Antiretroviral therapy: efficacy and toxicity in the ARES study and responses in the male genital tract
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Selwyn Lowe
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Selwyn Henry Lowe

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Antiretroviral therapy: Efficacy and toxicity in the ARES study and responses in the male genital tract

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit van Amsterdam op gezag van de Rector Magnificus Prof. dr. D.C. van den Boom ten overstaan van een door het college voor promoties ingestelde commissie,
in het openbaar te verdedigen in de Aula der Universiteit op vrijdag 5 oktober 2007, te 12:00 uur

door

Selwyn Henry Lowe

geboren te Nieuw Nickerie, Suriname
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              Prof. dr. K. Brinkman
              Prof. dr. P. Speelman
              Dr. K. Boer
              Dr. A. Verbon

Faculteit der Geneeskunde
To my mother Olga:
the one always facilitating

To my brother Oliver and sister Enith:
thank you for supporting

To my late sister Majoy:
I know you would be the proudest one;
thank you for the cover

To Juami, Alan, Dion, Evan, Afille, Aman:
it won’t come easy, the opportunity is yours
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Chapter 1

General introduction

Adapted from:
Antiretroviral therapy in previously untreated adults infected with the human immunodeficiency virus type 1: established and potential determinants of virological outcome

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Introduction

The standard antiretroviral therapy (ART) first given to individuals infected with the human immunodeficiency virus type 1 (HIV-1) is highly active antiretroviral therapy (HAART). HAART is not well defined and at present is usually a combination of at least three selected antiretroviral drugs. Primary aim of initial ART is to achieve a maximal and durable viral suppression. Maintaining the blood plasma HIV-1-RNA concentration (plasma viral load, pVL) below a detection limit of 50 copies/mL is currently recommended to achieve this goal\textsuperscript{1,4}, because it is associated with less virological failure than a level above 50 copies/mL and it may prevent the emergence of drug resistance despite ongoing low-level (residual) viral replication\textsuperscript{6-12}. If virological failure to the initial HAART regimen occurs, subsequent therapy is usually less effective due to accumulation of drug-resistance-associated mutations and cross-resistance amongst antiretroviral agents within the same class. Generally the subsequent therapy is also more cumbersome for the patient\textsuperscript{1,3,13-21}. A good initial HAART regimen is therefore of great importance. Considering factors which have been associated with the virological response to initial HAART could be of help in identifying patients with high and low risk for virological failure, and choosing an initial HAART regimen and the most appropriate moment to start therapy. For better insight of physicians who are not familiar with antiretroviral therapy a brief historical overview and global perspective of ART is given.

Antiretroviral therapy in historical and global perspective

In June 1981 the world first became aware of the acquired immunodeficiency syndrome (AIDS)\textsuperscript{22-24}. Major scientific breakthroughs achieved during subsequent years are summarized in Table 1.1 and in Table 1.2 an overview is given of the antiretroviral drugs and their date of licensing by the USA Food and Drug Administration (FDA)\textsuperscript{23-25}. A number of drugs that are currently under clinical evaluation are the NNRTI etravirine, the CCR5-blockers maraviroc and vicriviroc and the integrase inhibitor raltegravir. Now, in the year 2007, HIV can still not be cured with the available HAART regimens and there also is still no preventive vaccine available\textsuperscript{26}. 
Table 1.1  Historical overview of antiretroviral therapy.

<table>
<thead>
<tr>
<th>Year</th>
<th>Event</th>
</tr>
</thead>
<tbody>
<tr>
<td>1981</td>
<td>Recognition of first AIDS cases</td>
</tr>
<tr>
<td>1983</td>
<td>Identification of HIV-1 as the cause of AIDS</td>
</tr>
<tr>
<td>1984</td>
<td>Description of first AIDS cases in the Netherlands</td>
</tr>
<tr>
<td>1985</td>
<td>FDA approval of first commercial blood screening test</td>
</tr>
<tr>
<td>1986</td>
<td>Identification of HIV-2</td>
</tr>
<tr>
<td>1987</td>
<td>Introduction of antiretroviral therapy: zidovudine</td>
</tr>
<tr>
<td>1994</td>
<td>Reduction of HIV-1 transmission from mother to child</td>
</tr>
<tr>
<td>1995</td>
<td>Availability of standardised (commercial) HIV-1-RNA assay, which gave better understanding of HIV-1 viral dynamics</td>
</tr>
<tr>
<td>1996</td>
<td>Release of first protease inhibitors and introduction of HAART</td>
</tr>
<tr>
<td>1997</td>
<td>Ritonavir-induced pharmacokinetic enhancement of other PIs, which made dosing twice daily of many PIs possible; Recognition of a long-lived HIV cellular reservoir</td>
</tr>
<tr>
<td>1998</td>
<td>After induction therapy maintenance with two NRTIs or one or two PIs or one NRTI and one PI is insufficient; The virological response is more sustained when a blood plasma viral load nadir of 50 copies/mL is achieved; First descriptions of the lipodystrophy syndrome; Recognition of a strong improvement of survival in HAART era</td>
</tr>
<tr>
<td>1999</td>
<td>A high degree of drug adherence is needed to achieve a proper viral suppression; Demonstration of residual replication during HAART, which made clear that treatment with the current kind of HAART would be for life</td>
</tr>
<tr>
<td>2000</td>
<td>HAART can be relatively safely deferred until the CD4 count is nearing 200 cell/μL</td>
</tr>
<tr>
<td>2001</td>
<td>Recommendations to initiate HAART in HIV-1 infected adolescents and adults. Anno 2007 these recommendations are more or less the same</td>
</tr>
</tbody>
</table>

Note: AIDS=acquired immunodeficiency syndrome; FDA=the USA Food and Drug Administration; HAART=highly active antiretroviral therapy; HIV=human immunodeficiency virus; NRTI=nucleoside analogue reverse transcriptase inhibitor; PI=protease inhibitor.

In countries where HAART could be widely applied, the HIV-1-related morbidity and mortality has decreased tremendously since its introduction in 1996 and the treatment has appeared to be cost-effective. Basis for clinical improvement is a lasting adequate virological suppression which leads to immunological recovery. HAART is available to only a minority of the HIV-infected population. Estimates for the year 2006 are that at the global level about 4.3 million people became newly infected with HIV and 2.9 million (331 persons/hour) died of AIDS and that the number of people living with HIV continues to grow, from 35 million in 2001 to 39.5 million in 2006. Only less than two million HIV-infected persons (<5%; those living in high-income countries) have access to HAART. However, access to antiretroviral therapy in low- and middle income countries is in creasing, from 240,000 persons in 2001 to 1.3 million at the end of 2005. Antiretroviral therapy is based on HIV-1 subtype M (major) subtype B, which is the most prevalent in the Western world. Globally HIV-1 subtype B accounts only for 12% of new HIV-1 infections while this is 50%, 30% and 18% for HIV-1 subtypes C, A and circulating recombinant forms (CRFs). This may have therapeutic consequences, such
### Table 1.2 Licensed antiretroviral drugs and their date of approval by the Food and Drug Administration (FDA) of the USA.

<table>
<thead>
<tr>
<th>Class and generic name</th>
<th>Trade name</th>
<th>Date of FDA approval</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nucleoside/tide-analogue RTI</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zidovudine</td>
<td>Retrovir</td>
<td>19 Mar 1987</td>
</tr>
<tr>
<td>Didanosine (chewing tablet, os)</td>
<td>Videx</td>
<td>08 Oct 1991</td>
</tr>
<tr>
<td>Zalcitabine</td>
<td>Hivid</td>
<td>19 Jun 1992</td>
</tr>
<tr>
<td>Stavudine</td>
<td>Zerit</td>
<td>24 Jun 1994</td>
</tr>
<tr>
<td>Lamivudine</td>
<td>Epivir</td>
<td>17 Nov 1995</td>
</tr>
<tr>
<td>Zidovudine + lamivudine</td>
<td>Combivir</td>
<td>27 Sep 1997</td>
</tr>
<tr>
<td>Abacavir</td>
<td>Ziagen</td>
<td>17 Dec 1998</td>
</tr>
<tr>
<td>Didanosine (enteric coated caps)</td>
<td>Videx EC</td>
<td>31 Oct 2000</td>
</tr>
<tr>
<td>Zidovudine + lamivudine + abacavir</td>
<td>Trizivir</td>
<td>14 Nov 2000</td>
</tr>
<tr>
<td>Emtricitbine</td>
<td>Emtriva</td>
<td>02 Jul 2003</td>
</tr>
<tr>
<td>Abacavir + lamivudine</td>
<td>Kivexa, Epzicom</td>
<td>02 Aug 2004</td>
</tr>
<tr>
<td>Emtricitabine + tenofovir DF</td>
<td>Truvada</td>
<td>02 Aug 2004</td>
</tr>
<tr>
<td><strong>Nucleotide-analogue RTI</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tenofovir</td>
<td>Viread</td>
<td>26 Oct 2001</td>
</tr>
<tr>
<td><strong>Non-nucleoside RTI</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nevirapine</td>
<td>Viramune</td>
<td>21 Jun 1996</td>
</tr>
<tr>
<td>Delavirdine</td>
<td>Rescriptor</td>
<td>04 Apr 1997</td>
</tr>
<tr>
<td>Efavirenz (50-200 mg caps)</td>
<td>Stocrin, Sustiva</td>
<td>17 Sep 1998</td>
</tr>
<tr>
<td>Efavirenz (600 mg tablets)</td>
<td>Stocrin, Sustiva</td>
<td>01 Feb 2002</td>
</tr>
<tr>
<td><strong>Protease Inhibitor</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saquinavir (hard gel caps)</td>
<td>Invirase</td>
<td>06 Dec 1995</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>Norvir</td>
<td>01 Mar 1996</td>
</tr>
<tr>
<td>Indinavir</td>
<td>Crixivan</td>
<td>13 Mar 1996</td>
</tr>
<tr>
<td>Nelfinavir</td>
<td>Viracept</td>
<td>14 Mar 1997</td>
</tr>
<tr>
<td>Saquinavir (soft gel caps)</td>
<td>Fortovase</td>
<td>07 Nov 1997</td>
</tr>
<tr>
<td>Amprenavir</td>
<td>Agenerase</td>
<td>15 Apr 1999</td>
</tr>
<tr>
<td>Lopinavir + ritonavir (133/33 mg caps, os)</td>
<td>Kaletra</td>
<td>15 Sep 2000</td>
</tr>
<tr>
<td>Atazanavir</td>
<td>Reyataz</td>
<td>20 Jun 2003</td>
</tr>
<tr>
<td>Fosamprenavir</td>
<td>Lexiva</td>
<td>20 Oct 2003</td>
</tr>
<tr>
<td>Lopinavir + ritonavir (200/50 mg tablets)</td>
<td>Kaletra</td>
<td>28 Oct 2005</td>
</tr>
<tr>
<td>Tipranavir</td>
<td>Aptivus</td>
<td>22 Jun 2005</td>
</tr>
<tr>
<td>Darunavir</td>
<td>Prezista</td>
<td>23 Jun 2006</td>
</tr>
<tr>
<td><strong>Fusion Inhibitor</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enfuvirtide</td>
<td>Fuzeon</td>
<td>13 Mar 2003</td>
</tr>
<tr>
<td><strong>Multi-class combination products</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Efavirenz + emtricitabine + tenofovir DF</td>
<td>Atripla</td>
<td>12 Jul 2006</td>
</tr>
</tbody>
</table>

Note: RTI=reverse transcriptase inhibitor; caps=capsule; os=oral solution.  

as susceptibility to antiretroviral drugs, response to HAART, and rate and pattern of the emergence of drug resistance.
What is the virological response to current HAART?

A HAART regimen is currently considered adequate when after 3-6 months a pVL of <50 copies/mL is achieved and this level of viral suppression is maintained\(^1\)\(^-\)\(^3\)\(^,\)\(^47\). In case of a baseline pVL >750,000 copies/mL it may take longer than 6 months to reach the concentration of <50 copies/mL\(^48\)\(^,\)\(^49\).

In Table 1.3 an overview is given of the virological response rates reported in published prospective studies in chronic HIV-1-infected, previously untreated adults and adolescents. HAART regimens that are mostly used and studied are those of two nucleoside-analogue reverse transcriptase inhibitors (NRTIs) with either a (ritonavir-boosted) protease inhibitor (PI) or non-nucleoside reverse transcriptase inhibitor (NNRTI) or a third NRTI. As will be discussed later the latter type of regimen is nowadays considered insufficient for initial ART.

Table 1.3 demonstrates that the studies with initial HAART are very heterogeneous. This table and others\(^50\)\(^-\)\(^53\) show that according to an “intention-to-treat” (ITT) analysis between 20%\(^54\) and 88%\(^55\) of patients achieve a pVL below the limit of detection at 48 weeks of therapy or later with an average of 50%. Possibly this is about 70% with once-daily-dosed regimens and regimens with a low pill burden\(^51\)\(^,\)\(^56\)\(^-\)\(^61\). The “intention-to-treat” analysis gives insight into the overall success of a certain HAART regimen and should include discontinuation of the regimen due to virological failure and/or adverse events and/or intolerance. However, treatment failure is not uniformly defined in the several studies (Table 1.3). The “on treatment” (OT) virological success at long term reflects more selectively the virological failure rate of the regimen. In a systematic overview of studies on initial HAART published between 1994 and July 2004, based on an ITT analysis the percentage of patients having a pVL <50 copies/mL after 48 weeks of HAART improved from 41% before 1998 to 64% in the studies published between 2003-2005. In this study the pill count was not a significant predictor for the 48 week virological success rate and NNRTI and boosted PI-containing regimens were superior to unboosted PI-containing and triple NRTI regimens\(^52\). Treatment options are possibly better after failing an initial boosted PI-containing regimen than failing an initial NNRTI-containing one\(^53\).

It should be considered that in the general clinical practice the virological response rate to the first HAART regimen could be less than in prospective, randomised studies, but probably in clinical practice the virological success rate has also greatly improved over time\(^61\)\(^,\)\(^62\). As the knowledge and treatment possibilities evolve and patient management and therapy have improved (Table 1.1 and 1.2) there is a historical bias in the effectiveness figures of HAART regimens\(^61\).
Table 1.3 Overview of published prospective studies in which initially HAART is used.

<table>
<thead>
<tr>
<th>Ref.</th>
<th>Recruitment period</th>
<th>FU</th>
<th>HAART regimen</th>
<th>N</th>
<th>Baseline CD4</th>
<th>VL</th>
<th>OT</th>
<th>ITT</th>
<th>% VL&lt;50 c/ml (…)</th>
<th>+Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>304</td>
<td>07/94 – 07/96</td>
<td>52 w</td>
<td>AZT-3/DDI-2/NVP</td>
<td>51</td>
<td>395</td>
<td>17732</td>
<td>ns</td>
<td>51 (&lt;20 c)</td>
<td>139</td>
<td></td>
</tr>
<tr>
<td>54</td>
<td>01/95 – 05/96</td>
<td>48 w</td>
<td>AZT-3/DDI-2/NVP</td>
<td>32</td>
<td>37.5</td>
<td>5.8Log</td>
<td>ns</td>
<td>&lt;250000 ns</td>
<td>101</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>37.5 (&lt;400 c)* ns</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>45.5 (&lt;400 c)* ns</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>11/95 – 04/97</td>
<td>52 w</td>
<td>AZT-3/3TC/IDV</td>
<td>52</td>
<td>281</td>
<td>4.7Log</td>
<td>60</td>
<td>46 (&lt;20 c)</td>
<td>178</td>
<td></td>
</tr>
<tr>
<td>305</td>
<td>07/96 – 12/97</td>
<td>48 w</td>
<td>AZT-250/3TC/IDV</td>
<td>35</td>
<td>267</td>
<td>5.01Log</td>
<td>ns</td>
<td>66 ns</td>
<td>168</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>D4T/3TC/IDV</td>
<td>34</td>
<td>313</td>
<td>5.21Log</td>
<td>ns</td>
<td>59</td>
<td>232</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>D4T/DDI-2,1/IDV</td>
<td>37</td>
<td>277</td>
<td>5.00Log</td>
<td>ns</td>
<td>48</td>
<td>166</td>
<td></td>
</tr>
<tr>
<td>101</td>
<td>12/96 – 01/98</td>
<td>48 w</td>
<td>D4T/3TC/IDV</td>
<td>101</td>
<td>408</td>
<td>4.59Log</td>
<td>85</td>
<td>49* ns</td>
<td>142</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AZT-3,2/3TC/IDV</td>
<td>103</td>
<td>391</td>
<td>4.47Log</td>
<td>73</td>
<td>47* ns</td>
<td>110</td>
<td></td>
</tr>
<tr>
<td>102</td>
<td>12/96 – 07/97</td>
<td>48 w</td>
<td>D4T/DDI-2/IDV</td>
<td>102</td>
<td>422</td>
<td>3.1623</td>
<td>ns</td>
<td>70</td>
<td>214</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AZT-3,2/3TC/IDV</td>
<td>103</td>
<td>391</td>
<td>4.47Log</td>
<td>73</td>
<td>47* ns</td>
<td>110</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>ns</td>
<td>48 w</td>
<td>AZT-3/3TC/NFV</td>
<td>99</td>
<td>284</td>
<td>231884</td>
<td>83</td>
<td>ns (&lt;400 c)</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AZT-3/3TC/NFV-500</td>
<td>97</td>
<td>307</td>
<td>300705</td>
<td>56</td>
<td>ns (&lt;400 c)</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>01 – 04/97</td>
<td>52 w</td>
<td>D4T/3TC/RTV</td>
<td>33</td>
<td>640</td>
<td>23481</td>
<td>ns</td>
<td>88 (&lt;20 c)</td>
<td>131</td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>01 – 09/97</td>
<td>48 w</td>
<td>AZT-2/3TC/EFV</td>
<td>154</td>
<td>350</td>
<td>60256</td>
<td>90</td>
<td>64</td>
<td>201</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AZT-2/3TC/IDV</td>
<td>148</td>
<td>341</td>
<td>61660</td>
<td>79</td>
<td>43</td>
<td>185</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>EFV/IDV</td>
<td>148</td>
<td>344</td>
<td>56234</td>
<td>75</td>
<td>47</td>
<td>180</td>
<td></td>
</tr>
<tr>
<td>91</td>
<td>02/97 – 11/98</td>
<td>48 w</td>
<td>AZT-2/3TC/APV-1200</td>
<td>116</td>
<td>442</td>
<td>4.61Log</td>
<td>79</td>
<td>34</td>
<td>128</td>
<td></td>
</tr>
<tr>
<td>103</td>
<td>05/97 – 10/98</td>
<td>48 w</td>
<td>D4T/3TC/IDV</td>
<td>100</td>
<td>417</td>
<td>4.3Log</td>
<td>80.3</td>
<td>55.0</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>D4T/DDI/IDV</td>
<td>96</td>
<td>417</td>
<td>4.3Log</td>
<td>79.0</td>
<td>44.0</td>
<td>238</td>
<td></td>
</tr>
<tr>
<td>306</td>
<td>06/97 – 10/98</td>
<td>52 w</td>
<td>D4T/3TC-2/IDV</td>
<td>32</td>
<td>708</td>
<td>4.3Log</td>
<td>ns</td>
<td>84 (&lt;200 c)</td>
<td>186</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>D4T/3TC-2/IDV</td>
<td>96</td>
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- "VL: RNA-pcr
- <4 weeks AZT, DDI, D4T, DDC
- 3TC-, NNRTI-, PI-naive
- 28 days AZT, DDI, D4T, DDC
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Table 1.3 continued  Overview of published prospective studies in which initially HAART is used.

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Note: ABV=abacavir 300 mg b.i.d.; AZT-3=zidovudine 200 mg t.i.d.; AZT-2=AZT 300 mg q.d.; AZT-250=AZT 250 mg b.i.d.; CBV=Combivir (zidovudine/lamivudine 300/150 mg) b.i.d.; DDI-1=DDI 400 or 250 mg q.d.; DRT=stavudine 30 or 40 mg b.i.d.; FTC=emtricitabine 200 mg q.d.; KVX=Kivexa (abacavir/lamivudine 600/100 mg) q.d.; 3TC=lamivudine 150 mg b.i.d.; 2-3NRTIs=2 or 3 nucleoside-analogue reverse transcriptase inhibitors; TDF=Tenofovir disoproxil fumarate 245 mg q.d.; TZV=Trizivir (zidovudine/lamivudine/abacavir 300/150/300 mg) b.i.d.; EFV=efavirenz 600 mg q.d.; NVP=nevirapine 200 mg b.i.d.; APV=amprenavir 1200 mg b.i.d.; ATZV-200,400,500,600=atazanavir 200 or 400 or 500 or 600 mg q.d.; FAPV=Fosapenavir; IDV=indinavir 800 mg q.d.; LPV=r=lopinavir/ritonavir b.i.d.; KLT=Kaletra® (lopinavir/ritonavir 400/100 mg) b.i.d.; NFV=nefuvir 750 mg t.i.d.; NFV-2=NFV 1250 mg b.i.d.; NFV-500=NFV 500 mg t.i.d.; RTV=ritonavir 600 mg b.i.d.; r=low dose ritonavir; SQV=saquinavir; Sgc=soft gelatin capsule; Plac=placebo; Ref(reference); N=number of patients included; FU=follow up period in weeks (w) or months (m); CD4=CD4+ cell count, preferably the median; VL=viral load=blood plasma [HIV-1 RNA] copies/mL in Log₁₀ or linear count, preferably the median; % VL <50 c/ml=Percentage with VL <50 c/ml with reverse transcriptase PCR from Roche Pharmaceuticals; (...)=If other limit of detection is used; *=Other HIV-1 RNA assay, see remarks; OT=on treatment; ITT=intention to treat; ns=not stated; +∆=increase of CD4+ T-cell count from baseline.
Baseline characteristics that can be of influence on the virological response

In ART-naive individuals with a chronic HIV-1 infection several factors present at baseline have been associated with the virological response to HAART. These factors can be classified as being related to the host (genetic make-up, adherence), the virus (drug resistant virus, HIV-1 subtype), the drug, the treatment team or a combination of these. Some factors have been more extensively investigated because they are easily available, e.g. the CD4 count, pVL, blood plasma drug levels and patient adherence. The impact of these determinants on virological response is clear and established. On the other hand, e.g. HIV pharmacogenetics, which can elucidate factors at baseline that are associated with proper drug concentrations, toxicity and virological response, is still in its infancy. In the following we describe the factors associated with the virological response to initial HAART starting with the two most extensively investigated factors.

1: A low CD4+ T-lymphocyte cell count

A low CD4+ T-lymphocyte cell count (CD4 count) is usually associated with a high pVL. This makes it unclear which of these two is mostly associated with the virological response. Retrospective cohort studies have shown that patients with a baseline CD4 count <200 cells/µL have a worse virological response and more progression to HIV-related morbidity than patients with a higher baseline CD4 count. In five randomised studies an association was found between the baseline level of the CD4 count or pVL and the virological success at 48 and 96 weeks. The question is whether this difference in response is due to a difference in biology (host-virus-drug interactions, potency of the regimen) or in behaviour (e.g. that patients with a low CD4 count are likely more difficult to treat (initially)). Deferring initial HAART until the CD4 count is between 200 and 350 cells/µL is associated with a minimally increased risk of AIDS compared to starting at a CD4 count >350 cells/µL, depending on the pVL, as the risk of AIDS is higher when the pVL is higher. As a result, since 2001 most guidelines advice adolescents and adults with a chronic HIV-1 infection to start HAART when their CD4 count is nearing 200 cells/µL in order to postpone possible long-term side effects of HAART (e.g. lipodystrophy syndrome and cardiovascular diseases). However, the height of the CD4 count at initiation of HAART corresponds with the ultimate CD4 count achieved during long term suppressive therapy. Although all primary and secondary prophylaxis against opportunistic HIV-related infections can safely be discontinued when the CD4 count is sustained
above 200 cells/µL\textsuperscript{89}, it appears reasonable to strive for a CD4 count as close to normal as possible\textsuperscript{48}.

2: A high blood plasma viral load

For ART consisting of two NRTIs and either a (boosted) PI or a NNRTI there is conflicting evidence from studies (Table 1.3) whether the risk of virological failure is increased\textsuperscript{9,54,56,58,74-78,90-99} or not\textsuperscript{56,58,90,93,98,100-111} if the pVL at baseline is high. On theoretical grounds there could be an increased risk of virological failure when the pVL is very high. The virological response after HAART is initiated in ART-naïve patients is biphasic with a rapid decay of the pVL in the first two weeks and a slower decay thereafter. Given that there is a constant first- and second-phase viral decay rate after initiation of HAART, more time is required to reach the level of <50 copies/mL with a higher baseline pVL\textsuperscript{48,49,92,107} thus increasing the window for early development of drug resistance\textsuperscript{4,112-116}. It is estimated that per HIV-1 replication cycle one to two base-pair transcriptional errors occur\textsuperscript{19,117,118}. Thus, there could be an increased risk of an (early) emergence of strains with one or more drug-resistance mutations if the pVL at baseline is high, especially when drugs with a low genetic resistance barrier (drugs for which only one or two specific mutations in the reverse transcriptase or protease gene are needed to cause significant resistance), such as lamivudine, tenofovir, atazanavir, nelfinavir and the NNRTIs, are components of a triple-drug regimen\textsuperscript{57,116,119,120}.

Adding a fourth active drug in the initial phase of therapy if the pVL is high (e.g. >300,000 copies/mL) is defendable and some guidelines did (do) recommend this strategy\textsuperscript{82}. A triple-class, five-drug regimen consisting of abacavir, lamivudine, zidovudine, nevirapine and indinavir is more potent than a two-class, three-drug regimen consisting of lamivudine, zidovudine and ritonavir, thus giving rise to the question what the optimum potency of a HAART regimen should be\textsuperscript{121-123}. However, the potency of a regimen is dependant on the drugs that compose it, as in one study the four drug regimen consisting of efavirenz, nelfinavir with either didanosine and stavudine or lamivudine and zidovudine was as effective as the triple drug regimen with efavirenz, lamivudine and zidovudine but more effective than the regimens of nelfinavir with either didanosine plus stavudine or lamivudine plus zidovudine or efavirenz with didanosine plus stavudine\textsuperscript{124,125}. Further, more drugs implicates more toxicity. An alternative is to use three drugs with a high genetic resistance barrier in the initial phase.

It has now become obvious that when the baseline pVL is high, (certain) convergent regimens consisting of three NRTIs as initial therapy are virologically inferior to divergent regimens consisting of two NRTIs and either a (boosted) PI or NNRTI (Table 1.3)\textsuperscript{1,3,47,103-105,126-131}. This inferiority is also the case at low pVL. Studies with triple NRTI’s for initial ART were even
prematurely aborted because of more virological failure e.g. studies with tenofovir, lamivudine and abacavir q.d.\textsuperscript{119,132}, or zidovudine, lamivudine and abacavir\textsuperscript{130,133}, or with didanosine, lamivudine and tenofovir\textsuperscript{130}. In one very small study with subjects with sustained control of pVL there was in the lymph node more viral evolution with subsequent development of drug resistance mutations in the subjects who were treated with dual or triple NRTIs alone compared to the divergent regimens\textsuperscript{134}. What needs to be clarified is whether switching to a triple NRTI regimen during proper virological suppression in the absence of previous suboptimal treatment or resistance is associated with more virological failure or not\textsuperscript{127,128,135-137} and whether these convergent NRTI regimens can still be applied when the baseline pVL is (very) low. Further evaluation is needed for a four drug convergent regimen consisting of Trizivir\textsuperscript{8} and tenofovir DF, which in a small study demonstrated comparable efficacy as Combivir\textsuperscript{8} plus efavirenz\textsuperscript{138}.

3: Early virological response

It has been demonstrated that the first phase pVL decay rate after initiation of HAART might be predictive for the potency of the regimen and the virological response at 2-3 months\textsuperscript{138,140}. Similarly a decrease in pVL by 1Log\textsubscript{10} at week 4 or a pVL of <500 copies/mL at week 4 or 8 of therapy has been associated with a favourable virological response at 24 or 48 weeks of therapy\textsuperscript{9,141}. However the short-term virological response may be affected by other factors such as drug adherence and drug concentrations\textsuperscript{142}, and the question is whether with the more potent NNRTI or boosted PI-containing regimens the early virological response is still associated with the long term virological response\textsuperscript{143,144}.

4: Primary infection with drug resistant virus

Of great concern for virological response to initial HAART is the presence of (archived) drug-resistant virus at baseline\textsuperscript{145-148}. The prevalence of HIV-1 viruses with one or more RT and/or protease resistance-associated mutations in recently infected individuals has increased in some parts of the world to 24\%\textsuperscript{145,149-154}. In Europe this is about 10\%\textsuperscript{155}. Thus depending on the local prevalence, initial HAART needs to be optimised based on empiricism or on genotypic resistance testing. It is estimated that baseline resistance testing may be cost-effective when the prevalence of drug resistance in the relevant drug-naïve population is \geq1-5\%\textsuperscript{156,157}. The International AIDS Society – USA Panel recommends a baseline drug resistance test for an HIV-1 infection that was acquired less than two years ago in areas with a drug resistance prevalence of more than 5\%\textsuperscript{158} and the European recommendation is at a prevalence of \geq10\%\textsuperscript{159}. 
Chapter 1

5: Syncytium-inducing and non-syncytium-inducing phenotype of the virus

In the early asymptomatic phase of HIV-1 infection non-syncytium-inducing (NSI) (macrophage or CCR5-tropic) virus variants predominate. In the later course of the infection syncytium-inducing (SI) (T-cell or CXCR4-tropic) variants emerge. This switch in phenotype predominance coincides with a faster progression of the infection. In vitro and in vivo, zidovudine preferentially inhibits NSI variants, while didanosine preferentially inhibits SI variants. For lamivudine and the protease inhibitor ritonavir an equal inhibition of NSI and SI variants was observed. For the other NRTIs and the NNRTIs the effect on NSI and SI has not been compared. The difference in inhibition of NSI and SI variants by zidovudine and didanosine is probably due to differences in intracellular phosphorylation to the active triphosphate. Zidovudine and stavudine are preferentially phosphorylated in activated cells, while didanosine, lamivudine and zalcitabine are preferentially phosphorylated in resting cells. Activated CD4+ cells express more CCR5 receptors and resting cells express more CXCR4, thus giving a rationale for the divergent inhibition of NSI and SI variants by zidovudine or stavudine and didanosine or lamivudine or zalcitabine, respectively. This difference in inhibition is likely of no importance when current HAART is applied, but may be relevant for the use of CCR5-blockers.

6: HIV-1 subtype

Based on genetic divergence in the env, gag and pol region, HIV-1 is phylogenetically divided into an M (major), O (outlier) or N (non-M, non-O or new) subtype or clade. These three subtypes are further subdivided into several subtypes or clades. The O and N subtypes are rare and mainly restricted to West-Africa. The M subtype accounts for >90% of reported HIV/AIDS cases. Until now eleven M subtypes and sub-subtypes have been identified (A1-3, B, C, D, F1-2, G, H, J and K) and at least 21 circulating recombinant forms (CRFs) and unique recombinant forms (URFs). Recombination between HIV-1 strains is mostly a result of superinfection and is being reported with increasing frequency world wide. In Western Europe, the America’s, Australia and New Zealand subtype B is the most prevalent, while the non-B subtypes are mainly prevalent in the other continents. However, due to travel and migration within the western world these differences in prevalence are changing, i.e. in Eastern Europe, up to 1996 the dominant subtypes were B, C and G, while by 2003 subtype A1 accounted for 90% of all infections.
Within the M subtype inter-clade variation in env is 20-30%, in pol about 10% and in gag much less than 10%. The pol gene encodes for reverse transcriptase, RNase, protease and integrase. The extent of polymorphism between HIV-1 subtypes in protease and reverse transcriptase positions is 53% and 48%, respectively. These inter-clade variations are or can be accompanied by differences in biological behaviour (transmission, viral fitness, target cell entry, disease progression), susceptibility to antiretroviral drugs, response to HAART, and rate and pattern of emergence of drug resistance. Parallel to this, the HIV-1 subtype Outlier is naturally resistant to NNRTIs due to different amino acids at RT position 181. Also, HIV-2 is naturally resistant to the NNRTIs because in the wild-type virus amino acid substitutions associated with drug-resistance mutations in HIV-1 (at position 181, 188 and 190) are already present. Furthermore, there is a faster emergence of the multi-NRTI-resistance mutation Q151M and a rapid emergence of genotypic drug resistance (D30N) to the PI nelfinavir in HIV-2.

Prospective studies comparing the virological response among HIV-1 subtypes are lacking. Studies on response to antiretroviral therapy according to non-B HIV-1 subtypes have been performed in (resource poor) geographic areas where these subtypes predominate and in limited populations in (resource rich) geographic areas where the B subtype is prevalent. These studies demonstrate a clinical, immunological and virological response to HAART in the non-B subtypes comparable to that of the B subtype. The evolving resistance associated mutations in failing regimens appear to be the same. However, there are many (polymorphic) mutations with unknown effects and there might be inter-subtype difference in resistance pathways, e.g. a more rapid selection of K65R in subtype C when tenofovir or didanosine are used, a greater impact of (naturally occurring) minor protease mutations (polymorphisms) (V82F in subtype AE in response to exposure to indinavir) and greater impact of mutation M64V, I54V, V82A and L90M in vitro on resistance to nelfinavir and lopinavir in subtype C. Despite these differences the drug resistance interpretation algorithms for subtype B appear to be applicable to the non-B subtypes, although the discordances between the algorithms can be large. For enfuvirtide, a synthetic peptide of 36 amino acids which inhibits HIV-1 but not HIV-2 fusion to the CD4+ cell by interacting with the heptad repeat (HR) 1 region of viral gp41, recognized resistance mutations emerge in amino acid residue positions 36-45 of this region. Within the non-B subtypes there is a wide variability in the enfuvirtide complementary HR1 36 residue positions, but especially the positions 36-45 are conserved. Up till now no naturally occurring resistance mutations in HR1 have been detected in the non-B subtypes and the susceptibility of the non-B subtypes appears to
remain intact. The gp41 HR 2 region is also important for HIV-1 fusion, and mutations in this region appear also to be relevant [183]. In the non-B subtypes there is a greater variability in the HR2 than in HR1 region, e.g. 10-16 amino acid changes in subtype C, but for this subtype the susceptibility to enfuvirtide is not affected by these changes 39,171,184. For the chemokine CCR5 antagonists it may be relevant that expression of CXCR4 during the course of the HIV infection might differ, e.g. in contrast to the B subtype this is rare in subtypes A and C 39. Thus, current data on the non-B subtypes and HAART are reassuring, but more data are needed and the impact of the different HIV-1 M subtypes on the virological response to initial HAART needs to be established.

7: Through concentration of PIs and NNRTIs in blood plasma and the intracellular concentration of triphosphorylated NRTIs

Retrospective and prospective studies have shown a correlation between the blood plasma (through) drug concentration of PIs and NNRTIs and the virological response 17,185-189. Minimally effective drug concentrations have been defined, and nowadays therapeutic drug monitoring (TDM), the pro-active regular measurement of drug concentrations, is considered a standard during PI and NNRTI treatment in some countries 1,3,185-187,190. With the advent of boosted PI-containing regimes, the role of TDM for these regimens is controversial because of better drug concentrations and the lack of prospective studies demonstrating that TDM improves clinical outcome 47. As for some unboosted PIs, a positive association was found between the intracellular concentration of triphosphorylated NRTIs and the virological response 191,192.

8: Adherence to therapy

Proper adherence to the dosing interval and administration requirements of the drugs is one of the most important factors for a durable virological success. In pretreated patients adherence to therapy of at least 90-95% is needed to properly suppress HIV-1 for a prolonged time 193-197. However, one study demonstrated that while in treatment experienced patients treated with an unboosted PI (nelfinavir, indinavir, saquinavir or ritonavir) an adherence rate of 90-95% was needed to maintain the pVL <400 copies/mL, a lesser adherence rate (54%-100%) was also as effective in the NNRTI (efavirenz or nevirapine) group 143. For initial therapy with NNRTI or boosted PI-containing HAART probably a less strict level of adherence is needed 143,144, but one study, in which the initial regimen was not mentioned, demonstrated the contrary 198. Factors that influence adherence to therapy are dosing frequency 199,200, pill-burden 50,56, acute and long-term toxicity of the drugs 16,201-203, and socio-cultural factors 204. Before initiating HAART it is essential that the patient is willing and cooperative on taking the drugs and that he/she is well informed, instructed and
aware of the drug intake requirements, adverse effects and consequence of non-adherence. Frequent consultations and monitoring of drug levels, pVL and adherence in the early phase of therapy might be useful for this purpose.

9: Pharmacodynamic interaction between the antiretroviral drugs

Stavudine and zidovudine, both thymidine analogues, have a proven antagonistic effect. The combination of efavirenz, didanosine and tenofovir, the latter two drugs being adenosine/adenine analogues, is associated with early virological failure. The cause of this antagonism is unclear, but theoretically it could be due to steric hindrance at the enzyme active site or due to interference in the metabolic pathway of the drug. The virological inferiority of a regimen consisting of only three NRTIs could also be due to antagonism or a low genetic barrier to resistance. On the other hand, genotypic resistance to lamivudine (mutation M184V) can (partially) reverse resistance to zidovudine and improve the virological response to tenofovir. Possibly, resistance to NRTIs causes (in vitro) hypersusceptibility to NNRTIs and vice versa. Another example of increased susceptibility that can occur is the presence of the N88S protease gene mutation and improved virological response to amprenavir. Whether such in-vitro hypersusceptibilities result in clinical benefit remains to be proven.

10: Genetic make-up of the patient

Certain polymorphisms of chemokines (SDF-1 3’a, G protein b3 subunit 825T), chemokine- receptors (CCR5-delta32, CCR2-V64I, CCR5-promotor allel 59029-G), and certain HLA-alleles (B57, B27, Bw4, B*5701, B14, C8) are associated with a slower progression of the HIV-1 infection, while CCR5-promotor allel 59029-A and other HLA-alleles (B35, Cw4, DQB1*0402) are associated with a faster progression of the infection. Some of these factors are associated with a better (CCR5-delta32) or worse (the combination of wild-type CCR5, wild-type CCR2 and homozygous CCR5-promotor allel 59029-A) response to ART. Studies on single nucleotide polymorphism (SNP) of the multi-drug resistance transporter 1 (MDR1) gene at exon 26 position C3435T (allelic genotypes CC, CT and TT) on response to HAART are conflicting. Other SNPs have been associated with an increased drug exposure, slower drug clearance and toxicity, but the impact on the viral response to HAART was not clear or evident. Examples are cytochrome P450 (CYP) 2B6 (G516T) and efavirenz exposure and short term neurotoxicity, presence of the HLA-B*5701 genotype and abacavir hypersensitivity, and uridine diphosphate-glucuronosyltransferase 1A1 and atazanavir associated hyperbilirubinemia. Some of the (single
nucleotide) polymorphisms are more prevalent in certain ethnic groups, and thus may be of importance for selecting the components of the HAART regimen\textsuperscript{71,225}.

11: Experience and knowledge of the treating physician

A better survival was associated with both a greater HIV-treatment experience of the treating physician and a better adherence to therapy of the patient\textsuperscript{226}. These two factors even outweighed the worse outcome if HAART was started at a CD4 count below 50 cells/µL\textsuperscript{226}. Although not evaluated, the improved outcome was probably partly due to a better virological response because of a better instruction and management of the patient by the more HIV-experienced physician.

Sanctuary sites during antiretroviral treatment

A viral sanctuary site is an anatomical site which is highly impermeable to (some) antiretroviral drugs, and in which viral replication continues during treatment, thus allowing development and/or selection of drug-resistant strains\textsuperscript{227-234}. If recirculation of the virus from the sanctuary site occurs this can lead to systemic virological failure. Anatomical sites that are generally considered sanctuary sites are the central nervous system (CNS), because of the blood-brain and blood-cerebrospinal fluid (CSF) barrier\textsuperscript{230,235-238} and the male genital tract (MGT), because of the so called “blood-testis-barrier”\textsuperscript{227-234,239,240}. Discordant antiretroviral drug resistance mutations have indeed been observed in the CSF\textsuperscript{237,238}. Contrary to the CNS the lumen of the MGT is an excretory/paracrine organ, with different site specific characteristics and therefore it should be reconsidered whether the MGT is a viral sanctuary site or not.

For clarification a viral sanctuary site must be distinguished from a viral compartment and a viral reservoir. A viral compartment is an anatomical site in which the virus in untreated patients evolves distinctively from other anatomical sites or the main pool of infected cells, due to differences between the major cell types sustaining viral replication\textsuperscript{228}. A viral reservoir is a cell type or anatomical site in which a replication-competent virus persists much longer than in the main pool of infected cells that sustain the infection, and this cell type or anatomical site can replenish the pool of infected cells\textsuperscript{233,234}. The main cellular viral reservoir is the pool of latently infected resting CD4+ T-cells\textsuperscript{234}. 

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Suggestions for further studies

What needs to be further evaluated is whether there are other factors relevant for the virological response, what are early markers for long-term virological response, how regimens with a low, middle and high genetic resistance barrier compare to each other, how the virological response in non-B subtypes compare to subtype B, what the role is of viral sanctuary sites and what level of virus suppression is needed to prohibit virological failure. As long as HIV cannot be cured, the latter factor will really define what should be considered as HAART. However, improving adherence to therapy is likely the most relevant measure to achieve a durable virological success\textsuperscript{241}.

Since the risk of HIV-related morbidity and mortality is substantially increased as long as the CD4 count is below 200 cells/µL, strategies to raise the CD4 count more rapidly above this threshold (e.g. type of HAART regimen, interleukin-2) are also being explored\textsuperscript{242}. Chronic hyperactivation of the immune system might be an important cause of CD4+ cell loss\textsuperscript{243}. Considering the toxicity of HAART and the importance of a high level of adherence to HAART, it is also worthwhile to evaluate inhibition of this hyperactivation. Non-HAART strategies to evaluate the inhibition of this hyperimmunoactivation have also been explored, but have disappointing outcomes\textsuperscript{244}.

Outline of this thesis

In this thesis we study several aspects of highly active antiretroviral therapy of drug-naïve HIV-1 infected adults and we also study the role of the male genital tract as a supposedly viral sanctuary site.

In Chapter 2 the virological, immunological and toxicological results of the Antiretroviral Regimen Evaluation Study (ARES) are presented. Aim of this study, that was designed in 1998-1999, was to evaluate whether with once daily dosing of HAART virological success can be improved compared to a standard twice-daily dosed regimen. The HIV/ART-associated body fat redistribution / lipodystrophy syndrome (LDS) and dyslipidemia are major long term adverse events which can compromise adherence to therapy. In Chapter 3 changes in body composition, assessed by whole body dual energy X-ray absorptiometry (DEXA) scans, are evaluated in patients from the ARES study.

In Chapter 4 the current opinion that the male genital tract (MGT) is a sanctuary site for HIV during treatment is challenged. Based on the existing literature we postulate that the MGT is not a virological sanctuary site for HIV and likely not a source for virological failure of ART.
In Chapter 5 we report the HIV-1-RNA and drug concentrations in seminal plasma during 48-96 weeks of suppressive first-line HAART containing drugs that do not achieve therapeutic concentration in seminal plasma. This study confirms our hypothesis that the MGT is not a sanctuary site for HIV and that the source of HIV in semen is a spillover from the blood/extraluminal tissue. That the MGT is likely not a sanctuary site is in our view in part explained by the fact that the immunosuppressive state that is present in the lumen of the MGT precludes viral replication. To substantiate our hypothesis that there is a strong immunosuppressive state within the lumen of the MGT we describe in Chapter 6 the state of activation of lymphocytes in semen.

With the improved survival of HIV infected patients, procreation of these patients has become an important issue. Spermatozoa contain many mitochondria and the NRTI didanosine is one of the drugs with a high mitochondrial toxicity in vitro. In Chapter 7 we evaluate the quality of semen and the concentration of didanosine and tenofovir in seminal plasma. Tenofovir is one of the leading NRTIs, but data on its penetration in seminal plasma is lacking. These data are also lacking for didanosine. Didanosine is associated with mitochondrial toxicity and as mitochondria are abundant in spermatozoa, good penetration of this drug in to seminal plasma might affect semen quality.
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Chapter 1


Chapter 1


Chapter 2

Comparison of two once-daily regimens with a regimen consisting of nelfinavir, didanosine, and stavudine in antiretroviral therapy-naïve adults: 48-week results from the Antiretroviral Regimen Evaluation Study (ARES)

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Chapter 2

Abstract

Background
To improve the dosing frequency and pill burden of antiretroviral therapy we compared two once-daily-dosed regimens to a twice-daily-dosed regimen.

Method
HIV-1 infected, antiretroviral drug-naïve adults were randomised to either twice-daily nelfinavir and stavudine and once-daily didanosine (regimen A) or simplified once-daily dosed antiretroviral regimens consisting of nevirapine, didanosine and lamivudine (regimen B) or saquinavir, ritonavir, didanosine and lamivudine (regimen C).

Results
At 48 weeks of therapy, the proportion of patients with a blood plasma HIV-1-RNA concentration (pVL) <50 copies/mL by intention-to-treat analysis was 42.3%, 50.0% and 56.5% for regimens A (n = 26), B (n = 22) and C (n = 23), respectively. The time to a pVL <50 copies/mL for the first time was significantly shorter in regimen C, and there was significantly more progression to CDC events in regimen B. These differences are possibly due to differences in baseline characteristics. Adverse events were lowest for regimen C; more signs associated with mitochondrial toxicity occurred in regimen A. Increase in CD4 count was comparable between arms.

Conclusion
No statistically significant difference in efficacy was found between the two investigated once-daily dosed treatment regimens (B and C) and the reference (A). Regimen C possibly had a better virological response and less toxicity than regimens A and B.
Introduction

The aim of highly active antiretroviral therapy (HAART) given to previously untreated individuals infected with the human immunodeficiency virus type 1 (HIV-1) is to achieve a maximal and durable viral suppression. At present a blood plasma HIV-1-RNA concentration (plasma viral load, [pVL]) below the limit of detection of 80 copies/mL is considered appropriate for this goal. Several factors have been associated with the virological response to initial HAART. Of these factors, the adherence to therapy is one of the most important. The dosing frequency and pill burden are important factors for adherence to therapy.

To investigate options for improvement of the dosing frequency and pill burden of HAART in previously untreated HIV-1 infected adults the Antiretroviral Regimen Evaluation Study (ARES) was performed. The objective of this study was to compare the antiviral efficacy and tolerability of once-daily dosed (q.d.) regimens with a frequently used twice-daily dosed (b.i.d.) regimen. At the time of development and implementation of the protocol the protease inhibitor (PI) nelfinavir in combination with two nucleoside reverse transcriptase inhibitors (NRTIs) was considered one of the standard initial HAART regimens.

Method

Study design

This was a randomised, parallel arm, open-label, multicentre study comparing the efficacy and safety of three antiretroviral regimens: a once-daily dosed (q.d.) non-nucleoside reverse transcriptase inhibitor (NNRTI) based (regimen B), or q.d. PI-based (regimen C) or b.i.d. NNRTI plus PI based regimen (D) with a frequently used twice-daily-dosed PI based regimen (A, the reference regimen). The regimens were:

A: nelfinavir 1250 mg b.i.d., didanosine 400 or 250 mg q.d. and stavudine 40 or 30 mg b.i.d.
B: nevirapine 400 mg q.d., didanosine 400 or 250 mg q.d. and lamivudine 300 mg q.d.
C: saquinavir soft gelatin capsule (sgc) (Fortovase®) 1600 mg q.d., ritonavir 100 mg q.d., didanosine 400 or 250 mg q.d. and lamivudine 300 mg q.d.
D: nelfinavir 1500 mg b.i.d., nevirapine 200 mg b.i.d., didanosine 400 or 250 mg q.d., stavudine 40 or 30 mg b.i.d. and abacavir 300 mg b.i.d.
Chapter 2

The dose of didanosine and stavudine depended on whether the body weight was below (250 mg and 30 mg, respectively) or above 60 kg (400 mg and 40 mg, respectively). Up to October 2000, didanosine chewing tablets were used and thereafter the enteric-coated capsules were used. Didanosine was administered in a fasting state, defined as minimally one hour before or two hours after a meal. Nelfinavir and saquinavir sg were administered with food. Nevirapine had a lead-in dose of 200 mg q.d. during the first 14 days. There were three randomisation strategies: patients could choose to be randomised to one of these four antiretroviral regimens, or to regimens A, B and C, or to regimens A and D. This strategy was chosen to give patients the opportunity either to avoid the intensive, high pill burden and more toxic regimen D or to increase their chance to be randomised to regimen D.

The primary study objective was to compare the proportion of participants with a pVL below the level of detection between the b.i.d. PI-based reference regimen and the other three regimens after 48 and 96 weeks of treatment. Secondary objectives were to compare with the reference regimen (a) the time to the pVL lower limit of detection (50 copies/mL) for the first time, (b) virological failure, (c) treatment failure, and (d) changes in the CD4+ T-lymphocyte (CD4) count.

Seven hospitals in the Netherlands participated in the ARES study. The study was approved by the institutional review board of each participating site. All participating patients gave their written informed consent.

Study patients

Patients were eligible for the study if they were naïve for antiretroviral drugs and had an indication to start antiretroviral therapy. Other inclusion criteria were an age of 18 years or older, a pVL ≥5,000 copies/mL, and, for women of child-bearing potential, a negative urine pregnancy test within 28 days of baseline assessment and use of contraception with a barrier method. Exclusion criteria were an expected non-compliance with the protocol; excessive alcohol and elicited drug use; a history of pancreatitis, grade 3 or 4 hyperbilirubinemia, and / or elevated aspartate aminotransferase (AST) and / or alanine aminotransferase (ALT) according to the AIDS Clinical Trial Group (ACTG) toxicity grading scale; a hemoglobin level <6.3 mmol/L for men and <5.7 mmol/L for women; a neutrophil count <1.0 x 10⁹/L; a platelet count <75 x 10⁹/L; pregnant and / or breast-feeding women; presence of a severe medical condition; and concomitant use of drugs which are contraindicated with use of PIs and / or NNRTIs.
Assessments

Visits according to the protocol were screening (up to 28 days before baseline); baseline; weeks 2, 4, 8, 12, 16, 20, 24, 36, 48, 60, 72, 84 and 96; at virological failure; and at premature discontinuation of the study medication. At all these visits, blood was drawn for pVL measurement. Except for weeks 2, 12, and 20, blood was drawn for CD4 count; except for weeks 2, 16, and 20, blood was drawn for standard haematology and chemistry; and except for screening, baseline, and weeks 2, 16, and 20, blood was drawn for PI or NNRTI blood plasma concentrations. At baseline and weeks 24, 48, 72, and 96, blood was drawn in a fasting state, which was defined as an interval of at least six hours after the last meal.

The pVL was measured with a local assay at screening and from baseline onwards with the Amplicor HIV-1 Monitor 1.5 and ultra-Amplicor Cobas reverse transcriptase polymerase chain reaction assay (Roche Molecular Systems, Inc., New Jersey, USA) with a lower limit of detection of 50 copies/mL.

Virological failure was defined as one of the following: (a) a pVL >50 copies/mL and having a drop of the pVL <2Log_{10} at week 12 of treatment, (b) not achieving a pVL <200 copies/mL within 24 weeks of treatment, (c) not achieving a pVL <50 copies/mL within 48 weeks of treatment, (d) a pVL rebound from <50 copies/mL to ≥200 copies/mL on two consecutive measurements within six weeks, or (e) after an initial decrease a pVL increase of ≥0.5Log_{10} on two consecutive measurements.

Treatment failure was defined as a composite of virological failure and / or change of the allocated regimen, whichever came first. Adverse events were graded according to the ACTG toxicity grading-scale.

Change of randomisation procedure and termination of the study

In March 2000, the first patient was enrolled in the study. Because inclusion lagged behind and another study already had demonstrated an improved virological response with intensified treatment, randomisation to treatment arm D was discontinued in November 2000. At discontinuation of the randomisation to arm D, 21 patients were included in the study. Nine, eight, and four patients had then chosen randomisation strategies 1, 2, and 3, respectively, and seven, five, six, and three patients were randomised to regimens A, B, C, and D, respectively. In November 2002, the steering committee decided to stop enrolment in the study and in August 2003 to discontinue the study prematurely. The reasons for these decisions were, first, the expectation that the desired goal of 90 (30 in each arm) eligible patients would not be achieved within a proper period of time; second, the results of the ACTG 384 study; third, the indication from the interim analysis that statistically significant differences were not likely to be found; and, fourth, the concern of the
Independent Data Monitoring Committee (IDMC) about different rates of
treatment failure. In the ACTG 384 study, there was significantly more severe
or dose modifying toxicity in the regimen containing didanosine and stavudine
compared to the regimen containing lamivudine and zidovudine\textsuperscript{9,10}. At the
moment of discontinuation of the study, all patients had a follow-up of at least
48 weeks.

Statistical analysis

A sample size of 90 HIV-1-infected patients, 30 in each study arm, was
estimated to be sufficient to demonstrate a 30\% difference in the proportion of
participants with an undetectable viral load between arms B and C and arm A,
a two-sided significance level of 0.025 and a power of 0.80.

Interim analyses were performed on week 12 and 24 data, respectively, and
were presented to an IDMC. At both occasions, the IDMC concluded that the
data gave no reason to prematurely discontinue the study. However, the IDMC
had concerns about different, but not statistically significant, rates of treatment
failure; therefore, after the second interim analysis, they recommended that the
analysis of the events be continued sequentially.

A sequential analysis is a continuous interim analysis of the available data.
After each new treatment failure, the cumulative data are tested for evidence to
stop or continue the study. The stopping rule can be that (1) enough evidence
is reached to demonstrate a significant difference in failure rates or (2) enough
evidence is reached to make it likely that the anticipated difference will not be
found when the study is continued. On average, a sequential analysis requires
fewer patients to come to a decision than an analysis based on fixed sample
size design with the same characteristics (alpha, power and expected
difference). A sequential design guarantees the type 1 error (alpha) and the
power whenever a decision to stop the study is made. After stopping the study,
the effect estimate (e.g. the odds ratio [OR]) and its confidence interval (CI)
have to be adjusted for the sequential monitoring of the data, because after a
sequential analysis both the parameter estimate (i.e. the logarithm of the OR)
and its standard error are biased\textsuperscript{11}. The computer program PEST was used to
perform the sequential analysis\textsuperscript{12}.

Sequential analysis of week 24 data

Two parallel sequential analyses (regimen A vs. B; regimen A vs. C) were
performed using a double triangular test at each time point when a treatment
failure was observed before or at week 24. The design for the sequential
analyses was based on the same assumptions as for the original trial, that is,
assumed proportions of treatment failures of 40\% for regimen A and 10\% for
regimens B and C, respectively, a two-sided alpha of 0.25 and a power of 0.80.
Based on stopping rule 2 the comparison of regimen A versus B could be stopped after the evaluation of 49 patients, (18 in A and 14 in B) and the comparison of regimen A versus C could be stopped after the evaluation of 67 patients (24 in A and 23 in C). Both sequential analyses (A vs. B and A vs. C) of the observed data could be stopped with acceptance of the null hypothesis, meaning that the hypothesized treatment difference of 30% was not found. At the time the sequential analysis indicated that the study could be stopped (for A vs. B after evaluation of, in total, 49 patients and for A vs. C after evaluation of, in total, 67 patients), 4 more patients had been included in the trial. These patients were incorporated into the final study evaluation. Thus, the OR are estimated based on the data from 71 patients: 26 in arm A, 22 in arm B, and 23 in arm C. The estimated adjusted OR for treatment failure for regimen A versus B was 1.53 (97.5% CI, 0.33 – 8.99; the unadjusted OR was 1.44). For regimen A versus C, the adjusted OR was 3.15 (97.5% CI, 0.80 – 12.92; the unadjusted OR was 2.59). Therefore follow-up of the participating patients for longer than 48 weeks was discontinued.

Analysis of week 48 data

Data are presented as means and standard deviations or medians with interquartile range. Comparisons were made between the two once-daily regimens (B and C) versus regimen A. The intention to treat (ITT) population was used for the analyses with missing data equals failure wherever applicable.

Chi-square tests and Kruskal-Wallis tests were performed on categorical and continuous data, respectively. Time to undetectability was tested by the log rank test. Data on CD4 count were tested by repeated measurements using a generalized linear model (PROC MIXED of SAS software [SAS version 8.02, SAS Institute, Cary, North Carolina, USA]). The safety analyses were performed for the ITT population, but only adverse events that occurred while the patient was using the allocated treatment were considered. Taking into account multiple testing by Bonferroni, a two-sided p value <0.025 was considered statistically significant.

Results

Baseline characteristics and follow up

From March 2000 until October 2002, 71 patients were randomised. In Table 2.1, the baseline characteristics are given. Remarkably, the median CD4 count in arm B was lower than in arm A and C (115 vs. 190 and 300 cells/µL), but this
Table 2.1 Baseline characteristics of patients randomised to arm A, B and C.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Treatment arm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Number of patients</td>
<td>26</td>
</tr>
<tr>
<td>Mean age (years) (SD)</td>
<td>42.3 (9.8)</td>
</tr>
<tr>
<td>Number male/female (%)</td>
<td>20/6 (77/23)</td>
</tr>
<tr>
<td>Number with CDC A/B/C (%)</td>
<td>16/5/5 (62/19/19)</td>
</tr>
<tr>
<td>Median plasma HIV-1-RNA (Log_{10} copies/mL) (IQR)</td>
<td>5.1 (4.7-5.4)</td>
</tr>
<tr>
<td>Median CD4 (cells/µL) (IQR)</td>
<td>190 (110-261)</td>
</tr>
</tbody>
</table>

Note: arm A: nelfinavir, didanosine, stavudine; arm B: nevirapine, didanosine, lamivudine q.d; arm C: saquinavir sgc, ritonavir, didanosine, lamivudine q.d.; CDC=Centers for Disease Control and Prevention; IQR=25%-75% interquartile range; SD=standard deviation.

was not statistically significant. In arm C, the pVL was lowest (median 4.8Log_{10} copies/mL) and the percentage of asymptomatic patients relatively high (70%).

Virological and immunological efficacy

Effect on HIV load

In ITT analysis of 48-week data, the proportion of patients with a pVL <50 copies/mL was 42.3%, 50.0%, and 56.5% for regimens A, B and C, respectively (Table 2.2; Figure 2.1). The OR (and CI) for regimen A versus B and A versus C was 1.57 (0.49-5.05) and 2.06 (0.65-6.51), respectively. For virological failure, the proportion was 50%, 45.5% and 39.1%, respectively; for treatment failure, the proportion was 65.4%, 54.6%, and 47.8%, respectively (Table 2.2). These proportions were not statistically significantly different between regimens B and C and regimen A. However, the time to first measurement of a pVL <50 copies/mL in ITT analysis was statistically significantly different between regimen A and C (p = 0.01), but not between regimen A and B (p = 0.68) (Figure 2.2).

Effect on CD4 cell count

The mean increase of the CD4 count from baseline to week 48 of therapy in ITT analysis was 117 cells/µL, 196 cells/µL, and 168 cells/µL for regimen A, B, and C, respectively, and was not statistically significantly different between regimen B or C and regimen A (Table 2.2). The increase in CD4 count was mainly within the first 12 weeks of therapy (Figure 2.3). At 24 weeks of therapy, the absolute mean CD4 count was 326, 278, and 481 cells/µL for regimens A, B, and C, respectively; at 48 weeks, it was 333, 352, and 456 cells/µL, respectively (Figure 2.3).
Table 2.2 48-week results: efficacy (virological and immunological), serious adverse events, new CDC events and permanent change of allocated regimen (ITT).

<table>
<thead>
<tr>
<th>Treatment arm</th>
<th>p value comparison with A</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (n=26)</td>
<td>B (n=22)</td>
</tr>
<tr>
<td><strong>Virological efficacy</strong></td>
<td></td>
</tr>
<tr>
<td>Percentage with pVL&lt;50 copies/mL</td>
<td>42.3</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Virological failure, number (%)</td>
<td>13 (60.0)</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment failure, number (%)</td>
<td>17 (65.4)</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Immunological efficacy</strong></td>
<td></td>
</tr>
<tr>
<td>Mean increase CD4 count (cells/µL)</td>
<td>117</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Serious adverse events</strong></td>
<td></td>
</tr>
<tr>
<td>Number serious clinical adverse events</td>
<td>7</td>
</tr>
<tr>
<td>Number of patients with one or more serious clinical adverse events (%)</td>
<td>6 (23.1)</td>
</tr>
<tr>
<td>Number serious laboratory adverse events</td>
<td>8</td>
</tr>
<tr>
<td>Number of patients with one or more serious laboratory adverse events (%)</td>
<td>7 (26.9)</td>
</tr>
<tr>
<td>Number of patients with one or more serious adverse events (%)</td>
<td>8 (30.8)</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>New CDC events</strong></td>
<td></td>
</tr>
<tr>
<td>Number of new CDC category B events</td>
<td>0</td>
</tr>
<tr>
<td>Number of new CDC category C events</td>
<td>2</td>
</tr>
<tr>
<td>Number of patients with one or more new CDC events (%)</td>
<td>2 (7.7)</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Change of allocated therapy</strong></td>
<td></td>
</tr>
<tr>
<td>Number of patients with permanent change of allocated regimen (%)</td>
<td>15 (57.7)</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Reason for change of regimen (number)</strong></td>
<td></td>
</tr>
<tr>
<td>Virological failure</td>
<td>1</td>
</tr>
<tr>
<td>Adverse events</td>
<td>9</td>
</tr>
<tr>
<td>Clinical progression of HIV</td>
<td>1</td>
</tr>
<tr>
<td>Low drug levels</td>
<td>2</td>
</tr>
<tr>
<td>Lost to follow up</td>
<td>1</td>
</tr>
<tr>
<td>Patient withdraw</td>
<td>1</td>
</tr>
<tr>
<td>Interaction with comedication</td>
<td>0</td>
</tr>
<tr>
<td>Start with different regimen</td>
<td>0</td>
</tr>
</tbody>
</table>

Note: arm A: nelfinavir, didanosine, stavudine; arm B: nevirapine, didanosine, lamivudine q.d; arm C: saquinavir sgc, ritonavir, didanosine, lamivudine q.d.; CDC=Centers for Disease Control and Prevention. ITT=intention to treat. * One patient had clinical progression of a HIV-related disease, hepatitis, and virological failure at the same time.
serious clinical adverse events

Serious adverse events

Serious clinical adverse events

In arm A, seven serious clinical (grade 3 or 4) events occurred in six patients. In four patients, these adverse events were related to mitochondrial toxicity (peripheral neuropathy, 1; pancreatitis, 1; lactic acidosis, 1; lipodystrophy, 1). The fifth patient had recurring sickle cell bone crisis and has been described in a case report. The sixth patient had general malaise and a pneumonia. In arm B, 12 serious clinical adverse events occurred in eight patients. These adverse events concerned rash (3 patients), fever (3), dyspnoea (1), dyspepsia (1), a
process in the right cerebrum (1), pneumonia (1), general malaise (1) and an elective abortion (1). Of the three cases with rash, two were related to use of nevirapine and one to clindamycin. In arm C, six serious clinical events occurred in 3 patients and these concerned one each of rash, epigastric pain, pancreatitis, insomnia, fatigue and general malaise.

The proportion of patients with at least one serious clinical adverse event during 48 weeks of treatment with regimens A, B, or C was 23.1% (6/26), 36.4% (8/22) and 13.0% (3/23), respectively (Table 2.2). All adverse events were ACTG grade 3, except for the case of pancreatitis in arm C. The differences between regimen B or C and regimen A were not statistically significant.

**Serious laboratory adverse events**

In arms A, B, and C 8, 10 and 3 grade 3 or 4 laboratory adverse events occurred, respectively, in seven, five, and two patients, respectively. The serious laboratory adverse events were anaemia, neutropenia, thrombocytopenia, and increases in liver-biochemistry. All serious laboratory adverse events occurred within the first 24 weeks of therapy. The proportion of patients with at least one serious laboratory adverse event was 26.9%, 22.7%, and 8.7% for regimens A, B, and C, respectively (Table 2.2). The differences between regimen B or C and regimen A were not statistically significant.

Overall, 8 (30.8%), 12 (54.6%), and 4 (17.4%) patients in arm A, B, and C, respectively, had at least one serious clinical or laboratory adverse event. The differences were not statistically significant compared to arm A (Table 2.2).

**New CDC events**

New CDC (Centers for Disease Control and Prevention) B or C events that occurred after 8 weeks of antiretroviral therapy were 2, 15, and 5 for regimen
A, B, and C, respectively (Table 2.2). According to ITT analysis, the incidence of patients with at least one new CDC event was 7.7%, 40.9%, and 17.4% for regimens A, B, and C, respectively (Table 2.2). The difference between regimen B and regimen A was statistically significant ($p = 0.02$). The new CDC events emerged predominantly within the first 24 weeks of therapy. The most common CDC B event was oral candidiasis (8 cases, 6 of which were in arm B). There was no predominant CDC C event.

Change of allocated therapy

The percentage of patients in whom the initial therapy was changed during the period of follow-up were not statistically significantly different between study groups (57.7%, 50%, and 30.4% for regimens A, B, or C at 48 weeks, respectively; Table 2.2). It is important to note that therapy was not changed in all cases of virological failure, adverse event, or new diagnosed CDC event.

Discussion

The main purpose of this study was to examine the efficacy and tolerability of once-daily dosed NNRTI (nevirapine) or PI (saquinavir/ritonavir)-based regimens compared to a frequently used HAART regimen. The combination of nelfinavir, didanosine, and stavudine would not be used anymore, but at the time this study was started, this regimen was considered as an adequate standard and reference regimen. No statistically significant differences were found in the percentages of patients with a pVL <50 copies/mL at 48 weeks between both once-daily dosed regimens and the control regimen. However, because of the small sample size, the randomisation strategy, and the discontinuation of arm D, relevant differences between the groups might exist. The time to achieve a pVL <50 copies/mL was statistically significantly shorter in the once-daily dosed PI-based regimen (C) than in the control regimen. Further, we observed more CDC events in arm B. This might be explained by the fact that patients randomised to arm B had a more progressive HIV infection at baseline than those randomised to arms A and C. Cohort studies have shown that virological failure and progression of CDC events are found more frequently in patients with a baseline CD4 count of <200 cells/µL compared to those with a higher CD4 count$^{13,14}$. In addition, a higher baseline pVL might be associated with more virological failure$^4$. There were possibly more adverse events associated with mitochondrial toxicity in regimen A. The limited size of the study did not allow for a valid statistical analysis between arms B and C.
Although this study is hampered by its small size, the efficacy results of the three different regimens are comparable to those observed in other (randomised) prospective studies\(^4\). The virological success to initial HAART as defined by a pVL <50 copies/mL after 48 weeks of therapy according to an ITT analysis varies between 20% and 88\(^4,6,15-17\). The older studies demonstrate a virological success of about 50\(^4,5\), whereas the more recent studies demonstrate a virological success of 70-80%\(^4,6,17\).

In our study, the virological success of regimen A was 42.3%. The ITT virological success of nelfinavir-containing HAART regimens varies between 32% and 65.2% of cases\(^3,4,16-24\). For the combination of nelfinavir (750 mg three times a day), didanosine, and stavudine a 48-week response of 32-39% has been reported\(^21,22\). In these two studies, the median baseline CD4 count and pVL was >340 cells/µL and >4.74Log\(_{10}\), respectively\(^21,22\).

We observed a virological success of regimen B of 50.0%. For nevirapine-containing HAART the ITT virological success varies between 20% and 73%\(^3,4,15,25-33\). In the study with the least virological success the median baseline CD4 count was 37.5 cells/µL and the pVL ≥250,000 copies/mL\(^15\). In studies in which nevirapine once daily was used, the ITT virological success varied between 40% and 70%\(^26,28,31-33\). In these studies, the dual NRTI backbone was not didanosine and lamivudine and the median baseline CD4 count and pVL were ≥200 cells/µL and >4.34Log\(_{10}\), respectively. In the largest study done, the 2NN study, in ITT analysis a pVL <50 copies/mL after 48 weeks of therapy was achieved in 70% of cases with the combination of nevirapine once daily, stavudine and lamivudine twice daily. In this treatment arm the baseline CD4 count and pVL was 200 c/µL and 4.7Log\(_{10}\) copies/mL\(^33\).

Published prospective studies with once-daily ritonavir-boosted saquinavir and two NRTIs as initial antiretroviral therapy and with a follow-up of at least 48 weeks are limited\(^34,35\). Preliminary results of a randomised, prospective study, the FOCUS study, involving 75 patients in the treatment arm with saquinavir 1600 mg q.d., ritonavir 100 mg q.d., and 2 NRTIs, demonstrated in ITT analysis at 24 and 48 weeks of therapy that 60% and 51% of the patients had a pVL <50 copies/mL, respectively\(^36,37\). In this treatment arm, the median baseline CD4 count and pVL were ≥322 cells/µL and 4.70Log\(_{10}\) copies/mL, respectively. In the FOCUS study, the compared regimen consisting of efavirenz and two NRTIs was probably more effective at 48 weeks than the saquinavir treatment. In this efavirenz-containing treatment arm, 77 patients were included with a baseline CD4 count and pVL of 326 cells/µL and 4.77Log\(_{10}\) copies/mL, respectively and the ITT virological success (pVL<50 copies/mL) was 71\%\(^36\). In our study, the baseline CD4 count and viral load and ITT virological success at
24 and 48 weeks of therapy (52.2% and 56.5%, respectively) were comparable to the FOCUS study. With saquinavir hard gelatin capsules (hgc) the virological success at 24 weeks was even better, 92%.

In conclusion, the two investigated once-daily dosed treatment regimens (B and C) did not statistically significantly differ in efficacy from the reference regimen (A), and the virological response rate (50%) of this reference regimen was comparable to that reported in literature. However, because of the small sample size, differences between the treatment arms might still exist, although they may be smaller than expected. The efficacy was possibly better and toxicity less in arm C, but this arm had better baseline characteristics. In arm A there was possibly more mitochondrial toxicity. In arm B, there was statistically significantly more progression to CDC events, which might be related to the worse baseline characteristics. It is possible that the efficacy of arm A and B would be better and progression of CDC events in arm B would be less if treatment was started at a baseline CD4 count of >200 cells/µL, as current guidelines recommend1-3,14. One could question whether the efficacy of the once-daily dosed regimens used in this study, regimens B and C, is good enough, taking into account the much better virological success that can be achieved with the more recent HAART regimens4,6,17,39. However, regimen C performed well in our study, and this regimen can be further improved. For instances saquinavir hgc of 500 mg will soon become available. A once-daily dosed regimen consisting of saquinavir 2000 mg boosted with low dose ritonavir (100 mg) may have a greater efficacy than regimen C and comparable toxicity40.

Acknowledgment

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Chapter 3

Stavudine- but not didanosine as part of HAART contributes to peripheral lipoatrophy. A substudy from the Antiretroviral Regimen Evaluation Study (ARES)

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Submitted for publication
Chapter 3

Abstract

Purpose
To objectively assess changes in body fat distribution in a subgroup of antiretroviral therapy naïve participants in a randomised comparative trial of regimens including a nucleoside analogue backbone of didanosine with either lamivudine or stavudine.

Method
Whole body dual energy X-ray absorptiometry (DEXA) scans were performed at baseline and week 48 and 96 of therapy in all 19 patients from one of the sites participating in the Antiretroviral Regimen Evaluation Study (ARES). Patients had been randomised to receive nelfinavir/didanosine/stavudine (n = 8), nevirapine/didanosine/lamivudine (n = 7), or ritonavir-boosted saquinavir/didanosine/lamivudine (n = 4).

Results
In an intent-to-treat analysis, patients allocated to didanosine plus stavudine-containing treatment after 96 weeks had lost a median of 1825 grams of total limb fat, as compared to a median gain of 1639 and 403 grams in those randomised to the didanosine/lamivudine plus nevirapine or saquinavir-containing regimens, respectively. These changes in limb fat were statistically significantly different when comparing patients allocated to stavudine-containing treatment with both other two treatment arms combined (p = 0.01).

Conclusion
This study suggests that didanosine/lamivudine, when combined with either nevirapine or ritonavir-boosted saquinavir over 96 weeks of therapy is possibly not associated with limb fat atrophy, in contrast to when treatment contained didanosine, stavudine and nelfinavir combined.
Introduction

The pathogenesis of antiretroviral therapy (ART) associated adipose tissue redistribution remains incompletely elucidated. Peripheral fat loss (lipoatrophy) is a distinguishing feature, and exposure to thymidine analogue reverse transcriptase inhibitors has consistently been found to be one of the major risk factors for lipoatrophy development. The risk is greater with stavudine- compared to zidovudine-containing regimens. The results of one study have suggested that the protease inhibitor may also contribute to lipoatrophy development. The increased risk of peripheral lipoatrophy, particularly with the use of stavudine, is also nicely illustrated by several trials which demonstrated an at least partial reversal of subcutaneous fat loss, following the substitution of stavudine by abacavir or tenofovir. Given that formerly stavudine was often combined with didanosine, it is not surprising that some studies have also reported an increased risk of lipoatrophy for this particular combination, but to what extent didanosine contributes to this risk remains unclear. One observational study which confirmed the risk associated with stavudine, did not find didanosine to be an independent risk factor. Interestingly, in a trial comparing a combination of emtricitabine/didanosine/efavirenz with stavudine/didanosine/efavirenz in antiretroviral naïve patients investigator-assessed lipodystrophy over at least 48 weeks of follow-up was reported in only 1/286 patients (0.4%) in the emtricitabine group compared to in 17/285 patients (6%) in the stavudine group, suggesting a limited contribution from didanosine. Thus far however, changes in peripheral fat measured by objective assessment of body composition in patients initiating antiretroviral therapy for the first time with regimens containing didanosine but not stavudine, have not been reported. We assessed changes in body fat distribution by whole body dual energy X-ray absorptiometry (DEXA) scan over 96 weeks follow up in a subgroup of participants enrolled in a randomised, comparative multicenter trial of nelfinavir/didanosine/stavudine (NFV/ddI/d4T), nevirapine/didanosine/lamivudine (NVP/ddI/3TC) or ritonavir-boosted saquinavir/didanosine/lamivudine (SQV-r/ddI/3TC) in antiretroviral therapy naïve patients. The results of the main trial have previously been reported. The present substudy offered a unique opportunity to look at changes in body composition in a group of patients on different ddI containing regimens and is unlikely to be performed again anytime soon in the near future, given that the first choice reverse transcriptase nucleoside/-tide analogues (NRTI’s) which are currently recommended as part of first-line regimens include abacavir, emtricitabine, lamivudine, tenofovir and zidovudine.
Chapter 3

Patients and method

In the ARES study antiretroviral-therapy-naïve HIV-1 infected adults were randomised between one of the following three regimens: A) NFV/ddI/d4T: nelfinavir (NFV) 1250 mg twice daily (b.i.d.), didanosine (ddI) once daily (q.d.) and stavudine (D4T) b.i.d., or B) NVP/ddI/3TC: nevirapine (NVP) 400 mg q.d. (during the first 14 days 200 mg q.d.), didanosine q.d. and lamivudine (3TC) 300 mg q.d. or C) SQV-r/ddI/3TC: saquinavir (SQV) soft gelatin capsules (sgc) 1600 mg q.d., ritonavir (r) 100 mg q.d., didanosine q.d. and lamivudine 300 mg q.d. Didanosine and stavudine were dosed 250 or 400 mg q.d. and 30 or 40 mg b.i.d., respectively, depending on whether the body weight was less or greater than 60 kg. Other main eligibility criteria were a HIV-1-RNA plasma concentration (pVL) ≥5,000 copies/mL and absence of any severe medical conditions and/or severe laboratory abnormalities. There were no restrictions with respect to CD4+ T-lymphocyte (CD4) count. The pVL was measured with the Amplicor HIV-1 monitor 1.5 and ultra-Amplicor Cobas reverse transcriptase polymerase chain reaction (RT-PCR) assay (Roche Molecular Systems, Inc., NJ, USA) and/or the Versant HIV-1-RNA 3.0 branched-DNA assay (Bayer Corporation) both with a lower limit of detection of 50 copies/mL.

All nineteen participants enrolled into the main trial at the Academic Medical Centre (AMC) in Amsterdam gave separate informed consent to undergo DEXA scanning within the 4 week period prior to commencing trial treatment, and after 48 and 96 weeks of therapy. All scans were performed with the same Hologic scanner, model QDR 4500W (S/N 47802). Quality assurance procedures were routinely conducted every week. For whole body calibration a step phantom was used. Whole body analysis was performed in accordance with the instructions of the manufacturer. Regions of interest were determined by standardized landmarks, providing measures of regional trunk fat, regional extremity fat and lean body mass.

All analyses were performed as intention to treat. Two patients did not undergo a DEXA scan at week 96 and at week 48 and 96 respectively. For the analysis these missing data were not imputed but considered as missing. Data are presented as medians and range or interquartile ranges (IQRs) or number and percentages as applicable. Comparisons were made across all three treatment arms as well as between the stavudine containing arm and both non-stavudine containing arms combined. Absolute and relative differences in changes over time between the groups were tested by the Kruskall Wallis test for non parametric data. A two-sided p-value <0.05 was considered statistically significant. Since this was an exploratory substudy an a priori power calculation was not performed.
Results

Nineteen patients were enrolled into the ARES study at the AMC, of whom eight, seven and four patients were randomised to NFV/ddI/d4T, NVP/ddI/3TC and SQV-r/ddI/3TC, respectively. In all but two patients, a complete series of DEXA scans were performed at baseline, week 48 and 96. One patient randomised to NVP/ddI/3TC only underwent DEXA scanning at baseline and week 48. This patient had a right sided paralysis due to progressive multifocal leukoencephalopathy (PML) at the time of the week 48 scan. He became wheel-chair dependent and visually disabled, which precluded performing a scan at week 96. A second patient allocated to SQV-r/ddI/3TC only had a DEXA scan at baseline. After 36 weeks of therapy he returned to his country of origin and was lost to follow-up.

Table 3.1 shows the baseline characteristics of patients from the substudy (n=19) and ARES main study (n=71). Patients in the substudy allocated to NVP/ddI/3TC were younger and had a lower body weight, body mass index (BMI) and CD4 count. None of the patients in the substudy however had HIV associated wasting according to the Centers for Disease Control and Prevention (CDC) classification. Compared to all participants of the main study the patients of the present substudy had a comparable baseline pVL and CD4 count. In the substudy there were relatively more patients with CDC category C disease and the patients in the SQV-r/ddI/3TC arm of the subgroup were slightly older than the patients randomised to the same arm of the main study.

Table 3.2 shows the virological and immunological responses to treatment, which were reasonably comparable between arms. In Table 3.3 details of the type, timing and reason of treatment change, as well as subsequently prescribed antiretroviral therapy are provided. Six of eight patients allocated to
Table 3.2  Virological and immunological response to antiretroviral therapy.

<table>
<thead>
<tr>
<th>Regimen</th>
<th>Number (%) with pVL &lt;50 copies/mL at</th>
<th>Median (range) CD4 count, cells/µL at</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>week 24</td>
<td>week 24</td>
</tr>
<tr>
<td></td>
<td>(n=8)</td>
<td>(n=8)</td>
</tr>
<tr>
<td>NFV/ddI/d4T</td>
<td>4 (50)</td>
<td>310 (60-520)</td>
</tr>
<tr>
<td>NVP/ddI/3TC</td>
<td>3 (43)</td>
<td>200 (60-470)</td>
</tr>
<tr>
<td>SQV-r/ddI/3TC*</td>
<td>4 (100)</td>
<td>350 (190-600)</td>
</tr>
<tr>
<td></td>
<td>week 48</td>
<td>week 48</td>
</tr>
<tr>
<td></td>
<td>(n=7)</td>
<td>(n=7)</td>
</tr>
<tr>
<td></td>
<td>6 (75)</td>
<td>6 (75)</td>
</tr>
<tr>
<td></td>
<td>7 (100)</td>
<td>6 (86)</td>
</tr>
<tr>
<td></td>
<td>4 (100)*</td>
<td>7 (100)*</td>
</tr>
<tr>
<td></td>
<td>week 72</td>
<td>week 72</td>
</tr>
<tr>
<td></td>
<td>(n=4)</td>
<td>(n=4)</td>
</tr>
<tr>
<td></td>
<td>6 (75)</td>
<td>6 (75)</td>
</tr>
<tr>
<td></td>
<td>7 (100)</td>
<td>7 (100)</td>
</tr>
<tr>
<td></td>
<td>4 (100)*</td>
<td>3 (75)*</td>
</tr>
<tr>
<td></td>
<td>week 96</td>
<td>week 96</td>
</tr>
<tr>
<td></td>
<td>(n=4)</td>
<td>(n=4)</td>
</tr>
<tr>
<td></td>
<td>7 (88)</td>
<td>7 (88)</td>
</tr>
<tr>
<td></td>
<td>7 (100)</td>
<td>7 (100)</td>
</tr>
<tr>
<td></td>
<td>3 (75)*</td>
<td>3 (75)*</td>
</tr>
</tbody>
</table>

Notes: DDI=didanosine; D4T=stavudine; 3TC=lamivudine; NFV=nelfinavir; NVP=nevirapine; R=low dose ritonavir; SQV=saquinavir; * One patient was lost to follow up after 36 weeks of treatment.

NFV/ddI/d4T changed randomised treatment (five patients because of treatment limiting adverse events) after a median of 8.5 months (range 2-16 months). Of the seven patients on NVP/ddI/3TC four changed treatment after a median of 5.3 months (range 1-7 months), which included the discontinuation of didanosine in one patient. In the SQV-r/ddI/3TC arm treatment remained unchanged, but one patient was lost to follow-up after 36 weeks.

Figure 3.1 shows the changes in total limb fat of the individual patients as well as the median changes per treatment arm. Table 3.4 summarizes the median absolute and relative change from baseline in total limb, arm, leg and truncal fat, as well as in lean body mass (LBM) per treatment group. At baseline the median amount of total limb, arm and leg fat and LBM differed substantially across the three arms (Table 3.4), with the highest amount of limb fat and LBM in the NFV/ddI/d4T and SQV-r/ddI/3TC arm, respectively. The highest baseline amount of truncal fat likewise was found in the NFV/ddI/d4T arm, whereas this was lowest in the SQV-r/ddI/3TC arm.

In patients randomized to NFV/ddI/d4T total limb fat had decreased progressively by 26% after 96 weeks. Arm fat decreased by 23% and leg fat by 32%. In both of the other treatment groups the total limb fat increased by 35% and 17%, respectively, during the first 48 weeks and continued to do so out to 96 weeks in those allocated to NVP/ddI/3TC (by a total increase of 48%), but not in those randomized to SQV-r/ddI/3TC in whom the increase only remained 6% after 96 weeks of follow up. The percent change in total limb (p = 0.01), as well as in arm (p = 0.02) and leg fat (p = 0.01) was significantly different when comparing the stavudine containing regimen to both non stavudine containing regimens combined, except for the change in arm fat at week 48 (p = 0.12). One patient in the NFV/ddI/d4T arm had an aberrant weight loss of about 5 kg at 48 weeks which was nearly regained at 96 weeks (Figure 3.1). Since the
changes in limb and total fat are given as medians with interquartile ranges (Table 3.3) and a non-parametric test was used, the observed significant difference in limb and total fat between the stavudine containing regimen and both non stavudine containing regimens is not likely to be driven by this aberrant patient. Median truncal fat increased from baseline to week 96 by 13%, 35% and 10% in the NFV/ddI/d4T, NVP/ddI/3TC, and the SQV-r/ddI/3TC groups, respectively. In the SQV-r/ddI/3TC group the truncal fat decreased between week 48 and week 96 from 43.9% to 10.0%, while in the two other arms a continuous increase in truncal fat was observed. Between the NVP/ddI/3TC and SQV-r/ddI/3TC arm there were no statistically significant differences in change of fat content in any of the body regions. Furthermore, no statistically significant differences were observed in the change in LBM throughout the study period between the stavudine containing versus the non stavudine containing study groups.
Figure 3.1 Change from baseline in total limb fat for each individual patient as well as median change per treatment arm.
Table 3.4  Baseline total limb, arm, leg and truncal fat content and lean body mass, as well as absolute and relative changes from baseline during treatment (medians and interquartile ranges).

<table>
<thead>
<tr>
<th>Regimen</th>
<th>A NVP/ddI/3TC (n=8)</th>
<th>B NVP/ddI/3TC (n=7)</th>
<th>C SQV/ddI/3TC (n=4)</th>
<th>p-value A vs B+C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median total limb fat,</td>
<td>7321 (4535-9423)</td>
<td>5737 (1865-9861)</td>
<td>4565 (3207-7299)</td>
<td>NA</td>
</tr>
<tr>
<td>(IQRs)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median total arm fat,</td>
<td>2719 (1399-3224)</td>
<td>1843 (517-2401)</td>
<td>1525 (944-2284)</td>
<td>NA</td>
</tr>
<tr>
<td>(IQRs)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median total leg fat,</td>
<td>4513 (3219-6293)</td>
<td>3894 (1348-7239)</td>
<td>3040 (2263-5015)</td>
<td>NA</td>
</tr>
<tr>
<td>(IQRs)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median truncal fat,</td>
<td>7991 (3652-9959)</td>
<td>6073 (2179-7179)</td>
<td>5494 (3968-7280)</td>
<td>NA</td>
</tr>
<tr>
<td>(IQRs)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median LBM, grams (IQRs)</td>
<td>51015 (33735-58448)</td>
<td>48860 (31457-60063)</td>
<td>58274 (43590-77263)</td>
<td>NA</td>
</tr>
<tr>
<td>Change in total limb fat</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 48</td>
<td>-137 (-4711-2208)</td>
<td>742 (-206-4809)</td>
<td>790 (355-1928)</td>
<td>0.01</td>
</tr>
<tr>
<td>(range)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% (range)</td>
<td>-3.9 (-35.8-62.6)</td>
<td>35.1 (-4.5-135.5)</td>
<td>17.3 (4.9-60.1)</td>
<td>0.01</td>
</tr>
<tr>
<td>Change in total arm fat</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 48</td>
<td>9 (-1648-489)</td>
<td>273 (-68-1132)</td>
<td>205 (-215-581)</td>
<td>0.21</td>
</tr>
<tr>
<td>(range)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% (range)</td>
<td>0.4 (-40.1-57.3)</td>
<td>20.5 (-7.2-77.0)</td>
<td>13.4 (-9.4-61.6)</td>
<td>0.12</td>
</tr>
<tr>
<td>Change in total leg fat</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 48</td>
<td>-222 (-1216-790)</td>
<td>289 (-168-1196)</td>
<td>-51 (-85-742)</td>
<td>0.04</td>
</tr>
<tr>
<td>(range)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% (range)</td>
<td>-23.3 (-42.7-28.6)</td>
<td>44.4 (-17.8-73.6)</td>
<td>-2.2 (-6.2-78.6)</td>
<td>0.02</td>
</tr>
<tr>
<td>Change in truncal fat</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 48</td>
<td>-231 (-3063-1719)</td>
<td>651 (-138-3677)</td>
<td>589 (570-1347)</td>
<td>0.01</td>
</tr>
<tr>
<td>(range)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% (range)</td>
<td>-8.8 (-33.8-64.6)</td>
<td>37.1 (-3.8-160.2)</td>
<td>19.3 (11.4-59.5)</td>
<td>0.01</td>
</tr>
<tr>
<td>Change in LBM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 48</td>
<td>945 (-1687-5112)</td>
<td>1044 (-71.2-5475)</td>
<td>821 (-1531-2956)</td>
<td>1.00</td>
</tr>
<tr>
<td>(range)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% (range)</td>
<td>13.4 (-13.9-83.6)</td>
<td>35.2 (-1.9-56.1)</td>
<td>10.6 (-24.0-89.1)</td>
<td>0.60</td>
</tr>
</tbody>
</table>
| Notes: DDI=didanosine; DD4T= stavudine; 3TC=lamivudine; NVP=nelfinavir; NVP=nevirapine; R=low dose ritonavir; SQV=saquinavir; LBM=lean body mass; IQRs=25%-75% interquartile ranges; * One patient did not had a DEXA scan at week 96. ^ One patient was lost to follow up after 36 weeks of therapy.

The absolute changes in fat content of the different regions of the body were not statistically significant within each of the treatment groups. However in the NFV/ddI/d4T arm there was a clear trend towards loss of total limb fat (p = 0.08) after 96 weeks of treatment, whereas such an observation was less pronounced in the NVP/ddI/3TC arm (p = 0.13) and not observed in the SQV-r/ddI/3TC arm (p = 0.64). Within the NVP/ddI/3TC arm there was a trend towards gain of arm fat at 96 weeks (p = 0.09). However, a significant decrease in the median percent change in leg fat from baseline was found in the NFV/ddI/D4T arm at 96 weeks (-31.6%, p = 0.05), while in the NVP/ddI/3TC arm there was a significant median percent increase in limb fat a 48 weeks
(median 35.1%, p = 0.03), in leg fat at 48 weeks (37.1%, p = 0.03) and LBM at 96 weeks (median 5.3%, p = 0.03). There were no significant changes in the SQV-r/ddI/3TC arm (Table 3.4).

Discussion

Given that lipoatrophy is only poorly and partially reversible once it has developed\textsuperscript{15-19}, it is key to identify combination antiretroviral regimens which are associated with the least likelihood of lipoatrophy development. Initial antiretroviral treatment regimens with a nucleoside/nucleotide backbone of tenofovir or abacavir plus lamivudine have demonstrated a lack of limb fat loss both by physician report, anthropometry and whole body DEXA during 2-3 years of follow up, when compared to regimens including a backbone of stavudine or zidovudine plus lamivudine\textsuperscript{11,12,14,21,27,28}. For example, in a prospective, randomised comparative trial investigator-defined lipodystrophy after 144 weeks of follow up had been reported in 19% of patients randomised to the combination of stavudine, lamivudine and efavirenz, but only in 3% allocated to tenofovir, lamivudine and efavirenz\textsuperscript{14}. Using whole body DEXA, significantly more total limb fat was observed in the tenofovir group (7.9 kg) than in the stavudine group (5.0 kg) at week 96. Unfortunately, baseline DEXA measurements were not performed in this trial. In the only randomised trial which compared didanosine-containing HAART regimens in the presence or absence of concomitant stavudine, and which included an assessment of investigator-defined lipodystrophy, lipodystrophy was reported at 60 weeks of follow-up in 0.4% and 6% of patients randomised to emtricitabine/didanosine/efavirenz or stavudine/didanosine/efavirenz, respectively\textsuperscript{23}. Objective measurements of body composition were not reported in the latter trial.

In spite of the significant differences in baseline limb fat between arms (being greatest in the NFV/ddI/D4T arm), using DEXA, a significant decrease in the median percent change in leg fat from baseline was only observed in the NFV/ddI/D4T arm, but not in either of the other two didanosine-containing arms. In the stavudine-containing arm the typical loss of limb fat as reported by numerous other studies was observed, despite the fact that five of eight patients in this treatment arm had discontinued stavudine after a median of 8.5 months. Of note, an aggravating effect on limb fat loss from the nelfinavir used in this arm of the trial cannot be ruled out\textsuperscript{11,12}. These results in view of the limited sample size should be interpreted with caution, but do seem to support the notion that first time initiation of antiretroviral therapy with regimens including didanosine but not stavudine possibly may reduce the risk of limb fat loss. Interestingly, whereas a continued gain in limb fat was observed when the stavudine-sparing, but didanosine-containing regimen included the non-
nucleoside reverse transcriptase inhibitor nevirapine as the “third” agent, some degree of limb fat loss was seen in the second year of treatment when the regimen included ritonavir-boosted saquinavir. This may suggest that ritonavir-boosted saquinavir might have contributed to loss of limb fat, in a manner similar to what has been reported for nelfinavir.\textsuperscript{11,12} As in other studies in our study there was a modest increase in truncal fat.\textsuperscript{11-13,29}

The good virological and immunological responses to antiretroviral therapy in the three arms (Table 3.2) suggests that the patients were adherent to their therapy and that the different change in fat distribution is not due to difference in treatment adherence.

Current guidelines for the treatment of adult HIV-1 infection\textsuperscript{25,26} list the combination of didanosine plus lamivudine or emtricitabine as backbone of initial treatment regimens rightfully only as an alternative, given the limited number of studies which have been performed with these particular backbones.\textsuperscript{23,30} Concerns with regard to long term complications of treatment and notably lipoatrophy of course are only one of the many factors which need to be considered when choosing between the different available options for the initial treatment of HIV-1 infection. Our findings, in spite of the limited number of patients we were able to study, nevertheless do provide a degree of reassurance that, when the use of didanosine plus lamivudine or emtricitabine for whatever reason is being considered as the NRTI backbone for a patient’s initial antiretroviral therapy regimen, the risk of lipoatrophy development is likely to be limited.

Conflict of interest and sponsorship

Prof. dr. P. Reiss discloses having received grants or honoraria for speaking engagements or advisorship from Boehringer Ingelheim, Bristol-Myers Squibb, Gilead Sciences, GlaxoSmithKline, Merck, Roche, Theratechnologies and Tibotec. Prof. dr. J.M.A. Lange discloses having received grants or honoraria for speaking engagements or advisorship from Abbott, Bayer, Boehringer Ingelheim, Bristol-Myers Squibb, GlaxoSmithKline, Merck, Roche, Agouron/Pfizer, Schering-Plough, Shire and Virco/Tibotec. The ARES study was an investigator-initiated trial sponsored by the National AIDS Therapy Evaluation Centre (NATEC) funded by the Netherlands Ministry of Health, Welfare and Sport, with additional financial support from Boehringer Ingelheim, Bristol-Myers Squibb and Roche Pharmaceuticals, in The Netherlands. These pharmaceutical companies had no influence on the design, analysis and reporting of the study results.
Chapter 3

References


Chapter 4

Is the male genital tract really a sanctuary site for HIV?
Arguments that it is not

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Chapter 4

Introduction

One concern with current antiretroviral therapy for infection with the human immunodeficiency virus (HIV) is that certain anatomical sites in the body are considered "sanctuary sites". What is commonly meant is that these sites are less permeable to antiretroviral drugs, allowing the selection and/or development of drug-resistant strains, and thereby representing an important source of virological failure of therapy\textsuperscript{1-8}. The central nervous system is considered such a sanctuary site\textsuperscript{4,9-11}, and because of the so-called "blood-testis barrier" (BTB), the male genital tract (MGT) is also considered to be such a site\textsuperscript{1-8,12,13}. This assumption is very important in view of the sexual transmission of drug-resistant HIV type 1 (HIV-1), which is rapidly increasing in some areas of the world\textsuperscript{14-21}. Following a review of the literature, however, we oppose the opinion that the MGT is a sanctuary site for HIV.

Definition of viral compartment, reservoir and sanctuary site

The terms “compartment”, “reservoir” and “sanctuary site” are not well defined and used interchangeably\textsuperscript{1-8}. With respect to the MGT, definitions can be proposed on the basis of virology, pharmacology, immunology and reproductive biology. We use the following definitions here: A \textbf{viral compartment} is an anatomical site in which the virus in untreated patients evolves distinctively from other anatomical sites or the main pool of infected cells, because of differences between the major cell types sustaining viral replication\textsuperscript{2}. A \textbf{viral reservoir} is a cell type or anatomical site in which a replication-competent virus persists much longer than in the main pool of infected cells that sustain the infection, and this cell type or anatomical site can replenish the pool of infected cells\textsuperscript{7,8}. The main cellular viral reservoir is the pool of latently infected resting CD4\textsuperscript{+} T cells\textsuperscript{8}. A \textbf{viral sanctuary site} is an anatomical site that is highly impermeable to (some) antiretroviral drugs, and in which viral replication continues during treatment, thus allowing development or selection of drug-resistant strains\textsuperscript{1-8}.

The misleading concept of the blood-testis-barrier

Conclusions about HIV in the MGT are mainly based on an analysis of semen, 90% of which consists of plasma and 10% of cells. Seminal plasma derives mainly from the seminal vesicles (60%) and prostate glands (30%), and therefore reflects fluids distal to the vas deferens (\textbf{Appendix 4.1})\textsuperscript{22-28}. 

80
HIV-1-RNA in seminal plasma derives mainly from the distal part of the MGT, which is supported by the finding that vasectomy has no effect on the seminal plasma HIV-1-RNA concentration\textsuperscript{26}, and that proper target cells for HIV in the seminiferous tubules are absent\textsuperscript{22-24,30,31}. Approximately 90\% of leukocytes in semen are derived from the epididymis\textsuperscript{12,32-34}.

It is well known that many antiretroviral drugs do not reach adequate concentrations in seminal plasma and as a consequence no truly highly active antiretroviral therapy is achieved within the lumen of the MGT with many regimens (\textit{Table 4.1})\textsuperscript{2-5,7,35,36}. Drugs with a protein-binding of less than 90\% are the ones with a moderate to good concentration in the seminal plasma, suggesting that diffusion is the primary driving force for drug transport to the lumen of the MGT (\textit{Table 4.1})\textsuperscript{2-3}. However, active cellular drug transport, e.g. by P-glycoprotein, could also be important\textsuperscript{3,37-40}.

On the basis of histological and physiological studies, and the molecular weight of free and protein-bound antiretroviral drugs, it is unlikely that there is an important endothelial barrier to the current antiretroviral drugs in the testicular interstitial tissue (\textit{Figure 4.1}, and \textit{Table 4.1})\textsuperscript{23,41-52}. Therefore, opposing the suggestion by others\textsuperscript{42,43}, we argue that the testicular extraluminal tissue is not a drug-deprived area and sanctuary site.

The blood-testis-barrier (BTB), which is formed by the continuous layer of abluminally located Sertoli cells and the tight junctions between them (\textit{Figure 4.1})\textsuperscript{22-26,53,54}, is relevant for drug concentrations within the lumen of the seminiferous tubules because tight junctions hamper the intercellular diffusion of hydrophilic solutes at least a hundred times more than other intercellular junctions\textsuperscript{45}. The BTB is located only in the seminiferous tubules, which is just a fraction of the total length of the MGT of about six meters (\textit{Figure 4.1} and \textit{Appendix 4.1})\textsuperscript{22-26}. Also about 90\% of the seminiferous tubule fluid is resorbed in the epididymis\textsuperscript{23-25}. Therefore the BTB is not an explanation for the subtherapeutic drug concentrations in seminal plasma. HIV particles can get into the lumen of the seminiferous tubules, possibly through transcytosis\textsuperscript{55-68}. However, within the normal seminiferous tubule leukocytes, the target cells for HIV, appear to be absent\textsuperscript{22-24,30,31}. Therefore, antiretroviral drug concentrations “behind” the BTB are not relevant and as a consequence the lumen of the seminiferous tubules normally cannot be a sanctuary site for HIV. In the advanced stage of HIV infection histological changes in the seminiferous tubules occur and this may affect the BTB\textsuperscript{13,57,66,69,70}. 
Table 4.1 Characteristics and concentrations in blood and semen plasma of licensed antiretroviral drugs.

<table>
<thead>
<tr>
<th>Drug Type</th>
<th>Drug Name</th>
<th>MW * (Dalton)</th>
<th>L, H</th>
<th>Blood plasma</th>
<th>Semen plasma</th>
<th>Semen / blood plasma ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Protein Binding (%)</td>
<td>C max dos (µg/mL)</td>
<td>C min dos (µg/mL)</td>
</tr>
<tr>
<td>Nucleoside / -tide</td>
<td>Abacavir</td>
<td>NA</td>
<td>670.8</td>
<td>L [2]</td>
<td>50 *</td>
<td>2.87 [172]</td>
</tr>
<tr>
<td></td>
<td>Emtricitabine</td>
<td>NA</td>
<td>247.2</td>
<td>ND</td>
<td>&lt;4 *</td>
<td>1.8 *</td>
</tr>
<tr>
<td></td>
<td>Lamivudine</td>
<td>NA</td>
<td>229.3</td>
<td>H [2]</td>
<td>16-36 *</td>
<td>1.5 *</td>
</tr>
<tr>
<td></td>
<td>Stavudine</td>
<td>NA</td>
<td>224.2</td>
<td>H [2]</td>
<td>Negligible *</td>
<td>0.7-2.0 [174]</td>
</tr>
<tr>
<td></td>
<td>Zalcitabine</td>
<td>NA</td>
<td>211.2</td>
<td>H [2]</td>
<td>&lt;4 [2]</td>
<td>0.03 *</td>
</tr>
<tr>
<td></td>
<td>Zidovudine</td>
<td>NA</td>
<td>267.2</td>
<td>L [2]</td>
<td>&lt;38 *</td>
<td>1.8 *</td>
</tr>
<tr>
<td></td>
<td>Efavirenz</td>
<td>1.0</td>
<td>315.7</td>
<td>L [2]</td>
<td>99.5-99.75 *</td>
<td>12.9 *</td>
</tr>
<tr>
<td></td>
<td>Nevirapine</td>
<td>3.4</td>
<td>266.3</td>
<td>L [2]</td>
<td>60 [2]</td>
<td>5.74-6.69 [181]</td>
</tr>
<tr>
<td>Protease inhibitor</td>
<td>Amprenavir</td>
<td>0.4/1.2</td>
<td>505.6</td>
<td>L [2]</td>
<td>90 *</td>
<td>5.36 *</td>
</tr>
<tr>
<td></td>
<td>Atazanavir</td>
<td>ND</td>
<td>704.9</td>
<td>ND</td>
<td>80 *</td>
<td>3.15 *</td>
</tr>
<tr>
<td></td>
<td>Indinavir</td>
<td>0.10</td>
<td>711.9</td>
<td>H [2]</td>
<td>60 [2]</td>
<td>8.97 *</td>
</tr>
<tr>
<td></td>
<td>Lopinavir</td>
<td>1.0/4.0</td>
<td>628.8</td>
<td>ND</td>
<td>98-99 *</td>
<td>9.6 *</td>
</tr>
<tr>
<td></td>
<td>Nelfinavir</td>
<td>0.8</td>
<td>663.9</td>
<td>L [2]</td>
<td>99 [2]</td>
<td>4.0 *</td>
</tr>
<tr>
<td></td>
<td>Ritonavir</td>
<td>2.1</td>
<td>721.0</td>
<td>L [2]</td>
<td>98 [2]</td>
<td>11.2 ± 3.6 *</td>
</tr>
<tr>
<td></td>
<td>Saquinavir sgc</td>
<td>0.10</td>
<td>670.9</td>
<td>L [2]</td>
<td>&gt;97 *</td>
<td>1.0-2.2 *</td>
</tr>
<tr>
<td></td>
<td>Saquinavir hgc</td>
<td>0.10</td>
<td>670.9</td>
<td>L [2]</td>
<td>98 *</td>
<td>0.25 *</td>
</tr>
<tr>
<td>Fusion inhibitor</td>
<td>Enfuvirtide</td>
<td>ND</td>
<td>4,492</td>
<td>ND</td>
<td>92 *</td>
<td>4.59 *</td>
</tr>
</tbody>
</table>

Notes: * According to pharmaceutical companies' product information. MW, molecular weight; C max dos, maximal concentration during leg artis dosing interval; C min dos, minimal concentration during leg artis dosing interval; Eff Cmin, Effective minimal concentration, considered required for effective virus suppression. RTI, Reverse Transcriptase Inhibitor. H, hydrophilic; L, lipophylic; NA, not applicable. ND, no data. [ ] = references
Arguments against sanctuary site

Figure 4.1  
Microscopic anatomy of the seminiferous tubules.  
The blood capillaries are within the interstitium. The blood-testis-barrier is located in the seminiferous tubule between the Sertoli cells. Reprinted from “Principles of Anatomy and Physiology”, 9nd edition, Gerard J. Tortora and Sandra Reynolds Grabowski, editors. Chapter 28 “The Reproductive Systems”, page 981, © 2000, with permission from John Wiley & Sons, Inc.

Virological counter-arguments

Despite (expected) subtherapeutic seminal plasma drug concentrations, longitudinal\textsuperscript{71-94} and cross-sectional\textsuperscript{71-74,90,95-114} studies have shown that the virological response (HIV-1-RNA concentration) in seminal plasma parallels
that in blood plasma, and that the more suppressive the regimen, the better and more sustained the response. Such studies also show that compared to blood plasma, the median HIV-1-RNA concentration in the seminal plasma on average is about 10-fold lower. Nevertheless, there are some individuals in whom the HIV-1-RNA concentration in the seminal plasma is higher than in the blood plasma. This divergence is difficult to interpret in the absence of details concerning an individual’s stage of HIV infection and details on asymptomatic local infection/inflammation (which may have a prevalence of 8%\textsuperscript{114}) or leukocytospermia (idiopathic semen leukocytosis), which might influence local HIV production\textsuperscript{73,94,104,106,114,115}.

With properly suppressive antiretroviral therapy the concentration of HIV-1-DNA in peripheral blood mononuclear cells (PBMCs) and in non-spermatozoal seminal mononuclear cells (NSMCs) decreases, and it can drop to below the limit of detection in NSMCs, this in contrast to in PBMCs\textsuperscript{76,102,109,116-120}. Furthermore, HIV 2-long terminal repeat (LTR) circular-DNA is absent in NSMCs when HIV is properly suppressed\textsuperscript{99}. This absence indicates the arrest of viral replication\textsuperscript{121-123}. The combination of absence of HIV 2-LTR circular DNA with the presence of proviral-DNA\textsuperscript{99} suggests that infected cells in semen derive from blood, but become inactivated and unable to sustain HIV replication within the lumen of the MGT.

Studies on HIV-1 drug-related mutations have demonstrated that the mutations in blood and semen are homologous\textsuperscript{75,82,84,95-97,104,109,112,124-126}. Only in three studies did the mutations appear first in semen, in five out of 46 patients\textsuperscript{95-97,104,124}. In one study\textsuperscript{104} this was related to an (asymptomatic) local infection/inflammation, reflecting more virus replication during inflammation. Clearly, drug-resistant variants only rarely appear first in the semen compared with blood and (asymptomatic) local inflammation/infection might be a very important factor for emergence and transmission of drug resistant virus.

Divergent envelope evolution of HIV is not a phenomenon unique for the MGT, and probably merely reflects an adaptation to local circumstances\textsuperscript{101,127-133}. Even between the seminal plasma and seminal leukocytes phylogenetic differences have been demonstrated\textsuperscript{134}. As different genes are involved, envelope changes (compartmentalization) must not be made synonymous with the development of drug-resistance mutations\textsuperscript{75}.
Immunological counter-arguments

Under normal conditions no leukocytes are found in the lumen and epithelium of the seminiferous tubules, which is understandable given the immunogenicity of the process of spermatogenesis. The ampulla and tail of the epididymis (Appendix 4.1) are the sites where the spermatozoa are stored before ejaculation. As spermatozoa are very immunogenic, leukocytes have to become anergic. Several immunosuppressive mechanisms may play a role: the likely acidity of the epididymal and prostatic fluid; a lack of co-receptor B7 expression and presence of transforming growth factor beta and complement inhibitors; the preponderance of CD8+ (suppressor) T-lymphocytes over CD4+ (helper) T-lymphocytes within the luminal lining cells from the rete testis up to and including the prostate glands and seminal vesicles; possibly also a preponderance of CD8+ over CD4+ T-lymphocytes in semen; the presence of HIV-1-specific cytotoxic T-lymphocytes; the likely high expression of Fas ligand on spermatozoa, thus inducing the apoptosis of cells such as activated lymphocytes; and the strong immunosuppressive activity of seminal plasma and its components spermine and prostaglandins.

The consequence of an immunosuppressive state within the lumen and luminal lining cells of the MGT, especially in the epididymis, is that the local CD4+ cells are infected less easily and produce little or no HIV. The immunosuppressive state also implies a high barrier for the development of drug resistance, which is compatible with the finding that drug-resistant mutations only rarely appear first in the semen.

Hypothesis concerning the source of HIV in semen

The finding that vasectomy has no effect on the seminal plasma HIV-1-RNA concentration, the virological response to therapy in seminal plasma, and the immunological counter-arguments all indicate that it is not the seminal leukocytes, but rather the luminal lining cells and/or the blood/extraluminal tissue that are the source of HIV-1-RNA in seminal plasma (spillover). These two sources are fully exposed to antiretroviral drugs.

The size of the HIV particle could be an argument against spillover. However, leukocytes also cross cell membranes. Furthermore, some data suggest that residing tissue macrophages could also account for HIV in the seminal plasma, because these cells can have lumen-reaching dendritic processes.
A likely consequence of spillover could be that transmission of HIV and drug-resistant variants via semen does not occur in case of proper viral suppression in blood plasma (≤50 c/mL). An exception is possibly a minority of patients in whom circumstances exist that may be associated with (more) local HIV production. Relevant factors in this respect are (a)symptomatic local inflammation/infection, leukocytospermia and advanced-stage HIV infection. Treatment of local inflammation/infection could be an important measure for preventing development and transmission through semen of drug-resistant virus.

In conclusion, the HIV-1-RNA concentration gradient between blood plasma and seminal plasma, the parallel virological response to therapy in blood plasma and seminal plasma, including the decrease of proviral DNA and the absence of HIV 2-LTR circular DNA in NSMCs, vasectomy not influencing the seminal plasma HIV-1-RNA concentration, and the intraluminal immunosuppressive state are all strong arguments against intraluminal HIV production in the MGT. We therefore argue that the source of HIV in the semen is very likely outside the lumen of the MGT, i.e. spillover from luminal lining cells and/or the blood/extraluminal tissue, which are fully exposed to the antiretroviral drugs.

With respect to the definitions of viral compartment, reservoir and sanctuary site, the seminiferous tubule is neither a virological compartment nor a sanctuary site for HIV-1, because of the absence of appropriate target cells. The remainder of the lumen of the MGT is a viral compartment and reservoir for HIV-1 since proviral-DNA containing leukocytes are present, but this part is also not a virological sanctuary site because under normal circumstances replication is probably arrested as a result of the local immunosuppressive state.

There is a minority of patients in whom local factors are present that result in (more) HIV production within the lumen of the MGT, and thereby they are probably more prone to the development of drug-resistant HIV in the MGT. Relevant characteristics of these individuals are possibly semen leukocytosis and advanced stage of HIV infection. This warrants the aggressive screening for and treatment of sexually transmitted infections and local inflammation in these patients warranted.
Appendix 4.1

Schematic diagram of the male genital tract

From proximal to distal the lumen of the male genital tract is formed by the following structures: seminiferous tubules, straight tubules (tubuli recti), rete testis, efferent ductules (ductuli efferentes), epididymis, vas deferens (including ampulla and ejaculatory duct), seminal vesicle, prostate, bulbo-urethral gland, urethral gland (not in diagram) and urethra. Spermatogenesis occurs in the seminiferous tubules. In the ampulla and the (tail of the) epididymis the spermatozoa are stored. In the epididymis 90% of the fluid derived from the proximal part of the MGT is resorbed. During ejaculation consecutively fluids are added from the bulbo-urethral and urethral gland, the prostate and seminal vesicle. Only in the seminiferous tubules between the Sertoli cells tight junctions are present (blood-testis barrier). Reprinted from “Human Histology”, 2nd edition, Alan Stevens and James S. Lowe, editors, chapter 16 “Male reproductive system”, page 309, © 1997, with permission from Elsevier Science.
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No virological failure in semen during properly suppressive antiretroviral therapy despite subtherapeutic local drug concentrations

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Chapter 5

Abstract

Purpose
The aim of the study was to investigate whether drug resistance occurs earlier in seminal than in blood plasma with the use of such HAART regimens, of which only the two NRTIs achieve therapeutic concentrations in seminal plasma.

Method
Seminal and blood plasma of 12 patients, for 48-96 weeks on suppressive first-line therapy with saquinavir/ritonavir/didanosine/lamivudine, nelfinavir/didanosine/stavudine, or efavirenz/lamivudine/zidovudine, were prospectively evaluated for HIV-1-RNA resistance mutations and drug concentrations.

Results
Saquinavir, nelfinavir and efavirenz blood plasma concentrations were in the therapeutic range. Nelfinavir and efavirenz seminal plasma concentrations were below the limit of quantification. In only 2 of 9 seminal plasma samples, from 1 of 6 patients, the saquinavir concentration was above the minimum therapeutic level. The seminal plasma HIV-1-RNA concentration remained undetectable in all patients up to 96 weeks, and therefore drug resistance could not be demonstrated. Thus, despite suboptimal local drug concentrations no virological failure occurred in seminal plasma after prolonged first-line HAART.

Conclusion
This finding supports the hypothesis that the source of HIV in semen is a spillover from the blood/extraluminal tissue and that therefore seminal plasma drug levels may not be critical for viral suppression within the lumen of the male genital tract.
Introduction

Not all antiretroviral drugs penetrate well into the seminal plasma\(^1,2\). Theoretically, the resulting subtherapeutic drug concentrations within the lumen of the male genital tract (MGT) carry the risk for virological failure and emergence of drug resistant human immunodeficiency virus type 1 (HIV-1), especially when drugs with a low genetic barrier for resistance, such as lamivudine, efavirenz and nelfinavir, are components of the antiretroviral regimen.

During an ongoing randomised, prospective triple-arm study on first line antiretroviral therapy, the Antiretroviral Regimen Evaluation Study (ARES)\(^3\), we initiated a study on HIV in semen. In two of the arms of the ARES study, patients were treated with saquinavir/ritonavir/didanosine/lamivudine or nelfinavir/didanosine/stavudine. In extension of this, patients starting efavirenz and Combivir\(^\circledR\) (lamivudine 150 mg and zidovudine 300 mg) were also prospectively evaluated.

The aim of the study was to investigate whether drug resistance occurs earlier in seminal than in blood plasma with the use of HAART regimens of which only the two nucleoside analogue reverse transcriptase inhibitors (NRTIs) are likely to achieve adequate drug concentrations in the seminal plasma\(^1,2\). Earlier studies with treatment with one or two NRTIs only have demonstrated that the HIV-1-RNA concentration in seminal plasma remained detectable in most cases and is associated with drug resistance mutations\(^1,4-7\).

Method

Two groups of antiretroviral therapy-naïve HIV-1-infected patients were included in this study. One group consisted of patients who were participating in the ARES study and were randomised to treatment with either saquinavir/ritonavir/didanosine/lamivudine or nelfinavir/didanosine/stavudine and, the second group consisted of patients who started antiretroviral therapy with efavirenz and Combivir\(^\circledR\). For the present study, the protocol of the ARES study was amended when all patients had passed their ARES week 36 visit and thus semen was obtained from week 48 onwards. For patients starting efavirenz and Combivir\(^\circledR\) the same schedule for semen collection was used. Missing baseline semen samples were considered not to be crucial, because the aim of the study was to look for virological failure and genotypic resistance after long-term therapy. Vasectomy and a genitourinary tract infection (assessed by medical history, urinalysis and Chlamydia trachomatis light chain reaction assay in urine) within 8 weeks before semen collection were exclusion criteria. Patients gave their written informed consent before inclusion in this study.
Twelve patients who started one of the following regimens were included: (A) saquinavir soft-gelatin capsule formulation 1,600 mg, ritonavir 100 mg, didanosine 400 mg, and lamivudine 300 mg, all given once daily (n = 8), (B) nelfinavir 1,250 mg and stavudine 40 mg, both given twice daily, and didanosine 400 mg once daily (n = 2), or (C) efavirenz 600 mg once daily and Combivir® one tablet twice daily (n = 2). In all 12 included patients, the initial antiretroviral regimen remained unchanged during the follow up of 48-96 weeks.

For regimens A and B, blood plasma was collected for HIV-1-RNA measurement at week 0, 2, 4, 8, 12, 16, 20, 24 and every 12 weeks thereafter; for saquinavir and nelfinavir concentrations, blood plasma was collected similarly except at weeks 0, 2, 16 and 20. For regimen C HIV-1-RNA measurement was done at baseline, after one and three months of therapy, and thereafter every three months. Blood plasma drug levels for efavirenz were performed at the request of the treating physician. The difference between regimen A and B and regimen C in time points of collection of blood samples was due to the fact that regimens A and B were part of the ARES study and sample collection was more intensive in this study.

Collection of semen was done at week 48, 72, 84 and 96 of therapy. For regimen C only samples at week 48 and 72 could be obtained. Semen was obtained by masturbation and within two to four hours it was centrifuged at 1200 g for ten minutes. The supernatant was stored at -70°C until analysis was done. Blood samples for HIV-1-RNA measurement were obtained within two weeks of semen collection. We allowed this margin of two weeks to enhance patient cooperation.

The HIV-1-RNA concentration in blood plasma was measured using the Amplicor HIV-1 Monitor 1.5 assay (Roche Diagnostics; limit of detection [LoD] 50 copies/mL) in the patients who commenced regimen A or B and the Versant HIV-1-RNA 3.0 (bDNA) assay (Bayer Corporation; LoD 50 copies/mL) in patients who were using regimen C. The HIV-1-RNA concentration in seminal plasma was measured using the NucliSens HIV-1-RNA assay (bioMérieux) with an input of 200 µL seminal plasma and an ultrasensitive protocol adaptation resulting in a LoD of 50 copies/mL. If the result of the ultrasensitive assay was invalid, for example, due to inhibitors in the seminal plasma, the standard NucliSens HIV-1-RNA assay with a LoD of 400 copies/mL was used.

HIV-1-RNA detection in seminal plasma using the NucliSens assay has been evaluated in our laboratory. Dilution series of HIV-1-RNA were added to seminal plasma and analysed with this assay. The detection of HIV-1-RNA in seminal plasma appeared to be accurate (unpublished results).
Because of the limited volume in seminal plasma, only drug concentrations of saquinavir, nelfinavir and efavirenz were measured; these drugs were reported to have subtherapeutic concentrations in seminal plasma\textsuperscript{1,2,9}. Saquinavir, nelfinavir, the nelfinavir active metabolite hydroxy-t-butylamide (M8) and efavirenz concentrations in blood and seminal plasma were measured by previously described high-performance liquid chromatography methods\textsuperscript{10,11}. The lower limit of quantification for saquinavir, nelfinavir, and M8 was 0.054 mg/L and for efavirenz 0.24 mg/L. The collection of the blood plasma samples was not in parallel with the collection of semen. Therefore a seminal to blood plasma drug ratio could not be calculated.

Results

Twelve patients could be included in the study. Their baseline characteristics are given in Table 5.1. After the start of antiretroviral treatment, the patients’ blood plasma HIV-1-RNA concentration became undetectable (<50 copies/mL) for the first time at a median of 111 days. Some patients had virological "blips" during follow-up.

Table 5.1 Characteristics of the 12 patients at start of their antiretroviral regimen.

<table>
<thead>
<tr>
<th>Median age</th>
<th>40 years</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-1 CDC category (no. of patients)</td>
<td></td>
</tr>
<tr>
<td>A2</td>
<td>4</td>
</tr>
<tr>
<td>A3</td>
<td>3</td>
</tr>
<tr>
<td>B3</td>
<td>3</td>
</tr>
<tr>
<td>C3</td>
<td>2</td>
</tr>
<tr>
<td>Median CD4 count (range)</td>
<td>170 cells/µL (80-460)</td>
</tr>
<tr>
<td>Median bp HIV-1 RNA concentration (range)</td>
<td>123,500 c/mL (700-530,000)</td>
</tr>
<tr>
<td>Antiretroviral regimen (no. of patients)</td>
<td></td>
</tr>
<tr>
<td>saquinavir/ritonavir/didanosine/lamivudine</td>
<td>8</td>
</tr>
<tr>
<td>nelfinavir/didanosine/stavudine</td>
<td>2</td>
</tr>
<tr>
<td>efavirenz/lamivudine/zidovudine</td>
<td>2</td>
</tr>
</tbody>
</table>

Note: CDC=Centers for Disease Control and Prevention (classification system 1993); bp=blood plasma; c=copies.

In Table 5.2 the results of seminal and blood plasma HIV-1-RNA concentrations and seminal plasma drug concentrations for each patient are given. Patients could not deliver a sufficient volume of semen at all time points. Twenty-two semen samples were analysed for seminal plasma HIV-1-RNA concentration. At the moment of evaluation of the seminal plasma, the HIV-1-RNA concentration in blood plasma was undetectable or was <50 copies/mL in all patients except one. In 4 semen samples from 4 patients (2 at 72 weeks and 2 at 96 weeks and all using regimen A) the test result in the ultrasensitive assay was invalid because of inhibitors, but in the assay with a
Chapter 5

Table 5.2 Results of seminal and blood plasma HIV-1-RNA concentration and seminal plasma antiretroviral drug concentration for each patient by week of sampling since start of antiretroviral therapy.

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Regimen</th>
<th>SP/BP</th>
<th>SP HIV-1-RNA (copies/mL)</th>
<th>BP HIV-1-RNA (copies/mL)</th>
<th>SP [SQV] (µg/mL)</th>
<th>SP [NFV] (µg/mL)</th>
<th>SP [M8] (µg/mL)</th>
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Note: Regimen A=saquinvir/ritonavir/didanosine/lamivudine; regimen B=nelfinavir/didanosine/stavudine; regimen C=efavirenz/lamivudine/zidovudine; SP=seminal plasma; BP=blood plasma; [SQV]=saquinavir concentration; [NFV]=nelfinavir concentration; [M8]=nelfinavir hydroxy-l-butyramide concentration; [EFV]=efavirenz concentration; NP=not possible. *Result in standard NucliSens assay because the result in the ultrasensitive assay was invalid due to inhibitors.

LoD of 400 copies/mL the seminal plasma HIV-1-RNA concentration was undetectable. Drug resistance testing could not be performed, because the HIV-1-RNA concentration in seminal plasma was below the limit of detection in all cases.

The minimum effective blood plasma drug concentration (MEC) is 0.1, 0.8 and 1.0 mg/L for saquinavir, nelfinavir, and efavirenz, respectively<sup>3</sup>. There were 74, 21, and 10 blood plasma samples that were tested for saquinavir, nelfinavir and efavirenz levels, respectively. The median (range) drug level was 1.2 (0.0-9.6), 2.7 (0.4-4.5) and 1.3 (1.1-3.7) mg/L, respectively, and the median (range) interval between drug intake and blood collection was 3.5 (0.25-48.75), 0.75 (0-5.5) and 14 (12.5-17.0) hours, respectively.

In 17 seminal plasma samples, sufficient volume was available for a drug concentration measurement. The median (range) interval between drug intake
No virological failure in semen

and semen collection was 15.75 (0.5-23), 9.75 (8.25-10.5) and 11.25 (10.25-12.5) hours for saquinavir, nelfinavir, and efavirenz, respectively. In 13 of the 17 samples, the drug levels were well below the MEC as reported for blood plasma. In the remaining four seminal plasma samples, from two patients on regimen A, the seminal plasma concentration of saquinavir in one patient was 0.1 and 0.1 mg/L, 4 and 6 hours after drug intake, respectively, and in the other patient it was 0.59 and 1.11 mg/L, 21.5 and 22 hours after drug intake, respectively. The median (range) blood plasma saquinavir concentrations in these two patients were 1.6 (0.1-4.2) and 0.2 (0.0-2.3) mg/L, respectively, at a median (range) of 1.5 (0.25-12) and 23.5 (0.25-26) hours after drug intake.

Discussion

This is the first prospective study of HIV-1-RNA and antiretroviral drug concentrations in seminal plasma with a follow-up of 1-2 years. The main feature of the patients in this study was that their blood plasma HIV-1-RNA concentration was <50 copies/mL for a prolonged period. As far as data are available, abacavir, didanosine, lamivudine, stavudine, zidovudine, nevirapine, amprenavir and indinavir reach a moderate to good concentration in the seminal plasma. Lopinavir, nelfinavir, ritonavir, saquinavir and efavirenz do not reach therapeutic concentrations in seminal plasma. Therefore, it is assumed that in our patients only lamivudine plus either didanosine, stavudine, or zidovudine could reach a therapeutic concentration in seminal plasma. Indeed, nelfinavir and efavirenz seminal plasma concentrations were all below the MEC as reported for blood plasma, and in only two of nine samples (from one out of six patients) a therapeutic concentration of saquinavir was achieved. Despite these subtherapeutic seminal plasma drug levels, no virological failure occurred in seminal plasma over a period of 48-96 weeks.

The long interval between drug intake and semen collection might be a cause for the low seminal plasma saquinavir and nelfinavir levels. However, for saquinavir, low seminal plasma concentrations have been demonstrated also at shorter intervals (0-12 hours post dosing). For nelfinavir, seminal plasma concentrations have previously only been evaluated twelve hours postdosing; in this study, the levels were also very low. Further, these results and our data make clear that seminal plasma concentrations of these drugs are low during large parts of the dosing interval. Efavirenz has a long blood plasma half-life and therefore no large fluctuation in its seminal plasma concentration are expected.

The seminal plasma HIV-1-RNA concentration is in general about 1log\(_{10}\) lower compared to the blood plasma HIV-1-RNA. Thus, although baseline seminal
plasma HIV-1-RNA concentrations are lacking in our study, given the fact that
the median baseline blood plasma HIV-1-RNA was 123,000 copies/mL, it is
likely that baseline seminal HIV-1-RNA was above the LoD of 50 copies/mL in
most of our patients.

It is very well known that virological failure and emergence of drug resistance
rapidly occur if antiretroviral therapy consists of one or two NRTIs only.
Especially against lamivudine, which was part of two of the HAART regimens
used in this study, resistance occurs within a few weeks\textsuperscript{15,16}. Therefore, if
effective concentrations within the lumen of the MGT of all three drugs are
needed for viral suppression, virological failure and emergence of viral
resistance within the MGT would have been expected in our patients after 1-2
years of HAART. Despite suboptimal local drug concentrations, no virological
failure occurred in seminal plasma after prolonged first-line HAART. To explain
these results, one could argue that replication kinetics and target cells are
different in the MGT. In the brain, HIV replication was clearly suppressed
during the zidovudine-monotherapy period shown by decrease in HIV-dementia
in the early 1990s as well as by cerebral spinal fluid data\textsuperscript{17}. However, studies
with treatment with one or two NRTIs only have demonstrated that in seminal
plasma the HIV-1-RNA concentration remained detectable in most cases and is
associated with drug resistance mutations\textsuperscript{4-7}, and target cells other then CD4+
cells have not been clearly identified within the MGT\textsuperscript{18}.

The findings of the present study are therefore not compatible with the MGT
being a sanctuary site. Although differences in the HIV envelope between
blood and semen (and other body compartments) have been demonstrated in
untreated patients, changes in the HIV envelope (encoded by the env gene)
could merely be an adaptation to local (body compartment) conditions and
therefore do not necessarily correlate with evolution of drug resistance, which
is encoded by the pol gene\textsuperscript{1}. Several studies have demonstrated that when the
seminal plasma HIV-1-RNA concentration is below the LoD during HAART, HIV
can still be cultured from semen\textsuperscript{19,20}. This does not necessarily mean that there
is ongoing (cryptic) viral replication within the lumen of the MGT. The presence
of HIV in seminal plasma can also be explained by the presence of latently
infected resting memory CD4+ T cells or from spillover from the blood. Using
2-LTR circular-DNA to monitor cryptic HIV-1 infection, a recent study also
suggested that cryptic HIV-1 infection may not occur in the MGT during
effective HAART\textsuperscript{21,22}. Recently we have hypothesized that the source of HIV in
seminal plasma is from outside the lumen of the MGT, that the MGT is likely
not a sanctuary site for HIV, and that antiretroviral drug levels within the lumen
of the MGT may not be critical for local viral suppression\textsuperscript{1}. The results of the
present study support this hypothesis, but larger prospective studies are
needed to verify our findings. Especially the studies with protease-mono maintenance that are currently being conducted with lopinavir/ritonavir in several centres might give additional insight in HIV replication in the MGT.

Acknowledgment

Thanks to Nicolette Hulshoff and Vincent Bekker for their technical assistance. There are no potential conflicts of interest of the authors. A number of the reported patients were enrolled in the ARES study, which was an investigator-initiated trial sponsored by the Dutch National AIDS Therapy Evaluation Centre (NATEC), funded by the Netherlands Ministry of Health, Welfare and Sports, with additional financial support from Boehringer Ingelheim, Bristol-Myers Squibb and Roche Pharmaceuticals, in The Netherlands. These pharmaceutical companies had no influence on the design, analysis and reporting of the study.
Chapter 5

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Chapter 6

Activated lymphocytes in semen of HIV-1 positive and HIV negative men

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Submitted for publication
Chapter 6

Abstract

Background
With respect to the human immunodeficiency virus (HIV) two phenomena characterize a viral sanctuary site: absent or very low local drug concentrations and the presence of susceptible and virus replicating and producing cells. In the male genital tract (MGT), often considered to be a sanctuary site, low or absent drug concentrations have indeed been described. It is currently unknown however, whether the MGT also contains HIV-susceptible cells.

Objective
To determine the presence of HIV-susceptible cells in paired blood and semen samples of eight HIV-1/2 negative men, eight HIV-1 positive men without HAART and eight men on HAART.

Method
Seminal lymphocytes and blood lymphocytes were stained with monoclonal antibodies against CD45, HLAdr and CD38, and analysed using flow cytometry. Cells that were positive for all these markers were considered to be susceptible to HIV infection.

Results
In all cases we found activated lymphocytes in semen. The number of these cells was extremely low and the percentage of activated cells was non-significantly lower in semen than in blood. The percentage of activated lymphocytes in blood was 10% (IQR 8-14) in HIV-negative men, 17% (IQR 11-20) in HIV-positive men on HAART and 25% (IQR 20-27) in untreated HIV-positive men (p < 0.0001, ANOVA).

Conclusion
Although present in low numbers, seminal lymphocytes appear to be activated. Whether these cells are present at sites in the MGT that are exposed to inadequate drug levels and whether the number of these cells is sufficiently high to be clinically relevant with respect to the selection or development of drug-resistant strains remains to be determined.
Introduction

In human immunodeficiency virus (HIV) infection, a viral sanctuary site is characterized as an anatomical site which is highly impermeable to (some) antiretroviral drugs, and in which viral replication continues during treatment, thus allowing local selection and/or development of drug-resistant strains\(^1\text{-}^3\). Most investigators consider the male genital tract (MGT) a sanctuary site for HIV\(^4\), since most protease inhibitors (PIs), as well as the non-nucleoside reverse transcriptase inhibitor (NNRTI) efavirenz, do not penetrate well into the seminal plasma\(^4\text{-}^8\).

Besides the absence of sufficient local concentrations of antiretroviral drugs however, the presence of susceptible and virus replicating and producing cells, i.e. activated CD4 positive lymphocytes and macrophages (CD4\(^+\) cells), is also a prerequisite for the development of drug-resistant strains\(^9\). Whether such cells are also present in the MGT is to our knowledge currently unknown\(^4\).

We here report an explorative study on the presence of activated lymphocytes in semen from healthy semen donors, asymptomatic, antiretroviral therapy naïve HIV-1 infected men and HIV-1 infected men using HAART.

Method

Patients

Between September 2005 and October 2006 healthy men voluntary donating semen in a donor program at the Centre for Reproductive Medicine of the Academic Medical Centre (AMC), Amsterdam, the Netherlands, were recruited for this study. All donors were proven negative for HIV-1/2, hepatitis B and C, HTLV and Chlamydia trachomatis infection.

In addition, HIV-1 positive men who were antiretroviral therapy naïve because of sufficient CD4 counts (no HAART group), and HIV-1 infected men using effective first-line HAART for at least 12 weeks (HAART group) were recruited from the HIV outpatient clinic of the AMC. All men were enrolled in longitudinal studies evaluating the effects of HIV-1 or antiretroviral therapy on semen parameters. Exclusion criteria were known causes of male infertility, including a vasectomy, a history of mumps orchitis and a history of chemo- or radiotherapy. Patients were asked for symptoms of genitourinary infections and a ligase chain reaction (LCR) (Abbott diagnostics, Illinois, U.S.A.) was performed on first void urine to exclude active Chlamydia trachomatis infection in all patients.

The study was approved by the Institutional Review Board of the AMC and all patients and donors gave written informed consent.
Preparation of samples

Paired blood and semen samples were obtained on a single visit. Four-and-a-half mL of blood was collected in an ethylenediaminetetraacetic acid (EDTA) tube. Semen was collected after masturbation and semen analysis was performed within one hour after ejaculation according to the World Health Organisation (WHO) manual for routine semen analysis\textsuperscript{10}. The semen was diluted 1:1 with Hanks balanced salt solution (HBSS; Sigma) and centrifuged for 10 minutes at 350 g at room temperature. The supernatant was removed and stored at -80°C. After resuspension of the pellet in HBSS, the number of spermatozoa and round cells, which contain immature germ cells, leukocytes and other non spermatozoal seminal cells, was determined. The percentage of spermatozoa with a normal morphology and the nature of the non spermatozoal cells was determined on a semen smear by using the Diff Quick\textsuperscript{10} staining procedure.

Immunofluorescence staining

To detect lymphocytes in seminal plasma and blood samples we used anti-CD45 antibodies. To determine whether these lymphocytes were activated, we stained cell suspension with anti-CD38 and anti-HLAdr assuming that activated lymphocytes express both CD38 and HLAdr\textsuperscript{11}. Staining was performed as follows: 20 µL of monoclonal antibodies CD 45 -PE-Cy5, HLA-dr allophycocyanin (APC) and CD38 R-phycocerthrin (R-PE), was added to aliquots of 200 µL sperm suspension and 100 µL of EDTA whole blood. As a control, anti-CD45 PE-Cy5 alone was added to another 200 µL sperm suspension and 100 µL of EDTA whole blood. All monoclonal antibodies were purchased from Becton Dickinson (BD biosciences, San Diego, USA). Following vortex mixing and 20 minutes incubation in the dark at room temperature, 2 mL of ammonium chloride (8.3 g/L) was added to the triple stained and control samples. After 10 minutes PBS-plus, i.e. PBS supplemented with 10% pasteurised plasma solution (GPO; Sanquin, Amsterdam, the Netherlands) was added to these ammonium chloride treated samples and the sample was centrifuged at 350g for 5 minutes. The supernatant was discarded and this wash step was repeated. Finally, cell pellets were resuspended in 0.5 mL of PBS-plus with 1% of paraformaldehyde, to fixate the cells, and stored at 4°C until flowcytometric analysis. In order to be able to establish gates for lymphocytes in the flowcytometric analysis (see below), a semen sample was spiked with blood derived lymphocytes stained with anti-CD45 as described previously\textsuperscript{12}. Briefly, lymphocytes were extracted from EDTA blood, by adding an aliquot of 100 µL of EDTA blood to 2 mL ammoniumchloride at room temperature. After incubation for 10 minutes HBSS was added and the sample was centrifuged for...
Lymphocytes in semen

10 minutes at 350 g at room temperature. The supernatant was discarded and the pellet was resuspended in HBSS. Fifty µL of the blood suspension was then used to spike 100 µL of unstained spermatozoa suspension. The mixed sample was then stained with anti-CD45 as described above.

Flow cytometry analysis

All stained cell suspensions were analysed with LSRII (Becton Dickinson, San Diego, USA) using CELL Quest and FACSDiva software. Data were displayed in a dot plot on the basis of the linear forward (FSC) and side scatter (SSC) properties of the cells, with lymphocytes having high forward scatter (FSC) and very low side scatter (SSC) characteristics. The semen sample spiked with blood lymphocytes was used to set the gates for tracking and, subsequently, the unspiked sample, containing only a few lymphocytes, was analysed. The stained cells were expressed as a percentage of the cells with lymphocyte characteristics based on FSC and SSC characteristics and expression of CD45. Inactivated cells expressed CD45 only, whereas activated cells expressed CD45, CD38 and HLAdr.

Statistical analysis

The median percentage of activated blood and seminal CD45+ cells was calculated. To detect differences in immune-activation of blood and seminal lymphocytes between the three study groups a one-way ANOVA test with post hoc testing (Bonferroni) was performed. In addition, a non-parametric test for paired samples was performed to compare per group immune-activation of seminal lymphocytes and blood lymphocytes (Wilcoxon’s rank). Statistical analyses were performed using SPSS v. 12.0.2 software (SPSS Inc., Chicago, II, USA).

Results

Eight healthy HIV-1/2-negative semen donors (HIV negative), eight HIV-1 infected therapy-naïve men (no HAART) and eight HIV-1 infected men using HAART (HAART) were enrolled. All men were asymptomatic for genitourinary infections and none had Chlamydia infection.

The baseline characteristics of the 24 patients are described in Table 6.1. Median CD4 counts in the no HAART and in the HAART group were 390 (IQR 325 - 450) and 330 (IQR 225 - 430) cells/µL respectively. The median blood plasma HIV-1 RNA concentration in the no HAART and in the HAART group was 111,018 (interquartile range (IQR) 7,824 - 135,210) copies/mL and <50 (IQR <50 - 77) copies/mL, respectively. All men using HAART had a blood
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<td>Concentration of spermatozoa (x10^9/mL)</td>
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<td>Progressive motility (%)</td>
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<td>Total number of round cells (x10^9/mL)</td>
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Data are presented as medians with interquartile ranges, unless otherwise stated. ND= not determined, NA= not applicable; * as determined by Diff Quick® staining (Flow cytometry has a much lower detection level than this visual staining procedure).

plasma HIV-1 RNA level below 100 copies/mL. Semen samples contained an average of 2x10^6/mL round cells, with a negligible number of leukocytes.

The median percentages of CD45+ blood and seminal cells that expressed both CD38 and HLA-dr are displayed in Figure 6.1. Median percentages of CD45+CD38+HLA-dr+ cells in blood in the no HAART, HAART and the HIV-negative group were 25% (IQR 20-27), 17% (IQR 11-20) and 10% (IQR 8-14), respectively. These differences were statistically significant (p < 0.0001, ANOVA). Post hoc testing revealed statistically significant differences between all groups: no HAART versus HAART: p = 0.003, HAART versus HIV-negative: p = 0.04, no HAART vs HIV-negative: p < 0.001 (Bonferroni).

Median percentages of CD45+CD38+HLA-dr+ cells in semen in the no HAART, HAART, and the HIV-negative group were 16% (IQR 12-21), 14% (IQR 10-21) and 11% (IQR 5-19), respectively. These differences were not statistically significant (p = 0.48, ANOVA).

The percentage of CD45+CD38+HLA-dr+ cells in semen samples was lower than in the paired blood samples in the no HAART and HAART groups, but not statistically significant (p = 0.16 and p = 0.67, respectively, Wilcoxon’s rank, Figure 6.1). In the HIV-negative group the percentages of CD45+CD38+HLA-dr+ cells in semen and blood were comparable.
Figure 6.1 Percentage of CD45+ cells in blood and semen expressing both CD38 and HLA-dr.

Note: The difference between groups with respect to the percentage of double-positive cells in blood was highly significant (p<0.0001, one-way ANOVA), the difference between groups in semen was not significant (p = 0.48, one-way ANOVA)

Discussion

In this first explorative study, we showed that lymphocytes in seminal plasma, although present in very low numbers, express the activation markers CD38 and HLAdr. Although the percentage of lymphocytes expressing CD38 and HLAdr was generally higher in blood than in the paired semen samples, these differences were not statistically significant. There was a non-significant trend of decreased immune activation in seminal lymphocytes when comparing the no HAART, HAART and HIV-negative groups, that paralleled the significant decrease observed in the paired blood samples. This is in line with previous reports that demonstrated that there is a state of immune-activation caused by HIV, which is suppressed by effective HAART\textsuperscript{13}. 

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Our study has several limitations. First, we used CD45 and FACS forward scatter (FSC) and side scatter (SSC) properties to localize lymphocytes. Ideally, CD4 monoclonal antibodies would have been used, but pilot experiments showed that, in agreement with literature, these antibodies did not work well in semen (data not shown)\textsuperscript{12}. Second, we used ejaculated semen. Since biochemical changes in semen take place rapidly after ejaculation, our findings in the ejaculate may not reflect the situation of seminal fluid and cells within the MGT\textsuperscript{15}. In addition, lymphocytes in ejaculated semen could very well be blood derived and might not be present in parts of the MGT that are impermeable to antiretroviral drugs. The most ideal study to evaluate the activation state of CD4+ cells in the male genital tract would be to perform tissue biopsies of several parts of the MGT in healthy and in HIV infected men, but such studies are not clearly not feasible\textsuperscript{4}. Third, we cannot exclude an effect of semen processing on lymphocyte activation. Finally, during local infections numbers and activation state of lymphocytes in semen may be significantly higher\textsuperscript{4}.

In conclusion, we demonstrated the presence of activated lymphocytes in semen of HIV-1/2-negative men, HIV-1 infected therapy-naïve men and HIV-1 infected men using HAART. In general, the number of these cells was extremely low and the activation state was lower in semen as compared to paired blood samples. However, if such cells are present in the MGT at sites of low or absent antiretroviral drug concentrations, this might facilitate the selection or development of drug-resistant strains. Whether the number of these cells is sufficiently high to be clinically relevant in this respect remains to be determined.
References

Chapter 7

Semen quality and drug concentrations in seminal plasma of patients using a didanosine or didanosine plus tenofovir containing antiretroviral regimen

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Ther Drug Monit, 2007, accepted for publication
Data on concentrations of didanosine (ddI) and tenofovir (TFV) in seminal plasma are sparse. Subtherapeutic drug concentrations within the lumen of the male genital tract (MGT) may have implications for selection and transmission of drug-resistant human immunodeficiency virus strains. On the other hand, sufficient penetration of these drugs into the MGT has potential toxic effects on the spermatozoa and their precursors. In the current study, the authors obtained paired semen and blood samples at variable timepoints after drug intake from 30 HIV-1-infected patients using a ddI (n = 15) or ddI + TFV (n = 15) containing antiretroviral regimen. Didanosine and TFV concentrations were measured in seminal and blood plasma and semen quality was assessed. Both ddI and TFV penetrated well into seminal plasma. Whereas blood plasma ddI concentrations dropped to near or below the lower limit of quantification of 0.017 µg/mL nine hours after drug intake, the ddI concentration in seminal plasma remained detectable during the whole dosing interval with a median of 0.20 and 0.21 µg/mL in the ddI and ddI + TFV groups, respectively. Tenofovir was detectable during the whole dosing interval in both blood and seminal plasma, with a median concentration of 0.12 and 0.25 µg/mL, respectively, and a median seminal-to-blood-plasma-ratio of 3.3. Semen quality was within the normal range according to the criteria of the World Health Organization, except for the percentage of progressively motile sperm, which was low in both groups of patients. The authors conclude that ddI and TFV penetrate well into seminal plasma and that the reduced sperm motility deserves further study.
Introduction

Not all antiretroviral drugs penetrate sufficiently into the lumen of the male genital tract (MGT). Subtherapeutic drug concentrations may allow local selection of drug-resistant human immunodeficiency virus (HIV)\textsuperscript{1,2}. On the other hand, sufficient penetration into seminal plasma exposes the spermatozoa and their precursors to potentially toxic effects of antiretroviral drugs. In this context, it is of importance to note that several antiretroviral drugs, including didanosine (ddl), are associated with mitochondrial toxicity\textsuperscript{3}. Because mitochondria are abundant in spermatozoa, and necessary for their progressive motility, these drugs may thus affect sperm motility. Available data concerning the concentration of ddl and tenofovir (TFV) in seminal plasma are sparse. There is only one study that suggests that ddl accumulates in seminal plasma\textsuperscript{4}. Similarly, TFV concentrations have only been studied in four patients using tenofovir disoproxil fumarate (TDF); this study suggested accumulation of TFV in seminal plasma\textsuperscript{5}. Didanosine and TDF have a pharmacokinetic interaction which makes it necessary to reduce the dosage of ddl when it is used in combination with TDF; in patients with a bodyweight of 60 kg or more the recommended dose of ddl is 250 mg instead of 400 mg once daily\textsuperscript{6,7}. No studies exist on the effect on seminal plasma drug levels when these two drugs are used simultaneously.

The purpose of the current cross-sectional study were to assess the penetration of ddl and TFV into seminal plasma, in patients using a ddl or ddl plus TDF-containing antiretroviral regimen and to evaluate semen quality in these patients.

Materials and methods

Patients were eligible for this study if they used ddl (enteric-coated) or ddl (enteric-coated) plus TDF as part of their current potent combination antiretroviral regimen (also called highly active antiretroviral therapy, HAART) for at least six weeks and were adherent to their therapy. Exclusion criteria were vasectomy, a genitourinary tract infection (assessed by urinalysis and \textit{Chlamydia trachomatis} ligase chain reaction assay in urine within eight weeks before semen collection, and a negative medical history on the day of semen collection), an intercurrent medical condition and renal insufficiency. The study was approved by the institutional review board and all patients gave written informed consent.

Semen was produced by masturbation after at least two days of sexual abstinence. The ejaculate was collected in a sterile container and analyzed within one hour. All semen analyses were performed according to the World
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Health Organization (WHO) guidelines for routine semen analysis\(^8\). After liquefication at 37°C, semen volume and pH were measured. Subsequently, concentration and motility of spermatozoa were assessed using a counting chamber (Léjà products B.V., Nieuw Vennep, The Netherlands) and the percentage of spermatozoa with a normal morphology was determined by counting 100 Quick diff\(^\text{®}\) -stained spermatozoa. The whole semen sample was then centrifuged at 1200 g for 10 minutes and the supernatant, consisting of seminal plasma, was stored at -20°C until analysis of drug concentrations. Within two hours before or after semen collection, a venous blood sample was taken in heparinized tubes for measurement of the blood plasma ddl and TFV concentrations. Heparinized blood was centrifuged at 1200 g for 10 minutes and the plasma was stored at -20°C. Time of last intake of the drugs, production of the semen sample and blood collection were recorded. As time point of the seminal-to-blood-plasma-drug ratio, the mean of the interval between drug intake and semen or blood collection was taken.

Concentrations of ddl in blood plasma were measured using a previously described validated high-performance liquid chromatography (HPLC) method with ultraviolet light detection\(^9\). The lower limit of quantification (LLOQ) was 0.017 µg/mL. Concentrations of ddl in seminal plasma were determined using the same method with an adapted sample preparation i.e., solid phase extraction was used as described for urine\(^10\). The seminal plasma LLOQ with this method was also 0.017 µg/mL and the calibration curve was linear over a range of 0.017 to 5.19 µg/mL. Recovery after extraction from seminal plasma was 108% and accuracy ranged from 101% to 109%, whereas intraday and interday precision were 4.3% and 10.1%, respectively (data not shown).

Tenofovir blood and seminal plasma concentrations were measured by a previously described HPLC method with fluorimetric detection\(^11\). The tenofovir LLOQ was 0.015 µg/mL for blood plasma and 0.048 µg/mL for seminal plasma. Both ddl and TFV assays were externally validated by the Quality Assurance Program for Clinical Measurement of Antiretrovirals of the AIDS Clinical Trials Group (ACTG)\(^12\).

Descriptive statistics were performed using SPSS statistical programs version 11.5.1. (SPSS; Woking, Surrey, UK).

Results

From January till November 2004, 30 patients were included, 15 using ddl and 15 using ddl plus TDF-containing HAART. Baseline characteristics of the patients are presented in Table 7.1. All patients weighed more than 60 kg and
their daily dose of ddI was 400 mg (ddI-only group) and 250 mg (ddI + TDF group) as recommended. Of the patients using ddI only, 14 had a blood plasma HIV-1 RNA concentration (pVL) of less than 50 copies/mL (Versant HIV-1-RNA [branched-DNA] assay; Bayer Corporation, Tarrytown, New York, USA) in the eight weeks before semen collection. One patient had a virological blip of 78 copies/mL 6 weeks before semen collection. In the patients using ddI plus TDF, two had a detectable pVL around the time of semen collection (1042 and 472 copies/mL, respectively).

Data on drug intake and drug concentrations are shown in Figures 7.1 through 7.3. In most patients in both groups, the ddI seminal plasma concentrations were higher than the blood plasma concentrations (median concentration 0.20 µg/mL versus 0.09 µg/mL in the ddI-only group and 0.21 µg/mL versus 0.02 µg/mL in the ddI plus TDF group) (Figure 7.1). In both groups, the ddI blood plasma concentrations peaked at about two to seven hours after drug intake, whereas after 14 to 16 hours the ddI blood plasma concentrations were all below the LLOQ. For seminal plasma, in both groups, the ddI concentration

Table 7.1  Characteristics of patients using didanosine (ddI) or ddI plus tenofovir DF-(TDF) containing antiretroviral regimen.

<table>
<thead>
<tr>
<th></th>
<th>All patients</th>
<th>ddI</th>
<th>ddI+TDF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>30</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Age (years)</td>
<td>43 (38-52)</td>
<td>43 (39-53)</td>
<td>42 (37-48)</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>77 (70-86)</td>
<td>73 (66-84)</td>
<td>78 (75-87)</td>
</tr>
<tr>
<td>Present CD4 count (cells/µL)</td>
<td>515 (398-663)</td>
<td>520 (410-620)</td>
<td>510 (360-670)</td>
</tr>
<tr>
<td>Number with pVL &lt;50 RNA copies/mL</td>
<td>26</td>
<td>14</td>
<td>12</td>
</tr>
<tr>
<td>Time on current HAART (months)</td>
<td>18 (11-30)</td>
<td>28 (17-37)</td>
<td>12 (7-18)</td>
</tr>
<tr>
<td>Time on HAART (months)</td>
<td>64 (37-95)</td>
<td>53 (34-83)</td>
<td>90 (42-96)</td>
</tr>
<tr>
<td>Time on ART (months)</td>
<td>76 (37-122)</td>
<td>61 (34-113)</td>
<td>104 (42-122)</td>
</tr>
</tbody>
</table>

Note: Data are expressed as median and interquartile range where applicable. HAART=highly active antiretroviral therapy; ART=antiretroviral therapy (includes non-HAART and HAART regimens)

Table 7.2  Semen quality parameters.

<table>
<thead>
<tr>
<th></th>
<th>ddI</th>
<th>ddI+TDF</th>
<th>Normal values*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstinence period (days)</td>
<td>2 (2.0-3.0)</td>
<td>2 (2.0-4.0)</td>
<td>&gt;2</td>
</tr>
<tr>
<td>Volume (mL)</td>
<td>2.5 (1.40-3.00)</td>
<td>2.2 (1.70-4.00)</td>
<td>&gt;7.5</td>
</tr>
<tr>
<td>pH</td>
<td>7.5 (7.2-7.5)</td>
<td>7.5 (7.5-7.5)</td>
<td>&gt;7.2</td>
</tr>
<tr>
<td>Spermatozoa concentration (x10⁶/mL)</td>
<td>50 (40.0-73.0)</td>
<td>65 (50.0-108.0)</td>
<td>&gt;20</td>
</tr>
<tr>
<td>Spermatozoa motility (%)</td>
<td>Progressive (grade a+b) (%)</td>
<td>22 (15.0-46.0)</td>
<td>26 (16.0-47.0)</td>
</tr>
<tr>
<td></td>
<td>Slow (grade c) (%)</td>
<td>15 (11-25.0)</td>
<td>9 (7.0-15.0)</td>
</tr>
<tr>
<td></td>
<td>Immobile (grade d) (%)</td>
<td>53 (42-72)</td>
<td>59 (45.0-70.0)</td>
</tr>
<tr>
<td>Spermatozoa with normal morphology (%)</td>
<td>34 (26.0-41.0)</td>
<td>41 (35.0-64.5)</td>
<td>&gt;30</td>
</tr>
</tbody>
</table>

Note: Data are expressed as median and interquartile ranges. ddI=didanosine; TDF=tenofovir disoproxil fumarate. *Reference 8.

Semen and didanosine and tenofovir
peaked at approximately 12 to 13 hours after drug intake, whereas after 15 to 18 hours, the ddI concentrations were all in the lower range (Figure 7.1). In the patients from the ddI-only group and the ddI plus TDF group in whom a seminal-to-blood-plasma-ddI ratio could be calculated, this ratio clearly increased during the course of the dosing interval. Because of plasma concentrations less than the LLOQ, from 16 hours onward, a seminal-to-blood-plasma-ddI ratio could not be calculated (Figure 7.3).
Tenofovir was detectable in all blood and seminal plasma samples of the patients using ddi plus TDF with a median concentration of 0.12 µg/mL and 0.25 µg/mL, respectively. There was a clear relationship between the interval after TDF intake and the TFV concentration in blood plasma (correlation coefficient -0.8, p = 0.01) (Figure 7.2). There was no clear relationship between the interval after TDF intake and the TFV concentration in seminal plasma (Figure 7.2). In most patients, concentrations of TFV in seminal plasma were higher than in blood plasma, with a median seminal/blood plasma ratio of 3.3 (range 0.9-49.2) (Figure 7.3).

Data on semen quality are given in Table 7.2. The semen volume was on the lower side of normal values and the percentage of progressively motile sperm was below World Health Organization criteria in both groups. 
Chapter 7

Discussion

This study is the largest on ddi and tenofovir concentrations in seminal plasma and, with respect to the combination of ddi and tenofovir, the first published study. In this study we found that both ddi and TFV penetrate well into seminal plasma. In contrast to blood plasma ddi concentrations, the ddi concentration in seminal plasma remained detectable during a larger part of the dosing interval and compared with blood plasma, the concentration peak in seminal plasma was approximately eight hours later. During the last part of the dosing

Figure 7.3 Seminal plasma (sp) to blood plasma (bp) ratio of didanosine (ddi) and tenofovir (TFV).

*Note: Dotted line indicates ratio=1.*
Semen and didanosine and tenofovir

interval also, the ddI concentrations in seminal plasma drop to low levels. So, with some delay, the ddI concentrations in seminal plasma parallel those of blood plasma, explaining the increasing seminal-to-blood-plasma-ddI ratios during the course of the dosing interval (Figures 7.1 and 7.3). For indinavir not boosted with ritonavir and stavudine, both also antiretroviral drugs with a short blood plasma half-life (approximately 1.5 hours), the same delay between the blood and seminal plasma concentrations have been found. Our data also suggest that concurrent use of tenofovir-DF, with a dose reduction of ddI as recommended, has no apparent effect on the blood and seminal plasma ddI concentrations.

Our results are in line with previous small studies on ddI and TFV in seminal plasma, respectively. Furthermore, our results confirm previous findings that antiretroviral drugs with a protein binding of less than 90% penetrate well into the seminal plasma; ddI and TFV both have a protein binding of less than 10%.

The potential downside of good penetration is that spermatozoa and their precursors are exposed to more of the antiretroviral drugs. Most studies suggest that semen parameters are normal during asymptomatic HIV-1 infection. However, a direct effect of HIV-infection on semen volume and sperm motility has been noted before. Our current study suggests that there was a tendency toward a lower semen volume and a decreased percentage of progressively motile sperm in antiretroviral therapy exposed HIV-1 infected patients. The patients were, on average, already six years on antiretroviral treatment, so the influence of previous regimens and that of other drugs in the current regimen, or the HIV infection itself, cannot be excluded.

Data describing the effects of antiretroviral therapy on semen quality are limited. Only one longitudinal study has been performed on semen parameters before and during HAART, but the four to twelve weeks follow up of this study may have been too short to evaluate possible adverse effects on semen quality.

Thus, larger and more extended prospective longitudinal studies are needed to elucidate any potential detrimental effects of antiretroviral combination therapy on semen quality.

Acknowledgement

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Chapter 8

Summary and general discussion

Antiretroviral therapy:
Efficacy and toxicity in the ARES study
and responses in the male genital tract
Summary and general discussion

In 1996 highly active antiretroviral therapy (HAART) for infection with the human immunodeficiency virus type 1 (HIV-1) was introduced. While due to HAART the HIV related morbidity and mortality decreased drastically, it soon also became clear that treatment would be lifelong and that more effective, more tolerable, and safer drug regimens were needed. Besides potency of the combined antiretroviral drugs a very important factor for effective antiretroviral therapy is strict adherence to the regimen, for more than 95%. In addition to factors related to the patient, adherence is predominantly compromised by dosing frequency and acute and long term side effects of the drugs.

In search to improve existing antiretroviral regimens, the Antiretroviral Regimen Evaluation Study (ARES) was designed in 1998-1999. This was in a context of only a few years experience with HAART and limited insight in long term adverse effects of HAART, i.e. mitochondrial toxicity. The study design was outrun by new developments and insights in antiretroviral therapy, which made it necessary to terminate the study prematurely because one of the regimens became contraindicated.

In ARES two once daily dosed regimens consisting of nevirapine, didanosine and lamivudine and saquinavir, ritonavir, didanosine and lamivudine, respectively, were compared with an at that time commonly used twice daily dosed regimen consisting of nelfinavir, didanosine and stavudine. By some the latter regimen was considered the (international) standard at that time. In Chapter 2 (Comparison of two once-daily regimens with a regimen consisting of nelfinavir, didanosine and stavudine in antiretroviral therapy-naïve adults: 48-week results from the Antiretroviral Regimen Evaluation Study [ARES]) the findings of the ARES study are presented. The study showed that the intention-to-treat virological success (a blood plasma HIV-1 RNA concentration = viral load (pVL) <50 RNA copies/mL) of the three regimens at 48 weeks of therapy was about 50%, with difference in the rate and type of adverse events between the three regimens. A limitation of the ARES study is the small number of patients included and the results should be interpreted cautiously. In a historical perspective the ARES study did demonstrate that one of the standards for HAART of the late 1990's could be improved by either of the once daily dosed regimens. These two once daily dosed regimens must not be disqualified because they had a comparable virological success as the outdated “standard” of about 50%, while current HAART regimens achieve a virological success of about 70%. In the 2NN study it was demonstrated that with a NRTI backbone consisting of stavudine and lamivudine the pVL reduction to <50 copies/mL with once daily dosed nevirapine was similar to that of efavirenz, efavirenz being one of the current...
standard components of a HAART regimen. A once daily dosed regimen consisting of saquinavir boosted with low dose ritonavir needs further study, since in the ARES study there was an indication that it was a safe regimen and since, due to a new formulation the pill burden has been reduced. With the once daily regimen of saquinavir 2000 mg boosted with 100 mg ritonavir the drug exposure, including the through or minimum concentration, is significantly better compared to the 1600/100 mg q.d. dose\textsuperscript{8,9}. Only one other study also used eight 200 mg saquinavir soft gelatin capsules (sgc) boosted with 100 mg ritonavir\textsuperscript{10-12}. Other clinical studies with antiretroviral drug naïve patients starting boosted once daily saquinavir and with a follow up of at least 48 weeks are lacking. In the mentioned study, in the intention-to-treat analysis at 48 weeks 51% versus 71% in the saquinavir sgc and efavirenz group, respectively had a blood plasma HIV-1 viral load <50 copies mL\textsuperscript{-1} and adverse events leading to drug discontinuation occurred in 15% versus 8%, respectively\textsuperscript{12}.

Body fat redistribution, consisting of central adiposity and/or peripheral lipoatropy is the major long term adverse effect of HAART, and it was first described in 1998\textsuperscript{13}. Central adiposity is, with the exception of nelfinavir\textsuperscript{14,15}, associated with the use of protease inhibitors, and peripheral lipoatrophy with the use nucleoside analogue reverse transcriptase inhibitors (NRTI’s), in particular with stavudine and to a lesser degree with zidovudine\textsuperscript{16}. Data on didanosine and lipodystrophy are limited\textsuperscript{17,18}. In Chapter 3 (Stavudine- but not didanosine as part of HAART contributes to peripheral lipoatrophy. A substudy from the Antiretroviral Regimen Evaluation Study [ARES]) the results of a substudy from ARES focussing on body fat redistribution are presented. This study is the first randomized, prospective study in which whole body dual energy X-ray absorptiometry (DEXA) was used to evaluate the effect on fat redistribution of HAART containing didanosine with and without stavudine. This substudy showed that didanosine might be associated with peripheral lipoatrophy much less in absence of stavudine. Again the results must be interpreted with caution because of the small number of patients included, and the arm with stavudine and didanosine also contained nelfinavir. Since didanosine is dosed once daily the finding that this drug might be less toxic as assumed extends the possibilities for once daily dosed (first line) HAART regimens and thereby possibly improved drug adherence. At present in USA guidelines\textsuperscript{19,20} the nucleoside/nucleotide analogue reverse transcriptase inhibitor (NRTI) components tenofovir or zidovudine or abacavir are preferred for initial HAART regimens, and didanosine (in combination with lamivudine or emtricitabine) is recommended as an alternative, because of supposed toxicity and limited data compared to these NRTI’s. In light of limited experience with didanosine in once daily dosed HAART regimens and the adverse events of the other once
daily dosed NRTI’s, such as abacavir related hypersensitivity and tenofovir related nephropathy, further studies with didanosine in once daily dosed HAART regimens are warranted.

Body compartments where penetration of some antiretroviral drugs is limited are a threat for durable HIV suppression, due to the (theoretically) increased risk for selection or development of drug resistant virus. The male genital tract (MGT) is considered such a sanctuary site for HIV. In Chapter 4 (Is the male genital tract really a sanctuary site for HIV? Arguments that it is not) definitions are proposed for a viral compartment, reservoir and sanctuary site with respect to HIV, since these terms are unjustly used interchangeably. A **viral compartment** is an anatomical site in which the virus in untreated patients evolves distinctively from other anatomical sites or the main pool of infected cells, because of differences between the major cell types sustaining viral replication. A **viral reservoir** is a cell type or anatomical site in which a replication-competent virus persists much longer than in the main pool of infected cells that sustain the infection, and this cell type or anatomical site can replenish the pool of infected cells. Finally a viral **sanctuary site** is an anatomical site which is highly impermeable to (some) antiretroviral drugs, and in which viral replication continues during treatment, thus allowing the development and/or selection of drug-resistant strains. In chapter 4 arguments are given why the MGT might not be a sanctuary site for HIV. The arguments are that within the MGT there is a strong immunosuppressive state which prohibits local viral replication and that HIV in semen derives from a spillover from the blood. With respect to the definitions of viral compartment, reservoir and sanctuary site, the seminiferous tubule is neither a virological compartment nor a sanctuary site for HIV-1, because of absence of appropriate target cells. The remainder of the lumen of the MGT is a viral compartment and reservoir for HIV-1, since proviral-DNA containing leukocytes are present, but this part is also not a virological sanctuary site because under normal circumstances replication is likely arrested due to the local immunosuppressive state. Contradictory findings are likely due to a small group of patients with semen leukocytosis, in whom local factors are present that result in (more) HIV production within the lumen of the MGT, and thereby they are probably more prone to development of drug-resistant HIV in the MGT. That the MGT is likely not a sanctuary site for HIV has especially consequences for sexual transmission of (drug resistant) HIV, since it is expected that with a blood plasma viral load <50 copies/mL the seminal plasma viral load will also be undetectable, except for possibly the patients with semen leukocytosis.
In Chapter 5 (No virological failure in semen during properly suppressive antiretroviral therapy despite subtherapeutical local drug concentrations) a study is presented which was based on the concept that the MGT is a sanctuary site for HIV and that with the regimens studied virological failure in semen and emergence of drug resistant virus would readily be demonstrated. Patients from the ARES study using the regimens saquinavir, ritonavir, lamivudine and didanosine or nelfinavir, stavudine and didanosine respectively and patients using efavirenz, lamivudine and zidovudine were included. In these regimens only the two NRTI's achieve therapeutic concentrations in seminal plasma. Contrary to what was expected virological failure in semen did not occur despite low drug levels of saquinavir, nelfinavir and efavirenz in seminal plasma. The blood plasma VL in the patients was <50 copies/mL. Taken together the findings of this study support the hypothesis stated in chapter 4 that HIV in semen is a spillover from the blood, that therefore with a blood plasma viral load <50 copies/mL the seminal plasma viral load will also be below the limit of detection, and that the MGT is likely not a sanctuary site for HIV.

To substantiate that the target cells for HIV in semen are immunosuppressed, CD38 and HLA-dr expression, markers for immunoactivation of (blood) lymphocytes, were evaluated in peripheral blood and semen lymphocytes of eight asymptomatic antiretroviral therapy naïve HIV-1 infected men, eight HIV-1 infected men using effective antiretroviral therapy and eight healthy HIV seronegative semen donors. In Chapter 6 (Activated lymphocytes in semen from HIV-1 positive and HIV negative men) the results of this study are presented. In the semen the lymphocytes were present at very low numbers and in the HIV-1 infected patients the percentage of activated cells was statistically non-significantly lower in semen compared to the blood. That the seminal lymphocytes were activated, does not exclude the presence of an immunosuppressive state in the lumen of the MGT since the activation could be due to post-ejaculatory biochemical changes and/or the processing of the semen. To evaluate the immune state of the lumen of the MGT studies different from ours, e.g. using biopsies, are needed.

The improved life expectancy of HIV infected patients make the wish of these patients to procreate and raise their children more feasible. Data on the effect of HAART on semen quality are however limited. NRTI's are associated with mitochondrial toxicity and mitochondria are abundant in spermatozoa and necessary for their progressive motility. The question therefore is whether NRTI's may affect sperm quality. To exert a toxic effect the spermatozoa and their progeny must be exposed to these drugs. Didanosine is one of the NRTI's associated with substantial mitochondrial toxicity. For didanosine and
tenofovir data on their concentration in seminal plasma is very limited. In Chapter 7 (Semen quality and drug concentrations in seminal plasma of patients using a didanosine or didanosine plus tenofovir containing antiretroviral regimen) the results are presented of a study in which the drug concentrations of didanosine and tenofovir in seminal plasma were measured and the semen quality assessed. The findings were that didanosine and tenofovir penetrate well into seminal plasma and that the percentage of progressively motile spermatozoa was below normal as given by the criteria of the World Health Organization. Thus, HIV and/or HAART may effect sperm quality. The patients were on average already six years on antiretroviral treatment, so the influence of previous regimens and that of other drugs in the current regimen, or the HIV infection itself, cannot be excluded. Data describing the effects of antiretroviral therapy on semen quality are limited. Only one longitudinal study has been performed on semen parameters before and during HAART, but the four to twelve weeks follow up of this study may have been too short to evaluate possible adverse effects on semen quality. Thus, larger and more extended prospective longitudinal studies are needed to elucidate any potential detrimental effects of antiretroviral combination therapy on semen quality. Taken into account that HIV in semen is likely a spillover from the blood, that antiretroviral drug concentrations in semen might not be critical for suppression of HIV in the MGT and that antiretroviral drugs may affect semen quality, HAART regimens that do not penetrate into the MGT are possibly preferable in the context of procreation.

Future directions

Since adherence is an important factor for successful initial and subsequent antiretroviral therapy, once daily dosed antiretroviral regimens must be further explored with regard to effectivity and toxicity. At present several antiretroviral drugs are available which can be dosed once daily, such as the NRTI's abacavir, didanosine, emtricitabine, lamivudine, tenofovir, the non-nucleoside reverse transcriptase inhibitors (NNRTI's) efavirenz and nevirapine and the protease inhibitor (PI) atazanavir, probably saquinavir and fosamprenavir and for a restricted indication lopinavir. Randomised controlled trials comparing several of the possible initial once daily dosed regimens, and including sequential DEXA scans, should reveal the preferred regimens. To further elucidate whether the lumen of the male genital tract is a sanctuary site for HIV the state of activation of the target cells for HIV, the CD4+ T-lymphocytes and macrophages, within the lumen must be further explored and likely this means histological studies, since otherwise the question will remain how ejaculation itself influences the state of activation of seminal leukocytes.
Also, since the life expectancy of HIV infected patients has improved, the effect of HIV and HAART on procreation needs further exploration. Concerning male reproductive capacity, matters that need to be clarified are 1) whether HIV and HAART affect sperm quality and if so, is this clinically relevant and which component of semen is relevantly affected, 2) in case antiretroviral drugs affect spermatozoa (motility), is there a significant difference between drugs that do and do not penetrate well into semen. The currently available NRTI's penetrate well into semen. Ritonavir-boosted lopinavir and efavirenz do not penetrate well into semen and when the combination of these drugs is compared with a NRTI containing HAART regimen in antiretroviral therapy naïve men, the effect of drug penetration on sperm quality could be tested. Finally, 3) in case HIV and HAART affect semen quality, is there an additive or synergistic decrement with washing of semen and is this of concern for the chance of a successful fertilisation. A prospective study, matched for age and other non-seminal fertility related factors, comparing semen quality between HIV negative, HIV positive but ART-naïve and HIV positive, HAART using men could help elucidate the effect of HIV and HAART on semen quality.
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Chapter 8

Chapter 9

Samenvatting

Antiretrovirale therapie:
Effectiviteit en toxiciteit in de ARES studie
en respons in het mannelijk genitaal stelsel
Samenvatting

In juni 1981 verscheen de eerste publicatie over het Acquired Immunodeficiëntie Syndrome (AIDS), in het Nederlands het verworven immuundeficiëntie syndroom. In de eerste 15 jaren nadien bestond de behandeling van deze infectie met het humaan immuundeficiëntie virus type 1 en/of 2 (HIV-1 en 2), twee retrovirusse die in 1983 en 1986 respectievelijk werden ontdekt, uit het behandelen en onderdrukken van de complicaties, te weten opportunistische infecties en kwaadaardige gezwellen. In deze periode was de mortaliteit van AIDS zeer hoog. Behandeling door middel van het remmen van het HIV zelf, zo geheten antiretrovirale therapie (ART), werd geïntroduceerd in 1987, toen zidovudine, een nucleoside-analoog reverse transcriptase remmer/inhibitor (NRTI) werd toegepast. Vervolgens werden andere NRTI’s ontwikkeld en alleen of in combinatie toegepast, echter met een gering effect op het klinische beloop van de HIV infectie en AİDS. 1996 is een belangrijk jaar voor antiretrovirale therapie, waar het toen mogelijk werd om de concentratie van het virus in het bloed te meten en daarmee het effect van verschillende antiretrovirale regimenes kon worden geëvalueerd, maar ook doordat een nieuwe klasse van HIV-remmers, de protease remmers/inhibitors (PI’s), werden geïntroduceerd en het bleek dat met het combineren van een PI met twee NRTI’s het HIV krachtig werd onderdrukt. In de periode 1996-1998 werd een derde klasse van HIV-remmers geïntroduceerd, te weten de non-nucleoside reverse transcriptase remmers/inhibitors (NNRTI’s). Ook de combinatie van een NNRTI en twee NRTI’s bleek het HIV krachtig te onderdrukken. Een combinatie van HIV-remmers die het virus krachtig onderdrukt wordt Highly Active Antiretroviral Therapy (HAART) genoemd. In de huidige standaard is de combinatie van twee NRTI’s met één (een met ritonavir geboosterde) PI of NNRTI. Door het gebruik van HAART is het beloop van de HIV infectie en de kans op overlijden aan AİDS dramatisch verbeterd. In 2003 werd een vierde klasse van HIV-remmers geïntroduceerd, te weten de fusieremmer enfuvirtide en anno 2007 zijn nog twee nieuwe klassen in klinisch onderzoek, te weten HIV-coreceptor CCR5 blokkers en integrase remmers. Enfuvirtide wordt wegens het gebruiksongemak alleen bij virologisch falen van de initiële HAART in combinatie met andere HIV-remmers ingezet, terwijl de plaatsbepaling van de CCR5-blokkers en integrase remmer nog onduidelijk is.

Volgens een schatting van de World Health Organization (WHO) zijn er in 2006 4,3 miljoen personen geïnfecteerd geraakt met HIV-1, overleden er 2,9 miljoen personen (331 per uur) aan AIDS en leven er 39,5 miljoen mensen met een HIV-1 infectie, waarvan circa twee miljoen (<5%) in de Westerse wereld. Genezing van een HIV infectie is tot nu toe niet mogelijk, daar het virus langdurig in een rusttoestand in de gastheercel, bepaalde witte bloed
lichtaampjes, de CD4+ T-lymfocyt en macrofagen, aanwezig blijft en HAART daar geen effect op heeft. Het doel van HAART is daarom, om het HIV-1 krachtig en het liefst permanent te onderdrukken. De huidige routinematige testen om de virusconcentratie in het bloedplasma te meten (de HIV-bloedplasma viral load, pVL) kunnen een concentratie van 50 virussen per milliliter (mL) of hoger meten. Bij gebrek aan een gevoeliger routinematige detectiemethode is het streven bij het gebruik van HAART derhalve om de pVL onder de detectiegrens van 50 deeltjes per mL te houden (pVL<50 RNA kopieën/mL). Behalve met een goede prognose correleert een pVL<50 k/mL ook met minder resistentievorming van het virus ten opzichte van een hoger waarde, m.n. een pVL>400 k/mL. Tussen 1996 en 2005 is het succes van HAART gemeten aan een pVL<50 k/mL na 48 weken behandeling gestegen van circa 45% naar circa 70% van de patiënten die hiermee startten. Dit resultaat berust op onderzoeken bij HIV-1 subtype B, het meest voorkomende subtype in de Westerse wereld wat op wereldniveau voor 12% oorzaak is van een HIV-1 infectie (naast subtype B zijn tenminste 31 andere subtypen en combinaties van subtypen; subtype C en A komen met 50% en 30% respectievelijk wereldwijd het meeste voor). Dat met het eerste HAART regieme het HIV krachtig en langdurig wordt onderdrukt, is van wezenlijk belang, daar indien het virus doorbreekt en resistent wordt de vervolgbeslissing moeilijker en veeleisender is en in het algemeen ook minder effectief is door kruisresistentie binnen de klassen van de HIV-remmers. Factoren die in meer of mindere mate of mogelijk geassocieerd zijn met het succes van de eerste behandeling met HAART zijn de mate van immuniteitsstoornis t.g.v. de infectie gemeten aan de bloedconcentratie van de CD4+ T-lymfocyt (CD4+ cel), de pVL, de vroege virologische respons na start van HAART, het reeds aanwezig zijn van resistent virus voor start van HAART, het syncytium-inducerend en niet-syncytium-inducerend fenotype van het HIV-1, het HIV-1 subtype, bloedplasma en intracellulaire concentratie van de HIV-remmers, farmacodynamische interacties tussen de HIV-remmers, therapietrouw van de patiënt, genetische factoren van de patiënt en kennis en ervaring van de behandelend arts.

Door het gebruik van HAART is de prognose van met HIV-1 geïnfecteerde personen zeer sterk verbeterd. Kinderwens en voortplanting is daarmee ook een reële mogelijkheid geworden. Voor wat betreft sperma is het effect van HIV-remmers op de kwaliteit hiervan en een succesvolle bevruchting nog niet duidelijk. Verder vormt het lumen (de inwendige holte) van het mannelijke geslachtsorgaan een barrière voor een aantal HIV-remmers, hetgeen van belang kan zijn voor het ontstaan van resistent virus lokaal en het doorgeven van al of niet resistent virus.
In dit proefschrift wordt op verschillende aspecten van een HIV-1 infectie ingegaan. Ten eerste worden twee eenmaal daags gedoseerde behandel-schema's vergeleken met een standaard tweemaal daags gedoseerd schema, met als achtergrond dat vereenvoudiging de therapietrouw en daarmee de effectiviteit van het eerst gegeven HAART regieme ten goede komt.

In Hoofdstuk 2 wordt de Antiretroviral Regimen Evaluation Study (ARES) beschreven. In deze studie bleken de te onderzoeken eenmaal daags gedoseerde regiemes bestaande uit 1) de NRTI's didanosine en lamivudine en de NNRTI nevirapine of 2) de NRTI's didanosine en lamivudine en de PI saquinavir geboosterd met ritonavir even effectief als de tweemaal daags gedoseerde standaard bestaande uit de NRTI's didanosine en stavudine en de PI nelfinavir, met een 48 weeks behandel succes van 50%. De aard en mate van bijwerkingen was echter verschillend tussen de 3 behandelarmen; er was meer mitochondriale toxiciteit in de nelfinavir arm, meer ziekte progressie in de nevirapine arm en de minste bijwerkingen in de saquinavir arm. Daar het een klein onderzoek betrof kunnen geen zwaarwegende conclusies uit deze studie worden getrokken. Het nelfinavir bevattende regieme wordt tegenwoordig niet meer gebruikt, wegens de bijwerkingen en het thans bestaan van effectievere en veiliger behandelregiemes.

Een belangrijke langetermijn bijwerking van de behandeling met HAART is het lipodystrofie syndroom (LDS), een veranderde vetverdeling van het lichaam, waarbij er enerzijds afname is van onderhuids vet van de armen, benen, billen en het gelaat (perifere lipoatrofie) en anderzijds toename is van vet in de buik en eventueel ook van de nek en borsten (centrale adipositas). Daarnaast kunnen er bij het LDS ook afwijkingen zijn in het bloedvet, -suiker -insuline gehalte. In Hoofdstuk 3 wordt in een substudie van de ARES studie middels een objectief diagnostiek onderzoek (whole body dual energy X-ray absorptiometry (DEXA) scanning) geëvalueerd of er een verschil is in het ontstaan van en mate van ontstaan van een veranderde lichaamsvetverdeling. Uit dit onderzoek bleek dat met het regieme bestaande uit didanosine, stavudine en nelfinavir meer lipoatrofie voorkwam ten opzichte van de andere twee regiemes (gecombineerd). In tegenstelling tot wat tot nu toe werd gedacht is de HIV-remmer didanosine mogelijk veel minder geassocieerd met lipodystrofie. Ook hier geldt, dat geen zwaarwegende conclusies uit deze kleine studie kunnen worden getrokken.

Een tweede aspect waar in Hoofdstuk 4 van dit proefschrift op wordt ingegaan is of het lumen van mannelijk genitaal stelsel (MGS) al of niet een veilige schuilplaats, een “sanctuary site”, voor HIV-1 is daar dit een barrière vormt voor een aantal HIV-1 remmers. Uit deze literatuur studie blijkt allereerst dat de termen virologische “sanctuary site”, “compartment” en “reservoir” door elkaar
worden gebruikt en wordt een voorstel gedaan tot een eenduidig gebruik van deze termen. Argumenten die in de literatuur worden geopperd ten faveure van het MGS als een sanctuary site zijn het bestaan van de bloed-testis barrière, een andere evolutie van de HIV-1 envelope in semen t.o.v. bloed en andere lichaams compartimenten, het aanwezig zijn van resistentie HIV-1 in semen en soms een hoger concentratie van HIV-1 in semenplasma t.o.v. bloedplasma. In dit hoofdstuk worden tegenargumenten gegeven, zoals het alleen bij de tubuli seminifiri (Appendix Hoofdstuk 4), het deel van het MGS waar de zaadcellen worden geproduceerd, aanwezig zijn van de bloed-testis barrière, en het bestaan van een immuunonderdrukkende situatie in het lumen. Het aanwezig zijn van een immuunsuppressieve status in het lumen van het MGS heeft als consequentie dat het HIV wordt geïnactiveerd en ook geen nieuwe CD4+ cellen kan infecteren. Voorzichtig wordt geconcludeerd dat de tubuli seminifiri waarschijnlijk geen virologisch compartiment, reservoir of sanctuary site zijn, daar de gastheercellen, de CD4+ cellen, daar niet aanwezig (horen te) zijn, en dat het overige lumen van het MGS wel een compartiment en reservoir is, daar er CD4+ cellen afkomstig uit het bloed aanwezig zijn die (inactief) virus bevatten, maar waarschijnlijk geen sanctuary site daar door de ter plaatse aanwezig immuunsuppressie het HIV virus niet actief is en het al of niet aanwezig zijn van HIV-remmers in het lumen waarschijnlijk niet van belang is voor het onderdrukken van het HIV-1 in het lumen en dat waarschijnlijk in het lumen geen resistentievorming kan ontstaan. De hypothese wordt gesteld, dat HIV in semen een “spillover” is van het bloed en dat waarschijnlijk alleen in een minderheidspopulatie, in geval van mannen met semen leukocytosis, er sprake is van lokale HIV replicatie en dan dus het MGS wel een virologische sanctuary site is.

In hoofdstuk 5 en 6 wordt verder ingegaan op deze hypothese. **Hoofdstuk 5** betreft een studie waarbij HIV-1 in semenplasma wordt geëvalueerd tijdens behandeling met HAART, waarbij sommige componenten van het HAART regieme een onvoldoende concentratie in het semenplasma bereiken. Desondanks de onvoldoende HIV-1 remmer concentratie bleek het HIV virus in semenplasma toch goed onderdrukt, hetgeen de hypothese ondersteunt dat het lumen van het MGS waarschijnlijk geen sanctuary site is voor HIV. **Hoofdstuk 6** betreft een onderzoek met de markers CD38 en HLA-dr naar de immuunactivatie status van semen lymfocyten, die o.a. de CD4+T-lymfocyten, de gastheer cellen voor HIV, bevatten. Dit onderzoek toont aan dat een zeer gering aantal geactiveerde lymfocyten in semen aanwezig zijn en dat deze activatie mogelijk minder is ten opzichte van het bloed. De aanwezigheid van geactiveerde lymfocyten in semen sluit het bestaan van een immuun-suppressieve status in het lumen van de MGS niet uit maar de activatie kan zijn.
veroorzaakt door biochemische processen na de ejaculatie en/of door het bewerkingssproces van semen. Andersoortig onderzoek, bijvoorbeeld gebruikmakend van weefselonderzoek, is nodig om de immuunstatus van het lumen van het MGS te onderzoeken.

Het derde aspect wat in dit proefschrift wordt onderzocht is dat van de concentratie van de NRTI's didanosine en tenofovir in semenplasma. Tot nu toe ontbraken gegevens hierover of was dit beperkt onderzocht. Het belang van het al of niet goed doordringen van HIV-remmers in semen is het blootstellen van de zaadcellen aan de schadelijkheid van deze middelen. Didanosine wordt met name geassocieerd met meer schade aan de mitochondriën, de energiecentrale van de cel. Voor hun beweeglijkheid bevatten zaadcellen veel mitochondriën. Uit het onderzoek in Hoofdstuk 7 blijkt dat didanosine en tenofovir goed doordringen in semenplasma. Voorts was het percentage progressief beweeglijke zaadcellen verlaagd volgens de criteria van de World Health Organization (WHO). De betekenis hiervan is onduidelijk, maar kan geassocieerd zijn met een verminderde fertiliteit.

Samengenomen, gelet op het gegeven dat de concentratie van HIV-remmers in semenplasma mogelijk niet van kritisch belang is voor het remmen van HIV in semen en gelet op de mogelijke toxiciteit van deze middelen op zaadcellen, heeft het vanuit het oogpunt van voortplanting de voorkeur om te kiezen voor middelen die niet in het lumen van het MGS doordringen. Verbetering van de effectiviteit, veiligheid, verdraagbaarheid en therapietrouw van HAART is nog steeds nodig.
Dankwoord
Dankwoord

“I have fought against white domination, and I have fought against black domination. I have cherished the ideal of a democratic and free society in which all persons live together in harmony and with equal opportunities. It is an ideal which I hope to live for and to achieve. But if needs be, it is an ideal for which I am prepared to die for.” (Nelson Mandela. An extract from his Rivonia trial speech, Pretoria Supreme Court, 20 April 1964)

HIV/AIDS is nog steeds een stigmatiserende aandoening. Ook met HIV geïnfecteerde personen hebben een droom, willen gelijke kansen en hopen op een goede toekomst. Met zeer effectieve en veilig “highly active antiretroviral therapy” is dit misschien wel haalbaar.

Dit proefschrift is tot stand gekomen dankzij de directe en indirecte bijdrage van vele personen. Hoewel de farmaceutische industrie en subsidie-verstrekkers nodig zijn om nieuwe geneesmiddelen te ontwikkelen en uit te testen en er onderzoekers nodig zijn om de studies te begeleiden en de resultaten hiervan te interpreteren, zonder de bereidwilligheid en opofferingsgezindheid van patiënten en controle personen om te participeren in (prospectieve, gerandomiseerde, dubbel blinde, placebo gecontroleerde) onderzoeken, zou het inzicht in een goede en verbetering van de medicamenteuze behandeling van een humane aandoening beperkt of niet mogelijk zijn. Primair veel dank aan de patiënten en controle personen die de studies in dit proefschrift mogelijk hebben gemaakt en de mede-onderzoekers, helpers, sponsoren en co-auteurs van deze studies.

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Dankwoord

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Curriculum vitae
Curriculum vitae

Antiretroviral therapy: Efficacy and toxicity in the ARES study and responses in the male genital tract

Curriculum vitae


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