Investigating the clinical and mechanistic impact of combination antiretroviral therapy upon platelet activation

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Statement of Originality

The work presented in this thesis is my own unless otherwise stated. Any work from other authors is appropriately indicated in the text and references list.

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Abstract

Combination antiretroviral therapy has substantially increased the life expectancy of people living with HIV. There is now a focus on the management of non-infectious comorbidities in this population, with cardiovascular disease being one of the leading causes of mortality.

Use of the nucleoside reverse transcriptase inhibitor, abacavir, has been shown to increase the relative risk of myocardial infarction by 1.9 times versus individuals not on abacavir, though there has been no confirmation of causality, nor a credible mechanism identified.

Abacavir-induced increased platelet reactivity has been suggested as an underlying mechanism, although this has not been confirmed experimentally.

In this thesis, a randomised controlled trial was conducted to investigate the effect on platelets of switching from an abacavir containing antiretroviral therapy to an abacavir free regimen. This was combined with *in vitro* studies and a mouse model of thromboembolism examining the effect of abacavir and lamivudine on platelet activation in the absence of HIV infection, as well as a mechanistic investigation into the effect on nitric oxide signalling and reactive oxygen species (ROS) production.

A switch away from abacavir resulted in reduced platelet reactivity in response to collagen and increases in collagen receptor GPVI, both surface and plasma soluble forms.

Acute administration of abacavir and lamivudine *in vitro*, had no effect on platelet aggregation but abacavir did significantly increase expression of the activation marker CD63 following collagen addition. Collagen-evoked platelet responses were augmented in the *in vivo* model following abacavir administration but not with lamivudine individually or in combination. No effects of antiretrovirals on platelet nitric oxide signalling or ROS production was seen.

These results suggest that abacavir enhances platelet activation *via* the collagen pathway, independently of HIV-associated factors and this may be mediated by increased dense granule secretion. However, further investigation is needed to understand the underlying mechanism.

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Abbreviations

3TC	Lamivudine
AA	Arachidonic acid
ABC	Abacavir
ACD	Acid Citrate Dextrose
ADAM	A disintegrin and metalloproteinase
ADP	Adenosine diphosphate
AIDS	Acquired immune deficiency syndrome
ALT	Alanine transaminase
ANOVA	Analysis of variance
APC	Allophycocyanine
ART	Antiretrovial therapy
ARV	Antiretroviral
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
ATV	Atazanavir
AUC	Area under the curve
AZT	Zidovudine
BHIVA	British HIV Association
BIC	Bictegravir
BL	Baseline
c	Cobicistat
CAB	Cabotegravir
CBV	Carbovir
CBV-TP	Carbovir triphosphate
CCR	C-C motif chemokine receptor
CD	Cluster of differentiation
CDC	Center for Disease Control and Prevention
cGMP	Cyclic guanosine monophosphate
Cgk	cGMP-dependent protein kinases

CI	Confidence interval
COX	Cyclooxygenase
CRF	Circulating recombinant form
CVD	Cardiovascular diseasae
CXCR	C-X-C motif chemokine receptor
CYP	Cytochromes P450
d4T	Stavudine
D:A:D	Data Collection on Adverse Events of Anti-HIV Drugs
DAG	Diacylglycerol
ddI	Didanosine
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
DOR	Doravirine
DRV	Darunavir
DTG	Dolutegravir
DTS	Dense tubular system
DUOX	Dual Oxidase
EACS	European AIDS Clinical Society
ECG	Electrocardiogram
EDTA	Ethylenediaminetetraacetic acid
EFV	Efavirenz
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ETV	Etravirine
EVG	Elvitegravir
FDC	Fixed dose combination
FITC	Fluorescein isothiocyanate
FOS	Fosamprenavir
FTC	Emtricitabine
GFR	Glomerular filtration rate
GP	Glycoprotein

GTP	Guanosine triphosphate
HBV	Hepatitis B virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigens
HR	Heptad repeat
hsCRP	High Sensitivity C -Reactive Protein
HTLV	Human T-cell lymphotrophic virus
ICAM	Intercellular adhesion molecule
IP3	Inositol triphosphate
INSTI	Integrase strand transfer inhibitor
IRAG	IP3R-associated cGMP kinase substrate
ITAM	Immunoreceptor tyrosine-based activation motif
LAMP	Lysosomal-associated membrane protein
LMIC	Low- or Middle-Income Country
LPV	Lopinavir
LTA	Light transmission aggregometry
LTR	Long terminal repeat
MFI	Mean fluorescence intenstiy
MI	Myocardial infarction
NADPH	Nicotinamide adenine dinucleotide phosphate
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NO	Nitric oxide
NOS	Nitric oxide synthase
NOX	NADPH Oxidase
NRTI	Nucleoside reverse transcriptase inhibitor
OCS	Open canicular system
PAC	Procaspase activating compound
PAR	Protease-activated receptor
PDGF	Platelet-derived growth factor
PGE	Prostaglandin E2
PE	Phycoerythrin

PI	Protease inhibitor
PI3K	Phosphoinositide 3-kinase
PIC	Pre-integration complex
PIP2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PLWH	People living with HIV
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
RAL	Raltegravir
ROS	Reactive oxygen species
RPV	Rilpivirine
RTV or r	Ritonavir
SEM	Standard error of the mean
sGC	Soluble guanylate cyclase
SQV	Saquinavir
TAF	Tenofovir alafenamide
TDF	Tenofovir disoproxil fumarate
TRAP	Thrombin receptor activator peptide
TXA2	Thromboxane A2
URF	Unique recombinant form
VAMP	Vesicle-associated membrane protein
VASP	Vasodilator-stimulated phosphoprotein
VCAM	Vascular cell adhesion molecule
WHO	World Health Organization
WT	Wildtype
XL- CRP	Cross linked collagen related protein

Chapter 1: Introduction

1.1 HIV epidemiology and virology

The human immunodeficiency virus (HIV) is a lentivirus, which, left untreated, leads to AIDS (acquired immunodeficiency syndrome).

The first cases of AIDS were reported in 1981 in young men who have sex with men, and intravenous drug users in the US, who presented with *Pneumocystis carinii* pneumonia (1, 2).

In 1983 Barré-Sinoussi *et al* identified a T-lymphotropic retrovirus, distinct from human T-cell leukaemia viruses (HTLV), as a likely cause of AIDS (3). Following the identification of the virus (later known as HIV) diagnostic tests were developed- an enzyme-linked immunosorbent assay (ELISA) to detect IgG antibodies to the virus , a radioimmunoprecipitation assay to the core protein, P24, and western blot to detect antibodies to viral antigens. Outcomes of patients were, however, poor due to limited treatment options. At this time the median time from seroconversion to death would have been an estimated 10 years (4, 5)

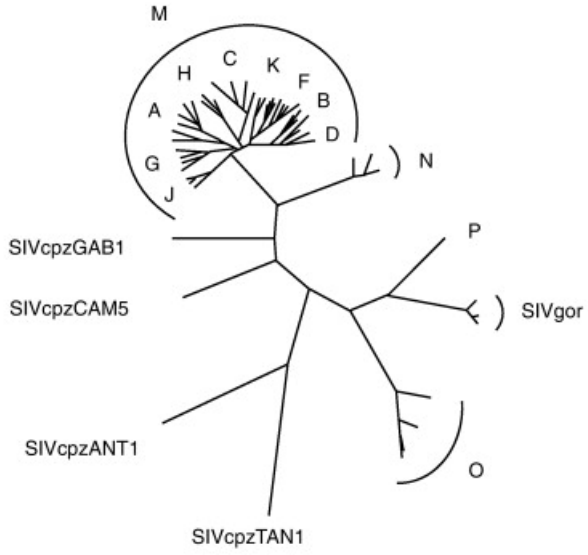
HIV is a leading cause of death globally and is the number one cause of death in southern Africa. At the end of 2020 an estimated 37.7 million (30.2- 45.1 million) people were living with HIV. Of these, 25.4 million are in the WHO African Region (6). Life expectancy has, however, improved dramatically thanks to a greater understanding of the virus and advances in antiretroviral therapy (ART), though there is still no cure.

There are over 23 million people currently on ART and, due to the effectiveness and accessibility of the treatment, there is now an ageing population of people living with HIV (7). In the United States in 2018, over 50% of those living with HIV were over 50 years old (8, 9).

In the UK in 2019 98% of those in HIV care were receiving ART and almost 45% were over 50 (10). This trend is seen globally where, in 2016 80% of all PWLH over 50 were from low- and middle-income countries (LMICs) (11, 12).

There are 2 major subtypes of HIV: HIV-1 and HIV-2. HIV-2 is likely to have emerged from sooty mangabey monkeys and is predominantly seen in west Africa, although there are increasing infections being seen around the world, particularly India and the US (13)

Figure 1.a Phylogenetic tree of HIV-1 and simian immunodeficiency viruses (SIV)

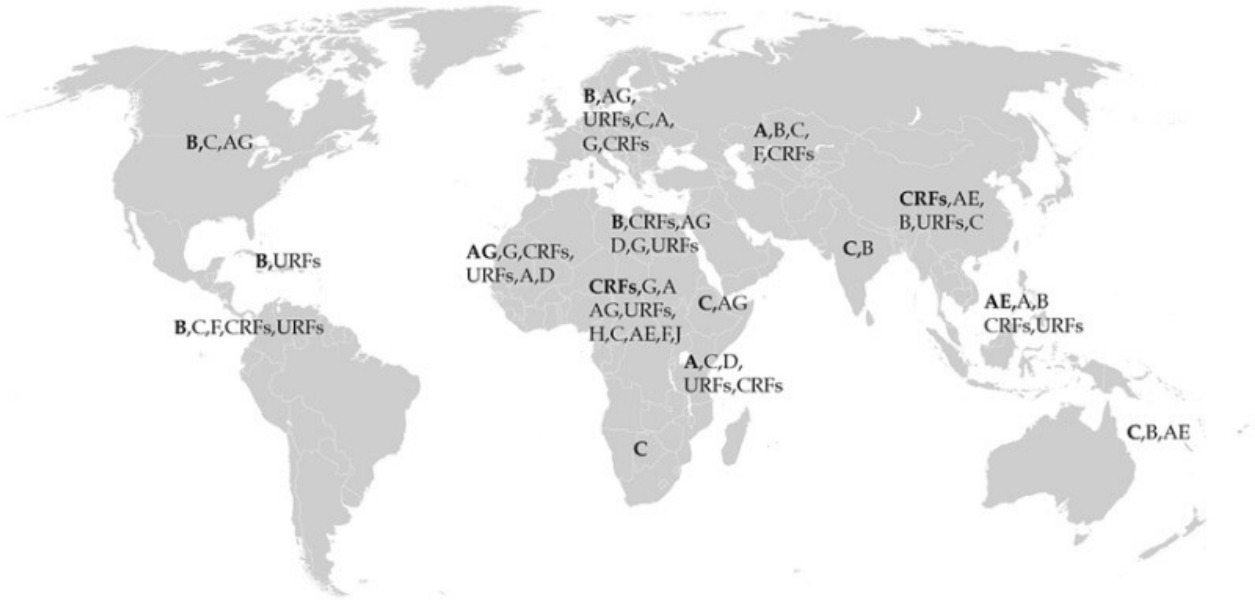


A rootless phylogenetic tree of HIV-1 and simian immunodeficiency virus based on polymerase sequences of SIV and HIV-1 reference sequences. Shown here are SIVcpzGAB1, SIVcpzCAM5, SIVcpzANT1, SIVgor and subtypes A-K from HIV-1 group M and HIV-1 groups N, O and P are also shown. Adapted from: Hemelaar, *Trends in Molecular Medicine* (2012) (14)

This thesis will describe HIV-1. HIV-1 can be subdivided into 4 groups: M (major), O (outlier), N (non-M, non- O) and group. M is the most dominant group and can be divided into 9 clades or subtypes (A-D, F-H, J,K) (**Figure 1.a**). Subtypes are also able to combine, leading to a circulating recombinant form (CRF), of which there are at least 132 and several unique recombinant forms (URF) (15) (16)

Subtype C is the most common subtype, accounting for ~48% of infections and it predominates in sub–Saharan Africa. Subtype B makes up approximately 11% of infections and is the dominant subtype in western Europe and the Americas (17) (**Figure 1.b**).

Figure 1.b Global distribution of HIV-1 subtypes



Global distribution of HIV-1 subtypes. The predominant subtype is in bold followed by other subtypes that have been reported. CRFs: circulating recombinant forms. URF: unique recombinant forms. Based on circulating subtype data from 2004-2007. Image taken from Sarabia I, Bosque A. *Viruses*. 2019 (17)

1.2 HIV infection

Transmission of HIV is via infected body fluids and is predominantly transmitted through sexual contact (18, 19). HIV can also be acquired perinatally and through injection drug use, though these routes of transmission are less common. In the UK in 2021 92% of individuals receiving HIV care acquired HIV via sexual transmission, 1.7% through vertical transmission and 1.5% through injection drug use (20).

HIV infects $CD4^+$ cells and, in the initial stages, after crossing the mucosal barrier, HIV targets partially activated $CD4^+$ T cells, then infecting activated $CD4^+$ T cells where it rapidly replicates, before moving to the gut associated lymphoid tissue (21, 22). At the gut associated lymphoid tissue there is a dramatic depletion of $CD4^+$ T cells and the increased viral replication results in immune activation (23, 24).

Between 2-4 weeks post transmission, the virus replicates exponentially, and plasma viral load peaks to around 10^6 - 10^7 HIV-1 RNA copies/mL in what is known as the acute phase (21, 25). The infected individual will experience flu like symptoms and generalized lymphadenopathy, and HIV antibody levels also become detectable at this stage (22, 26).

The adaptive immune response is then initiated, effected primarily by $CD8^+$ cytotoxic T cells which results in a substantial reduction in viral load of approximately 2 to 4 $\log_{10}$ copies/mL where it plateaus to a level known as the set point and enters the chronic phase of infection (25, 27). Untreated, the individual can live for up to approximately 6-10 years but during this time there is notable immune dysregulation, increased general inflammation, the steady depletion of $CD4^+$ T cells and rise of viral load (21).

The $CD4^+$ T cell count drops by approximately 50 to 75 cells per year with HIV infection (25) and when the count drops to under 200 cells/µL (normal range: 500 cells – 1200 cells per/µL), described

by the Centers for Disease, Control and Prevention (CDC) as AIDS, the risk of opportunistic infection increases substantially and this and ultimately leads to death (21, 28).

1.3 HIV structure and life cycle

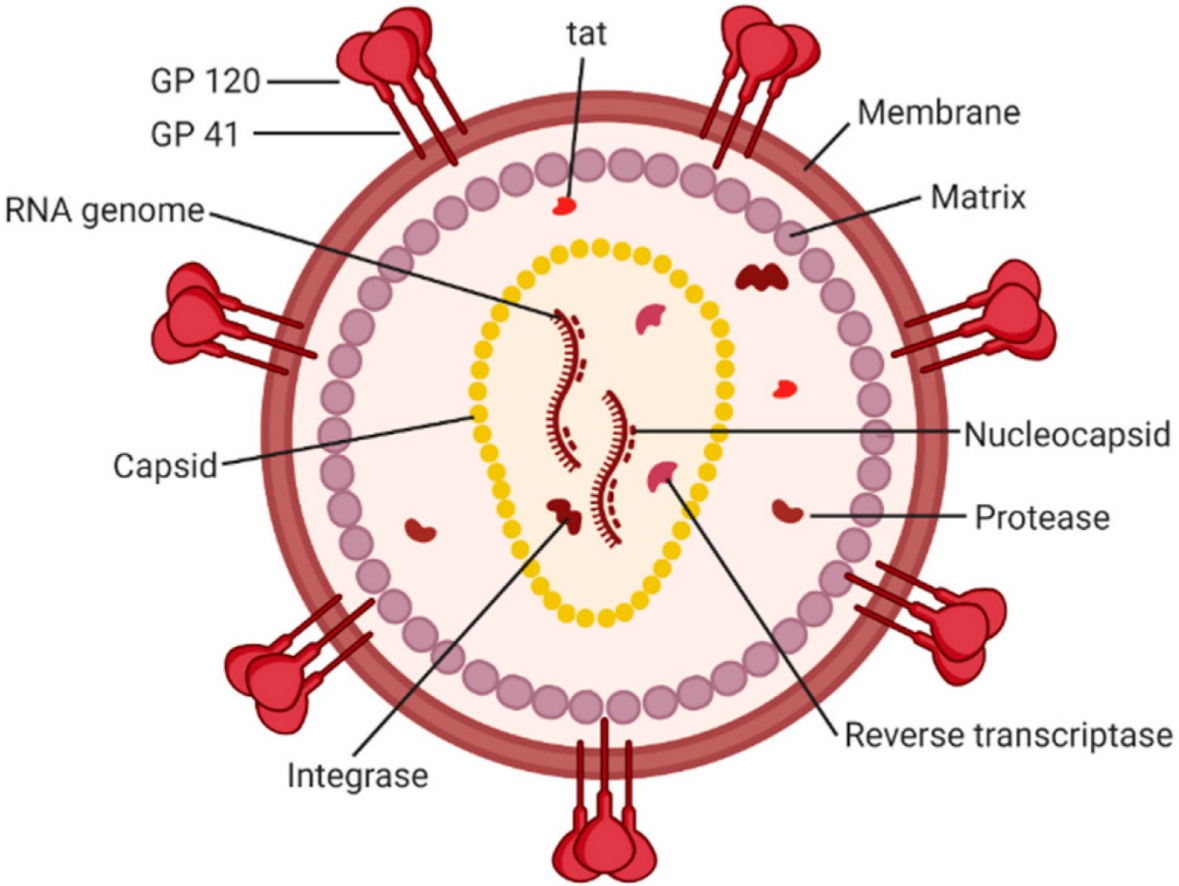
1.3.1 HIV structure

The mature HIV virion (**Figure 1.c**) has a diameter of 100 nm. The outer lipid membrane contains trimers of Env proteins and, anchored to the membrane are trimers of gp120 surface proteins, non-covalently bound to a trimer of gp41 transmembrane proteins (29).

The outer capsid membrane is comprised of the matrix protein and the cone- shaped capsid composed of P24 protein. The capsid core has 2 main functions: to maintain the viral genome in a closed environment for an efficient reverse transcription process and to protect the viral genome from host defences (30).

Within the capsid there are 2 copies of unspliced HIV RNA genome as well as nucleocapsid protein, in addition to the enzymes protease, reverse transcriptase and integrase. There are also 6 accessory proteins: Nef, Vif Vpr, Rev, Tat and Vpu, which are essential for viral survival through the modification of the local environment (31).

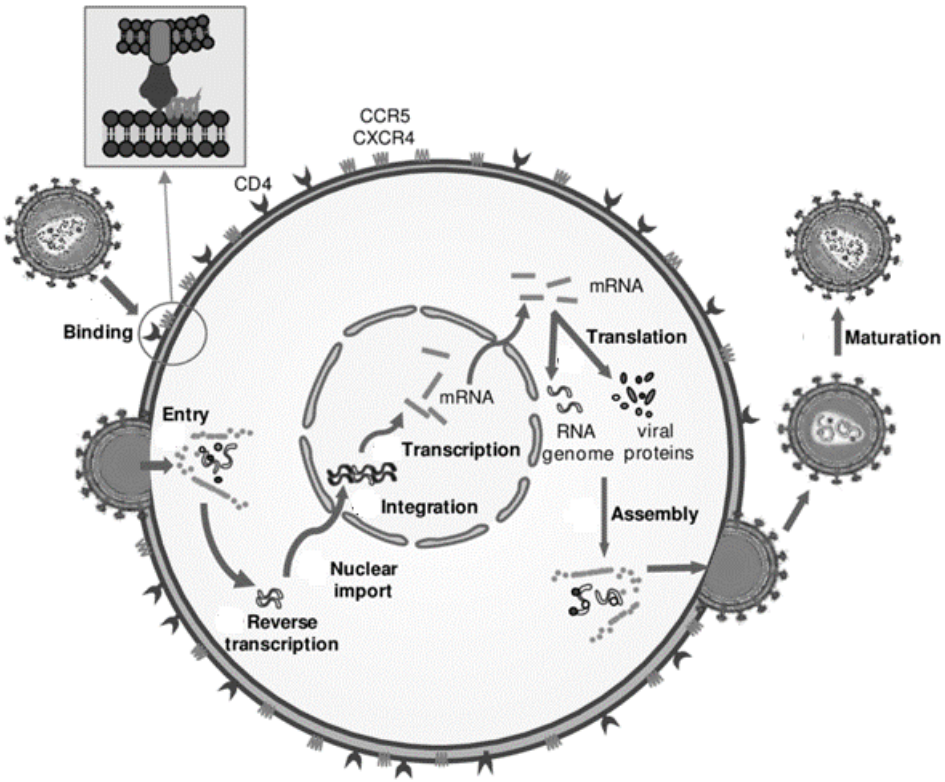
Figure 1.c The HIV Virion



A schematic of the structure of the HIV virion detailing the envelope, structural, enzymes and regulatory proteins. Image taken from Rossi E, Meuser ME, Cunanan CJ, Cocklin S. Life. 2021 (32)

1.3.2 HIV life cycle

Figure 1.d The HIV-1 life cycle



A schematic of the HIV-1 life cycle. HIV-1 binds to $CD4^+$ cells via GP120 and engagement with chemokine co receptor 4 or 5 (CXCR4, CCR5). The viral capsid enters the cell and disassembles releasing viral proteins and RNA. Reverse transcription gives rise to HIV-1 DNA, and the preintegration complex facilitates nuclear import. Viral DNA is then inserted into the host genome where RNA polymerase yields messenger RNA encoding for viral proteins. Following translation, envelope proteins are transported to the host cell surface membrane and other viral proteins are transported in complexes. After budding, protease cleaves the polyproteins, yielding the mature viral proteins which form a mature, infectious virion. Image adapted from Herrera-Carrillo E, Berkhout B. Viruses. 2015 *(33)*.

1.3.2.1 Fusion

HIV principally targets CD4+ T helper cells but can also infect macrophages and dendritic cells (21, 34) The HIV life cycle lasts 1-2 days and can be described in distinct stages (**Figure 1.d**). The mature virion first binds to the CD4 receptor of the host cell via gp120. Membrane fusion is initiated through binding to a coreceptor: Chemokine co receptor 4 or 5 (CXCR4 or CCR5) (35).

Binding leads to a conformational change in gp41, resulting in the formation of a channel, and the N terminus inserts into the plasma membrane of the target cell, completing the fusion process (29, 35). The viral capsid subsequently translocates to the cytoplasm where it is believed to disassemble aiding in the process of reverse transcription (36).

1.3.2.2 Reverse transcription

Reverse Transcription begins with the binding of a host cell tRNA primer, which is complimentary to the 5' end of the viral genome. Subsequent extension by reverse transcriptase leads to the formation of the minus strand DNA. There is a polypurine tract that is unable to be cleaved by RNase H and this sequence acts as the primer for the formation of the plus strand DNA. This DNA molecule anneals to the minus strand and extends to form the long terminal repeat (LTR) at the end of the DNA (37)

1.3.2.3 Integration

The viral protein, integrase, catalyses the integration of viral DNA into the host DNA. The preintegration complex (PIC), comprising integrase and other cellular proteins, including reverse transcriptase, matrix protein, vpr and nucleocapsid protein enters the host cell nucleus (38). The process of integration begins with the exposure of the 3' hydroxyl groups following the removal of nucleotides from each end of viral DNA. The viral DNA is inserted into the host DNA and the integration

intermediate is repaired by host cell enzymes, resulting in a 5bp duplicated sequence each side of the integrated provirus (39, 40).

1.3.2.4 Transcription

The integrated provirus is the template for synthesis of viral RNA. Once the provirus is integrated, HIV-1 produces spliced mRNAs encoding tat and rev and, following this initial period, transcription increases and mRNAs encoding Env, Vif, Vpr, Vpu and the Gag-Pol polyprotein are produced (10).

1.3.2.5 Assembly and budding

The Gag polyprotein mediates assembly, budding and maturation of the HIV-1 virion and the processes of membrane binding, virion assembly and RNA packaging are thought to take place simultaneously (41).

The virion lipid membrane and glycoprotein are acquired through budding from the plasma membrane. Env is glycosylated and later processed by furin to give rise to gp41 and gp120, which is transported in vesicles to the plasma membrane. (41)

The Gag polyprotein precursors are also transported to the plasma membrane. During this process 2 copies of positive sense single-stranded viral RNA are recruited. This complex at the host membrane brings about membrane curvature, resulting in the formation of a bud. Env glycoproteins, integrase, protease and reverse transcriptase are also packaged (42, 43). The immature particles then bud and protease is activated, which cleaves the gag polyprotein precursors to produce the matrix, capsid, nucleocapsid, p6, protease, reverse transcriptase and integrase proteins. The proteins then reconfigure to form a mature virion (41).

1.4 Antiretroviral therapy

Since the elucidation of the HIV life cycle, there has been great progress in drug development (44). The first approved antiretroviral drug was zidovudine (formerly known as Azidothymidine or AZT), a nucleoside reverse transcriptase inhibitor (NRTI) that reduced viral load, slowed the progression of the disease, leading to an increased life expectancy of 5.5 months (45). Whilst this was a significant breakthrough, viral suppression was not maintained and there were a number of adverse side effects- including anaemia and liver toxicity, and the cost of a course of treatment was prohibitive (46, 47).

In the mid 90s agents from a new class of antiretrovirals, protease inhibitors (PI), were approved, including saquinavir and ritonavir. The use of a PI in combination with NRTIs proved to be much more effective than monotherapy. With these regimens, HIV RNA was supressed to undetectable levels, CD4 cell count improved, and survival was extended (44, 48).

Following this, another class of drugs was introduced to the market- non nucleoside reverse transcriptase inhibitors (NNRTI) and, though commonly quickly resulting in the development of resistance when administered as monotherapy, in combination with other antiretroviral agents it was shown to be very effective (49).

Prescribing antiretrovirals in combination (most commonly as a triple drug combination) became the standard of care due to its effectiveness and following these recommendations, morbidity and mortality decreased substantially (49, 50).

Today there are over 25 antiretroviral agents across 6 different drug classes, targeting the various stages of the virus life cycle (44) (**Figure 1.d)**. The effectiveness of cART has led to a markedly increased life expectancy of people living with HIV, resulting in an aging population in this group, in both resource rich and low resource settings (51, 52).

As there is now a wider variety of ART available (**Table 1.a**), a more tailored approach can be taken, taking into consideration comorbidities, polypharmacy and tolerability (53)

1.4.1 Nucleoside reverse transcriptase inhibitors

NRTIs are commonly used in cART, often forming a 'backbone' of treatment. Typically NRTIs are given with one or two other antiretroviral agents from one of the other classes (54).

NRTIs are prodrugs and are phosphorylated once inside the cell, giving rise to their active anabolite. The active form of NRTIs act as deoxyribonucleotide triphosphates (dNTP) and are incorporated into the Viral DNA during the reverse transcription process. Since they do not have a 3' hydroxyl group, the following dNTP cannot bind and the chain is terminated (54, 55).

1.4.2 Non-nucleoside reverse transcriptase inhibitors

NNRTI act by binding to reverse transcriptase, forming a hydrophobic pocket near the active site, leading to a conformational change, reducing the activity of the enzyme and thus inhibiting DNA synthesis (56). NNRTIs are often administered in combination with NRTIs and are widely used due to their specificity and favourable toxicity profile (57).

1.4.3 Integrase inhibitors

Integrase strand transfer inhibitors (INSTI) are the most recent class of antiretroviral. They prevent the incorporation of viral DNA into the host genome by inhibiting the binding of the PIC to the host DNA (58). The divalent metal ions Mg^{2+} and Mn^{2+} in the active site of integrase are crucial to the activity of the integrase enzyme. INSTIs act by binding the active site Mg^{2+} ions, thereby impairing the integrase enzyme, competitively inhibiting it from binding host DNA (59, 60).

Second generation INSTIs are thought to have a high barrier to resistance and are well tolerated and are therefore often administered with NRTIs.

1.4.4 Protease inhibitors

Protease inhibitors (PI) act later in the virus life cycle, targeting HIV Proteases, preventing the production of mature virions. (44) The hydroxyl groups of the inhibitor forms a hydrogen bond with the carboxyl group of the aspartate (Asp) residues Asp-25 and Asp-25', blocking the active site (61).

Whilst effective, these drugs are associated with a number of metabolic effects (although less common in newer PIs) including hyperlipidaemia and insulin resistance (54).

Darunavir has a high potency in inhibiting HIV-1 and HIV-2 as well as a high resistance barrier due to the Hydrogen bonds formed with the protease backbone amide nitrogen atoms of Asp-29 and Asp-30 (61-63).

PIs are commonly administered with ritonavir or cobicistat to increase the drug concentration and lengthen the half-life, through the inhibition of the cytochrome P450 enzyme, CYP3A4 (64, 65). Boosted PIs have a high genetic barrier to resistance and usually need more than one major mutation to reduce efficacy, therefore, virological failure for those on PI- based therapy is often due to non-adherence (66).

1.4.5 Entry inhibitors

1.4.5.1 CCR5 antagonists

Maraviroc is the only approved CCR5 antagonist and works by blocking the entry of the virus to the host cell (44). HIV-1 enters the cell following the binding of the CD4 receptor and co receptor, CCR5. Maraviroc selectively binds to CCR5, causing a conformational change which results in the inhibition of the engagement of GP120 and CCR5; preventing viral entry (67-69). As there is a need for costly tropism testing prior to treatment initiation and bi daily dosing, maraviroc is not often prescribed (44).

1.4.5.2 Fusion inhibitors

Enfurvitide binds the first heptad repeat (HR1) of GP41, preventing the conformational change and entry pore formation necessary for the virus and host cell membrane fusion process (70). Enfurvitide is administered subcutaneously twice daily and is recommended for use in patients where other treatments have failed (54).

Table 1.a Currently licenced antiretroviral drugs and abbreviations

Antiretroviral Class	Drug names and abbreviations
Nucleoside reverse transcriptase inhibitors (NRTI)	Zidovudine (AZT) Lamivudine (3TC) Abacavir (ABC) Tenofovir disoproxil fumarate (TDF) Tenofovir alafenamide (TAF) Emtricitabine (FTC)
Non-nucleoside reverse transcriptase inhibitors (NNRTI)	Doravirine (DOR) Efavirenz (EFV) Etravirine (ETV) Neviripine (NVP) Rilpivirine (RPV)
Integrase inhibitors (INSTI)	Bictegravir (BIC) Dolutegravir (DTG) Elvitegravir (EVG) Raltegravir (RAL) Cabotegravir (CAB)
Protease inhibitors (PI)	Atazanavir (ATV) Darunavir (DRV) Lopinavir (LPV) fosamprenavir (FOS-APV) lopinavir (LPV) ritonavir (RTV) saquinavir (SQV) tipranavir (TPV)
Fusion inhibitors	Enfurvitide (T-20)

CCR5 antagonists	Maraviroc (MVC)
Pharmacokinetic enhancers	cobicistat (c) low-dose ritonavir (r)

1.5 Treatment guidelines

HIV treatment guidelines vary between regions and countries and, over the past 20 years there have been changes in line with the improvements and availability of ART, the management of HIV and a greater understanding of the virus. Although the guidelines are now more closely aligned, there was previously a clear difference in availability of ART (and thus treatment guidelines) in resource rich versus resource limited countries. The variations in guidelines remain, however, and are due to differing public health priorities, licence agreements and resources of the region or country (71).

The WHO guidelines consider resource limitations, focus on public health and feasibility, and are targeted towards those in resource limited settings. In 2003 WHO guidelines recommended the NRTIs stavudine or zidovudine to be used with Lamivudine plus an NNRTI – either nevirapine or efavirenz. At this time there were an estimated 6 million people living with HIV in the developing world but only 230,000 with access to ART. Though there was a move away from these drugs in industrialised nations in favour of less toxic alternatives, these cheaper drugs allowed for increased access, with the hope that 3 million would be on ART by the end of 2005 (72).

Today WHO guidelines recommend that the first line treatment should be dolutegravir plus 2 NRTIs (tenofovir disoproxil fumarate+ lamivudine for adults and adolescents and abacavir + lamivudine for children and infants), and, as an alternative regimen efavirenz at 400mg (low dose) should be administered with 2 NRTIs (73).

Guidelines in resource rich settings are often based on the most recent evidence-based findings and a wider range of ART are suggested and available.

In the US the department of health and human services (DHHS) guidelines state those beginning treatment should start on bictegravir/tenofovir alafenamide and emtricitabine or dolutegravir plus (emtricitabine or lamivudine) plus (tenofovir alafenamide or tenofovir disoproxil fumarate).

Dolutegravir/lamivudine is also recommended except for individuals with a HIV-1 viral load of >50000 copies/mL or Hepatitis B virus co infection. dolutegravir/abacavir is also a recommended treatment provided the individual is HLA-B*5701 negative and without chronic hepatitis B virus coinfection (74)

The European Aids Clinical Society (EACS) guidelines recommend 2 NRTI plus 1 INSTI as initial treatment- abacavir/lamivudine + dolutegravir (or abacavir/lamivudine/dolutegravir) OR tenofovir alafenamide/emtricitabine/bictegravir (or tenofovir alafenamide/emtricitabine or tenofovir disoproxil/emtricitabine + dolutegravir). Raltegravir can also be given in place of dolutegravir with tenofovir alafenamide/emtricitabine or tenofovir disoproxil/emtricitabine. Other recommended regimens include 1 NRTI+ INSTI or 2NRTI + NNRTI (75).

This thesis will focus on the United Kingdom treatment guidelines (**Table 1.b**). The British HIV Association guidelines currently recommend initial treatment as 2 NRTI plus one agent from the NNRTI, boosted PI or INSTI class of antiretoviral drug classes. The preferred NRTI backbone is comprised of tenofovir disoproxil fumarate and emtricitabine OR tenofovir alafenamide and emtricitabine plus dolutegravir. The alternative suggested NRTI backbone is abacavir and lamivudine plus dolutegravir, provided the individual is HLA B*5701 negative and their cardiovascular risk score is less than 10%.

In 2008 the recommendations were that 2 NRTI should be used with efavirenz as the first line treatment with there being no preference over tenofovir disoproxil fumarate and emtricitabine or abacavir/lamivudine. The alternative treatment was a ritonavir boosted PI as a 3rd Agent (lopinavir, fosamprenavir, atazanavir, saquinavir)

In 2013 guidelines still recommended tenofovir disoproxil fumarate/emtricitabine as the first line NRTI backbone but abacavir/lamivudine as an alternative choice of treatment. Ritonavir boosted atzanavir or darunavir, efavirenz, raltegravir and cobicistat boosted elvitegravir were considered acceptable first line 3rd agents.

The recommendations in the 2022 guidelines under consultation recommend an INSTI- based three drug regimen – tenofovir disoproxil fumarate/emtricitabine or tenofovir alafenamide/emtricitabine with dolutegravir OR abacavir/lamivudine/dolutegravir. tenofovir alafenamide/emtricitabine/bictegravir is also recommended as first line treatment, as is dolutegravir/lamivudine on the condition that there is no lamivudine resistance, the baseline viral load is <500000 copies/mL, baseline CD4 count is >200 cells/mm^3 , no active hepatitis B infection and the individual is hepatitis B virus immune if at risk (76). Ritonavir boosted atazanivir, cobicistat boosted elvitegravir, rilpivirine and efavirenz are no longer recommended as first line treatments. The changes in the guidelines can be seen in **Table 1.b**.

Table 1.b BHIVA guidelines on antiretroviral treatment for adults living with HIV-1 from 2006-2022

Year	Preferred	Alternative
2006	AZT/3TC, ABC/3TC, TDF/FTC + EFV, LPV/r, FOS/r	SQV/r ddI
2008	TDF/FTC, ABC/3TC + EFV	ddI, AZT LPV/r, FOS/r, ATV/r, SQV/r,
2013	TDF/FTC + ATV/r, DRV/r, EFV, RAL, EVG/c	ABC/3TC RPV, LPV/r, FOS/r, NVP
2015	TDF/FTC or TAF/FTC + ATV/r, DRV/r, DTG, EVG/c, RAL, RPV,	ABC/3TC EFV
2022 **(under consultation)**	TDF/FTC or TAF/FTC + DTG TAF/FTC/BIC DTG/3TC ABC/3TC/DTG	DRV/r, DRV/c, DOR, EFV, RAL,

1.6 HIV and cardiovascular disease risk

With the success of ART there is now an aging population of people living with HIV and more of a focus on non-AIDS related morbidities. Cardiovascular disease (CVD) is one of the leading causes of mortality (77) and is in fact observed at higher rates than in the general population, with those living with HIV being two times more likely to develop CVD than the general population (78). The reasons are numerous and complex but can be broadly grouped into HIV associated chronic inflammation, traditional lifestyle factors and cART toxicity.

1.6.1 HIV-associated inflammation

In the investigation of the increased risk of CVD in people living with HIV, there has been a recent focus on HIV associated immune activation and inflammation. Untreated HIV infection results in increased immune activation, which persists even following viral suppression (79).

Results from the Strategies for Management of Anti-Retroviral Therapy (SMART) study suggested that HIV may increase the risk of CVD. This trial compared two treatment strategies – episodic use of ART based on CD4 count versus continuous ART, where the primary endpoint was opportunistic infection or death from any cause, and the secondary endpoint was major cardiovascular, renal or hepatic disease (80).

Contrary to expectations, participants in the drug conservation (episodic ART) group had a higher rate of major CVD events versus the viral suppression (continuous ART) group (1.3 vs 0.8 events per 100 person years) (80).

Further investigations have shown that the CVD risk markers interleukin-6 (IL-6), high sensitivity C - reactive protein (hsCRP) and D-dimer are raised in people living with HIV compared to the general population (81).

HIV itself has also been shown to activate endothelial cells, resulting in the increased expression of MCP-1, ICAM-1, VCAM-1 and e-selectin, and subsequent recruitment of inflammatory monocytes and T cells potentially driving vascular damage and CVD (82).

Monocytes are implicated in CVD progression and have been proposed to promote atherosclerosis, coagulation, and plaque destabilization (83, 84), and non-classical ($CD14^{+}$ $CD16^{++}$) and intermediate ($CD14^{++}$ $CD16^{+}$) monocytes are found in higher proportions in people living with HIV (84, 85). These activated monocytes express the procoagulant molecule tissue factor (TF), CD62P and secrete the proinflammatory cytokines IL-6 and IL-18. HIV-1 monocyte activation may contribute to a hypercoaguable state in people living with HIV whether on treatment or treatment naïve, increasing the risk of CVD (84-86).

1.6.2 Traditional risk factors

Traditional risk factors for CVD are high in people living with HIV, contributing to the overall increased risk. Smoking is a major risk factor for CVD and rates among people living with HIV are 2-3 times higher than the general population and 70% of MI in people living with HIV can be attributed to smoking (87).

Hypertension is another leading risk factor for CVD and older individuals living with HIV may develop hypertension at an increased prevalence than their counterparts in the general population. This has been demonstrated in two studies where the mean and median age was 45±8 years and 55 years respectively (88, 89) and cART may also contribute to increased hypertension. A meta-analysis of 49 studies showed that there was a higher prevalence of hypertension in PLWH on ART versus the general population (34.7% v 30.8%) (90, 91), and with an aging population it is expected that hypertension in PLWH will become more common (92).

Excessive alcohol consumption and illicit drug use is often reported as more prevalent in people living with HIV than the general population, increasing the risk of CVD (93). Alcohol abuse can result in dyslipidaemia, increases CRP and hypertension, all leading to plaque formation (94). Drug use has also

been shown to be higher in subpopulations of people living with HIV, with cocaine abuse being associated with cardiovascular events. Studies have shown that cocaine can lead to coronary artery calcification, an early step in plaque formation and atherosclerotic cardiovascular disease (95).

1.6.3 cART toxicity

Several studies have suggested that some antiretroviral agents are associated with an increased risk of CVD, although mechanistic data has not been conclusive.

PI use was initially implicated in increased risk of CVD in people living with HIV due to their well-known metabolic effect (96, 97). These adverse effects include the induction of dyslipidaemia, insulin resistance as well as endothelial dysfunction (97-99).

The data collection on adverse events of anti-HIV drugs (D:A:D) study was an international, prospective multi cohort study with over 30,000 participants. The study was designed to determine whether there is an independent association between cART use and risk of CVD. (100)

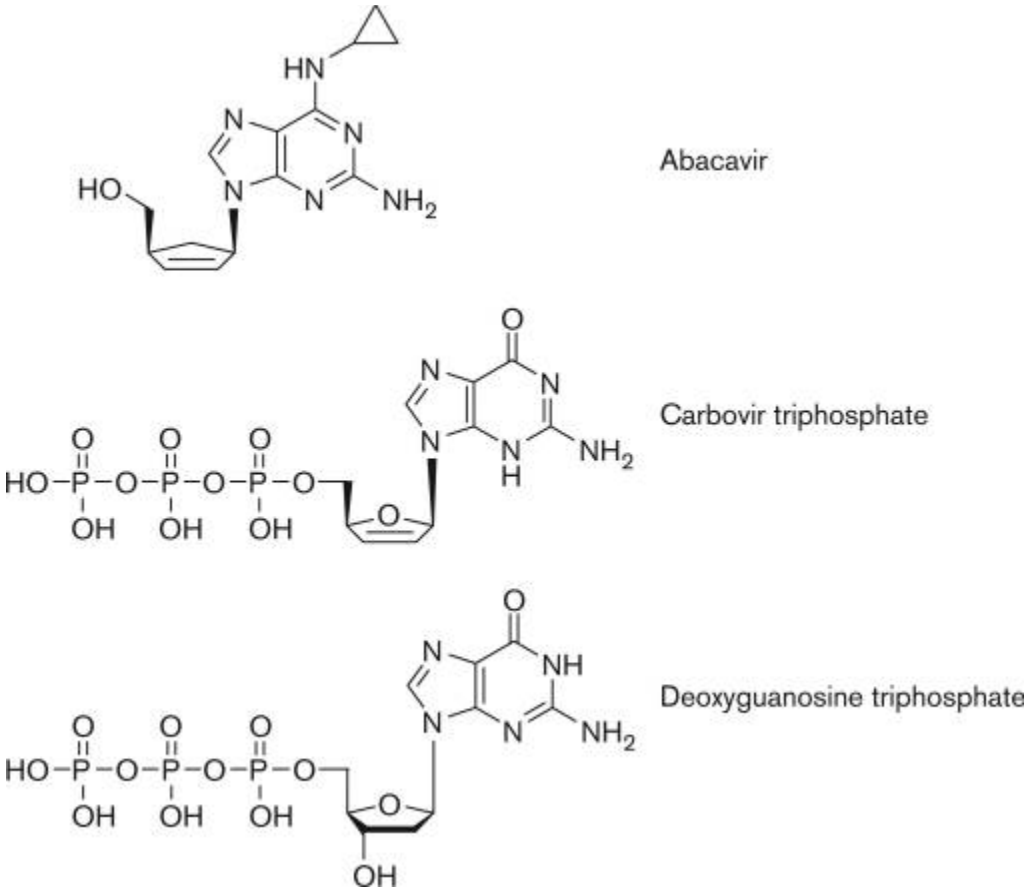
Analysis showed that there was an association between PI exposure and increased risk of MI, however the mechanism was not clear, with dyslipidaemia being likely only partly implicated in this observed association (100, 101).

The findings from the D:A:D study suggested that NRTI use was also associated with in increased risk of CVD and, in particular, myocardial infarction (MI). The thymidine analogues stavudine and zidovudine had previously been shown to cause dyslipidaemia and insulin resistance but analysis demonstrated that exposure to the adenosine analogue, didanosine and the guanosine analogue, abacavir, resulted in a 1.49 and 1.9 fold higher relative risk of MI respectively, compared to those not using those drugs. These observations were not associated with dyslipidaemia and was shown to reverse 6 months after stopping use of abacavir (102, 103).

1.7 Abacavir

Abacavir is a carbocyclic 2'-deoxyguanosine, or guanosine analogue, and is principally metabolised by the liver via two main pathways: the uridine diphosphate Glucuronosyltransferase and the alcohol dehydrogenase pathways. Abacavir exhibits its effects through the active anabolite, carbovir triphosphate (CBV-TP) by inhibiting HIV reverse transcriptase, preventing the addition of nucleotides in the DNA extension process (104).

Figure 1.e Chemical structure of abacavir, carbovir triphosphate and deoxyguanosine triphosphate



Chemical structures for abacavir, carbovir triphosphate and deoxygunaosine triphosphate. The lack of a 3' hydroxyl group on the deoxyribose ring of carbovir triphosphate (a guanosine analogue and active metabolite of abacavir) results in the termination of the DNA chain since no nucleotides can be added. Image taken from Barbarino JM. *et al*. *Pharmacogenet Genomics*. 2014 (105)

Abacavir rapidly enters $CD4^+$ T cells and human erythrocytes by passive diffusion. Once inside the cell abacavir is phosphorylated by adenosine phosphotransferase to abacavir monophosphate and cellular kinases serially phosphorylate this molecule giving rise to the active anabolite CBV-TP (**Figure 1.e**) (106).

CBV-TP is incorporated into the DNA chain and, due to the absence of a 3' OH nucleotide, nucleotides are prevented from being added, halting DNA synthesis (105). CBV-TP exhibits high selectivity for HIV-RT, avoiding the binding of DNA polymerases and the resulting toxicity that can be seen in other NRTIs (105).

Abacavir does not inhibit cytochrome P450 enzymes, there are few drug interactions, and is generally well tolerated but genetic screening is advised prior to the use of abacavir due to a hypersensitivity reaction observed in approximately 5-8% of the caucasian population (104). Those with human leukocyte antigen B (HLA-B) *57:01 allele experience a hypersensitivity reaction which is the result of abacavir non-covalently binding to the cleft of HLAB*57:01 with high affinity, changing the shape of the binding cleft and thus the repertoire of self peptides that can bind. With this change of self peptides, $CD8^+$ T cells are activated, resulting in so called abacavir hyperensitivity syndrome (107). Despite hypersensitivity screening, abacavir has been shown to be cost effective, asssuming similar virologic efficacy with tenofovir- based therapy (108).

All considered, abacavir is a well tolerated, effective treatment and is still recommended as a treatment option in combination with dolutegravir and lamivudine in the US, EU and UK (74-76, 109).

1.8 Abacavir and cardiovascular disease

The D:A:D study first highlighted the signal of increassed risk of myocardial infarction, with recent use of abacavir (i.e within the prior 6 months) and this risk was shown to be eliminated once usage of abacavir was stopped. This was also seen with didanosine at a lower relative risk but not in other NRTIs (102). As it was an observational study, there were a number of confunding factors including a channeling bias, where indivduals were prescribed abacavir in favour of other NRTIs. This is because they were already at a higher risk of CVD of other NRTIs as they were known to cause dyslipidaemia. There was also the criticism of that those with poorer renal function are likely to suffer from hypertension and diabetes and therefore have an inherently higher CVD risk (110). Subsequent analysis addressing the confounding resulted in a higher hazard ration of 1.98 (vs 1.90 in the first study) and other studies examining a potential channeling bias in patients with renal dysfunction showed that there is an over 2 fold increase in the risk of CVD for those using abacavir (111).

There have been several studies following the D:A:D study exploring an asociation between abacavir use and CVD, including cohort studies (110-114), randomised controlled trials (115-118) and meta analyses (119-123) but a consensus has not been reached.

Dorjee and colleagues reported that the hazard ratio of CVD events was raised (1.18-1.43 per year) for current, recent and cumulative exposure to abacavir regardless of prior CVD risk factors (123) and the SMART/INSIGHT study, a cohort study, examined biomarkers, ischemic electrocardiogram changes, as well as rate of CVD events, and found that abacavir use was associated with increased risk of CVD, potentially through inducing vascular inflammation (124).

The Simplification of antiretroviral therapy with Tenofovir-Emtricitabine or Abacavir-Lamivudine trial (STEAL) study was an open-label multi centre phase IV randomised controlled study to examine the

safety and efficacy of tenofovir disoproxil fumarate/emtricitabine vs abacavir/lamivudine and, though Non-AIDS events were a secondary endpoint, results showed that abacavir/lamivudine was associated with more cardiovascular events than with tenofovir disoproxil fumarate/emtricitabine use (125)

A cohort study in the US that followed over 8000 individuals from 1998-2011 from the initiation of antiretroviral therapy until death, end of study or health plan disenrollment also showed a positive association with abacavir usage and increased risk of CVD (111). Results from the study demonstrated that abacavir was associated with an over 2 times risk of CVD after correcting for underlying risk fators and any channeling bias (111).

Other studies have shown no association between abacavir and CVD. One study collected data from 52 GlaxoSmithKline sponsored clinical trials totalling 14,174 individuals who had received >24 weeks of abacavir and those who hadn't (n= 9502 vs n= 4672). Participants were well matched at baseline demographically and with respect to clinical characteristics. Brothers and colleagues (119) observed no increased risk of MI with abacavir therapy, however, there were weaknesses in the study, including a lack of randomised controlled trial data with only 12 of the 52 studies being randomised controlled studies, not all of the studies adjusted for confounding factors and the wide variety of study design endpoints, definitions and comparators make it difficult to come to a clear conclusion or to rule out an association. As the relative rate of MI was low, the study was also low powered given the number of subjects included and the authours acknowledged that more clarification and understanding is needed regarding the suggested association between ABC and CVD (119).

In the AIDS Clinical Trials Group (ACTG) Logitudinal Linked Randomized Trials Group (ALLRT), individuals are enrolled who have been randomised to receive ART under the various ACTG trials. Investigators analysed data from 5056 individuals randomly assigned to initiate abacavir or other antiretroviral treatment in prospective clinical trials, in order to understand increased CVD risk

associated with abacavir use, and attempt to address the confounding and channeling bias seen in the cohort studies performed. Results showed no evidence of an increased risk of MI in those using abacavir vs those on alternative treatments over a period of 6 years (122).

The lack of randomised controlled trial data and mechanistic data have led to continued confusion over the potential effect of abacavir use on the cardiovascular system. A randomised trial to assess a direct link, where the primary outcome would be MI would be controversial but conducting a switch study would be a good alternative and give greater insight.

The latest analysis of the D:A:D cohort, when taking into consideration confounding factors and the channeling bias, the intial observation was preserved albeit at a lower level (103). Given the central role of platelets in MI and the fact that the observed association was reversible, it has been suggested that platelet dysfunction, specifically changes in activation, might be responsible for the reported increased risk of MI with abacavir use (126).

1.9 Platelets

Platelets are anucleated cells measuring ~ 2.0 – 4.0 µm in diameter and with a mean volume of approximately 8.5 – 9 femtoliltres (127). They are derived from megakaryocytes in the bone marrow and primarily act to maintain haemostasis and repair the endothelium where, at the site of vascular injury, platelets will localise and form a haemostatic plug to prevent haemorrhage (128). Secondary roles also include angiogenesis and in innate immunity (128, 129).

1.9.1 Platelet structure

Despite being cell fragments, platelets have a complex structure (**Figure 1.f**). The platelet can be divided into 4 main zones: The peripheral zone, the sol-gel zone, organelles and the membrane zone (130).

The peripheral zone incorporates the extra and intracellular membrane, covered by a glycocalyx coat, and the opening of the open canicular system (OCS), linked to the surface. This OCS is involved in the transport of molecules into and out of the platelet and is especially important in Ca^{2+} signalling, granule release and the uptake of proteins necessary for platelet function (131, 132).

The plasma membrane is a phospholipid bilayer containing cholesterol, glycolipids and glycoproteins important for signalling and trafficking, as well as cation pumps to maintain homeostasis (128, 132).

The sol-gel zone is found beneath the peripheral zone and is a transparent, gel like matrix containing mictrotubles and actin filaments, forming a cytoskeleton and, seemingly maintains structural integrity of the platelet. The actin and microtubles constrict following platelet activation, resulting in platelet shape change and granule secretion (130, 132).

The Oganelle zone is located in the centre of the platelet and consists of α granules, dense granules and lysosomes, as well as mitochondria. α-granules are spherical or oval shaped and measure between

200 and 500 nm in diameter, numbering about 40 -80 per platelet (128, 133). They contain proteins involved in haemostasis, inflammation and wound healing, including P-selectin (CD62P), which is expressed on the platelet surface upon activation. Also contained within α-granules is GPIIbIIIa, GPIV (CD36), thrombospondin, von Willebrand Factor (vWf), as well as platelet derived growth factor (PDGF) and platelet factor 4 (133).

Dense granules (so called because of their electron dense cores) are thought to form in megakaryocytes. They are less numerous (~3-6 per platelet) and are smaller than α-granules, at 150 nm in diameter (134, 135). Dense granules contain adenosine triphosphate (ATP), adenosine diphosphate (ADP), serotonin, calcium ions, and lysosomal integral membrane protein (LIMP) or CD63 (128, 132). The release of these contents are key to the process of platelet aggregation and forming a haemostatic plug, and the importance of this role in seen in individuals with with Hermansky-Pudlak syndrome, an autosomal receessive disorder, characterised by a reduction or absence of dense granules resulting in a haemorrhagic syndrome (136, 137).

The function of platelet lysosomes is not well known but may play a role in receptor cleavage and degradation of platelet aggregates and of proteins ingested by phagocytosis (138). They contain hydrolases as well as CD63 and lysosomal- associated membrane proteins LAMP-1 and LAMP-2 (135).

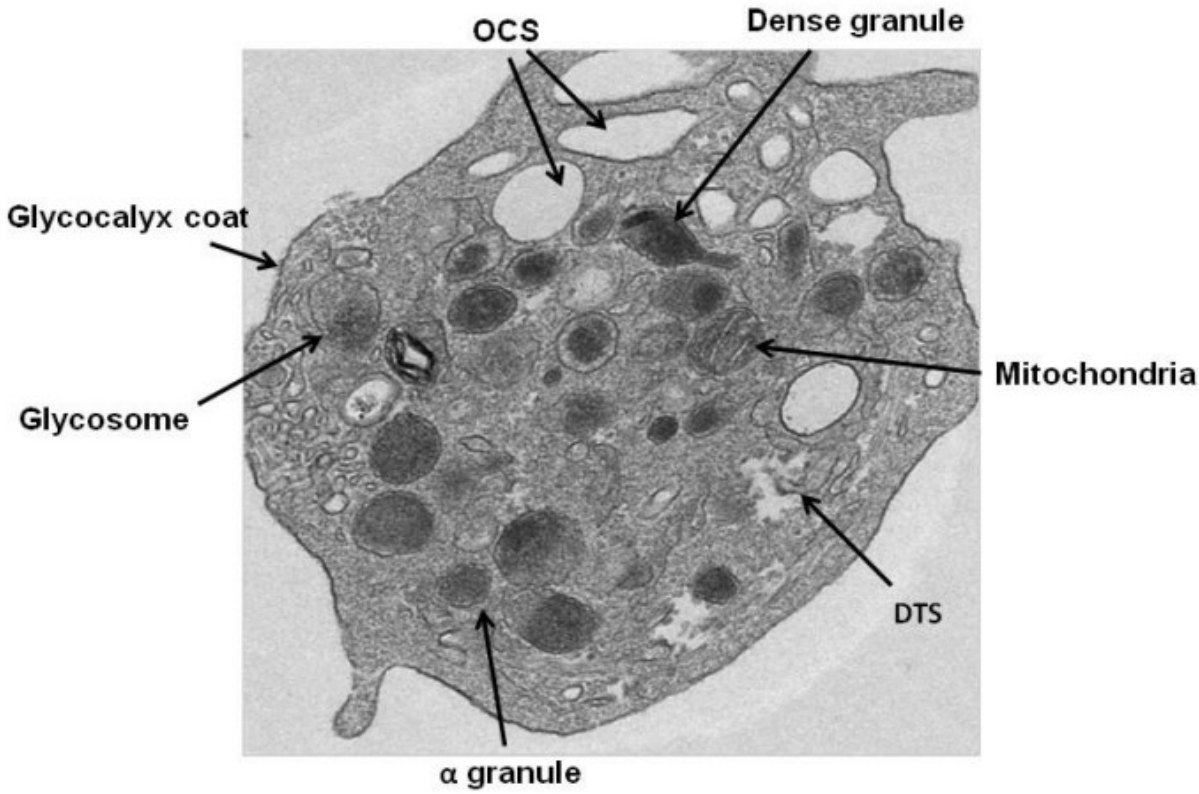
Finally mitochondria, though few in number, provide the energy source necessary for platelet function, especially the requirements for activation and granule exocytosis and the mitochondria may also play a role in providing the platelet with calcium (130, 135).

Their role is also key in the process of platelet apoptosis, where platelet stress leads to the inhibition of the prosurvival protein BCL-2 and the activation of BAK and BAX. These proteins act on the mitochondrial membrane, increasing permeability and resulting in the release of cytochrome c from

the mitochondria. This initiates the apoptotic caspase cascade with the end result being cell death (139, 140).

The membrane zone contains the dense tubular system (DTS); a membrane system with an important role in platelet activation regulation. Originating from the megakryocyte endoplasmic reticulum, the DTS is a store of calcium (that is released during platelet activation), thromboxane synthase, prostaglandin G and H and cyclooxygenase (130, 135).

Figure 1.f Transmission electron microscopy image showing platelet structure



Transmission electron microscopy image of a platelet showing glycocalyx coat, Glycosome, alpha granules, dense granules, mitochondria, the open canicular system (OCS) and dense tubular system (DTS). (Emerson, unpublished)

1.9.2 Platelet function

The primary function of platelets is to help maintain haemostasis. At ths site of vascular injury a haemostatic plug will form after platelets undergo 3 stages: adhesion, activation and aggregation (130).

Where there is damage to the vasucular wall, the sub endothelial matrix proteins are exposed, including vWf, collagen type IV, proteoglycans, nidogen-1, laminin and fibulin (141). Plasma vWF first binds exposed collagen via the type A domain, A3, and GP1bα of the GPIb-IX-V protein complex on the platelet surface binds to the immobilised vWF A1 domain (142, 143). This reversible engagement result in a rolling motion of platelets along the extracellular matrix and the platelet surface protein, GPVI is able to bind its ligand, collagen, and stable adhesion is brought about by the binding of

The platelet receptors GPIb-IX-V and GPVI bind vWF and collagen respectively. GPIb-IX can initiate adhesion and the development of a thrombus under high shear conditions (144). vWF, also found in plasma and α-granules , in addition to promoting platelet adhesion, has an alternative role of binding clotting factor VIII (145).

GPIbα of the GPIb-IX-V protein complex binds to the A1 domain of the exposed vWF, following vascular trauma (143). This reversible engagement results in a rolling motion of platelets along the extracellular matrix and other platelet receptors can then bind their ligands, namely GPVI which binds collagen and integrin glycoprotein (GP) IIb/IIIa ($\alpha_{IIb}\beta_3$).

GPVI binding its agonist, collagen , leads to inside out activation of GPIb/IIIa. This protein, unable to bind vWF on inactivatied platlets, can now bind vWF, fibrinogen or fibronectin with high affinity (142) and this is incredibly important for platelet-platelet interaction and thrombus stability (132, 143, 146).

GPVI signalling initiates platelet activation and is brought about via several pathways. ADP from dense granules acts in an autocrine and paracrine fashion resulting in platelet shape change, activating GPIIbIIIa, thromboxane A2 is also synthesised and released, promoting platelet aggregation, and P-selectin released from α-granules also aids in the recruitment of platelets and leukocytes (130, 147).

With the activation of GPIIbIIIa and interaction with vWF and fibrinogen, processes known as pseudopods extend from the platelets, aiding in aggregation. Tissue factor (TF) or factor III with factor VII and activated facor VIII stimulates the production of thrombin and converts fibrinogen to fibrin, resulting in a network stabalising and strengthening the haemostatic plug and forming a clot (145, 147).

1.10 Platelet agonists and signalling

1.10.1 Collagen

There are over 20 forms of collagen encoded for in the human genome. In the vessel wall expression of 9 of these has been identified, of which type I and type III are most common in the extracellular matrix and are exposed where there is damage to the vessel wall. Collagens are made up of repeating GXY motifs (G being glycine and X and Y, commonly proline, and hydroxyproline) which comprise a single left-handed helix and 2 right handed chains of around 1000 amino acids as well as non helical telopeptide extensions and fibrilar collagen is formed from the crosslinking of these chains (148).

There are a number of platelet collagen receptors; the two primary receptors being $\alpha_2\beta_1$ and GPVI, with integrin $\alpha IIb\beta_3$ as an indirect receptor through vWF (149). The integrin $\alpha_2\beta_1$ is key to platelet adhesion and there are about 2000-4000 molecules per platelet acting in a Mg^{2+}/Mn^{2+} dependent manner. When activated, $\alpha_2\beta_1$, undergoes a conformational change to a high affinity state via inside out signalling (148, 150) and It may also have a role in stabalising the thrombus and facilitating GPVI - collagen interactions and potentiate the resulting signalling and response (151).

GPVI, another direct collagen receptor, is from the immunoglobulin superfamily, encoded for by the GP6 gene on chromosome 19q13. 4 and only expressed on platelets and megakaryocytes, with each platelet expressing approximately 3000-4000 molecules (150) (**Figure 1.g**). It is comprised of two

extracellular immunoglobulin-like domains, D1 and D2, linked to a transmembrane domain by a mucin-like stalk (152). Collagen binds to the D1 domain of GPVI via the GPO motif and this engagement is crucial in the initial formation of a thrombus following damage to blood vessels (152). GPVI can also bind collagen related peptides and convulxin, the snake venom peptide which, due to it's multimeric structure, produces the strongest response of the GPVI ligands due the frequency of GPO motifs and its multimeric structure allowing for the cross linking of 4 GPVI receptors (153).

The transmembrane domain of GPVI possesses an arginine group necessary for association with the Fc receptor γ (FcRγ); a homodimer linked by a disulphide bridge with two Immunoreceptor Tyrosine-based Activation Motifs (ITAM) (148, 150). The Src kinases, Fyn and Lyn, phosphorylate the ITAM upon activation and this then provides the docking site for Syk, a tyrosine kinase, through the Src homoloy-2 (SH2) domain, which then phosphorylates the adaptor proteins SH2 domain containing leukocyte protein of 76 kDa (SLP-76) and the Linker for Activation of T cells (LAT) which, itself, serves as a docking site for proteins including phosphatidylinositol-3 kinase (PI3K) and phospholipase Cγ2 (PLCγ2) (154, 155). PI3K phosphorylates phosphatidylinositol 4,5 bisphosphate (PIP2) forming phosphatidylinositol-3, 4, 5-triphosphate (PIP3) which can then bind to the pleckstrin homology (PH) domain of phospholipase C γ2 (PLCγ2) (155). Brutons tyrosine kinase (Btk) acts to phosphorylate and activate PLCγ2 which cleaves PIP2 into inositol trisphosphate (IP_3) and diacylglycerol (DAG)ultimately resulting in the intracellular release of Ca^{2+} and activation of protein kinase C (PKC) the release of secondary agonists such as ADP and thromboxane A2 , and aggregation (152, 156).

In humans, GPVI deficiency results in a mild bleeding disorder and mice that do not express GPVI have diminsihed collagen responses, and reduced adhesion and thrombus formation (157).

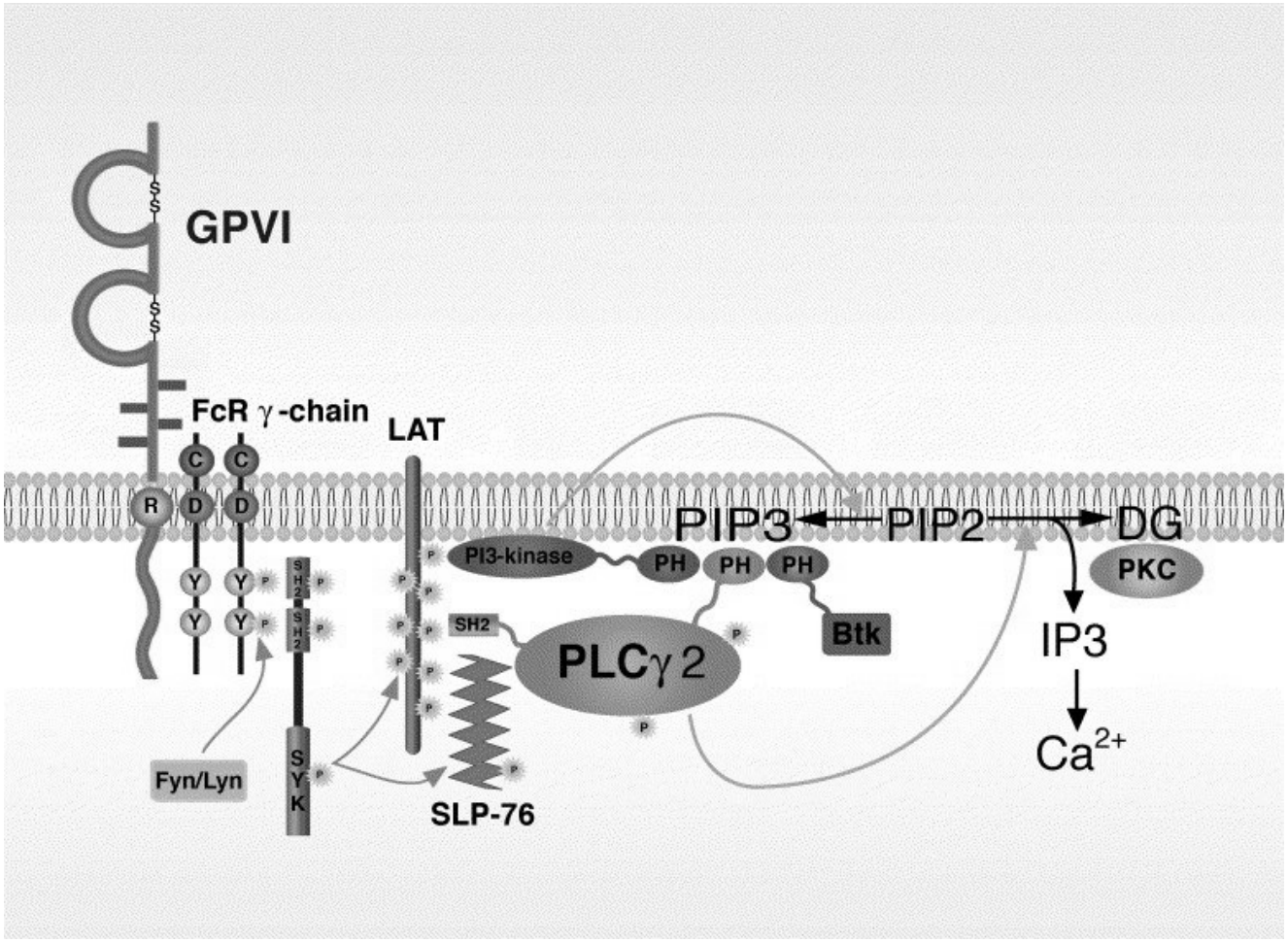
In the case of artherothrombosis, platelets can bind via GPVI to the collagen present on eroded plaque, resulting in inappropriate activation and thrombus formation which can occlude blood vessels (158). In exploring this, Kuijjpers and colleagues demonstrated that GPVI deficiency resulted in reduced thrombus formation in $ApoE^{-/-}$ mice and this has been confirmed since (159). Given the impact on

thrombus generation, limited bleeding risk and exclusivity to platelets GPVI has been identified as a target for peventing thrombosis (158, 160).

Collagen engagement with GPVI also results in ADAM10 mediated GPVI shedding, where soluble GPVI (sGPVI), a 55 kDa soluble fragment, is released. This is an irreversable process and has been shown to be raised in those with rheumatoid arthritis and acute ischaemic stroke (161, 162) and PLWH have also been shown to have higher levels of sGPVI compared to a healthy control population though interestingly this decreased prior to a coronary artery event (163, 164). This shedding is likely to be a mechanism of regulation of adhesion and activation and sGPVI can serve as a marker of platelet activation (165) (152, 161).

Glycoprotein Ib-V-IX is an indirect collagen receptor, where it binds vWF bound to collagen. Circulating vWF binds to exposed collagen from damaged vessel walls via the A3 domain. This causes a conformational change in vWF allowing GPVI-V-IX to bind; a key process in platelet adhesion and the initiation of thrombus formation (150, 166).

Figure 1.g GPVI structure and signalling pathways



GPVI structure and signalling pathways. GPVI engagement of collagen leads to Fyn and Lyn activation and and the subsequent phosphorylation of ITAM on the FcRγ chain with which Syk engages through the SH2 domain an in turn phosphorylates the adaptor molecules SLP-76 and LAT. This acts as a docking site for PI3-Kinase and PLCγ2 which catalyses the the hydrolysation of PIP2 generating IP3 and DG ultimately resulting in the release of Ca^{2+}, the activation of PKC and subsequent platelet activation. Abbreviations: ITAM: Immunoreceptor Tyrosine-basedActivation Motifs, SH2: Src homoloy- 2 , LAT: Linker for Activation of T cells, PI3- Kinase: Phosphatidylinositol 4,5-bisphosphate, PIP3: Phosphatidylinositol (3,4,5)-trisphosphate, PLCγ2: phospholipase C gamma 2 , IP3: Inositol trisphosphate, DG: diacylglycerol , PKC: Protein kinase C. Image adapted from Moroi M, Jung SM. *Thromb Res*. 2004 (155)

1.10.2 Adenosine diphosphate

ADP is released from damaged cells and from stimulated platelets where it is released from dense granules (167, 168), and platelet exposure to ADP results in shape change, degranulation and aggregation in a response mediated via the purinergic receptors $P2Y_1$ and $P2Y_{12}$ (169).

$P2Y_1$ receptors which are Gq coupled, are expressed throughout the body and there are approximately 150 per platelet. On binding this receptor ADP stimulates the phospholipase C pathway and is important for platelet shape change and aggregation (170, 171). Phospholipase C activation leads to hydrolysis of PIP2 and the resulting generation of IP3 and DAG leads to Ca^{2+} release and activation of PKC. This ultimately results in the synthesis of the platelet agonist thomboxane A2 (TxA2) and granule secretion in what is a positive feedback mechanism (168, 172-174).

1.10.3 Thromboxane A2

TxA2 is a metabolite of arachidonic acid (AA) belonging to the eicosanoid family and is released upon platelet activation, acting as a positive feedback mediator (175).

AA is converted to prostoglandin H2 in a COX-1 mediated proces and, via the action of thromboxane synthase, is finally converted to TxA2.

TxA2 binding its receptor, TP, results in platelet activation, shape change, aggregation and degranulation. TP is a G- coupled receptor and primarily activates Gq, G12 or G13 (176). PLCβ acts on PIP2, leading to the release of DAG and IP3, which results in the PKC activation and Ca^{2+} release. G13 coupled receptors act via the Rho/Rho kinase pathway and platelets deficient in G13 and Gq have been shown to lack the ability to undergo shape change in response to TxA2 and thrombin, even at maximal agonist concentrations (172).

TxA2 induced platelet shape change is mediated via the Rho/Rho kinase sinalling pathway. Ultimately, phosphorylation of the myosin light chain by myosin light chain kinase, leads to cycling of the actin

and myosin and platelet contraction in a proportional manner, as well as the formation of new actin filaments (175, 177)

1.10.4 Thrombin

Thrombin is central to the process of platelet activation and clot formation and is generated from its precursor molecule Prothrombin. Factor Xa interacts with the cofactor protein, factor Va, to form the platelet membrane bound prothrombinase complex, and this enzyme complex cleaves the prothrombin molecule at Arg^{271} and Arg^{320} to produce thrombin (178).

Thrombin acts via G-protein coupled receptors known as proteae- activated receptors (PARs), of which there are 4 mamalian isoforms: PAR_1, PAR_2, PAR_3 and PAR_4 (179). These receptors are characterised by seven transmembrane helices which are linked by three extracellular loops and three intracellular loops (179, 180).

Thrombin activates human platelets by first cleaving PAR_1 and PAR_4 at specific sites at the N terminus, exposing a new terminus which acts as a tethered receptor activated ligand (179, 180)

PAR_1 activates platelets at low thrombin concentrations, whereas PAR_4 requires higher thrombin concentrations to mediate platelet activation (172, 181). PAR_1 has a hirudin-like sequence which is suggested to be responsbile for the difference in receptor activity. Hirudin is a potent thrombin inhibitor and binds it with high affinity (182). The Hirudin-like domain found in PAR_1 near the cleavage site but absent in PAR_4 also has a high affinity for thrombin and is therefore able to activate PAR_1 at lower concentrations (172, 183). This result in Ca^{2+} release, platelet aggregation and degranulation, which includes release of ADP, TxA2, serotonin and epinephrine(171, 172, 179)

GPIb-IX-V is another receptor for thrombin and binding of the GPIbα subunit may facilitate cleavage and therefore activaition of PAR_1 (179). Inhibition of the thrombin binding sites of GPIbα have been shown to reduce platelet reactivity in response to thrombin with regard to activation, secretion and aggregation, demonstrating the importance of GPIbα in thrombin induced platelet responses (184).

1.11 Reactive oxygen species and platelets

Platelet responses have been shown to be influenced by reactive oxygen species (ROS) produced from platelets and the endothelium (185). Platelet activation results in the production of ROS via the NADPH oxidases (Nox) and by mitochondria, and include superoxide, hydrogen peroxide, hydroxyl radicals and peroxynitrate; all deemed to be important for full platelet activation (185, 186).

There are seven mammalian Nox isoforms: Nox 1-5, DUOX1 and DUOX2. Nox 1 and 2 are thought to be the isoforms primarily responsible for platelet ROS production in humans, becoming activated once platelet agonists bind their correponding receptor, whereupon a protein complex is formed (187). Nox1 forms a complex with $p22^{phox}$, NOXO1, NOXA1 and Rac1, and appears to be implicated in collagen induced platelet activation (186, 188). A pharmacological blockade of Nox1 inhibits platelet responses to collagen, reducing aggregation, adhesion and thrombus formation, as well as the expression of platelet surface markers of activation. Thrombin responses are preserved however, and similar outcomes are seen in $Nox1^{-/-}$ platelets (189).

Nox2 is thought to play an important role in thrombin-evoked platelet activation but can also undergo activation via GPVI signalling, where it forms a protein complex comprising $p22^{phox}$, $p40^{phox}$, $p47^{phox}$,$p67^{phox}$ and Rac1 (187, 188). Patients with X-linked granulomatous disease have a Nox2 deficiency and have reduced ROS production and platelet responses (190).

Rac1 GTPase is key to the production of ROS via both Nox1 and Nox2, where Rac GTP binding to NOXA1 and $p67^{phox}$ respectively, increases Nox activation. Inhibition of Rac1- $p67^{phox}$ has been shown to reduce ROS production and thus platlet activation, particularly via the thrombin-PAR pathway (190). Understanding redox regulation in platelets is important given the association of cardiovascular disease with ROS production and platelet hyperactivity (185).

1.12 Platelets in myocardial infarction

MI is a leading cause of death in the west and is characterised by myocardial tissue death as the result of reduced oxygenated blood to the area, following occlusion of the coronary arteries (191-193).

Platelets are key to the pathogenesis, where rupture of atherosclerotic plaque leads to platelet activation and aggregation, following the exposure of subendothelial matrix proteins (194). Where there is occlusion of the blood vessel, increased shear rates lead to the activation and conformational change of vWF that allows for the binding of GPIb-IX-V and platelet aggregation, which can lead to complete occlusion of the vessel (195).

Platelets can also contribute to plaque progression by releasing molecules such as fibrinogen and P selectin, which is translocated to the membrane, promoting the recruitment of monocytes and lymphocytes, as well as the release of platelet derived growth factor, also stimulating atherosclerotic plaque formation (141).

Given the central role of platelets in MI, it is essential that haemostasis is tightly controlled, as dysregulation, where there is platelet hyperreactivity, can result in thrombosis.

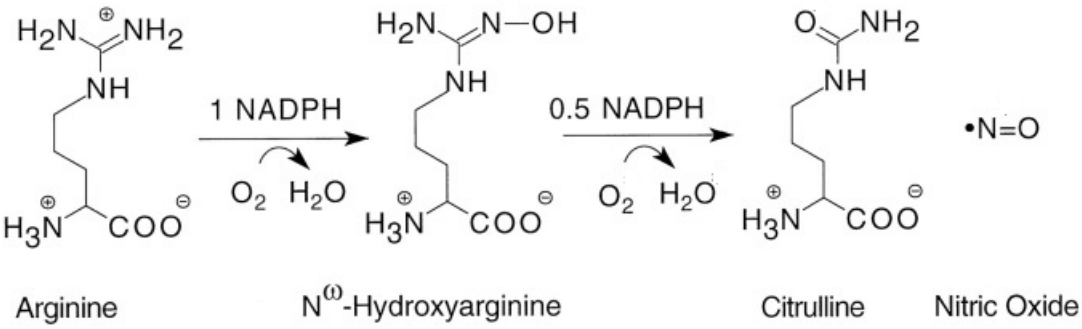
1.13 Nitric oxide and platelet inhibition

Platelet activation and aggregation is regulated to a large degree by nitric oxide (NO), making it a key molecule in maintaining overall haemostasis (196).

NO is produced by the hydroxylation and oxidation of L-arginine in a two-step process, catalysed by NO synthase (NOS), of which there are 3 mammalian isoforms: neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS). ENOS is stimulated by increases in Ca^{2+} but also by oestrogen, TNFα and, crucially, shear stress. Increases in shear stress result in the phosphorylation of eNOS, activating the enzyme (197).

NOS first hydroxylates L-arginine to N^{ω}- hydroxy-L-arginine and then oxidises this molecule to produce L-citrulline and NO (**Figure 1.h**) (198).

Figure 1.h Nitric oxide synthesis



Nitric oxide synthesis by nitric oxide synthase. Image adapted from Stuehr D, & Pou S, Rosen GM. *J Biol Chem.* 2001 (198).

Production of NO results in vasodilation and inhibition of platelet activation and aggregation, mediated soluble guanylyl cyclase (sGC). NO binds to the Fe^{2+} of the haem group in the active site of sGC, activating the enzyme, which in turn catalyses the production of cGMP. cGMP activates cGMP-dependent protein kinases (cGK), of which cGK type I (cGKI) predominates in platelets. CGKI acts on vasodilator- stimulated phosphoprotein (VASP) and IP_3 receptor associated cGMP kinase substrate

(IRAG) where they can then exhibit antithrombotic effects likely inhibiting activation of the fibrinogen receptor and inhibiting Ca^{2+} release respectively (199, 200).

1.14 Abacavir and platelet activation

Given the role of platelets in MI and the reversible nature of the association observed in abacavir use and MI, it has been hypothesised that abacavir-induced platelet dysregulation may be a causative factor (126, 201).

Many of the studies investigating the effect of ART on platelets have examined *ex vivo* or *in vitro* platelet aggregation by light transmission aggregometry (LTA) (126, 201). LTA is considered the gold standard test for assessing platelet function, where platelet agonists are added to platelet rich plasma (PRP) and placed between a light source and sensor in the aggregometer. As the platelets aggregate, the sample clears and more light is able to pass through. The apparatus allows for light transmission to be measured over time (202, 203).

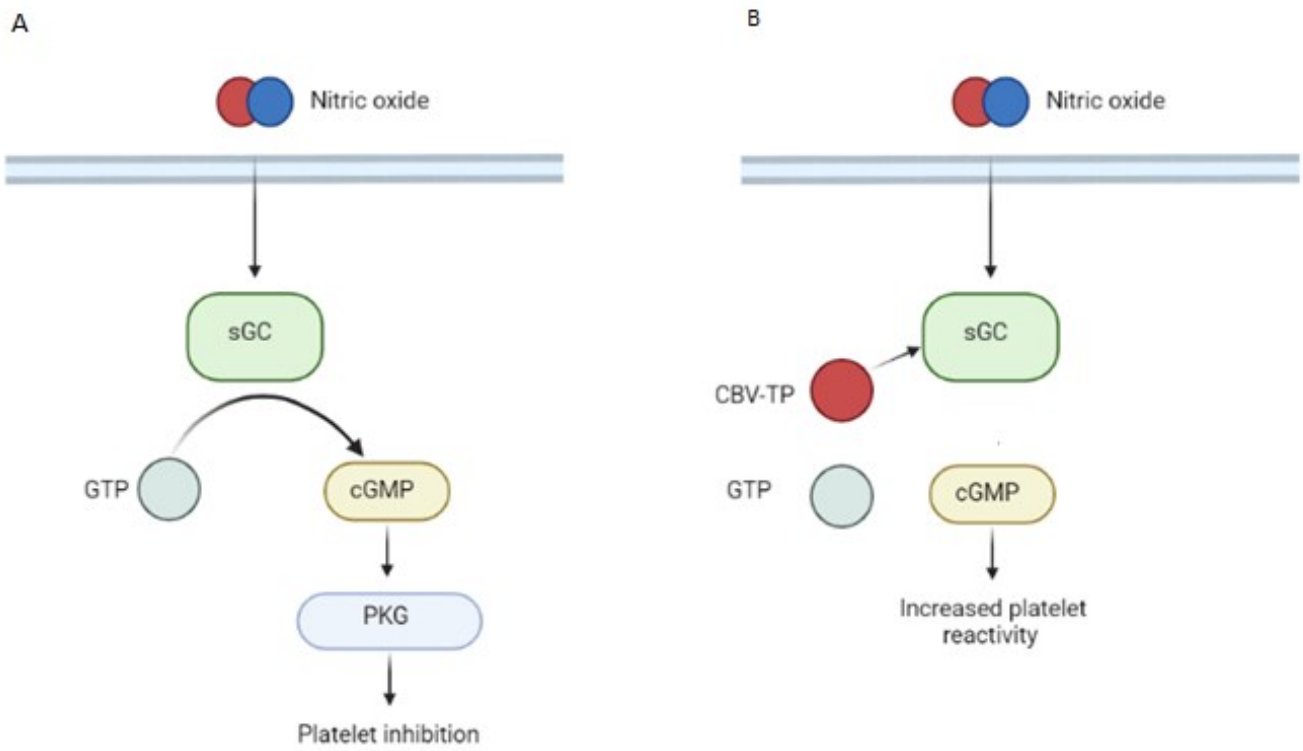
Studies examining the effect of abacavir on platelets have generally reported increased activation of platelets following abacavir use or addition of abacavir or its active metabolite to platelets (126, 201, 204) however, study design has varied widely. There were studies using samples from people living with HIV (126, 201, 205) and others using platelets from healthy volunteers, where abacavir is introduced *in vitro* (204, 206, 207). There have also been differences in drug pre incubation time and concentration, where in some cases this was at supraclinical concentrations (201, 207). Additionally, many of the studies were non-randomised, resulting in the potential for selection bias, and baseline characteristics of participants varied as well as agonists and agonist concentrations. With such a lack of consistency in experimental design, it is difficult to make a comparison and establish a clear conclusion. Consequently, a pharmacological mechanism for the observed increase in platelet activation and aggregation is yet to be elucidated.

1.15 Potential mechanism of abacavir induced platelet activation

Abacavir is a prodrug and is metabolised within the cell, where it undergoes deamination and serial phosphorylation steps to produce the active metabolite, CBV-TP (208). CBV-TP is similar in structure to guanosine triphosphate (GTP) and given this similarity it has been suggested that CBV-TP may cause a disruption in the inhibitory NO-sGC-cGMP signalling pathway (**Figure 1.i**) (204).

Baum *et al* and Falcinelli *et al* demonstrated reduction in cGMP production in response to abacavir *in vivo* and *in vitro*. Baum and colleagues showed competitive inhibition of sGC by CBV-TP, although the drug concentrations used were much higher than would be seen clinically (201, 204). Taylor and colleagues also showed an interruption in the NO-sGC-cGMP signalling pathway through measuring platelet aggregation following the incubation of platelets with ABC and the NO donor SNAP (206).

Figure 1.i The nitric oxide signalling pathway and proposed mechanism for abacavir-induced increased platelet reactivity



Nitric oxide signalling pathway and a proposed mechanism for abacavir-induced increased platelet reactivity. (A) Under normal conditions nitric oxide diffuses into the platelet and acts on soluble guanylate cyclase (sGC). sGC once activated catalyses the formation of cyclic GMP (cGMP) from Guanosine triphosphate (GTP). cGMP activates protein kinase (PKG), the action of which results in inhibition of platelet activation and aggregation. (B) Where Carbovir triphosphate (CBV-TP) (the active anabolite of abacavir) is present, it is proposed that given its structural similarity to GTP, there is competitive inhibition of sGC, leading to a reduction in cGMP and the downstream antithrombotic effects resulting in a more reactive platelet phenotype. Image created with BioRender.com

1.16 Summary

The life expectancy of PLWH on stable treatment is now comparable with that seen in the general population. Non- AIDS related comorbidities in this cohort are a focus of clinicians, as PLWH are at higher risk than the general population, with cardiovascular disease being one of the most prevalent causes of death (209, 210).

Large cohort studies have shown that use of the antiretroviral, abacavir, increases the risk of myocardial infarction compared to other antiretrovirals (103, 113, 211, 212). However, despite many subsequent studies attempting to clarify this, there is no clear consensus.

Increased activation of platelets may be implicated in this observed increased risk of MI but due to the varied nature of investigations to date (small or inappropriate study populations, non-randomised studies, differing experimental techniques and measurements) it has been difficult to come to a clear conclusion.

In my thesis I will use data from a randomised controlled clinical trial examining platelet aggregation and activation following a switch away from an abacavir based regimen. I will also explore the effect on platelets of the antiretrovirals abacavir and lamivudine (the antiretroviral commonly combined with abacavir) singularly and in combination, *in vitro* and in a mouse model of thromboembolism. Finally, I will explore a potential mechanism for antiretroviral induced platelet activation, looking at redox control of platelet function by examining the effect of abacavir, lamivudine and abacavir + lamivudine on nitric oxide donor mediated inhibition of platelet aggregation as well as the effect on ROS production.

1.17 Hypotheses

The hypotheses of this thesis are:

Hypothesis 1

Switching from an abacavir containing to a non-abacavir containing cART regimen will be associated with a reduction in platelet activation and aggregation in people living with HIV.

Hypothesis 2

Using *in vitro* and *in vivo* models, platelet activation and aggregation will be increased in the presence of abacavir but not other NRTIs.

Hypothesis 3

Preincubation of platelets with abacavir will reduce NO-donor mediated inhibition of platelet aggregation and will increase collagen-induced reactive oxygen species production compared to platelets preincubated with vehicle control and lamivudine.

Chapter 2: Materials and Methods

2.1 GS-US-311-1717 platelet sub-study

The GS-US-311-1717 Platelet Sub-study was a substudy of a phase 3b, randomised, double blind, switch study to evaluate emtricitabine/tenofovir alafenamide in HIV-1 positive subjects, virologically suppressed on regimens containing abacavir plus lamivudine. The study was registered with ClinicalTrials.gov, number NCT02469246 and funded by Gilead Sciences Inc. The study was carried out in accordance with the Declaration of Helsinki and approved by the National Research Ethics Service Committee south east coast – Brighton and Sussex. All participants gave written informed consent.

The GS-US-311-1717 platelet sub-study was designed by Professor Patrick Mallon and Professor Dermot Kenny and was carried out across 4 sites in Dublin and London. Platelet aggregation and flow cytometric analysis of samples in Dublin was carried out by Robert Maughan at UCD, and I performed the aggregation assays and flow cytometry for samples in London. Analysis and interpretation of the results for the platelet aggregation, platelet markers and sGPVI measurement was carried out by the team in Dublin. I analysed and interpreted the maximum aggregation data from the subjects recruited in London.

2.1.1 Participants

Participants were recruited from 4 study sites: Mater Miscordiae University Hospital and St James Hospital in Dublin and St Mary's Hospital and King's College Hospital in London.

Eligible participants were aged ≥18 years, receiving abacavir/lamivudine in combination with a third agent for ≥6months prior to the screening visit with plasma HIV-1 RNA levels <50 copies/mL for 6 months prior to the screening visit. Participants also had to have no clinically significant abnormalities on electrocardiogram (ECG), estimated glomerular filtration rate (eGFR) of ≥50mL/min, liver transaminases (AST and ALT) and serum amylase ≤5x upper limit of normal and total bilirubin of 1.5mg/dL or less. Females were eligible if they were not pregnant or of non childbearing potential. Those who were, had to agree to use a highly effective method of contraception, practise abstinence

or be non heterosexually active for the duration of the study and for 30 days after discontinuation of the study drug.

Those diagnosed with an AIDS-defining condition 30 days prior to screening, had positive hepatitis B surface antigen results, experiencing decompensated cirrhosis, or were receiving bisphosphonate for treatment of bone disease were excluded from the study. Those with a positive serum pregnancy, implanted defibrillator or pacemaker, and those with a history of malignancy within the last 5 years were also ineligible for the study. Individuals with a serious infection that needed antibiotics or antifungal treatment, or any other clinical condition or therapy deemed by the investigator to make the subject unsuitable for the study or prevent compliance with dosing requirements, were also not enrolled, nor were those partaking in other clinical trials or taking drugs that could potentially interact with the study drugs.

2.1.2 Primary outcome measures

Changes in population derived agonist concentrations inducing 50% platelet aggregation between groups at week 4 and week 12.

2.1.3 Secondary outcome measures

Between group differences in platelet surface expression of GPVI, CD42b and P-Selectin at baseline and week 12 pre and post stimulation with CRP-XL

2.1.4 Sample size

Based on 80 subjects (40 per arm) and previous data from 5µM epinephrine-evoked platelet aggregation in abacavir treated subjects, this study would provide 65% power to determine a change of 12% in platelet aggregation in participants switching to emtricitabine/tenofovir alafenamide as being statistically significant based on $P<0.05$.

2.1.5 Study design

The study was a substudy of a randomised, double-blind, multicentre, active controlled trial to assess the safety and efficacy of switching from abacavir/lamivudine to emtricitabine/tenofovir alafenamide fixed dose combination (FDC) tablet versus continuing abacavir/lamivudine in virologically suppressed HIV-1 RNA (<50 Copies/mL) subjects on a stable regimen for ≥6 months prior to the screening visit.

Subjects were randomly assigned, in a 1:1 ratio, according to a computer generated sequence (block size 4), to switch from abacavir/lamivudine (600/300mg) to emtricitabine/tenofovir alafenamide (200/10 or 25mg) + placebo to match abacavir/lamivudine or continue with abacavir/lamivudine + placebo to match emtricitabine/tenofovir alafenamide. Randomisation was stratified by the third agent (boosted PI versus other agents).

Treatment arm 1: emtricitabine/tenofovir alafenamide + placebo to match abacavir/lamivudine. Third agent remains the same.

Treatment arm 2: abacavir/lamivudine + placebo to match emtricitabine/tenofovir alafenamide. Third agent remains the same.

2.1.6 Study procedures

2.1.6.1 Screening visit

The screening visit occurred within 30 days of the baseline visit, this visit included a detailed explanation of the study and a discussion about trial safety and any potential side effects before signing an informed consent form. A detailed medical history was obtained, including a history of HIV-1 related events and prior ARV drug exposure within 30 days of the screening visit. Volunteers underwent a complete physical examination; vital signs were checked, and body weight and height measured. Blood samples were collected for haematology, biochemistry, plasma HIV-1 RNA and, CD4+ T cell count, Hep B surface antigen serology and serum pregnancy test, where applicable. Urine was also collected for urinalysis and chemistry.

2.1.6.2 Day 1

Blood was collected for a chemistry profile and metabolic assessment, a haematology profile, CD4+ T cell count, plasma HIV-1 RNA and hepatitis C virus antibody (HCVAb) serology. Blood was also collected for platelet aggregation analysis, platelet surface marker analysis and for the measurement of soluble markers of platelet activation.

A subject number was assigned, and the subject was randomised via the interactive unique web response system (IWRS) and the study drug was dispensed in a blinded fashion.

2.1.6.3 Follow up visits

Study visits were completed within ±2 days of the specified timepoint with respect to the day 1 visit. At each visit there was a review of adverse events (AE), changes in any concomitant medications, and vital signs were checked. At week 4 and week 12 blood was collected for the platelet aggregation assay and platelet activation soluble marker analysis. At week 12 an additional sample was taken for the measurement of platelet surface markers.

Urine was collected for chemistry, unrinalysis, evaluation of renal function and a pregnancy test where applicable. Blood was also collected for a chemistry profile, metabolic assessments, pharmacokinetic analysis, plasma HIV-1 RNA, evaluations of inflammation and bone turn over assessment.

2.1.6.4 Plate-based platelet aggregation assay

Blood was collected from subjects using a 21 gauge needle into 4 x 10.0 mL S-Monovette® blood collection system containing 3.2% sodium citrate (Sarstedt UK, Leicester, UK), giving a final ratio of 1:9 (citrate:blood). Blood was rested for 30 minutes, centrifuged at 170g for 10 minutes and platelet rich plasma subsequently transferred to a reagent reservoir. 1mL of the PRP was centrifuged at 1600g for 2 minutes leaving platelet poor plasma.

180µl of PRP was added to increasing concentrations of the platelet agonists: arachidonic acid, collagen, epinephrine, adenosine diphosphate (all, Bio/Data Corporation; Horsham, PA) and Thrombin Receptor Activator Peptide 6 (Sigma Aldrich; Dorset, UK) in a 96-well plate. Agonists were diluted with trisodium citrate 13 mM, NaCl 123 mM and glucose 33 mM buffer solution (all, Sigma Aldrich; Dorset, UK), pH 7.0, to achieve the desired concentration. The final concentrations were AA 0.006-5mg/mL, collagen 0.002-0.19mg/mL, ADP 0.156-20µM, epinephrine 0.001-20µM and TRAP-6 0.156-20µM. The negative control was PRP alone and positive control was the PPP **(Figure 2.a**).

Platelet aggregation was measured as light absorbance using a SpectraMax® M2 multidetection microplate reader (Molecular Devices; Sunnyvale, CA). The plate was read 7 times at 3 minute intervals at 572nm. Percentage aggregation was calculated with respect to the 0% control (PRP) and the 100% control (PPP) light absorbance values. The percentage aggregation values for each concentration of agonist were plotted as dose response curves. All samples were assayed within 2 hours of blood collection.

Figure 2.a Agonist concentrations and plate layout for plate based platelet aggregation assay

	AA (mg/mL)	Collagen (mg/mL)	ADP (µM)	Epinephrine (µM)	TRAP (µM)	
A	0.006	0.002	0.156	0.001	0.156	PRP
B	0.012	0.004	0.313	0.005	0.313	PRP
C	0.023	0.009	0.625	0.020	0.625	PRP
D	0.047	0.018	1.250	0.078	1.250	PRP
E	0.094	0.036	2.500	0.313	2.500	PPP
F	0.188	0.071	5.000	1.250	5.000	PPP
G	0.375	0.143	10.000	5.000	10.000	PPP
H	0.500	0.190	20.000	20.000	20.000	PPP

Schematic representation of the agonist concentrations and plate used for the 96-well platelet aggregation assay. % aggregation was calculated with respect to 0% and 100% (PRP and PPP) controls.

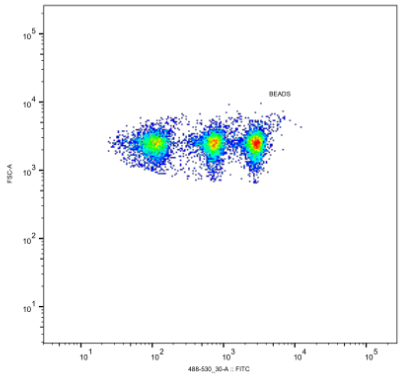
2.1.6.5 Platelet surface marker assay

Surface expression of the platelet glycoproteins CD42b (GPIb) was measured by single colour flow cytometry using a BD LSRFortessa™ flow cytometer (BD Biosciences, Belgium) and the Biocytex PLT Gp/Receptors kit (Biocytex, ID 7004, Marseille, France) according to the manufacturer's instructions. Calibration beads coated with known quantities of mouse IgG molecules (5,900, 36,000, and 133,000) were stained with polyclonal anti mouse IgG-FITC, the mean fluorescence intensity was measured, and a calibration curve was produced. For samples, the number of glycoprotein molecules per platelet was determined by converting the mean fluorescence intensity (MFI) into the corresponding number of sites, based on the calibrated bead curve after subtracting the negative isotypic control measurement. Quantitative differences in platelet activation were determined by measuring GPVI and CD62P expression in the presence or absence of cross-linked collagen related peptide (CRP-XL) (**Figure 2.b**).

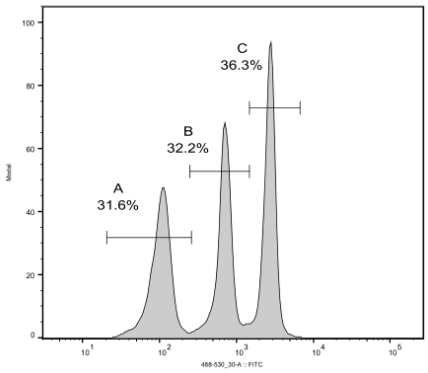
10µL of 1:8 diluted PRP (PRP:Biocytex reagent 1) was incubated with CRP-XL or buffer alone , GPVI antibody clone 4B8 (Donated by Professor Dermot Kenny, Royal College of Surgeons in Ireland) and CD62P-PE (BD Biosciences; Belgium) were added and incubated at room temperature in the dark for 10 minutes. 10µL of FITC antibody was then added to the GPVI antibody-bound sample and incubated for a further 10 minutes. Samples were diluted to 1mL with reagent 1 and analysed within 2 hours. A minimum of 10000 counts were recorded for analysis. Data were analysed using FlowJo software (v10.4; LLC, Ashland, OR, USA)

Figure 2.b Platelet surface marker quantification

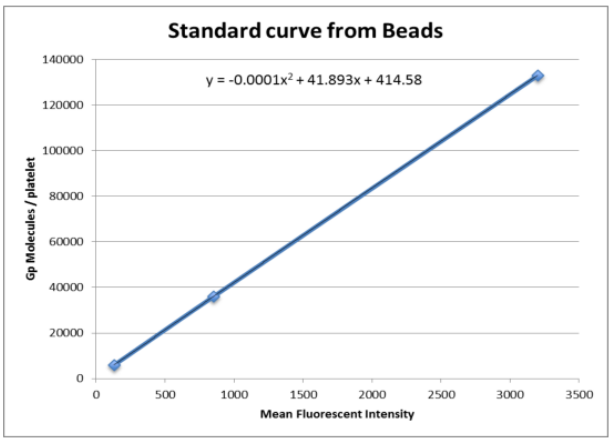
A



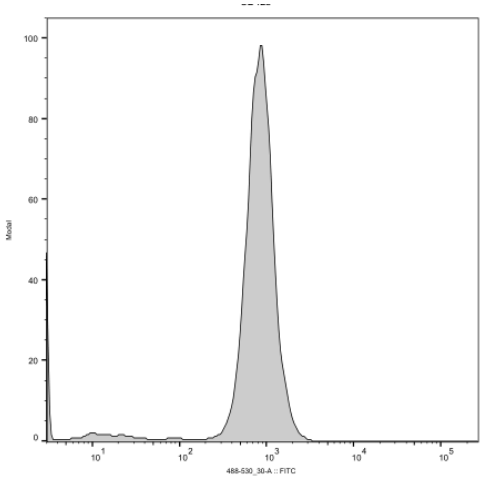
B



C



D



3 bead populations with known quantities of mouse IgG molecules (5,900, 36,000, and 133,000) (A and B) were incubated with FITC-conjugated anti mouse IgG and analysed by flow cytometry. The mean fluorescence intensity was plotted against the known numbers of antigenic sites to make a standard curve (C). Test sample surface marker (D) quantities per platelet could then be determined by the mean fluorescence intensity.

2.1.6.6 Measurement of soluble GPVI

Soluble GPVI (sGPVI) measurement was carried out on samples from the main study population of 545 taken at baseline, week 4, week 12, week 24 and week 48 by the team in Dublin. Blood was collected into 6mL BD K2EDTA Vacuette ®, centrifuged at 1500 g for 15 minutes and the plasma collected.

96 well binding plates from MesoScale Discovery (MSD) (Rockville, MD) were coated with sheep anti-human GPVI polyclonal antibody (R&D Systems, Abingdon, UK) 5% MSD Blocker A was added to block the plate for 1hr at room temperature and washed 3 times with 150µL PBS/0.05% Tween. 25µL platelet poor plasma from was added to the wells and samples were incubated at room temperature with vigorous shaking for 1 hour. The plate was subsequently washed 3 times with PBS/0.05 Tween and 25µL biotinylated sheep anti-human GPVI antibody at 1µg/mL (diluted in 1% MSD Blocker A) was added to each well. The plate was incubated at room temperature or 1 hour shaking at 650 rpm and then washed 3 times with PBS/0.05% Tween. 150µL 2x read buffer was added to each well and the plate was read on an MSD Quickplex SQ120 plate scanner as per the manufacturer's instructions.

2.1.7 Data and statistical analysis

Platelet aggregation was determined from the light absorbance values using the PRP control sample at time 0 as the 0% aggregation control and the PPP control sample as the 100% aggregation control. To calculate the mean platelet aggregation, the mean value for light absorbance at the last 3 timepoints for each agonist concentration was determined and dose response curves were plotted (**Figure 2.b**). 4 parameter logistic regression was used to calculate the log EC_{50} (i.e. the concentration of platelet agonist needed to induce a 50% platelet aggregation) and between group variances were determined by F test.

Platelet surface marker data are presented as arithmetic mean ± standard error of mean [SEM] and percentage changes where appropriate. Differences between the groups at baseline and week 12, and pre and post stimulation with CRP were compared by Wilcoxon rank sum test.

The primary endpoint for the sGPVI measurement was change to week 48 and differences between the group analysed using mixed effects regression models.

2.2 *In vitro* laboratory materials and methods

2.2.1 Platelet preparation

Blood was taken from volunteers (without any chronic illnesses nor having taken any non steroidal anti-inflammatories in the previous 14 days) into a syringe containing 1:9 volume ACD using a 1 gauge butterfly cannula. Informed consent was obtained from all donors before procedures were carried out and ethical approval was given by the Imperial College London Research Ethics Committee.

Blood was centrifuged at 175g for 15 min to obtain PRP. Where indicated, to produce washed platelet suspensions, PRP was transferred to a falcon tube and, following the addition of 150µL ACD and 5µL 2µM prostaglandin E_1 (PGE_1), centrifuged at 1400g for 10 min. The supernatant was discarded, and the platelet pellet was gently resuspended in modified Tyrode's HEPES buffer (in mM: 134NaCl, 2.9 KCl, 20 HEPES, 0.34 Na_2PO_4, 12 $NaHC0_3$, 1 $MgCl_2$, 5 glucose; pH 7.4) and centrifuged twice with the addition of 5µL 2µM PGE1 in between steps. The total platelet count was determined using the Accuri™ C6 flow cytometer (BD Oxford), UK) and platelets were then diluted to a concentration of $2.5X10^8$ μL^{-1} with Tyrode's HEPES buffer and allowed to rest at room temperature for 30 mins.

2.2.2 96 well platelet aggregation assay

Platelets were incubated with ARV agent (US Biologicals, Massachusetts, USA) or vehicle control for 30 min at 37°C and added to a 96 well plate (VWR, Leicestershire, UK) containing platelet agonists at a range of concentrations: ADP 0.1-30µM, TRAP-6 0.1-30µM (Sigma-Aldrich, Dorset, UK), Collagen 0.1-30 µg mL^{-1} (Takeda, Osaka, Japan) . Thrombin 0.03-10UmL^{-1} (Sigma-Aldrich, Dorset, UK) was used in place of TRAP-6 where washed platelets were used.

Absorbance at 595nm was measured for 16 mins at 20 second intervals with shaking for 7 seconds before each reading using a Power Wave XS plate reader (BioTek, Swindon, UK) the temperature was maintained at 37°C for the course of the experiment. Maximum aggregation was calculated as percentage change in absorbance versus baseline.

2.2.3 Platelet activation markers

PRP was incubated with drug or vehicle control and subsequently diluted 1:200 in Tyrode's HEPES buffer containing PAC-1 (Cat#340507, RRID: AB_2230769), CD62P (Cat#550888, RRID: AB_398475) and CD63 antibodies (Cat#556019, RRID: AB_396297) (BD Biosciences, Belgium). Samples were analysed using the Accuri™ C6 plus flow cytometer (BD biosciences). Following 30 seconds of data acquisition, collagen, ADP or TRAP6 was added (10µgmL^{-1} or 10µM respectively) and data collected for a total of 5 minutes. Data were analysed using FlowJo (v10.4; LLC, Oregon, USA). The relative increase in fluorescence was calculated.

2.2.4 Light transmission aggregometry

Following incubation with C_{max} concentration of drug or vehicle control, 425µL of PRP was transferred to siliconised glass cuvettes and placed in a Chrono-log 490 aggregometer (Chrono-log, Lab Medics, Abingdon, UK). Where applicable PRP was incubated with 5µM S-Nitroso-N-acetylpenicillamine (SNAP) alone or following incubation with 20µM 1H-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (both for 3 minutes). 5µg/mL collagen was then added, and light transmission measured over 5 minutes using the Chono-log software (Chrono-log Coorperation, Haverton, PA, USA).

2.2.5 Reactive oxygen species measurement

Washed platelets were diluted to $2.5x10^8$ platelets mL^{-1} in Tyrode's HEPES buffer and incubated with C_{max} concentration of drug or vehicle control and 10µM 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) (Molecular Probes, Eugene, OR, USA) for 15 min in the dark. Samples were analysed using the Accuri™, where data was acquired for 30 sec prior to the addition of collagen or thrombin. The change in fluorescence was measured from baseline over 5 min and relative change in fluorescence was calculated using FlowJo.

2.3 *In vivo* laboratory materials and methods

2.3.1 Mice

Male C57BL/6 mice weighing ~20-25g were obtained from Harlan (Bicester, UK) and given one week to acclimatise to conditions prior to any procedures being carried out. Mice were kept in IVC cages (2-5 per cage) with regular rodent chow and water within the Central Biomedical Services unit at Imperial College London. Procedures were carried out under the Home office project licence PPL 70/8791 and approved by the Imperial College London Animal Welfare and Ethical Review Board.

2.3.2 Mouse platelet preparation

Blood from terminally anaesthetised mice (10mL $Kg^{-1}$of 25% (w/v) urethane administered i.p)) (Sigma-Aldrich, Dorset, UK) was collected by cardiac puncture using a 25G needle into a 2mL syringe containing 200µL ACD (Sigma-Aldrich, Dorset, UK). Mice were terminated by cervical dislocation.

Blood was centrifuged at 300 g for 3 minutes and PRP transferred to a fresh tube. 400µL of Tyrode's/ACD solution (10:1) with 2.5µM PGE_1 was added to the remaining blood and centrifuged again for 3 minutes at 300 g. The PRP was combined with that from the original centrifugation and centrifuged at 200 g for 2 minutes to pellet the red blood cells and PRP underwent an additional

centrifugation (1500 g for 7 minutes). The pelleted platelets were resuspended in 1mL Tyrode's/ACD/PGE_1 solution.

Platelets were incubated for 10 minutes with 1.8 MBq ^{111}Indium tropolone (^{111}In), centrifuged at 1500g for 5 minutes and washed gently and resuspended in Tyrode's buffer (in mM: 137NaCl, 2.7 KCl, 0.14 Na_2PO_4, 12 $NaHC0_3$, 5.5 glucose). The radiolabelled platelets were then rested for 30 minutes.

2.3.3 In vivo platelet monitoring

Following i.p. administration of urethane (10mL $Kg^{-1}$of 25% (w/v)) to anaesthetise recipient mice, they were assigned at random to receive either a single i.p. injection of drug or vehicle (saline). To compensate for the increased metabolic capacity versus humans, mice were administered a dose to achieve a C_{max} level 10x that observed in humans. Abacavir and lamivudine were administered at a concentration of 1,755µg.Kg^{-1} and 1,170 µg.Kg^{-1} respectively. 220µL of radiolabelled platelets were infused via the femoral vein using a 29G needle and left to equilibrate for 10 minutes. 30 minutes after the introduction of drug or vehicle Collagen was administered i.v. and platelet responses were monitored using a 1cm SPEAR (Single Point Extended Area Radiation) detector (eV Products, PA, USA) placed over the pulmonary vascular bed. Radioactive counts were measured continuously over 10 minutes using a UCS-20 spectrometer and software supplied by Mumed Systems (London, UK).

All experiments were conducted on a heat mat at 37°C to maintain the core body temperature of the mice. Following the completion of procedures, mice were terminated by cervical dislocation, and exsanguination was carried out as confirmation of death. I carried out the experimental work with Kirk Taylor for the lamivudine experiments and abacavir + lamivudine experiments, and Kirk Taylor carried out the abacavir experiments, which has since been published (206).

2.3.4 Data and statistical analysis

Data were analysed in GraphPad Prism and presented as the mean ± SEM. Where there were two group comparisons, data were tested by a paired student's *t* test with a two-tailed test of significance. For multiple comparisons where there were single or multiple variables, a one way or two way ANOVA were performed respectively with Tukey's multiple comparison post hoc analysis. For clarity, data were displayed as normalised values. A p value of <0.05 was considered to be statistically significant. * p≤0.05, ** p≤0.01, ns: not significant

2.4 Materials

Table 2.a Table of materials

Compound	**Supplier**
1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ)	Sigma-Aldrich (Dorset, UK)
2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA)	Molecular Probes (Eugene, OR, USA)
Abacavir sulfate	Selleckchem (Houston,TX, USA)
Adenosine Diphosphate (ADP)	Bio/Data Corporation (Horsham, PA, USA)
Adenosine Diphosphate (ADP)	Sigma-Aldrich (Dorset, UK)
Arachidonic Acid (AA)	Bio/Data Corporation (Horsham, PA, USA)
Biocytex PLT Gp/Receptors kit 7004	Biocytex (Marseille, France)
Carbovir Triphosphate	US Biologicals (Salem, MA, USA)
APC Mouse Anti-Human CD62P	BD Biosciences (Belgium)
PE Mouse Anti-Human CD63	BD Biosciences (Belgium)
Collagen (Type 1)	Bio/Data Corporation (Horsham, PA, USA)
Collagen (Type 1)	Takeda Pharmaceutical Company (Osaka, Japan)
Epinephrine	Bio/Data Corporation (Horsham, PA, USA)
Glucose	Sigma-Aldrich (Dorset, UK)
Human glycoprotein VI (GPVI) Antibody	R&D Systems (Minneapolis, MN, USA)
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Sigma-Aldrich (Dorset, UK)
Indium In 111 (tropolone) (In^{111})	Mallinckrodt Pharmaceuticals (Surrey, UK)
Lamivudine	Selleckchem (Houston, TX, USA)
Magnesium chloride ($MgCl_2$)	Sigma-Aldrich (Dorset, UK)
Mouse anti human (GPVI)	Donated by Professor Dermot Kenny, Royal College of Surgeons in Ireland
FITC Mouse Anti-Human PAC-1	BD Biosciences (Belgium)
PE Mouse Anti-Human CD62P (Clone AC1.2)	BD Biosciences (Belgium)
Potassium chloride (KCl)	Sigma-Aldrich (Dorset, UK)
S-nitroso-N-acetyl-penicillamine (SNAP)	Sigma-Aldrich (Dorset, UK)
Sodium bicarbonate ($NaHCO_3$)	Sigma-Aldrich (Dorset, UK)
Sodium Chloride	Sigma-Aldrich (Dorset, UK)
Sodium phosphate dibasic ($Na2HPO_4$)	Sigma-Aldrich (Dorset, UK)
Thrombin	Sigma-Aldrich (Dorset, UK)
Thrombin Receptor Activator Peptide 6 (TRAP-6)	Sigma-Aldrich (Dorset, UK)
Trisodium Citrate	Sigma-Aldrich (Dorset, UK)
Urethane	Sigma-Aldrich (Dorset, UK)
XL CRP	Donated by Professor Dermot Kenny, Royal College of Surgeons in Ireland
Acid citrate dextrose (ACD)	Sigma-Aldrich (Dorset, UK)
Tropolone	Alfa Aesar (Heysham, UK)
Prostaglandin E1 (PGE1)	Sigma-Aldrich (Dorset, UK)

2.5 Buffers

Table 2.b Table of buffers

Buffer	Components	Molarity
CSG buffer	Trisodium citrate NaCl Glucose	13 mM 123 mM 33 mM
Modified Tyrode's-HEPES buffer	NaCl KCl $NaHCO_3$ Na_2HPO_4 HEPES $MgCl_2$ Glucose	134 mM 2.9 mM 12 mM 0.34 mM 20.00 mM 1 mM 4.5 mM
Tyrode's (*in vivo*) buffer	NaCl KCl Glucose $NaHCO_3$ Na_2HPO_4	137 mM 2.70 mM 5.50 mM 12.0 mM 0.14 mM
TAP buffer	Tyrodes (*in vivo*) buffer ACD PGE1	(9 volumes) (1 volume) 2.5µM

2.6 Equipment

Table 2.c Table of equipment used

Equipment	Supplier
Chrono-log 400 aggregometer	Chrono-log, Lab Medics (Oxfordshire, UK)
Chrono-log Aggrolink software	Chrono-log Lab Medics (Oxfordshire, UK)
Power Wave XS plate reader	BioTek (Swindon, UK)
SpectraMax® M2 multidetection microplate reader	Molecular Devices (Sunnyvale, CA, USA).
Graphpad Prism	GraphPad (San Diego, CA, USA)
Siliconised glass cuvettes	Lab Medics (Oxfordshire, UK)
Microsoft Office Excel	Microsoft (Reading, UK)
Flow Jo	LLC (Ashland, OR, USA)
Accuri C6 Plus flow cytometer	BD Biosciences (Belgium)
BD LSRFortessa™ flow cytometer	BD Biosciences (Belgium)
UCS-20 Spectrometer	Mumed systems (London, UK)
Single Point Extended Area Radiation (SPEAR) detector	eV Products (Saxonburg, PA, USA)
Radioactive count software	Mumed systems (London, UK)
KC4 Data analysis software	BioTek (Swindon, UK)

Chapter 3: Clinical Results

3.1 Introduction

Since the introduction of cART there has been a dramatic decrease in AIDS related deaths and there is now a focus on the treatment of non-infectious co morbidities, where cardiovascular diseases, including MI is among the leading causes of death in people living with HIV (213, 214).

There are a number of contributing factors to this increased CVD risk, and these can be broadly categorised into HIV associated inflammation, lifestyle factors as well as HIV drug associated toxicity (215).

The DAD study was the first to report an association between exposure to the NRTI abacavir and MI (102) and many subsequent studies have been performed to explore this signal further. However, despite several studies, a consensus has not been reached (102, 110, 122, 216). This is due to a number of reasons, including poorly defined outcome measures or definitions, the nature of the studies conducted E.g., observational and retrospective where causality cannot be determined, and trials conducted with inappropriate study subjects (studies in cohorts that are unrepresentative of the affected population).

Given that the increased risk observed was reversible, one potential mechanism could be drug induced activation of platelets (126). Platelets are key mediators in MI, where they are involved in the initial formation of a thrombus and recruitment of leukocytes, which is also associated with acute thrombosis (141, 217)

The SWIFT study was the first prospective randomised controlled study to investigate the link between abacavir use and MI by investigating the effect of a switch away from an abacavir-based regimen to a tenofovir-based regimen on platelet function. The results showed that a switch away from abacavir to tenofovir disoproxil fumarate led to an increase in sGPVI concentrations, suggesting changes in intrinsic platelet function (218). However, this study only assessed platelet function through serum

markers of activation and not aggregometry- the gold standard measurement of platelet activation (202).

The GS-US-311-1717 study, sponsored and funded by Gilead Sciences, Inc, was a randomised, double blind, multicentre, active controlled study to evaluate the safety and efficacy of switching to fixed-dose emtricitabine/tenofovir alafenamide versus remaining on fixed-dose abacavir/lamivudine. This study presented the opportunity to perform a comprehensive examination of the effect of a switch away from abacavir/lamivudine to emtricitabine/tenofovir alafenamide on platelet function, through platelet aggregation assays and surface marker analysis. This substudy was the first randomised, in-depth comparison of platelet function in the presence and absence of abacavir in people living with HIV.

The first hypothesis of this thesis is that switching from abacavir containing to a non-abacavir containing cART regimen will be associated with a reduction in platelet aggregation and activation in people living with HIV. This was tested by assessing changes in platelet aggregation and activation in response to platelet agonists in subjects randomised to the GS-US-311-1717 platelet sub study.

In this sub study, participants were randomised in a 1:1 fashion to remain on abacavir/lamivudine plus a 3rd agent or switch to emtricitabine/tenofovir alafenamide plus a 3rd agent, and platelet activation was measured at baseline , week 4 and week 12 by a plate based light transmission aggregometry assay. Platelet function was measured by flow cytometric analysis of surface activation markers and soluble markers of activation.

The substudy aimed to recruit 80 subjects from the main study, across 4 sites: The Mater Misericordiae University Hospital and St James's Hospital in Dublin, and St Mary's Hospital and King's College Hospital in London. A sample size of 80 with 40 per arm was calculated to provide 65% power to determine a change of 12% in platelet aggregation in subjects switching to emtricitabine/tenofovir alafenamide as statistically significant ($p= \leq 0.05$).

Based on previously observed percentage changes in platelet aggregation following exposure to epinephrine (5µM) in abacavir treated subjects, a sample size of 80 subjects with 40 per arm was calculated to provide 80% power to determine a between group difference of 15% in platelet aggregation as statistically significant.

3.2 Hypothesis

Switching from an abacavir containing to a non abacavir containing cART regimen will be associated with a reduction in platelet activation and aggregation in people living with HIV.

3.3 Aims

The aims of this chapter were:

1) To examine the effect of switching from abacavir/lamivudine to emtricitabine/tenofovir alafenamide on platelet aggregation
2) To perform exploratory analysis on the effect of a switch from abacavir/lamivudine to emtricitabine/tenofovir alafenamide on platelet surface receptors GPVI, CD42b and the activation marker, P-selectin,
3) To examine the effects of switching from abacavir/lamivudine to emtricitabine/tenofovir alafenamide on the biomarker of platelet function: soluble glycoprotein VI

3.4 Methods

3.4.1 Study design and participants

GS-US-311-1717 platelet substudy participants were recruited from 4 study sites in London and Dublin from subjects already enrolled to the main study. Samples collected at King's College Hospital were transferred to the St Mary's Hospital site as soon as possible on a dedicated courier service to ensure they could be assayed within 2 hours. Samples collected in Dublin (Mater Misericordiae University Hospital and St James's Hospital) were transferred to the HIV molecular research group, MMHU and were also assayed within 2 hours of collection.

3.4.2 Platelet aggregation

PRP was prepared from participants within 2 hours of blood draw at baseline, week 4 and week 12 and added to increasing concentrations of 5 platelet agonists- arachidonic acid, collagen, ADP, epinephrine, and TRAP-6. Light absorbance was measured at 572 nm at 3 minute intervals over 21 minutes, using a Spectromax ® M2 multidetection microplate reader (Molecular Devices; Sunnydale, CA)

Percentage aggregation was determined using the mean of the last 3 absorbance readings with respect to the 0% aggregation control (PRP) and 100% aggregation control (PPP). Dose response curves were plotted using the percentage aggregation values at each agonist dose.

3.4.3 Surface marker analysis

Expression of the surface markers CD42b, p-selectin and GPVI was measured at baseline and week 12 by single colour flow cytometry using the PLT Gp/Receptors kit (Biocytex, ID 7004, Marseille, France).

A standard curve was constructed based on the MFI measured following FITC labelled antibodies binding known quantities of the corresponding antigenic sites on beads and the platelet surface

markers were quantified by converting the MFI into the corresponding number of sites using the calibration curve (**Figure 2.b.C**).

Differences in P-selectin surface induction and GPVI shedding were examined by measuring expression in the presence and absence of the highly potent agonist XL-CRP (6.25µg/mL). Data were analysed using FlowJo software (v10.4; Oregon, USA) as % inducted (P-selectin) or % shedding (GPVI).

3.4.4 Soluble GPVI analysis

sGPVI was measured in platelet poor plasma collected from participants enrolled to main study at baseline, week 4, week 12, week 24 and week 48. 96 well binding plates from Meso Scale Discovery were coated with sheep anti-human GPVI polyclonal antibody (R&D Systems, Abingdon UK) overnight, non-specific binding was prevented by adding 5% MSD blocker and incubating for 1 hour at room temperature. Plates were washed 3 times with 150µL PBS/0.05% Tween, and 25µL of undiluted platelet poor plasma. Sample were incubated at room temperature, shaking for 1 hour. Following another washing step, 25µL biotinylated sheep anti human GPVI antibody (1µg/mL) was added to each well, the plate was incubated at room temperature for 1 hour shaking and washed 3 times. 150 µL 2x read buffer was added to each well and the plate was read on a Meso Scale Quickplex SQ120 Plate Scanner.

3.5 Results

3.5.1 Participants

556 participants were recruited to the GS-US-311-1717 study and within this main study, 61 participants were recruited to the platelet sub study (43 participants from London and 18 from Dublin). 32 were randomised to remain on the abacavir/lamivudine regimen and 29 to switch to emtricitabine/tenofovir alafenamide. I carried out the platelet aggregation and flow cytometry for the participants from London and the experimental work on samples from patients recruited in Dublin

was carried out by Robert Maughan. Statistical analysis and plotting of graphical data was carried out by the team in Dublin.

Baseline characteristics were comparable between the 2 groups (**Table 3.a**). The median (IQR) age was 50 (49-53) in those randomised to the emtricitabine/tenofovir alafenamide arm and 49 (38-54) in those remaining on abacavir/lamivudine, representing an older cohort of people living with HIV receiving care in the UK. The participants had been stable on an abacavir/lamivudine regimen for a median of 8 years (emtricitabine/tenofovir alafenamide arm: 3-10 years and abacavir/lamivudine arm: 4-10 years) and the median platelet counts were within the normal range of 150-450 x 10^9/L. Participants were well matched for CVD and CVD risk factors.

Table 3.a GS-US-311-1717 platelet sub study participant baseline characteristics

	Emtricitabine/Tenofovir Alafenamide n=29	**Abacavir/Lamivudine n=32**
Age (years) (range)	50 (43, 53)	49 (38, 54)
Female (%)	28	31
White (%)	52	60
CD4 count (cells/mm^3)	659 (503, 833)	616 (512, 774)
Duration on abacavir/lamivudine (years)	8 (3, 10)	8 (4, 10)
Platelet count (10^9/L)	203 (186, 235)	218 (190, 265)
Current smoker (%)	17	22
Hyperlipidaemia (%)	31	41
Hypertension	28	34
Diabetes (%)	3	3
Cardiovascular disease (%)	3	6

Data are median (IQR) or %, unless otherwise specified

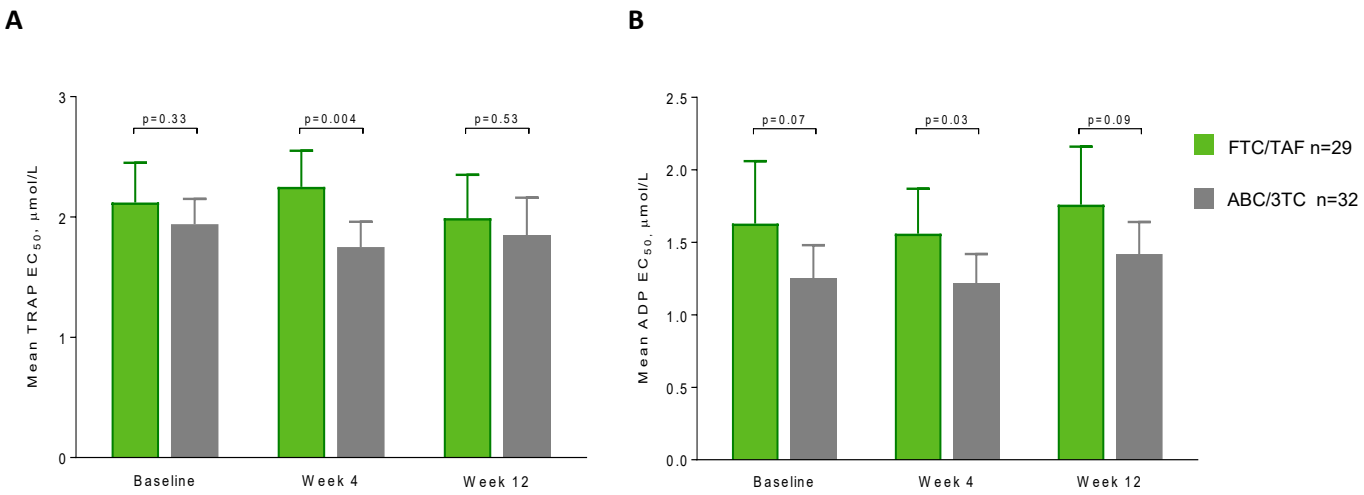
3.5.2 Platelet aggregation

To examine any changes in platelet reactivity following a switch away from abacavir and lamivudine over 12 weeks, platelet aggregation was assessed in response to varying concentrations of agonists (TRAP, ADP, collagen, arachidonic acid, and epinephrine) and EC_{50} determined. EC_{50} was defined as the concentration of agonist required to induce 50% of maximal aggregation. A lower EC_{50} indicates a

more reactive platelet profile, where a lower concentration of agonist is needed to induce 50% max aggregation.

There were no between group differences in platelet aggregation in response to any of the agonists at baseline. At week 4 the mean EC_{50} for TRAP was significantly lower for those in the abacavir/lamivudine arm versus the emtricitabine/tenofovir alafenamide arm (p=0.04), however, this did not persist to week 12 (**Figure 3.a A**). This was also seen when looking at the $P2Y_1$ and the $P2Y_{12}$ pathways, where the ADP EC_{50} was significantly lower in the abacavir/lamivudine arm at week 4 (p=0.03) but no difference was observed at week 12 **(Figure 3.a B**).

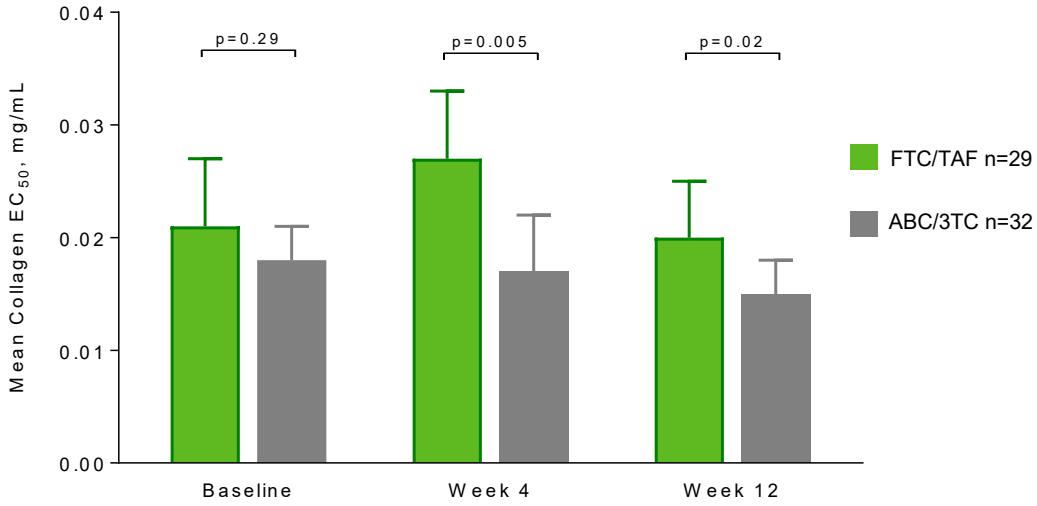
Figure 3.a Participant mean TRAP and ADP EC_{50} at baseline, week 4 and week 12



Platelet aggregation from trial participants was assessed upon exposure to TRAP (0.156- 20µM) (A) and ADP (0.156 – 20 µM) (B) at week 0 and week 4 and 12 post randomisation. Green bars represent participants in the emtricitabine/tenofovir alafenamide (FTC/TAF) arm and grey bars the abacavir/lamivudine (ABC/3TC) arm. Participants on emtricitabine/tenofovir alafenamide arm displayed increased *ex vivo* platelet aggregation in response to TRAP and ADP at week 4 but not at week 12 Data are displayed as mean TRAP(A) and mean ADP(B) EC_{50} values, between-group differences were compared by 4 parameter logistic regression.

In response to collagen, platelet aggregation for those on the emtricitabine/tenofovir alafenamide arm had reduced platelet aggregation (evidenced by higher mean EC_{50}) at both week 4 (p=0.005) and week 12 (p=0.02) **(Figure 3.b)**. There were no differences in platelet aggregation between the groups at any timepoint in response epinephrine and arachidonic acid.

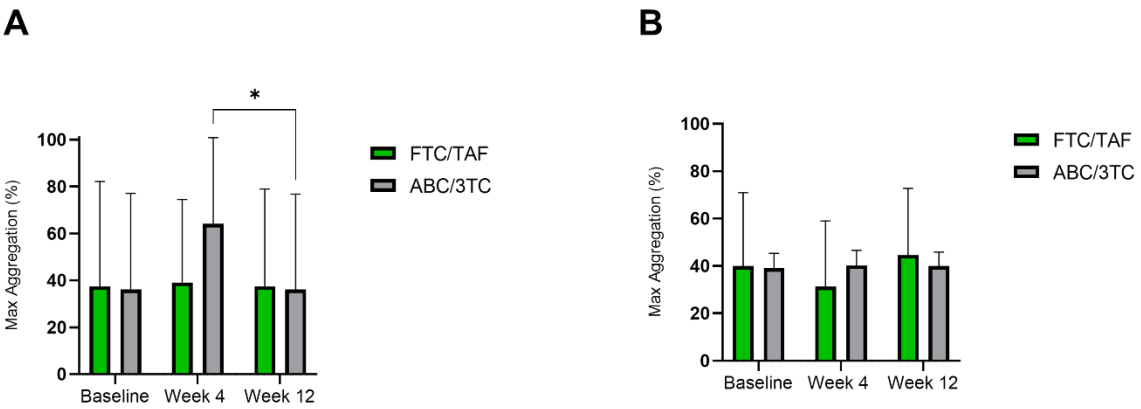
Figure 3.b Participant mean collagen EC_{50} at baseline, week 4 and week 12



Platelet aggregation from trial participants was assessed upon exposure to collagen (2- 190µg/mL) at week 0 and week 4 and 12 post randomisation. Data are displayed as mean collagen EC_{50} values, green bars represent participants in the FTC/TAF arm and grey bars the ABC/3TC arm Participants on FTC/TAF arm displayed decreased *ex vivo* platelet aggregation in response to collagen at week 4 and 12. between-group differences were compared by 4 parameter logisitic regression.

An important aspect that was not included in the final analysis for the platelet sub study was an examination of the platelet maximal response to agonists, which would give a picture of the capacity of response and any changes following a switch away from abacavir. As a sub analysis I took the baseline, week 4 and week 12 platelet aggregation data from the St Mary's Hospital participants (n=18; emtricitabine/tenofovir alafenamide arm: 7; abacavir/lamivudine: 11) and compared the maximal platelet responses to arachidonic acid, epinephrine (**Figure 3.c**), collagen, ADP and TRAP (**Figure 3.d**).

Figure 3.c St Mary's Hospital cohort maximal aggregation in response to arachidonic acid and epinephrine at baseline, week 4 and week 12



Platelet aggregation was assessed upon exposure to arachidonic acid (A) (0.006-0.5 mg/mL) and epinephrine (B) 0.001 – 20 µM at baseline, week 4 and week 12 post randomisation. Data are displayed as mean maximum aggregation in response to arachidonic acid and epinephrine for St Mary's Hospital trial participants (FTC/TAF: green; ABC/3TC: grey). Between group differences were compared by independent T-test and differences over time compared by 2-way ANOVA.

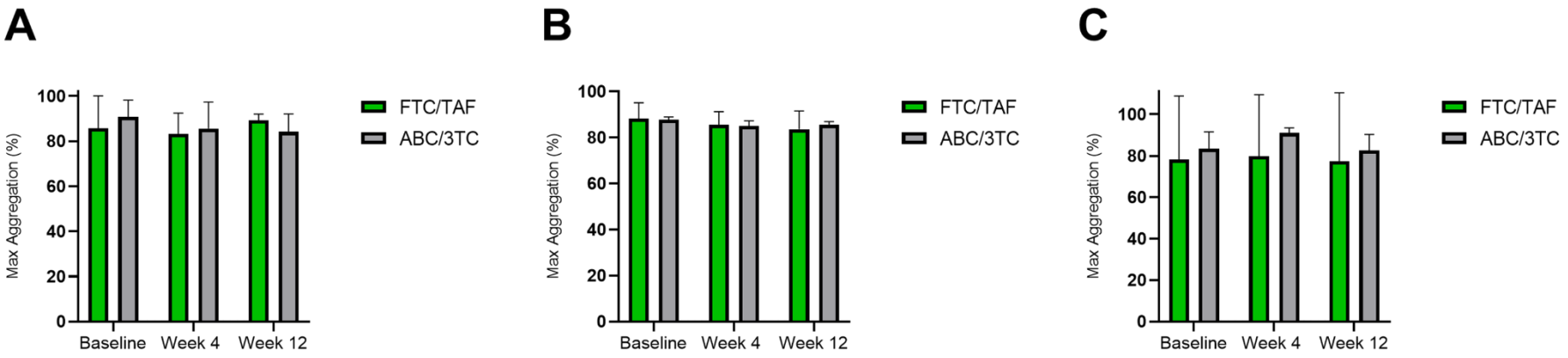
Aggregation responses to arachidonic acid and epinephrine were variable in both arms, at all visits, resulting in a mean max aggregation that was lower than would be expected. In the response to arachidonic acid, mean max platelet aggregation for those in the emtricitabine/tenofovir alafenamide arm was baseline: 37.54% ± 44. 66, week 4: 39.13% ± 35.37, week 12: 37.48% ± 41.49. In the abacavir/lamivudine arm max mean aggregation was baseline: 36.15 ± 41.00, week 4: 64.18 ± 36.68, week 12: 49.74 ± 41.80. There was no difference between the 2 arms but the mean max aggregation in the abacavir/lamivudine arm was significantly higher in week 4 vs week 12 64.18 ± 36.68 v 36.17 ± 40.66 (p= 0.0420).

The average max epinephrine responses were similarly reduced with all mean max aggregation figures below 50%. Emtricitabine/tenofovir alafenamide arm (baseline: 39.94 ± 31.04, week 4: 31.28 ± 27.74, week 12: 44.68 ±28.11) versus abacavir/lamivudine (baseline: 39.10 ±20.87, week 4: 40.27 ± 21.10, week 12: 39.92 ±19.66).

The mean max aggregation for samples in response to collagen, ADP and TRAP (**Figure 3.d**) were much higher with an overall mean max aggregation of 89.58%. There were no differences in mean max aggregation between the arms at each time point nor any differences in platelet responses in those who switched to emtricitabine/tenofovir alafenamide over ethe 12 weeks. However there was a non significant trend where platelets from those in the emtricitabine/tenofovir alafenamide group displayed a lower mean max aggregation than platelets from those remaining on abacavir (FTC/TAF v ABC/3TC, week 4: 79.79 ± 29.64 v 90.94 ± 8.38 ; week 12: 77.28 ± 33.09 v 82.51 ± 25.79).

Whilst differences were seen in the EC_{50} data responses in the overall dataset for collagen and TRAP, within the St Mary's cohort no differences were seen in the capacity of platelet responses between the two groups over the 12 weeks.

Figure 3.d St Mary's Hospital cohort maximal aggregation in response to collagen, ADP and TRAP at baseline, week 4 and week 12



Platelet aggregation was assessed upon exposure to collagen (A) (0.002-0.19 mg/mL) and ADP (B) (0.156 – 20 µM) and TRAP (0.156 – 20 µM) (C) at baseline, week 4 and week 12 post randomisation. Data are displayed as mean maximum aggregation in response to arachidonic acid and epinephrine for St Mary's Hospital trial participants (FTC/TAF: green; ABC/3TC: grey). Between group differences were compared by independent T-test and differences over time compared by 2-way ANOVA.

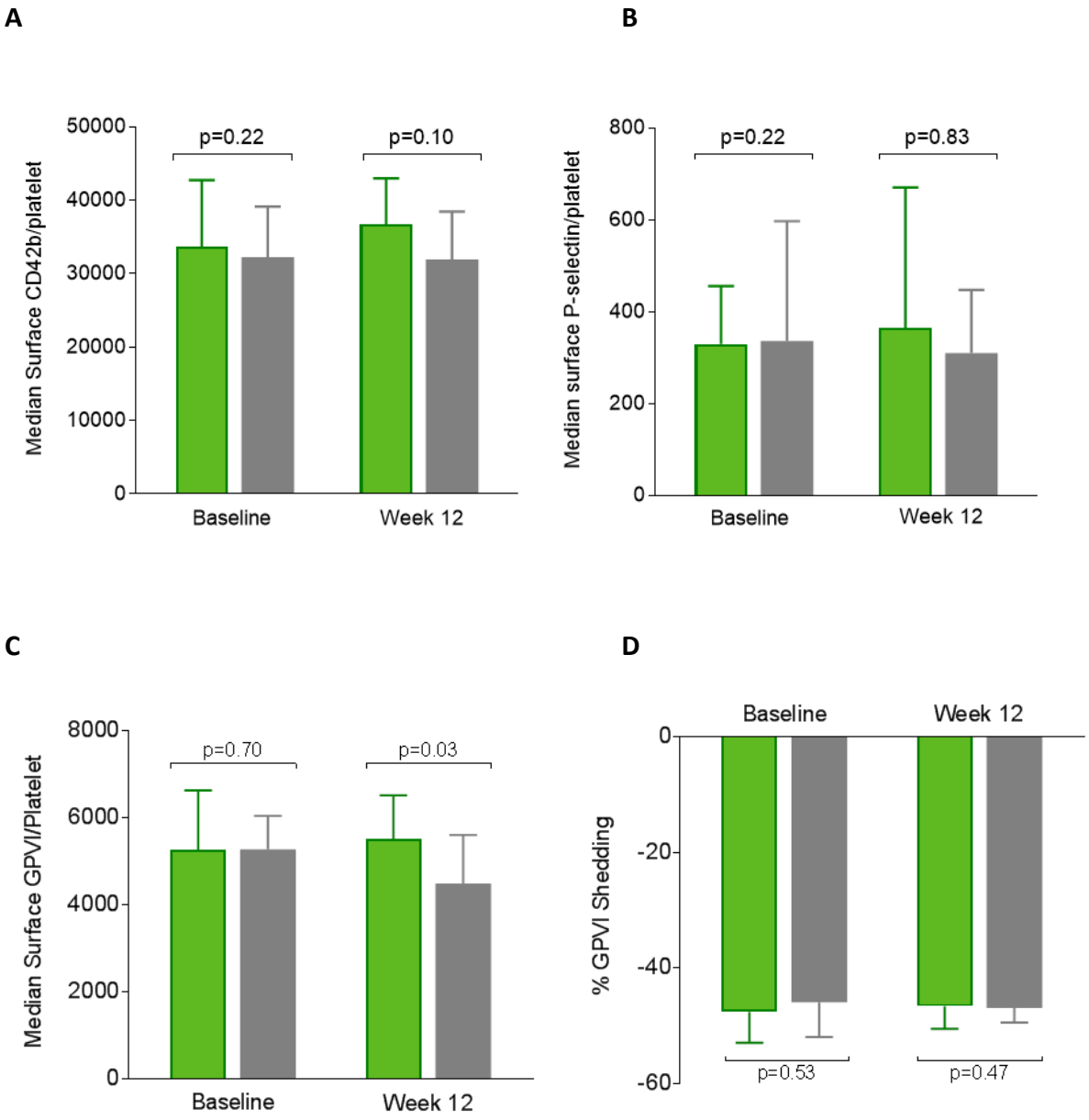
3.5.3 Platelet surface marker analysis

To gain a better understanding of the effect on platelets following a switch away from an abacavir/lamivudine based regimen to a emtricitabine/tenofovir alafenamide regimen, we examined the expression of the platelet surface makers CD42b, GPVI, and the activation marker, P selectin, by flow cytometry at baseline and week 12.

CD42b is the largest subunit of the vWf receptor complex, GP1b-V-IX (219) and expression has been shown to decrease upon platelet activation (220). P-selectin and GPVI surface expression is also altered upon activation of platelets. P selectin, released from α granules, rapidly translocates to the platelet surface whereas GPVI, upon binding its ligands E.g., collagen, convulxin or XL-CRP, undergoes cleavage and shedding of the extracellular domain. We hypothesised that a switch away from an abacavir based regimen would result in a decrease in platelet activation and we therefore would have expected to see an increase in expression of GPVI and a decrease in P-selectin and CD42b expression.

We saw no difference between the groups in expression of CD42b or P-selectin at baseline or week 12 (**Figure 3.e A&B**) and when examining expression of the collagen receptor, GPVI, at baseline there was no difference between the groups. However, at week 12, the median surface GPVI/ platelet was significantly higher in the emtricitabine/tenofovir alafenamide arm versus the abacavir/lamivudine arm: 5.52 x 10^3 (4.51, 6.52 CI); 4.49 x 10^3 (4.06, 5.61 CI) respectively (p= 0.03) (**Figure 3.e C**)

Figure 3.e Platelet surface marker analysis



Quantification of platelet CD42b, P-selectin and GPVI was determined by flow cytometry at baseline and week 12. Based on calibrated bead curve, mean fluorescence intensity was converted into absolute numbers of surface receptors per platelet (A-C). Percentage of GPVI shedding was determined by measuring GPVI expression pre and post addition of cross-linked collagen related peptide (6.25 µg/mL) (D) Participants on FTC/TAF arm had higher expression of GPVI but not CD42b or P-selectin at week 12 and this is not mediated by changes in receptor shedding

3.5.4 Soluble GPVI analysis

Table 3.b GS-US-311-1717 main study participant baseline characteristics

	emtricitabine/tenofovir alafenamide n=273	**abacavir/lamivudine n=272**
Age (years) (range)	52 (46, 57)	52 (45, 57)
Female (%)	15	22
White (%)	73	72
CD4 count (cells/mm^3)	644(487, 841)	700 (550, 891)
Duration on abacavir/lamivudine (years)	8 (3, 11)	8 (4, 11)
Platelet count (10^9/L)	220 (182, 254)	218 (181, 259)
Current smoker (%)	27	23
Hyperlipidaemia (%)	47	50
Hypertension	39	40
Diabetes (%)	12	13

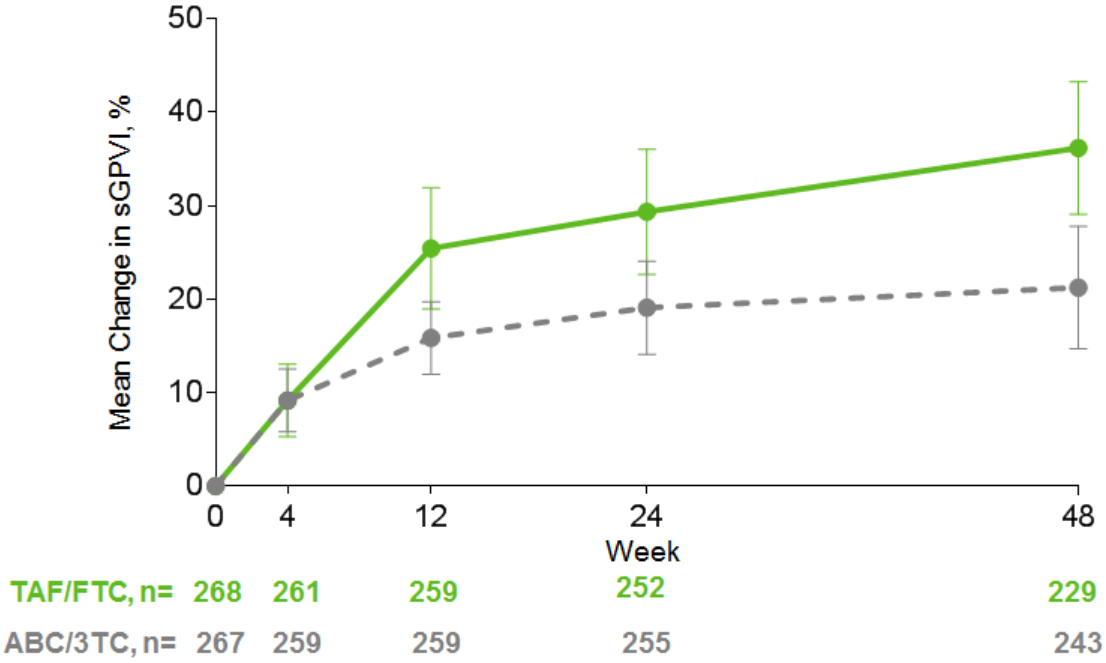
Data are median (IQR) or %, unless otherwise specified

GPVI is cleaved following ligand binding, and this cleaved portion can serve as a marker of platelet hyperactivity which has been shown to be altered in various diseases (221). Since we observed changes in platelet GPVI following a switch away from abacavir and lamivudine, we sought to determine whether this corresponded with changes in sGPVI levels but over a longer period of 48 weeks, and to do this we used the stored serum from the main study.

Of the 556 enrolled to main GS-US-311-1717 study, samples were available from 545 participants (98%). The baseline characteristics were, on balance, well matched although the emtricitabine/tenofovir alafenamide arm had fewer women (**Table 3.b**). This analysis was carried out in Dublin. At baseline, the sGPVI concentration was comparable between the groups. The median concentration in the emtricitabine/tenofovir alafenamide arm was 0.736 ng/mL (IQR 0.520, 1.274 ng/mL) and the abacavir/lamivudine arm was 0.846 ng/mL (IQR 0.527, 1.451 ng/mL) (p=0.18). At week 4 there was also no significant difference between the groups but at week 12 there was a significantly greater increase in sGPVI concentration in those in the emtricitabine/tenofovir alafenamide arm versus those in the abacavir/lamivudine arm and this persisted to week 48. Over 48 weeks there was a 14.7% (95% CI: 4.1, 26.3, p=0.005) greater increase in sGPVI levels in the emtricitabine/tenofovir

alafenamide group versus the abacavir/lamivudine arm. Interestingly there were also increases in mean sGPVI levels in the abacavir/lamivudine arm to week 48 but this was less pronounced than in the emtricitabine/tenofovir alafenamide arm and began to stabilise after week 24 (**Figure 3.f**).

Figure 3.f Mean percentage change in soluble GPVI to week 48



sGPVI was measured in platelet poor plasma of the whole study population of 545 subjects by electrochemiluminescence. sGPVI concentrations were measured at baseline, week 4, week 12, week 24 and week 48. Differences were analysed using linear mixed models. Participants on FTC/TAF arm had greater increase in sGPVI to week 48

3.6 Discussion

Recent abacavir use has been shown to significantly increase the risk of MI, however there is no consensus on the mechanism (103). The fact that this risk was seen to be reversible following cessation of abacavir treatment, and the role of platelets in the pathogenesis of MI, led many to examine the effect of abacavir on platelets (103). Of the studies investigating the effect of abacavir on platelet

activation, the results generally indicate an increase in platelet activation following abacavir use or addition. However, comparing studies becomes difficult as some use samples from people living with HIV with *in vivo* abacavir exposure (201), and others using samples from HIV negative individuals with drug added *ex vivo* (204); often in these cases abacavir or its metabolite was added at supraclinical concentrations (207). The overwhelming majority of studies were also non-randomised and therefore subject to confounding bias.

The GS-US-311-1717 platelet sub study gave a unique opportunity to investigate the effect on platelets of a switch away from abacavir to an alternative therapy. This was one of the first randomised trials that allowed for a comprehensive examination of platelet activation and function in those on an abacavir based regimen. One of the only other randomised controlled trials to examine this was the SWIFT study, where investigators saw a significant increase in sGPVI following a switch away from abacavir/lamivudine (218, 222). The study was limited in that only soluble biomarkers of platelet function were measured and not platelet aggregometry- which is the gold standard test for platelet function- nor were platelet bound markers measured (222).

The GS-US-311-1717 platelet substudy allowed for a comprehensive examination of platelet function following a switch away from abacavir/lamivudine to emtricitabine/tenofovir alafenamide including soluble and platelet bound markers as well as platelet aggregometry.

The aims of this chapter were:

1) **To examine the effect of switching from abacavir/lamivudine to emtricitabine/tenofovir alafenamide on platelet aggregation.**
2) **To perform exploratory analysis on the effect of a switch from abacavir/lamivudine to emtricitabine/tenofovir alafenamide on platelet surface receptors GPVI, CD42b and P-selectin.**
3) **To examine the effects of switching from abacavir/lamivudine to emtricitabine/tenofovir alafenamide on biomarker of platelet function, soluble glycoprotein VI.**

The 96-well plate-based aggregometry assay allowed us to assess several distinct platelet activation pathways simultaneously, giving a broad view of platelet aggregation as well as avoiding the risk of variability through sample deterioration, which can occur in less efficient, cuvette-based protocols.

Through this assay we demonstrated that at week 4 those on the abacavir/lamivudine arm had a lower mean ADP and TRAP EC_{50} versus those on the emtricitabine/tenofovir alafenamide arm, however this did not persist to week 12. Those remaining on abacavir/lamivudine also had a significantly lower mean collagen EC_{50} at week 4 and week 12 than those who switched to emtricitabine/tenofovir alafenamide. This lower collagen EC_{50} indicates a more reactive platelet profile, where a lower concentration of agonist (collagen) is required to elicit a 50% aggregation response. A switch away from abacavir/lamivudine to emtricitabine/tenofovir alafenamide therefore, resulted in decreased collagen-induced platelet reactivity.

When assessing platelet function, often only the agonist EC_{50} or the capacity of response is considered, however this does not give a full picture. The EC_{50} gives an indication of the platelet sensitivity of platelets to agonists whereas the maximal aggregation shows us the capacity of response (223). Therefore, to gain a better understanding of the effect of a switch from abacavir/lamivudine to emtricitabine/tenofovir alafenamide on platelet function, it is necessary to also measure both the sensitivity and the capacity of response.

In the GS-US-311-1717 platelet sub-study analysis only the agonist EC_{50} were considered. To explore further, I used the data from one of the London sites, St Mary's Hospital, to examine the capacity of platelet responses. There were 18 participants from the St Mary's site with the full data set (11 remaining on abacavir/lamivudine and 7 randomised to receive emtricitabine/tenofovir alafenamide) and I looked at the maximal aggregation at each time point.

I saw no differences in max aggregation between the two groups at any time point nor in the emtricitabine/tenofovir alafenamide group over the 12 weeks. There was however a difference in the abacavir/lamivudine group in response to arachidonic acid, where the max aggregation in response to

arachidonic acid was significantly higher in week 4 versus week 12 (64.18 ± 36.68 v 49.74 ± 39.88., p= 0.0420). Since there were no changes to this group i.e., these participants remained on abacavir/lamivudine, I would have expected the aggregation to remain similar, but this reflects the observed variable responses to arachidonic acid amongst participants. The abacavir/lamivudine arm week 4 and week 12 arachidonic acid-evoked max aggregation interquartile ranges were 4.23-88.73 and 3.89-89.64 respectively. The emtricitabine/tenofovir alafenamide week 4 and 12 max aggregation interquartile ranges in response to arachidonic acid were 5.72-87.53 and 1.56-77.25 respectively. This variability may have been due to operator error where air bubbles were introduced into the well whilst mixing PRP and arachidonic acid or insufficient mixing.

Responses to epinephrine were also generally lower than expected; emtricitabine mean max aggregation in the emtricitabine/tenofovir alafenamide group (baseline, week 4, week 12) was: 39.94 ± 31.04, 31.28 ± 27.74, 44.68 ± 28.11 and in the abacavir/lamivudine group (baseline, week 4, week 12): 39.10 ± 20.87, 40.27 ± 21.10, 39.92 ± 19.66. Though epinephrine is often described as a weak agonist, I would still have expected the max aggregation to be higher especially given the maximum agonist used was double that suggested in established 96 well aggregation assays (224). It is important to note the low numbers in this sub analysis, making it difficult to compare results, especially given the variability in responses.

Platelet responses to collagen, TRAP and ADP were much more consistent, but again with the low numbers it is very difficult to determine any genuine changes and it's therefore important to analyse the max aggregation responses in the entire data set to establish this.

Platelet agonists within a population can vary widely and have traditionally been characterized as high and low responders (225, 226) but within these groups there are distinct phenotypes (223). Exploring both sensitivity and capacity of response and grouping participants at baseline could allow us to see any subtle changes following a switch away from abacavir/lamivudine.

For a more detailed examination of platelet activation, we used flow cytometry to quantify the platelet markers GPVI, P selectin and CD42b, with GPVI and P selectin measured in the presence and absence of XL-CRP.

The von Willebrand factor receptor, CD42b is essential in platelet adhesion to the vascular wall following injury and is abundantly expressed on the platelet surface. Upon activation, CD42b will decrease in expression as it is re distributed to the open canicular system (142) . However, we saw no between group differences in CD42b expression at baseline or week 12.

Platelet surface P selectin expression in contrast increases when platelets are activated (133). Given the hypothesis, we would have expected a decrease in surface P selectin expression following a switch away from abacavir treatment, however, we saw no difference in expression at either time point.

With collagen-evoked aggregation we observed that there was a less reactive platelet profile following a switch away from abacavir/lamivudine up to 12 weeks post switch and when we carried out flow cytometric analysis of the collagen receptor, GPVI, we saw there were significant between group differences at week 12, where the median surface GPVI molecules per platelet was significantly lower in the abacavir/lamivudine group compared to the emtricitabine/lamivudine group.

Collagen-GPVI engagement is an important step in thrombus formation at the site of vascular injury and initiates platelet activation and aggregation (148). GPVI expression has been linked with coronary artery disease and myocardial infarction (227) and individuals with GPVI levels below the normal level exhibit reduced platelet shape change and aggregation and increased bleeding (228). The regulation of GPVI expression is through ectodomain shedding, mediated by the metalloproteinases ADAM 10 and ADAM 17, as well as localisation from intracellular stores to the surface membrane (229, 230).

At rest, platelet GPVI is largely intact however upon collagen bonding it is cleaved, releasing a 55kDa fragment known as sGPVI (165).

The results from this study show increased GPVI expression and greater increases in sGPVI levels with a switch away from abacavir/lamivudine. Given the associations with abacavir and MI, and GPVI/sGPVI with thrombosis, a decrease in GPVI and sGPVI with a switch away from abacavir might have been expected, however the opposite was seen. The sGPVI results are, however, in line with those seen in the SWIFT trial where, following a switch from abacavir to tenofovir disoproxil fumarate based treatment, sGPVI levels were significantly increased (222). Since we also saw increased platelet reactivity in response to collagen, it appears abacavir may lead to alterations in the GPVI signalling pathway and GPVI regulation, and although our results indicate shedding mechanisms were not altered following a treatment switch, HIV nef protein (still detectable in those receiving ART) signalling has been shown to activate and release ADAM10 containing vesicles (231, 232), so sGPVI may be inherently higher and shedding increased/dysfunctional in these subjects. Another explanation for decreased platelet GPVI in the group remaining on abacavir may be due to permanently increased platelet activation as is seen in patients with atrial fibrillation (164). The results may also suggest changes in reorganisation of GPVI or in the mechanism connected to the storage pool and following up longer term (especially with platelet GPVI expression) will give better understanding and to see if these changes are genuine or just seen initially before returning to similar levels. With sGPVI levels it would be beneficial to see absolute levels for plasma concentration and pair this with the platelet surface GPVI expression to compare. It would also be worthwhile exploring the GPVI signalling pathway and determining any differences in expression of the active form of Src or levels of phosphorylated PLCγ2, as has been seen in obese patients, who display platelet hyperactivity (233).

It should be noted that the participants for the platelet substudy were from the larger non-inferiority switch trial, comparing abacavir/lamivudine and emtricitabine/tenofovir alafenamide, so it cannot be discounted that any changes could be due to the tenofovir alafenamide-based treatment rather than the effect of switching away from an abacavir treatment. A small phase 1 trial has been conducted exploring the effects of emtricitabine/tenofovir alafenamide treatment on platelet aggregation and activation over one month, and no changes were seen from baseline (206), but more investigation is

needed. Since the second drug was also switched in the GS-US-311-1717 platelet sub study (lamivudine to emtricitabine) the potential effects of these drugs should also be considered, as well as the 3rd agents it is therefore difficult to attribute any changes to one drug in particular.

In conclusion this chapter showed that a switch away from abacavir based treatment and to tenofovir alafenamide based treatment resulted in reduced platelet reactivity in response to collagen, greater expression of surface GPVI and greater increases in sGPVI, suggesting potential platelet dysfunction induced by abacavir use.

Chapter 4 will assess the effect of abacavir and lamivudine on platelets in the absence of HIV associated factors.

Chapter 4: The Functional Effect of ART on Platelets *In Vitro*

4.1 Introduction

Platelet function can be impacted by several factors including platelet production, activation, aggregation, and platelet metabolic pathways (145).

Bone marrow defects such as in myelodysplastic syndrome and chronic lymphocytic leukaemia, can lead defective platelet production and platelet receptor function (234) and, HIV infection itself can lead to thrombocytopenia through bone marrow suppression, infection of megakaryocytes and immune-mediated destruction of platelets (235), though this often improves once on stable ART treatment (236). The first generation NRTIs zidovudine and stavudine have been shown to cause bone marrow suppression and mitochondrial dysfunction (237-239), and whilst not affecting platelet count, increased platelet activation has been observed through increased ROS production (240). Newer NRTIs such as abacavir are not thought to cause any bone marrow toxicity (241).

The various interconnected and complex platelet activation pathways are key to overall platelet function, and any disruption in these pathways can cause changes in overall function. In this chapter I explored this by looking at platelet activation markers and platelet aggregation in response to ART and different platelet agonists.

In evaluating the effect of ART on platelets in people living with HIV, there can be confounding factors despite having well matched cohorts, within the context of a randomised controlled trial. Viral induced platelet activation, the effect of smoking and recreational drugs and the differing 3rd agents can make it difficult to attribute any changes to ART directly or elucidate a potential mechanism. Additionally, in the GS-US-311-1717 platelet sub study as there was a double switch and the participants were taking the drug in combination, we cannot be certain that alterations in platelet function are due to one drug.

To explore the direct effect of single antiretroviral agents on platelet function and remove confounding factors from the virus itself, in this chapter, platelet aggregation and activation was

assessed in samples from healthy volunteers following the addition of abacavir or lamivudine alone and abacavir and lamivudine in combination. Specifically, platelet aggregation was measured by 96 well light transmission aggregometry, using a range of platelet agonists and a range of drug concentrations, and platelet activation was be assessed by flow cytometry.

4.2 Hypothesis

Using *in vitro* models, platelet activation and aggregation will be increased in the presence of abacavir but not other NRTIs.

4.3 Aims

1) To investigate the effect of in vitro administration of abacavir, lamivudine and abacavir + lamivudine on platelet aggregation in samples from healthy volunteers.
2) To investigate the effect of in vitro administration of abacavir, lamivudine and abacavir + lamivudine on CD62P, CD63 and PAC-1 in samples from healthy volunteers.

4.4 Methods

4.4.1 Platelet aggregation

PRP or washed platelets were prepared from healthy volunteers within 2 hours blood draw and antiretroviral agent(s) or vehicle (water) were added and incubated at 37°C for 30 minutes. Plasma or washed platelets were added to increasing concentrations of ADP, Collagen or TRAP-6 (thrombin for washed platelets) and light absorbance was measured. Max aggregation was determined as percentage change in absorbance from baseline.

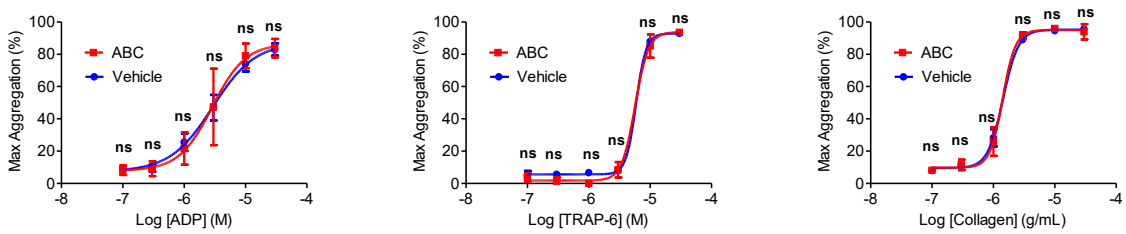
4.4.2 Platelet activation markers

PRP or washed platelets isolated from healthy volunteers was diluted in Tyrode's HEPES buffer to a concentration of 2.5 x 10^8 platelets mL^{-1} and incubated with antiretroviral agent(s) or vehicle (water) for 30 minutes and subsequently incubated with fluorescently labelled PAC-1, CD62P and CD63 antibody. Fluorescence measured using the Accuri ™ C6 plus flow cytometer.

4.5 Results

4.5.1 Platelet aggregation

Figure 4.a Platelet aggregation following abacavir preincubation plasma C_{max} levels (PRP)

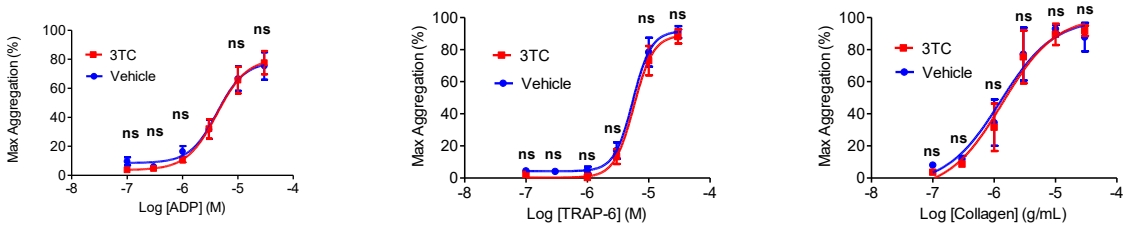


Concentration-response curves of platelet aggregation in PRP from volunteers without HIV (n=5) in response to ADP (0.1-30µM) TRAP-6 (0.1-30 µM) (b) and collagen (0.1-30 µg/ml). following PRP incubation with abacavir at C_{max} concentration or vehicle control (H_2O) for 30 minutes at 37°C. Aggregation was measured using a 96 well assay. *P< 0.05, significantly different from vehicle; two-way ANOVA was used for analysis

In assessing the direct effect of antiretrovirals on platelets from healthy volunteers, as expected maximum platelet aggregation increased in a dose dependant manner (respective to agonist concentration) however there were no observed differences in aggregation of platelets pre - treated with plasma C_{max} concentration of abacavir for 30 minutes versus vehicle (ADP EC_{50}: 2.86µM, 2.92µM; TRAP-6 EC_{50}: 5.68 µM v 5.90 µM ; collagen EC_{50}: 1.42 µg mL^{-1} ,1.44 µg mL^{-1}).

Nor were there were any significant differences in the capacity of response of platelets. The maximum aggregation of platelets treated with abacavir versus vehicle were as follows: ADP: 86.69% v 87.44%; TRAP-6: 93.76% v 92.90%; collagen: 95.02% v 95.28% (**Figure 4.a**).

Figure 4.b Platelet aggregation following lamivudine preincubation at plasma C_{max} levels (PRP)

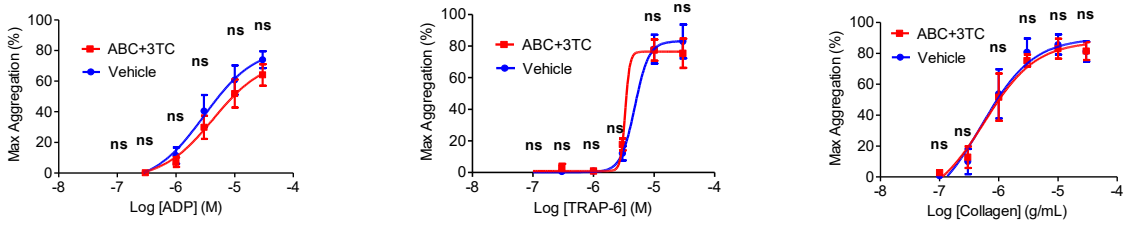


Concentration-response curves of platelet aggregation in PRP from volunteers without HIV (n=5) in response to ADP (0.1-30µM) TRAP-6 (0.1-30 µM) (b) and collagen (0.1-30 µg/ml). following PRP incubation with lamivudine at C_{max} concentration or vehicle control (H_2O) for 30 minutes at 37°C. Aggregation was measured using a 96 well assay. *P< 0.05, significantly different from vehicle; two-way ANOVA was used for analysis

This was also the observation in platelets pre-treated with plasma C_{max} concentrations of lamivudine. There were no differences when compared to platelets treated with vehicle (ADP EC_{50}: 5.37µM, 5.38µM; TRAP-6 EC_{50}: 5.51 µM v 5.72 µM; collagen EC_{50}: 5.87 µg mL^{-1}, 5.90 µg mL^{-1}) (**Figure 4.b**).

With platelets from healthy volunteers pre treated with abacavir + lamivudine in combination, there was a trend where platelets treated with the drugs exhibited lower max aggregation following exposure to ADP ad TRAP-6 (ADP: 74.86% v 82.44%; TRAP-6: 76.45% v 83.13%) (**Figure 4.c**).

Figure 4.c Platelet aggregation following abacavir + lamivudine preincubation at plasma C_{max} levels (PRP)

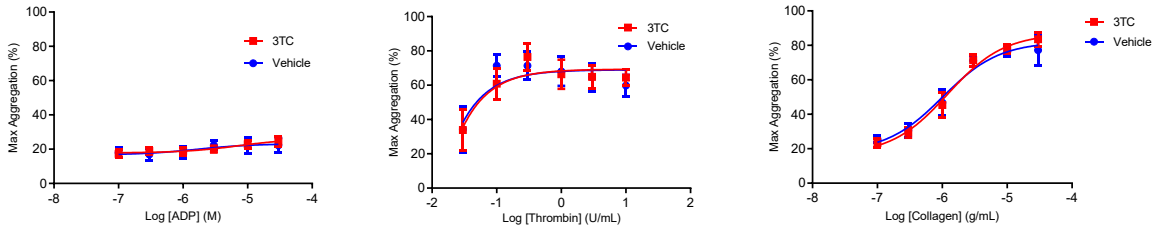


Concentration-response curves of platelet aggregation in PRP from volunteers without HIV (n=5) in response to ADP (0.1-30µM) TRAP-6 (0.1-30 µM) (b) and collagen (0.1-30 µg/ml) following PRP incubation with abacavir+ lamivudine at C_{max} concentration or vehicle control (H_2O) for 30 minutes at 37°C. Aggregation was measured using a 96 well assay. *P< 0.05, significant difference from vehicle; two-way ANOVA was used for analysis

Both abacavir and lamivudine exhibit low to moderate protein binding but to get a clearer understanding of the direct effect of abacavir and lamivudine on platelets, I carried out the 96 well aggregation assay using washed platelets.

ADP responses were substantially reduced due to the desensitisation of the P2Y1 receptor through the washing process (Max aggregation: vehicle 23.14% v 3TC 26.19%; vehicle 20.97% v ABC 19.55%) (**Figure 4.d left**).

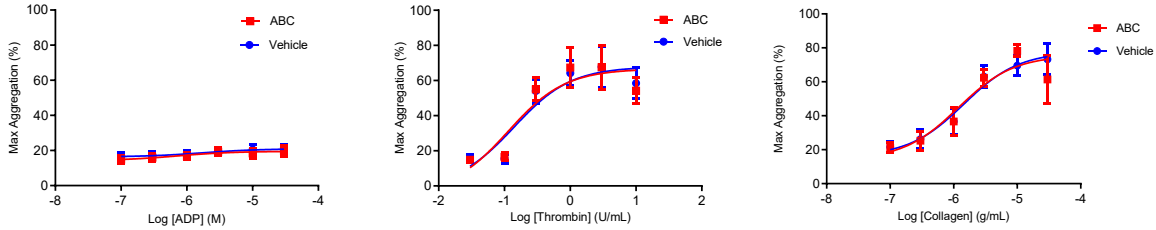
Figure 4.d Platelet aggregation following lamivudine preincubation at plasma C_{max} levels (washed platelets)



Concentration-response curves of platelet aggregation in washed platelets from volunteers without HIV (n=5) in response to ADP (0.1-30µM), thrombin (0.03-10 U/ml) (b) and collagen (0.1-30 µg/ml) following PRP incubation with lamivudine at C_{max} concentration or vehicle control (H_2O) for 30 minutes at 37°C. Aggregation was measured using a 96 well assay. *P< 0.05, significant difference from vehicle; two-way ANOVA was used for analysis

There were no differences in aggregation responses of platelets pre incubated with drug (3TC or ABC) or vehicle following thrombin or collagen exposure (**Figure 4.d and 4.e centre and right**). The effect on washed platelets of ABC and 3TC in combination was not investigated.

Figure 4.e Platelet aggregation following abacavir preincubation at plasma C_{max} levels (washed platelets)

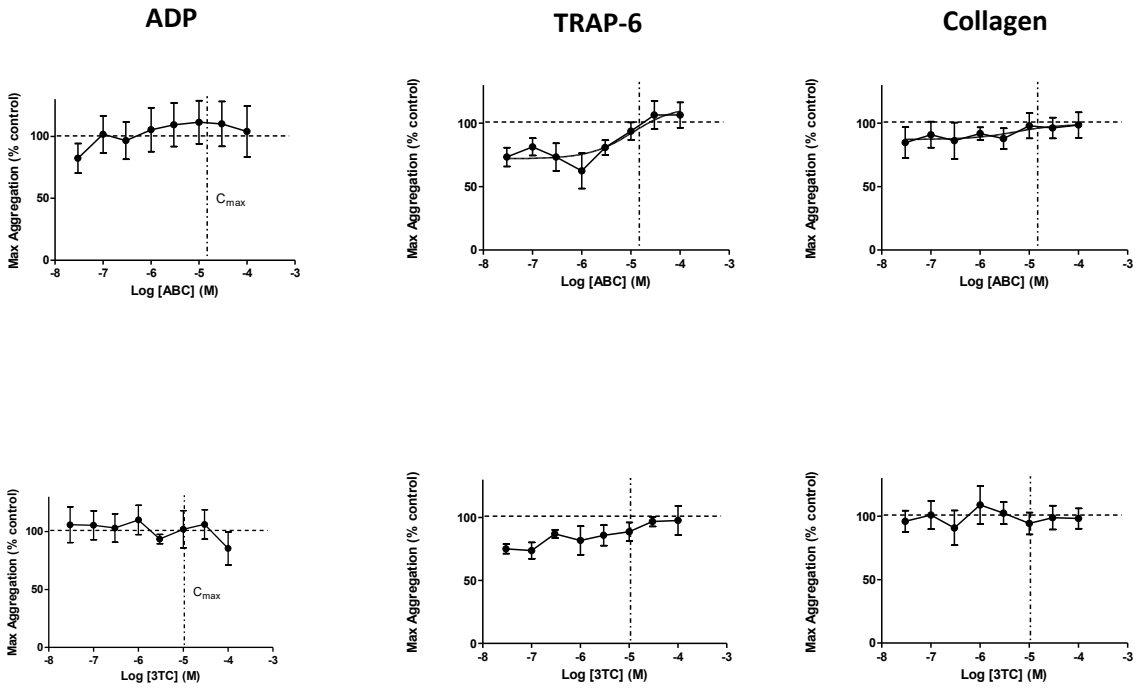


Concentration-response curves of platelet aggregation in washed platelets from volunteers without HIV (n=5) in response to ADP (0.1-30µM), thrombin (0.03-10 U/ml) (b) and collagen (0.1-30 µg/ml) following PRP incubation with abacavir at C_{max} concentration or vehicle control (H_2O) for 30 minutes at 37°C. Aggregation was measured using a 96 well assay. *P< 0.05, significant difference from vehicle; two-way ANOVA was used for analysis

From the dose response curves, I was able to determine each agonist EC_{50} concentrations. The approximate EC_{50} concentrations were as follows: ADP: 3µM, TRAP-6: 6µM, collagen: 1.5 µg mL^{-1}. I then used these agonist EC_{50} concentrations to assess any changes in aggregation when PRP was incubated with varying concentrations of drug, both above and below published plasma C_{max} (abacavir 12.7 µM; lamivudine 8.7 µM) (**Figure 4.f**)

There were no observed differences seen in the maximum aggregation when compared to vehicle control in platelets preincubated with abacavir and lamivudine, although there was a general positive trend with the increase in abacavir, appearing to plateau at concentrations past $C_{max.}$ (**Figure 4.f top**)

Figure 4.f Agonist induced platelet aggregation responses following drug incubation at varying concentrations



Platelets were treated *in vitro* with abacavir (top panel) and lamivudine (bottom panel) at a range of concentrations above and below plasma C_{max} (indicated with the vertical dotted line). Platelet aggregation was induced by EC_{50} concentrations of the agonists ADP (left), TRAP-6 (centre) and collagen (right). No difference in maximum aggregation was observed between platelets incubated with each drug concentration and control (n=5).

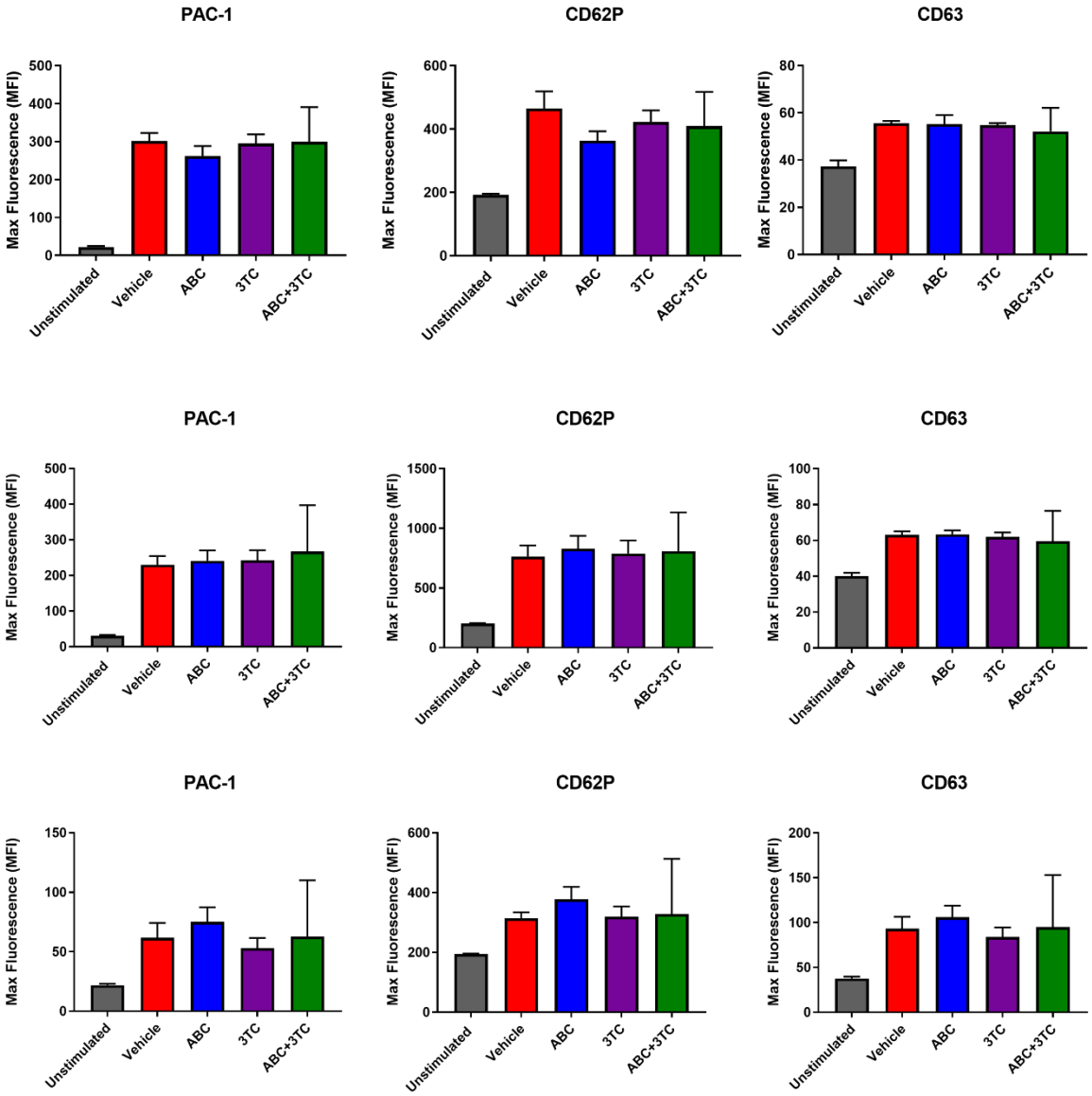
4.5.2 Platelet activation markers

Having seen no effect of ARVs on platelet aggregation using end point assays (i.e. after 15 minutes), I decided to examine platelet activation at an earlier stage of the process, using of flow cytometry.

The expression of markers for alpha granule release (CD62P), dense granule release (CD63) and integrin $\alpha_{IIb}\beta_3$ activation (PAC-1) was measured following 30 minutes of incubation with drug or vehicle control and the addition of platelet agonist (ADP 10µM, TRAP-6 10µM, Collagen 10µg mL^{-1} based on concentrations established previously in the lab). Fluorescence was measured for 210 seconds post agonist addition and the max fluorescence from baseline was determined (**Figure 4.g**). When looking at max fluorescence, all markers were increased following agonist addition, however the baseline fluorescence and scale of change differed for each marker. ADP and TRAP-6 (top and centre panels) elicited a much higher response than collagen (bottom panel) except for CD63 which was comparable in response to all agonists (CD63 max fluorescence in platelets incubated in vehicle control in response to ADP: 55.56 ± 3.20, TRAP-6: 63.02 ± 6.35 collagen: 93.25 ± 41.83).

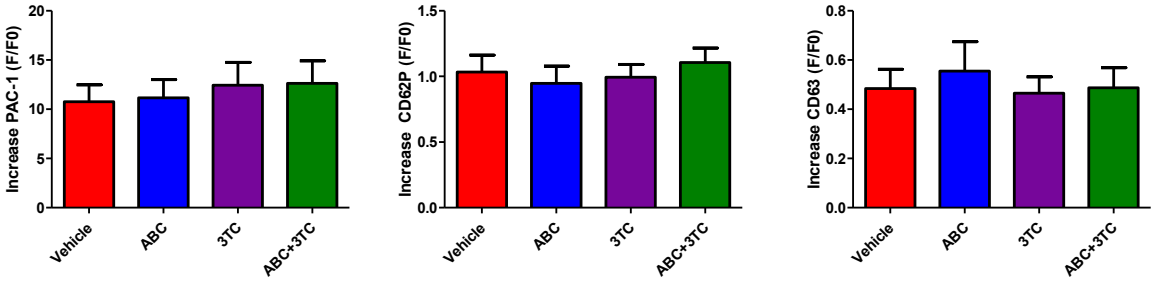
Max fluorescence for PAC-1 and CD62P for platelets incubated with vehicle control and stimulated with ADP was 301 ± 67.52 and 464.6 ± 171.70 respectively. For platelets incubated with vehicle control and stimulated with TRAP-6 the max fluorescence for PAC-1 and CD62P was 229.9 ± 72.84 and 764.8 ± 275.1 respectively. There was no statistical difference in max fluorescence in platelets preincubated in any of the conditions, though there was a trend for those preincubated with abacavir to have higher fluorescence compared to platelets preincubated with vehicle control (H_2O) in response to collagen for PAC-1, CD62P and CD63.

Figure 4.g Platelet activation marker expression max fluorescence



Platelet PAC-1, CD62P and CD63 expression was measured by flow cytometry following preincubation with C_{max} concentrations of ABC (12.7µM), 3tc (8.7µM), ABC+3TC or vehicle control (H_2O) and exposure to ADP (10µM) (Top) TRAP-6 (10µM) (Middle) and Collagen (10µg ml^{-1}) (Bottom) or unstimulated (grey bars) (n=9). Median fluorescence intensity was measured, and maximum values recorded after 210s. Data are expressed as mean ± standard error of the mean (SEM), one-way ANOVA with Tukey's *post hoc test*.

Figure 4.h Platelet activation marker expression in response to ADP

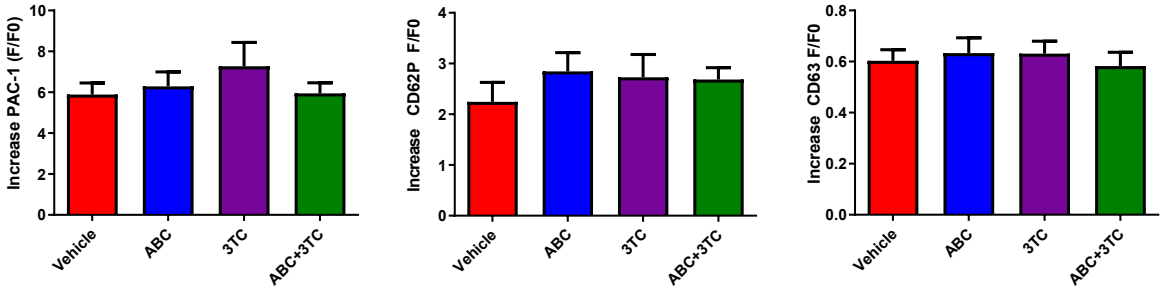


Platelet PAC-1, CD62P and CD63 expression was measured by flow cytometry following preincubation with C_{max} concentrations of ABC (12.7µM), 3tc (8.7µM), ABC+3TC or vehicle control and exposure to ADP (10µM). (n=9) Data are expressed as mean ± standard error of the mean (SEM), one-way ANOVA with Tukey's *post hoc test*.

Since there was quite a wide variation in the fluorescence between markers and between participants, to help in the comparison of data, I normalised the data and looked at the increase in fluorescence from baseline.

Following the addition of ADP and TRAP – 6 no significant difference was seen in PAC-1 binding, CD63 expression nor CD62P expression between platelets from healthy volunteers pre incubated with vehicle control (H_20), ABC, 3TC or ABC+3TC (**Figure 4.h and 4.i**).

Figure 4.i Platelet activation marker expression in response to TRAP-6

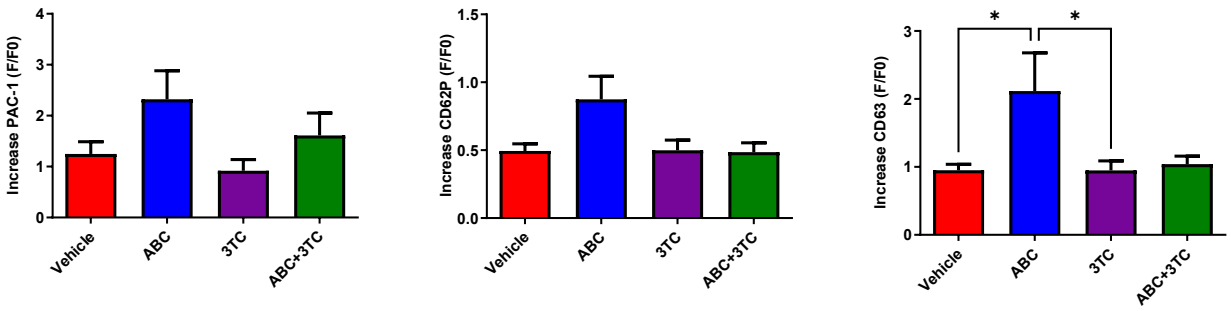


Platelet PAC-1, CD62P and CD63 expression was measured by flow cytometry following preincubation with C_{max} concentrations of ABC (12.7µM), 3tc (8.7µM), ABC+3TC or vehicle control and exposure to TRAP-6 (10µM). (n=9) Data are expressed as mean ± standard error of the mean (SEM), one-way ANOVA with Tukey's *post hoc test*.

Following exposure to collagen (10 µg mL^{-1}), platelets pre incubated with plasma C_{max} concentration of abacavir exhibited a significant relative increase in expression of the dense granule marker, CD63 (2.12 ± 0.56) compared to platelets incubated with vehicle control (0.95 ± 0.09) and 3TC (0.95 ± 0.14). There was no difference in CD63 expression in platelets incubated with abacavir+lamivudine (**Figure 4.j centre & 4.k**).

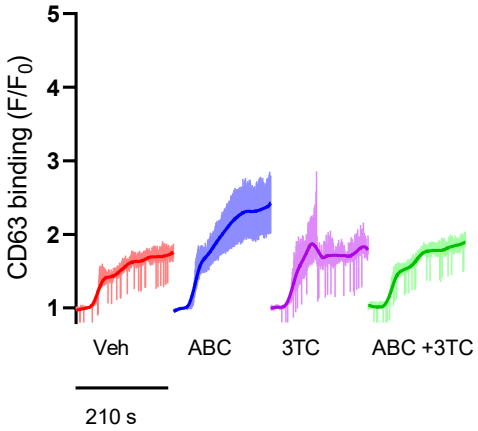
PAC-1 binding and CD62P expression were unaffected by preincubation of abacavir, lamivudine or abacavir + lamivudine, however there was a trend for platelets incubated with abacavir to exhibit higher PAC-1 binding and CD62P expression (**Figure 4.j left & centre**).

Figure 4.j Platelet activation marker expression in response to collagen



Platelet PAC-1, CD62P and CD63 expression was measured by flow cytometry following preincubation with C_{max} concentrations of ABC (12.7µM), 3tc (8.7µM), ABC+3TC or vehicle control and exposure to Collagen (10µg ml^{-1}). ABC treatment increased collagen induced dense granule release (n=9) Data are expressed as mean ± standard error of the mean (SEM), one-way ANOVA with Tukey's *post hoc test* (* denotes P<0.05.)

Figure 4.k CD63 expression in response to collagen



CD63 surface expression was recorded over 210s. After 30s to ensure baseline was stabalised, collagen (10µg ml^{-1}) was added to platelets that has been preincubted with abacavir (ABC), lamivudine (3TC) abacavir+lamivudine (ABC+3TC) or vehicle (veh) control for 30 minutes at 37°C, and binding responses measured. Collagen addition led to increased CD63 expression in all conditions; platelets treated with abacavir displayed increased CD63 expression compared to vehicle control treated platelets (n≥7).

4.6 Discussion

The aim of this chapter was to determine the direct effect of abacavir, lamivudine, and abacavir and lamivudine in combination on platelet aggregation and activation in platelets from healthy volunteers. The different activation pathways were investigated using the platelet agonists ADP ($P2Y_{12}$) TRAP-6/ thrombin (PAR1/PAR4) and collagen (GPVI) in a 96 well aggregation assay and flow cytometry.

The 96 well assay showed that platelets incubated in plasma C_{max} concentrations of abacavir, lamivudine or abacavir + lamivudine had no effect on maximum aggregation at any of the agonist concentrations when compared to platelets incubated with vehicle control (H_20). To avoid any interference of plasma proteins, experiments were also conducted using washed platelet suspensions. No difference was observed between platelets incubated with drug and those incubated with vehicle control.

Despite the effectiveness of the model for a broad view of the effect of ARV on platelet aggregation generally, it is a poor model of physiological conditions of those who have been on ART long term. From the experiments using a range of platelet agonist concentrations, each agonist EC_{50} was determined, and platelets were incubated with drug at concentrations 30- 100 X plasma C_{max}. No changes were seen in aggregation and was therefore not in agreement with the hypothesis.

Given that any differences were likely to be subtle, I used flow cytometry to examine any effect of drug on the activation markers: activated αIIbβ3, CD62P and CD63.

At all conditions the activation markers increased in expression following stimulation to varying degrees, but the most pronounced increases were seen in PAC-1 and CD62P expression. The MFI for CD62P did have quite high readings even before stimulation 194.5 ± 1.79, 191.9 ± 3.65, 203.6 ± 2.63. CD63 had a much less pronounced increase in expression following agonist addition than in the other

markers. This can most likely be explained by the lower relative numbers of dense granules to alpha granules (5.5 ± 0.5 versus 50 ± 5 per platelet) (242). There was no difference in max fluorescence although there was a trend with collagen addition where platelets pre incubated with abacavir had higher max fluorescence versus platelets incubated in vehicle; this difference however was modest.

Given baseline fluorescence differences with markers and between participants, I normalised the data and compared the relative increase in fluorescence post against addition. Pre exposure to (C_{max}) abacavir in vitro led to increased dense granule secretion relative to those incubated in vehicle control (1.12 ± 0.56) and lamivudine (1.17 ± 0.14). The trend remained for platelets preincubated with abacavir, showing a higher relative increase in PAC-1 and CD62P expression. This observation was only seen in response to collagen and not ADP or TRAP-6 and agrees with data from Taylor *et al* who showed incubation with abacavir caused an increase in CD63 expression following the introduction of collagen. This also corresponds with the data from the GS-US-311-1717 platelet sub-study showing an effect on platelet aggregation and activation along the collagen-GPVI pathway.

CD63 is a marker for dense granule release which is key in the process of haemostasis. These granules contain ADP, ATP, epinephrine and serotonin and release of these molecules at the site of vascular injury result in further platelet aggregation and vasoconstriction (243). Increased expression would suggest a higher reactive profile of platelets exposed to abacavir. To explore this further it would be helpful to measure specific components of the dense granules such as ATP, ADP, or serotonin in PRP following the addition of collagen. This could also be measured in people living with HIV on ART to assess the impact in this population and with the use of an anti-CD63 antibody, we can understand the impact more clearly.

Taylor *et al* (206) also showed an increase in CD62P expression following abacavir exposure but despite seeing this trend, it did not reach significance in my investigation. No difference was seen in platelets incubated with abacavir, lamivudine nor abacavir and lamivudine in combination. Baum *et al* (204) also saw increases in CD62P following abacavir pre incubation, however this was along the ADP-

$P2Y_{12}$ pathway where concentrations of abacavir were much higher (50µg $mL^{-1)}$ and incubation periods were longer (1h). No differences were seen in platelet CD62P expression nor soluble CD62P in the 1717 study.

Collagen is not generally recommended for use in flow cytometry due to its structure, as it does not interact with platelets in a consistent way and means that very high concentrations are needed to activate even a small proportion of platelets (148, 244). This may account for the reduced response I observed when compared to stimulation with other agonists, and in exploring this pathway further it would be worthwhile to carry out this experiment with a direct GPVI agonist such as CRP. Though I did see differences using collagen, CRP which would give a stronger, more consistent response and may confirm the differences I have seen.

There are a few limitations with this aspect of my investigation. These experiments do not take into account the role of the endothelium in platelet activation and were conducted with platelets in isolation. Second, these experiments were conducted on samples from non -randomised, young (< 35 years) healthy individuals, with low numbers (n= ≤ 10) therefore caution must be taken with any implications for the clinical setting based on this data. The mechanism for the observed association between abacavir and MI is likely to be subtle and multifactorial and any effect on platelets are unlikely to be seen in the 96 well assay following acute administration of the drug.

Acute administration of drug does not accurately represent physiological conditions of an individual taking ART long term nor the platelet exposure to drug. abacavir is a prodrug and is metabolised intracellularly to CBV-TP, however, intraplatelet concentrations are unknown. Data from studies exploring membrane permeation show abacavir to rapidly cross the membrane of erythrocytes and CD4 T-lymphoblastoid $CD4^+$ CEM cell (245). However, within the cell, there are a number of phosphorylation and deaminase steps, and different cell types have varying degrees of activation. Understanding the intraplatelet pharmacokinetics is important to give a clear picture of the impact of ART on platelet activation.

In my experiments I incubated platelets with drug for 30 minutes but it would be useful to incubate with drug for up to 2 hours and assess the platelet aggregation and activation markers. This was conducted by Baum *et al*, who saw time dependent increases in CD62P following ADP addition. Beyond 2 hours or even repeated dosing would be ideal but one is limited by the viability of platelets ex vivo.

It should also be noted that these experiments assume intraplatelet metabolism of ARVs. Some studies have conducted in vitro experiments with CBV-TP exploring the effect on platelet activation and cGMP inhibition (201, 206) however these have been at concentrations much higher than those seen physiologically. Additionally, CBV-TP is likely to only be encountered intracellularly; whether it can be internalised is debatable, given the ionic triphosphate group which traps the drug within the cell (246). As a result, I decided not to carry out the experiments using CBV-TP or lamivudine triphosphate, and instead use the prodrug.

Liquid chromatography mass spectrometry could be used to determine the intraplatelet concentration of CBV-TP and this would allow us to know if the platelet is taking up the ARV, if it is being metabolised, to what extent and how comparable this is to platelets of those undergoing long term ART.

In conclusion, this chapter demonstrated that platelets incubated with abacavir at pharmacological concentrations resulted in increased dense granule release in response to collagen. Neither lamivudine nor abacavir +lamivudine has any effect on platelet aggregation or activation in any of the conditions. In the next chapter, to gain a clearer understanding of the effect of ART on platelets in a whole organism setting, I used an *in vivo* model of platelet aggregation.

Chapter 5: The Functional Effect of ART on Platelets *In Vivo*

5.1 Introduction

Previously we saw that acute administration of abacavir and lamivudine and abacavir + lamivudine had no effect on aggregation *in vitro* but abacavir treatment did lead to increased activation, as seen through increased CD63 expression, in response to collagen.

Platelet activation and aggregation is heavily influenced by the endothelium, both directly and via endothelium-derived mediators (143) so it is therefore pertinent when investigating the effect of ART on platelets to also include this factor.

In investigating the effect of antiretrovirals on platelets, there is apparently only one study other than Taylor *et al* (166) that used a mouse model. Collado-Diaz *et al* employed the use of a ferric chloride-induced murine model of thrombosis and found that abacavir promoted thrombosis formation in a dose dependant manner and was the result of interference with purinergic signalling. The subsequent treatment with a P2X7 receptor antagonist removed the prothrombotic effects of abacavir and the effects were not seen at all in P2rX7 KO mice (201).

Since many of the investigations into the effect of antiretrovirals on platelets used *in vitro* or *ex vivo* techniques, I decided to use a mouse model of *in vivo* platelet aggregation, which allowed me to investigate the effect of ART in a whole-organism context and therefore gain a more realistic view with the important aspect of an intact endothelium.

5.2 Hypothesis

The hypothesis of this chapter is:
Using an *in vivo* model, platelet aggregation will be increased in the presence of abacavir but not other NRTIs.

5.3 Aim

The aim of this chapter is:

To investigate the effect of abacavir, lamivudine and abacavir + lamivudine on *in vivo* platelet aggregation using a mouse model of thromboembolism.

5.4 Methods

5.4.1 Platelet radiolabelling

Washed platelets were prepared from donor mice and incubated with Indium^{111}- tropolone for 10 minutes. Radioactive counts were recorded, and platelets were subsequently washed and resuspended in buffer before radiolabelling efficiency was calculated.

5.4.2 Agonist dose response

Wild-type male C57BL/6 mice were injected IP with drug or vehicle control (saline), anaesthetised and left for 30 mins. Radiolabelled platelets were injected via the femoral vein and 10 minutes was given to allow for circulation. Collagen (50µg Kg^{-1}) was injected IV and radio counts were measured using a SPEAR detector for 10 minutes. Data were analysed using Excel and GraphPad Prism 8.

Figure 5.a Measurement of platelet responses in a mouse model of thromboembolism



Anaesthetised wild-type (WT) C57bl/6 mice had radiolabelled washed platelets from donor mice transfused and were injected IP with drug or vehicle control, and 50µgKg^{-1} collagen was administered. Counts were measured using a SPEAR detector placed over the thoracic region for 10 minutes (left). The equipment set-up for the measurement of radiolabelled platelet responses (right).

5.5 Results

5.5.1 Platelet radiolabelling efficiency

Table 5.a Platelet radiolabelling efficiency

	Radioactive counts prior to addition of In^{111} (Counts/8 seconds)	Radioactive counts after labelling (Counts/8 seconds)	Efficiency of labelling
ABC experiment	39200 ± 4409	29400 ± 3803	75%
3TC experiment	116820 ± 93488	85440 ± 66818	73.14%
ABC+3TC experiment	1634 ± 217	893.4 ± 190	54.38%

Platelets from a donor mouse were isolated washed and incubated for 10 minutes with In^{111}-troponlone. Radiation counts were recorded, and platelets were washed in *in vivo* buffer. Radioactive counts were recorded, and efficiency of labelling was calculated (n=≥4)

Radioactive counts were wide ranging across the 3 experimental conditions, with the mean initial and post labelling counts for the abacavir + lamivudine experiments measuring 100x fewer than those for the lamivudine experiments. A labelling efficiency of over 70% was achieved in both the lamivudine and the abacavir alone experiments, whereas efficiency for the abacavir + lamivudine experiments were at 54.38% (**Table 5.a**).

5.5.2 Agonist dose response

The use of a mouse model of thromboembolism allowed us to examine the effect of drug in a whole organism setting. The mice were dosed via I.P injection with the prodrug to replicate conditions seen in ART users as much as possible and this was at 10X C_{max} to account for the higher metabolism of the mouse versus humans. Radiolabelled platelets were introduced into the mice and collagen administered (50 µg mL^{-1}) via the femoral vein after 30mins of drug administration according to the established protocol (206, 247). For the drug to be administered via the same route it would be quite technically difficult to complete 3 injections and as a result, IP administration was chosen which allowed for a quick uptake into the bloodstream.

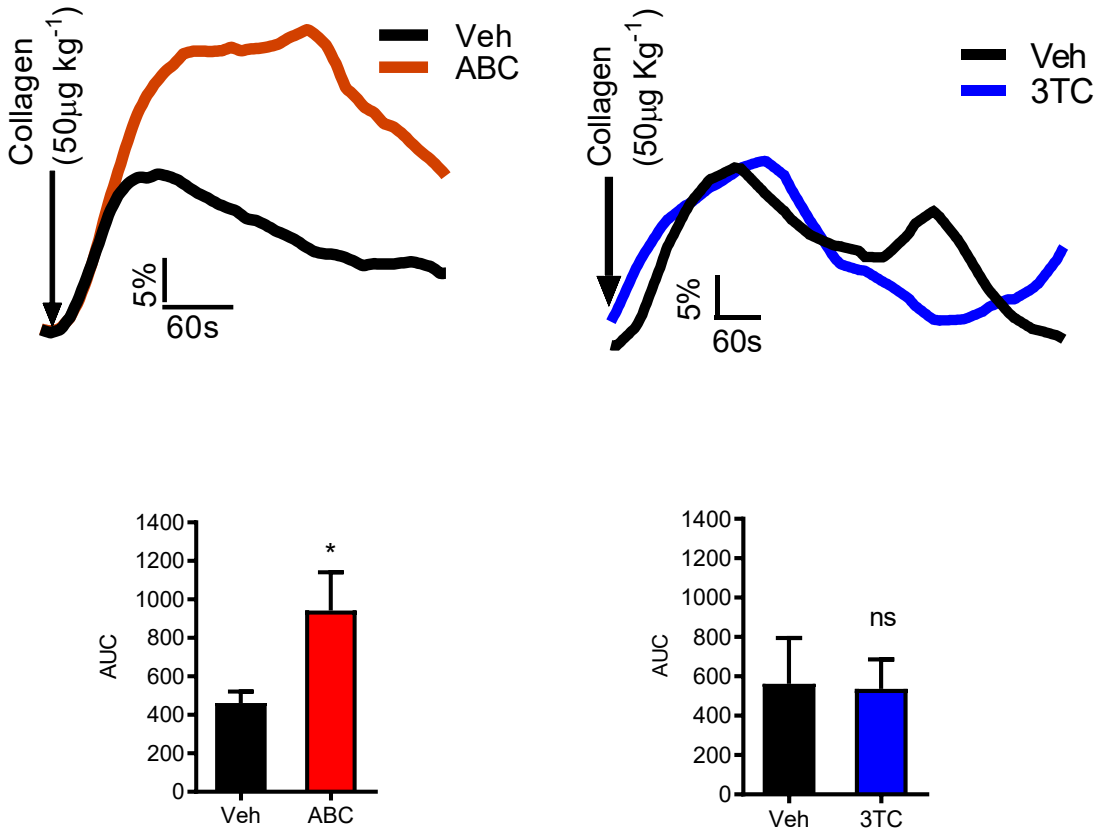
Following agonist administration, the platelets aggregate and collect in the pulmonary vasculature where a probe, positioned over the area, could detect changes in radioactive counts (**Figure 5.a**). The response to collagen is presented as percentage increase in radioactive counts from baseline and area under the curve (AUC).

Abacavir at a concentration of 30µg mL^{-1} led to greater peak response measured as an increase from baseline counts. The AUC for abacavir treated mice was significantly greater than that of the vehicle control treated mice 941.7 V 461.6, 95% CI [27.99, 932.2] (**Figure 5.b left**) These experiments were carried out by Kirk Taylor (206).

Lamivudine pre treatment (20µg mL^{-1}) had no effect on collagen evoked platelet aggregation (AUC vehicle: 562.4 3tc: 536.8) (**Figure 5.b right**).

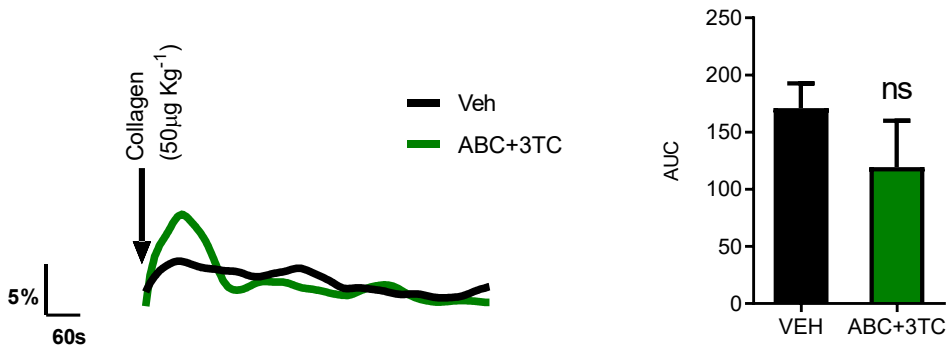
Abacavir + lamivudine had no effect on platelet aggregation however peak values and AUC were notably lower (both in mice treated with drug and vehicle control) when compared to those seen in experiments where abacavir and lamivudine were administered individually (**Figure 5.c**). This is potentially due to a higher than expected decay which would result in lower initial radioactive counts and thus labelling efficiency.

Figure 5.b In vivo platelet aggregation following abacavir or lamivudine treatment.



Platelet aggregation responses were monitored *in vivo* using terminally anaesthetised mice. Mouse platelets were isolated, radiolabelled (1.8MBq indium tropolone) and transfused into a recipient mouse. Abacavir (left; 30µgmL^{-1}) or lamivudine (right; 20µgmL^{-1}) was administered i.p. for 30min. A bolus of 50µgKg^{-1} collagen was injected (arrows) and responses were monitored for 10min. Experiments were conducted using wild-type (WT) C57bl/6 mice. The area under the curve (AUC) was calculated for the duration of the experiment and representative traces and summary data are shown. Changes in the maximum % increase in platelet aggregation (top panel) and AUC (bottom) were measured. Abacavir treatment increased collagen-evoked platelet responses while lamivudine had no effect on platelet aggregation *in vivo* (n≥4). Paired, two tailed Students' *t*-test was used for analysis. *$P < 0.05$.

Figure 5.c In vivo platelet aggregation following abacavir + lamivudine treatment



Platelet aggregation responses were monitored *in vivo* using terminally anaesthetised mice. Mouse platelets were isolated, radiolabelled (1.8MBq indium tropolone) and transfused into a recipient mouse. Abacavir (30µgmL^{-1}) and lamivudine (20µgmL^{-1}) were combined and administered i.p. for 30min. A bolus of 50µgKg^{-1} collagen was injected (arrow) and platelet response monitored for 10min. Experiments were conducted using wild-type (WT) C57bl/6 mice. The area under the curve (AUC) was calculated for the duration of the experiment (right) and representative traces (left) and summary data are shown. Changes in the maximum % increase in platelet aggregation and AUC were measured. ABC+3TC treatment had no effect on collagen-evoked platelet responses *in vivo* (n=6). Paired, two tailed Students' *t*-test was used for analysis.

5.6 Discussion

The aim of this experiment was to investigate the effect of abacavir, lamivudine and abacavir + lamivudine *in vivo* with the use of an established mouse model of thromboembolism (247). Having seen no effect of drug on platelet in isolation, I was able to investigate if there is an effect on platelets in a whole organism setting with any effect of interplay between platelets and endothelium, either directly or indirectly.

A key step of platelet activation *in vivo* is adherence at the site of injury on a vessel wall (143). It is therefore important to consider the influence of the endothelium in the investigation of ART on platelet activation. Whilst the aspect of subendothelial matrix protein exposure can be replicated *in vitro*, it is difficult to recreate the effect of endothelial derived NO, a negative regulator of platelet activation.

In my investigation, the radiolabelling of platelets proved to be inconsistent, and the counts varied widely between experimental groups, although, the labelling efficiency for platelets used in the abacavir and lamivudine alone experiments was over 70%. For reliability it's suggested that a labelling efficiency of over 70% should be achieved (248).

The efficiency for the platelets used in the abacavir + lamivudine experiments was 54.38%. This can often indicate damage to platelets or, more likely in this case, since it was seen repeatedly, there may have been unexpected or increased radio decay of indium prior to use. As a result of the low radio counts and labelling efficiency it is difficult to observe any true changes in platelet aggregation.

With treatment of abacavir and lamivudine alone, 50µg mL^{-1} collagen administration led to a rapid accumulation of platelets in the pulmonary vasculature. lamivudine had no effect on platelet aggregation however collagen evoked platelet aggregation in abacavir treated mice was significantly higher versus vehicle. This finding agrees with the data in GS-US-311-1717 clinical trial and *in vitro* flow cytometry data where abacavir treatment/pre incubation led to increased platelet activation and aggregation and the collagen-GPVI pathway appears to be implicated.

This investigation appears to be one of the only studies to show the effect of abacavir on platelets *in vivo*. The overwhelming majority of other studies investigating this and a potential mechanism for the observed association with abacavir and MI are *in vitro* or *ex vivo* and in cohorts that are largely unaffected by the observed outcome. It has been therefore difficult to make any comprehensive conclusions.

The only other *in vivo* models used in the investigation of this have been by De Pablo *et al* and Collado-Diaz *et al* who showed abacavir promoted leukocyte-endothelial interactions and increased platelet-endothelial binding. In these investigations it was concluded that abacavir induced alterations were through the ATP-$P2X_7$ receptor pathway, given abacavir's similarity in structure to endogenous purines such as ATP and metabolites (249, 250).

To examine the observed effect of abacavir on platelets *in vivo* further, since the effect was seen along the collagen pathway, between group sGPVI can be measured in plasma following dosing and collagen administration, which would allow for us to compare with the results of the GS-US-311-1717 clinical trial.

On dosing, we used 10x published abacavir and lamivudine plasma C_{max} which was in accordance with previous laboratory practices and based on an estimate, given that mice have a higher metabolism than humans, and it must be noted that the plasma drug concentration achieved was not measured. The necessary dose could be determined more accurately using an established method of dose translation to ensure that the dose given is appropriate, considering metabolic differences and surface area and this would give a more reliable animal equivalent dose. For this, the body surface area (BSA) normalization method would be a much more suitable method (251).

The routes of administration in my investigation could also be examined further. I used IP administration, based on previous work published in the lab and the fact that this is a reliable method of administration allowing for quick absorption into the bloodstream. This gives a good indication of acute drug administration but would differ from the long term drug intake that one would see in HIV therapy.

To represent what is seen clinically, daily dosing of mice can be carried out and samples taken after an extended period, though oral dosing in this context can be unpredictable. Despite seeing an effect of abacavir on platelet aggregation 30 minutes post treatment, long term treatment would likely result in a more pronounced effect and if we could accurately mimic the conditions seen in ART users, this would be more worthwhile in understanding the longer term effect of ART on platelets.

In my experiments it is assumed that the drug dose is comparable to that seen in humans but can be a more accurate calculation. Conducting a time course experiment and measuring plasma drug concentration using high performance liquid chromatography would give a clearer idea of whether the amount of time before agonist administration is enough and whether the dose is appropriate and

comparable to that seen clinically. If I were to conduct dosing over days, tail vein sampling could be carried to monitor drug levels.

In summary, the data presented in this chapter show that abacavir administration led to increased platelet aggregation *in vivo* compared to vehicle control (saline) treatment in response to collagen administration. Lamivudine treatment had no effect on platelet aggregation responses and abacavir + lamivudine treatment will need to be re-examined due to poor labelling efficiency but showed no significant change versus vehicle treatment.

As C57BL/6 mice are an inbred strain, we can say that the only independent variable is the drug and we can therefore be confident that it is the effect of the drug that led to increased platelet aggregation.

We have seen that abacavir treatment influences platelet activation and aggregation *in vitro* and *in vivo*. In the next chapter I will examine a potential mechanism driving this observed effect.

Chapter 6: Mechanistic Investigation on the Impact of ART on Platelet Activation

6.1 Introduction

Several studies have been conducted investigating the effect of abacavir (as well as other ARVs) on platelets. With abacavir specifically, the purpose has often been to further the understanding regarding the reported association between recent abacavir use and myocardial infarction (103). One potential mechanism previously reported implicated the active metabolite of abacavir, CBV-TP, which is similar in structure to guanosine triphosphate (GTP) (204). GTP is converted to cyclic guanosine monophosphate (cGMP) by soluble guanylyl (sGC) and cGMP has wide ranging anti platelet effects. However, given the structural similarity of CBV-TP to GTP, it has been suggested that it competitively inhibits sGC, preventing the production of cGMP and the downstream antithrombotic effects. This has been investigated, however, only looking at the direct impact on purified sGC, where CBV-TP was shown to inhibit Bovine sGC, but it is unclear whether this is the case intra platelet (204).

Since platelets do not produce NO in large quantities, I used the nitric oxide donor (NO), S-Nitroso-N-acetylpenicillamine (SNAP) donor as well the sGC inhibitor,1H-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), to examine the effect of ART on the NO mediated platelet inhibition. Given previous data, abacavir treatment of platelets would be expected to cause reduced NO mediated inhibition of platelets. To determine whether abacavir affects sGC, the level of inhibition can be compared to the sGC specific inhibitor, ODQ. In this chapter light transmission aggregometry assays were caried out to investigate the effect of ART on the NO signalling pathway.

It has also been shown that thrombus formation is influenced by oxidative stress and reactive oxygen species are involved in platelet activation and function (252) and ROS and endothelium-derived NO appear to be in fine balance, necessary for the regulation of platelet activation. Furthermore, ROS can be produced through the collagen-GPVI signalling pathway (186, 253). Since all the effects of abacavir on platelets so far in the investigation have been along the collagen pathway, I hypothesised that any abacavir-mediated platelet dysfunction may be due to dysregulation in the GPVI pathway or modulation of platelet redox states. In this chapter, the indicator for ROS, 2',7'-

dichlorodihydrofluorescein diacetate (H2DCFDA), was used to investigate this further. H2DCFDA is able to detect hydroxyl radicals, peroxynitrate anion, hydrogen peroxide, peroxyl radical and to a lesser extent superoxide anion (254).

6.2 Hypothesis

The hypothesis of this chapter is:

Preincubation of platelets with abacavir will reduce NO-donor mediated inhibition of platelet aggregation and will increase collagen-induced reactive oxygen species production compared to platelets preincubated with vehicle control and lamivudine.

6.3 Aims

The aims of this chapter were to:

1) Investigate the effect of abacavir, lamivudine and abacavir + lamivudine on the NO-donor mediated inhibition of platelet aggregation *in vitro*
2) Investigate the effect of abacavir, lamivudine and abacavir + lamivudine on platelet ROS production *in vitro*

6.4 Methods

6.4.1 Light transmission aggregometry

PRP from healthy volunteers was incubated with vehicle control (H_20), abacavir, lamivudine or abacavir+lamivudine for 30 minutes at 37°C. Samples were incubated with the NO donor, 5µM SNAP alone or SNAP and then 20µM of the soluble guanylyl cyclase inhibitor ODQ for 3 minutes each. 5µg mL^{-1} was added and light transmission was recorded over 5 minutes.

6.4.2 Reactive oxygen species measurement

Washed platelets from healthy volunteers were incubated with vehicle control (H_20), abacavir, lamivudine or abacavir+lamivudine, and H2DCFDA for 15 minutes in the dark. Change in fluorescence was measured by flow cytometry over 5 minutes following the addition of agonist.

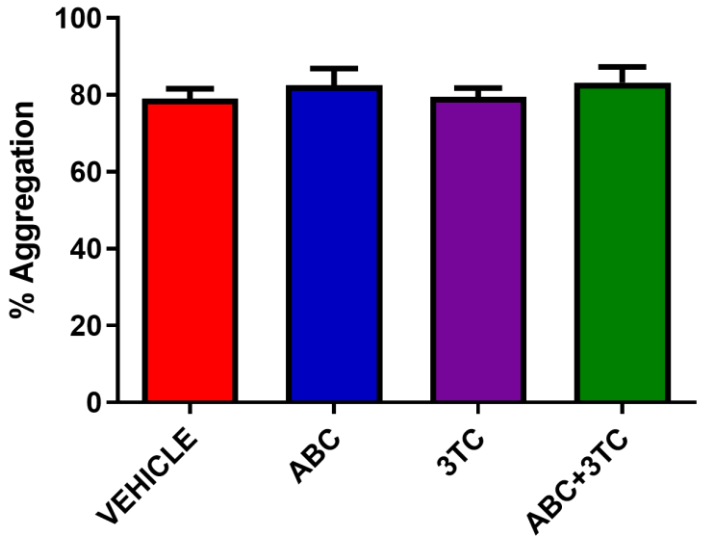
6.5 Results

6.5.1 Light transmission aggregometry

Light transmission aggregometry is the gold standard assay when measuring platelet aggregation and allows for the detection of any subtle changes (255).

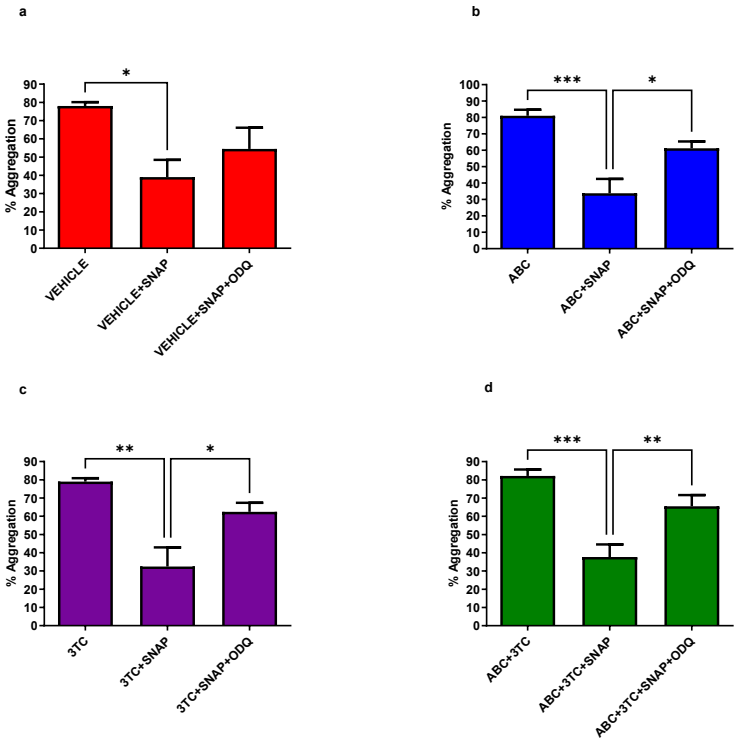
There was no difference in collagen-evoked platelet aggregation of platelets from healthy volunteers that had been pre treated with vehicle control (81.25% ± 2.93) or plasma C_{max} concentration of abacavir (81.20% ± 3.61), lamivudine (78.6% ± 2.02) or abacavir+lamivudine (82.17 ± 3.50) (**Figure 6.a)**. I would have expected platelets incubated with abacavir to have augmented aggregation compared to platelets incubated with vehicle control, following collagen addition, and the agonist concentration of 5µg/mL should have been appropriate based on my previous experiments to see any enhancement in aggregation, but this potentially could have been reduced.

Figure 6.a Max platelet responses following drug incubation



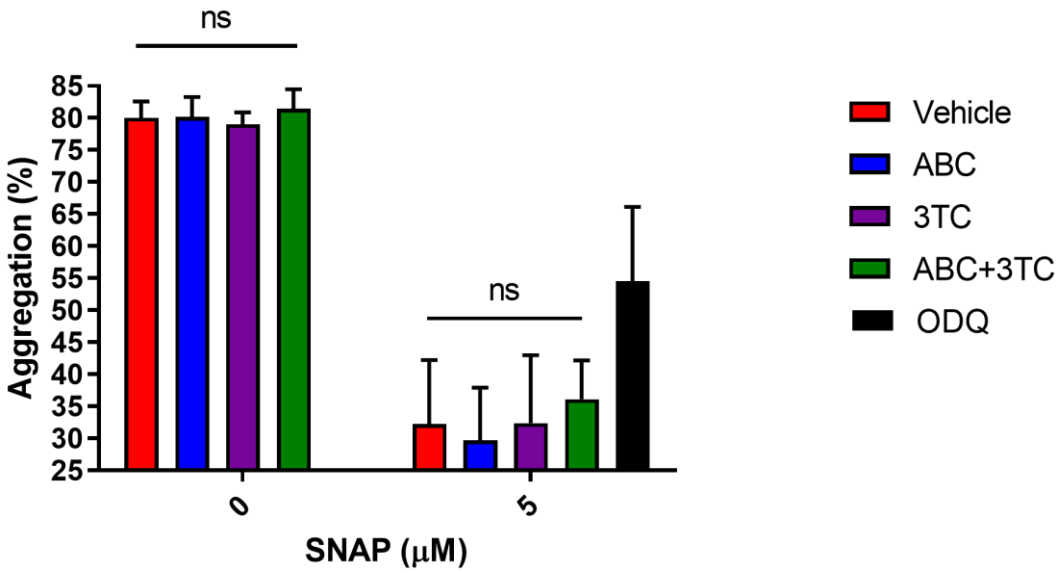
Aggregation responses to 5µg/mL collagen were assessed by light transmission aggregometry. PRP was incubated with C_{max} level drug or vehicle control for 30 mins and aggregation responses to collagen were measured by light transmission aggregation. Data are expressed as mean ± standard error of the mean (SEM), n=≥4 One-way ANOVA with Tukey's post hoc multiple comparisons test was used for analysis

Figure 6.b Platelet aggregation responses in the presence of a nitric oxide donor



Aggregation responses to 5µg/mL collagen were assessed by light transmission aggregometry. Platelets were incubated with the NO donor S-Nitroso-N-acetylpenicillamine (SNAP) (5µM) +/- 20µM 1H-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), the soluble guanylate cyclase inhibitor. Aggregation responses were recorded for platelets preincubated with vehicle (a), or plasma C_{max} levels of abacavir (b), lamivudine (c) or abacavir + lamivudine (d). Incubation with SNAP caused a significant reduction in aggregation in all conditions and ODQ addition rescued the response. Data are expressed as mean ± standard error of the mean (SEM), n=≥4. One-way ANOVA with Tukey's *post hoc* test was used for analysis. *P< 0.05, **P< 0.01, ***P< 0.005.

Figure 6.c Collated aggregation responses in the presence of a nitric oxide donor



Combined aggregation responses to 5µg/mL collagen were assessed by light transmission aggregometry. Platelets were incubated with or without the S-Nitroso-N-acetylpenicillamine (SNAP) (5µM) +/- 20µM 1H-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one (ODQ). Aggregation responses were recorded for platelets preincubated with vehicle (a), or plasma C_{max} levels of abacavir (b), lamivudine (c) or abacavir + lamivudine (d). Incubation with SNAP caused a significant reduction in aggregation in all conditions and ODQ addition rescued the response. ART incubation did not affect SNAP mediated inhibition of platelet aggregation. Data are expressed as mean ± standard error of the mean (SEM), n=≥4.

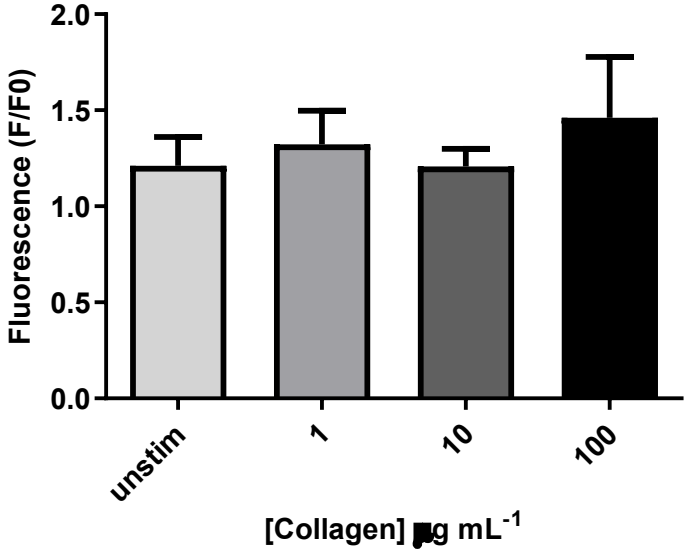
I then introduced the NO donor, SNAP and, as expected, aggregation was significantly inhibited in all cases (mean aggregation: vehicle 28.50%± 12.00, abacavir 27.20 % ± 9.64, lamivudine 32.25 % ± 13.63 %, and abacavir + lamivudine 33.33%± 6.31) (**Figure 6.b & 6.c)**. However, when comparing the different conditions, there were no significant differences in the level of inhibition of samples (**Figure 6.c)**. Again, I would have expected platelets incubated with abacavir to display reduced inhibition i.e. have a higher max aggregation compared to platelets incubated with vehicle, lamivudine and potentially abacavir and lamivudine, based on my previous findings.

This inhibition was partially recovered by ODQ, indicating the successful inhibition of sGC (aggregation: 54.5%± 11.62).

6.5.2 Reactive oxygen species measurement

Having seen no effect of ART on the NO donor mediated inhibition of platelets, I examined the effect on ROS generation. To establish the optimal collagen concentration to use in subsequent experiments, I conducted a collagen concentration-response experiment. ROS generation was measured by flow cytometry in response to collagen addition at 1, 10 and 100 µg mL^{-1}. No difference was seen in fluorescence at any concentration of collagen administered verses unstimulated (**Figure 6.d**) which went against expectations. As the collagen concentration increased, I would have expected an increase in ROS production and therefore fluorescence but there was no difference from the measurement in unstimulated platelets.

Figure 6.d Platelet reactive oxygen species production in response to collagen

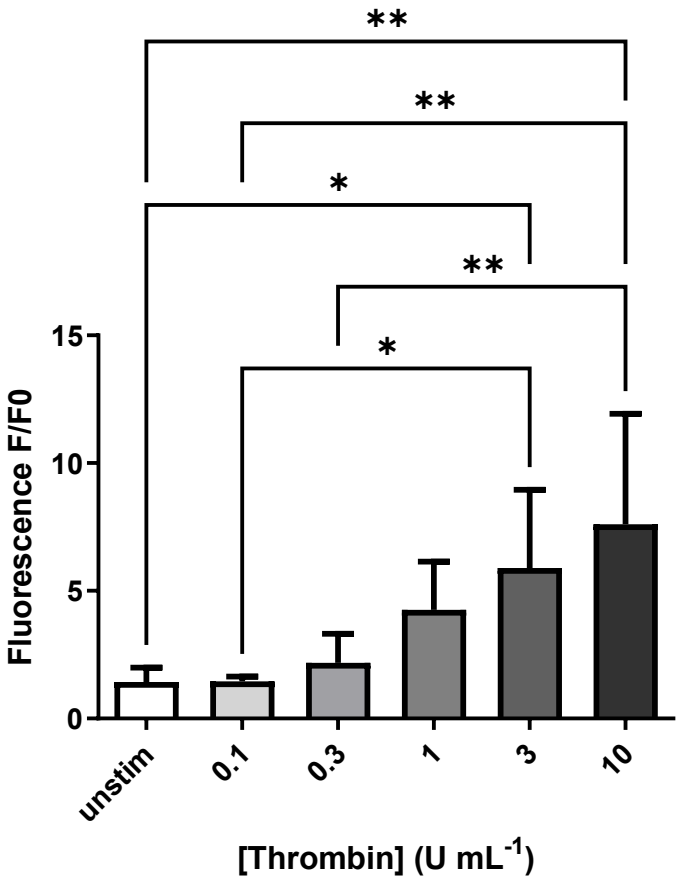


Platelet reactive oxygen species (ROS) production in response to rising concentration of collagen (1µg ml^{-1}, 10 µg ml^{-1}, 100 µg ml^{-1}) was assessed using the fluroscent ROS indicator, H2DCFDA. No difference was seen in fluorescence at any concentration of collagen administered verses unstimulated. n=≥4. One-way ANOVA with Tukey's *post hoc* test was used for analysis.

Having failed to see any increase in collagen induced increase in ROS, I focused on an alternative pathway of platelet ROS generation, that is, via thrombin signalling. NADPH oxidase 1 (NOX1) is the main source of GPVI induced ROS production, but thrombin, which signals through platelet activation receptor 1 and 4 (PAR 1 and PAR 4) has also been shown to induce ROS production, also via NOX1 (256). I therefore investigated the impact of ART on platelet ROS production following thrombin addition.

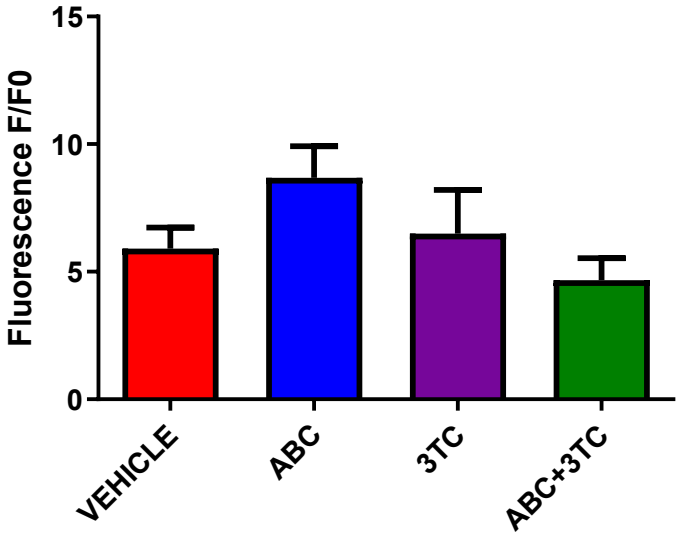
To determine the optimal concentration, a thrombin concentration curve was carried out. The concentrations tested were 0.1, 0.3, 1, 3 and 10 U mL^{-1}. ROS production increased in line with thrombin concentration (**Figure 6.e**) and based on the results, a thrombin concentration of 1U mL^{-1} was chosen as it would allow for the detection of any potentiation in ROS production whilst also giving a reasonable response.

Figure 6.e Platelet reactive oxygen species production in response to thrombin



Platelet reactive oxygen species (ROS) production in response to rising concentration of thrombin (0.1 U ml^{-1}, 0.3 U ml^{-1}, 1 U ml^{-1}, 3 U ml^{-1}, 10 U ml^{-1}) was assessed using the fluroscent ROS indicator, H2DCFDA. Fluorescence (F/F_0) increased in line with thrombin concentration. n=≥4. One-way ANOVA with Tukey's *post hoc* test was used for analysis.

Figure 6.f Platelet reactive oxygen species production following ART preincubation



Platelet reactive oxygen species (ROS) production in response to 1 U mL^{-1} thrombin was assessed using the fluroscent ROS indicator, H2DCFDA, following preincubation of washed platelets with vehicle, abacavir (ABC), lamivudine (3TC) or abacavir + lamivudine (ABC+3TC). No difference was seen in fluorescence between any conditions tested. n=5. One-way ANOVA with Tukey's *post hoc* test was used for analysis.

Washed platelets from healthy volunteers were incubated with vehicle, abacavir, lamivudine or abacavir+lamivudine, and H2DCFA. 1U mL^{-1} thrombin was added and ROS production measured. No difference in ROS production was seen between any of the conditions, although there was a trend of platelets pre incubated with abacavir to produce more ROS in response to thrombin. Platelets incubated with abacavir displayed an 8.68 ± 1.25 fold change in fluorescence from baseline, whereas the fold change from baseline for platelets incubated with vehicle (H_20) was 5.91 ± 0.81 in response to 1U mL^{-1} thrombin. For platelets incubated with lamivudine and abacavir + lamivudine, thrombin induced addition resulted in a 6.50 ± 0.68 and 4.47 ± 0.68 fold increase in fluorescence from baseline respectively (**Figure 6.f**).

6.6 Discussion

Abacavir has been shown to increase platelet activation in previous studies (126, 201, 204, 206, 250) but there is a lack of clear mechanistic data. Previous chapters have shown increases in platelet activation and aggregation linked to abacavir use or exposure and this chapter investigated a potential mechanism for the apparent abacavir mediated platelet activation.

Previous mechanistic studies have pointed to increased P2X7 signalling as a reason for increased platelet activation (257), whilst Baum *et al* suggested disruption of the NO-cGMP pathway, thus reducing platelet inhibition. In that study, CBV-TP was shown to inhibit sGC (204) and, though it was assumed that the intra platelet concentration of CBV-TP is equivalent to that measured in lymphocytes previously, it nonetheless shows competitive inhibition of sGC and prevention of antithrombotic downstream effects.

Taylor *et al* also investigated the effect of ART on this pathway and demonstrated that CBV-TP reduced inhibition of platelets. Platelet aggregation was measured after platelets were treated with drug and incubated with the NO donor, SNAP. CBV-TP treatment resulted in reduced SNAP mediated inhibition of aggregation compared to vehicle control (206).

Abacavir is converted intracellularly to CBV-TP and becomes trapped within the cell (246, 258) and concentrations extracellularly are unknown, nor is anything published regarding cell- much less platelet- membrane permeation characteristics. Carbovir (CBV) has been shown to be slower to enter cells compared to abacavir, dependent on nucleoside transporters, and is also subject to inhibition by adenine (245) and, as a result, I decided not to use CBV-TP in this study. My investigation does therefore assume that abacavir in taken up by the platelet and metabolised within the 30 minutes of incubation. NRTIs can be metabolised within erythrocytes (259) but, as mentioned previously, it would be beneficial to measure intraplatelet drug concentration and through this it would be clear to see if the concentration, time of incubation and drug choice was appropriate.

To investigate a possible mechanism for the observed abacavir induced activation of platelets, I incubated platelets with abacavir alone, lamivudine alone or abacavir + lamivudine and investigated the effect on the NO-signalling pathway following collagen addition. SNAP was able to significantly inhibit platelet aggregation, however, there was no difference in platelet SNAP-mediated inhibition of those incubated with the different drugs. ODQ reversed the response, showing successful inhibition of sGC.

I would have expected abacavir incubation to cause a reduction in inhibition of platelet aggregation, however the concentration of SNAP used in the experiments (5μM) might have been too high to see any effects of the abacavir. This concentration had been used in previous experiments carried out in the laboratory, but it would be worthwhile to carry out these experiments using different concentrations from which, a dose response curve can be constructed where any differences could be seen by a shift in the curves.

Another limitation of the study is the unknown element of the appropriate length of time of the platelet-drug incubation, and how much drug is taken up and metabolised to potentially act on sGC. A further experiment could be to examine the production of cGMP in platelet lysates following treatment with drug and SNAP. Where Taylor and colleagues saw a reduction of inhibition of platelet aggregation following incubation with CBV-TP versus vehicle control, measurement of cGMP production could be a confirmatory experiment of disruption in the NO-cGMP pathway.

Platelet function is heavily maintained by NO/ROS balance. Given the active metabolite of abacavir has been suggested to interfere in the NO signalling pathway, it was relevant to examine any effect on platelet ROS as well.

ROS play a key role in platelet activation and aggregation as well as regulation of the signalling pathways (260) and excess or increased production of ROS has been shown to be associated with various disease states including hypertension and diabetes, increasing the overall risk of thrombosis (185, 253).

GPVI signalling has been implicated in ROS generation and NADPH oxidases (Nox) are key sources of ROS where binding of GPVI with its agonist leads to the activation of tumour necrosis factor receptor associated factor (TRAF4) which binds to the intracellular tail of GPVI and interacts with Nox1/2 promoting the production ROS (252). ROS generation does not appear to correlate with GPVI surface expression levels and can also be mediated *via* PAR 4 receptor signalling (256).

Abacavir use has been shown to enhance ROS production through interruption of the electron transport chain (261) but there is also evidence of abacavir exposure resulting in a reduction of the marker of Nox2 activation, sNox-dp when compared to zidovudine use. This was seen in a small, randomised pilot study where participants switched from zidovudine/lamivudine or tenofovir disoproxil fumarate/emtricitabine to abacavir/lamivudine (262). The numbers were small, and zidovudine is known to increase mitochondrial dysfunction and increase ROS (261, 263) but it demonstrated that ART can affect ROS production. However, this hasn't been examined extensively in the context of abacavir use.

Given that GPVI signalling can lead to ROS generation, and, in this study I have seen changes in activation and aggregation along the GPVI signalling pathway, I examined GPVI mediated ROS production in response to ART.

Using the fluorescent dye H2DCF-DA, I was unable to measure any differences in platelet ROS production in response to a range of collagen concentrations and this may have been because collagen can cross link with itself and form pseudo aggregates, and that collagen fibres are large which can lead to inconsistent platelet activation, making it problematic in flow cytometric analysis (264-266). Use of XL-CRP would give a stronger, more consistent response and would allow for a clearer examination of GPVI mediated ROS generation.

I then looked at thrombin induced ROS generation but did not see a difference in any of the conditions tested versus vehicle although there was a trend for platelets incubated with abacavir to display higher fluorescence following thrombin addition compared to platelets incubated in all other conditions.

Overall, I was unable to prove the hypothesis that preincubation of platelets with abacavir will reduce NO-donor mediated inhibition of platelet aggregation and will increase collagen-induced reactive oxygen species production compared to platelets preincubated with vehicle control and lamivudine.

The tight control of platelet redox balance is important to maintain healthy platelet function and platelets are also able to synthesise NO (platelet derived NO) albeit at lower levels than endothelial cells (267) which helps in the maintenance of the redox balance (187).

GPVI signalling, as well as inducing activation and aggregation also results in NOS activation and subsequent NO production *via* Src family kinases and PKC (268). An interference in the GPVI signalling cascade by abacavir may result in dysregulation in redox control and the development of a prothrombotic state and may provide a mechanism for the observed increase in platelet activation and aggregation in response to abacavir, though further investigation would need to be done to determine if and to what extent this is the case.

Chapter 7: General Discussion

7.1 Introduction

The success of ART has meant that people living with HIV have a higher life expectancy than prior to the ART era, and there is now an aging population in this cohort (269).

As a result, there is an increased focus on non-AIDS related comorbidities in this group, particularly CVD, which is a leading cause of mortality, occurring at a higher rate than in the general population – a trend observed in both resource rich and resource poor settings (77, 78).

This increased CVD risk fundamentally can be attributed to traditional cardiovascular risk factors, HIV induced immune activation and inflammation, and antiretroviral toxicity. ART had formally been associated with CVD; this was particularly focussed on the 1st generation PI, which were known to cause dyslipidaemia, insulin resistance and endothelial dysfunction (98, 99), and the earlier thymidine analogue, stavudine and zidovudine, which had similar adverse metabolic effects (102, 240).

The D:A:D study, an international prospective multicohort study of over 30000 participants, aimed to investigate the incidence of serious non-AIDS conditions in people living with HIV and the association with ART (102). Analysis from the study suggested PI use was associated with increased risk of MI and, though the mechanism was unclear, the result was unsurprising, given the known adverse events (100, 270). A more unexpected outcome was the observed association with the adenosine analogue, didanosine and the guanosine analogue, abacavir, where those using the latter were shown to have a 1.9 fold increase in relative risk of MI verses those on other therapies. Additionally, this association was also shown to be removed once abacavir therapy was stopped (103). This reversible element and the fact that MI risk was associated recent use, make it unlikely to be the result of dyslipidaemia.

Platelet dysfunction has been reported as a potential causative factor, given the role of platelets in MI. However, despite several studies examining this, there is no clear consensus, with some supporting the hypothesis and others not(126, 201, 207).

A reason for the disagreement in the field is the lack of a clear mechanism, though one suggested mechanism which has been examined by a few groups is the disruption of the NO-sGC-cGMP pathway by carbovir triphosphate, the active metabolite of abacavir (204, 206).

Though not as widely prescribed today, abacavir is still part of the alternative treatments in the prescribing guidelines in the UK (76), Europe (75) and US (74), especially in those with poorer renal function and it is therefore important to clearly understand the associated risk.

7.2 Discussion of hypothesis 1

Switching from an abacavir containing to a non-abacavir containing cART regimen will be associated with a reduction in platelet activation and aggregation in people living with HIV.

The GS-US-311-1717 platelet sub study allowed for the examination of the effect on platelets of a switch away from an abacavir/lamivudine-based therapy to emtricitabine/tenofovir alafenamide.

In response to collagen the EC_{50} for the participants who switched to emtricitabine/tenofovir alafenamide was significantly higher than that for those in the abacavir/lamivudine arm at both week 4 and week 12, indicating a reduction in platelet aggregation. There were no differences in CD42b or P selectin expression, but median GPVI molecules/ platelet were significantly higher in the emtricitabine/tenofovir alafenamide arm versus the abacavir/lamivudine arm.

These results prove the hypothesis that platelet aggregation and activation is decreased with a switch away from an abacavir-containing to a non-abacavir containing regimen, specifically along the collagen-GPVI pathway. Alterations in this pathway were further seen with the soluble marker results, where those in the emtricitabine/tenofovir alafenamide arm had a greater increase in sGPVI to week 48.

This was one of the first randomised controlled trials addressing the association of ART and comorbidities in people living with HIV, much less the effect of abacavir on platelets. Many of the studies before were observational and therefore open to selection bias and often retrospective, relying heavily on soluble markers of platelet activation from stored samples (103, 112, 115, 118, 121).

This study provided a comprehensive overview of platelet activation and aggregation, through the use of a 96 well aggregation assay, platelet surface markers of activation and soluble markers of activation, carried out in an appropriate population. I.e., one who is likely to be more affected by MI (median age: ~ 50 years).

Limitations are recognised in that the recruitment targets were not achieved (61 v 80) making the study slightly underpowered. Second, the use of the 96 well platelet aggregometry assay, which, while effective in assessing aggregation via many pathways of activation in parallel, it does miss the details that can be seen in light transmission aggregometry, particularly the kinetic responses. This includes platelet shape change and patterns such as reversible and biphasic aggregation, which would all give a greater insight into the effect of drug on platelets. That said, to carry out light transmission aggregometry on all samples would be labour intensive, would require much more plasma from each subject and is subject to operator variation. However, analysis that would add to this work would be to include the max aggregation seen at all timepoints, which would give greater insight into platelet function and any changes that are potentially being missed. I was able to analyse the samples from subjects from St Mary's but saw no differences, the numbers were however small (n=18; emtricitabine/tenofovir alafenamide arm: 7; abacavir/lamivudine: 11). Analysis of the entire substudy group would allow for bigger numbers and a greater chance of seeing any genuine changes in the capacity of platelet response following a switch away from abacavir/lamivudine.

It should be noted that since there is a double drug switch (abacavir/lamivudine to tenofovir alafenamide/emtricitabine), any changes seen cannot be completely attributed to a switch away from

abacavir but may in fact be due to a switch away from lamivudine or due to the switch to tenofovir alafenamide/emtricitabine.

Additionally, 3rd agents were not taken into consideration in the analysis in this study. Recent studies have demonstrated that exposure to atazanavir or darunavir in combination with abacavir/lamivudine and, raltegravir in combination with tenofovir/emtricitabine leads to an increased risk of MI (211), and another study has shown integrase inhibitor initiation is associated with increased incidence of CVD in the first 2 years of us (271). Taylor and colleagues have reported enhanced alpha and dense granule release as well as integrin activation in response to collagen in platelets preincubated with darunavir versus platelets preincubated with vehicle control (272). It will be important to explore 3rd agents further to assess the impact on platelets individually, any combined effect, and how this might have influenced the results of the study.

7.3 Discussion of hypothesis 2

Using in vitro and in vivo models, platelet aggregation will be increased in the presence of abacavir but not other NRTIs.

Subsequently, I aimed to test the second hypothesis by assessing the effect of abacavir, lamivudine and abacavir + lamivudine on platelet activation and aggregation using platelets from healthy volunteers, in *in vitro* experiments. Additionally, a mouse model of thromboembolism was used to determine the effect of ART on platelets in a whole organism setting.

The *in vitro* aggregation experiments did not show any change in aggregation after pre incubation with abacavir, lamivudine or abacavir + lamivudine, this was at a range of agonist and drug concentrations (abacavir + lamivudine was not tested following no changes in platelet response seen with the individual drugs at a range of concentrations). In exploring the effect further, no effect in response to collagen was seen using the more sensitive method, light transmission aggregometry.

Whilst no effect was seen in *in vitro* aggregation, an effect was observed in platelet activation, where platelets pre incubated with abacavir at C_{max} concentrations had increased relative expression of the marker of dense granule release, CD63, specifically in response to collagen, versus platelets pre incubated with vehicle control. No change was seen following preincubation with lamivudine nor with abacavir + lamivudine. There was also a trend for platelets preincubated with abacavir to have increased CD62P expression and PAC-1 binding in response to collagen but not other agonists.

These results should be taken with some caution since conditions are far removed from physiological conditions, this particularly with respect to the acute administration of drug (published plasma C_{max} concentrations incubated for 30mins) which is unlikely to mimic general conditions and possibly limits the drug-platelet interactions that would take place in those taking medication. It would be useful to carry out a dose-response experiment and explore the effect of increased periods of drug incubation, although with the latter, one is limited in the length of incubation prior to analysis due to reduced platelet responses. The International Society on Thrombosis and Haemostasis recommend completing platelet function experiments within 4 hours of blood draw (273), and when assessing platelet responses to arachidonic acid and ADP it has been suggested that experiments should be completed within 2 hours of blood draw (274).

CD63 translocates to the plasma membrane following platelet activation and dense granule release (275), and this process is a critical step in the process of aggregation especially, particularly in the recruitment of platelets. To confirm and explore this finding further, it would be useful to measure ATP release through a luciferin-luciferase assay or serotonin through a ^{14}C serotonin release assay (276). Additionally, since dense granules are a large store of Ca^{2+} ions in platelets, enhanced Ca^{2+} release in response to collagen, following abacavir pre incubation could be a mechanism through which we see the increased platelet aggregation in the clinical study. Whether abacavir exposure truly results in enhanced dense granule-specific release, or it is general platelet activation, needs to be explored further. Whilst my results show dense granule release was increased, Taylor *et al* (206)

showed increased alpha granule release and no effect on dense granule release. My results did show a trend towards increased CD62P expression and PAC-1 binding in response to collagen, though this did not reach significance. What is consistent, however, and in line with the results of the clinical trial, is that the observed increased aggregation and activation is in response to collagen.

Given the importance of the endothelium in haemostasis and the modulation of platelet function, in chapter 5 I investigated the effect of abacavir, lamivudine and abacavir + lamivudine in a mouse model of aggregation (247). This allowed for the examination of platelet aggregation in response to ART, taking into account the impact of vascular mediators such as nitric oxide. The *in vivo* results showed that treatment with abacavir led to a significant increase in platelet aggregation versus vehicle control (saline) treatment, again in response to collagen. Lamivudine treatment had no effect on platelet responses and, somewhat against expectation but in keeping with prior *in vitro* results, abacavir + lamivudine had no effect on platelet aggregation. It should be noted that there was poor radio labelling efficiency (54.38%) making it difficult to determine any true changes in aggregation.

Another limitation is the drug concentration achieved in the mouse model. Due to the high metabolism of the mouse, 30X plasma C_{max} was administered I.P for 30 minutes. To establish whether this is an appropriate dose, enough time to be metabolised, and physiologically relevant, it would be beneficial to carry out microsampling to understand the pharmacokinetic profile through high-performance liquid chromatography. Moreover, it would be pertinent to measure the intra platelet drug concentration, which has not been examined in this context. To achieve a more appropriate representation of daily ART dosing, rather than bolus I.P administration of drug, donor mice could be dosed over many days and microsampling carried out to measure platelet activation markers, as well as the *in vivo* aggregation assay.

In examining the effect of ART on platelets, few studies have utilised mouse models. Collado Diaz and colleagues have used a ferric chloride model and found abacavir treatment to enhance thrombus formation (250). Although this is a well-established model of thrombosis, its relevance to the complex

process of arterial thrombosis is questioned and the terminal nature, with occlusion as an end point is somewhat crude, not allowing for the examination of subtle changes (277, 278).

The refined model used in this thesis allowed for a more physiologically relevant assessment of platelet function in response to ART and, taken in consideration with the *in vitro* activation marker findings, the results suggest that abacavir may increase platelet activation aggregation specifically along the collagen signalling pathway.

7.4 Discussion of hypothesis 3

Preincubation of platelets with abacavir will reduce nitric oxide-donor mediated inhibition of platelet aggregation and will increase collagen-induced reactive oxygen species production compared to platelets preincubated with vehicle control and lamivudine.

This hypothesis was tested in chapter 6. Nitric oxide is an important molecule in the regulation of platelet activation, and disruption of the Nitric oxide-sGC-cGMP pathway has been proposed as a potential mechanism for the observed association of abacavir use and increased risk of MI, where carbovir triphosphate (the active anabolite of abacavir) inhibits sGC, thereby reducing the production of cGMP and downstream antithrombotic effects (204).

Baum *et al* (204) showed that carbovir triphosphate competitively inhibits sGC, likely due to the structural similarity to its natural substrate, GTP. Falcinelli and colleagues (201) demonstrated increased *ex vivo* platelet reactivity 2hr post abacavir intake where the NO donor, SNAP EC_{50} was significantly greater versus the EC_{50} prior to abacavir intake. Additionally, intraplatelet cGMP levels were reduced following abacavir ingestion and similar results were seen *in vitro* where platelets were preincubated with carbovir triphosphate (201) and, Taylor and colleagues showed that preincubation of platelets with carbovir triphosphate reduced SNAP induced platelet inhibition in response to ADP (206).

Besides Falcinelli *et al* whose *ex vivo* results showed a change in cGMP and changes in SNAP EC_{50} in response to abacavir (201), other reported effects observed regarding abacavir and the NO signalling pathway have been where carbovir triphosphate has been added to platelets *in vitro*. While the results are clear, the physiological relevancy must be taken into consideration, as, *in vivo,* abacavir is converted to carbovir triphosphate in a multistep process intracellularly, and very little, if any, of the active anabolite will be present in the plasma or extracellular milieu. The intracellular concentration achieved following pre incubation is unknown, especially since, once inside the cell the ionic phosphate groups prevent the molecule from leaving the cell (246, 258).

To explore this potential mechanism further, in chapter 6, I pre incubated platelets with abacavir, lamivudine and abacavir + lamivudine and measured aggregation in response to collagen following incubation with the NO donor, SNAP. In testing these conditions, I remained consistent with previous experiments and, as much as possible I was able to subject platelets to the environment following intake of cART. I saw significant inhibition of platelet aggregation with the addition of SNAP, as expected, which was partially recovered with the addition of the sGC inhibitor, ODQ, but there were no differences in inhibition of aggregation between any of the pre incubation conditions.

Limitations, again, include the uncertainty of intraplatelet drug concentration, especially in this case, carbovir triphosphate, and whether 30 mins of preincubation with prodrug is enough time for any changes to be observed versus the much longer-term exposure that would be seen in an individual on ART. Since it is unclear whether this is enough time for abacavir to even be metabolised, it may be beneficial to carry out a time course experiment. Additionally, it could be worthwhile to examine cGMP production or/and VASP phosphorylation to see the effects further downstream following ART treatment. It will also be important to carry out these experiments with a lower concentration of SNAP as the concentration used may have been too high to observe any effect of drug.

ROS production in platelets is important for activation and thrombus formation, and dysregulation could lead to platelet hyperactivity, and is associated with coronary artery disease (185). Collagen induces superoxide anion and hydrogen peroxide via the GPVI-TRAF4-NOX pathway (252), thus I investigated the role of ART on platelet ROS generation in response to collagen using an H2DCF-DA flow cytometric assay.

I was unable to see a difference in ROS generation (as indicated by fluorescence change) in platelets following the addition of collagen at a range of concentrations, however, this was most likely due to the size of fibrous collagen and irregularity in the way it is presented to platelets in solution, causing difficulties in flow cytometric analysis (148). Cross linked CRP is a much more potent agonist of GPVI (as a result of the more numerous and consistent GPO motifs) (279), often preferred to collagen and

may result in clearer results, allowing for the observation of any potentiation in ROS generation following ART preincubation. Another explanation for a lack of a defined ROS generation response with an increase in collagen concentration may have been due to dye leak from platelets previously loaded with dye.

Having been unable to see an effect in ROS generation at a range of collagen concentrations, I explored the effect of ROS generation via the thrombin-PAR pathway where I saw an increase in ROS in a dose dependent manner, however, following this I saw no significant changes in ROS generation in response to preincubation with abacavir, lamivudine or abacavir + lamivudine. There was, however, a trend for platelets incubated with abacavir to display increased ROS production following agonist addition. Time allowing, I would have explored ROS generation further using different GPVI agonists (XL-CRP, convulxin), exploring the effect of GPVI and PAR dual stimulation on ROS, and examining the property of platelets of those using abacavir and other ART to generate ROS.

I was therefore unable to prove the 3rd hypothesis but showed another aspect of this field of research that can be explored further – the effect of ART on the redox control of the platelet function. In examining the role of abacavir on platelets specifically, it is possible that increased platelet activation and aggregation could be due to an interruption in the NO signalling pathway as well as increased GPVI mediated increased ROS generation.

7.5 Impact of the results and recommendations for future work

These results of this thesis suggest abacavir treatment can augment platelet activation via the collagen pathway, independently of potential HIV associated confounding factors. These data are in alignment with the data from the clinical trial conducted which also suggest abacavir use may alter platelet responses via the collagen-GPVI signalling pathway. These results may shed some light on the uncertainty surrounding the topic of a causal relationship between abacavir use and increased risk of MI, by strengthening the hypothesis of platelet dysfunction as a mechanism for this association, however details of this need to be clarified with more experimental mechanistic data.

As people living with HIV continue to get older and the ageing population grows, the prevalence of non-AIDS co morbidities, including CVD, will increase. It is therefore pertinent to understand the additional risks associated with ART exposure in order to provide the best possible care.

Since the results of the D:A:D study that showed abacavir use was associated with increased risk of MI, many studies have explored this further, but a clear conclusion has not been reached and, crucially, a mechanism hasn't been established.

Several studies have explored the role of platelet dysfunction, with varying outcomes, owing to several reasons previously discussed in this thesis and, through the use of *in vitro* and *in vivo* experiments and a randomised controlled trial, results suggest an effect of abacavir use on platelets and point to platelet dysfunction as a mechanism for the observed results seen in the D:A:D study.

Since starting this investigation, abacavir is now prescribed less frequently and those previously on abacavir based therapies are being moved onto alternative therapies. At the beginning of my investigation, abacavir based treatment (typically abacavir/lamivudine + 3rd agent) was an alternative to the preferred NRTI backbone of tenofovir/emtricitabine (280) as it was considered a metabolically 'clean' drug with a favourable bone and renal safety profile versus TDF, as well as being cost effective (281, 282). Recently, treatment guidelines have changed to suggest abacavir use only in coformulation

with lamivudine and dolutegravir, this is due to CVD risk and observed increased rates of virologic failure versus tenofovir/emtricitabine with efavirenz or atazanavir/ritonavir (76, 216).

BHIVA recommend most people should be on a preferred first line therapy, which now includes the 2 drug regimen, dolutegravir/lamivudine (76). In two randomised controlled trials, dolutegravir/lamivudine showed non-inferior efficacy versus tenofovir/emtricitabine + dolutegravir and no increased risk of resistance (76, 283).

To avoid the documented adverse effects of some NRTIs – abacavir and tenofovir disoproxil fumarate, especially - there is much interest in NRTI reducing regimens such as dolutegravir/lamivudine and, as it is non-inferior to current 3 drug regimens, it is recommended as an acceptable and indeed preferred regimen to switch to, especially for those on abacavir/lamivudine.

tenofovir disoproxil fumarate-based regimens are a recommended treatment for those who have resistance and those with hepatitis B virus (HBV) co infection (284), and is the treatment of choice for HBV due to its potency and high barrier to resistance (285, 286). Abacavir has no HBV activity however, therefore those on abacavir are more likely to be switched to dolutegravir/lamivudine whereas those on tenofovir disoproxil fumarate-based regimens with resistance or HBV will often remain on this regimen (76, 284).

Overall, there may be a move away from abacavir based therapy in adults towards nucleoside sparing regimens, but abacavir based regimens are still recommended in children with HIV (75, 287) to avoid the adverse bone and renal effects of tenofovir disoproxil fumarate (288). The research into abacavir-associated CVD risk in children and adolescents living with HIV is limited and it is pertinent to explore this area in more detail, given the inherent increased risk in people living with HIV. Perinatally infected children commonly have increased dyslipidaemia, raised markers of endothelial dysfunction and subclinical atherosclerosis (289). Furthermore, adolescents with HIV have a noted poor adherence to ART, with resulting elevated HIV-associated CVD risk factors (290, 291). Exploring the effect of long

term abacavir use on platelets in infants to understand any additional CVD risk would therefore be beneficial.

It is also important to understand the effect of newer therapies on the cardiovascular system and platelets. The RESPOND cohort is a prospective multicentre collaboration with more than 32 000 people living with HIV enrolled and recent analysis showed initiation of INSTI was associated with increased incidence of CVD with 2 years of exposure (271).

Use of ritonavir boosted darunavir has also been shown to be associated with increased risk of CVD (101) and the effect on platelets has also been investigated, where Taylor *et al* saw increased integrin activation and alpha and dense granule release in response to acute darunavir exposure (272). Additionally, Loelius and colleagues demonstrated a considerable increase in the prostanoid, prostaglandin E2, which has been shown to potentiate the platelet aggregatory response to agonists (292).

The techniques described in this thesis give a comprehensive analysis of platelet function in response to ART and warrant use in the investigation of other ART, especially INSTI and contemporary PI.

7.6 Overall summary and conclusions

In this thesis I have demonstrated that acute abacavir treatment *in vitro* leads to an increase in dense granule release, and a trend of increased alpha granule release and PAC-1 binding compared to platelets treated with vehicle control in response to collagen, which is indicative of increased platelet activation. Furthermore, we saw increased platelet aggregation in a mouse model of thromboembolism following treatment of abacavir compared to vehicle control in response to collagen. In a multisite randomised controlled study, we showed that switching away from an abacavir-based regimen resulted in decreases in platelet reactivity in response to collagen, increases in the expression of GPVI at week 12 and increases in soluble GPVI to week 48. *In vitro* studies

exploring the effect of abacavir, lamivudine and abacavir + lamivudine on redox regulation of platelets however showed no effect on NO signalling or thrombin-induced ROS generation, though there was a trend for platelets preincubated with abacavir to display increased thrombin-induced ROS generation versus platelets preincubated with vehicle control.

These data suggest abacavir treatment may increase the platelet activation in the absence of HIV, however, more investigation is needed to understand the mechanism, though it appears to be along the collagen- GPVI pathway.

The techniques used can be applied to contemporary antiretroviral agents both individually and in combination to understand their impact on platelet function.

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24	figure	Figure 1. Global distribution of HIV-1 subtypes	Sarabia I, Bosque A. HIV-1 Latency and Latency Reversal: Does Subtype Matter? Viruses. 2019; 11(12):1104	© 2019 by the authors. Licensee MDPI, Basel, Switzerland	n/a	yes	Licensed with a Creative Attribution 4.0 International (CC BY 4.0)
27	figure	Figure 2.	Rossi E, Meuser ME, Cunanan CJ, Cocklin S. Structure, Function, and Interactions of the HIV-1 Capsid Protein. Life. 2021; 11(2):100.	© 2021 by the authors. Licensee MDPI, Basel, Switzerland.	n/a	yes	Licensed with a Creative Attribution 4.0 International (CC BY 4.0)
28	figure	Figure 2. Steps of the HIV-1 replication cycle that can be targeted by gene therapy	Herrera-Carrillo E, Berkhout B. Bone Marrow Gene Therapy for HIV/AIDS. Viruses. 2015; 7(7):3910-3936.	© 2015 by the authors; licensee MDPI, Basel, Switzerland	n/a	yes	Licensed with a Creative Attribution 4.0 International (CC BY 4.0)
44	figure	Fig. 2 Structures for abacavir, CBV-TP, and deoxyguanosine triphosphate (dGTP).	Barbarino JM, Kroetz DL, Altman RB, Klein TE. PharmGKB summary: abacavir pathway. Pharmacogenet Genomics. 2014 May;24(5):276-82.	Copyright © 2014, © 2014 Wolters Kluwer Health | Lippincott Williams	18.11.22	yes	Written permission
57	figure	Figure 3. Model of the activation pathways induced by GPVI.	Moroi M, Jung SM. Platelet glycoprotein VI: its structure and function. Thromb Res. 2004;114(4):221-33.	Copyright © 2004 Elsevier Ltd.	18.11.22	yes	Written permission

62	figure	Figure 1. Stepwise NO synthesis by NOS.	Stuehr D, Pou S, Rosen GM. Oxygen reduction by nitric-oxide synthases. J Biol Chem. 2001 May 4;276(18):14533-6.	© 2001 ASBMB. Currently published by Elsevier Inc; originally published by American Society for Biochemistry and Molecular Biology.	n/a	yes	Licensed with a Creative Attribution 4.0 International (CC BY 4.0)
128	figure	Figure 4a	Taylor KA, Smyth E, Rauzi F, Cerrone M, Khawaja AA, Gazzard B, Nelson M, Boffito M, Emerson M. Pharmacological impact of antiretroviral therapy on platelet function to investigate human immunodeficiency virus-associated cardiovascular risk. Br J Pharmacol. 2019 Apr;176(7):879-889	© 2019 The British Pharmacological Society	14.08.2023	yes	Written permission