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Chapter 1

General introduction

Excerpts of this chapter have been published [2]:

Smith RL, de Boer R, Brul S, *et al*. (2012) Premature and accelerated aging: HIV or HAART? Front Genet 3:328. doi: 10.3389/fgene.2012.00328

General introduction

1. HIV infection and AIDS

1.1 Background and epidemiology

The first signs of a deadly new infectious disease appeared in 1981 in New York and Los Angeles, USA, with the emergence of *Pneumocytis carnii* pneumonia and Kaposi's sarcoma among previously healthy homosexual men and injection drug users. *Pneumocytis carnii* pneumonia and Kaposi's sarcoma are rare and chiefly opportunistic diseases, before only known to occur in the severely immune compromised. Soon thereafter, more cases of these opportunistic infections rose amidst sex workers, partners of infected men, and Haitian migrants [3]. In 1983 the cause for the rise in these immune deficiency-related diseases was identified as a novel retrovirus similar to human T-lymphotropic viruses and named the acquired immune deficiency syndrome (AIDS) virus. In 1986 it was officially designated as the human immunodeficiency virus (HIV) [4].

HIV is a *Retrovirus* belonging to the *Lentivirus* genus and comes in two types; HIV-1 and HIV-2. HIV-1 is closely related to the common chimpanzee and Western lowland gorilla simian immunodeficiency viruses SIV_{cpz} and SIV_{gor} respectively. HIV-2 is much less common than HIV-1 and was isolated from AIDS patients in West Africa in 1986. HIV-2 is similar to the sooty mangabey monkeys SIV_{sm} which inhabit Central and West Africa. HIV-1 is the predominant type worldwide and has particularly become well-known as a causative agent for AIDS.

To date, HIV infection is one of the most important global health issues, with approximately 35 million people infected worldwide and 2.1 million newly infected each year [5]. Particularly in Sub-Saharan Africa the HIV disease burden is very high with an approximate prevalence rate of 5.2% [3]. HIV infection has challenged the public health systems of every country infected and can justly be named the most significant of modern pandemics [6,7].

1.2 AIDS

Although the course of a HIV-1 infection varies from patient to patient, in general, infection follows a common pattern. After initial infection the virus replicates exponentially during a period of approximately three to six weeks. As the viral load increases the innate immune response becomes activated after a period of one to two weeks, followed by a humoral response after four to eight weeks post infection [8]. Symptoms are similar to those presented in patients with acute infections. This initial phase of infection is followed by a decrease in viral load after the onset of the immune response and viral titers can settle to constant values for several years. This phase is known as the chronic or asymptomatic phase wherein the infected immune cells show rapid turnover. Particularly $CD4^+$ T-cells decrease in number as they are primary targets for HIV-1. The third phase is characterized by a decline in T-cell number to a degree that viral load can no longer be kept stable which consequently leads to a peak in viral load. Clinically, HIV-1 infection is classified as AIDS when the host has less than 200 $CD4^+$ T cells per µL blood [8]. T-cells are not the only immune cells to be targeted by HIV-1, other $CD4^+$ cells such as monocytes, macrophages and dendritic cells are also known to be susceptible to infection, albeit to a lesser extent than T-cells [9]. Due to the depletion of the immune system's CD4+ cells the immune system of

the patient becomes compromised to the extent that opportunistic pathogens can easily infect the host. If HIV-1 infection is left untreated it can lead to immune-incompetence and acquired immune deficiency syndrome (AIDS), which quickly leads to death.

1.3 HIV structure

HIV-1 is approximately 120nm in diameter and appears spherical in shape due to its phospholipid bilayer envelope. From this bilayer a number of spikes protrude which consist of glycoproteins gp41 and gp120. Within the bilayer envelope a protective matrix of p17 proteins enclose a conical capsid composed of structural capsid p24 proteins. The capsid contains two copies of positive single-stranded RNA, which are coated with the nucleocapsid protein, p7. The capsid also contains the viral enzymes; reverse transcriptase, integrase and protease. Together with the capsid these components make up the core of the virus [3] (Figure 1).

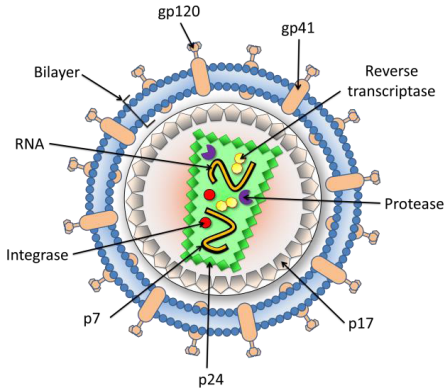

Figure 1. Diagram of a mature HIV-1 virion. The viral RNA (orange) is coated by p7 proteins (black) and together with the viral reverse transcriptase (yellow), integrase (red) and protease (purple) is enclosed in the conical capsid (green) which is made up of structural capsid p24 proteins. A protective matrix of p17 proteins ensures structural integrity of the core. Collectively the core and protective matrix are enveloped by a lipid bilayer (blue), which contains transmembrane glycoprotein gp41 and glycoprotein gp120. Figure adapted from Teixeira *et al*. 2011 [11].

1.4 HIV life cycle

Once an individual is infected, HIV-1 replication preferably takes place in several steps. In the first step, the virion attaches itself to the host cell with the help of gp120 which engages with the host cell's outer membrane CD4 molecules. Depending on the HIV strain, a conformational change initiated by gp120-CD4 binding ensures anchorage of the virion onto the host cell by interacting with either chemokine co-receptor CCR5 or CXCR4. Fusion of the virus with the host cell is mediated by gp41, whereafter the capsid is disrupted and the viral core contents are introduced into the host cells cytoplasm. The infectious cycle of HIV-1 continues with the reverse transcription of the viral RNA by the viral reverse transcriptase. Once transcribed the resulting viral DNA is transported into the infected cell's nucleus, is processed and incorporated into the host DNA with the aid of the viral integrase. Once the viral DNA has been inserted into the host genome, infection of that specific cell is permanent. The integrated viral DNA, now known as a provirus, is transcribed and translated by the host machinery to synthesize viral proteins and single stranded RNA for new virions. After processing and assembly of these components at the plasma membrane, the new virions bud off and mature with the aid of the viral protease, completing the HIV-1 life cycle (Figure 2). Release of the infectious viral particles causes rupture and consequently destruction of the host-cell.

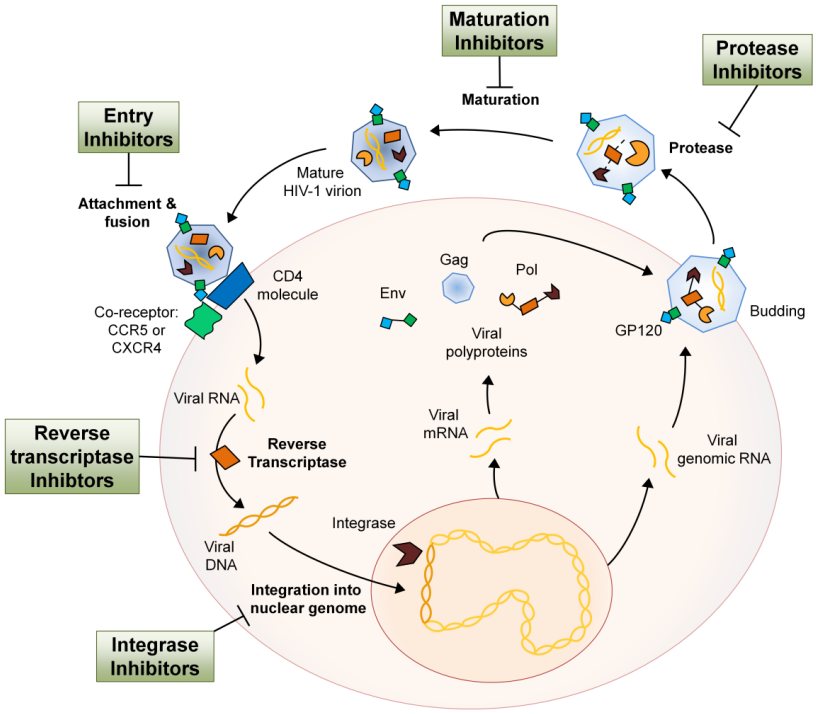

Figure 2: The HIV-1 life cycle and the antiretroviral drug class intervention points [2]**.** Entry inhibitors interfere with viral entry into the host cell and are comprised of a complex group of drugs with multiple mechanisms of action. By inhibiting several key proteins that mediate the process of virion attachment, coreceptor binding and fusion, virus spreading can be mitigated [12]. NRTIs imitate endogenous deoxyribonucleotides and have a high affinity for the viral reverse transcriptase, thus facilitating incorporation into the viral DNA strand during synthesis. NRTI incorporation results in transcription termination as they all lack the 3'-OH group necessary for phosphodiester bond formation in DNA strand elongation [13]. NNRTIs are compounds that fit into the allosteric 'pocket' site of the HIV-1 reverse transcriptase and disrupt its enzymatic activity, selectively blocking HIV-1 transcription [14]. Integrase inhibitors bind cofactors of the viral integrase that are essential in host DNA interaction and therefore block insertion of proviral DNA into the host genome [15]. Protease inhibitors bind the viral protease active site with high affinity and therefore inhibit cleavage of viral polypeptides and subsequent maturation of the virion after budding from the host cell [16]. HIV-1 maturation inhibitors act much like protease inhibitors in that they inhibit the processing of the HIV-1 polypeptides. However, maturation inhibitors do not bind the protease but rather the polypeptide itself, rendering it non-cleavable [17]. The relative size of different components has been altered for pictorial clarity.

2. Highly Active Anti-Retroviral Therapy (HAART)

Since the discovery of HIV-1 as a cause for AIDS, many antiretroviral pharmaceuticals have been developed to target viral replication. The first to be developed and prescribed as singular therapy was zidovudine (AZT) in 1987. Therapy with one drug however led to viral resistance and ineffective treatment. For the treatment of HIV-1 infection today there are six different classes of anti-HIV drugs. Each class of drug acts on a particular aspect of the viral life cycle (Figure 2), and are used in unison to increase therapy efficacy, overcome problems of tolerance, and decrease emergence of viral resistance. The major classes include the Entry Inhibitors (EIs), the Nucleoside Reverse Transcriptase Inhibitors (NRTIs), the Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs), and the Protease Inhibitors (PIs). The additional two anti-HIV drug classes are the Maturation Inhibitors (MIs) and Integrase Inhibitors (IIs), of which most compounds are still in clinical development.

Since 1996 the combination of at least three antiviral drugs, preferably from at least two different classes, has become standard practice and is known as Highly Active Anti-Retroviral Therapy (HAART). A typical HAART regimen consists of two NRTIs combined with a PI or NNRTI, sometimes supplemented with one drug from another class [18,19]. Without antiretroviral therapy HIV-infected patients usually die within years because of immune system failure. Due to HAART however, early death is prevented, allowing HIV-patients to live decades as long as medication is continued [20]. The therapeutic use of a combination of drugs was a major advance in HIV therapy and has significantly improved the quality and length of patient lives.

2.1 Nucleoside Reverse Transcriptase Inhibitors

The first class of antiretroviral drugs to be utilized in the clinic was the NRTIs. To date they are considered the 'backbone' of HAART (Table 1). With the exception of Tenofovir which is a nucleotide analogue, NRTIs are analogues of endogenous 2'-deoxy-nucleosides. NRTIs, however, miss the 3'-hydroxyl group on their ribose moiety which is necessary for chain elongation during reverse transcription (Figure 3). Due to their affinity for the viral reverse transcriptase, NRTIs act as chain terminators, suppressing the viral reverse transcriptase and consequent viral RNA to viral DNA transcription [13,21] (Table 5).

Table 1. The nucleoside reverse transcription inhibitor (NRTI) class. The approval date indicates FDA approval date [6]. * FLT trials were stalled in phase II due to potential side effects relating to bone marrow toxicity. However, FLT has shown to be very effective in suppressing NRTI-resistant HIV-1 mutants and trials have been resumed [22]. The NRTIs used in this study (didanosine, zalcitabine, zidovudine, stavudine & alovudine) are highlighted.

Nucleoside analogue		Drug name	Other names/Abbreviations	Approval date
Purine	Adenosine	Tenofovir disoproxil fumarate	TDF; 2-Cyclopentene-1-methanol, 4-(2-amino-6-(cyclopropylamino)-9H-purin-9-yl)-, (1S-cis)-	2001
	Guanosine	Abacavir	ABC; 2-Cyclopentene-1-methanol, 4-(2-amino-6-(cyclopropylamino)-9H-purin-9-yl)-, (1S-cis)-	1998
	Inosine	Didanosine	ddI; 2',3'-dideoxyinosine	1991
Pyrimidine	Cytidine	Emtricitabine	FTC; 2'-deoxy-5-fluoro-3'-thiacytidine	2003
		Lamivudine	3TC; 2',3'-dideoxy-3'-thiacytidine	1995
		Zalcitabine	ddC; 2',3'-dideoxycytidine	1992
	Thymidine	Alovudine	FLT; 3'-deoxy-3'-fluorothymidine	*
		Stavudine	d4T; 2',3'-didehydro-2',3'-deoxythymidine	1994
		Zidovudine	AZT; 3'-azido-3'-deoxythymidine	1987

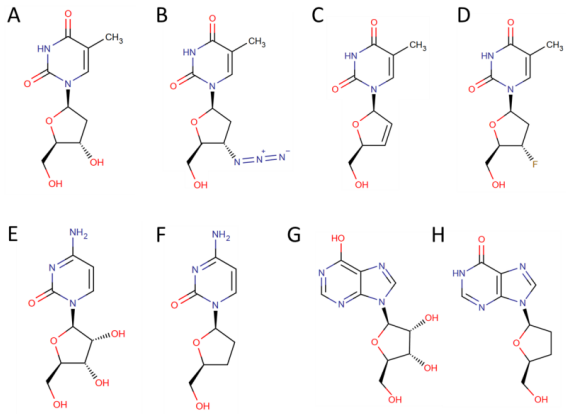

Figure 3. Molecular structure of NRTIs used in this study and their endogenous analogues. A: Deoxy-thymidine, B: AZT, C: d4T, D: FLT, E: Cytidine, F: ddC, G: Inosine, H: ddI. NRTIs are analogues of endogenous nucleosides (A, E & G). NRTIs (B-D, F & H), however, miss the 3'-hydroxyl group on the ribose moiety which is necessary for chain elongation during reverse transcription.

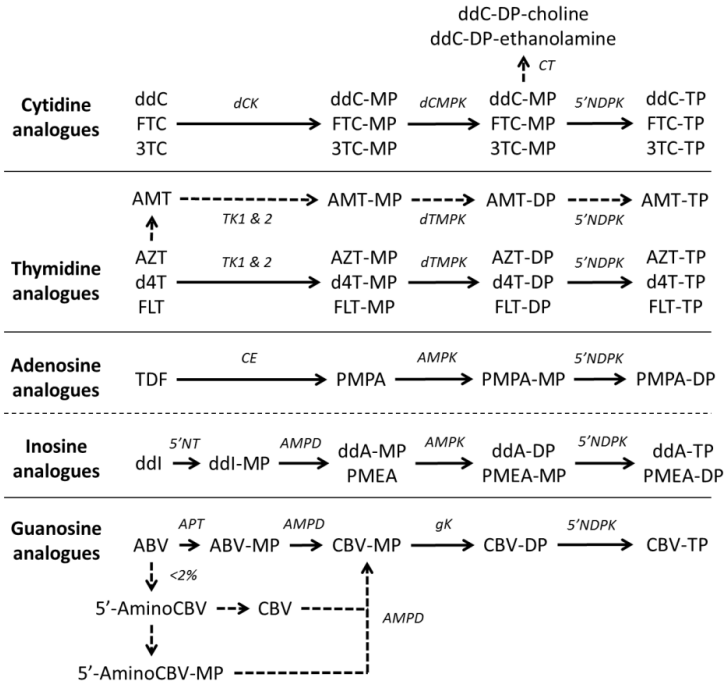

Figure 4. Intracellular phosphorylation pathways of NRTIs. AMPD, adenosine monophosphate deaminase; AMPK, adenosine monophosphate kinase (adenylate kinase); AMT, 3'-amino-3'-deoxythymidine; APT, adenosine phosphotransferase; CBV, carbovir; CT, cytidylyl transferase; dCK, deoxycytidine kinase; dCMPK, deoxycytidinemonophosphate kinase; ddA, 2'3'-dideoxyadenosine; dTMPK, deoxythymineoxythymine monophosphate kinase (thymidylate kinase); DP, diphosphate; gK, guanylate kinase; MP, monophosphate; PMEA, adefovir (a nucleotide RTI); PMPA, tenofovir (PMPA DP is a triphosphate analogue); TK1, thymidine kinase 1; TK2, thymidine kinase 2; TP, triphosphate; 5'NDPK, 5'nucleoside diphosphate kinase; 5'NT, 5' nucleotidase. Adapted from Kakuda 2000; Stein and Moore 2001; and Anderson *et al*. 2004 [23–25].

Before NRTIs can be incorporated into the elongating viral DNA strand and inhibit chain elongation they must become triphosphorylated. The host cell mediates the sequential enzymatic phosphorylation steps generating active dideoxynucleoside analogue triphosphates (ddNTPs) which compete with endogenous deoxynucleotide triphosphates (dNTPs) (Figure 4) for substrate binding to the viral reverse transcriptase. The NRTIs affinity for the viral reverse transcriptase, the ratio of ddNTP to endogenous dNTP and the error-prone nature of the viral reverse transcriptase contribute to the functional ability of the viral reverse transcriptase to incorporate NRTIs into the proviral DNA [23].

2.2 Protease Inhibitors

PIs were the second class of antiretroviral drugs to be administered in the clinic for HIV-1 infection. PIs are designed to inhibit the HIV-1 protease-mediated cleavage of the viral Gag and Gag-Pol precursor polypeptides and inhibit viral enzyme maturation. HIV-1 protease inhibition hinders the production of mature infectious virus particles which is an essential step in viral infectivity (Figure 2). This is achieved through high affinity competitive binding to the viral protease [16]. Besides their inhibitory effects on the viral protease, PIs have also been reported to reduce HIV-1 protease-induced apoptosis of $CD4^+$ cells [26].

The HIV-1 protease belongs to the aspartyl protease family, whose active form is a symmetrical homodimer that contains two aspartic acid residues at its active site. More specifically, a substrate-binding cleft is formed between the two identical subunits that each consist of 99 amino acids. The active site, containing the two aspartic acids at position 25 of each subunit, is located at the centre of the cleft. The substrate-binding cleft interacts with Gag and Gag-Pol polypeptides through recognition of the asymmetric shape of the peptide substrates and not by recognition of a specific amino acid sequence [16,27].

To date, nine PIs have been approved for clinical use (Table 2). The first PI drug to be approved for clinical use by the FDA in the USA was Saquinavir, in 1995 [27]. Between 1995 and 1997 three other PI drugs were approved – namely Ritonavir, Indinavir and Nelfinavir – and together with Saquinavir they comprise the first generation PIs (Figure 5A-D). Because of problems with viral drug resistance, toxicity and low bioavailability of these drugs in patients, a second generation of PIs was introduced between 1999 and 2006, which partly solved these problems. Second generation PIs include Amprenavir (and its improved pro-drug Fosamprenavir), Lopinavir, Atazanavir, Tipranavir and Darunavir. These nine PIs, with the exception of Tipranavir which is characterised by a dihydropyrone ring (Figure 5E), are competitive peptidomimetic inhibitors and their cleavage by the viral protease is hindered by a hydroxyethylene core [27] (Figure 5A-D).

Table 2. The protease inhibitor (PI) class. The approval date indicates FDA approval date [16,27]. The PIs used in this study (Indinavir, Nelfinavir, Ritonavir, Saquinavir) are highlighted. * Amprenavir was approved in 1999 and the prodrug Fosamprenavir in 2003.

Inhibitor type	Drug name	Trade names/Abbreviations	Approval date
Peptidomimetic (hydroxyethylene core)	Saquinavir	SQV; Fortovase; Invirase	1995
	Ritonavir	RTV; Norvir	1996
	Indinavir	IDV; Crixivan	1996
	Nelfinavir	NFV; Viracept	1997
	Fosamprenavir*	FPV; FOS-APV; Lexiva; Telzir	1999
	Lopinavir	LPV; ABT	2000
	Atazanavir	ATV; ATZ; Latazanavir; Reyataz; Zrivada	2003
	Darunavir	DRV; Prezista	2006
Non- peptidomimetic (dihydropyrone rine)	Tipranavir	TPV; Aptivus	2005

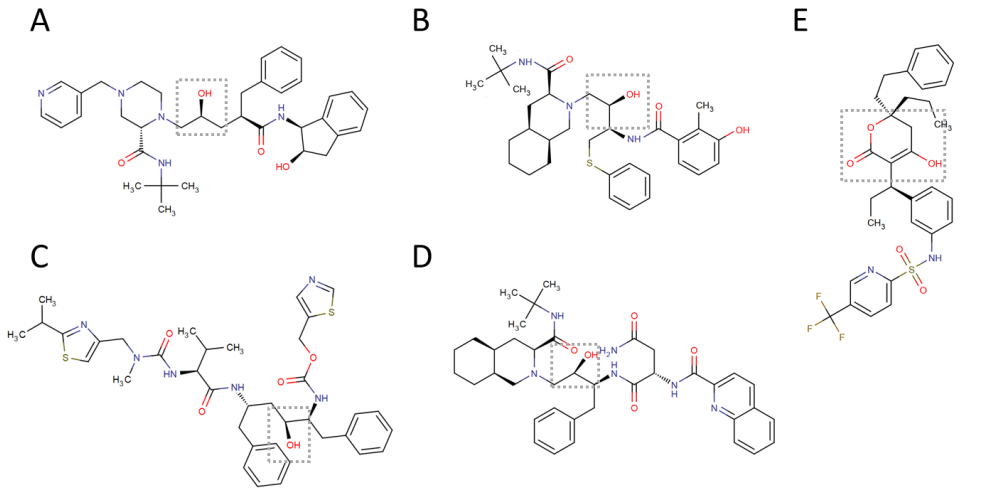

Figure 5. Molecular structure of PIs used in this study: A: Indinavir, B: Nelfinavir, C: Ritonavir, D: Saquinavir. Their hyroxyethylene cores and Tipranavir's dihydropyrone ring (E, is not used in this study) and are indicated with dashed boxes [27].

The introduction of PIs was considered a major advance of the past two decades in anti-retroviral therapy, not only because of their clinical potency, but also because they markedly increased the physicians' drug repertoire when applied in HAART regimens. Indinavir was the first drug to be administered in 1997 in combination with two NRTIs as a triple drug-combination therapy (HAART), leading to increased viral suppression and a significantly lower mortality of AIDS patients. A second major advance in PI use came when Ritonavir was recognized to improve bioavailability and half-life of concomitantly administered PIs through inhibition of intestine and hepatic cytochrome P450 3A4. This 'boosting' of antiretroviral therapy with low concentrations of Ritonavir led to the suppression of HIV-RNA to below 200copies/mL in 80% of patients and importantly enabled lower concentration use and fewer daily dosages for concomitant drugs [27].

2.3 Non-NRTIs, Entry inhibitors, Integrase inhibitors & Maturation Inhibitors

NRTIs and PIs are the most commonly used drug classes and most HAART regimens include them. Other less frequently used classes include NNRTIs, EIs, IIs and MIs. NNRTIs are compounds that fit into the allosteric 'pocket' site of the HIV-1 reverse transcriptase and disrupt its enzymatic activity, selectively blocking HIV-1 transcription [14]. Entry inhibitors interfere with viral entry into the host cell and are comprised of a complex group of drugs with multiple mechanisms of action. By inhibiting several key proteins that mediate the process of virion attachment, co-receptor binding and fusion, virus spreading can be mitigated [12]. Integrase inhibitors bind cofactors of the viral integrase that are essential in host DNA interaction and therefore block insertion of proviral DNA into the host genome [15]. HIV-1 maturation inhibitors act much like protease inhibitors in that they inhibit the processing of the HIV-1 polypeptides. However, maturation inhibitors do not bind the protease but rather the initially synthesized large polypeptide itself, rendering it non-cleavable [16,17].

2.4 Development of new compounds

True eradication of HIV-1 cannot be achieved with the technologies now at hand. Viral rebound follows discontinuation of therapy which means that therapy must be taken for a lifetime. Even though administration of antiretroviral drugs occurs with great care to diminish pill burden and improve efficacy, viral drug resistance and drug toxicity still interfere with successful chronic treatment. Additionally, drugs specialized at HIV-1 replication-inhibition in children and the elderly are scarce [6]. Whilst continuing to refine current treatment strategies, new targets for therapy are also required. Besides the expansion of the afore-mentioned antiretroviral drug classes [13,28], new paradigms in treating HIV-1 also exist. In short, these include proteosomal inhibitors, specific small molecule antagonists of Vif, Small interfering RNA molecules, and RNase H-mediated retrovirus-destruction triggered by oligodeoxynucleotides ("siDNA"). For a review see Broder 2010.

3. The antiretroviral drug burden

3.1 Background

Nowadays cocktails of antiretrovirals has become standard therapy and has led to the near successful inhibition of viral replication and, as a consequence, to a momentous decrease in morbidity and mortality of HIV-1 patients [6]. Overshadowing this celebrated success, however, is the problem that near complete inhibition of viral replication demands that patients continue daily therapy for the rest of their lives so as to prevent rapid virus rebound [29]. Besides this pill burden, HIV-1 infected patients are afflicted with drug induced adverse events, some of which can be life threatening [30,31]. Typical initial adverse events include hypersensitivity reactions such as rash and gastrointestinal toxicity, which mostly abate after time. Chronic drug exposure, however, can lead to more severe symptoms such as lipodystrophy, myopathy, neuropathy, hepatic steatosis, hepatitis, lactic acidosis, insulin resistance, neutropenia, and an increased risk for myocardial infarction [19,32] (Table 3). Patients suffering from (severe) adverse events are required to alter their therapy regime, likely only to delay the reoccurrence of new or similar adverse events. To make matters worse, even with successful and tolerated regimes, HIV-1 patients receiving antiretrovirals can show signs of premature and accelerated ageing [2] (See Chapter 1, Paragraph 3.9).

Table 3. Summary of common toxic effects of NRTIs and PIs. Gastrointestinal (GI) toxicity = abdominal pain, nausea, emesis, diarrhea; GI upset = nausea, emesis and abdominal pain; dyslipidemia = abnormal amount of lipids in the blood, including hypercholesterolemia and hypertriglyceridemia. ++++, strongest association with mitochondrial toxicity, + weakest association with mitochondrial toxicity: adapted from Apostolova *et al*. 2011; and Margolis *et al*. 2014 [19,32]. The data presented here should be considered with caution, as there are discrepancies among studies regarding some of these adverse events.

Antiretroviral class		Common toxicities	Potential for mitochondrial toxicities
NRTI	Abacavir (ABC)	Hypersensitivity reaction, cardiovascular risk, dyslipidemia	+
	Alovudine (FLT)	Myelosuppression, hepatotoxicity	++++
	Didanosine (ddI)	Pancreatitis, peripheral neuropathy, cardiovascular risk, dyslipidemia, insulin resistance, hepatotoxicity, lactic acidosis	++++
	Emtricitabine (FTC)	Mild headache, rash, GI upset	+
	Lamivudine (3TC)	Constitutional symptoms, dyslipidemia, pancreatitis, peripheral neuropathy	+
	Stavudine (d4T)	Lipoatrophy, pancreatitis, peripheral neuropathy, dyslipidemia, lipohypertrophy, insulin resistance, hepatotoxicity, lactic acidosis	++++
	Tenoforvir (TDF)	Fanconi's syndrome, renal insufficiency, GI upset	+
	Zalcitabine (ddC)	Thrombocytopenia, anemia, pancreatitis, cardiomyopathy, peripheral neuropathy, lactic acidosis, hepatotoxicity	++++
	Zidovudine (AZT)	Myelosuppression, lipoatrophy, lipohypertrophy, (cardio)myopathy, dyslipidemia, insulin resistance, hepatotoxicity, lactic acidosis	++
PI	Atazanavir (ATV)	GI toxicity, dyslipidemia, unconjugated hyperbilirubinemia, lipohypertrophy	+
	Darunavir (DRV)	GI toxicity, rash, lipohypertrophy, hepatotoxicity	unknown
	Fosamprenavir (FPV)	GI toxicity, risk for myocardial infarction, rash, lipohypertrophy, dyslipidemia	unknown
	Indinavir (IDV)	GI toxicity, dyslipidemia, unconjugated hyperbilirubinemia, nephrolithiasis, insulin resistance, lipohypertrophy	++
	Lopinavir (LPV)	GI toxicity, dyslipidemia, risk for myocardial infarction, insulin resistance, lipohypertrophy	+
	Nelfinavir (NFV)	GI toxicity, insulin resistance, lipohypertrophy, dyslipidemia, hypersensitivity reaction, rash	++
	Ritonavir (RTV)	GI toxicity, dyslipidemia, risk for myocardial infarction, insulin resistance, lipohypertrophy	+++
	Saquinavir (SQV)	GI toxicity, cardiovascular risk, insulin resistance, lipohypertrophy	+
	Tipranavir (TPV)	GI toxicity, dyslipidemia, intracranial hemorrhage, rash, hepatotoxicity	unknown

3.2 Mitochondrial toxicity as a common pathway for antiretroviral induced adverse events

Most adverse events witnessed by patients using antiretroviral therapy seem to be related to tissues with high-energy demand and show a strong similarity to hereditary mitochondrial diseases [33]. Indeed, after introduction of HAART to treat HIV-1 infection, it quickly became apparent that mitochondrial toxicity is likely a major reason for antiretroviral-related adverse events [34]. Mitochondria are organelles with a unique double membrane structure that play a vital role in the life cycle and fitness of the cell. As they produce most of the cell's adenosine 5'-triphosphate (ATP), harbour the citric acid cycle, β-oxidation and the respiratory chain, they are often termed 'cellular power houses' [35]. Importantly, mitochondria are also versatile signalling organelles, fundamental to cellular function by maintaining the cell's redox balance and regulating apoptosis [36–38]. Therefore, a perturbation of any of these functions impairs cellular life-expectancy and has been shown to have tissue and systemic repercussions including accelerated ageing [39].

3.3 NRTI related mitochondrial toxicity

Mitochondrial failure caused by the antiretroviral therapy class of NRTIs has been extensively studied. As NRTIs mimic endogenous nucleosides and can compete for polymerase binding it was quickly reasoned that NRTI-TPs

might inhibit cellular polymerases. In line with this, NRTI-TPs were found to have affinity for cellular polymerases [40] (Table 4). Upon examination as early as 1989 it became apparent that NRTI-TPs more readily inhibit mtDNA polymerase gamma (γ) than other polymerases, albeit with varying affinity [21,41,42] (Table 4 & 5). As polymerase-γ is responsible for mtDNA replication and repair, inhibition thereof results in low mtDNA copy numbers, and mutated and/or truncated mtDNA.

Table 4. NRTI K_i values for human cellular polymerases. Polymerases were purified from HeLa S3 cells. ND, not determined. Km values for each enzyme were determined using endogenous nucleotide substrates. Adapted from Martin *et al*. 1994 [40].

Substrate	Inhibitor	Polymerase-α		Polymerase-β		Polymerase-γ		Polymerase-ε	
		K_i (μM)	K_i/K_m	K_i (μM)	K_i/K_m	K_i (μM)	K_i/K_m	K_i (μM)	K_i/K_m
dTTP	AZT-TP	140 ± 20	58	290 ± 20	180	8.7 ± 0.7	51	400 ± 50	130
	d4T-TP	120 ± 10	50	1.2 ± 0.3	0.75	0.048 ± 0.005	0.28	59 ± 9	20
	FLT-TP	8.8 ± 0.9	3.6	1.7 ± 0.3	1.1	0.036 ± 0.004	0.21	ND	ND
dGTP	CBV-TP	6.9 ± 0.9	7.7	340± 30	240	14 ± 2	100	410 ± 80	160
dCTP	ddC-TP	90 ± 20	64	1.2 ± 0.2	0.86	0.015 ± 0.003	0.09	70 ± 10	29
dATP	ddA-TP	64 ± 8	49	1.1 ± 0.2	0.92	0.018 ± 0.002	0.11	67 ± 9	22

Table 5. Discrimination of NTRIs by polymerase-γ and the wild type HIV-1 reverse transcriptase. Human polymerase-γ and wild type HIV-1 reverse transcriptase (RT) were recombinant and purified from *E. coli*. Discrimination is determined by the binding preference for the correct versus the incorrect nucleoside triphosphate during polymerization. Effective discrimination is represented as a high value and a high value for polymerase-γ (pol-γ) over HIV-1 reverse transcriptase therefore indicates the effectiveness of the analogue to be a more specific HIV-1 reverse transcriptase inhibitor. Adapted from Johnson *et al*. 2001 [21].

Analog	HIV-1 reverse transcriptase		Pol-γ
	DNA	RNA	
AZT-TP	3.4	1.4	37.103
d4T-TP	0.56	1.17	7.4
CBV-TP	34	7.4	902.777
ddC-TP	15.7	4.5	2.9
3TC-TP	61.5	149	2.857
ddA-TP	5	4.5	4
PMPA-DP	6.1	7.1	11.4

Because each cell can harbour multiple copies of mtDNA, between 1000 and 1.000.000 copies depending on their energy need and thus dependency on mitochondrial function, a natural heterogeneity exists within mitochondria of any given cell, and among cells of a particular tissue [43]. This phenomenon is further amplified by the unpredictable segregation of mtDNA during cellular replication and the malleability of mitochondria through the dynamic processes of mitochondrial fission and fusion. This genomic diversity and sheer number of mtDNA copies causes there to be a threshold effect for the expression of mitochondrial dysfunction. More specifically, a mitochondrion will only show evidence of dysfunction when a large population of its mtDNA has a mutation load that exceeds a threshold (usually >80%: 90% for tRNA point mutations and 60% for large scale mtDNA deletions). This threshold effect can therefore often mask the severity of mtDNA depletion or degradation and may explain tissue specificity and clinical presentation of NRTI induced mitochondrial toxicity and the onset of side effects [44]. Interestingly, whole mtDNA sequencing has shown that NRTIs can also hasten the clonal expansion of pre-existing mutations in mtDNA as depleted mtDNA pools display accelerated digression from their original genetic content. This can lead to an irreversible increase in the frequency of

deficient mitochondria and the premature onset of progressive multi-organ failure mirroring those seen much later in life caused by normal aging [45,46].

Besides the direct competitive inhibition of mtDNA replication by NTRI-TPs, NRTI-MPs have been found to obstruct base excision repair and proofreading capabilities of polymerase-γ [42,47,48]. Interestingly, mice with impaired polymerase-γ proofreading ability show rapid accumulation of mtDNA mutations leading to disrupted mitochondrial function, a variety of ageing phenotypes and early death [49]. The exonucleolytic ability of polymerase-γ appears to be dependent on the NRTI analogue. For example, although AZT is one of the NRTIs least likely to be incorporated into mtDNA due to its relatively low affinity for polymerase-γ, it is the NRTI which is most ineffectively removed [50].

Taken together, polymerase-γ is responsible for mitochondrial DNA (mtDNA) replication and repair, and inhibition results in reduced mtDNA copy numbers and/or truncated mtDNA [47]. As mtDNA encodes for essential components of the mitochondrial respiratory chain, depletion in mtDNA quality and quantity likely impedes mitochondrial oxidative phosphorylation and consequently mitochondrial function. Indeed, Pan-Zhou *et al.* showed that in HepG2 cells NRTIs can influence mtDNA encoded polypeptide synthesis and activity of respiratory chain enzyme complexes, although the analogues differ in their inhibitory capacity and there are signs of selectivity in the extent of inhibition of individual polypeptides [51]. Diminished oxygen consumption, reduced membrane potential, increased lactate/pyruvate ratios and altered mitochondrial morphology have all been witnessed in mitochondria from HIV-1 patients, mice, worms and cell-lines receiving NRTIs [2,52,53]. This central mechanism underlying the toxic effects of NRTIs was postulated as the 'polymerase-γ theory' in 1995 (Lewis and Dalakas 1995; Figure 6).

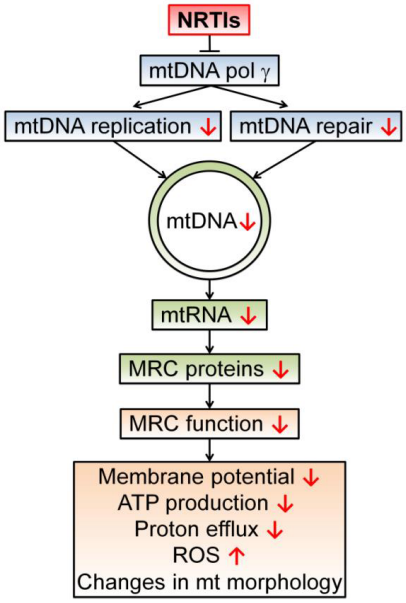

Figure 6: The polymerase-γ theory [2]**.** NRTIs compete with endogenous nucleotides and nucleosides for transcriptase binding. Due to the surplus and high affinity of NRTIs for polymerase-γ, NRTIs are frequently incorporated into the new DNA strand which results in chain termination as they all lack the 3'-OH group necessary for phosphodiester bond formation in DNA strand elongation. This results in a reduced number of mtDNA molecules and possibly a reduction in mtDNA encoded proteins, essential components of the mitochondrial respiratory chain (MRC) complexes. In turn, this leads to disrupted electron transport through the MRC and a concomitant reduction in proton efflux, reducing the membrane potential and ATP production by the mitochondrion. This disturbed mitochondrial function can result in augmented ROS production and morphological changes. Disturbed mitochondrial function due to polymerase-γ inhibition has been proposed as a central mechanism for NRTI induced adverse events [30,50].

3.4 NRTI toxicity beyond the polymerase-γ theory

Although the polymerase-γ theory clarified the cause of many adverse events witnessed in patients receiving NRTIs, a large body of evidence has accumulated over the years which suggests that there are modes to NRTI mitochondrial toxicity that lie beyond simple inhibition of polymerase-γ [32,54]. Initial doubt was placed in the polymerase-γ theory because affinity with, and concurrent inhibition of, polymerase-γ by NRTIs does not linearly correlate with clinical manifestations of mitochondrial toxicity. Moreover, newer NRTIs which have low affinity for polymerase-γ can still cause mtDNA depletion and mitochondrial dysfunction [13].

Here besides, mitochondrial toxicity caused by NRTIs does not necessarily follow the chronological steps of the polymerase-γ theory. Not every case of mtDNA depletion leads to changed expression levels or activity of mitochondrial respiratory chain proteins [49,55]. Similar observations were made in knockout mice deficient of the mtDNA transcription factor *Tfam*, which show reduced mtDNA content but normal levels of mitochondrial transcripts and mtDNA encoded respiratory chain subunits [56]. Conversely, altered mitochondrial gene transcription and impaired respiratory chain activity have been observed in the absence of mtDNA depletion during NRTI exposure [57,58]. In addition, respiratory chain complex activity was shown to decrease significantly in the absence of mtDNA or polypeptide synthesis depletion [51].

3.4.1 Adaptation of mtDNA encoded transcripts

Expression profiles of mitochondrial mRNA possibly explain these occurrences as they have been shown to adjust, both in a peripheral blood mononuclear cell line and mice upon exposure to NRTIs. These adjustments likely reflect cellular adaptation to pressure on the mitochondrial transcriptional machinery [59,60]. Indeed, polymerase-γ deficient nematodes compensate for their mtDNA replication deficiency by up-regulating mitochondrial transcripts and mice that have lowered mtDNA copy number show normal levels of mtDNA-encoded transcripts and proteins [61]. Furthermore, primate cell lines have been shown to adapt to mtDNA expression inhibition by increasing the stability of mtDNA-encoded transcripts and proteins [62].

Such adaptations are likely to prove successful only for short periods of time. Continued suppression of mtDNA transcription for longer periods will eventually result in MRC failure due to the perturbed turnover of mtDNA encoded transcripts and MRC proteins. This is likely especially the case, when under constant inhibition, polymerase-γ generates truncated mtDNA copies or during transcription can no longer reach the end of its templates, creating non-polycistronic transcripts [63]. Furthermore, these components remain susceptible to ROS induced damage that increases upon deterioration of MRC function [2]. To allow for compensation of mtDNA-encoded proteins mitochondria likely increase fusion so as to complement defective components that have accumulated in individual mitochondria during NRTI pressure on the mtDNA replicative machinery [52,61]).

3.5 Major theories of NRTI toxicity beyond polymerase-γ inhibition

In an elegant review, Apostolova *et al*. illustrate that mitochondrial toxicity of antiretroviral drugs often goes beyond the polymerase-γ theory, as disruption of many other mitochondrial mechanisms has been discovered upon exposure to NRTIs [32]. The major theories for toxicity beyond polymerase-γ inhibition are described below.

3.5.1 NRTI pharmacokinetics

Most NRTIs are hydrophilic which inhibits their diffusion into cells in large quantities. NRTIs therefore depend upon nucleoside transporters to transpose across biological membranes. In humans, cells can directly import unphosphorylated NRTIs via the equilibrative nucleoside transporter (ENT2) [64]. Besides nucleoside transporters, organic anion and cation transporters as well as multi-drug resistant proteins have been reported to transport NRTIs, although their exact function in NRTI related toxicity remains to be truly evaluated. Studies of the role of such transporters may, however, shed light upon specific drug effects and the susceptibility of specific tissues to NRTI toxicity as the distribution of such transporters differs among tissues [53]. Evidence suggests that NRTIs can alter activity and expression of multidrug resistance-related proteins (MRP) and in this way modify drug absorption and elimination in a tissue specific manner. Moreover, different NRTIs were seen to inhibit different MRPs. This observation may explain the superior efficacy of combinational therapy but also the enhanced toxicity of specific antiretroviral combinations [65]. Indeed, the transport of nucleosides and nucleotides into mitochondria, or other subcellular compartments, has been proposed as a major determinant of mitochondrial effects of NRTIs [66]. There are many transport systems available for nucleosides depending on their phosphorylation state. For instance, di- and tri-phosphorylated NRTIs can be transported into the mitochondria via the deoxynucleotide carrier (DNC) [67]. NRTIs have been proposed to affect DNC stoichiometry and consequently disrupt the homeostasis of native nucleotides [50].

3.5.2 Direct inhibition of (mitochondrial) enzymes

NRTIs, in particular AZT, are known to directly inhibit the mitochondrial respiratory chain complex I, (NADH dehydrogenase), complex II (succinate dehydrogenase), and complex V (F_1,F_0-ATPase) [68–70]. Most other enzymes that show inhibition by NRTIs depend on nucleosides or nucleotides as substrates or cofactors for their activity. For example, AZT is known to competitively inhibit the ADP/ATP translocator, also known as the adenine nucleotide translocator (ANT). ANT is solely responsible for the transport of cytosolic ADP into the mitochondrial matrix in exchange for ATP, and AZT exposure of isolated rat liver mitochondria caused a rapid significant decline of cellular ATP levels [71,72]. AZT is also known to interact with adenylate kinase, hexokinase, and fructose-6-phosphokinase [73]. Additionally, AZT-MP has been found to inhibit protein glycosylation, *N*-linked protein glycosylation in particular, by directly competing with several pyrimidine sugars for transport into Golgi membranes. The accumulation of AZT-MP in the cytosol likely unbalances the nucleotide gradient on which the enzymatic activity of the sugar tranferases depends [74].

3.5.3 NRTI thymidine analogue phosphorylation

To become incorporated into the viral DNA, NRTIs need to become tri-phosphorylated (Figure 4). The cellular energy state of any given cell type has the most influence on this process, as has been well described for thymidine analogues. Thymidine kinase 1 (TK1) is cytosolic and is particularly active in replicative tissues, especially during S-phase. TK2 is constitutively expressed, is predominantly localised in the mitochondria, and is the major thymidine kinase in mitotically quiescent tissues such as the heart [75]. Different thymidine analogues have varying affinity for TK1 and TK2. AZT and FLT were shown to be efficiently phosphorylated by TK1 compared to endogenous deoxythymidine, whereas d4T was not. TK2 can phosphorylate AZT, although poorly,

whereas FLT and d4T were not phosphorylated by TK2 [23,76]. The differences in TK expression during cellular replication activity and the varying abilities of NRTI thymidine analogues to be phosphorylated by TK's may give insight into the susceptibility of specific tissues for NRTI toxicity, although more research is needed.

Besides the TK1 supported de novo synthesis of deoxyribonucleotides, TK1 and TK2 are needed for thymidine salvage with the end product being deoxythymidine-TP. Deoxythymidine-TP is a feedback inhibitor of TK1 and an allosteric inhibitor of ribonucleotide reductase where it shifts ribonucleotide reductases' specificity from pyrimidine to purine reduction. Therefore a rise in the deoxythymidine-TP pool causes an imbalance in pyrimidine and purine pools, inhibiting DNA metabolism and repair. An imbalance in nucleoside and nucleotide pools has been proposed as a major cause for NRTI induced toxicity [77,78]. For example, changes in cellular thymidine kinase kinetics by interaction with NRTI thymidine analogues have been linked to cardiomyopathy and lipodystrophy [32]. Conversely, mitochondrial dysfunction has itself been shown to lead to a decrease in deoxynucleoside pools which causes a delay of replication fork progression and double strand breaks, causing genomic instability such as chromosomal translocations and rearrangements [79].

Thymidine analogues have also been shown to inhibit TK1 and TK2 phosphorylation of endogenous deoxythymidine. TK1 is particularly inhibited by d4T, and FLT and d4T can effectively inhibit TK2 [76,80]. AZT has also been shown to inhibit TK2 in rat heart mitochondria and in this way AZT likely limits the supply of normal deoxypyrimidine phosphates resulting in loss of replication fidelity and mtDNA depletion [81]. AZT exposure in human osteosarcoma (U2OS) cells led to the reduction of mitochondrial TK2 and deoxyguanosine kinase (dGK) protein levels. dGK complements TK2 function in the mitochondria and both are vital to mitochondria as deficiency in either causes severe mtDNA depletion [82]. Interestingly, ddI also has been found to down-regulate TK2 and dGK whilst TK1 and the cytosolic deoxycytidine kinase were not affected [83]. In addition, nucleoside diphosphate kinase (NDPK) resides within the mitochondrial intermembrane space and is an important enzyme for the tri-phosphorylation of all NRTI-DPs (Figure 4). Valenti *et al*. have shown that AZT can competitively inhibit NDPK in isolated rat liver mitochondria at clinically effective concentrations [84].

NRTIs can also inhibit cellular replication by a phenomenon known as the "thymidine block". Cells exposed to a high concentration of thymidine undergo S-phase arrest. Thymidine is a strong allosteric inhibitor of ribonucleotide reductase which converts a fraction of the cellular pool of ribonucleotides into deoxyribonucleotides. In this way thymidine reduces the affinity of ribonucleotide reductase for its pyrimidine ribonucleotide substrates; uridine diphosphate and cytidine diphosphate. Inhibition of cytidine diphosphate conversion to deoxycytidine diphosphate depletes deoxycytidine triphosphate pools, thus causing S-phase arrest. Hematopoiesis in bone marrow and thymus is especially sensitive to thymidine block at physiological concentrations of thymidine, causing replication stress in proliferating T-cells, B-cells and erythroid precursors [85]. It is not surprising then that bone marrow toxicity and neutropenia are common adverse events in patients receiving thymidine analogues (Table 3).

Finally, the use of the hosts' enzymes to accomplish NRTI activation and nucleoside pool imbalance can put demands upon normal nucleoside homeostasis resulting in over-activation of these enzymatic processes.

Indeed, HIV-1 patients taking NRTIs often show higher rates of NRTI phosphorylation than normal nucleosides [24]. *In vitro* experiments have also shown that cellular activation can be increased by amplified rates of NRTI and endogenous nucleoside phosphorylation. Cellular activation corresponds to an over expression of cytokines, which is often seen in patients, and links NRTI use to a pro-inflammatory state and adverse events [24].

3.5.4 Reactive oxygen species

Many of the adverse events seen in patients receiving NRTIs have specifically been linked to oxidative stress [86,87]. For example, cardio-vascular disease, ageing, central nervous system disorders, inflammation, and metabolic and lipodystrophy syndromes have all been found to be related to ROS caused by antiretroviral treatment [88,89]. HIV-1 patients treated with antiretroviral therapy have been shown to have significantly higher serum oxidant levels compared to therapy naive HIV-1 patients and uninfected controls. Underscoring the effect of antiretroviral therapy on oxidative stress, patients who strictly adhere to therapy guidelines have increased oxidative status compared to those who do not closely follow therapy [90]. Many *in vitro* experiments have also shown that NRTIs can cause ROS production.

Of the theories for NRTI toxicity beyond the polymerase-γ theory, oxidative stress is considered to be the most conspicuous [87]. This is especially the case when taking into account that ROS can further worsen mitochondrial function by causing oxidative damage to lipids, MRC proteins, and mtDNA [91,92]. Moreover, polymerase-γ is very sensitive to oxidative damage and modification of its amino acid residues by oxidation causes a decrease in DNA-binding ability and polymerase activity [93].

One could argue that MRC malfunction in consensus with the line of events proposed by the polymerase-γ theory would logically lead to increased ROS levels, making an increase in oxidative stress a secondary effect. Interestingly, however, mitochondrial malfunction and augmented ROS production have been observed with NRTIs in the absence of mtDNA depletion, which is in contradiction to the chronology of events proposed in the polymerase-γ theory [68].

3.6 PI related mitochondrial toxicity

In general, PIs have been linked to various side-effects ranging from mild – such as gastrointestinal irritations, headache and fatigue – to severe, such as cardiovascular complications and metabolic abnormalities including hyperlipidemia, lipodystrophy and insulin resistance (Table 3). These severe symptoms are usually observed after chronic PI based therapy [26,27,94]. Although a direct mechanism for PI toxicity has yet to be verified, PI adverse events, much like NRTIs, are similar to those observed in patients suffering from mitochondrial diseases, implying that PIs induce mitochondrial dysfunction [33]. In support of this, a reduction of mitochondrial membrane potential ($\Delta\Psi_{mt}$) has been observed in fibroblasts after long-term treatment with Indinavir (IDV) and Nelfinavir (NFV), accompanied with decreased expression of the mitochondrial-encoded cytochrome c oxidase subunit II (COX2) and increased ROS production from the mitochondria [95]. Studies have also demonstrated that PIs affect mitochondrial morphology [96–98] and increase mtDNA content [58]. The precise chronology of events leading to PI induced mitochondrial dysfunction, however, is - to date - unknown. The major theories underpinning PI mitochondrial toxicity are described below.

3.6.1 Reactive oxygen species

A principal theme within PI-induced mitochondrial dysfunction is the elevation of ROS [32]. Several human and animal studies have clearly established a link between PI usage and increased ROS production in different cell- and tissue types (reviewed in Reyskens & Essop, 2014 [99]). For example, high levels of ROS were found after 30 minutes exposure to PIs in human peripheral blood mononuclear cells (PBMCs) [100]. Increase of ROS production has also been reported after treatment of different cell-systems such as human pulmonary artery endothelial cells and human umbilical vein endothelial cells with some PIs [32]. The widely used antiretroviral 'booster' Ritonavir (RTV) has been shown to cause increased oxidative stress in arterial endothelium, and through this mechanism is expected to stimulate cardiovascular disease [101]. Caron *et al*. demonstrated that skin fibroblasts treated with IDV or NFV had a premature ageing phenotype, alterations in expression of MRC proteins, and ROS hyper-production [95]. RTV and Lopinavir (LPV) have been shown to induce elevated ROS production by mitochondria from HL-1 cardiomyocytes, possibly via depolarization of mitochondrial membrane potential [102].

The exact role of oxidative stress in PI-induced mitochondrial dysfunction and development of adverse events is still unclear. Although most studies suggest a mitochondrial origin for PI induced ROS production, others have indicated the presence of extra-mitochondrial ROS and hypothesize that cytosolic ROS is the initial trigger that leads to mitochondrial malfunction [99]. For instance, RTV treated porcine carotid arteries showed elevated levels of ROS likely through increased NAD(P)H oxidase activity and a consequent elevation in superoxide production [101]. Elevated cytosolic ROS by NFV has been linked to a decreased activity and gene expression in Cu/ZnSOD but not MnSOD [99]. Additionally, Chandra *et al*. found decreased levels of glutathione and Cu/ZnSOD in a rat pancreatic insulinoma cell line (INS-1) after exposure to NFV, suggesting that PIs affect the antioxidant defence mechanism [103]. Reyskens and Essop therefore proposed that a diminished cytosolic antioxidant defence system leads to an increase in cytosolic ROS, which then raises ROS levels in the inter-mitochondrial membrane space causing enhanced leakage of ROS from the mitochondria and a perturbation of MRC complex activity [99]. Although the proposed chronology is plausible, more research is needed to precisely elucidate the chain of events that leads to mitochondrial dysfunction.

3.7 Contradictory effects of PIs

Individual PIs have been found to have different or even contradictory effects, depending on their concentration and in which model system they are applied. For instance, treatment of lymphocytes with low concentrations (<10nM) of NFV, LPV, IDV or Saquinavir (SQV) followed by stimulation of the cells to undergo apoptosis, yielded a protection from apoptosis-related $\Delta\Psi_{mt}$ loss and ROS production. When treating the cells with higher drug concentrations (>10µM) the effects were reversed, leading to accelerated apoptosis [96]. Additionally, there are indications that increased ROS production only occurs after exposure to a certain threshold concentration of PIs. For example, neither SQV nor Atazanavir (ATV) in concentrations up to 20µM enhanced ROS levels or negatively affected antioxidant defence systems in INS-1 cells [103]. In primary human skeletal myotubes, a higher concentration (40µM) of SQV and ATV, however, did significantly increase ROS production [104]. Indeed, the model system in which PIs are tested also influences the outcome of adverse events. In human pulmonary artery endothelial cells treated with RTV and IDV at their plasma concentrations

(15µM and 13.5µM respectively), exposure reduced their $\Delta\Psi_{mt}$ [105]. Incubation of the parasite *Leishmania* with 40µM of NFV resulted in complete loss of $\Delta\Psi_{mt}$ [97]. On the other hand, no effect on $\Delta\Psi_{mt}$ was observed when HeLa cells were treated with 10µM NFV or RTV, but the drugs did cause extensive mitochondrial fragmentation [106].

Concentration dependant effects may be related to the well documented inhibition of cytochromes P450 and ABC transporters by PIs. All the PIs currently available inhibit CYP3A4, with RTV being the most potent and SQV the weakest [107]. CYP3A5 and 3A7 are also inhibited by RTV, NFV, IDV, APV, and SQV *in vitro*, albeit with varying potency [108]. RTV and SQV also inhibit CYP2C9 in human liver microsomes *in vitro* [109]. SQV, NFV, RTV, APV, and IDV are known to inhibit the ABC transporter P-glycoprotein and MRP-related transporters with varying affinity [110–112] and RTV, SQV, and NFV have been found to effectively inhibit the breast cancer resistance protein (BCRP) [113]. Taken together, PIs have been found to modify activity and expression of active drug transport systems which in turn may alter drug absorption, elimination, and tissue distribution. The difference in expression of drug transporters between cell types, especially *in vitro*, may explain the concentration dependent and model system specific effects seen with PI treatment. Moreover, expression variation between tissues of patients may clarify why PI toxicity is particularly evident in specific cell types, such as adipocytes (Table 3).

3.8 Theories on the mechanistic basis of PI induced toxicity

Due to their ability to inhibit the viral protease, PIs have been speculated to also inhibit endogenous proteases or other enzymes with similar catalytic sites to the HIV-1 viral protease. Theories on the mechanistic basis of PI induced toxicity have particularly arisen in an attempt to understand PI induced metabolic syndromes such as lipodystrophy and insulin resistance, which affect up to 80% of patients on PIs [114].

3.8.1 The low density lipoprotein-receptor-related protein

The aminoacid sequence of the HIV-1 protease catalytic site is 63% homologous with a lipid binding domain in the low density lipoprotein-receptor-related protein (LPR). Besides LPRs importance in post-prandial chylomicron clearance in the liver, LPR is also co-expressed with lipoprotein lipase (LPL) on capillary endothelium where it cleaves fatty acids from circulating triglycerides enabling free fatty acid entry into adipocytes for storage as fat. Inhibition of LPR by PIs likely then initiates hyperlipidaemia as circulating lipids in the blood rise. Also LPR perturbation increases the occurrence of insulin resistance by, for example, disruption of glucose and lipid oxidation pathway competition in skeletal muscle [115].

PIs may also be linked to deregulation of fatty acid metabolism by inhibiting 20S proteasomal degradation of newly synthesized apolipoprotein B (ApoB). ApoB is the principal structural component of triglyceride and cholesterol-rich plasma lipoproteins, and secretion of these lipoproteins is negatively-regulated by proteasomal degradation of nascent ApoB [116]. IDV, RTV and SQV have been shown to inhibit proteasomal ApoB degradation and, surprisingly, also decrease secretion of ApoB related lipoproteins by impairing cholesteryl ester synthesis and triglyceride transfer to ApoB through inhibition of the triglyceride transfer protein [116]. This imbalanced regulation of lipoprotein particle turnover together with the implicated LRP inhibition may contribute to the hyperlipidemia onset in patients on PIs.

3.8.2 The cytoplasmic retinoic-acid binding protein type 1

The HIV-1 protease catalytic site is also 58% homologous to a C-terminal region of the cytoplasmic retinoic-acid binding protein type 1 (CRABP-1). CRABP-1 binds almost all intracellular retinoic acid and presents it to CYP450 3A isoforms which catalyse the conversion of retinoic acid to cis-9-retinoic acid. Cis-9-retinoic acid is the sole ligand of the adipocyte nuclei retinoid X receptor (RXR) which works in a heterodimer with peroxisome-proliferator-activated receptor type gamma (PPAR-γ) to inhibit adipocyte apoptosis and upregulate adipocyte differentiation and proliferation. RXR and PPAR-γ agonists improve abnormal insulin sensitivity and hyperlipidaemia, and inhibition of CRABP-1 by PIs has been suggested to be a cause of insulin resistance and lipodystrophy in HIV-1 patients [115].

3.8.3 The mitochondrial processing protease

The mitochondrial processing protease (MPP) is a metalloendoprotease localised in the mitochondrial matrix and catalyses the proteolysis of N-terminal mitochondrial targeting signals from nuclear encoded mitochondrial proteins. It does so by utilisation of a glutamic acid residue in its active site which recognises targeting sequences based on their secondary and tertiary structure, like the viral protease [117]. The activity of MPP is essential for the viability of eukaryotic cells and lack of cleavage by the MPP results in dysfunctional or non-functional mitochondrial proteins, hindering mitochondrial function. Mukhopadhyay *et al.* demonstrated that IDV and APV significantly inhibit MPP processing in yeast, and protein import was also found to be inhibited by IDV [118]. Since only supra-physiological concentrations of PIs significantly inhibited MPP activity, the clinical relevance may be limited. However, PIs may reach higher concentrations *in vivo* when boosted with RTV and may accumulate in the mitochondria over time. In both cases a PI concentration may be reached to an extent that MPP is inhibited. PIs are also highly hydrophobic and therefore may concentrate in adipose tissue predisposing dysfunction and adverse events such as lipodystrophy [118].

3.8.4 The integral membrane metalloprotease ZMPSTE24

ZMPSTE24 is an integral membrane zinc metalloprotease that processes prelamin A which provides nuclear structural and functional integrity. Exposure to LPV, RTV and TPV inhibited the activity of the purified ZMPSTE24 yeast ortholog Ste24p [119]. In cultured fibroblasts, IDV, NFV, TPV and LPV inhibited ZMPSTE24 and consequent prelamin A maturation leading to dysmorphic nuclei and the accumulation of the farnesylated precursor of prelamin A [95,120]. The accumulation of improperly processed farnesylated prelamin A caused mitochondrial dysfunction, increased oxidative stress and premature senescence [95,121]. Interestingly, prelamin A accumulation has also been shown to cause genomic instability [122,123].

3.9 HAART treated HIV-patients age prematurely

Without antiretroviral therapy HIV-infected patients usually die within years because of immune system failure. Thanks to HAART however, early death is prevented, allowing HIV-patients to live decades as long medication is continued [20]. It was recently estimated that more than 50% of HIV-infected patients in the United States will be over the age of 50 in 2015 [124]. Even though this gain in lifespan is celebrated as a success, data show that the life expectancy of treated patients remains shorter than that of the normal population [125]. Life expectancy for

treated HIV-patients is dependent on the age at which antiretroviral therapy is started and is estimated to be 10-30 years less than that of the uninfected [126]. Several studies have also observed that co- and multi-morbidities, like cardiovascular disease, diabetes, and osteoporosis, which are normally witnessed later on in life as a result of natural ageing, were increasingly prominent among the HIV-infected population [127,128]. These observations led to the hypothesis that the HAART treated HIV-infected population is ageing more rapidly, a phenomenon now known as premature and accelerated ageing.

3.9.1 Theories for premature and accelerated ageing in HAART treated patients

There are several factors that influence life-span of the HIV-infected, but have limited effects on progression of premature and accelerated ageing phenotypes. These include lifestyle risk factors such as smoking, drinking, and illicit drug use, which are prevalent across the HIV-infected population [129]. Illicit drug use for example, is associated with poorer medication adherence and lesser immunological and virological control [130]. Additionally, co-infection, such as with viral hepatitis, is common among the HIV-infected population and is known to decrease life expectancy [131]. HIV-1 patients also run a greater risk for adverse drug interactions due to the increase in 'pill-burden' to combat comorbidities [132]. Moreover, both natural ageing or HIV-1 infection cause changes in gastrointestinal tract, liver and kidney function that collectively affect the pharmacology of administered drugs [133]. None of these factors however can directly be related to causing the premature and accelerated ageing phenotype witnessed in treated HIV-patients [134].

Most research in this relatively new field focuses on how HIV-1 infection depletes $CD4^+$ cell counts and exhausts the patient's immune system [9,135]. In this way, HIV-infection itself if left untreated has been shown to convert the immune system of a young individual into one similar to someone 40 years older [136]. This theory of an accelerated ageing process of the immune system is called immunosenescence and is characterized by continuous immune provocation and systemic low-grade inflammation, which predisposes patients to comorbidities and natural ageing symptoms more frequently seen in the elderly [137,138].

The immunosenescence theory of ageing has substance when considering untreated patients, as it principally focusses on viral effects. However, this theory is less plausible for treated patients as HAART has proven highly successful in swiftly replenishing $CD4^+$ cell counts and reducing viral-load to barely detectable limits [139]. Additionally, various antivirals have been shown to induce inflammatory signals and it is therefore plausible that if an altered immune-organization is seen in HAART treated patients it is due to antiretroviral therapy [121,140,141]. The influence HAART has warrants thorough investigation as HIV-patients take HAART daily and for the rest of their lives. Very few premature and accelerated ageing studies in the HIV-infected population, however, focus upon the influence that antiretroviral drugs have on ageing and age-related comorbidities. Accordingly, no consensus has arisen as to why the successfully treated HIV-infected population shows signs of premature and accelerated ageing.

3.9.2 Is HAART involved in immunosenescence?

With the success of HAART in viral suppression the question arises whether HIV-1 is the sole plausible cause for immunosenescence in HIV-treated patients. HAART, which is taken daily for lifelong periods, is probably also

responsible for immune system malfunction. Besides that various antivirals have been shown to induce inflammatory signals [121,140,141], senescent cells have also been shown to change their phenotype, secreting proinflammatory cytokines and contributing to systemic low-grade inflammation [142]. The systemic exposure and relatively high concentration of antiretrovirals likely affects all cell types, immune system cells included. The direct relationship between antiretroviral drugs and inflammation needs to be addressed further.

Hematopoietic progenitor and lymphoblastoid cell toxicity of NRTIs may explain immune cell depletion independent of inflammation [143–145]. Moreover, a decline in mitochondrial genetic integrity in hematopoietic progenitor cells could also explain continued immune dysfunction upon cessation of therapy. MtDNA levels do recover in patients after discontinuation of HAART [146], but due to generation of somatic mutations by antiretrovirals and ROS, it is likely that replenished mtDNA harbours mutations predisposing the recuperating mitochondria to continued dysfunction [45].

3.9.3 HAART-related mitochondrial toxicity in ageing

Antiretroviral therapy as an explanation for premature and accelerated ageing was first mentioned in studies wherein clinical symptoms of ageing were shown to correlate with adverse side effects of antiretroviral therapy [147]. For example, cardiovascular disease, diabetes, kidney and liver disease, metabolic disorders, osteoporosis and lipodystrophy have all been associated with HAART [124,148]. Accelerated Tau deposition, a marker for neurodegenerative diseases such as Alzheimer's and Parkinson's, has also been shown to be elevated in patients receiving HAART compared to HIV-infected non-treated patients [149]. These symptoms collectively seem to be related to tissues with high-energy demand and show a strong similarity to hereditary mitochondrial diseases [33]. Indeed, after introduction of HAART to treat HIV-1 infection, it quickly became apparent that mitochondrial toxicity is a major reason for antiretroviral-related adverse events [34]. The specific influence of HAART upon mitochondria and ageing however is often not addressed.

Due to the apparent relationship between mitochondrial dysfunction and exposure to antiretroviral drugs, HAART-induced mitochondrial dysfunction likely plays a role in most, if not all complications associated with premature and accelerated ageing [150,151]. In consensus, an accumulation of mitochondrial DNA (mtDNA) mutations, increased mitochondrial oxidative stress and a decrease in mitochondrial energy metabolism are all important contributors to ageing [152]. Mitochondria therefore play dominant roles in ageing and marked effects of HAART upon mitochondria likely accelerate these effects. HAART is known to influence mtDNA integrity, alter mitochondrial morphology and function, induce oxidative stress, inflammation and cell senescence, and HAART is also directly connected to the emergence of ageing symptoms and comorbidities. With the increase in life expectancy it has only recently become clear that HIV-1 patients are suffering from symptoms of ageing ahead of time. Strong correlations exist between antiretroviral drug-induced mitochondrial toxicity and premature and accelerated ageing which are discussed below (Figure 7: A schematic representation of our current framework of analysis).

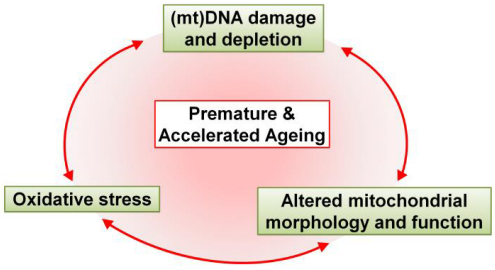

Figure 7: Schematic representation of the major effects of antiretroviral drugs that drive premature and accelerated ageing [2]. Antiretroviral drugs cause mtDNA damage and depletion, oxidative stress and altered mitochondrial morphology and function. These alterations in the mitochondria contribute, either alone or in unison, to premature and accelerated ageing in HAART treated patients.

3.9.4 Drug induced accumulation of mtDNA damage

Because mitochondria contain their own DNA, mitochondrial genome integrity is essential for organelle function. The mtDNA encodes vital components of the mitochondrial respiratory chain and therefore damage to mtDNA is directly detrimental to energy metabolism and organelle fitness. Not surprisingly, cell senescence and ageing are associated with an increase in the amount of damaged mtDNA. Additionally, accumulation of mutations in mtDNA is known to increase with age, and aberrant mtDNA replication, specifically accumulation of point mutations, contributes to premature-and-accelerated-ageing phenotypes [153,154]. MtDNA repair is an essential mechanism to limit the deterioration of mtDNA integrity and polymerase-γ exhibits extremely high mtDNA replication fidelity, partly due to its exonuclease ability [155]. Moreover, polymerase-γ is needed for mtDNA mismatch repair and base-excision repair of oxidised or damaged bases [156]. NRTIs have been shown to inhibit mtDNA polymerase-γ replication and exonuclease activity, causing a decrease in mtDNA amount and quality; commonly known as the polymerase-γ theory [45,47,143]. FLT, known for its high toxicity, can cause DNA fragmentation and induce apoptosis [143]. Interestingly, AZT and d4T also disrupt telomerase maintenance and have telomere shortening effects, properties often related to cell senescence and ageing [157,158].

During HAART it is very likely that NRTIs and PIs augment each other's ability to steer the cell into premature senescence. This is especially the case when the 'booster' RTV is used in the HAART cocktail, as RTV inhibits CYP3A function by mechanism-based inhibition through direct interaction with the CYP3A heme group [159]. Interestingly, mtDNA damage has also been found to correlate with PI RTV use in human endothelial cell cultures in a dose-dependent manner [160]. Although mitochondria are the most important players in antiretroviral toxicities, outside these organelles PIs can cause accumulation of the farnesylated pro-senescence protein prelamin A.

3.9.5 Oxidative stress

Oxidative stress can be defined as an imbalance between production and detoxification of ROS, or similarly put; an imbalance between pro-oxidants and anti-oxidants. 'ROS' is a collective term encompassing many chemical species that are formed upon incomplete reduction of oxygen [161]. Typically, 'ROS' is used to refer to the initial species (superoxide anions or hydrogen peroxide) as well as their secondary reactive products [1]. In many studies, ROS and oxidative stress have been measured through indirect methods, such as fluorescent and

colorimetric studies, or via the detection of ROS 'footprints'. As such the actual oxidative stress inducing reactive species are not identified [162].

ROS, especially superoxide and hydrogen peroxide, are habitually produced in small quantities by mitochondria during oxidative phosphorylation. However, a decrease in, or malfunction of, mitochondrial proteins, due to diminished mtDNA for instance, can disrupt electron flow through the electron transport chain and cause increased ROS formation [163]. Consequently, this increase in ROS can damage mitochondrial components, such as the electron transport complexes, and hence induce even more ROS production [164]. A fundamental feature of ageing is a decline in mtDNA transcription and repair capacity which can lead to mitochondrial malfunction and set in motion a vicious cycle of enhanced ROS production [165].

An increase in oxidative stress, observed as increased oxidant and reduced antioxidant levels in serum, has frequently been associated with HAART in patients [90]. Several studies conclude that symptoms of ageing such as cardiovascular disease, lipodystrophy and insulin resistance are all influenced by antiretrovirally induced ROS production [86,166]. A common side effect of AZT, namely cardiomyopathy, is likely caused by stimulation of ROS production in heart and endothelial mitochondria [167,168]. Prompt heart injury has even been ascribed largely to 2',3'-dideoxycytidine (*zalcitabine* or ddC) induced ROS production, independent of mtDNA depletion or damage, a finding that emphasizes the impact of antiretroviral induced ROS toxicity [169]. Increased oxidation of lipids, mtDNA and the major antioxidant glutathione (GSH), further relate AZT to skeletal muscle myopathy [170]. d4T is known to cause oxidative stress in human hepatoma cells and may underlie hepatic steatosis and lactic acidosis, which are often experienced by patients on HAART [171]. Thymidine analogues have additionally been shown to cause cell senescence through an increase in oxidative stress and induction of mitochondrial dysfunction in human fibroblast cell lines and in subcutaneous adipose tissue from HAART patients [172].

PIs also have the potential to induce oxidative stress, although it is not always clear whether PI induced elevated ROS is produced at the mitochondrial level. The most clearly PI affected cell type is endothelial cells, although other cell types are also afflicted, and strong connections exist between drug toxicity and ROS production [173]. RTV and LPV can increase ROS production in human arterial endothelial cells [121] and are known to induce ROS through a perturbed mitochondrial function in cardiomyocytes [102]. IDV and NFV have been shown to elicit ROS production in skin fibroblast cultures *in vitro* and in patients' adipose tissue *in vivo* [58]. IDV and NFV have furthermore been shown to cause ROS production in human aortic endothelium and are thus involved in recruitment of mononuclear cells and exacerbation of inflammation, prerequisites for vascular complications [141]. Additionally, treatment with IDV or NFV was shown to cause increased mitochondrial ROS production and premature senescence in skin fibroblasts [95], and an IDV and AZT combination induces ROS mediated apoptosis in human brain microvascular endothelial cells [88]. Short-term treatment of NFV increases ROS generation and diminishes levels of GSH and the detoxification enzyme superoxide dismutase in a pancreatic insulinoma cell line [103]. Moreover, NFV has been linked to adipocyte insulin resistance through oxidative stress induced apoptosis and necrosis [174,175], which is noteworthy as the anti-apoptotic properties of PIs in a low-dose have been documented [96]. SQV however, was shown to cause apoptosis in human umbilical

vein endothelial cells via higher levels of ROS production [176]. SQV, IDV, NFV and RTV also elevate ROS in cerebral endothelial cells and interfere with proper blood brain barrier maintenance. Therefore, these PIs conceivably play a significant role in antiretroviral-induced neurological symptoms and could also increase viral entry into the central nervous system [177]. Collectively, these results indicate that oxidative stress is a powerful driving force behind antiretroviral induced toxicity and has important roles in premature-and-accelerated-ageing symptoms [87].

3.9.6 Altered mitochondrial morphology and function

Mitochondrial function, especially respiration and ATP production, has been demonstrated to decline with age and even be an important mediator of senescence [178]. Energy deficiency can cause a broad range of metabolic and degenerative diseases including ageing [179]. Mitochondrial processes for example play important roles in adipocyte differentiation and function, which in turn influence a wide array of homeostatic processes including insulin sensitivity and lipid accumulation [166].

Changes in mitochondrial structure and function are known to occur in age-associated disorders such as Parkinson's disease, sarcopenia and metabolic diseases, including heart-disease and diabetes mellitus [178,180]. Mitochondria are no longer considered as static spherical bodies, but highly dynamic organelles that readily fuse, divide, propagate and diminish according to cellular requirements. Mitochondrial morphology plays an essential role in mtDNA rescue, protein quality control and cell survival [181,182]. Certain distinct morphological changes in mitochondrial structure and organisation are therefore considered indicators of ageing in worms, mice and humans [183,184]. Specifically, mitochondria of aged individuals are often swollen and their structures contain less villous cristae, while the mitochondrial network is frequently disrupted [164].

Not surprisingly then, antiretroviral drugs are found to alter mitochondrial morphology and function, although specific mechanisms and the chronology of these events remain to be fully unravelled. Altered mitochondrial morphology might be considered a compensatory mechanism to help preserve mitochondrial functions under stress. Increased proliferation for example, may be an attempt of mitochondria to recover mtDNA and increase functional capacity under pressure [185]. However, evidence exists that the newly formed mitochondria could be non-functional [172]. Electron microscopy of AZT-treated striated skeletal muscle from rats, and AZT, ddC and ddI treated human hepatocytes show widespread mitochondrial swelling with poorly organized cristae [51,186]. Muscle biopsies from AZT treated patients give similar results with striking variations in mitochondrial size, shape and network organization [187]. AZT and d4T induce a rapid increase in mitochondrial proliferation in human fibroblasts [172], and their combination with or without IDV increase mitochondrial mass in both white and brown murine adipocytes [58]. Individual exposure of HeLa cells to NFV, RTV and SQV caused fragmentation of the mitochondrial network and decreased mitochondrial number and volume [98].

Murine adipocytes exposed to AZT, d4T and/or IDV displayed impaired mitochondrial function as measured by lower respiration rate and decreased ATP production [58,188]. AZT is also known to competitively inhibit the ADP/ATP antiporter in rat heart mitochondria and thus could contribute to the ATP deficiency syndrome

witnessed in patients [72]. Cells with diminished oxidative phosphorylation shift to glycolysis for their energy demands which results in accumulation of lactate and, if left untreated, can cause lactic acidosis. AZT, d4T or ddC treated human hepatoma cells show increased lactate concentrations and, in some cases, decreased activity of mitochondrial respiratory chain complexes [171]. An analysis of mitochondrial genes in adipose tissue and monocytes from HIV-negative subjects receiving dual NRTI therapy revealed a significant decrease in mitochondrial respiratory chain component expression [57]. AZT and IDV have additionally been found to suppress membrane potential and cause apoptosis in blood-brain barrier endothelial cells [88]. Moreover, PI-induced mitochondrial effects are typically related to an altered membrane potential [32]. A randomized, double-blind, placebo-controlled study found that short-term AZT exposure reduced mitochondrial function and insulin sensitivity in non-infected participants [189]. Additionally, a randomized clinical trial in non-symptomatic antiretroviral-naïve patients showed that long-term exposure to PIs or NNRTIs is associated with disrupted glucose transport as well as disrupted lipid metabolism with increased insulin resistance [190]. In conclusion, antiretroviral therapy has frequently been implicated in metabolic diseases as a result of mitochondrial dysfunction [166] and mitochondrial impairment is found in the absence of HIV infection.

3.10 HIV-1 induced mitochondrial toxicity

Non-HAART induced mitochondrial damage in myocardial tissue led to the finding that HIV-1 itself may affect mitochondrial function [191]. HIV-1 infection in untreated individuals has been associated with the development of (cardio) myopathy and distal symmetric polyneuropathy and nephropathy [192]. T-lymphocytes from untreated HIV-1 patients show depletion in $\Delta\Psi_{mt}$ and enhanced ROS levels that are likely responsible for the increase in apoptosis underlying these adverse events [193]. Indeed, apoptosis could largely be avoided by supplementation of antioxidants, implying mitochondrial dysfunction as a cause for HIV-1 induced lymphocyte toxicity [193,194]. The expected culprits include viral proteins such as transactivating regulatory protein *(tat)* and viral protein R *(vpr)* which have been reported to adversely affect mitochondrial function, mostly through mitochondrial transmembrane depolarization [195–199]. Additionally, previous studies have shown that HIV-1 infection alone can cause a decrease in mtDNA in several tissues including adipocytes and peripheral blood mononuclear cells [200,201].

These findings have substance when considering untreated patients, as they focus on viral effects. However, they become less relevant or even unlikely for treated patients, as HAART has proven highly successful in swiftly replenishing $CD4^+$ cell counts and reducing viral-load to barely detectable limits [139]. Additionally, as is the case for mtDNA depletion, antiretroviral induced toxicity clearly adds to the HIV-1 induced effects [146].

4. Outlook & Thesis outline

There are a number of questions that remain unanswered in the research field of antiretroviral induced toxicity, simply because we do not fully understand the effects of antiretroviral drugs individually, let alone in combination (Chapter 2 & 6). For example, questions remain about mitochondrial toxicity of NRTIs beyond the polymerase-γ theory (Chapter 4). Various cellular transport systems interact with NRTIs and once inside the cell NRTIs are actively phosphorylated from their pro-drug form [47] (Chapter 4). Additionally, the occasionally

divergent relationship between mtDNA copy number, respiratory chain protein levels and mitochondrial dysfunction needs to be explained (Chapter 2, 3 & 4). The influence NRTIs have on gene expression could give us insight into cellular adaptation to antiretroviral drugs (Chapter 4). Alleviation of oxidative stress could prove an easy way to improve the well-being of patients and delay the detrimental effects of antiretroviral drugs. Interestingly, most of the above mentioned ROS complications have experimentally been found to lessen upon co-administration of antioxidant compounds. Antioxidant- or mitochondria-directed supplementation may therefore benefit HAART patients, although thorough research remains to be done before any definitive advice can be given to patients [202] (Chapter 3 & 5).

4.1 Searching for the causes of NRTI and PI toxicity

In vivo approaches like mice or rat models have clarified toxic consequences of antiretroviral therapy, particularly in support of the polymerase-γ theory [186,203]. These approaches, however, are often costly, time consuming and ethically questionable. To circumvent these problems, efforts have been made to study drug specific responses *in vitro* and diverse cell-type cultures have been used with varying success to clarify mechanisms behind NRTI and PI related toxicity. Through these investigations it has become apparent that no clear link can be made between mitochondrial toxicity effects *in vitro* and symptoms from patients in the clinic. For example, tissue-specific cell cultures show no marked differences between specific NRTIs in their potential to inhibit mtDNA replication or cause mitochondrial dysfunction. Here besides, the immortality of some cell-lines places further doubt on the advantages of such *in vitro* approaches. This is especially the case for undifferentiated cell lines because their mitochondria strongly differ in metabolism from differentiated cells [204]. In this study, we use *Caenorhabditis elegans* as a model system to study the mechanisms and timing of antiretroviral therapy induced (mitochondrial) toxicity.

4.2 Choice of antiretroviral drug classes used in this study

For this study first-generation NRTIs and PIs (Table 1 & 2) were selectively chosen as these drugs have been broadly studied and most are still frequently prescribed. It is increasingly important to understand the mechanisms behind NRTI and PI toxicity because there are limited prospects for alternative therapies to battle HIV/AIDS, the number of HIV-1 infected continues to grow, and importantly, due to the prolonged lifespan of HIV-1 patients receiving antiretrovirals, the incidence of antiretroviral related toxicities will continue to rise. Moreover, the possible introduction of FLT into the physician's arsenal to battle HIV-1 resistance and continued prescription of first-generation antiretrovirals, particularly in low-income countries, demands that mechanisms of toxicity by these compounds are addressed [13,205].