADP or TRAP-6, or 10 μ g/mL of collagen. Figure 4 shows no significant differences between treatment groups following TRAP-6 or collagen-evoked activation. Fold change of CD63 was not significantly different between INSTI and control groups in all three agonist-induced platelet activation. In only one instance, the DTG group activated with ADP, exhibited an apparently small but statistically significantly higher fold change in CD62P compared to the DMSO group (p value = 0.04). This observation was not consistent between agonists despite a broader effect in light transmission data. Thus, aggregation responses were not associated with changes in conventional markers of platelet activation weakening the evidence of a link between drug exposure and platelet activation. We then progressed to studies in people with HIV to explore if the *in vitro* effects on platelets were replicated in patients on effective INSTI-based therapies.

Pilot study from people living with HIV on BIC/FTC/TAF or DTG/3TC

Table 1 shows the demographics of the 16 patients recruited in the study upon consent, eight from each treatment group. The table demonstrates participant profiles including age, ethnicity, sex at birth and co-medications for every participant individually. All participants had a viral load of less than 50 copies. **Figure 5** illustrates platelet aggregation profiles from people living with HIV on BIC/FTC/TAF or DTG/3TC. We made comparisons between treatment groups and no statistical difference was observed at any data point. Thus, although an effect was seen when drugs were applied *in vitro*, this was not replicated in an ex vivo setting using the same method of laboratory analysis.

CONCLUSION

Given the higher risk of developing CVD [1, 2, 4], greater understanding of the underlying causes and treatment of cardiovascular co-morbidity in people with HIV is required. There are numerous clinical studies and observations that CVD risk associates with ART use [1, 2] but, in contrast, other studies failed to confirm earlier reported associations [3]. Understanding the effects of individual antiretroviral components of ART combinations is important because it potentially allows personalization of prescribing practice to reduce the detrimental impact of ART on the cardiovascular system. Platelets and endothelial cells are key drivers of thrombotic CVD and vascular disease, and their function can be characterized in great detail. We have previously explored the effects of NRTIs on platelets and cultured endothelial cells in studies involving the application of individual drugs at clinically relevant concentrations [4, 5]. With a big shift towards INSTIs over the last decade [15], and relatively little known about their off-target effects on cellular drivers of CVD such as platelets, we focused this study on popular INSTI-based regimens BIC/FTC/TAF and DTG/3TC, considering both the effects of individual drugs and therapeutic combinations. We focused on well-established models of platelet activation and therapeutically relevant drug concentrations. Within our study, we used both *in vitro* and *ex vivo* studies and we have identified key strengths and limitations of both studies that should be considered when interpreting data comparing off-target-effects of clinically approved therapeutic drugs.

Initial studies focused on light-transmission aggregometry assays which are considered the “gold-standard” model of platelet activation [6], correlating well with thrombotic activity in a range of settings from anti-thrombotic drug development and platelet signalling [7] to validating pollution-associated CVD risk [8]. We conducted studies in blood from HIV-negative volunteers and exposed their ART-naïve PRP to clinical maximum concentrations (C_{max}) of DTG and BIC, alone or in relevant clinical combinations *in vitro*. We used established methods of acute drug exposure that increase the likelihood of any observed effect being pharmacological in nature and reversible upon switching to an alternative therapeutic [4].

Our results show a significantly higher aggregation profile in DTG-treated platelets compared to BIC or DMSO. This profile was consistent across a range of platelet agonists and could be seen consistently in experimental settings involving both individual application of drugs and therapeutic combinations. Given that platelet aggregometry simply measures light transmission through platelet-rich plasma, we wanted to confirm that observed differences reflected changes in active aggregation rather than either an artifactual effect on light transmission or a passive agglomeration effect leading to changes in light transmission independent of canonical platelet activation pathways [9]. Platelet activation is mediated via $\alpha_{IIb}\beta_3$, a transmembrane receptor for fibrinogen that mediates platelet-to-platelet aggregation. We demonstrated that DTG-enhanced light transmission was abolished by the $\alpha_{IIb}\beta_3$

antagonist, eptifibatide, indicating a $\alpha_{IIb}\beta_3$ -mediated effect and ruling out passive agglomeration processes. Although most of the significant changes in platelet aggregation occurred with DTG, we observed that BIC-treated platelets showed an augmented aggregation response relative to DMSO at 3 μ M TRAP-6. Thus, both DTG and BIC can affect platelet aggregation when applied *in vitro*. It is not clear, however, if these changes are limited to *in vitro* aggregometry or have broader significance. We therefore progressed to additional well established flow cytometric platelet assays.

Platelet aggregation is driven by underlying signalling events involving receptor activation and processes such as degranulation which drive thrombotic events and contribute to CVD. We therefore explored platelet degranulation *via* flow-cytometric analysis of surface expression of CD62P and CD63 as indices of α -granule and δ -granule release respectively. Our data demonstrate an apparently small but statistically significant increase in CD62P release in DTG-treated platelets following stimulation with ADP. This effect was isolated to one marker and was not seen with other agonists. Thus, although there were widespread effects on platelet aggregation measured as light transmission, these effects were not consistently associated with established markers of platelet activation. It is important to highlight that this study explores off-target effects of drugs that are designed to target viral activity. Their effects on mammalian cells are likely to be non-specific and may not be related to established mechanistic pathways. Thus, although the platelet assays that we use are well established when looking at platelet-targeting drugs, any effect on an individual assay, even one that is considered “gold-standard” should be interpreted with caution and we recommend that mechanistic studies incorporate a combination of functional assays to confirm that any effect is observed consistently. The absence of an effect on established markers of activation in our study means that it is not possible to link any of the drugs under observation with a consistent effect on platelet and therefore cardiovascular function.

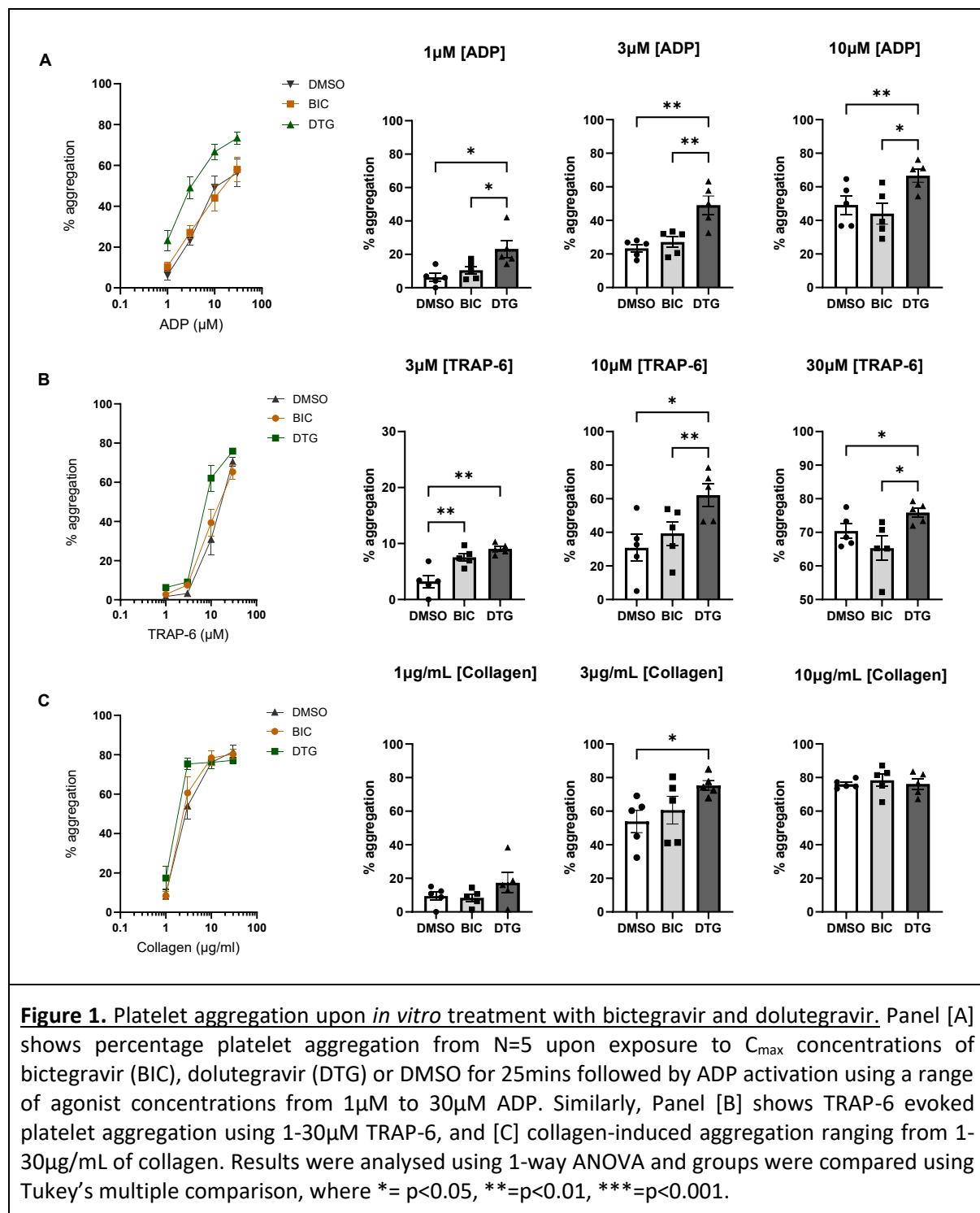
Our study involved *in vitro* application of individual drugs and drug combinations in an experimental design that allowed drugs to be compared to vehicle controls and with each other in platelet samples from an individual donor. For the aggregometry assays, each comparison was made on an individual 96-well plate. This single-variable approach does have mechanistic advantages, but it is distant from the patient setting. A key limitation of our study is that *in vitro* findings, even if they had been found consistently across the assays employed, may not be reflected in people. Acute drug exposure is mechanistically useful but is very different from long-term daily drug intake. Furthermore, studying individual cell types in isolation is mechanistically useful but risk over-interpretation of effects that have not been confirmed in a real-world, patient setting. It is therefore unclear if the observed changes in platelet activation *in vitro* translate into the development and progression of CVD. Ultimately platelet studies should include analysis involving people and this is time-consuming and expensive. Nonetheless, we conducted a pilot study to compare platelet profiles in PLWH on BIC/FTC/TAF or DTG/3TC. Comparator groups were roughly matched for basic demographics such as age and sex

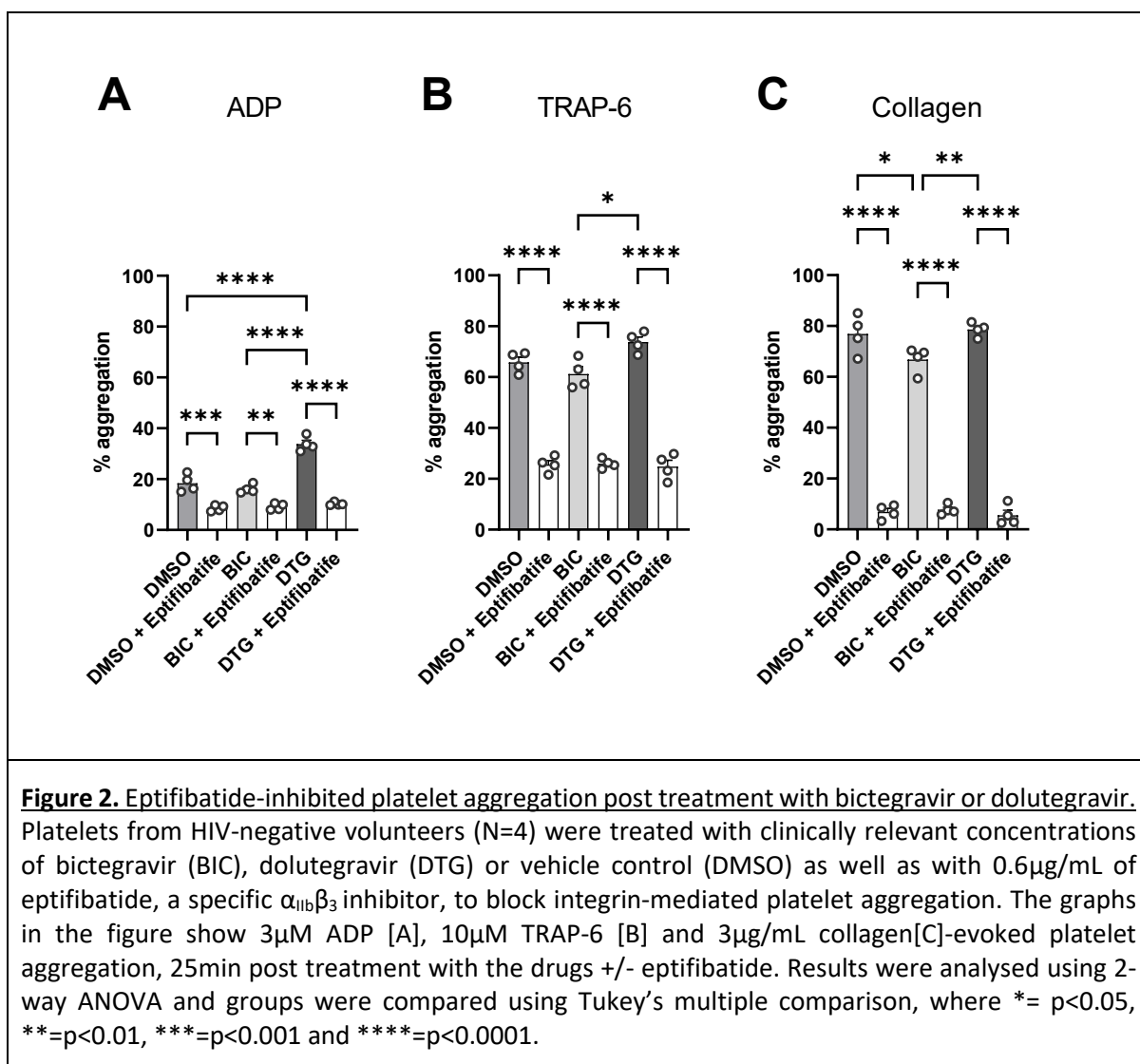
although there were differences in co-morbidity that we have summarized, and which are inherent in real-world settings.

We focused on light transmission aggregometry as this was the most affected parameter in our *in vitro* studies and we did not see any significant changes between our patient cohorts. Post-study power calculations were conducted to calculate appropriate sample size for the clinical study using the online tool OpenEpi (<https://www.openepi.com/SampleSize/SSMean.htm>). For an 80% power and a 95% confidence level, given the ratio of the two treatment populations is 1, the recommended sample size is 182, 91 participants in each treatment group. Data from the 3µg/mL collagen-induced platelet aggregation (Figure 5C) was used for this calculation. Thus, our study was under-powered and it should only be seen as a pilot.

Future platelet studies involving people with HIV should therefore carefully consider the sample sizes required to deliver powered analysis. An alternative approach could be an interventional study in which participants switch their drug regimen. This would permit intra-individual analysis and a more realistic cohort size. Further mechanistic studies could also involve more physiologically relevant assays incorporating shear flow at physiological and pathological rates as well as assays incorporating multiple cell types and animal studies to explore platelet function and thrombus formation at a whole organism level.

In summary, BIC and DTG are commonly prescribed INSTIs with a shared mechanism of action to block retroviral integration. The possibility of differing off-target effects led us, and others [12], to hypothesize that they may have different effects on biological structures such as the cardiovascular system. In this study, we have shown that it is relatively easy to detect differences between different therapeutic reagents using individual assays. However, it is important to work comprehensively across a range of mechanistic assays and to include studies in people before linking observations with clinical significance. In this study, an effect on platelet aggregation using a well-established assay was not reflected in other *in vitro* assays nor in pilot patient studies. Our study provides useful insight into the design of studies to explore the off-target effects of drugs used in the treatment of HIV as well as providing useful reflections on the interpretation of data comparing different antiretrovirals. Further studies involving modelling of human pathophysiology involving people with HIV are recommended.





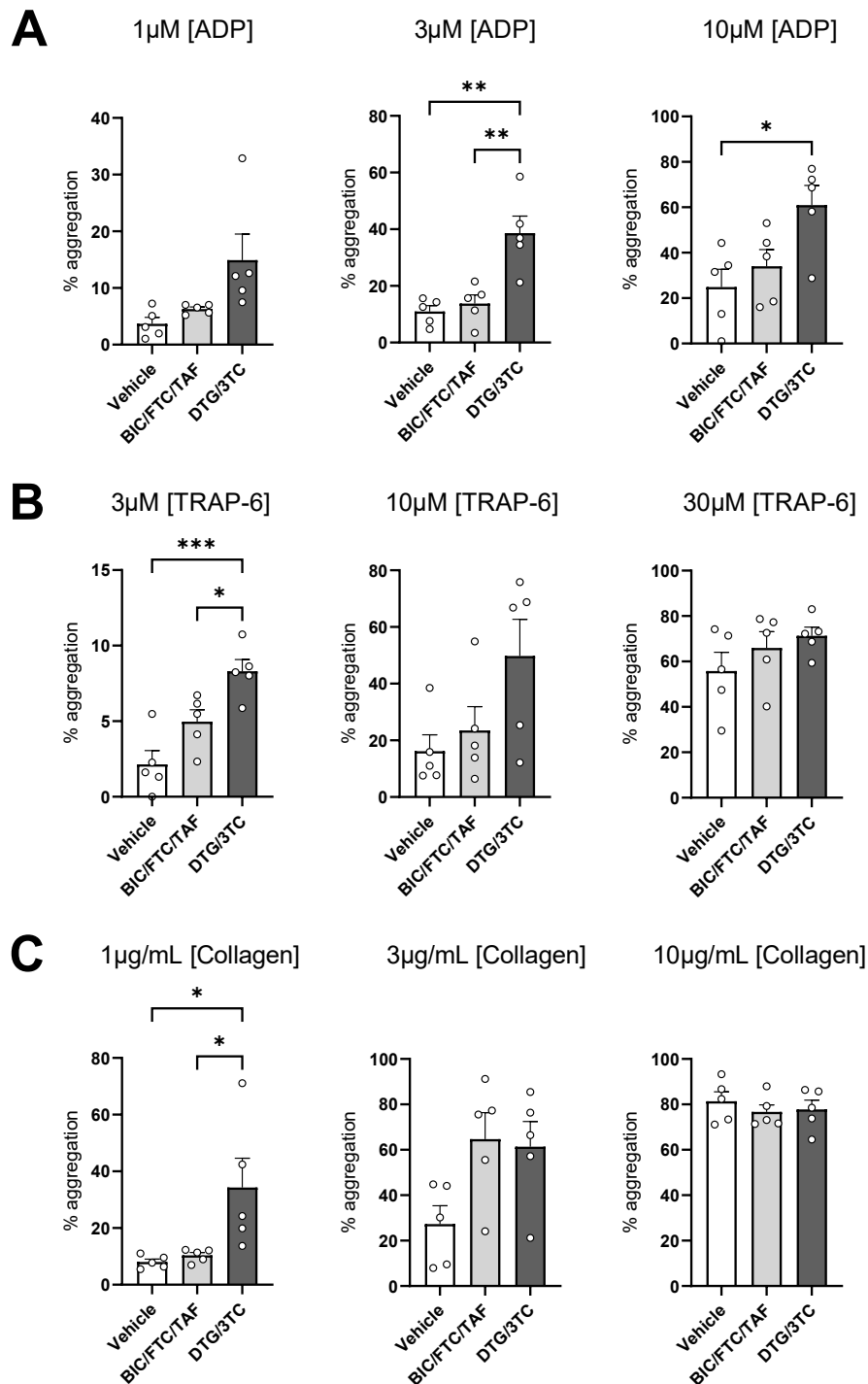
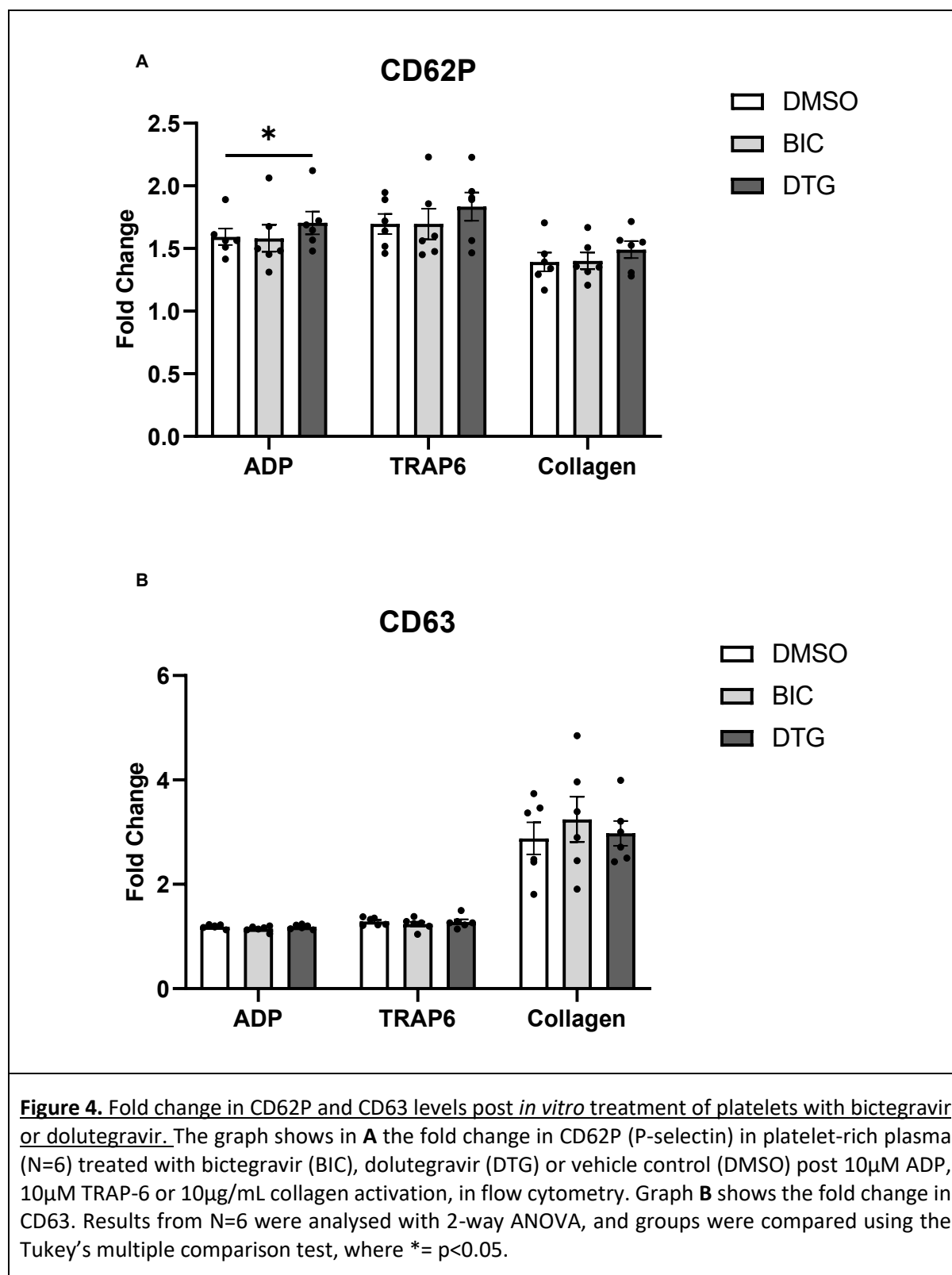


Figure 3. Aggregation profiles of BIC/FTC/TAF- or DTG/3TC-treated platelets. Top row [A] shows aggregation profiles of ADP-evoked platelet activation at increasing agonist concentrations from left to right, post *in vitro* treatment of platelets from healthy volunteers (N=5) with clinically relevant concentrations of BIC/FTC/TAF, DTG/3TC or vehicle (DMSO). Row [B] indicates TRAP-6-induced platelet aggregation and row [C] the collagen-induced, at increasing concentrations from left to right. Results were statistically analysed using 1-way ANOVA and groups were compared in Tukey's multiple comparison, where * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$.



	BIC/FTC/TAF	DTG/3TC
N (sample size)	8	8
Age (mean years $\pm$ SD)	53.1 ($\pm$ 5.9)	53.1 ($\pm$ 5.8)
Ethnicity	Caucasian (6) Black (African) (1) Not stated (1)	Caucasian (5) Multiple/ Mixed Ethnicity (1) Not stated (2)
Sex at birth (M/F)	8/0	8/0
Viral load (copies/mL)	< 50 copies (8)	< 50 copies (8)
Co-medications	P1. atorvastatin, perindopril, amlodipine P2. citalopram, acyclovir P3. none P4. atorvastatin P5. metformin, amlodipine, ramipril, glitazone P6. none P7. atorvastatin P8. finasteride, escitalopram	P9. propecia, minoxidil P10. atorvastatin, colecalciferol, doxycycline lansoprazole, zoledronic acid P11. none P12. sertraline P13. sertraline P14. none P15. atorvastatin, alendronic acid P16. vitamin D

Table 1. Patient demographics from BIC/FTC/TAF and DTG/3TC treatment groups. The table displays the demographics of the sixteen people with HIV recruited in the pilot study, including age, sex at birth, ethnicity and co-medications. P in co-medications stands for participant.

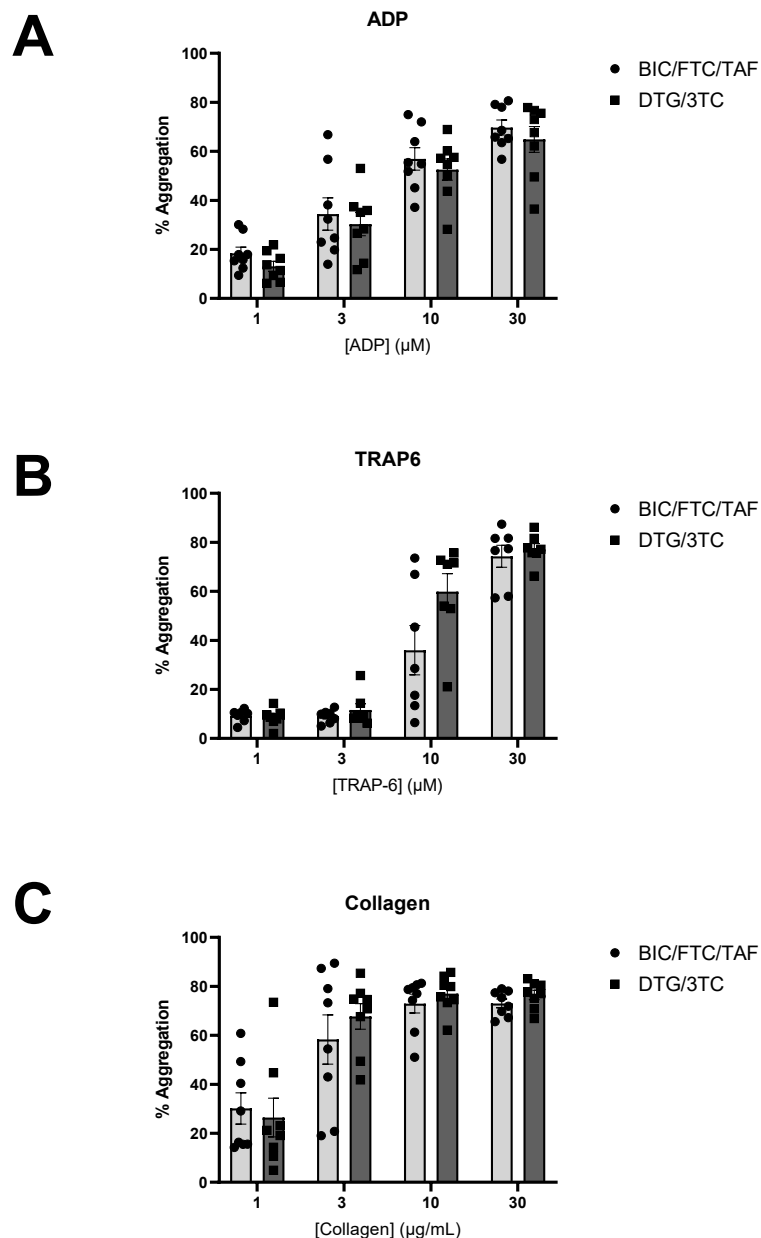


Figure 5. Aggregation profiles of platelets from people living with HIV on BIC/FTC/TAF or DTG/3TC. The figure shows in **[A]** the ADP-induced platelet aggregation in platelet-rich plasma (PRP) from people with HIV, N=8 on BIC/FTC/TAF and N=8 ON DTG/3TC. Row **[B]** demonstrates TRAP-6-induced aggregation in PRP from people with HIV on the two treatment groups, and row **[C]** the collagen-induced one. Results are analysed using 2-way ANOVA and groups were compared in Tukey's multiple comparison. No statistically significant differences were observed.

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Appendix B

Macros developed to automate the analysis of confocal images to obtain MFI values:

```
run("Set Measurements...", "area mean min integrated display redirect=None decimal=3");
fn=getTitle();
roiManager("reset");
//run("Clear Results");
run("Select None");
getDimensions(ImageWidth, ImageHeight, ImageChannels, ImageSlices, ImageFrames);
getVoxelSize(px, py, pz, unit);
//cell count
Roi.setDefaultGroup(1);
selectWindow(fn);
run("Duplicate...", "title=["+fn+" Nuclei count] duplicate channels=1");
run("Gamma...", "value=0.50");
run("Subtract Background...", "rolling=50");
run("Gaussian Blur...", "sigma=2");
run("Threshold...");
resetThreshold();
setAutoThreshold("Default dark");
setOption("BlackBackground", false);
run("Convert to Mask");
run("Watershed");
run("Analyze Particles...", "size=30.00-Infinity exclude summarize add");
close();
RoiManager.selectGroup(1);
roiManager("combine");
roiManager("add");
roiManager("delete");
//create cell mask
Roi.setDefaultGroup(2);
selectWindow(fn);
run("Z Project...", "start=1 projection=[Max Intensity]");
run("Gamma...", "value=0.50");
```

```

run("Despeckle");
//run("Gaussian Blur...", "sigma=2");
setThreshold(9544, 65535, "raw");// threshold value for cells
waitForUser("Check threshold");
run("Create Selection");
roiManager("Add");
close();
roiManager("select", 0);
roiManager("rename", "Nuclei");
roiManager("select", 1);
roiManager("rename", "Cells");
//analyse channel 2
selectWindow(fn);
run("Duplicate...", "title=["+fn+" CD31] duplicate channels=2");
roiManager("Select", 1);
run("Measure");
close();
//analyse channel 3
selectWindow(fn);
run("Duplicate...", "title=["+fn+" ICAM1] duplicate channels=3");
roiManager("Select", 1);
run("Measure");
close();
//analyse channel 4
selectWindow(fn);
run("Duplicate...", "title=["+fn+" VE-CAD] duplicate channels=4");
roiManager("Select", 1);
run("Measure");
close();
Roi.setDefaultGroup(0);
selectWindow(fn);
roiManager("Show All without labels");

```