The 'Triple Study': viral dynamics and immune reconstitution in HIV-1 infection during potent antiretroviral therapy
Notermans, D.W.

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CHAPTER II

DECREASE OF HIV-1 RNA LEVELS IN LYMPHOID TISSUE AND PERIPHERAL BLOOD DURING TREATMENT WITH RITONAVIR, LAMIVUDINE AND ZIDOVUDINE


Objectives: Triple-combination treatment of HIV-1 infection using two reverse transcriptase inhibitors and a protease inhibitor, can result in significant and sustained decreases in the quantity of viral RNA in peripheral blood. Lymphoid tissue, however, constitutes the major reservoir of HIV in infected patients. Study of the viral burden in these tissues has provided additional insight in the efficacy of antiretroviral treatment.

Design: Patients were randomized into two groups in order to study differences in the development of resistance to reverse transcriptase inhibitors. Group I started treatment with all three drugs simultaneously. Group II started with ritonavir monotherapy, aiming at initial reduction in virus production before the addition of lamivudine and zidovudine three weeks later.

Methods: Changes in the amount of HIV in plasma and tonsillar lymphoid tissue during 24 weeks of treatment with ritonavir, lamivudine and zidovudine were studied by reverse transcriptase polymerase chain reaction.

Results: Thirty-three antiretroviral-naive HIV-infected patients were included for analysis. After 24 weeks, median CD4⁺ cell count increased by \(152 \times 10^6/L\) and median plasma viral RNA levels decreased by at least \(2.87 \text{log}_{10}\text{ copies/mL}\). In 88% of the patients remaining on treatment, plasma RNA levels were below the quantification limit of the assay used (mean, \(2.4 \text{log}_{10}\text{ copies/mL}\)). The lymphoid tissue viral burden, ranging from 9.16 to 8.52 \text{log}_{10}\text{ copies/g} at baseline, was markedly reduced with at least \(2.1 \text{log}_{10}\text{ copies/g}\) by week 24 in five patients analyzed. Eight patients (24%) withdrew because of side-effects. In one patient in group II, ritonavir and lamivudine resistance-associated mutations developed.

Conclusions: Treatment with this triple antiretroviral drug combination produced a durable and strong decrease in HIV-1 RNA burden in both plasma and lymphoid tissue.

INTRODUCTION

Triple-drug combination treatment of HIV-1 infection using two reverse transcriptase (RT) inhibitors and a protease inhibitor can result in significant and sustained decreases of peripheral blood viral RNA.\(^{[1-3]}\)

The HIV-1 protease inhibitor ritonavir is one of the most potent antiretroviral drugs currently licensed \(^{[4,5]}\) and a decrease in HIV-RNA levels of \(1.94 \text{log}_{10}\text{ copies/mL}\) after eight weeks of monotherapy has been achieved.\(^{[6]}\) In most patients, monotherapy with ritonavir does not result in a sustained reduction of HIV replication and drug-resistant virus may emerge.\(^{[6,7]}\)

The potency of lamivudine (3TC) plus zidovudine (ZDV) has been demonstrated in several clinical trials.\(^{[8,11]}\) This combination rapidly selects for the amino acid M184V mutation in the RT enzyme, conferring phenotypic resistance against 3TC.\(^{[11]}\) Since the speed of resistance
development is theoretically dependent on the level of viral replication, it was hypothesized that lowering the viral turnover with a protease inhibitor before starting these nucleoside analogues will delay the development of resistance.

Lymphoid tissues constitute the major reservoir of virus and infected cells in HIV-infected patients, with the greatest viral burden consisting of virions associated with follicular dendritic cells. The palatine tonsils have been shown to be representative of the distribution of HIV in the lymphoid tissue, are readily accessible, and can be used for serial biopsies. Studies of changes in the amount of HIV in lymphoid tissue compared with peripheral blood during antiretroviral treatment have been reported, but in most of these studies patient follow-up was of limited duration and the antiretroviral therapy used did not contain a protease inhibitor. Therefore, lymphoid tissue has not been thoroughly studied during strong and sustained suppression of HIV replication.

In this study we report on the changes of viral RNA levels in tonsillar lymphoid tissue in comparison with peripheral blood during triple therapy of ritonavir, 3TC and ZDV. The effects of lowering the viral turnover with a protease inhibitor before starting the nucleoside analogues were studied by randomly assigning patients into two groups. Group I started treatment with all three drugs simultaneously, while group II started with ritonavir monotherapy, followed by the addition of lamivudine and zidovudine 3 weeks later.

METHODS

Patients and study treatment

Antiretroviral-naive HIV-1-infected patients, aged ≥ 18 years were eligible for inclusion if they had CD4⁺ lymphocyte counts of at least 50 x 10⁹/L and a plasma HIV-RNA level of at least 30,000 (4.48 log₁₀) copies/mL. Patients were recruited from three referral centers for HIV infection in The Netherlands from January to April 1996. Study participants were randomized into two groups: group I (immediate triple therapy) received an open-label triple combination of ritonavir 600 mg twice daily, 3TC 150 mg twice daily and ZDV 300 mg twice daily; group II (delayed triple therapy) received ritonavir monotherapy for 3 weeks before 3TC and ZDV were added, also on open label basis. An adaptive randomization method, minimization, was used based on CD4⁺ lymphocyte counts (< 200 or ≥ 200 x 10⁹ cells/L) as well as plasma HIV-RNA levels (< 5 log₁₀ or ≥ 5 log₁₀ copies/mL). Ritonavir was started at 300 mg twice daily and escalated to full dose in four days.

Women with a positive pregnancy test within 14 days of randomization were excluded, as were patients requiring medication that was contraindicated with the use of ritonavir (according to the package insert; Abbott, Abbott Park, IL, UAS). Presence of accessible tonsils was not mandatory. All patients gave written informed consent.
Laboratory Methods

Blood was sampled at weeks -2, -1, 0, 0.3 (day 3), 1, 1.3 (day 10), 2, 3, 4, 6 and 8, and every four weeks thereafter. Tonsillar biopsies were obtained from all patients in the study with accessible tonsillar tissue within 14 days prior to start of therapy and 24 weeks thereafter. Biopsy samples were frozen immediately and stored at -70°C.

Indicators of treatment efficacy included plasma HIV-RNA levels and absolute CD4+ lymphocyte counts. RNA levels were assessed using a commercially available RT polymerase chain reaction (PCR) assay, following the manufacturers instructions (Amplicor-HIV Monitor Test, Roche Molecular Systems, Branchburg, NJ, USA), with a variable lower quantification level (LQL). Mean ± SD plasma LQL for this study was 251 ± 148 (2.4 ± 0.2 log

Frozen tonsillar biopsy specimens were sectioned into 15 µm slices. Eighty slices of each specimen (mean total weight, 10 mg) were added to 1 mL of guanidine thiocyanate containing lysis buffer. After homogenization, 10 µL of each sample was added to 1 mL of new lysis buffer with internal standard RNA (Amplicor HIV Monitor Test) and 50 µL of silica suspension to bind nucleic acid. After centrifugation, the silica pellet was washed five times (twice with guanidine thiocyanate based wash buffer, twice with 70% ethanol and once with acetone). Subsequently, nucleic acid was eluted using 50 µL of Tris-EDTA based elution buffer. Five microlitres of this nucleic acid solution was added to 45 µL of specimen diluent (Amplicor HIV Monitor Test) and the samples were amplified using RT-PCR (Amplicor HIV Monitor Test), following the manufacturers protocol, for quantification of total viral nucleic acid (RNA plus DNA). Simultaneously, 5 µL of nucleic acid solution was amplified omitting the RT step (incubation for 30 minutes at 60°C) and thereby omitting the amplification of viral RNA. Since the assay did not include an internal DNA standard, the assessment of HIV DNA was qualitatively only. The amount of nucleic acid was expressed as copies/g of tissue, based on the internal RNA standard.

Genotypic resistance analysis of the protease and RT gene was performed at baseline, at week 3 and when possible at week 24. RNA isolated from 100 µL of serum was reverse-transcribed and PCR amplified. Subsequently, 5 µL of this PCR product was subjected to a nested PCR for 25 cycles with three different primer sets extended with the SP6 and T7 recognition site. A fivefold dilution of this nested PCR product was sequenced in one direction with dye-labeled T7 primers and analyzed on an automated sequencer (model 373, Applied Biosystems Inc., Foster City, CA, USA).

Statistical analysis

HIV-RNA levels were log_{10} transformed. Viral RNA levels below the LQL of the assay were assigned the corresponding cut-off values. Baseline values of HIV-RNA levels and CD4+ cell counts were calculated as the mean values of three pre-treatment assessments: two weeks and one week.
prior to treatment, and day of treatment initiation. The screening assessment was not included for the calculation of baseline to prevent potential regression to the mean. Patients withdrawn from study treatment were followed until a change of their antiretroviral treatment.

Linear repeated measures models were used to detect differences between the two treatment arms with repeated unequally spaced observations within the patients (ProcMixed of SAS version 6.11; SAS Institute, Cary, NC, USA).

The difference in proportions of patients reaching HIV-RNA levels below the LQL were analyzed with a two-tailed Fisher's exact test. Safety parameters were analyzed using Wilcoxon rank-sum tests for differences between the two groups and changes from baseline at week 12.

Protocol approval

The protocol was approved by the Scientific Advisory Board of the Dutch National AIDS Treatment Evaluation Centre and by the Institutional Review Boards of the participating centers.

RESULTS

Thirty-four patients were randomly assigned to treatment. One participant withdrew prior to the start of treatment and was excluded from further analysis. Eight persons withdrew before week 24, and three of the withdrawn persons were lost to follow-up. Baseline characteristics are shown in Table 1.

<table>
<thead>
<tr>
<th>No. participants (men/women)</th>
<th>Group I</th>
<th>Group II</th>
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</thead>
<tbody>
<tr>
<td>Median age (years)</td>
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<td>40</td>
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<td>Injecting drug use</td>
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<tr>
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<td>1</td>
</tr>
<tr>
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<td>6</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>4</td>
</tr>
<tr>
<td>Median (IQR)</td>
<td>5.27 (5.00-5.72)</td>
<td>5.37 (4.88-5.63)</td>
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<tr>
<td>Plasma RNA (log_{10} copies/mL)</td>
<td>177 (120-230)</td>
<td>134 (94-245)</td>
</tr>
<tr>
<td>CD4+ cell count (× 10^3/L)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Plasma HIV-RNA levels

Within the first three weeks, group I showed a significantly greater decline in plasma load (median, -2.09 log_{10} copies/mL) compared to group II (median, -1.91; p = 0.0079) (Fig. 1). The levels continued to decline at a slower rate, stabilizing at about 2.8 log_{10} copies/mL below baseline by week 12. When analyzed for the full 24 weeks of treatment, no significant difference remained between the two groups (p = 0.54).

Twelve (86%) out of 14 patients from group I and 11 (69%) out of 16 in group II achieved an HIV load below LQL at 24 weeks in an intention-to-treat analysis (p = 0.40 for the two treatment arms). An on-treatment analysis showed that this was achieved in 11 (92%) out of 12 patients in group I and in 11 (85%) out of 13 in group II (Fig. 2).

Fig. 1. Plasma viral load. HIV-RNA levels expressed as log_{10}-transformed changes compared with baseline. Median values and quartiles for the immediate (I) and delayed (II) groups are shown. The number of analyzed patients at each timepoint is indicated below the graph.

Tonsillar tissue HIV nucleic acid load

Paired tonsillar biopsies, obtained pre-treatment and at week 24, were available for (RT)-PCR analysis from five individuals. In all five patients, total HIV nucleic acid levels in the tissue declined substantially. Baseline values ranged from 9.16 to 8.52 log_{10} copies/g and declined to 6.88-5.88 log_{10} copies/g or lower at week 24 (Fig. 3). The ratio of optical density values of PCR with and without RT-step ranged between 197 and 1754 at
baseline, strongly suggesting that HIV-1 DNA contributed a minor fraction of the total amount of HIV nucleic acids in the lymphoid tissue at that timepoint. In these five patients, plasma HIV-RNA levels decreased substantially to levels below the LQL by week 24 (Fig. 3).

![Graph showing percentage of patients with RNA levels below the quantification limit.](image)

Fig. 2. Percentage of patients with RNA levels below the quantification limit. Viral load was measured using reverse transcriptase polymerase chain reaction (Amplicor HIV Monitoring Test), which had a mean quantification limit of $251 \pm 148$ (2.4 ± 0.2 log10) copies/mL in this study. The immediate (I) and the delayed (II) triple groups are shown in an intention-to-treat and an on-treatment analysis.

**Genotypic resistance**

At baseline one patient had two mutations in the RT gene (M41L and T215D). Mutations at these positions are associated with ZDV resistance,[23] although the phenotypic resistance of this combination, with the aspartic acid at position 215, has not been established. The plasma HIV-RNA levels in this patient declined to values below the LQL, similar to the levels in the other participants. None of the patients had the M184V mutation in RT, which is associated with 3TC resistance.[24] No mutations were present at baseline at position 82 of protease, where the first mutations are described to occur in the development of ritonavir resistance,[6,7,27] but variation was seen at positions 36 or 71 or both in six patients.
After three weeks, none of the patients had an M184V mutation in RT. One patient in group II, starting with ritonavir monotherapy, developed a V82A mutation in protease at week 3. After an initial decline in viral RNA levels, this patient later had acquired additional protease (I84V) and RT (M184V) mutations, probably due to poor compliance. It was not possible to sequence the HIV genome at week 24 in any other subject, due to the very low levels of plasma HIV RNA.

**CD4**+ lymphocytes

At week 24, group I had a median increase in absolute CD4+ lymphocytes of 180 \times 10^3/L and the delayed group of 99 \times 10^3/L (Fig. 4). After correction for baseline values, the difference between the groups was not statistically significant during the 24-week period (p = 0.11).

**Adverse- and HIV-related events**

Apart from the recurrence of oral candidiasis in two patients no other HIV-related events occurred.

The most common adverse events involved the gastro-intestinal tract (diarrhea and nausea), and eight patients (24%) withdrew from the study because of adverse events (Table 2). Peri-oral paresthesia and flushes, commonly associated with ritonavir, were frequently seen (67% and 36%, respectively), but did not lead to withdrawal and rarely to dosage changes. To Prevent development of resistance, ritonavir dosage was not lowered below 500 mg twice daily, except in the case of dose escalation after a prolonged interruption.
Fig. 4. Changes in T-cell subsets. Median changes in absolute CD4+ lymphocyte counts ($\times 10^6$/L) are shown. The number of analyzed patients at each timepoint is indicated below the graph.

Table 2. Adverse events, reported in at least 20% of patients. *Excluding withdrawn patients. Dose reductions include slower dose escalations of ritonavir in case of adverse events occurring directly upon initiation of therapy, but do not include ritonavir dose escalation after ritonavir interruption for more than 1 month.
No statistically significant difference was found between the two groups at 12 weeks for alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, amylase, triglycerides, cholesterol, hemoglobin, white blood cell count or neutrophils. Triglyceride levels rose from a median of 1.85 mmol/L at baseline to 3.99 mmol/L at week 12 in group I and from 1.32 to 3.21 mmol/L in group II. One patient experienced a grade 4 rise in plasma triglycerides, prompting treatment interruption for 10 days. Cholesterol levels rose from 4.80 to 5.80 mmol/L in group I and from 3.90 to 5.05 mmol/L in group II.

**DISCUSSION**

The combination of ritonavir, 3TC and ZDV has potent antiviral activity in both plasma and lymphoid tissue, lasting for at least 24 weeks. The decline in HIV-RNA levels showed a biphasic pattern, with a steep decline of approximately two log₁₀ during the first three weeks and a slower decline thereafter. This second phase probably reflects continuing but declining recruitment of viral particles into the circulation from long-lived virus-producing cell populations such as macrophages, gradual activation of latently infected cells, and release of trapped virions from tissue. With 3TC-ZDV combination therapy in antiretroviral-naïve patients, an initial decline of approximately 1.5 log₁₀ copies/mL has been achieved.

Immediate triple therapy during the first three weeks in this study (group I) resulted in an HIV-RNA level that was slightly (0.18 log₁₀ copies/mL) but significantly lower than that achieved with ritonavir monotherapy in group II. This implies that a greater rate of HIV-RNA decline can be achieved with more powerful combinations.

The strong decline of the HIV load in lymphoid tissue shows that the changes measured in plasma load also take place in lymphoid tissue. Exact calculations of the rates of decline in lymphoid tissue were not possible due to the limited number of measurements and the RNA levels falling below LQL at week 24 in two patients. The RT-PCR assay used in this study was designed for use in plasma and the characteristics for use in tissue samples have not been fully determined. The viral load in lymphoid tissue consists, to a large extent, of virus particles associated with follicular dendritic cells, and therefore of genomic RNA. The measurements of viral nucleic acids in the lymphoid tissue with RT-PCR have to be considered a composite of messenger RNA and genomic RNA in a DNA background. Comparison of the results of RT-PCR with and without the RT step suggests the relative contribution of DNA at baseline was minor, in accordance with other studies. The relative contribution of the different nucleic acids may change with therapy, as reflected by the lower ratio of optical density values of PCR with and without the RT-step found on week 24. Nevertheless, the amount of nucleic acids was calculated in copies/g of tissue based on the internal RNA standard only, because the lack of an internal DNA standard did not allow a further distinction. In the same
study population the tissue RNA load has been measured using in situ hybridization and a load decline of the same order of magnitude was found.\[32\] Pre-existing resistance did not play a role in response to treatment. Sequencing of the protease gene and part of the RT gene showed a possible pre-treatment ZDV resistance mutation in one patient only, and plasma RNA levels in this patient declined at the same rate as in the other patients. Some variation in the protease gene was found at baseline in a few patients, although not at the key position 82 \[6,7\] and the mutations discovered did not influence the decline of viral load. By week 3, no further RT mutation associated either with ZDV or 3TC resistance was found. Even though the absence of resistance mutations at week 24 in all patients but one could not be fully proven, because the amount of viral RNA available was insufficient for sequencing, it is unlikely that patients with a strong and persistent reduction of HIV-RNA levels harbored resistant virus. This indicates that a potent combination of three antiretroviral compounds may prevent the occurrence of resistance.

The majority of patients were able to tolerate the regimen, even though a considerable number of adverse events occurred without a clear difference between the two groups. A slower dose escalation than used in this study might diminish the occurrence of the events during the first few weeks of treatment.

The median increase in peripheral CD4\(^+\) lymphocytes in this study parallels that observed with other triple drug combinations including a protease inhibitor.\[31,33\] Strong suppression of HIV-RNA levels in both peripheral blood and lymphoid tissue is presumed to be essential to obtain durable control over HIV infection.\[33,34\] Our study demonstrates that this can be achieved by combining ritonavir with 3TC and ZDV. At present, several other potentially powerful combinations are under investigation. Analyzing the effects of these combinations on the lymphoid viral replication should be an essential part of these evaluations, at least until it has been established more definitively that long-lasting suppression of HIV in the peripheral blood is associated with declines in the lymphoid tissue. Longer follow-up is warranted to determine whether the antiretroviral effects in both plasma and lymphoid tissue are sustained.

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REFERENCES


**APPENDIX**

**In addition to the study authors, the Ritonavir/3TC/ZDV Study Group included the following persons: Elly Baan, Esther M. van Egmond, Joep M.A. Lange, M.D., Remko van Leeuwen, M.D., Gerrit Jan Weverling, M.D. (Academic Medical Centre, Amsterdam, The Netherlands); Ashley T. Haase, M.D. (University of Minnesota, Minneapolis, MN, USA); Keith Henry, M.D. (HIV Program, St. Paul-Ramsey Medical Center, St. Paul, MN, USA); Frank Miedema, Ph.D., Maarten Koot, Ph.D., Marijke Roos, Nadine G. Pakker, Peter T.A. Schellekens, M.D. (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands).**