Maternal and infant factors and lymphocyte, CD4 and CD8 cell counts in uninfected children of HIV-1-infected mothers

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Objective: To evaluate the effects of antiretroviral treatment (ART) for mother-to-child transmission of HIV and infant/maternal characteristics on total lymphocytes (TLC) and lymphocyte subsets in uninfected children of HIV-1-infected mothers.

Design: The European Collaborative Study followed 1663 uninfected children from birth until at least 8 years of age using a standard protocol.

Methods: Smoothers (running medians) illustrated patterns of immune markers over age by ART exposure and race. Associations between lymphocyte parameters and maternal/infant characteristics were quantified in linear regression analyses using *z*-scores obtained after modelling $\log_{10}$-transformed TLC, CD4 and CD8 cell counts using the LMS method. Cox proportional hazard models assessed time to TLC, CD4 and CD8 cell counts below the defined cut-off. Covariates included prematurity, gender, race, drug withdrawal and ART exposure.

Results: Overall, black children had lower TLC, CD4 and CD8 cell counts than white children, and an increased risk of TLC, CD4 and CD8 cell counts below the cut-off. ART exposure was associated with TLC levels (but not with TLC below the cut-off for lymphopenia), with reduced CD4 cell counts in the first year of life, and with reduced CD8 cell counts until at least 8 years of age. Duration and intensity of ART exposure was associated with TLC levels.

Conclusion: The effect of ART exposure in fetal and early life on TLC and CD8 cell counts was prolonged until at least 8 years. These results add to the growing list of adverse effects associated with ART used as prevention of mother-to-child transmission of HIV.

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Introduction

With antiretroviral (ART) as prophylaxis, mother-to-child transmission rates have been reduced to < 1% [1,2]. However, adverse effects following ART exposure during fetal and early life in uninfected children have been reported, including mitochondrial and haematological toxicity [1,3,4].

Although haematological toxicity can apparently affect all cell lineages [5], long-term effects of ART exposure in early life for uninfected children have only been shown

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for neutrophils [6]. Lymphocytes are important in the defence against viruses but also indirectly in bacterial infections [7]. It would, therefore, be of interest to assess longer-term effects of ART exposure on lymphocytes, CD4 and CD8 cells.

This paper reports the analysis of data from a European longitudinal birth cohort and estimates the effect of gender, race, prematurity and ART exposure on levels and patterns of CD4 and CD8 cells and total lymphocytes count (TLC) over the first 8 years of life in uninfected children born to HIV infected mothers.

Methods

The European Collaborative Study is a prospective birth cohort study that has been ongoing since 1986 [8,9]. Data relating to children born to HIV-1-infected women were collected from birth, using a standard clinical and laboratory protocol, with frequent follow-up in the first 2 years of life. Thereafter, uninfected children were seen yearly; after about age 8 years phlebotomy became less frequent. Parental consent was obtained and the study approved by local ethics committees.

A child was considered to be uninfected if antibody negative after 18 months of age and presumed uninfected if RNA or DNA polymerase chain reaction was negative on two occasions or if no virus antigen had ever been identified [9]. Laboratory tests including RNA viral load assay, white blood cell count and differentiation were carried out locally according to standard procedures that did not vary between centres.

Prematurity was defined as gestational age < 37 weeks. Race was determined on the basis of maternal ethnicity; more than 90% of black mothers had recently immigrated from subSaharan Africa. Maternal illicit drug use (IDU) was based on self-report and/or neonatal drug withdrawal symptoms. ART exposure was classified as none or any, and categorized as *in utero* exposure and/or neonatal ART prophylaxis. Maternal ART regimen nearly always included zidovudine (90%). Differences in regimens were evaluated, comparing mono and combination therapy and regimens including protease inhibitors (PI) or non-nucleoside reverse transcriptase inhibitors (NNRTI).

Maternal CD4 cell count information has been collected routinely only since 1992, based on the sample closest to delivery and categorized in three groups: < 200, 201–500 and $> 500 \times 10^6$ cells/l. The TLC and CD4 cell counts of infants were grouped by age-appropriate values below which clinical symptoms become more common. The cut-off for TLC was 2500×10^6 cells/l at < 18 months of age and 1500×10^6 cells/l after 18 months; these values indicate lymphopenia generally and correspond with World Health Organization cut-offs for ART eligibility in HIV-infected children [10,11]. For CD4 cells, we used the cut-offs for infected children recommended by the Centers for Disease Control and Prevention (CDC) classification at class II (moderate immune suppression) and class II (severe immunodeficiency) [12,13]. Currently, there are no established cut-offs for CD8 cells associated with clinical symptoms; CD8 cut-offs were calculated from those for CD4 for CDC class II by using a CD4/CD8 ratio of 1.5, resulting in a cut-off of 500×10^6 cells/l in the first year, 333×10^6 cells/l for ages 1 to 5 years and 133×10^6 cells/l after 5 years [14–16].

Statistical methods

TLC pattern and levels over age were visualized by smoothers with and without stratification. Smoothers are running medians, best representing the data but not allowing formal statistical comparison. Therefore, the associations between various factors and TLC, CD4 and CD8 cells were investigated in regression analyses using the z-score (SD) of individual measurements. These were obtained after modelling $\log_{10}$-transformed TLC, CD4 and CD8 cell counts using LMS [6,17]. The LMS method summarizes the changing age distribution of a variable by three curves representing the median (M), the coefficient of variation (S) and the skewness (L); these curves are fitted as cubic splines by non-linear regression using all measurements. The total study population formed the standard from which z-scores for individual measurements were calculated. The z-scores are no longer age dependent and provide a way to maximize data. In linear regression analyses, the repeated measurement nature of the data was accounted for by linear mixed effects models [18]. The goodness of fit of the models was assessed with the likelihood-ratio test and Akaike's Information Criterion [19].

Kaplan–Meier survival curves visualized time to lymphopenia and progression to levels of CD4 or CD8 cell counts below the defined cut-off; associations between variables and time to lymphopenia were quantified in Cox proportional hazard models [20,21].

Data entry and management were carried out using Microsoft Access XP (Redmond, Washington, USA). Statistical analyses were carried out using STATA 8.2, 2003 (Stata Corp., College Station, Texas, USA).

Results

By June 2004, 1663 uninfected children were enrolled, with 8945 assessments of TLC, CD4 and CD8 cell counts. Observations after 8 years of age were sparse and not included in the analyses. The median number of observations was three (range, 1–29). There were 4285

Table 1. Association of *z*-scores for cell counts of total lymphocytes and CD4 and CD8 cell subsets in uninfected children with gender, race, antiretroviral therapy exposure and *in utero* exposure to maternal injecting drug use.

		Total lymphocyte count *z*-score		CD4 cell count *z*-score		CD8 cell count *z*-score	
Variable	No.	Univariable coefficient (*P* value)	Multivariable coefficient (*P* value)	Univariable coefficient (*P* value)	Multivariable coefficient (*P* value)	Univariable coefficient (*P* value)	Multivariable coefficient (*P* value)
Ethnicity							
White	1200						
Black	463	−0.383 (< 0.001)	−0.304 (< 0.001)	−0.394 (< 0.001)	−0.367 (< 0.001)	−0.393 (< 0.001)	−0.261 (< 0.001)
Gender							
Female	818						
Male	845	−0.098 (0.007)	−0.100 (0.004)	−0.183 (< 0.001)	−0.183 (< 0.001)	−0. 013 (0.723)	−0.019 (0.593)
Prematurity							
Yes	348						
No	1315	−0.048 (0.284)	−0.061 (0.168)	0.014 (0.761)	−0.009 (0.845)	−0.042 (0.350)	−0.088 (0.049)
Antiretroviral therapy exposure							
No	870						
Yes	793	−0.236 (< 0.001)	−0.132 (< 0.001)	−0.163 (< 0.001)	−0.043 (0.293)	−0.354 (< 0.001)	−0.270 (< 0.001)
Maternal injecting drug use							
No	1400						
Yes	263	0.245 (< 0.001)	0.108 (0.001)	0.175 (0.001)	0.045 (0.388)	0.260 (< 0.001)	0.096 (0.051)

Multivariable regression analysis allows for ethnicity, gender, prematurity, antiretroviral therapy exposure and maternal injecting drug use.

measurements for white and 1815 for black children between 0 and 18 months, 2296 measurements for white and 194 measurements for black children between 18 months and 5 years, and 340 measurements for white and 15 measurements for black children between 5 and 8 years of age. About half of the children (48%) had been exposed to ART (Table 1).

Patterns of lymphocytes over age

To reflect the changing characteristics of mothers enrolling in the European Collaborative Study, with more heterosexually acquired HIV, subSaharan origin and increasing use of ART over time, smoothers were constructed separately for black and white children and for ART exposure (Fig. 1a,b).

Levels of all three markers were lower in black than in white children (Fig. 1). ART-exposed white children had consistently lower levels than unexposed children. This was also apparent for black children, although their data became sparse after about 3 years of age, reflecting more recent enrolment. A similar picture was seen for CD8 cell counts for black and white children; however, the effect of ART exposure on CD4 cell count was concentrated in the first year of life.

Lymphocyte counts and maternal/infant variables

Uni- and multivariable regression analyses of *z*-scores were used to quantify associations between maternal/infant factors and TLC, CD4 and CD8 cells. For TLC, the association with race was the strongest and most significant of all factors investigated (Table 1). The effects of ART exposure, gender and maternal IDU, although statistically significant, were smaller, especially in multivariable analysis, while there was no statistically significant association with prematurity.

Race was also the main factor associated with CD4 cell levels (Table 1). Again, boys had significantly lower levels, but the effect of ART exposure and maternal IDU was only significant in univariable analysis.

In contrast to TLC and CD4 cell counts, the effect of ART exposure on CD8 cell count was of similar magnitude as the effect of race. CD8 cell counts did not differ significantly by gender, while children born to mothers who were IDU during pregnancy had borderline higher CD8 cell counts, as did premature children (Table 1).

Exposure to antiretroviral therapy during fetal and early life

The association of ART with TLC, CD4 and CD8 cell count was further investigated in subanalyses assessing the effect of duration, timing and type of ART. Children whose mothers started ART during the third trimester of pregnancy had significantly higher TLC than children of mothers who started ART in the first trimester [first trimester reference coefficient taken as 0; second trimester coefficient, 0.125 ($P = 0.132$); third trimester coefficient, 0.222 ($P = 0.017$)]. This was also seen for CD4 cell counts but not for CD8 cell counts.

The effect of ART exposure (none, *in utero*, neonatally, or *in utero* plus neonatally) on levels of TLC was only significant for *in utero* plus neonatal exposure. This was also true for CD4 cell count in univariable analysis, but not significantly so in multivariable analysis. CD8 cell count was reduced in children with only neonatal exposure and in those with neonatal plus *in utero*

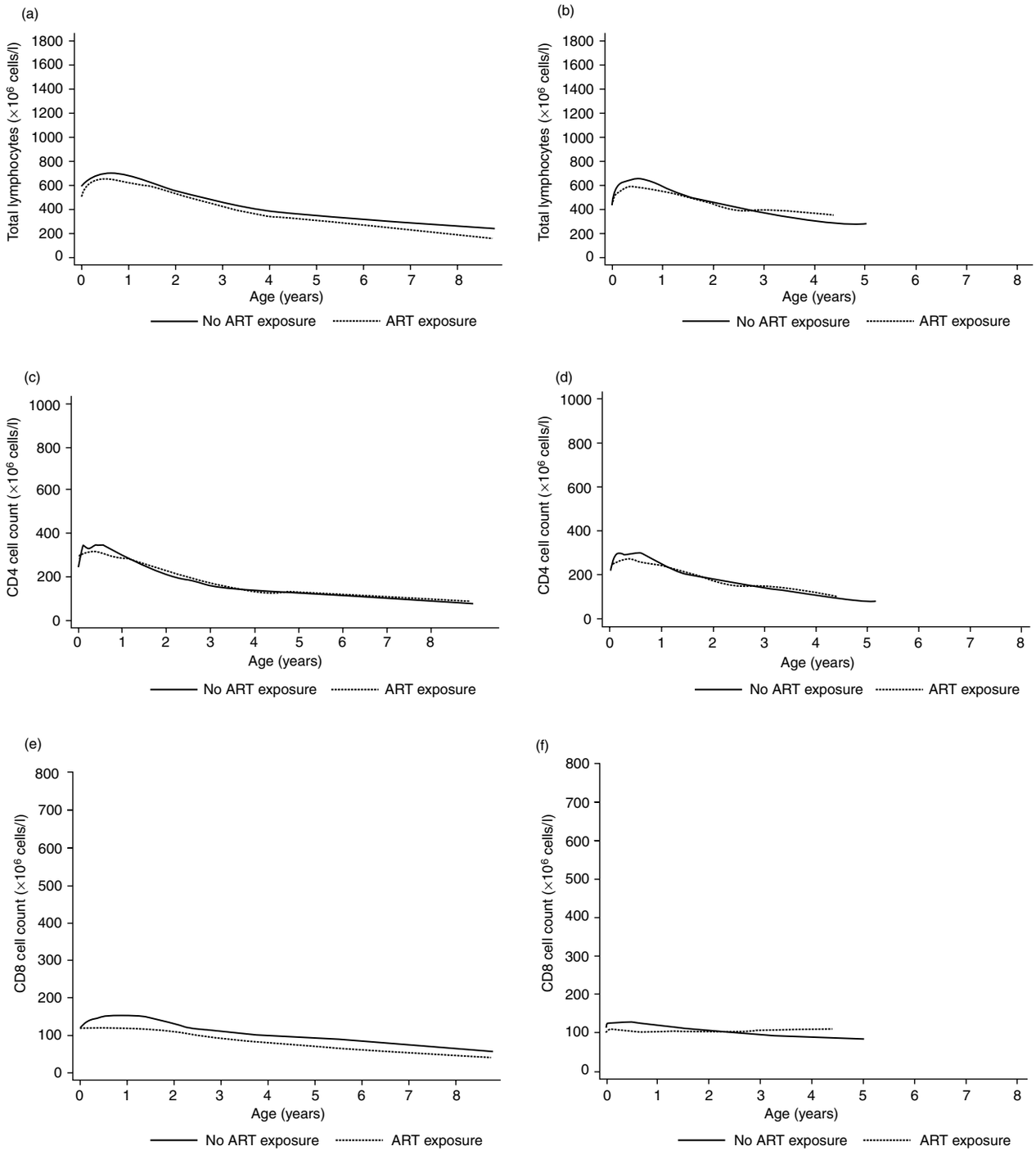

Fig. 1. Immunological parameters in white and black children by exposure to ART. (a) Total lymphocytes in white children; (b) total lymphocytes in black children; (c) CD4 cell count in white children; (d) CD4 cell counts in black children; (e) CD8 cell count in white children; (f) CD8 cell count in black children. ART, antiretroviral therapy.

exposure, with comparable coefficients. The *z*-scores for TLC in children exposed to combination therapy were 0.161 lower ($P = 0.053$) than *z*-scores of children only exposed to monotherapy; *z*-scores for CD8 cell counts in children only exposed to monotherapy were 0.247 higher ($P = 0.003$) than children exposed to combination therapy; No significant differences were observed in CD4 cell counts. There was no evidence to suggest that the type of drugs within combination therapy (nucleoside reverse transcriptase inhibitor, with non-nucleoside

reverse transcriptase inhibitor or protease inhibitor) affected levels of any of the three markers.

Maternal immune status

Maternal CD4 cell counts were available for 731 children with 2224 measurements: counts were $< 200 \times 10^6$ cells/l for 103, $200-500 \times 10^6$ cells/l for 366, and $> 500 \times 10^6$ cells/l for 262 children.

In univariable analysis, children of mothers with CD4 cell counts $> 500 \times 10^6$ cells/l had significantly higher TLC z-scores than those of mothers with CD4 cell counts $< 200 \times 10^6$ cells/l (coefficient, 0.308; $P < 0.001$); the coefficient was of a similar magnitude in multivariable analysis. This was also true for CD4 cell counts: a maternal CD4 cell count $> 500 \times 10^6$ cells/l was associated with a 0.437 increase in CD4 z-score in the child ($P < 0.001$) compared with that in children of mothers with CD4 cell count of $< 200 \times 10^6$ cells/l. The difference in the child's z-scores for CD4 cells with a maternal CD4 cell count of $200-500$ and $< 200 \times 10^6$ cells/l did not reach statistical significance (coefficient, 0.128; $P = 0.159$). In multivariable analysis, race and maternal immunity were the most important and significant factors associated with z-scores for TLC and CD4 cell counts. No statistically significant association was observed between maternal CD4 cell count levels and child CD8 cell counts; race and ART exposure remained the most important factors for CD8 cell counts.

Effect of age

To investigate whether the associations between maternal or infant variables and TLC, CD4 or CD8 cell counts were age related, a categorized age variable was introduced in the multivariable model, relating to measurements before 18 months of age, between 18 months and 5 years and after 5 years of age (based on the pattern over age illustrated by the smoothers). The goodness of fit of all three models (TLC, CD4 and CD8 cells) improved significantly with the introduction of the age variable, with substantial reductions in Akaike's Information Criterion (from 22 305 to 22 246 for TLC; from 21 693 to 21 650 for CD4 cells and from 21 857 to 21 745 for CD8 cells). In all three models, age was

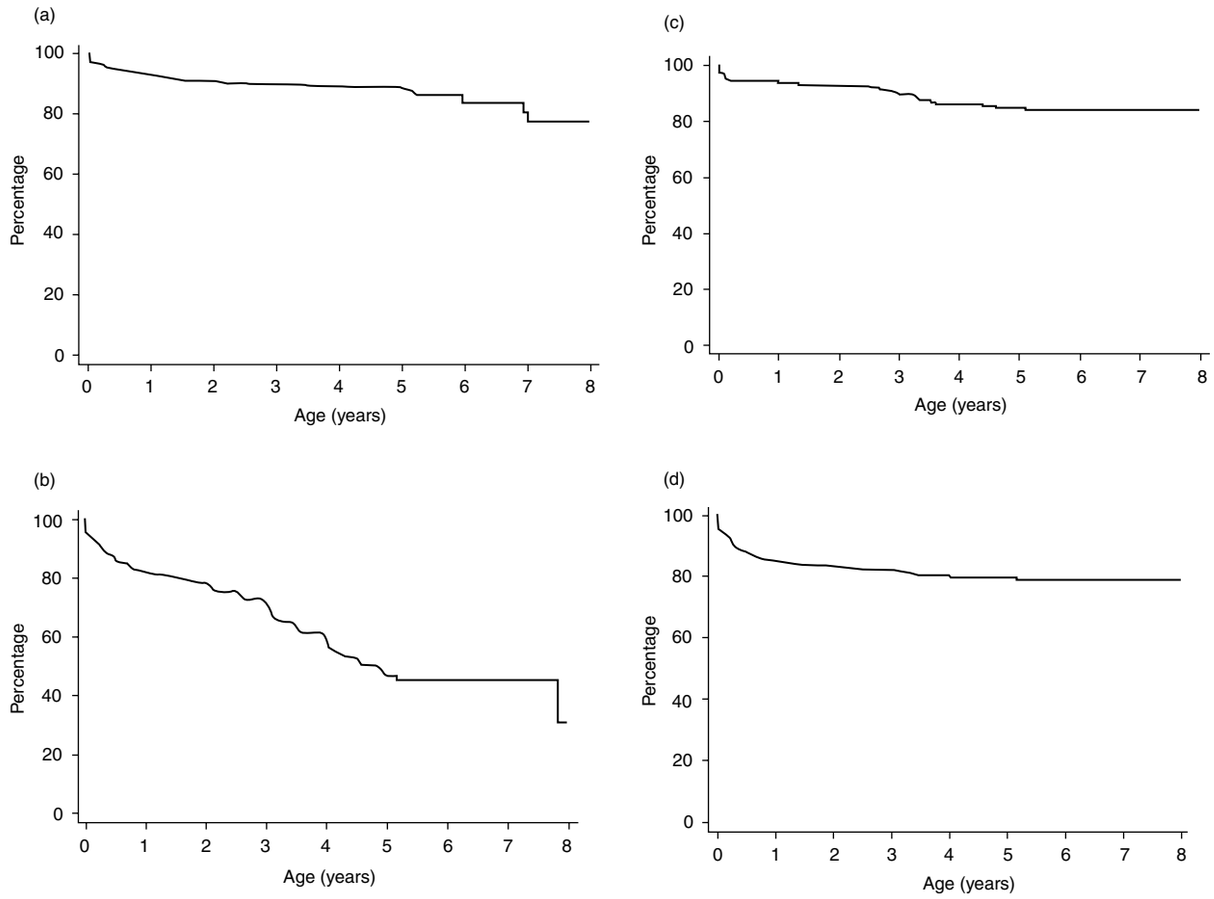

Fig. 2. Changes in lymphocytes over time. (a) Percentage of children with lymphocytes below the cut-off (see Methods). (b) Percentage of children reaching a CD4 cell count below the defined cut-off for CDC class II. (c) Percentage of children reaching a CD4 cell count below the defined cut-off for CDC class III. (d) Percentage of children reaching a CD8 cell count below the defined cut-off (see text for details). CDC, Centers for Disease Control and Prevention.

Table 2. Time to lymphopenia as determined by TLC, and low CD4 and CD8 cell counts in uninfected children by gender, race, antiretroviral therapy exposure and *in utero* exposure to maternal injecting drug use.

		Total lymphocyte count		CD4 cell count CDC stage II		CD4 cell count CDC stage III		CD8 cell count	
Variable	No.	Univariable ratio (*P* value)	Multivariable ratio (*P* value)	Univariable ratio (*P* value)	Multivariable ratio (*P* value)	Univariable ratio (*P* value)	Multivariable ratio (*P* value)	Univariable ratio (*P* value)	Multivariable ratio (*P* value)
Ethnicity									
White	1200								
Black	463	1.63 (0.008)	1.74 (0.008)	1.98 (< 0.001)	2.02 (< 0.001)	1.570 (0.089)	1.470 (0.208)	2.33 (< 0.001)	1.90 (< 0.001)
Gender									
Female	818								
Male	845	1.32 (0.108)	1.29 (0.158)	1.29 (0.010)	1.29 (0.009)	1.328 (0.245)	1.405 (0.192)	1.13 (0.328)	1.18 (0.214)
Prematurity									
Yes	348								
No	1315	1.61 (0.055)	1.66 (0.050)	1.10 (0.456)	1.10 (0.472)	1.439 (0.289)	1.473 (0.294)	1.29 (0.144)	1.33 (0.108)
Antiretroviral therapy exposure									
No	870								
Yes	793	1.39 (0.073)	1.29 (0.215)	1.27 (0.027)	1.09 (0.492)	1.285 (0.359)	1.192 (0.559)	2.07 (< 0.001)	1.73 (< 0.001)
Maternal injecting drug use									
No	1400								
Yes	263	1.15 (0.501)	1.30 (0.287)	0.95 (0.693)	1.03 (0.834)	0.133 (0.680)	1.029 (0.937)	0.64 (0.024)	0.86 (0.484)

Multivariable analysis allows for ethnicity,gender, prematurity, antiretroviral therapy exposure and maternal injecting drug use.

statistically significantly associated with *z*-scores, which were lower in the older two categories than the younger one, mimicking the pattern of decreasing counts over age illustrated by the smoothers. The *z*-scores coefficients of measurements at 18 months to 5 years of age and at 5–8 years of age, respectively, were -0.1582 ($P < 0.001$) and -0.2599 ($P < 0.001$) for TLC, -0.1301 ($P < 0.001$) and -0.2156 ($P < 0.001$) for CD4 cell counts, and -0.2193 ($P < 0.001$) and -0.3170 ($P < 0.001$) for CD8 cell counts. The coefficients and *P* values of all other factors changed only marginally with inclusion of an age variable, suggesting that the effects, including that of ART exposure, observed earlier apply to the whole age range and are not only concentrated in the early part of life; this corresponds with the graphs for the raw data (Fig. 1).

Lymphocyte counts below the cut-off

To examine the potential clinical relevance of the differences observed, a Kaplan–Meier analysis visualized time to lymphopenia or low CD4 or CD8 cell counts, with a Cox proportional hazards analysis quantifying the effect of maternal/infant characteristics; TLC, CD4 and CD8 cell counts were grouped by age-appropriate values below or above which clinical symptoms become more common [10,11]. A total of 131 children had ever been diagnosed with lymphopenia before 8 years of age; 431 children ever had CD4 cell counts indicating moderate immunodeficiency (CDC class II) and 62 had severe immunodeficiency (CDC class III); and 231 children had ever had reduced CD8 cell counts.

At 1 year of age, an estimated 8% of children would have been lymphopenic at least once, increasing to an estimated 13% at 5 years of age (Fig. 2a). CD4 cell counts below the CDC class II or class III cut-offs, respectively, would have occurred in an estimated 18% and 8% of children by 1 year of age and in 53% and 16% by 5 years of age (Fig. 2b,c). An estimated cumulative 14% of children would have had an event below the CD8 cut-off at 1 year and 20% at 5 years of age (Fig. 2d).

In Cox proportional regression analysis, race was significantly associated with lymphopenia: at any age, the risk of lymphopenia was 1.74 times higher in black than in white children allowing for gender, prematurity, ARV exposure and maternal IDU (Table 2). This was also true for CD4 and CD8 cell count, where black children were again about twice as likely to have had an event. Children exposed to ART were 1.73 times more likely to have CD8 cell counts below the cut-off than unexposed children in multivariable analysis. Although ART exposure was associated with an increased risk of lymphopenia, this did not reach statistical significance and for low CD4 cell counts the association was marginal (Table 2).

In the subanalysis investigating maternal immunity, children born to mothers with CD4 cell counts $> 500 \times 10^6$ cells/l were at a lower risk of lymphopenia than children born to mothers with CD4 cell counts $< 200 \times 10^6$ cells/l (ratio, 0.406; $P = 0.018$); a similar effect was seen for low CD4 cell counts in the child, with a similar ratio.

However, although one low measurement was relatively common, more than one was less so. Of the 65 children with lymphopenia during infancy, only three had two or more low TLC counts. Of the 289 children with CD4 cell counts below the cut-off for CDC class II, 70 children had two or more count below the cut-off, but only five had more than one observation below the cut-off for CDC class III. Two or more CD8 cell counts below the

cut-off were observed in 31 of the 217 children, and in 19 (8.8%) children three times or more. There was some evidence that ART exposure and race were associated with an increased likelihood of more than one CD8 or CD4 cell count measurement below the cut-off, but the differences did not reach statistical significance owing to lack of power with limited sample size.

Discussion

These data from the European Collaborative Study, a large European birth cohort study, have provided an opportunity to analyse in uninfected children born to HIV-infected mothers the associations between maternal/infant characteristics and lymphocytes, including CD4 and CD8 cell subsets, from birth up to 8 years of age. Le Chenadec and colleagues [5] were the first to show a long-term effect of ART prophylaxis on haematopoietic parameters, but their data did not go beyond 18 months of age. We have previously shown that the association with ART exposure and reduced neutrophil cell counts persisted until at least 8 years [6], and now show that ART exposure in fetal and early life is also associated with long-term reductions in lymphocytes and CD8 cell counts, although the effect on CD4 cell counts is concentrated in early childhood. We also investigated associations between haemoglobin and ART exposure, and detected, similar to the French study and earlier analyses by the European Collaborative Study [5,22,23], only a short-term effect that resolved after the end of exposure (data not shown). The clinical relevance of these associations remains unclear in the light of the lack of a statistically significant association between ART exposure and the risk of lymphopenia or low CD4 cell counts, and the finding that few of the children ever diagnosed with a TLC, CD4 or CD8 measurement below the defined cut-off had more than one such low value.

In a previous publication [9], we showed that patterns and levels of lymphocytes, CD4 and CD8 cells in uninfected children differed by race. In a more detailed analysis using *z*-scores and quantifying the associations in a manner not possible previously, we have confirmed that race was the most important factor associated with all three immunological markers overall. In multivariable analyses, lymphocytes and subsets were approximately 0.3 SD (*z*-score) lower in black children than in white children. Black children, mostly with mothers recently arrived from subSaharan Africa, were also significantly more likely (about two times) to have a lymphopenic event or achieve CD4 or CD8 cell counts below the cut-offs. This suggests for the first time that lymphocyte levels are not just decreased in black children overall but that they are also more likely to reach critically low levels, which may put them at clinical risk in terms of general childhood infections [10].

Gender was associated with levels of lymphocytes and CD4 cell counts, but not CD8 cell counts. As was known from earlier studies [9], boys had lower levels than girls. However, this difference was not substantial enough to be translated into an increased risk of lymphopenia or low CD4 or CD8 cell counts. Maternal IDU has been described by others to be associated with higher levels of lymphocytes during childhood [5], and we here confirm those findings.

Since other studies suggested an association between maternal immunity and levels of the immunological markers in their children, we investigated this in a subanalysis [5]. We showed that children born to mothers with CD4 cell counts $< 200 \times 10^6$ cells/l had significantly lower CD4 cell counts and a higher risk of reaching CD4 cell counts below the cut-off than children born to mothers with CD4 cell counts $> 500 \times 10^6$ cells/l; this was also seen for lymphocytes, although not to the same magnitude. For CD8 cells, there was no clear association. This is similar to results showing that, irrespective of ART exposure, CD4 cell counts, in particular, were decreased in cord blood from children born to HIV-infected women [24]. In this paper by Nielsen *et al.*, decreased thymic output of CD4 cells was said to be suggestive of impaired progenitor function. In the context of our findings, these results may suggest that the altered immune setting of HIV-infected mothers (e.g., imbalance of T helper 1 and helper 2 cytokines) during pregnancy may have long-term consequences for the thymic output of CD4 cells of the child.

Combination ART exposure had a stronger effect on TLC and CD8 cell levels than monotherapy, in line with results from other studies [5], but the type of drug within the combination [PI or NNRTI in addition to the nucleoside reverse transcriptase inhibitors (NRTI)] was not associated with immunological markers. This is in line with the known haematological adverse effects of NRTI [25], particularly on haemoglobin and neutrophilic granulocytes [5,23]. Investigation of a possible treatment duration or intensity effect showed that, for all three markers, children exposed both *in utero* and neonatally had consistently lower levels than those exposed in one period only. Additionally, there was a marginal effect of timing of initiation of ART during pregnancy, with exposure to ART from the first trimester being associated with lower TLC and CD4 cell counts than exposure from the third trimester, which is consistent with the transplacental passage of NNRTI and NRTI [3,26,27].

Why there are distinct differences in the duration of the effect of ART exposure on different haematological and immunological markers is difficult to explain, but these differences could result from effects of ART exposure on haemato-progenitor cells at different levels, for example bone marrow or thymus [23,24,28]. This should be investigated in an experimental setting. With respect to

CD8 cell counts, CD8 cell counts generally rapidly decrease after initiation of ART in HIV-infected patients. This has always been thought to be provoked by a rapid decrease in viral load and removal of the viral stimulation; however, other mechanisms may play a role [29]. There are suggestions that ART alters the function of CD8 cells; this, combined with a depressing effect, would have clinical relevance and influence the capacity of a child to fight general viral infections [30]. Again, this association should be investigated further.

The statistical models used in this study have their limitations individually but in combination give a more complete picture. The smoothers visualize the patterns and differences over age, while the regression analyses allowed the relative importance of the variables to be quantified. The cut-offs used to indicate lymphopenia and low CD4 cell counts were based on generally applied cut-offs; however, these were generated using data predominantly from white children [10,12,13]. Cut-offs for CD8 cell counts were inferred from various sources specifically for the analyses presented here, because of a lack of pertinent information [14,15]. Future research needs to investigate the appropriateness and clinical relevance of the CD8 cut-offs and provide age-related CD8 standards for children in a general population.

We show here for the first time that exposure to ART in fetal and neonatal life is associated with long-term decreased TLC and CD8 cell counts in uninfected children born to HIV-infected mothers, which may also be clinically relevant. This adds to the growing list of possible adverse events after early-life ART exposure, such as mitochondrial abnormalities [4] and increased risk of premature delivery [31]. ART as prophylaxis is essential in preventing mother-to-child transmission and the benefits still greatly outweigh these long-term adverse consequences of ART exposure in early life. More research is required and, in particular, continuation of the follow up of ART-exposed uninfected children beyond 18 months of age to inform the discussion on ART in the prevention of mother-to-child transmission.

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References

1. Mandelbrot L, Landreau A, Rekacewicz C, Berribi A, Benifla JL, Burgard M, *et al*. **Lamivudine-zidovudine combination for prevention of maternal-infant transmission of HIV-1.** *JAMA* 2001; **285**:2083–2093.
2. European Collaborative Study. **Are girls more at risk of intra-uterine-acquired HIV infection than boys?** *AIDS* 2004; **18**:344–347.
3. Mofenson LM, Munderi P. **Safety of antiretroviral prophylaxis of perinatal transmission for HIV-Infected pregnant women and their infants.** *AIDS* 2002; **30**:200–215.
4. Blanche S, Tardieu M, Rustin P, Stama A, Firtion G, Giraru-Vigneron N, *et al*. **Persistent mitochondrial dysfunction and perinatal exposure to antiretroviral nucleoside analogues.** *Lancet* 1999; **354**:1084–1089.
5. Le Chenadec J, Mayaux MJ, Guihenneuc-Jouyaux C, Blanche S. **Perinatal antiretroviral treatment and hematopoiesis in HIV – uninfected infants.** *AIDS* 2003; **17**:2053–2061.
6. European Collaborative Study. **Levels and patterns of neutrophil cell counts over the first 8 years of life in children of HIV-1 infected mothers.** *AIDS* 2004; **18**:2009–2017.
7. Janeway CA, Travers P, Walport M, Shlomchick M. *Immunobiology, The Immune System in Health and Disease*. 5th edn. New York: Garland; 2001.
8. European Collaborative Study. **HIV-infected pregnant women and vertical transmission in Europe since 1986.** *AIDS* 2001; **15**:761–770.
9. European Collaborative Study. **Are there gender and race differences in cellular immunity patterns over age in infected and uninfected children born to HIV-infected women?** *J Acquir Immune Defic Syndr* 2003; **33**:635–641.
10. Stiehm ER, Ochs HD, Winkelstein JA. *Immunologic Disorders in Children*. 5th edn. Philadelphia, PA: Elsevier/Saunders; 2004.
11. World Health Organization. *Treat 3 Million by 2005*. Geneva: World Health Organization; 2003; 31–39. http://www.who.int/hiv/pub/prev_care/en/arvrevision2003en.pdf
12. Centers For Disease Control and Prevention. *Guidelines for the Use of Antiretroviral Agents in Pediatric HIV Infection*. Bethesda, MD Centers For Disease Control and Prevention HIV/AIDS Treatment Information Service; 2001.
13. Ammann A. **Pediatric human immunodeficiency virus infection.** In: *Immunological Disorders in Children*. Edited by Stiehm ER, Ochs HD, Winkelstein JA. Philadelphia, PA: Elsevier/Saunders; 2004. pp. 878–951.

14. Lugada ES, Mermin JH, Kaharuza F. **Population-based hematologic and immunologic reference values for a healthy Ugandan population.** *Clin Diag Lab Immunol* 2004:29–34.
15. Shearer WT, Rosenblatt HM, Gelman R, Oyomopito R, Plaeger S, Stiehm ER, *et al*. for the Pediatric AIDS Clinical Trials Group. **Lymphocyte subsets in healthy children from birth through 18 years of age: the Pediatric AIDS Clinical Trials Group P1009 Study.** *J Allergy Clin Immunol* 2003:973–980.
16. Uppal SS, Tewari SC, Verma S, Dhot PS. **Comparison of CD4 and CD8 lymphocyte counts in HIV-negative TB patients with those in normal blood donors and the effect of antitubercular treatment: hospital-based flow cytometric study.** *Clin Cytometry* 2004; **61**:20–26.
17. Cole TJ, Green PJ. **Smoothing reference centile curves: the LMS method and penalized likelihood.** *Stat Med* 1992; **11**:1305–1319.
18. Laird NM, Ware JH. **Random-effects models for longitudinal data.** *Biometrics* 1982; **38**:963–974.
19. Akaike H. **A new look at statistical model identification.** *IEEE Trans* 1974; **AU-19**:716–722.
20. Kalbfleish JD, Prentice RL. *The Statistical Analysis of Failure Time Data*. New York: Wiley; 2004.
21. Cox DR, Oakes D. *Analysis of Survival Data*. London: Chapman & Hall; 1984.
22. European Collaborative Study. **Exposure to antiretroviral therapy in utero or early life: the health of uninfected children born to HIV-infected women.** *J Acquir Immune Defic Syndr* 2003; **32**:380–387.
23. Shah MM, Li Y, Christensen RD. **Effects of perinatal zidovudine on hematopoiesis: a comparison of effects on progenitors from human fetuses versus mothers.** *AIDS* 1996; **10**:1239–1247.
24. Nielsen SD, Jeppesen DL, Kolte L, Clark DR, Sorensen TU, Dreves AM, *et al*. **Impaired progenitor cell function in HIV-negative infants of HIV-positive mothers results in decreased thymic output and low CD4 counts.** *Blood* 2001; **98**:398–404.
25. Dainiak N, Worthington M, Riordan MA. **3′-Azido-3′-deoxythymidine (AZT) inhibits proliferation in vitro of human haematopoietic progenitor cells.** *Br J Haematol* 1988; **69**:299–304.
26. Mandelbrot L, Peytavin G, Firtion G, Farinotti R. **Maternal–fetal transfer and amniotic fluid accumulation of lamivudine in human immunodeficiency virus-infected pregnant women.** *Am J Obstet Gynecol* 2001; **184**:153–158.
27. Olivero OA, Shearer GM, Chouquet CA, Kovacs A, Baker R, Stek AM, *et al*. **Corporation of zidovudine into cord blood DNA of infants and peripheral blood DNA of their HIV-1 positive mothers.** *Ann N Y Acad Sci* 2000; **918**:262–268.
28. Luzuriaga K, Sullivan J. **Prevention of mother-to-child transmission of HIV Infection.** *Clin Infect Dis* 2005; **30**:466–467.
29. Bohler T, Walcher J, Holzl-Wenig G, Geiss M, Buchholz B, Linde R, *et al*. **Early effects of antiretroviral combination therapy on activation, apoptosis and regeneration of T cells in HIV-1-infected children and adolescents.** *AIDS* 1999; **13**:779–789.
30. Trabattoni D, Piconi S, Biasin M, Rizzrdini G, Migliorino M, Seminari E, *et al*. **Granule-dependent mechanisms of lysis are defective in CD8 T cells of HIV-infected, antiretroviral therapy-treated individuals.** *AIDS* 2004; **18**:859–869.
31. European Collaborative Study, The Swiss Mother and Child HIV Cohort Study. **Combination antiretroviral therapy and duration of pregnancy.** *AIDS* 2000; **14**:2913–2920.

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