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Polymorphisms of innate immunity genes influence disease progression in HIV-1-infected children

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Toll-like receptors (TLRs) and defensins (DEFs) play a crucial role in the host's innate immunity and may influence HIV-1 disease progression. We investigated the impact of *TLR9* +1174G > A, 1635A > G and *DEFβ1* −44C > G, −52G > A single nucleotide polymorphisms on the clinical outcome of 95 HIV-1-infected children. The *TLR9* 1635AG genotype and *TLR9* [G;G] haplotype were associated with rapid disease progression, whereas the *DEFβ1* −44CG genotype and *DEFβ1* [G;G] haplotype correlated with a better clinical outcome.

HIV-1-infected children and adults exhibit a high degree of variability in their rate of disease progression but, in the absence of highly active antiretroviral therapy (HAART) children progress more rapid than adults [1]. Host's factors likely contribute to the variability in clinical outcome. Genetic variants of innate immunity genes may affect virus–host interactions and impact disease progression; this effect may be of particular interest in children who acquire infection when the adaptive immune response is still under development.

Toll-like receptors (TLRs) and defensins (DEFs), initiators and effectors of innate immunity, may influence HIV-1 pathogenesis. Genetic variants of *TLR9* have been associated with the rate of disease progression in adults [2–4]. Specific variants of *TLR9* [5] and *DEFβ1* [6–8] were found to be associated with risk of mother-to-child transmission (MTCT) of HIV-1, but no data are available regarding their role in pediatric HIV-1 disease progression. The aim of this work was to investigate the impact of variability in *TLR9* and *DEFβ1* genes on the clinical outcome of HIV-1 infected children.

The study population included 95 HIV-1-infected children, born to HIV-1-seropositive mothers between 1984 and 1996. None of the mothers had undergone antiretroviral prophylaxis. The HIV-1-infected children attended the Pediatric Department of Padova University; their clinical and immunological status was defined according to the classification system of Centers for Disease Control and Prevention [9]. The endpoint of the study was defined as the onset of disease (stage C) or the initiation of HAART. The median (interquartile) follow-up from birth to endpoint was 87 (46–134) months.

Genomic DNA was extracted from peripheral blood mononuclear cells with the QIAamp DNA Blood mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Single nucleotide polymorphisms (SNPs) of *TLR9* and *DEFβ1* were analyzed by the TaqMan allelic discrimination assay (Applied Biosystems, Foster City, California, USA). Primers and probes used for *TLR9* [5] and *DEFβ1* [8] were previously described. Allele discrimination and genotype determination were based on the endpoint fluorescence measured by the Sequence Detection System software (Applied Biosystems). The accuracy of genotyping was confirmed by known DNA samples of each genotype and by direct sequencing of randomly selected samples as previously described [8].

The genotypes for each SNP were analyzed as a codominant variable and were also grouped according to the dominant, recessive or underdominant model. The C-free stage was defined as the time from birth to the onset of stage C or HAART entry. The probability of acquiring disease was calculated with the Kaplan–Meier method and the log-rank test was used to test for differences between genotype categories. Hazard ratios and their 95% confidence interval based on the Cox proportional hazards model were estimated to test the association between genotypes, haplotypes and risk of stage C. To estimate the haplotype effect on disease we used the Thesias program [10]. Values of P less than 0.05 were considered statistically significant and all tests were two-sided. Analyses were carried out in R (http://www.r-project.org/).

Results indicated that the *TLR9* 1174AA genotype tended to be associated with a better prognosis in the codominant model and was significantly associated with a slow disease progression in the recessive model, with both Kaplan–Meier ($P = 0.034$) and Cox analyses ($P = 0.042$) (Table 1). Conversely, a significant correlation was observed between the *TLR9* 1635AG genotype and rapid disease progression with both Kaplan–Meier ($P = 0.008$) and Cox analyses ($P = 0.009$) (Table 1). This result is in agreement with a previous finding in adults [2]; the underdominant model might explain this unusual association between a heterozygous status and a disadvantageous condition [11]. The *TLR9* [G;G] haplotype was significantly associated with a higher risk of rapid disease progression (Table 1); this disadvantage is consistent with a previous finding, indicating the association

Table 1. Polymorphisms in *TLR9* and *DEFβ1* genes and disease progression in HIV-1-infected children.

TLR9 SNP	Genetic model	Genotype	Events[a]/total number	Median[b] (95% CI)	*P* value (log-rank)	HR[c] (95% CI)	*P* value
1174; rs352139	Codominant	AA	6/22	199 (128;–)	0.054	1	
		GA	20/44	124 (79;173)		3.15 (1.17–8.46)	0.022
		GG	12/29	– (92;–)		2.15 (0.75–6.13)	0.152
	Recessive	AA	6/22	199 (128;–)	0.034	1	
		GA + GG	32/73	140 (92;–)		2.67 (1.04–6.90)	0.042
1635; rs352140	Codominant	GG	6/23	199 (166;–)	0.025	1	
		AA	14/39	– (122;–)		1.35 (0.51–3.56)	0.544
		AG	18/33	114 (70;153)		2.90 (1.12–7.51)	0.028
	Underdominant	GG + AA	20/62	199 (124;–)	0.008	1	
		AG	18/33	114 (70;153)		2.36 (1.23–4.53)	0.009
TLR9; rs352139; rs352140		Haplotype	Estimated frequency			HR (95% CI)	*P* value
		A;G	0.47881			1	
		G;A	0.35647			0.82 (0.45–1.47)	0.503
		A;A	0.10629			0.79 (0.39–1.58)	0.498
		G;G	0.05842			2.97 (1.09–8.17)	0.033
DEFβ1 SNP	Genetic model	Genotype	Events/total number	Median (95% CI)	*P* value (log-rank)	HR (95% CI)	*P* value
−44; rs1800972	Codominant	CG	7/24	173 (140;–)	0.020	1	
		CC	30/70	124 (92;199)		2.61 (1.13–6.01)	0.024
−52; rs1799946	Codominant	GA	20/50	173 (124;–)	0.416	1	
		AA	7/17	116 (52;–)		1.80 (0.74–4.35)	0.192
		GG	11/28	122 (94;–)		1.14 (0.54–2.41)	0.731
DEFβ1; rs1800972; rs1799946		Haplotype	Estimated frequency			HR (95% CI)	*P* value
		A;C	0.44681			1	
		G;C	0.42553			0.87 (0.51–1.49)	0.616
		G;G	0.12766			0.34 (0.14–0.83)	0.016

DEF, defensin; SNP, single nucleotide polymorphism; TLR, Toll-like receptor.
[a]Stage C according to Centers for Disease Control and Prevention classification [9].
[b]Median time to stage C, in months, and 95% confidence interval (95% CI) were estimated by Kaplan–Meier method.
[c]Hazard ratio (HR) and 95% CI were estimated by Cox analysis.

of this haplotype with a higher risk of MTCT of HIV-1 [5]. The *DEFβ1* −44CG genotype was associated with a slower disease progression compared with the −44CC genotype with both Kaplan–Meier ($P = 0.020$) and Cox analyses ($P = 0.024$) (Table 1). The *DEFβ1* −52 SNP, which has been found to play a role in MTCT of HIV-1 [8], did not influence clinical progression (Table 1). However, the *DEFβ1* [G;G] haplotype, previously found to be protective against MTCT of HIV-1 [8], was also found to be associated with a better disease outcome (Table 1).

Overall, these results support a role of genetic variability of innate immunity genes in the clinical outcome of pediatric HIV-1 infection. The mechanisms by which these genetic variants may influence HIV-1 disease are still unknown. DEFβ1 is mainly produced by epithelial cells [12], and higher levels of DEFβ1 have been reported in exposed uninfected individuals than in seropositive patients [13]. Specific variants of *DEFβ1* may protect against disease progression by increasing DEFβ1 levels at the mucosa level [14]. Furthermore, loss of mucosal surface integrity, particularly in the gastrointestinal tract, leads to microbial translocation [15], which, along with HIV-1 viremia, induce immune activation, a hallmark of disease progression in children as well as adults [16,17]. *TLR9* and *DEFβ1* expression, both of which play important roles in controlling the overall responses of immune cells to pathogens [12,18,19], may modulate immune activation. Lower levels of TLR9 expression have been found in viremic versus aviremic HIV-1-infected patients [20]. Studies suggested that *TLR9* 1174 and 1635 SNP, although not inducing amino acid change, may affect TLR9 expression [21], and a role of these SNP in *TLR9* immune activation has been advanced [2–5]. In conclusion, specific genotypes and haplotypes in the *DEFβ1* and *TLR9* genes may affect the functional ability of their encoded proteins to modulate innate immunity and immune activation, thus contributing to the variability of clinical outcome in HIV-1-infected children.

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Conflicts of interest

There are no conflicts of interest.

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Regional differences in predictive accuracy of WHO immunologic failure criteria

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We compared the performance of the WHO immunologic criteria for treatment failure among Uganda and American patients. Antiretroviral treatment-naive patients with a CD4 T-cell count less than 200 cells/μl or AIDS at enrollment on a nonnucleoside reverse transcriptase inhibitors-based regimen for more than 1 year were selected. For all criteria, the positive predictive value was significantly higher in the American compared with the Ugandan patients. Population-specific guidelines should be developed using large African cohorts to identify more specific and sensitive criteria.

In industrialized countries, antiretroviral treatment (ART) efficacy is monitored through routine measurement of $CD4^+$ cell counts and plasma viral load. Guidelines from the WHO on ART for HIV infection in resource-limited settings recommend the use of immunologic monitoring when viral load testing is not available [1,2].

Previous studies from sub-Saharan Africa have shown that the proposed criteria are neither sensitive nor specific [3–7]. We suspected that one of the reasons for poor performance in African patients is the high incidence of opportunistic infections, which could consequently impair the immune reconstitution of patients on ART [8,9].

We sought to evaluate the performance of the WHO immunologic criteria for treatment failure in a Ugandan and an American cohort and to compare the predictive values of these criteria in identifying patients on first-line ART with viral failure. We also examined the relative contribution of opportunistic infections on the performance of the criteria among the Ugandan patients.

The Infectious Diseases Institute (IDI) Research Prospective Observational Cohort is a closed research cohort of 559 patients, enrolled at ART initiation between April 2004 and April 2005 in Uganda. Details of the cohort have been published elsewhere [10].

The Johns Hopkins HIV Clinical Cohort (JHHCC) is an open observational longitudinal cohort database of HIV-infected patients in the USA, established in 1989 with over 6000 patients enrolled, with majority initiated on ART between 1999 and 2003. Details of the cohort have been published elsewhere [11].

Patients selected from both cohorts were as follows: ART-naive at cohort enrollment; initiated on zidovudine/stavudine with lamivudine and efavirenz/nevirapine; baseline $CD4^+$ cell count less than 200 cells/μl or clinical criteria of AIDS; and on ART for at least 1 year.

We compared the characteristics of the patients at ART initiation in both cohorts. Categorical and continuous variables were compared using chi-square and Mann–Whitney tests, respectively. To compare the performance of the WHO criteria in both cohorts, we identified the proportion of patients fulfilling at least one of the three criteria: $CD4^+$ cell count less than 100 cell/μl after 12 months; more than 50% drop from $CD4^+$ cell count peak; and $CD4^+$ cell count lower than baseline [1]. We then obtained the proportion of patients with confirmed viral failure at the time they met any of the criteria. We calculated the positive and negative predictive values (PPV and NPV). Finally, we did a sensitivity analysis excluding patients who had ever had an opportunistic infection in the first year of ART in the IDI cohort. Ethical approval was obtained from the local Institutional Review Board committees.

We included 442 patients from the IDI cohort and 153 patients from the JHHCC. At ART initiation, a lower proportion of patients from the JHHCC were female (28.8 versus 69%, $P < 0.0001$), had a slightly lower median age [35 years, interquartile range (IQR)

Table 1. Proportion of patients fulfilling the immunological criteria for treatment failure and proportion of patients with confirmed virologic failure.

	Immunologic failure			Confirmed virologic failure		
$CD4^+$ cell count criteria	IDI, *n* (%)	JHHCC, *n* (%)	*P*	IDI, *n* (%)	JHHCC, *n* (%)	*P*
<100 cells/μl after 12 months	37/442 (8.1)	19/109 (17.4)	<0.0001	5/37 (13.5)	10/19 (52.6)	0.003
>50% drop from peak	50/442 (11.3)	63/153 (41.2)	<0.0001	13/50 (26.0)	53/63 (84.1)	<0.0001
Less than baseline	88/442 (19.9)	11/153 (7.2)	0.034	18/88 (20.4)	9/11 (81.8)	<0.0001

IDI, Infectious Diseases Institute; JHHCC, Johns Hopkins HIV Clinical Cohort.

30–41 years versus 39 years, IQR 35–44 years, $P < 0.001$], higher median BMI (22 kg/m^2, IQR, 19–26 kg/m^2 versus 20 kg/m^2, IQR 17–22 kg/m^2, $P < 0.0001$), lower median $CD4^+$ cell count per microliter (42 versus 102, $P = 0.064$) and a higher proportion initiated on zidovudine with lamivudine and efavirenz (39% versus 26%, $P = 0.012$). A larger proportion of the IDI cohort experienced an opportunistic infection in the first year of ART (28.7% versus 13.7%, $P = 0.0002$). Tuberculosis was the most common opportunistic infection, with (4.7%) in the IDI cohort compared with (0.6%) in the JHHCC ($P = 0.021$).

The proportion of patients who fulfilled each of the criteria and had confirmed virologic failure in the IDI cohort versus JHHCC were as follows: 1.1% versus 6.5% ($P = 0.0002$) for criterion 1, 2.9% versus 37.9% ($P < 0.0001$) for criterion 2 and 4.1% versus 5.9% ($P = 0.354$) for criterion 3.

As shown in Table 1, the PPV for virologic failure in the IDI cohort and the JHHCC, respectively, was 13.5% and 52.6%, for criterion 1; 26.0% and 84.1%, for criteria 2; and 20.4% and 78.9%, for criteria 3. The NPV for criteria 1, 2 and 3 in the IDI cohort and JHHCC, respectively, was 87.6% versus 85.5%, 93.6% versus 84.4% and 93.5% versus 71.8%.

In a sensitivity analysis including only IDI cohort patients (71.3%) who did not experience an opportunistic infection, there was no significant difference in the PPV of this subgroup compared with the entire cohort for criteria 1 and 2: criterion 1, 13.0% versus 13.5% ($P = 0.835$); criterion 2, 26.0% versus 20.4% ($P = 0.06$); but, a significant difference for criterion 3 (20.4% versus 10.2%, $P < 0.0001$).

The WHO immunologic criteria selected higher proportions of virologically failing patients in the American than in a Ugandan cohort and had higher PPVs. Our results suggest that this difference is not due to the high rate of diagnosed opportunistic infections in the African setting, even though severe bacterial diseases often seen could also affect immunological response. Other possibilities include undiagnosed and untreated opportunistic infections, poor nutrition and differences in HIV subtypes. Studies from the east African region suggest that subtype D is associated with a faster decline in $CD4^+$ cell count and increased risk of mortality compared with subtype A [12,13] due to increased $CD4^+$ cells activation and subsequent apoptosis [14]. It is important to evaluate the impact of host characteristics of the infected population on ART efficacy and viral subtype [15].

Published data suggest that, with immunological monitoring only, patients with confirmed viral failure are identified after significant accumulation of drug resistance [16].

Recent studies have shown that a viral load will reduce an early and unnecessary switch to second-line therapy, reduce accumulation of resistance and prevent drug resistance viral transmission by patients who are failing [17–19].

Our study limitations included, first, the differences in the data used in both cohorts; however, patients were identified during the roll-out of ART in both countries. Second, the younger age of JHHCC patients compared with IDI cohort patients could have influenced the higher immune response in JHHCC, as has been shown previously [20,21].

In summary, our data highlight the need for viral load measurements when identifying treatment failure in HIV-positive individuals on ART. Further research into the risk factors for the limited performance of these WHO immunologic criteria in resource-limited settings is needed. Population-specific guidelines should be developed using large African cohorts to identify more specific and sensitive criteria.

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Conflicts of interest

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Prevalent tuberculosis and mortality among HAART initiators

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The effect of tuberculosis on mortality in people initiating highly-active antiretroviral therapy (HAART) remains unclear; here, we strengthened a previous cohort analysis. Multivariate Cox proportional hazards models were used to assess the association of baseline tuberculosis and time to all-cause mortality among HAART initiators. In reanalysis, treatment for tuberculosis at time of

HAART initiation remained unassociated with increased risks of all-cause mortality, with adjusted hazard ratios ranging from 1.00 to 1.09.

In 2010, Straetemans *et al.* [1] published a meta-analysis of the effect of tuberculosis (TB) on mortality in HIV-positive people. In a subanalysis of six studies, they found an overall hazard ratio of 1.08 [95% confidence interval (CI) 0.91–1.27] for the effect of TB on all-cause mortality in HIV-positive individuals, where at least 50% of the cohort reported use of highly-active antiretroviral therapy (HAART). Nonetheless the authors concluded that insufficient data were available to draw strong conclusions about the effect of TB on all-cause mortality among individuals receiving HAART. The largest contributor to that subanalysis was a 2009 report in this journal by Westreich *et al.* [2], which examined the effect of prevalent pulmonary tuberculosis being treated at time of HAART initiation on time to mortality among patients all of whom were initiating HAART in Johannesburg, South Africa. We found an adjusted (weighted) hazard ratio of 1.06 (95% CI 0.75–1.49) in these individuals, indicating no increased risk of death among those with prevalent tuberculosis at time of HAART initiation.

Although our findings were in line with those of at least some other studies [1,3–5], our study had two related limitations. First, there was a high rate of loss to follow-up in our study; second, we had a low recorded incidence of mortality. We attempted to account for these effects [2] by using inverse probability of censoring weights [6]. However, inverse probability of censoring weights rely on assumptions that data are censored (missing) at random [7–9], an assumption which, similar to an assumption of no uncontrolled confounding [10], is not verifiable. Because the publication of our report, additional mortality information has been obtained from the South African National Death Registry [11] for a subset of patients in the database. This allowed an opportunity to enhance our analysis with better mortality data [11], extend follow-up by 18 months, and check the validity of modeling assumptions and overall results from the original publication in three analyses.

Details on TB screening, treatment for TB and HIV at the Themba Lethu Clinic and in South Africa more generally, and on clinical care and research procedures of the clinic has been described in detail [2,11,12]. In the *original cohort analysis*, we examined impact of being in treatment for TB at baseline (initiation of HAART) on time to all-cause mortality among all patients initiating HAART at the Themba Lethu Clinic between 1 April 2004 and 31 March 2007 [11]. In the *updated analysis*, we used the same set of individuals and same time frame, but updated vital status outcomes and dates of death. In the *extended analysis*, we extended follow-up (including opportunity for death) until 1 October 2008, allowing up to 18 additional months of follow-up in all participants. In the original report [2] we found no changes in estimates of effect when using inverse probability of censoring weights [6] compared to traditional adjusted Cox proportional hazards models, so these analyses used traditional Cox models, adjusted for confounding by factors as in the original report [2].

Results from reanalysis are summarized in Table 1. In the original cohort, the 7512 participants in the database experienced a recorded 298 deaths, 74 (25%) of which were in participants exposed to prevalent TB. There were 1423 participants recorded as lost to follow-up. The crude hazard ratio was 1.68 (95% CI 1.29–2.19), and the adjusted was 1.07 (95% CI 0.80–1.44). These are nearly identical to the originally reported results [2].

In the updated cohort, among 7512 participants there were 494 deaths recorded, of which 115 (23%) occurred in prevalent TB cases; and there were 882 participants recorded as lost to follow-up. The crude hazard ratio was 1.55 (95% CI 1.26–1.91), and the adjusted was 1.00 (95% CI 0.80–1.26).

In the extended cohort, among 7512 participants there were 666 deaths, of which 155 (23%) occurred in prevalent TB cases. There were 1460 participants recorded as lost to follow-up. The crude hazard ratio was 1.62 (95% CI 1.35–1.94), and the adjusted hazard ratio was 1.09 (95% CI 0.90–1.33).

This reanalysis of data from a large cohort of individuals initiating HAART in South Africa reaffirms earlier findings [2] that patients receiving active treatment for tuberculosis at HAART initiation were not at a higher risk of death compared to those not being treated for TB, demonstrating that these findings were robust to more-complete collection of previously missing data. One limitation of this reanalysis is that Fox *et al.* [11] were able

Table 1. Summary of results from reanalyses of effect of treated tuberculosis on all-cause mortality among patients initiating HAART in Johannesburg, South Africa.

Scenario	Deaths	Hazard ratio (95% confidence limits)	
		Crude	Adjusted
Original cohort analysis	298	1.68 (1.29–2.19)	1.07 (0.80–1.44)
Updated analysis	494	1.55 (1.26–1.91)	1.00 (0.80–1.26)
Extended analysis	666	1.62 (1.35–1.94)	1.09 (0.90–1.33)

to obtain vital registration data for only 42% of participants presumed lost to follow-up. Thus, more than 50% of those lost to follow-up may have in fact not had their vital status validated. Nonetheless, reanalysis only among those patients with valid medical identification numbers (those whose status would have been evaluated by Fox *et al.* if they had been presumed lost to follow-up; about 64% of all patients) yielded very similar results, with hazard ratio = 0.90 (95% CI 0.68–1.20) in the updated cohort and hazard ratio = 1.01 (95% CI 0.79–1.29) in the extended cohort.

High rates of patients becoming lost to follow-up are an unfortunate reality in both the practice and analysis of large-scale HIV clinical cohorts [13], and the missing data that results from these losses can be a significant challenge to the validity of the results of analyses in those cohorts [14–16]. When that missing data comprises missing outcome values, which are caused by the true value of the missing outcomes, not only are biased effect estimates likely [17], but the bias cannot generally be eliminated through analytic approaches such as inverse probability of censoring weights or multiple imputation [17] (although these approaches [18] as well as others [14–16] may help reduce bias). However, this bias will not be introduced when the true effect is null [17]; as this reanalysis demonstrates, the true effect is likely to be null, and our original report was likely unbiased.

In conclusion, our analysis substantially strengthens the evidence that TB treatment at time of HAART initiations is not associated with increased risk of mortality on HAART.

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Conflicts of interest

The authors have no financial, consultant, institutional or other conflict of interest to declare.

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