CONCISE COMMUNICATION

Comparing genotoxic signatures in cord blood cells from neonates exposed *in utero* to zidovudine or tenofovir

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Objectives: Zidovudine and tenofovir are the two main nucleos(t)ide analogs used to prevent mother-to-child transmission of HIV. *In vitro*, both drugs bind to and integrate into human DNA and inhibit telomerase. The objective of the present study was to assess the genotoxic effects of either zidovudine or tenofovir-based combination therapies on cord blood cells in newborns exposed *in utero*.

Design: We compared the aneuploid rate and the gene expression profiles in cord blood samples from newborns exposed either to zidovudine or tenofovir-based combination therapies during pregnancy and from unexposed controls ($n = 8$, 9, and 8, respectively).

Methods: The aneuploidy rate was measured on the cord blood T-cell karyotype. Gene expression profiles of cord blood T cells and hematopoietic stem and progenitor cells were determined with microarrays, analyzed in a gene set enrichment analysis and confirmed by real-time quantitative PCRs.

Results: Aneuploidy was more frequent in the zidovudine-exposed group (26.3%) than in the tenofovir-exposed group (14.2%) or in controls (13.3%; $P < 0.05$ for both). The transcription of genes involved in DNA repair, telomere maintenance, nucleotide metabolism, DNA/RNA synthesis, and the cell cycle was deregulated in samples from both the zidovudine and the tenofovir-exposed groups.

Conclusion: Although tenofovir has a lower clastogenic impact than zidovudine, gene expression profiling showed that both drugs alter the transcription of DNA repair and telomere maintenance genes.

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Introduction

Guidelines on treatment during pregnancy have gradually expanded from zidovudine monotherapy to various combinations of nucleos(t)ide analogs and either a protease inhibitor or a non-nucleoside reverse transcriptase inhibitor. The tenofovir–emtricitabine nucleos(t)ide analog combination is now widely prescribed (either before conception or during pregnancy) and has recently been recommended by the WHO [1,2].

Potential genotoxicity is a key issue for all currently marketed antiretroviral nucleos(t)ide analogs, since they can all integrate into human nuclear DNA and act as terminators of DNA replication [3–6]. However, the affinity of these nucleos(t)ide analogs for human nuclear and mitochondrial DNA varies from one molecule to another, and possibly from one cell type to another [4]. All of these molecules cross the placental barrier freely and some of them concentrate in the amniotic fluid [7,8]. Thus, exposure *in utero* might give rise to greater genotoxicity than that observed in adults or older children. This risk merits in-depth evaluation.

We and others identified biomarkers of genotoxicity in neonates exposed to zidovudine–lamivudine [9–12], including a high proportion of aneuploid mononuclear cells in cord blood and the abnormal expression of many DNA repair genes in hematopoietic stem and progenitor cells [12]. To date, few studies on the genotoxicity of tenofovir have been published. Some preclinical data suggest that tenofovir has a weak genotoxicity profile [13,14]. However, in two recent studies, tenofovir–emtricitabine combination was found to be more cytotoxic than zidovudine–lamivudine *in vitro*, and tenofovir was the most potent inhibitor of telomerase activity [15,16].

Here, we compared the aneuploid rate and the gene-expression profiles in newborns exposed *in utero* to either fixed-dose combinations of zidovudine–lamivudine or tenofovir–emtricitabine with a ritonavir-boosted protease inhibitor.

Patients, materials and methods

Umbilical cord blood was collected after written consent from non-HIV-1-infected and HIV-1-infected pregnant women having been treated with either a tenofovir or a zidovudine-based combination for at least 4 weeks during pregnancy. The noninfected status of infants born to HIV-1-infected women was checked by PCR. $CD34^+$ hematopoietic stem and progenitor cell (HSPC) and $CD34^-/CD3^+$ T-lymphocyte were sorted as previously described [12].

$CD3^+$ T cells were karyotyped as previously described [12] according to the 2009 International System for Human Cytogenetic Nomenclature guidelines [17].

The methods used in the transcriptome analysis of $CD34^+$ HSPCs are described elsewhere [12]. For $CD3^+$ T cells, hybridization was performed on HumanHT-12 v4.0 BeadChips (Illumina, Inc., San Diego, California, USA). Analyses were performed as previously described, with a few minor changes [18,19].

Gene set enrichment analysis was performed with GSEA software (http://www.broadinstitute.org/gsea/index.jsp) and reactome pathways derived from the Molecular Signatures Database (http://www.broadinstitute.org/gsea/msigdb/index.jsp).

Quantitative PCR reaction was performed in triplicate with SYBR Green, on 15 and 19 genes selected for validation in $CD34^+$ HSPCs in $CD3^+$ T cells, respectively. The fold-change [$2^{-\Delta\Delta}$ cycle threshold (CT)] was calculated by normalizing the CT values against the mean values of two housekeeping genes (*RPS13* and *SEC11a*) with stable expression levels according to the microarray data.

Statistical analysis (other than transcriptional profiling analysis)

All statistical analyses were performed with SAS software (version 9.2, SAS Institute Inc., Cary, North Carolina, USA).

Results

Characteristics of patients and controls

Umbilical cord blood was collected for eight HIV-1-uninfected newborns exposed to a zidovudine-based regimen, nine exposed to a tenofovir-based regimen and eight born to uninfected women. The three groups did not differ significantly in terms of maternal age, term of pregnancy or birth weight (data not shown). Infected mothers had no distinctive clinical or biological features relative to the overall data from the French national perinatal cohort. There was no significant difference between the zidovudine and the tenofovir groups in terms of the maternal HIV load. However, the $CD4^+$ cell count was higher in the zidovudine group because the duration of exposure to zidovudine *in utero* was shorter than that of tenofovir (tenofovir was more frequently initiated before pregnancy than zidovudine).

Cytogenetic studies

The proportion of aneuploid $CD3^+$ T cells in the zidovudine-exposed group ($n = 7$, mean ± SD: 26.3% ± 9.2) was twice that observed in the tenofovir-exposed group ($n = 8$, 14.2% ± 6.7; $P < 0.05$) and in the control group

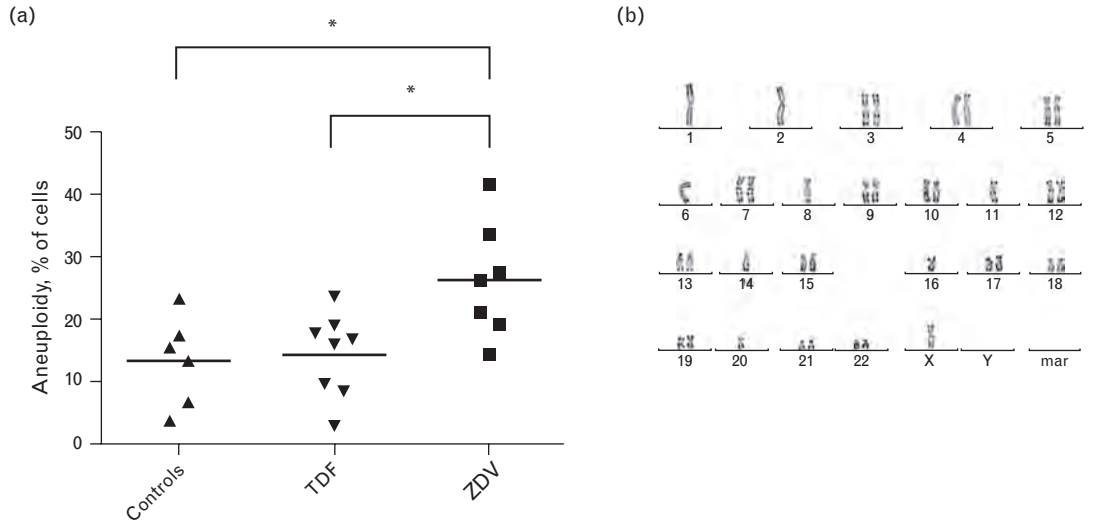

Fig. 1. The frequency of aneuploidy in CD3$^+$ cells stimulated with phytohemagglutinin for 72 h. (a) The aneuploidy rate was defined as the percentage of all scored cells that were aneuploid (i.e. with more or less than 46 chromosomes per metaphase) for each of the antiretroviral-exposed and control samples. Bars represent the mean values. TDF, tenofovir; ZDV, zidovudine. (*) $P < 0.05$. (b) An example of severe hypoploidy (37X, -Y, -1, -2,-6; -8, -11, -14, -16, -20).

($n = 6$, $13.3\% \pm 7.2$; $P < 0.05$) (Fig. 1a). All chromosomes were involved, and the alterations were randomly distributed (Fig. 1b).

Gene expression profiling of CD3$^+$ T cells and CD34$^+$ hematopoietic stem and progenitor cells

We used microarrays to examine the gene-expression profile of CD3$^+$ cells ($n = 7$ each for zidovudine, tenofovir and controls). The overall analysis of transcriptome profiles (as illustrated by heat maps; fold-change >1.5; $P < 0.05$) revealed a characteristic molecular pattern for each group, but with many more similarities between zidovudine and tenofovir samples than between zidovudine or tenofovir and control samples (Fig. 2a). Hierarchical clustering confirmed that the neonates could be segregated according to their in-utero exposure (except for two zidovudine and two tenofovir samples that segregated with control samples; data not shown); however, the number of genes that were differentially expressed when comparing the zidovudine and the tenofovir-exposed groups (3) was much lower than the corresponding values for the zidovudine-exposed vs. control samples (837) and for the tenofovir-exposed vs. control samples (852).

Gene set enrichment analysis confirmed that very similar sets of genes were affected by tenofovir and zidovudine in CD3$^+$ T cells (Suppl Table 1, http://links.lww.com/QAD/A668). The expression of genes involved in DNA/RNA synthesis, nucleotide metabolism, cell cycle, DNA repair and telomere maintenance in both the zidovudine and the tenofovir-exposed groups differed significantly from that observed in the control group.

The gene expression profiles of CD34$^+$ HSPCs were similar to those obtained for CD3$^+$ T cells. As illustrated for genes involved in telomere maintenance and DNA repair, further analysis of enrichment plots and heat maps gave similar results when comparing either the tenofovir or the zidovudine-exposed groups with controls (Fig. 2b).

These results suggest that zidovudine and tenofovir have an impact on both mature and immature hematopoietic cells.

Validation of the transcriptome profile with quantitative PCRs

To validate the microarray data, we used quantitative PCRs (qPCRs) to examine the expression of genes selected on the basis of their involvement in DNA repair, chromosome maintenance and the cell cycle; and their significant transcriptional deregulation in both zidovudine and tenofovir-exposed samples.

We found that 43% of the genes analyzed in CD34$^+$ cells and 72% of those analyzed in CD3$^+$ cells were deregulated in zidovudine and tenofovir-exposed samples ($P < 0.05$), confirming the microarray experiments. Furthermore, qPCR showed that most of the genes involved in the DNA damage response (nucleotide excision repair, mismatch repair, nonhomologous end-joining and homologous recombination) and chromosome maintenance were more strongly expressed in both zidovudine and tenofovir-exposed samples than in control samples (data not shown). When we compared zidovudine and tenofovir-exposed groups, some genes appeared to be expressed differentially (Fig. 2c); for

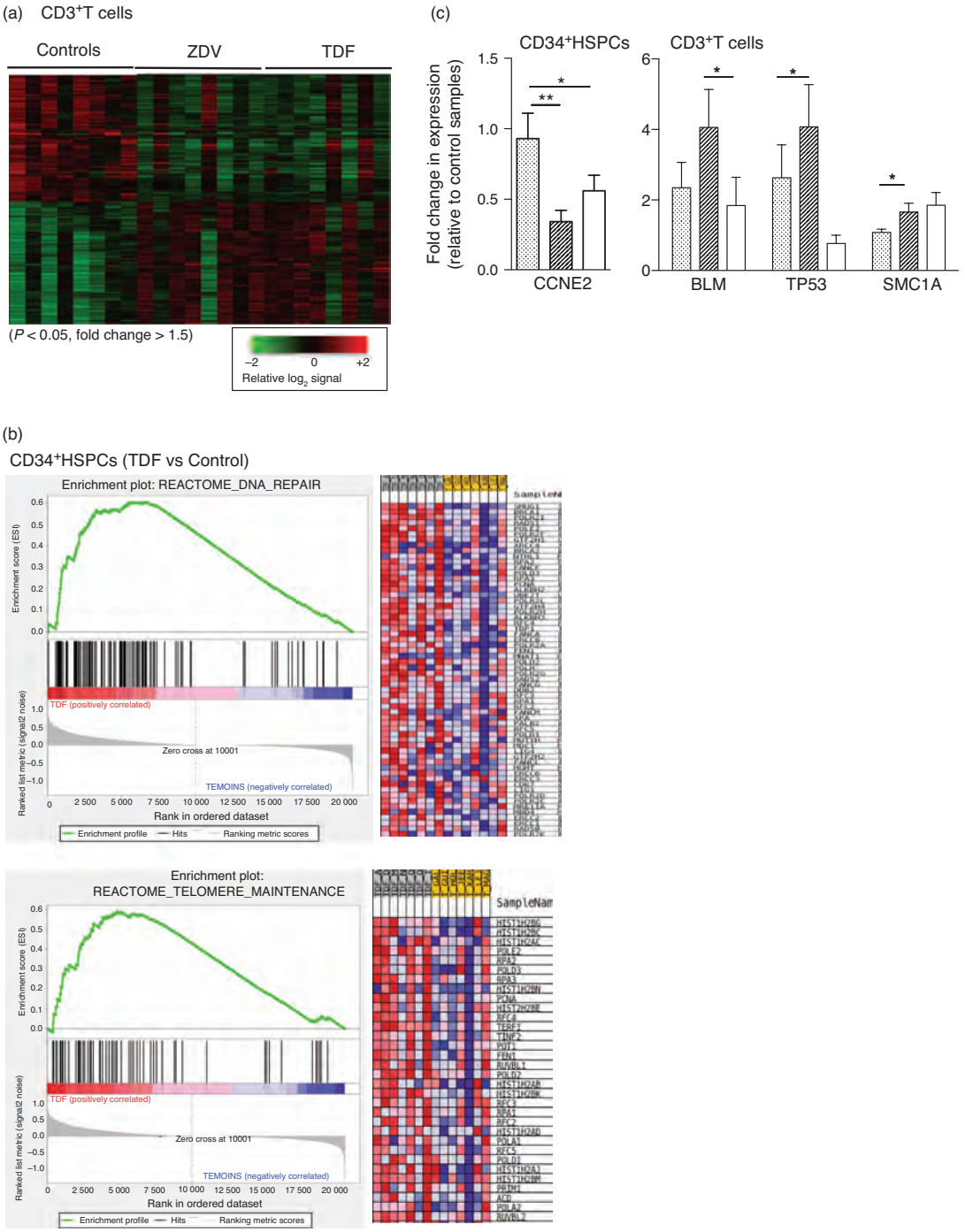

Fig. 2. Gene-expression profiles in cord blood cells. Affymetrix analysis of the gene-expression profiles of $CD3^+$ T cells from zidovudine (azidothymidine)-exposed (ZDV), tenofovir disoproxil fumarate-exposed (TDF) and control groups. Significant differences are based on a 1.5-fold difference in expression and P less than 0.05. (a) The heat map. (b) Enrichment plots and heat maps for telomere maintenance and DNA repair gene sets in GSEA are shown for $CD34^+$ HSPCs. Top left panel: the running enrichment score for the gene set as the analysis walks along the ranked list. Middle left panel: location of the genes from the telomere maintenance reactome within the ranked list. Bottom left panel: a plot of the ranked list of genes. Right panel: a heat map of the core enrichment genes (genes that appear in the ranked list before or at the peak in the enrichment score). The range of colors (red to blue) shows the range of expression values (high to low). (c) Validation of mRNA profiles by qPCR in $CD34^+$ HSPCs (left graph) and $CD3^+$ T cells (right graph) is shown for genes that are differentially expressed in the TDF and ZDV groups. The relative expression (mean ± SD) of genes differentially expressed in ZDV-exposed (dotted bars) and TDF-exposed (hatched bars) groups vs. control group (white bars) is shown. (*) $P < 0.05$; (**) $P < 0.01$. GSEA, gene set enrichment analysis; HSPC, hematopoietic stem and progenitor cell; qPCR, quantitative PCR.

example, *CCNE2* was more strongly expressed in $CD34^+$ cells from the zidovudine-exposed samples; and *BLM*, *TP53* and *SMC1A* were strongly expressed in $CD3^+$ cells from the tenofovir-exposed samples.

Discussion

Our results for the proportion of aneuploid cells in cord blood suggest that tenofovir-based combinations are less genotoxic for the fetus than zidovudine-based combinations. Preclinical data suggest that tenofovir has less clastogenic activity than zidovudine [13,14], which was confirmed here *in vivo* in newborns exposed *in utero*. The present results also independently confirmed our previous report of the clear-cut increase in aneuploidy induced by in-utero exposure to zidovudine–lamivudine [12]. Aneuploidy is primarily caused by the occurrence of centrosome and spindle abnormalities during mitosis, and is considered to be a predisposing factor for cancer. Although most aneuploid cells are probably eliminated by apoptosis, one cannot rule out the survival of some of them (notably stem cells) and thus the occurrence of a potentially oncogenic first event [20].

The reassuring aneuploidy data on the tenofovir-based combination are tempered by the results of the transcriptome analyses. We found that tenofovir exposure and zidovudine exposure are associated with altered gene expression in DNA repair and telomere maintenance pathways in $CD34^+$ HSPCs and $CD3^+$ T cells. The expression profiles of some key cell cycle genes also displayed distinctive features. In $CD34^+$ cells, zidovudine exposure was associated with overexpression of *CCNE2* (essential for cell cycle control in the late G1 phase and early S phase). It is noteworthy that overexpression of *CCNE2* has been reported in mammary epithelial cells treated *in vitro* with zidovudine [21]. *CCNE2* is known to be up-regulated in many tumors [22–24], which may contribute to chromosome instability and even tumorigenesis. *SMC1A* was down-regulated in $CD3^+$ T cells in the zidovudine-exposed group. The protein encoded by *SMC1A* is an important part of the kinetochore and is required for cohesion between sister chromatids [25]. It has been established that the frequency of spontaneous chromosome aberrations is significantly higher in *SMC1A*-mutated cell lines than in control cell lines [26]. We also found that exposure to tenofovir *in utero* was associated with dysregulated expression of key DNA repair genes, such as *BLM* and *TP53*. Despite these specific differences between the zidovudine and the tenofovir-exposed groups, a detailed reactome analysis (using enrichment plots) showed that the two groups exhibited very similar patterns of gene expression dysregulation.

One of the main unresolved questions in our hypothesis-generating study is the persistence over time of this molecular signature and its potential long-term clinical consequences. Olivero *et al.* [27] demonstrated the presence of centrosome amplification and micronuclei in mesenchymal cells up to 3 years after the in-utero exposure of monkeys to zidovudine. The few studies including children exposed to nucleos(t)ide analogs *in utero* have small sample sizes and short follow-up periods [28]. A continuous, long-term evaluation via international registries [29] is thus required. The use of drugs (of whatever class) capable of preventing mother-to-child HIV transmission without interfering with the fetal cells' transcription profile should be preferred.

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Author contributions: A.V. performed the experiments, analyzed the data and wrote the initial draft of the manuscript. T.S.S., W.C., S.L. and J.L. performed experiments and/or participated in analysis of the data. L.M. helped to design the study, monitored the patients and collected clinical and biological data. A.G.C. and E.A. monitored the patients and collected clinical and biological data. J.S. and M.C. conceived the study and analyzed the data. S.B. and I.A.S. conceived and conducted the study, analyzed the data and wrote the manuscript. All authors have seen and approved the final version.

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Conflicts of interest

There are no conflicts of interest.

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