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Discontinuation of secondary prophylaxis against penicilliosis marneffei in AIDS patients after HAART

Romanee Chaiwarith, Noppawan Charoenyos, Thira Sirisanthana and Khuanchai Supparatpinyo

A retrospective cohort study was conducted to determine the relapse rate of penicilliosis marneffei after the discontinuation of itraconazole secondary prophylaxis in 33 HIV-infected patients who received HAART and had CD4 cell counts of 100 cells/μl or greater for at least 6 months. The observed incidence of relapse of penicilliosis marneffei was zero cases per 641 person-months (95% confidence interval 0–0.6 cases per person-month) after a median follow-up of 18 months (range 6–45).

Penicilliosis marneffei, a systemic infection caused by the dimorphic fungus *Penicillium marneffei*, is most commonly found in southeast Asian countries, India, Taiwan, Hong Kong, and southern China [1]. It is the third most common AIDS-defining opportunistic infection in northern Thailand, accounting for 15–20% of presenting AIDS-related illnesses [2]. This disseminated fungal infection develops more commonly in HIV-infected patients who have CD4 cell counts of less than 100 cells/μl [3]. The common clinical manifestations include fever, anaemia, weight loss, and generalized skin papules with central umbilication [2]. A presumptive diagnosis could be made by the identification of characteristic septate yeast-like organisms under microscopic examination of the Wright's-stained samples of bone marrow aspirate, and touch smears of the skin biopsy and lymph node biopsy specimens. *P. marneffei* could be isolated from various clinical specimens including blood, skin, bone marrow, and lymph node. Treatment with intravenous amphotericin B for 2 weeks, followed by oral itraconazole for 10 weeks, is highly effective and safe [4]. A study from Chiang Mai University showed that 50% of patients had a relapse of penicilliosis marneffei within 6 months after the discontinuation of antifungal therapy [5]. A double-blind, placebo-controlled study by Supparatpinyo *et al.* [6] demonstrated that oral itraconazole 200 mg a day for secondary prophylaxis in AIDS patients reduced the relapse rate of penicilliosis marneffei from 57 to 0% ($P < 0.001$). A lifelong secondary prophylaxis with itraconazole has become the standard of care for this systemic fungal infection in Thailand.

Data from developed countries have shown that primary and secondary prophylaxis for several opportunistic infections can safely be discontinued when patients have received HAART and their CD4 cell counts are persistently greater than the values associated with a high risk of the development of specific opportunistic infections [7–10]. For penicilliosis marneffei, there has been only one observation by Hung *et al.* [11] in 14 HIV-infected patients who had received HAART with favourable immunological response and discontinued secondary prophylaxis. No relapse was reported after a median follow-up duration of 18 months. However, the sample size was relatively small and the level of CD4 cells at the time of antifungal discontinuation was not specified.

We conducted a retrospective cohort study at Chiang Mai University Hospital to evaluate the safety of discontinuation of secondary prophylaxis for penicilliosis marneffei in AIDS patients who were receiving HAART and had a sustained immunological response. The study was approved by the Faculty of Medicine, Chiang Mai University Ethical Committee. Inclusion criteria included: (i) patients were 15 years of age or older; (ii) had previously been documented as having culture-confirmed penicilliosis marneffei; (iii) were successfully treated with systemic antifungal therapy; (iv) had received oral itraconazole 200 mg a day for secondary prophylaxis; (v) received HAART, defined as two nucleoside reverse transcriptase inhibitors plus either a non-nucleoside reverse transcriptase inhibitor or a protease inhibitor; (vi) had a favourable immunological response to HAART, defined as having a CD4 cell count of 100 cells/μl or greater for at least 6 months; and (vii) had discontinued itraconazole secondary prophylaxis. The decision to discontinue itraconazole secondary prophylaxis was made by attending physicians with consultation from infectious disease specialists. This followed the recommendation from the Thai Ministry of Public Health that antifungal prophylaxis could be discontinued in patients who had CD4 cell counts of 100 cells/μl or greater for at least 6 months after antiretroviral therapy. All patients were scheduled for follow-up every 2 months to evaluate the evidence of relapse of penicilliosis marneffei. The relapse rate was expressed as the number of events per person-months of follow-up. Statistical analyses were performed using SPSS version 10.0 (SPSS for Windows, Rel. 13.0.1997; SPSS Inc., Chicago, Illinois, USA).

From March 2002 to December 2005, there were 33 HIV-infected patients with culture-confirmed penicilliosis marneffei who met all the eligibility criteria for the

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Table 1. Clinical characteristics of 33 HIV-infected patients with penicilliosis marneffei

Characteristic	$N = 33$
Mean age (range) years	35.6 (23–59)
Sex: male/female (no.)	19/14
Mode of HIV transmission (no.)	
Heterosexual	32
Blood transfusion	1
Duration of HIV infection, years (no.)	
≥ 10	4
5–10	11
< 5	18
HAART regimen (no.)	
Lamivudine, stavudine, nevirapine	25
Lamivudine, stavudine, efavirenz	3
Zidovudine, lamivudine, nevirapine	2
Zidovudine, lamivudine, efavirenz	1
Zidovudine, lamivudine, saquinavir, ritonavir	1
Lopinavir/ritonavir, saquinavir	1
CD4 cell counts: mean (range)	
Before initiation of HAART	50 cells/μl (10–340)
At the time of itraconazole discontinuation	250 cells/μl (130–640)
At the time of study completion	329 cells/μl (137–860)
No. of penicilliosis marneffei episodes	
Before initiation of HAART	0
Before initiation of the study	0
After itraconazole discontinuation	0

discontinuation of itraconazole secondary prophylaxis. The clinical characteristics of these patients are shown in Table 1. The median duration of antifungal secondary prophylaxis before study enrollment was 25 months (range 8–83). The median duration of HAART before the discontinuation of antifungal secondary prophylaxis was 13.0 months (range 6–49). The CD4 cell counts at the time of discontinuation of itraconazole secondary prophylaxis and the time of study completion were significantly higher than the baseline values before the initiation of HAART ($P < 0.05$). Plasma HIV-RNA measurement was performed in 26 patients; 24 of those (92.3%) had undetectable HIV viral loads at the end of the study. In one patient who had an HIV viral load of 53 307 copies/ml, the antiretroviral regimen was changed to lopinavir/ritonavir and saquinavir. The antiretroviral regimen was not changed in the other patient who had an HIV viral load of 2165 copies/ml.

At the time of study completion, none of the 33 patients were lost to follow-up. The median length of follow-up was 18.0 months (range 6–45), with a total follow-up of 641 person-months. Twenty-four patients (72.7%) had a follow-up duration of more than 12 months. Throughout the follow-up duration, no patients had symptoms or signs suggestive of systemic fungal infection. There was no relapse of penicilliosis marneffei; the observed incidence of relapse was zero cases per 641 person-months [95% confidence interval (CI) 0–0.6 cases per 100 person-months]. No patients died during the study period.

Our study has demonstrated the safety of the discontinuation of secondary prophylaxis in AIDS patients with a previous history of penicilliosis marneffei who had a sustained increase of the CD4 cell count to 100 cells/μl or greater after HAART. The patients were followed for a total of 641 person-months after the discontinuation of antifungal prophylaxis. There was no relapse among this group of patients, giving the relapse rate of 0.6 cases per 100 person-months at the upper limit of the 95% confidence interval. This is in marked contrast to the relapse rate of 11.5 cases per 100 person-months (95% CI 7.2–17.2) obtained in patients without secondary antifungal prophylaxis in one of our studies conducted before the HAART era [6]. In that study, 35 HIV-infected patients with successfully treated culture-confirmed penicilliosis marneffei who were not given secondary antifungal prophylaxis were followed for a total of 174 person-months. There were 20 relapses among the 35 patients.

Our study has the drawback of using a retrospective cohort and comparing the relapse rate with that obtained from a historical control group. However, because of the magnitude of the difference between the two relapse rates and the fact that the two studies were conducted at the same university hospital drawing from the same pool of patients, we are confident to conclude that it is safe to discontinue itraconazole secondary prophylaxis in HIV-infected patients with a previous history of penicilliosis marneffei who have started HAART and have had a CD4 cell count of 100 cells/μl or greater for at least 6 months. A randomized controlled trial should, however, be conducted to address this issue more definitively.

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Department of Medicine, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand.

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Three-year follow-up of protease inhibitor-based regimen simplification in HIV-infected patients

Esteban Martínez[a], Juan A. Arnaiz[a], Daniel Podzamczer[b], David Dalmau[c], Esteban Ribera[d], Pere Domingo[e], Hernando Knobel[f], Maria Leyes[g], Enric Pedrol[h], Luís Force[i], Elisa de Lazzari[a] and José M. Gatell[a]

Patients with sustained virological suppression on protease inhibitor (PI)-based therapy were randomly assigned to switch the PI to nevirapine ($n = 155$), efavirenz ($n = 156$), or abacavir ($n = 149$) and were followed for at least 3 years regardless of the discontinuation of assigned therapy. There was a higher probability of maintaining virological suppression after 3 years of follow-up with nevirapine or efavirenz than with abacavir. In contrast, abacavir showed a lower incidence of adverse effects leading to drug discontinuation.

The concept of simplification emerged as a need for adherent HIV-infected patients receiving successful complex or unconvenient antiretroviral regimens, and it has now been incorporated into the updated recommendations for the use of antiretroviral therapy [1]. The NEFA Study was a multicentre, randomized, open-label clinical trial. Eligible patients were HIV-1-infected adults who were receiving triple antiretroviral therapy consisting of at least one protease inhibitor (PI) plus two nucleoside reverse transcriptase inhibitors (NRTI), who had had plasma HIV-1-RNA levels of less than 200 copies/ml for at least 6 months, and who wished to change the PI component of their regimen. Patients were randomly assigned to receive nevirapine, efavirenz, or abacavir instead of the PI used in their current antiretroviral regimen while their NRTI remained unchanged. The study was initially designed to be a 48-week study [2], but it was extended to at least 3 years following recommendations from the European health authorities in studies of maintenance therapy with simplified regimens in patients showing adequate virological control [3].

The primary study endpoint was the occurrence of death, progression to AIDS, or a virological failure throughout the study period. Secondary endpoints were the CD4 cell count, the incidence of side effects, and plasma lipids. According to recently published recommendations on virological-based endpoints in antiretroviral drug switching trials, patients were followed for the entire duration of the trial regardless of the premature discontinuation of assigned therapy [4]. 'Switching equal to failure' intent-to-treat (considering as failures all patients who died, progressed to AIDS, had detectable viral loads, discontinued study medication, or were lost to follow-up) and as-treated analyses were performed.

Among 460 patients eligible for the study, 155 were assigned to nevirapine, 156 were assigned to efavirenz, and 149 were assigned to abacavir. The baseline characteristics of the patients were not significantly different among the groups. Approximately half of the patients in each arm had received therapies with one or two NRTI before PI-containing antiretroviral therapy. Indinavir ($n = 278$) and nelfinavir ($n = 135$) were the PI withdrawn in 90% of patients. Lamivudine with either stavudine ($n = 259$, 56%) or zidovudine ($n = 145$, 32%) were the combinations of NRTI most commonly used. The outcomes of the patients can be seen in Table 1. At 36 months, the Kaplan–Meier estimates to reach a protocol-defined endpoint in the nevirapine, efavirenz and abacavir arms were 33, 46, and 40% according to the intent-to-treat analysis (generalized log-rank test, $P = 0.068$). According to the intent-to-treat analysis, the Cox's proportional hazards survival regression showed a hazard ratio of failure of 0.817 [95% confidence interval (CI) 0.562–1.187] in the nevirapine arm and of 1.242 (95% CI 0.881–1.749) in the efavirenz arm compared with the reference hazard ratio of 1 in the abacavir arm ($P = 0.070$). In the as-treated analysis, the Kaplan–Meier estimates to reach a protocol-defined endpoint at

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Table 1. Outcome of therapy.

	Nevirapine ($n=155$)	Efavirenz ($n=156$)	Abacavir ($n=149$)
Death[a]	1	4	2
Progression to AIDS	0	1	2
Virological failure			
While taking study medication	10	11	29
After switching study medication	6	7	1
Lost to follow-up	8	12	9
Switched study medication without virological failure	26	37	17

[a]No death was attributable to antiretroviral therapy.

36 months in the nevirapine, efavirenz and abacavir arms were 7, 10, and 22% (generalized log-rank test, $P<0.001$), respectively. According to the as-treated analysis, the Cox's proportional hazards survival regression showed a hazard ratio of failure of 0.302 (95% CI 0.153–0.598) in the nevirapine arm and 0.472 (95% CI 0.260–0.858) in the efavirenz arm compared with the reference hazard ratio of 1 in the abacavir arm ($P=0.001$).

Among patients who had received previous single or double therapies with NRTI ($n=238$), the rate of virological failure while on study medication in the abacavir arm (25 of 69, 36%) was significantly higher that those in the nevirapine (six of 79, 8%) or efavirenz (10 of 90, 11%) arms ($P<0.001$). In contrast, there were no differences in the rates of virological failure among the three arms among patients without previous suboptimal therapy with NRTI ($n=222$): four of 76 patients (5%) in the nevirapine arm, one of 66 patients (2%) in the efavirenz arm, and four of 80 patients (5%) in the abacavir arm ($P=0.479$).

The incidence of adverse events leading to study discontinuation was significantly lower in the abacavir group ($n=13$, 9%) than in the nevirapine group ($n=29$, 19%) or the efavirenz group ($n=39$, 25%; $P=0.005$). Adverse effects leading to discontinuation were among those expected for each study drug, and interestingly most of them occurred early after the introduction of each study drug. Sixty-two of the 82 patients (77%) who discontinued study drugs did so in the first 12 months of the study, and most of them in the first weeks of therapy. An interesting observation emerging from the long follow-up in our study was that most adverse effects leading to the discontinuation of study drugs in the cases of nevirapine and abacavir were concentrated almost exclusively in the first weeks of the study. There was only one (1%) case of clinical hepatitis and four (3%) of increased aminotransferase levels leading to discontinuation of study drug in the nevirapine arm, and all these occurred in the first year of the follow-up. Interestingly, nevirapine-related exanthema and hepatitis have been reported less frequently in antiretroviral-experienced patients with virological suppression [5,6]. In the case of patients assigned to efavirenz, neuropsychiatric adverse effects were those most commonly leading to discontinuation, and they were scattered during follow-up. In general, the patients who discontinued efavirenz late during the study did so because of the persistence of neuropsychiatric effects rather than because of its severity. The median fasting plasma triglyceride values at each timepoint were not significantly different between the arms. The median fasting plasma cholesterol values at all but the month 30 follow-up visit ($P\leq 0.012$) were significantly lower in the abacavir than in the nevirapine and efavirenz arms.

In summary, simplification of PI-containing HAART in patients with sustained virological response had a higher probability of maintaining the suppression of viral replication after 3 years of follow-up when nevirapine or efavirenz were substituted for PI compared with abacavir. However, the rates of viral suppression among patients who had not had previous suboptimal therapy with NRTI were similar for the three drugs. In contrast, abacavir showed a lower incidence of adverse effects leading to discontinuation and caused a greater decrease in plasma cholesterol.

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Hospital Vall d'Hebron, Barcelona (Esteban Ribera, Manuel Crespo).

Hospital de Viladecans, Viladecans (Manuel Javaloyas).

[a]Hospital Clínic, Barcelona, Spain; [b]Hospital de Bellvitge, L'Hospitalet, Barcelona, Spain; [c]Hospital de Mútua de Terrassa, Terrassa, Spain; [d]Hospital de Vall d'Hebrón, Barcelona, Spain; [e]Hospital de la Santa Creu i Sant Pau, Barcelona, Spain; [f]Hospital del Mar, Barcelona, Spain; [g]Hospital Son Dureta, Palma de Mallorca, Spain; [h]Hospital General de Granollers, Granollers, Spain; and [i]Hospital de Mataró, Mataró, Spain.

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Stable pattern of HIV-1 subtype C Gag-specific T-cell responses coincides with slow rate of CD4 T-cell decline in HIV-infected Ethiopians

Aster Tsegaye[a], Leonie Ran[b], Dawit Wolday[a], Beyene Petros[c], Nening M. Nanlohy[b,e], Hailu Meles[a], Mulu Girma[a], Ermias Hailu[a], José Borghans[b,e], Frank Miedema[b,d,e] and Debbie van Baarle[b,e]

We studied HIV-1 clade C Gag-specific T-cell responses in five HIV-infected Ethiopians with a relatively slow (< 15 cells/μl per year) and five with a fast (> 45 cells/μl per year) CD4 T-cell decline longitudinally. Six study subjects had T-cell responses directed to one or more HIV-1 Gag peptides. The persistence of strong and broad anti-Gag cytotoxic T-lymphocyte responses was associated with a slow rate of CD4 T-cell decline and with human leukocyte antigen alleles from the B27 supertype.

Longitudinal data demonstrating the role of cytotoxic T lymphocytes (CTL) in the course of HIV -infection are available mainly from clade B-infected individuals and have revealed the persistence of strong and broad CTL responses in long-term non-progressors [1–3]. The breadth of CTL directed against HIV proteins was stable over time and was shown to be negatively correlated with viral load and disease progression [4]. Despite low CD4 T-cell numbers and a persistently activated immune system [5–7], HIV disease progression in clade C-infected Ethiopians is comparable to that in developed nations [8]. To compare individuals with fast and slow disease progression, HIV-specific T-cell responses were analysed in the course of HIV infection in 10 antiretroviral-naive HIV-1 subtype C-infected Ethiopian factory workers who participated in a long-term cohort study on HIV-1 incidence and progression [9,10].

Almost all subjects in the slow CD4 T-cell decline group had stable CD4 T-cell levels, varying from 100 to 966 cells/μl at the first HIV-positive visit and from 56 to 1061 cells/μl after 5 years of follow-up, with a maximum CD4 T-cell loss of 13 cells/μl per year (Fig. 1a), which is smaller than the median yearly CD4 T-cell loss in HIV-infected Ethiopians (32 cells/μl blood) [8]. The viral load ranged from 1.9 to 4.3 log and declined in two subjects (subjects A and D) and increased in three subjects (subjects B, C, and E). CD8 T-cell numbers remained stable between 500 and 600 cells/μl in two subjects

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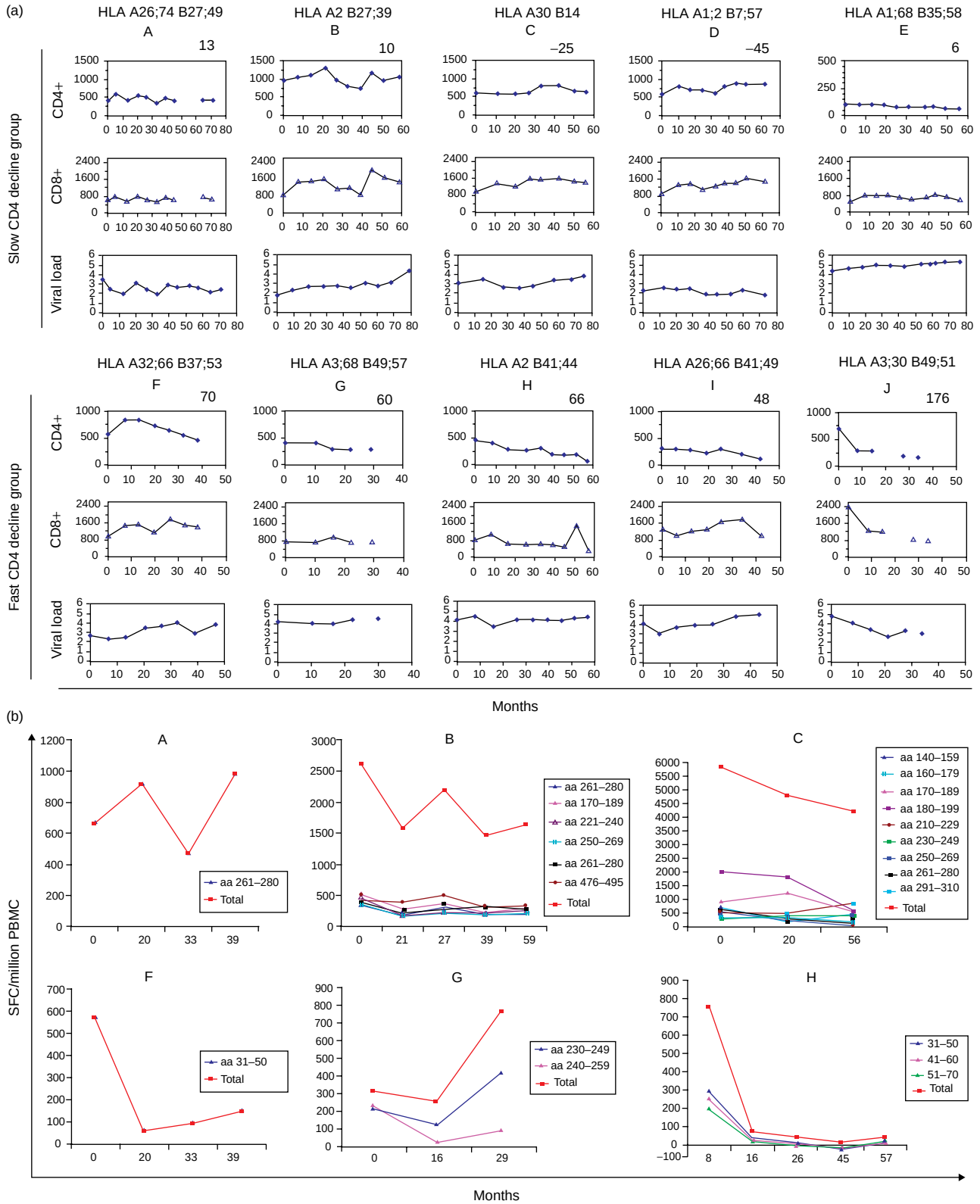

Fig. 1. CD4 and CD8 T-cell and viral load dynamics in subjects studied longitudinally and longitudinal analysis of HIV-1 subtype C Gag-specific cytotoxic T-lymphycyte responses in HIV-infected Ethiopians. (a) CD4 and CD8 T-cell (per μl blood) and viral load (log copies/ml) dynamics in subjects studied longitudinally ($n = 10$). Subjects A–E from the slow CD4 T-cell decline group (panel 1) and subjects F–J from the fast CD4 T-cell decline group (panel 2). Subjects F, G and J are incident cases. The human leukocyte antigen A and B type of each individual is indicated above the patient identification. The average CD4 T-cell loss/μl per

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(A and E) and increased in the other three subjects (75–95 cells/μl per year).

CD4 T-cell counts in individuals with a relatively fast CD4 T-cell decline ranged from 310 to 826 cells/μl at the first visit, and declined to 74–456 cells/μl at the last visit. The yearly CD4 T-cell loss in the fast CD4 T-cell decline group ranged from 48 to 176 cells/μl, which is significantly higher than that in the slow CD4 T-cell decline group ($P = 0.009$, Mann–Whitney test; Fig. 1a). CD8 T-cell counts declined in all subjects, which coincided with a severe depletion of the CD4 T-cell counts at the last timepoints analysed. The viral load increased in all cases except one.

The CD8 T-cell count in the fast CD4 T-cell decline group at intake was significantly higher (772–2429 cells/μl) than in the slow CD4 T-cell decline group (514–1000 cells/μl; $P = 0.047$). CD4 T-cell counts ($P = 0.92$) and viral load ($P = 0.14$) at intake were no different between the two groups (Mann–Whitney test).

Over a timeframe of 3–5 years, HIV-1 subtype C Gag-specific CTL responses were evaluated by IFN-γ enzyme-linked immunospot assay after stimulation with a total pool of 49 clade-C (isolate 96 ZM 651.8) specific synthetic Gag peptides (20 amino acids in length overlapping by 10 amino acids), and 14 subpools each containing seven peptides (final concentration 2 μg/ml for each peptide) to identify the specific peptide(s) responsible for the observed T-cell responses [11].

Six out of the 10 study subjects, three with a fast and three with a slow CD4 T-cell decline, showed CTL directed to one or more HIV-1 Gag peptides [responders, > 100 spots/10^6 peripheral blood mononuclear cells (PBMC); Fig. 1b], whereas the remaining four lacked detectable Gag-specific T-cell responses despite a good response to mitogens (phytohaemagglutinin; non-responders) confirming our previous cross-sectional study in which 40% of individuals also lacked detectable Gag-specific T-cell responses (A. Tsegaye *et al.*, 2006, in preparation). The lack of HIV-1 Gag-specific responses persisted over time during a follow-up of 4–5 years and was not explained by differences in stages of disease progression, as non-responsiveness was observed irrespective of the rate of CD4 T-cell decline.

The magnitude of the total anti-Gag CTL response was below 800 spot-forming cells (SFC)/10^6 PBMC in the fast CD4 T-cell decline group. In two of the individuals with fast CD4 T-cell decline, CTL responses declined to less than 200 SFC/10^6 PBMC. In contrast, in the slow CD4 T-cell decline group, the total response ranged from 600 to 6000 SFC/10^6 PBMC at the first timepoint, and persisted over time (Fig. 1b). Similarly, Betts *et al.* [12] showed a role for HIV-specific CTL in long-term survivors with a slow rate of CD4 T-cell loss (18 cells/μl per year).

Most peptides targeted by the slow CD4 T-cell decline group were from the P24 region of Gag, whereas responses against p17 (amino acids 31–70) dominated in the fast CD4 T-cell decline group. As a whole, more conserved epitopes that are less prone to CTL escape are found in the p24 region of Gag [13], and may thus give a persistent CTL response leading to CD4 T-cell preservation. Remarkably, all of the individuals with a slow CD4 T-cell decline responded to the p24 peptide ^{261}IYKRWIILGLNKIVRMYSPV^{280}, whereas none of the individuals in the fast CD4 T-cell decline group did, suggesting that a persistent response to this p24 peptide may be beneficial. The three individuals responding to this peptide shared human leukocyte antigen alleles belonging to the B27 supertype (Fig. 1b), making it likely that the response against this peptide is B27 restricted and suggesting a protective role for this supertype.

CTL responses from two of the three responders in the slow CD4 T-cell decline group were directed against

Fig. 1. (*Continued*)
year (△) is indicated for each individual within the CD4 cell plots. Subjects C and D showed increases in CD4 cell numbers. The X axis indicates time in months from entry into the cohort up to the last timepoint studied. (b) Longitudinal analysis of HIV-1 subtype C Gag-specific cytotoxic T lymphocyte (CTL) responses in HIV-infected Ethiopians. The magnitude of HIV-1-specific CTL responses directed against overlapping synthetic peptides spanning Gag, whose sequence corresponds to the Zambian 96 ZM651.8 strain, was determined in the six responder study subjects in the longitudinal analysis (subjects A–C, F–H). The magnitude of response is given as spot-forming cells per million input peripheral blood mononuclear cells (SFC/million PBMC). Subjects with slow CD4 T-cell decline throughout their follow up visit are shown in the upper panels and subjects with fast CD4 T-cell decline are shown in the lower panels. The X axis indicates the timepoints studied in months from entry into the cohort up to the last timepoint studied. Total indicates the response against the whole Gag clade C peptide pool. Separate peptide responses are indicated as amino acid sequences in p17 for which amino acids (aa) 31–50 = IKHLVWASRELERFALNPGL; aa 41–60 = LERFALNPGLLETSEGCKQI; aa 51–70 = LETSEGCKQIMKQLQPALQT and in p24 for which aa 140–159 = MVHQKLSPRTLN AWVKVIE; aa 160–179 = EKAFSPEVIPMFTALSEGAT; aa 170–189 = MFTALSEGATPQDLNTMLNT; aa 180–199 = PQDLNT MLNTVGGHQAAMQM; aa 210–229 = EWDRLHPVHAGPIAPGQMRE; aa 221–240 = GPIAPGQMREPRGSDIAGTT; aa 230–249 = RGSDIAGTTSTLQEQIAWM; aa 240–259 = STLQEQIAWMTSNPPIPVGD; aa 250–269 = TSNPPIPVGDIYKRWII LGL; aa 261–280 = IYKRWIILGLNKIVRMYSPV; aa 291–310 = EPFRDYVDRFFKTLRAEQAT and aa 476–495 = DREAL TSLKSLFGSDPLSQ.

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several Gag peptides (six and nine peptides) including the peptide ^{170}MFTALSEGATPQDLNTMLNT^{189}, which contains the most dominantly targeted epitope in South African individuals, TL9 [14,15]. In contrast, the breadth of the Gag-specific CTL response in the group with a relatively fast CD4 T-cell decline was narrow; the three responders in this group responded to one, two, and three peptides each.

Taken together, poor and declining CD8 T-cell responses mainly directed to p17 Gag coincided with fast CD4 T-cell decline, whereas high, persistent and broad CD8 T-cell responses mainly directed to p24 Gag correlated with slow CD4 T-cell loss. Our finding that T cells directed against dominant peptides persisted over time is promising for the design and development of effective vaccines appropriate for the local population. Furthermore, the identified peptides are part of the recently identified immunodominant epitope-rich long amino acid stretches in South African individuals [16], which contain multiple epitopes that are frequently targeted. Therefore, our data suggest that vaccines focusing on p24 Gag might be appropriate for subtype C-prevailing African regions.

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[a]Ethio-Netherlands AIDS Research Project, Ethiopian Health and Nutrition Research Institute, PO Box 1242, Addis Ababa, Ethiopia; [b]Department of Clinical Viro-Immunology, Sanquin Research at CLB and Landsteiner Laboratory, University of Amsterdam, Amsterdam, the Netherlands; [c]Department of Biology, Addis Ababa University, PO Box 1176, Addis Ababa, Ethiopia; [d]Department of Retrovirology, Academic Medical Center, Amsterdam, the Netherlands; and [e]Department of Immunology, University Medical Center Utrecht, Utrecht, the Netherlands.

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Tuberculous meningitis in HIV-infected patients: drug susceptibility and clinical outcome

Diego Cecchini[a], Juan Ambrosioni[a], Clarisa Brezzo[a], Marcelo Corti[a], Ana Rybko[b], Marcela Perez[b], Susana Poggi[b] and Marta Ambroggi[b]

The objective of this study was to identify prognostic factors of death in patients with tuberculous meningitis (TM) and show the impact of infection by multidrug-resistant strains on the outcome of this disease. We retrospectively analysed clinical charts of HIV-infected patients with culture-confirmed TM attending our institution during 1996–2004. The following variables were associated with death during hospitalization: neurological signs at admission, a CD4 T-cell count less than 50 cells/μl and infection by multidrug-resistant strains.

Meningitis is the clinical presentation of *Mycobacterium tuberculosis* infection with the highest mortality rate. Although HIV infection itself is considered a risk factor for death in patients with tuberculous meningitis (TM), prognostic factors in this population remain unclear [1,2].

Infection by multidrug-resistant strains poses a major challenge for the clinician as it is an additional predictor of mortality. Although the association between infection by these strains and HIV is well known, there is a paucity of data on its impact in the clinical outcome of TM [3,4].

The Infectious Diseases F.J. Muñiz Hospital is a 300-bed institution located in Buenos Aires city, Argentina, which successfully controlled a multidrug-resistant tuberculosis outbreak in the past decade [5,6]. With the aim of characterizing prognostic factors and showing the impact of multidrug-resistant strains on the mortality of HIV-infected patients with culture-confirmed TM, we undertook a retrospective study considering clinical and microbiological aspects.

We retrospectively analysed data from the clinical charts of HIV-infected patients with a confirmed diagnosis of TM by the isolation of *M. tuberculosis* from cerebrospinal fluid (CSF) attending our institution from 1996 to 2004. During this period, CSF samples were cultured in Lowenstein–Jensen medium or BACTEC. The susceptibility profile was analysed by either the proportion method or the BACTEC radiometric method according to standardized procedures [7–10].

The demographic characteristics, clinical presentation and outcome, neuroimaging findings, CSF alterations, and drug resistance patterns were recorded. Statistical analyses were performed using Statistix software 7.0. HIV infection was documented by HIV-1 antibody enzyme-linked immunosorbent assay and Western blot analysis. A multidrug-resistant strain was defined as *M. tuberculosis* that was resistant to, at least, isoniazid and rifampicin. The neurological status of patients was described according to the British Medical Research Council (BMRC) system for grading meningitis modified as follows: grade I, patient was conscious and without neurological signs; grade II, patients was confused or had neurological signs (meningeal or focal signs); and grade III, patient was stuporose or comatose. Univariate logistic regression analyses were performed to identify the independent variables that were associated with death during hospitalization. The list of potential variables included extraneural tuberculosis, previous or ongoing treatment for tuberculosis, the BMRC grade, the co-existence of another AIDS-defining illness, infection by a multidrug-resistant strain, the CD4 T-cell count, absence of CSF pleocytosis, CSF glucose level less than 30 mg/dl, a delay in the start of treatment greater than 24 h after hospitalization, and neuroimaging alterations. Only those variables with a significance level of $P < 0.05$ were considered to be candidate variables for the multivariate logistic regression analysis.

Of a total of 141 CSF samples with positive culture for *M. tuberculosis*, 101 corresponded to HIV-infected patients. The median age was 33 years, and 68% were men. Thirty-one (30%) had had previous incomplete treatment for pulmonary or extrapulmonary tuberculosis, and 13 (12.8%) were taking antituberculous drugs at the moment of TM diagnosis. The median of CD4 T-cell count was 53 cells/μl.

The BMRC clinical assessment rating for meningitis was grade I for 19 patients (18.8%), grade II for 74 patients (73.3%), and grade III for eight patients (7.9%). The onset of treatment was within the 24 h of admission in 79 patients (78.2%). In 56 patients (54.9%) *M. tuberculosis* was also isolated from extraneural samples (sputum, bronchoalveolar lavage, pleural effusion, suppurative lesions). Six patients had positive documented blood cultures. Twelve patients (11.8%) had a simultaneous diagnosis of another AIDS-defining illness, and 77.8% had neuroimaging alterations. The global mortality during hospitalization was 63.3% (64 patients).

Forty-nine isolates (48.5%) were not resistant to any drug and 42 (41.6%) were multidrug-resistant strains. Three patients had isolated resistance to isoniazid, four to streptomycin, and three to isoniazid plus pyrazinamide or ethambutol. Infection with multidrug-resistant strains was more frequent in patients with previous incomplete or ongoing non-compliant antituberculous treatment (odds ratio 3.09; 95% confidence interval 1.34–7.08; $P < 0.05$). The proportion of infection by these strains was similar in patients with less than 50 CD4 T cells/μl and in those with more than 50 cells/μl ($P > 0.05$).

According to the primary criteria in univariate analysis (Table 1), the following variables were associated with

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Table 1. Univariate analysis for the identification of independent variables associated with death during hospitalization in 101 HIV-infected patients with tuberculous meningitis.

Variable	Odds ratio (95% CI)	*P* value
Extraneural tuberculosis	1.07 (0.48–2.3)	1.00
Previous or ongoing antituberculous treatment	1.30 (0.58–2.89)	0.65
BMRC grade II or III at admission	3.49 (1.23–9.87)	0.02
Simultaneous AIDS indicador condition	2.01 (0.60–6.77)	0.38
Multidrug-resistant strain	3.54 (1.44–8.68)	0.00
Absence of CSF pleocytosis	0.96 (0.33–2.77)	1.00
CD4 T-cell count < 50 cells/μl	4.43 (1.48–13.2)	0.01
CSF glucose < 30 mg/dl	0.45 (0.19–1.07)	0.11
Onset of treatment > 24 h after admission	1.25 (0.39–4.00)	0.91
Neuroimaging alterations	3.50 (0.92–13.216)	0.11

BMRC, British Medical Research Council; CI, confidence interval; CSF, cerebrospinal fluid.

death during hospitalization: BMRC grade II or III at admission, a CD4 T-cell count less than 50 cells/μl and infection caused by multidrug-resistant strains. The three variables remained associated when included in a multivariate logistic regression model.

The high frequency of multidrug-resistant strains in our population allowed us to have an adequate number of patients to show statistically the impact of the infection with these strains on the mortality rate of the disease. The higher mortality rate in patients with multidrug resistant TM may be attributable to the failure of first-line drugs to control the infection and the delay before the susceptibility pattern is known and adequate therapy instituted. When instituted, the treatment of multidrug-resistant TM is less effective because second-line drugs have, with the exception of ethionamide, low CSF penetration [3,4]. In this clinical setting, investigation for new drugs with good CSF penetration is urgently needed.

HIV-infected patients with TM constitute an heterogeneous group. As shown in the multivariate analysis, a CD4 T-cell count less than 50 cells/μl is associated with a higher mortality rate. This fact is independent of the infection by multidrug-resistant strains and may suggest that HIV-infected patients included in series of TM should be stratified according to the CD4 T-cell count when analysing the mortality rate.

As a result of the retrospective design of our study, the evaluation of drug combinations for the treatment of multidrug-resistant strains and reporting the frequency of long-term disability in the surviving patients after hospitalization were not possible.

The identification of risk factors related to infection by multidrug-resistant strains was beyond the scope of this study. Although epidemiological data regarding a family history of tuberculosis and confinement to penal institutions could not be evaluated because of logistical constraints, our institution assists a marginalized population that can be reflected in the high frequency of non-compliance with treatment, as directly observed therapy is an uncommon policy in our country [11].

In conclusion, interventions that reduce the transmission of multidrug-resistant strains and improve adherence to first and second-line antituberculous regimens are required to reduce the burden of this disease among HIV-positive patients in Argentina.

[a]Infectious Diseases Division; and [b]'Abel J. Cetrángolo' Laboratory of Mycobacterias, Infectious Diseases 'Francisco Javier Muñiz' Hospital, Buenos Aires, Argentina.

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Specificity of anti-human leukocyte antigen antibody responses after immunization with Remune, an inactivated HIV-1 vaccine

Mark Page[a], Agatha Ojugo[a], Nesrina Imami[b], Gareth Hardy[b], Frances Gotch[b] and Neil Almond[a]

Antibody responses against human leukocyte antigen (HLA) classes I and II were detected in HIV-1 infected individuals who received a fixed inactivated HIV-1 (Remune) immunotherapy. The response was specific for HLA-B62 and HLA-DR4 concordant with the host cell line, HUT-78, used in vaccine production. These responses were not detected in HLA-B62 and HLA-DR4-positive individuals indicating that immunotherapy did not break tolerance to self-antigens.

Remune is an inactivated HIV-1 vaccine currently under clinical development for the immunotherapeutic treatment of HIV-1-positive individuals undergoing antiretroviral therapy [1]. This immunotherapy is designed to elicit antiviral immunity that will provide enhanced control of virus infection and consequently delay disease progression. It was recently reported that the immunotherapeutic administration of Remune elicits anti-human leukocyte antigen (HLA) antibodies in HIV-1-positive individuals [2]. These preliminary data corroborate previous observations that fixed inactivated SIV vaccines prepared in human T-cell lines were capable of eliciting potent anti-HLA responses in macaques [3–6]. Generating such responses in a xenogeneic setting would be anticipated; however, in an allotypic setting it raises the potential of autoimmunity. This study was designed to investigate these anti-HLA responses in greater detail.

Sera were collected from 35 HIV-1-positive male patients enrolled into a phase 1, randomized, controlled study conducted at the Chelsea and Westminster Hospital, London, UK [7]. All patients were initiated onto HAART for 17 weeks before randomization into four groups. Group A were maintained on HAART for a further 47 weeks; group B were maintained on HAART and received treatment with 5×10^6 units of IL-2 (Proleukin) subcutaneously, twice a day at least 8 h apart; group C were maintained on HAART and received treatment with IL-2 as above, plus Remune (100 μg) administered intramuscularly with Freund's incomplete adjuvant at weeks 17, 29, 41 and 53; and group D were maintained on HAART and received treatment with Remune as above. Serum samples were collected before the initiation of HAART, before randomization (week 17) and at weeks 41, 53 and 65.

HIV-1 immunogen (Remune) is derived from a Zairian HIV-1 isolate (HZ321), an intersubtype recombinant of clade A envelope and clade G Gag [8] propagated on the human T lymphoblastoid cell line (HUT-78) [9]. Virions in the extracellular supernatant of this culture were purified by ultrafiltration and ion-exchange chromatography. The antigen is then inactivated through a sequential application of β-propiolactone and ^{60}Co irradiation [1]. During this process the envelope protein (gp120) is removed.

Anti-HLA antibodies were characterized by Luminex using Lifematch antigen bead sets. HLA haplotypes of patients were assessed by polymerase chain reaction–sequence-specific primer as described previously [10].

No anti-HLA antibody responses were detected before the initiation of immunotherapy. Five patients were identified as having anti-class I responses after treatment, including one patient from group B who received HAART and IL-2 (F577), one patient from group C who received HAART, IL-2 and Remune (J565), and three patients from group D who received HAART and Remune (O152, R362 and S433). For the class I responses, bead set numbers 105 (A2, B49, B62, Bw4, Bw6, Cw3, Cw7), 112 (A25, A68, B51, B62, Bw4, Bw6, Cw15, Cw3), 137 (A2, A26, B62, Bw6, Cw3, Cw7), 156 (A11, A29, B56, B62, Bw6, Cw1, Cw3), 159 (A1, A11, B75, B8, Bw7, Cw7, Cw8) and 183 (A3, A33, B42, B63, Bw4, Bw6, Cw17, Cw5) were consistently reactive (mean fluorescence intensity >5000) with responding patient sera. In four of the bead sets (105, 112, 156, and 159) HLA B62 was the only common antigen present (Table 1).

Specific anti-HLA class II responses were detected consistently (mean fluorescence intensity $>20\,000$) against bead sets 126 (DR1, DR4, DR53, DQ5, DQ7), 128 (DR4, DR53, DQ7, DQ8), 134 (DR14, DR4, DR52, DR53, DQ5, DQ8) and 160 (DR4, DR53, DQ5, DQ8) by selected sera. A total of 13 patients were identified as responders. These included three patients from group B treated with HAART and IL-2 (B231, 0992 and F577), five patients from group C who were treated with HAART, IL-2 and Remune (H140, F652, O232, S566 and C425), and five patients from group D treated with HAART and Remune (S433, W425, B318, L685 and K235). HLA-DR4 is common to the four bead sets against which seroreactivity was detected (Table 1).

The generation of specific anti-HLA antibody after immunotherapy raises concern as to whether they represent auto-immune responses. Therefore we determined the HLA expression profiles of each patient in groups C and D and responding patients in group B (Table 1). A total of seven patients of those treated with Remune were demonstrated to express HLA-B62 or HLA DR4 alleles. None of these patients produced detectable antibody responses against their own alleles, which is reassuring with regard to concerns over the induction of autoantibodies.

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Table 1. HLA typing and anti-HLA antibody responses.

Patient ID	Anti-class I antibody response	HLA A and B typing	Anti-class II antibody response	HLA DR and DQ typing
Group B (HAART + IL-2) responders only				
B231	Negative	A2, A9, B49	DR4	DR11, DR13
O992	Negative	A3, A32, B7	DR4	DR8, DR11
F577	B62	A29, A68, B7, B35	DR4	DR3, DR9
Group C (HAART + IL-2 + Remune)				
H140	Negative	A1, A26, B57, B27	DR4	DR1, DR7, DQ3, DQ5
F652	Negative	A1, A2, B35, B57	DR4	DR12, DR18, DQ5, DQ6
O232	Negative	A1, A2, B57, **B62**	DR4	DQ6
S566	Negative	A11, A29, B8, B44	DR4	DR3, DR7
C425	Negative	A2, A3, B7, B55	DR4	DR15, DR3
J565	B62	A3, A29, B44, B35	Negative	**DR4**, DR7
F065	Negative	A3, A29, B44, B35	Negative	DR1, DR7
Group D (HAART + Remune)				
O152	B62	A1, A2, B8, B44	Negative	**DR4**, DR17, DQ2
C600	Negative	A2, A11, B8, **B62**	Negative	**DR4**, DR13, DQ6
H450	Negative	A1, A2, B7, B35	Negative	DR1, **DR4**
R362	B62	A1, A3, B7, B8	Negative	DR3, **DR4**
S433	B62	A11, A68, B44, B55	DR4	DR15, DR11
W524	Negative	A1, A29, B8, B58	DR4	DR3, DR13
B318	Negative	A2, A24, B18, B35	DR4	DR1, DR13
L685	Negative	A1, A26, B37, B41	DR4	DR10, DR13
K235	Negative	A3, A25, B39, **B62**	DR4	DR11, DR13

HLA, Human leukocyte antigen. Alleles in bold are present on HUT-78. All subjects receiving Remune (groups C and D) are presented along with three subjects in group B who alone showed an anti-HLA response.

This paper confirms the observation of Fernandez-Cruz *et al.* [2] that patients treated with Remune, a whole inactivated HIV-1 vaccine, elicit antibodies against HLA class I and class II. Although the bead sets used to detect anti-HLA antibodies contain multiple specificities in each well, it is possible to deconvolute the responses detected against HLA to a single allele. As a result, antibodies against both class I (HLA-B62) and class II (HLA-DR4) were detected specifically. The specificities of the responses were consistent with the allelic phenotype of the HUT-78 cell line (A1, B62, Cw3, DR4 and DQ3 [8]) used to propagate the vaccine virus.

The higher response rate against HLA-DR4 compared with that against HLA-B62 is likely to reflect a dose–response effect because of the reported higher content of HLA-DR (11.7%) compared with class I (0.7%) on the vaccine particle [2].

It was surprising that similar anti-HLA antibody responses were detected in a small proportion of patients who were not vaccinated with Remune (group B) but were treated with IL-2. However, IL-2 is an immunostimulatory component and may have elicited non-specific but HLA crossreactive antibody responses.

The observations in this study may re-invigorate research into the potential of fixed inactivated HIV-1 as a prophylactic AIDS vaccine. Over 15 years ago, it was demonstrated that protection conferred by fixed inactivated SIV vaccines correlated with potent serological responses against host cell components incorporated into virions [11]. Subsequent studies indicated that immunization with HLA class I and class II derived from the human T-cell lines used in vaccine preparations could reproduce this protection [4–6]. There is, however, only a single report of similar protection being obtained in an allo-immune setting [12]. Further studies are needed to examine whether the anti-HLA antibodies generated by the administration of Remune possess antiviral properties.

[a]Division of Retrovirology, National Institute for Biological Standards and Control, Potters Bar EN6 3QG, UK; and [b]Department of Immunology, Imperial College, Chelsea and Westminster Hospital, London SW10 9NH, UK.

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Dynamics of HIV viral load in blood and semen of patients under HAART: impact of therapy in assisted reproduction procedures

Giovanni Battista La Sala[a], Elisabetta Pilotti[c], Alessia Nicoli[a], Silvana Pinelli[c], Maria Teresa Villani[a], Paola Ronzi[c], Enrico Zendri[c], Maria Carla Re[d], Giacomo Magnani[b] and Claudio Casoli[c]

We examined the efficacy and effect of HAART in HIV-1-infected men confronted with assisted fertilization procedures. We showed that HAART did not always reduce the HIV-1-RNA level in blood and semen compartments, and that a significant upward shift in mitochondrial DNA was observed in spermatozoa from a HAART-treated patient group compared with spermatozoa from HAART-untreated or HIV-1-uninfected groups ($P < 0.001$). These findings emphasize the negative role of HAART, but not of HIV-1 infection, in determining semen alterations.

A number of studies have demonstrated that HIV-1-RNA levels in blood and semen compartments decrease below the detection limits during HAART [1,2]. However, the presence of latently infected cells has been a major issue for the sustained control and eradication of HIV-1 from the body.

Some studies reported that HIV-1 compartmentalization tends to differ in semen and in blood [3–5]. In particular, several lines of evidence suggest that HIV-1 in semen arises from genital tissues rather than from blood, and the HIV-1 particles in semen are further subcompartmentalized between seminal leukocytes and seminal plasma [6–8]. It remains to be determined whether the semen compartment, like the blood compartment, harbours a latent reservoir after prolonged therapy. Some studies detected replication-competent HIV-1 virus in the semen compartment in a few patients after 1–2 years of therapy, whereas others reported the opposite findings [8–11]. It is therefore still controversial as to whether the semen compartment represents a major reservoir of latently HIV-1-infected cells.

In the majority of HIV-1-infected couples the infected partner is the man. In that case, semen washing can be applied to eliminate the risk of sexual transmission of the virus during assisted fertilization procedures [12]. The seminal viral load may decrease but is not eliminated in HIV-positive men taking HAART [13].

The safety of antiretroviral drug treatment is a key issue in the management of HIV-infected men belonging to HIV-1-serodiscordant couples. HAART may modify cell metabolism, and the risk of adverse effects on semen characteristics and pregnancies should be assessed. It is known that sperm DNA fragmentation associated with apoptosis can be a cause of recurrent pregnancy loss [14,15]. In particular, the role of the mitochondrial genome (mtDNA) in male infertility and an analysis of the implications of mtDNA on sperm mobility should be carefully considered [16]. The adverse effect observed during treatment with nucleoside analogue reverse transcriptase inhibitors seems to be linked to an alteration in mitochondrial activity [17,18].

The present study examined the efficacy of reducing HIV-1 replication in semen after prolonged HAART therapy, and its effect on the genomic material of the male gamete.

Nineteen patients were enrolled in the study, 11 had been on HAART therapy for 5 years, eight of them having developed antiretroviral drug resistance. The cellular and viral characteristics of the patients are listed in Table 1 [19,20]. CD4 cell counts ranged between 25 and 704 cells/μl; blood viral loads ranged between 3.5×10^2 and 4.8×10^5 copies/ml, whereas seminal viral loads ranged between less than 50 and 1.8×10^5 copies/ml. HIV-1 was detected by HIV-1-RNA analysis at least four times during the study period. HIV-1 RNA was detectable in blood samples at baseline, whereas viral load was below the detection limits in semen (< 50 copies/ml of semen) in seven out of 19 patients during therapy. In general, viral loads in blood and semen were stable throughout therapy in patients with available longitudinal

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Table 1. HIV-1 detection in the blood and semen compartments of infected men.

Patient no.	CD4 cell count/μl	Serum HIV-RNA (copies/ml)	Semen HIV-RNA (copies/ml)	*gag*-Nested PCR on spermatozoa	Semen mtDNA[a] (copy number)	HAART
1	526	3.2×10^5	1.6×10^4	Neg	6.2 ± 1.1	Lamivudine/zidovudine, nevirapine
2	633	1.28×10^3	1.5×10^3	Neg	7.8 ± 1.5	Lamivudine/zidovudine, nevirapine
3	481	8.17×10^4	< 50	Neg	6.8 ± 1.9	Zidovudine, amprenavir, lamivudine
4	632	4.84×10^5	< 50	Neg	9.1 ± 2.1	Lamivudine/zidovudine, didanosine
5	298	2.79×10^5	< 50	Neg	8.9 ± 1.3	Lamivudine/zidovudine, lopinavir
6	277	1.54×10^5	< 50	Neg	5.9 ± 0.9	Efavirenz, lopinavir, stavudine
7	577	8.16×10^4	5.6×10^3	Neg	10.7 ± 1.2	Lamivudine/zidovudine, tenofovir
9	313	522	< 50	Neg	9.4 ± 0.3	Nelfinavir, lamivudine
11	313	9.32×10^4	1.8×10^5	Neg	7.6 ± 1.2	Lamivudine/zidovudine, didanosine
12	269	345	< 50	Neg	7.9 ± 1.1	Lamivudine/zidovudine, nelfinavir
15	239	< 50	< 50	Neg	11.0 ± 2.2	Lamivudine/zidovudine, efavirenz
8	196	350	< 50	Neg	3.6 ± 1.3	No
10	458	1.3×10^5	3×10^6	Neg	2.8 ± 1.1	No
13	528	1.24×10^5	1.32×10^3	Neg	1.9 ± 0.9	No
14	548	1.73×10^4	2.4×10^3	Neg	3.1 ± 1.2	No
16	704	2.89×10^4	1.1×10^4	Neg	1.9 ± 1.4	No
17	409	7.2×10^4	3.6×10^2	Neg	3.2 ± 0.6	No
18	25	8.38×10^4	2.4×10^3	Neg	2.3 ± 0.8	No
19	217	4.59×10^4	7.8×10^2	Neg	2.6 ± 1.3	No

[a]Mitochondrial DNA/β-globin ratio. Mean ± SD. HIV-1-infected men donated their semen and blood samples after informed consent. All men were former injecting drug users. Semen and blood samples were taken on the same day and analysed within 2 h. Seminal plasma and spermatozoa pellets, HIV-1-RNA detection in specimens and nested polymerase chain reaction (PCR) of the HIV-1 *gag* gene in spermatozoa were performed as described [19]. mtDNA/β-globin real-time PCR quantification was carried out on DNA extracted from swim-up sperm samples (200 μl) as described [20]. The calculation of the mtDNA/β-globin ratio was performed taking into account the volume of the extracted DNA used as a template for PCR amplification (10/200 μl for mtDNA and 2/200 for β-globin). Normal mtDNA/β-globin ratio values ($n = 10$ healthy donors): 1.8 ± 0.7.

samples. The discrepancy between viral loads in blood and in semen in our study was thus probably caused by a compartmentalization process reflecting different active replication or evolution in these sites. In this connection, antiretroviral therapy did not reduce the HIV-1-RNA level in either blood or seminal plasma in some patients, whereas in others it reduced viral shedding in the semen, but not in the blood (see Table 1). HIV-1 isolates from blood and semen showed different drug resistance patterns, confirming a divergent expansion of the infection in the two sites. These data clearly demonstrate the concomitant presence of different strains generated under therapy.

The HIV-1-RNA viral load assessed in the blood to monitor HIV-1 patients during and after HAART treatment cannot be indicative of the viral load in the semen, and therefore it is not possible to evaluate the risk of HIV-1 transmission by in-vitro fertilization just by analysing HIV-1-RNA levels in the blood. Moreover, the probability of sexual transmission increases when the semen viral load is high [18]. These findings are of particular interest for assisted reproduction in discordant couples consisting of an infected man and an uninfected woman. In this case, semen washing and in-vitro fertilization are recommended [13]. We observed that the washing process reduced the original HIV-1-RNA semen load of the subjects by approximately three logs (data not shown), indicating that a seminal viral content below the detectable limit of molecular bioassay can be obtained only from sperm with a low HIV-1 load (< 1000 copies/ml).

The diagnosis of male infertility has primarily been based on traditional semen analysis, with a strong emphasis on the assessment of semen volume and sperm concentration, motility and morphology [21]. This study focused on the mtDNA content in semen after HIV infection and undergoing HAART therapy. The semen changes reported in infected men, such as lower ejaculate volume and sperm counts [21], were confirmed in our cohort. In addition, sperm DNA fragmentation was also tested by electrophoresis [22]. The HAART regimen and corresponding HIV-1-RNA semen load in enrolled subjects are shown in Table 1. Four patients were resistant to therapy, seven were responders, and eight, with high viral loads, were naive for HAART treatment. The determination of mtDNA/β-globin gene ratios using real-time polymerase chain reaction indicated that the mtDNA copy number in motile spermatozoa, HIV-1 negative for *gag*-nested polymerase chain reaction, ranged from 5.9 to 11.0 in subjects under HAART and from 1.9 to 3.6 in untreated subjects. Table 1 showed a significant upward shift of mtDNA in the sperm of the HAART-treated group with respect to controls ($P < 0.001$), whereas the HIV-1 untreated men presented a copy number distribution not significantly different from that of the control group. This difference was not statistically significant in relation to high or low plasma/semen viral loads. In no case was sperm DNA fragmentation associated with apoptosis detected (data not shown).

In conclusion, the level of sperm mtDNA is increased in HAART-treated subjects, thus raising the risk involved in assisted reproduction techniques. These findings

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emphasize the negative role of HAART, but not of HIV-1 infection, in determining semen alterations. These results are in line with the findings of May-Panloup *et al.* [20] who measured the mtDNA content in sperm samples presenting abnormal motility and morphology. Recent studies have suggested that nucleoside analogue reverse transcriptase inhibitor exposure could impair the mitochondrial energy-generating ability of spermatozoa [23].

This report highlights the importance of ongoing investigation into the use of HAART regimens in infected men in relation to the sexual transmission of HIV-1, and the prospect of assisted fertilization in HIV-discordant couples with seropositive men.

[a]Division of Obstetrics, Gynecology and Reproduction Medicine; [b]Division of Infectious Diseases, ASMN Reggio Emilia, Italy; [c]Department of Clinical Medicine, Nephrology and Health Sciences, University of Parma, Italy; and [d]Department of Clinical and Experimental Medicine, Microbiology Section, University of Bologna, Italy.

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