Levels and patterns of neutrophil cell counts over the first 8 years of life in children of HIV-1-infected mothers

European Collaborative Study*

Background: Antiretroviral drugs (ARV) as prophylaxis to prevent mother-to-child transmission of HIV results in decreased haematological parameters during and shortly after exposure, with recent data suggesting a more prolonged inhibition of haematopoiesis until at least 18 months.

Design: Data on 156 HIV-infected and 1533 uninfected children in the European Collaborative Study followed from birth until at least 8 years of age.

Methods: Smoothers and splines were used to elucidate patterns over age; linear mixed effects allowed for repeated measurements. Covariates included the child's HIV-1 infection status, prematurity, gender, race, drug withdrawal symptoms at birth and ARV exposure; effects on neutrophil count were quantified in regression analyses using *z*-scores (SD from mean) of neutrophil counts obtained after modelling untransformed values using the LMS method. For HIV-infected children, progression to AIDS and ARV therapy were also included.

Results: After approximately 4 months of age, neutrophil counts were consistently and substantially lower in HIV-infected children than in uninfected children; in both groups, black children had significantly lower counts than white children across the whole age range. In uninfected children, male gender and ARV exposure were associated with reduced neutrophil count until at least 8 years of age. In HIV-infected children, advanced disease and ARV treatment were significantly associated with neutrophil count.

Conclusion: A considerably longer effect of exposure to ARV was shown in uninfected children than previously thought and significant associations were shown between race and gender and neutrophil count, as previously observed for lymphocyte counts. The clinical relevance of these reduced levels of neutrophils requires further investigation.

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Keywords: HIV-1, haematopoiesis, antiretroviral medication, gender, race

Introduction

The widespread use of antiretroviral drugs (ARV) as prophylaxis to prevent mother-to-child transmission, has reduced this to less than 1% [1–3]. However, concern has been expressed about potential side effects of antenatal and neonatal exposure to ARV [4]. Infants born to HIV-infected mothers are exposed not only to maternal HIV-1 infection and decreased maternal immunity but also to ARV in fetal and neonatal life; both exposures may influence the developing immune system.

In vitro, ARV drugs, in particular zidovudine, induce

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suppression of the myeloid cell lineages of the bone marrow [5], and zidovudine has been associated with anaemia and neutropenia in treated HIV-infected adults [6]. There is limited evidence of adverse effects of ARV prophylaxis on the haematological system in children, although until recently these effects were presumed to be short term, with levels of haemoglobin and neutrophils returning to normal after cessation of exposure [1,2,4]. Recently, Le Chenadec *et al.* [7] showed that exposure to ARV *in utero* and postnatally was associated with lower levels of haematological parameters, in particular neutrophil counts, until 18 months of age, but no longer-term data were available.

Neutrophils are the most numerous and important cellular component of the innate immune response, and deficiencies in neutrophil function can lead to overwhelming bacterial infections. Their vital role in a competent immune system adds to the concern of possible long-term adverse effects of ARV exposure. Previous results from the European Collaborative Study showed gender and race differences in CD4 and CD8 T cell and absolute lymphocyte counts in uninfected and HIV-infected children, and gender and race may also be associated with neutrophil patterns and levels [8].

Here we report results of an analysis of data collected in a European longitudinal cohort study identifying patterns and levels of neutrophil cell counts over 8 years from birth in HIV-infected and uninfected children of HIV-infected mothers and estimate the effect of factors including gender, race, prematurity, ARV prophylaxis and treatment.

Methods

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

A child was classified as HIV infected after the onset of AIDS, the detection of virus or antigen in at least two blood samples (taken on separate occasions), or the persistence of antibody beyond 18 months of age. A child was presumed uninfected if at least two blood samples were antibody negative and no virus or antigen had ever been identified [8]. Laboratory tests including viral load assay and white blood cell count and differentiation were carried out locally according to standard procedures that did not vary between centres.

Prematurity was defined by a gestational age < 37 weeks. ARV exposure was classified as none or any and categorized as *in utero* exposure or neonatal ARV prophylaxis or both. Based on the availability of different ARV treatment regimens, the cohort was divided in three groups: children born before 1 January 1994 (before introduction of ARV prophylaxis), children born between 1994 and 31 December 1997 (use of mono or double combination ARV) and children born 1 January 1998 and after (when highly active antiretroviral therapy became more commonly used). Maternal ARV regimen nearly always included zidovudine (90%). ARV treatment for HIV-infected children was grouped as none, monotherapy or combination therapy.

The analyses were repeated for the subset of children for whom a maternal CD4 T cell count at delivery was available, since this information was collected routinely only since 1992. The maternal CD4 T cell count was from the sample obtained closest to delivery. CD4 T cell count was divided in three groups: < 250, 250–499 and $\geqslant 500 \times 10^6$ cells/l.

Statistical methods

Absolute neutrophil counts were $\log_{10}$ transformed to normalize the data. The structure of the data was visualized by a variable span smoother, which is a weighted average of the neutrophil count [9]. Natural cubic spline regression models [splines are a series of cubic functions joined together at specified time points (knots)] were used to model patterns of neutrophil counts with age; the position and number of knots of these spline models were determined by judging the shape of the splines against the smoother. Knots for the model using measurements for all children and for uninfected children were located at 1 week and at 1, 2 and 45 months; the model for HIV-infected children had knots at 1, 2 and 45 months.

Linear mixed effects were used to allow for repeated measurements and were fitted using maximum likelihood to allow testing for goodness of fit improvements. Once the general shape of the spline was determined, Akaike's Information Criterion was used to decide the random effects (to allow for between-child variability in neutrophil counts) and the covariates to be included in the models. Confidence intervals for predicted neutrophil values at 5 years were calculated using 1000 bootstrap [10] samples with replacement over the set of children.

Covariates included the child's HIV-1 infection status, prematurity, gender, race, drug-withdrawal symptoms at birth and exposure to ARV. As natural cubic spline models are non-parametric, the magnitudes of the coefficients cannot be interpreted in a direct way that is meaningful in the conventional quantitative sense. Therefore, the effects of these variables on the neutrophil count were quantified in uni- and multivariable regression analyses using *z*-scores (or SD) of neutrophil counts. The *z*-scores were obtained after modelling untransformed absolute neutrophil counts using the LMS method [11]. The *z*-scores are related to measurements that are no longer age dependent, as each measure is compared with the measure of a standard at that age; this provided a way to optimize the data available for analyses. Again the repeated measurement nature of the data was accounted for by using linear mixed effects.

In the *z*-score regression analyses of the HIV-1-infected children, two further variables were introduced: progression to AIDS and ARV therapy for the child. These two variables were time dependent; the first indicated whether the child had progressed to AIDS at the time the blood sample was obtained. The second variable indicated if the child was on none, monotherapy or combination ARV to treat his/her HIV-1 infection at least 1 month before the neutrophil count measurement was obtained.

Data entry and management were carried out using Microsoft Access XP (Redmond, Washington, USA). Natural cubic splines and supersmoothers were carried out using S-plus 2000 (Insightful, Seattle, Washington, USA). Regression analyses of the *z*-scores were carried out using STATA 2001 (Stata Corp., College Station, Texas, USA).

Results

By July 2003, a total of 2407 children were enrolled. Children with missing data for neutrophil counts or any of the covariates were excluded, and observations obtained for ages up to 8 years were considered since measurements at later ages were sparse. This resulted in 11 756 measurements from 1669 children. Of these, 1513 children (with 9293 measurements) were uninfected, and 156 (with 2463 measurements) were HIV infected. The median age at last visit was 1.72 years (1.58 years for uninfected and 5.0 years for HIV infected). The median number of observations was six (range, 1–26) for uninfected and 12 (range, 1–42) for HIV-infected children. Table 1 shows the distributions of the main covariates.

Table 1. Characteristics of the 1669 mothers and children.

Variable	No. (%)
Race	
Black	450 (27.0)
White	1219 (73.0)
Gender	
Female	801 (48.0)
Male	868 (52.0)
HIV status	
Infected	156 (9.3)
Not infected	1513 (90.7)
Gestational age < 37 weeks	
Yes	355 (21.3)
No	1314 (78.7)
Year of birth	
Before 1994	816 (48.9)
1994–1997	299 (17.9)
After 1997	554 (33.2)
Exposure to antiretroviral drugs	
No	905 (54.0)
Yes	764 (46.0)
Drug-withdrawal symptoms[a]	
No	1468 (88.0)
Yes	201 (12.0)
Maternal CD4 cell count ($\times 10^6$ cells/l)	
< 250	131 (20.6)
250–499	270 (42.5)
$\geqslant 500$	235 (36.9)

[a]In maternal injecting drug use.

Neutrophil levels and patterns

The pattern and levels of neutrophils over age varied significantly by the child's infection status (Fig. 1). To establish the structure of this variation, HIV-infected and uninfected children were modelled separately; models closely followed the smoothers from the raw data and the goodness of fit of the models was assessed as described in the Methods. In uninfected children, the neutrophil count decreased rapidly after birth reaching a nadir at 2 months, then increasing until approximately 7 months; after this, the increase was more gradual before stabilizing at 3.5 years. In HIV-infected infants, neutrophil counts at birth were similar but decreased less, stabilizing at 4 months, at a lower level than in uninfected children, with a further gradual decline after about 3.5 years of age (Fig. 1).

Black children had significantly lower neutrophil counts overall than white children ($P < 0.001$), but the difference decreased with increasing age among the uninfected, but not HIV-infected, children (Fig. 2). In uninfected children, boys had statistically significantly lower neutrophil counts ($P = 0.024$) over the entire age range than girls, although the difference was not large. In HIV-infected children, girls had lower neutrophil counts than boys until about 5 years of age, although this did not reach statistical significance ($P = 0.139$). ARV exposure was associated with lower neutrophil counts in uninfected children ($P < 0.001$) over the whole age range, but not significantly so in HIV-infected children ($P = 0.089$) (Fig. 3).

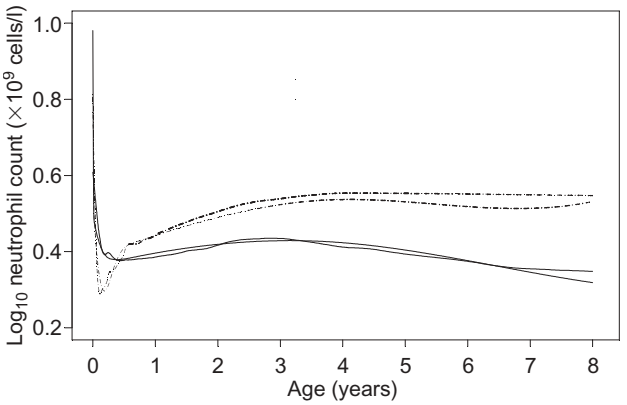

Fig. 1. Smoother and fitted spline models for HIV-infected (—) and HIV-uninfected (- - -) children.

For uninfected children, prematurity had a significant, though small, effect ($P < 0.001$): infants with gestational age < 37 weeks had a lower neutrophil count for approximately 7 months; thereafter their neutrophil levels were similar to those in full-term children. There was no significant association with prematurity in the HIV-infected children.

Further improvement in fitting of the spline models was achieved by allowing for several variables simultaneously. Table 2 shows the estimated value of the neutrophil counts obtained from the spline regression models illustrating the above associations. In uninfected children not exposed to ARV, the estimated difference between black and white boys was 616, 713 and 177×10^6 cells/l at 1 month, 1 year and 5 years of age, respectively (Table 2). In uninfected children exposed to ARV, the estimated differences between black and white children were less pronounced initially but larger at 5 years: 236, 526 and 300×10^6 cells/l, at 1 month, 1 year and 5 years, respectively. The difference between white girls and boys not exposed to ARV was relatively stable at 238×10^6 cells/l at 1 month and 298×10^6 cells/l at 5 years (Table 2). Differences between girls and boys were also influenced by ARV exposure, with marginal differences in white ARV-

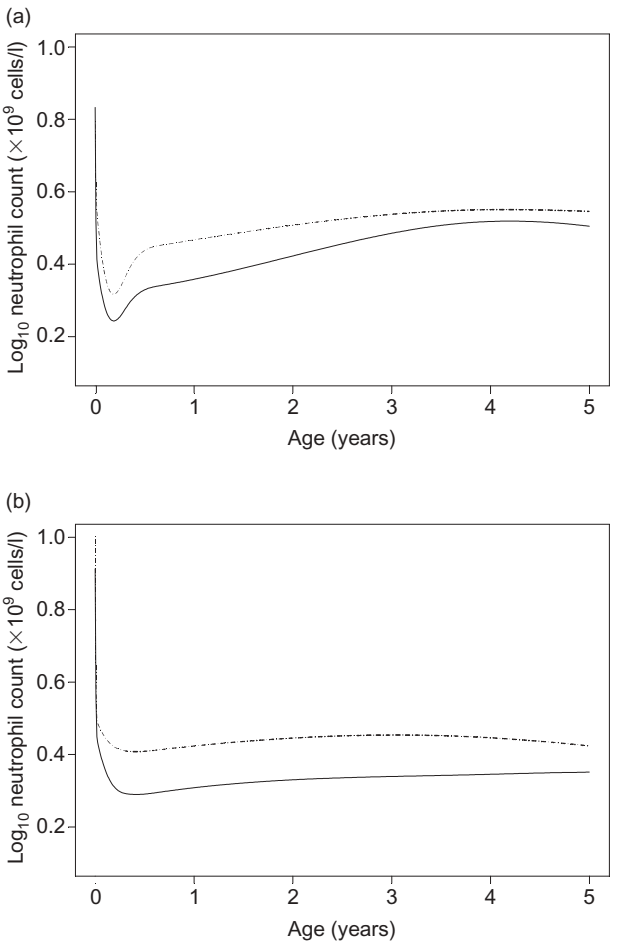

Fig. 2. Fitted spline models for black (—) and white (- - -) children. (a) HIV-negative children; (b) HIV-positive children.

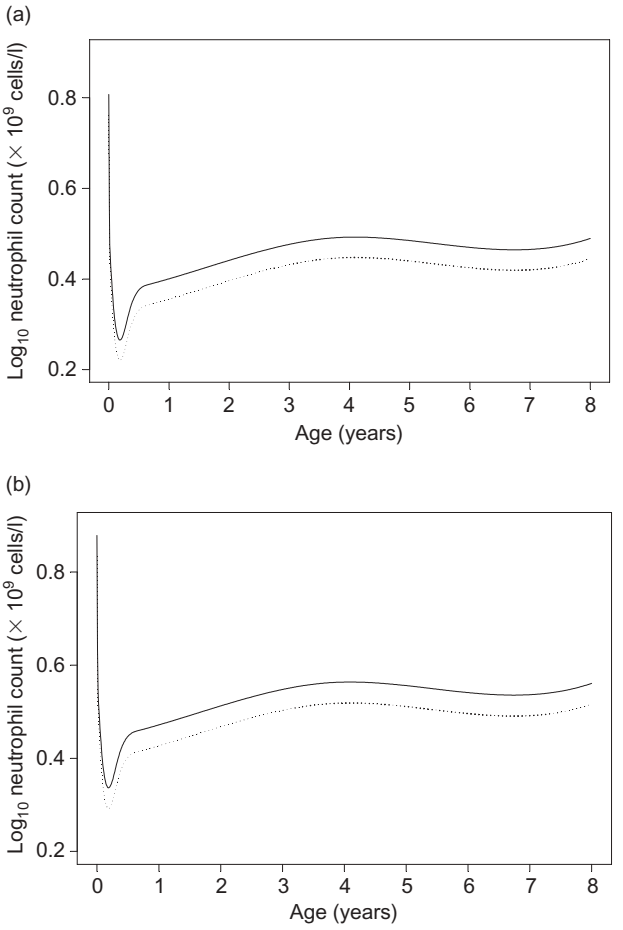

Fig. 3. Fitted spline models for uninfected children with (- - -) and without (—) exposure to antiretroviral therapy. (a) Black children; (b) white children.

Table 2. Estimated neutrophil cell counts in uninfected children by race from the natural cubic spline regression models.

	Neutrophil cell count in white children ($\times 10^6$ cells/l)			Neutrophil cell count in black children ($\times 10^6$ cells/l)		
Variable	1 month	1 year	5 years (CI)	1 month	1 year	5 years (CI)
Girls, ARV exposure						
No	3027	3084	3764 (3621–3907)	2359	2310	3570 (2848–4161)
Yes	2180	2766	2970 (2555–3464)	1940	2223	2658 (2128–3441)
Boys, ARV exposure						
No	2789	2841	3466 (3316–3593)	2173	2128	3289 (2627–3819)
Yes	2141	2710	2911 (2530–3392)	1905	2184	2610 (2104–3362)
Term, ARV exposure						
No	2491	2979	3571 (3460–3753)	2118	2445	3431 (2679–3865)
Yes	2269	2707	3246 (2506–3397)	1925	2223	3118 (2204–3327)
Premature, ARV exposure						
No	2300	2982	3559 (3352–3970)	1953	2447	3419 (2644–4078)
Yes	2091	2708	3234 (2669–11339)	1775	2224	3107 (1962–12381)
No ARV exposure, drug-withdrawal symptoms[a]						
No	2941	2969	3588 (3486–3748)	2254	2193	3288 (2820–3732)
Yes	3070	3099	3745 (3430–3862)	2352	2289	3432 (2820–3795)

CI, confidence interval; ARV, antiretroviral drug.
[a]In maternal injecting drug use.

exposed children: 39, 56 and 69×10^6 cells/l at 1 month, 1 year and 5 years, respectively.

In HIV-infected children, ARV prophylaxis exposure was not significantly associated with lower neutrophil counts. Injecting drug use of the mother during pregnancy resulting in drug-withdrawal symptoms in the infant was associated with increased neutrophil counts in both HIV-infected and uninfected children (Tables 2 and 3).

Regression analyses of *z*-scores

The spline models showed significant effects on levels of neutrophil counts by infants' HIV status, race, gender, ARV exposure, maternal injection drug use and prematurity, but these differences could not directly be quantified from these non-parametric models. Therefore, a *z*-score, which is age independent, was calculated for each neutrophil cell count and analysed in uni- and multivariable regression to quantify the differences by infants' HIV status, gender, race, prematurity, maternal injection drug use and ARV exposure. The short-term effect was evaluated based on measurements in the first 6 months of life, and the overall and long-term effect on data relating to at least age 8 years.

Association of z-score with child's HIV infection status

In univariable analysis of *z*-scores measured before 6 months of age, HIV-infected infants had significantly higher neutrophil counts than uninfected infants (coefficient, 0.148; $P = 0.03$), a reflection of the more exaggerated decline in neutrophil counts in uninfected than in HIV-infected children (Fig. 1). In multivariable analysis, this association was no longer statistically significant. The long-term effect of HIV infection was in the opposite direction: in univariable analyses of neutrophil *z*-scores, HIV-infected children had lower neutrophil counts (coefficient, -0.214; $P < 0.001$) than uninfected children across the full 8 years. This

Table 3. Estimated neutrophil cell counts in HIV-infected children by race from the natural cubic spline regression models.

	Neutrophil cell count in white children ($\times 10^6$ cells/l)			Neutrophil cell count in black children ($\times 10^6$ cells/l)		
Variable	1 month	1 year	5 years (CI)	1 month	1 year	5 years (CI)
No ARV exposure						
Girls	2569	2559	2487 (2394–2666)	2478	1903	1872 (1584–2344)
Boys	2832	2821	2741 (2623–3004)	2731	2098	2064 (1791–2585)
No ARV exposure/Drug-withdrawal symptoms[a]						
No	2812	2664	2624 (2474–2811)	3189	1942	1933 (1518–2472)
Yes	2965	2808	2766 (2553–3003)	3362	2047	2039 (1626–2568)

CI, confidence interval; ARV, antiretroviral drug.
[a]In maternal injecting drug use.

association remained statistically significant in multivariable analysis (coefficient, −0.269; $P < 0.001$). Further analyses were conducted separately for HIV-infected and uninfected children.

Uninfected children

ARV exposure and race were the most important determinants of neutrophil values in uninfected children (Table 4), persisting until at least age 8 years. Further distinction by timing of ARV (*in utero*, neonatal or both) and by maternal monotherapy and combination regimen showed effects of ARV exposure, although statistical significance was not reached because of small numbers in some categories. Black children had, on average, a *z*-score that was 0.30 lower than white children and ARV-exposed children had a *z*-score that was 0.15 lower than unexposed children. In analysis of measurements relating to the first 6 months only, the effect of race and ARV exposure were similar. The effect of prematurity was minimal and only significant in univariable analysis in the first 6 months of life, but not in multivariable analysis nor in analysis of neutrophil counts over the full 8 years (Table 4).

The effect of gender in the *z*-score analysis confirmed the marginal although significant differences observed in the modelling, with uninfected boys having lower neutrophil counts than girls (Table 4); in the analyses of the short-term effect, the association was of similar size and significance. The effect of injection drug use by the mother during pregnancy was associated with a marginal 0.032 increased *z*-score overall both in short-term and long-term analyses.

HIV-1-infected children

There was no significant short- or long-term effect of ARV exposure on neutrophil *z*-scores in the HIV-infected children (Table 4). Similar to uninfected children, race was associated with lower neutrophil *z*-scores: black children had a 0.32 lower score than white children in multivariable analysis of measurements until 8 years of age. The effect by gender was in opposite direction to that in uninfected children but did not reach statistical significance. Prematurity and maternal injection drug use were not significantly associated with neutrophil *z*-scores in the HIV-infected children.

A total of 53 HIV-infected children progressed to AIDS; in 93 HIV-infected children, ARV therapy was initiated. After progression to AIDS or initiation of ARV therapy, neutrophil counts declined and these

Table 4. Differences in neutrophil *z*-scores in uninfected and HIV-infected children by gender, race, antiretroviral drug exposure and treatment, using measurements over the first 8 years of life.

	Uninfected children[a]			Infected children[a]		
Variable	No.	Univariable coefficient (*P* value)	Multivariable coefficient (*P* value)	No.	Univariable coefficient (*P* value)	Multivariable coefficient (*P* value)
Gender						
Female	723			78		
Male	790	−0.058 (0.03)	−0.065 (0.03)	78	0.151 (0.12)	0.160 (0.09)
Ethnicity						
White	1101			118		
Black	412	−0.383 (< 0.001)	−0.296 (< 0.001)	38	−0.347 (0.002)	−0.320 (0.007)
Prematurity						
Yes	325			30		
No	1188	0.048 (0.21)	0.012 (0.34)	126	−0.021 (0.87)	0.032 (0.79)
Maternal injection drug use						
Yes	1258			134		
No	255	0.076 (< 0.001)	0.032 (0.02)	22	0.046 (0.32)	0.052 (0.25)
ARV exposure						
No	777			128		
Yes	736	−0.280 (< 0.001)	−0.150 (0.04)	28	−0.102 (0.44)	0.099 (0.65)
Birth cohort						
Before 1994	701			115		
1994–1997	271	−0.184 (< 0.001)	0.001 (0.989)	28	0.033 (0.80)	0.116 (0.51)
After 1997	541	−0.290 (< 0.001)	−0.044 (0.57)	13	−0.222 (0.24)	−0.120 (0.65)
AIDS						
No	–	–	–	149		
Yes	–	–	–	53	0.370 (< 0.001)	−0.236 (0.002)
ARV therapy						
None	–	–	–	155		
Monotherapy	–	–	–	87	−0.397 (< 0.001)	−0.355 (< 0.001)
Combination	–	–	–	22	−0.582 (< 0.001)	−0.584 (< 0.001)

ARV, antiretroviral drug.
[a]AIDS and antiretroviral therapy are time-dependent variables and so one child can contribute to both categories at different times.

variables were the main long-term determinants of neutrophil levels in HIV-infected children (Table 4).

Maternal immunity and infant neutrophil counts

The influence of maternal immune status on infant neutrophil levels was evaluated in a subset of uninfected children ($n = 597$) for whom maternal CD4 T cell counts were available. In the first 6 months of life, uninfected children of mothers with CD4 T cell counts $< 250 \times 10^6$ cells/l had lower neutrophil counts than those of mothers with $250-500 \times 10^6$ cells/l (coefficient, 0.161; $P = 0.037$) or $> 500 \times 10^6$ cells/l (coefficient, 0.102; $P = 0.193$). The long-term analyses showed a similar trend for offspring of mothers with CD4 cell counts of $250-499 \times 10^6$ cells/l (coefficient, 0.144; $P = 0.026$) and $\geqslant 500 \times 10^6$ cells/l (coefficient, 0.157; $P = 0.017$). These differences were borderline significant in multivariable analyses including the same factors as above in addition to maternal CD4 T cell count. Race and ARV exposure remained the most important and significant factors associated with neutrophil counts. In HIV-infected children ($n = 39$), maternal CD4 T cell counts were not significantly associated with neutrophil counts in either uni- or multivariable analyses.

Discussion

Using data from this large European cohort, levels and patterns of neutrophil cell counts were analysed in children born to HIV-infected mothers. This cohort presented a unique opportunity to clarify the effect on neutrophil counts of a child's HIV infection status and other factors. Neutrophil levels were initially high shortly after birth but declined (especially for uninfected children) and reached a nadir around 2 months before increasing again to reach a plateau around 3.5 years in uninfected children; at which age, levels in HIV-infected children started to decline. A nadir at 2 months is later than might be expected given that after stimulation with granulocyte–macrophage colony-stimulating factor the bone marrow normally replenishes neutrophil counts in the circulation in 14–20 days [12]. This might possibly indicate that the bone marrow of neonates has reduced capacity for restoring neutrophil levels after the release of neutrophils during and immediately after delivery. HIV-infected infants may possibly have an upregulated immune system in the first months of life and, therefore, continuously release more neutrophils.

HIV infection in children was associated with substantially decreased neutrophil counts at all ages after the first 4 months, with approximately a 0.25 unit SD difference overall. In HIV-infected children, progression to AIDS, ARV therapy initiation and black race were the main factors related to low levels of neutrophils, associated with an approximate 0.24–0.58 unit SD difference, which would at least partially explain the decline in neutrophil counts seen in HIV-infected children after 3.5 years of age. The effect of ARV therapy was more pronounced with combination therapy than with monotherapy. These results are consistent with results from studies in adults, which showed that neutropenia in HIV-1-infected patients was associated with ARV therapy and progression of disease [5,6,13,14].

Any effect on haematopoiesis of ARV exposure in fetal or early neonatal life in uninfected children had been presumed to be short term, with return to normal values at cessation of exposure until Le Chenadec *et al.* [7] showed an effect until at least 18 months. We were able to analyse levels up to at least 8 years of age and showed that exposure to ARV was associated with reduced neutrophil counts at all ages, regardless of race and gender, but only in uninfected children. It is unclear whether this effect persists into adolescence and adulthood as our data became too sparse after 8 years for meaningful analyses. Our estimated neutrophil cell counts at 1 month and 1 year of age were very similar to the estimates from the French perinatal cohort [7] .We compared the levels in uninfected children without exposure to ARV with only exposure *in utero*, only neonatal or both and with maternal monotherapy and combination therapy; although we saw an effect on neutrophil counts in each grouping of exposure to ARV, the number of measurements was too small to reach a reliable conclusion. However, our results were suggestive of a duration-dependent effect of ARV exposure on haematopoiesis, which would be consistent with the transplacental passage of nucleoside and non-nucleoside reverse transcriptase inhibitors [4,15,16].

Additional to the toxicity of ARV exposure is the effect of prematurity on neutrophil counts. Premature infants cannot eliminate drugs as well as term neonates and, therefore, may be more susceptible to the toxic effects of ARV drugs on the bone marrow [17,18]. In our study, premature infants exposed to ARV had the lowest neutrophil levels until approximately 7 months of age. Thereafter, there were no significant differences between premature and term infants, possibly because premature children have gained enough bone marrow reserve to catch up with term children [19,20].

Maternal immune status was only marginally associated with infant neutrophil counts in uninfected children and the effect was only borderline significant once the effect of other variables was allowed for. Maternal immune status may be associated with infant immune status, especially on CD4 T cells. Le Chenadec and colleagues [7] reported that maternal immunity influ-

enced haematological parameters, but this was only significant in lymphocytes. In our multivariable analyses, race and ARV exposure were dominant over maternal CD4 cell counts, suggesting that at most the effect is of limited relevance. Analyses in larger cohorts are required to investigate the association between markers of maternal and infant immunity.

Earlier findings from the European Collaborative Study showed a difference in lymphocyte counts associated with race and gender, with black children having lower lymphocyte counts than white children [8]. Here, we show that this is also true for neutrophils, with both uninfected and HIV-infected black children having lower neutrophil counts than white children. The decrease in the race difference with age in the uninfected children has, to our knowledge, not been shown before and may be clinically relevant. In adult studies, results of the effect of race on white blood cell counts are conflicting [6,21–24]. In our study, differences between black and white children were smaller in uninfected children exposed to ARV than in non-exposed children. Although marginal, the gender difference we found was in the same direction as previously observed for CD4 T cells [8].

We observed that the course of the neutrophil cell count fluctuated within a child over time (data not shown), which could indicate that the bone marrow remains responsive to infections even when overall levels are decreased. Investigation of the clinical implications of the lower neutrophil counts in subpopulations in our cohort was beyond the scope of this paper and detail of data available, but it would be the next step in understanding the clinical relevance of the differences.

The statistical models used in this study have their limitations but in combination give a more complete picture. Splines provide flexibility, which is crucial to replicate adequately both the rapid decrease in neutrophil counts for very early ages and the relative stability observed between 3 and 8 years. However, in these non-parametric models, differences are difficult to quantify directly. The regression analysis of *z*-scores (SD) allows the quantification of the differences but cannot be easily used to explore differences in patterns over time.

We confirm that in HIV-infected children ARV therapy and disease progression are associated with decreased neutrophil count. However, we show for the first time that among uninfected children, exposure to ARV is associated with long-term decreased neutrophil counts and thus add to the growing list of possible adverse events after early-life ARV exposure, such as mitochondrial abnormalities [25] and increased risk of premature delivery [26].

ARV prophylaxis is essential in preventing mother-to-child transmission and the benefits greatly outweigh the currently unquantified long-term clinical adverse consequences of ARV exposure in early life. However, more research is required, in particular continuation of the follow-up of exposed uninfected children beyond 18 months of age is essential to inform this discussion.

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Appendix

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