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Research article

Abacavir alters the transcription of inflammatory cytokines in virologically suppressed, HIV-infected women

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Abstract

Background: Abacavir (ABC) may be associated with a small, increased risk of myocardial infarction in HIV-infected adults, possibly related to cytokine-mediated inflammation.

Methods: To evaluate the induction of inflammatory cytokine transcription by ABC, we used samples from women randomized to receive zidovudine/lamivudine/ABC (Trizivir) or lopinavir/ritonavir and zidovudine/lamivudine (Kaletra/Combivir) from the third trimester through six-months postpartum for the prevention of mother-to-child transmission (PMTCT). Women were matched by CD4 count and baseline HIV RNA. All women attained viral suppression (<50 copies/ml) by the time of sampling.

Results: Four cytokines showed a difference in expression between the treatment arms, all in a proinflammatory direction for the ABC arm: CD40LG 1.82-fold, ($p = .027$); IL-8 3.16-fold ($p = .020$); LTA 2.82-fold, ($p = .008$); and CCL5 -1.67-fold, ($p = .035$). At 12-months postpartum, 6-months after antiretroviral discontinuation, cytokine expression was similar by treatment arm.

Conclusions: We conclude that ABC may upregulate proinflammatory cytokines at the transcriptional level in this population.

Keywords: HIV; abacavir; cytokine; transcription; antiretroviral; inflammation; AIDS.

To access the supplementary material to this article please see Supplementary Files under Article Tools online.

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Introduction

Abacavir (ABC) may be associated with a small, increased risk of myocardial infarction (MI) in HIV-infected adults, possibly related to cytokine-mediated inflammation; however, no consistent outcome has been found in studies reporting cytokine levels during ABC treatment.

Data for increased cardiovascular disease (CVD) with ABC use are mixed. The D:A:D study, with over 178,000 person-years of follow-up, reported that recent ABC use was associated with a 68% increased risk of MI [1]. In the SMART study, sub-group analysis showed that ABC, compared with other NRTIs, was associated with increased risk of MI, major cardiac event (MI, stroke and cardiovascular surgery) and an expanded CVD risk definition (including MI, stroke, heart failure and sudden death), although the use of ABC was not randomized [2]. In a nested case-control study from France, increased risk of MI was found in patients exposed to ABC for less than one year or with recent use, but not in others (OR, 2.01, 95% CI, 1.11 to 3.64) [3].

Other studies dispute these apparent associations. GlaxoSmithKline (GSK) published their cumulative analysis from 54 GSK-sponsored clinical studies and found no increased risk of MI with ABC use; overall incidence of MI was low and equal in both groups [4]. In ACTG A5001 (ALLRT), using data from 5056 treatment-naïve patients randomized in multiple clinical trials, there was no evidence that ABC was associated with either short- or long-term increased MI or CVD risk in patients randomized to ABC as their initial regimen [5]. Two meta-analyses recently conducted both suggest that there is no link between ABC use and CVD [6,7].

The mechanism for the increased cardiovascular risk suggested in some studies is unclear. It has been proposed that the risk may be independent of traditional cardiovascular risk factors since it has been largely confined to the period of actual ABC exposure [1]. Several studies have looked at putative mechanisms for the increased cardiovascular risk and suggest that increased platelet reactivity and enhanced leukocyte/endothelial cell interactions may be responsible [8–10]. The SMART study detected higher levels of the proinflammatory markers hsCRP and IL-6 in those receiving ABC compared with other NRTIs. However, whether changes in circulating inflammatory biomarkers associated

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with CVD occur during treatment with ABC has yet to be conclusively resolved [11–13].

In this study, we evaluated samples at two timepoints from a cohort of women who were randomized to either ABC-based or PI (protease inhibitor)-based antiretrovirals as part of a prevention of mother-to-child transmission (PMTCT) regimen. We measured transcription levels of selected inflammatory markers immediately following six to eight months of treatment and again at six months post-exposure to determine if these cytokines were differentially regulated in patients during and after treatment with ABC compared to those on a PI-based regimen.

Materials and methods

Study population

Samples were obtained from patients enrolled in the Mma Bana Study, a randomized, placebo-controlled clinical trial conducted by the Botswana–Harvard AIDS Institute (BHP) to determine the optimal antiretroviral therapy (ART) regimen to reduce vertical transmission in breastfeeding women [14]. This study enrolled 560 HIV-positive, treatment naïve, pregnant women randomized to either ABC+zidovudine+lamivudine (Arm A) vs. lopinavir/ritonavir+zidovudine+lamivudine (Arm B) from 26 to 34-weeks gestation to six months postpartum. Stored samples were available at 6-months postpartum (on treatment) and 12-months postpartum (off treatment). Study drugs were provided as previously reported [14]. The Health Research Development Committee of Botswana and the Human Subjects Committee of the Harvard School of Public Health approved the study protocol and amendments. An independent data and safety monitoring board reviewed safety and efficacy data approximately every six months. Participants were provided with study consent information in English and/or the local Setswana language and agreed to the use of stored samples for future research projects. The full study protocol is available as an appendix to the original parent study [14].

Subjects were chosen for this sub-study by allocating a random number (Microsoft Excel, RAND() function) to each patient ID in the ABC -based arm, ranking the associated number in ascending order and selecting the first 40 patients. Subjects from the PI-based arm were chosen by matching for CD4 and viral load. Clinical characteristics of the participants are provided in Table 1.

RNA purification

Plasma and buffy coat were separated by density gradient centrifugation shortly after collection. Buffy coat fractions were stored at -80°C and went through a single freeze–thaw cycle immediately prior to cellular RNA purification. Total RNA from buffy coat was extracted using a hybrid Trizol and Qiagen RNeasy mini kit protocol (Qiagen, Valencia, CA, USA); 250 μl of buffy coat was added to 1 ml Trizol BD (Sigma, St. Louis, MO, USA), vortexed briefly and incubated at room temperature for 15 minutes; 135 μl of 1-bromo-3-cholopropane was added and then vortexed before another 15-minute incubation. The crude lysate was centrifuged at 12,000g for 15 minutes in order to separate the phases. Afterwards, 500 μl of the aqueous phase was

removed and added to 500 μl 75% ethanol. The sample was then passed through an RNeasy spin following the manufacturer's protocol. Only RNA with an A260:A230 ratio >1.7 , an A260:A280 ratio >1.8 and a concentration >40 ng/ μl was considered of sufficiently high quality for use in real-time PCR.

RT² Profiler assay

Gene expression was quantified using an RT² Profiler inflammatory response and autoimmunity PCR array according to the manufacturer's instructions (SABiosciences, Frederick, MD, USA). The cycle time (C_q) values from these reactions were interpreted by using SABiosciences' PCR array data analysis tool to calculate the $\Delta\Delta C_q$ and the fold change [fold change = $2^{(-\Delta\Delta C_q)}$]. The RT² Profiler assay also verified the absence of genomic DNA and inhibitors of reverse transcription and real-time PCR to ensure consistency between samples.

Real-time PCR validation of results

cDNA synthesis was carried out with the Transcriptor High Fidelity kit (Roche, Randburg, South Africa) according to the manufacturer's protocol using oligo dT as the priming method and a extension temperature of 55°C . Real-time PCR was performed on cDNA using the DyNAmo HS SYBR Green qPCR kit (New England Biolabs, Ipswich, MA, USA). Primers were designed using Primer-BLAST (Table S1 in Additional file 1). Data were acquired in an ABI 7500 under the following cycling conditions: 95°C for 15 minutes followed by 45 cycles of 94°C for 10 seconds, annealing at 65°C or 67°C for 30 seconds and extension at 72°C for 32 seconds during which time the fluorescence was acquired. The optimal annealing temperature was determined empirically. Baseline fluorescence and cycle threshold were calculated automatically by the SDS software v1.4.

Data analysis

In-house qPCR assays for each patient were done in triplicate to determine mRNA transcripts levels for each cytokine. The median mRNA quantity for a cytokine in each arm was determined by transforming the C_q values using $2^{(-\Delta C_q)}$, after correction for primer efficiency using the Pfaffl method, and compared to give a fold-change in expression [15]. A p -value was calculated by Student's t -test after correction for outliers using Grubbs' test with an alpha of 0.05 for all statistical tests. Correction for multiple comparisons was carried out using the Benjamini–Hochberg method to control the false discovery rate [16].

Results

The preliminary cytokine analysis involved 10 individuals from each arm that were matched for CD4 count and viral load at baseline and were confirmed to have full virologic suppression (<50 RNA copies/ml) at six months postpartum (time of ART cessation). Cellular gene expression was quantified by RT² PCR Array and the average ΔC_q value was calculated for each gene in both treatment arms. The majority of cytokines on the PCR array were considered to have insufficient expression for analysis, which was defined as a C_q value >35 (Table S2 in Additional file 1).

Table 1. Clinical characteristics of study participants

	Arm A (zidovudine/lamivudine/ abacavir) (n = 30)	Arm B (zidovudine/lamivudine/lopinavir/ ritonavir) (n = 30)
Age at enrolment, median years (IQR)	29 (25 to 33)	25 (22 to 28)
HIV RNA levels at enrolment, median (log ₁₀ copies/ml)	3.98 (3.65 to 4.46)	3.90 (3.50 to 4.17)
CD4 ⁺ count at enrolment, median cells/μl (IQR)	410 (347 to 579)	393 (338 to 491)
HIV RNA levels at treatment cessation, median (log ₁₀ copies/ml)	50	50
CD4 ⁺ count at treatment cessation, median cells/μl (IQR)	591 (468 to 729)	585 (470 to 835)

IQR, interquartile range.

Eight out of 86 genes demonstrated a greater than 50% change in gene expression in those treated with ABC-based vs. PI-based antiretrovirals (Table S2 in Additional file 1). These genes were chosen for expanded analysis: *ccl2*, *ccl5*, *ccr7*, *cd40*, *cxcl1*, *cxcl5*, *il8* and *lta*. To account for false negatives, *nfk1*, *tollip*, *ifng* and *cd40lg* were included in the subsequent qRT-PCR despite showing <50% change in expression between arms in the preliminary analysis.

For the main analysis, 30 new individuals from each arm were similarly matched for CD4 count and viral load. Cellular RNA was purified from stored buffy coat taken at six months postpartum and from 24 patient-matched samples available at 12-months postpartum. Cellular gene expression was quantified by real-time PCR and the median ΔC_T value was calculated for each gene of the 12 genes in both treatment arms (Figure 1).

At six-months postpartum, those treated with an ABC-containing regimen showed significantly higher transcription of CD40 ligand (CD40LG), IL-8 and lymphotoxin alpha (LTA) and significantly lower CCL5 (RANTES) levels (Figure 1, Table 2a). The respective *p*-values were adjusted for multiple testing by the Benjamini–Hochberg approach. All observed changes in transcription were in a proinflammatory direction for ABC: CD40LG, 1.82 (± 0.35) fold (*p* = .027); IL-8, 3.16 (± 0.87) fold (*p* = .020); LTA, 2.82 (± 0.65) fold (*p* = .008); and CCL5, -1.67 (± 0.33) fold (*p* = .035). There was a non-significant trend for an increase in TOLLIP expression in the ABC-treated group (2.34 (± 0.86) fold, *p* = .14). By 12-months postpartum, when both arms had received no ART for 6-months, there was no difference in transcription of the five cytokines between the treatment arms (Figure 2, Table 2a).

At the six-month study visit when ART was discontinued, all participants were virologically suppressed, and by 12-months postpartum, all individuals had an expected rebound plasma viraemia. Within each arm, there was a significant increase at 12-months postpartum compared to 6-months in CCL5, CXCL1, CD40LG and IL-8 in both arms (Figure 2). At 12 months, LTA had significantly decreased transcription (-3.64 (± 1.03) fold; *p* = .001) in women treated with Trizivir compared with levels detected at six-months. In women treated with Kaletra/Combivir, there was no change

in transcription between timepoints (-1.03 (± 0.27) fold; *p* = .90) (Figure 3, Table 2b).

Figure 1 highlights the presence of outliers in each treatment arm that is greater than three standard deviations from the mean and were confirmed by Grubbs' test (*p* < .05). Removing these single outliers resulted in a true Gaussian distribution. A more conservative approach is to utilize a Mann–Whitney test, rather than a Student's *t*-test, to compare cytokine expression in the two treatment arms at 6 and 12 months, which reduces the influence of outlying values. A Mann–Whitney test would give a *p* = .41 for the differences in LTA transcription between the two treatment arms at six months, as compared to *p* = .08 that we determined using an established correction method. Exclusion of the outliers does not alter the final significance of the results at *p* < .05 (Table S3 in Additional file 1).

Discussion

This is the first report of differences in cytokine expression mediated at the transcriptional level in HIV-infected individuals treated with an ABC-containing regimen. Previous studies chose to assess circulating inflammatory mediators based on their reported associations with CVDs and systemic inflammation. Our approach allowed us to simultaneously profile the expression of a large number of cytokines in two treatment groups, and these results suggest a heightened transcriptional response for inflammatory mediators in those treated with ABC.

One of the four cytokines that showed a significant change in expression, CD40LG, has also been shown to be elevated in the plasma of an ABC-treated animal model [17]. CD40 ligand is expressed primarily on activated CD4⁺ T cells, platelets and vascular smooth muscle cells and, under inflammatory conditions, is also induced on monocytes and NK cells [18]. Ligation with CD40 leads to the production of reactive oxygen species and adhesion molecules that promote a local inflammatory reaction. Both of these molecules are increased in atherosclerotic plaques and their coupling triggers atherogenic signalling events and the upregulation of VCAM-1, ICAM-1, various interleukins, including IL-8 and members of the TNF superfamily such as LTA. LTA itself mediates a large

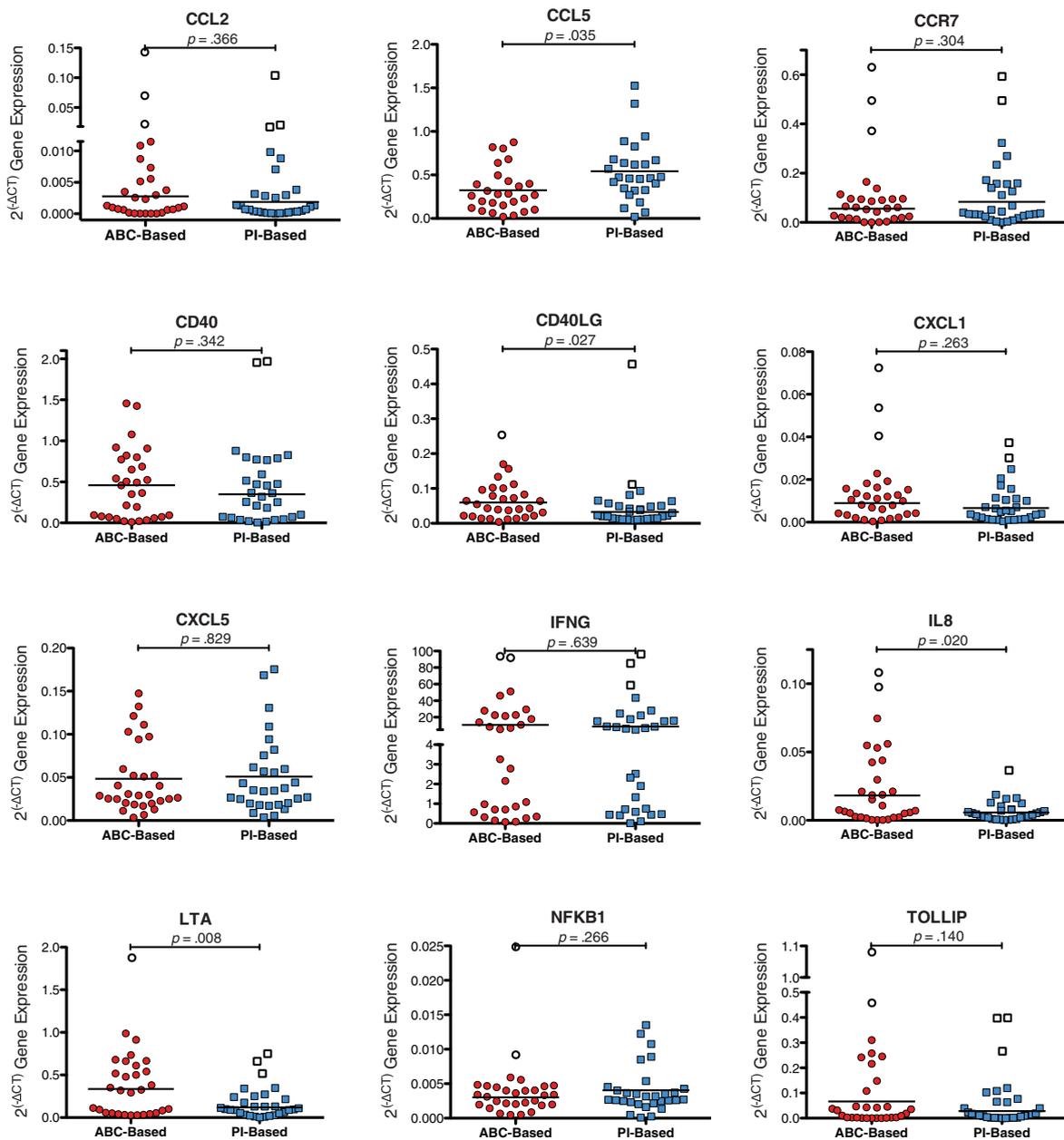


Figure 1. Differential expression of cytokine transcript during ART. The Y-axis plots the $2^{(-\Delta C_t)}$ value, with a greater value indicative of higher gene expression. Differential expression of cytokine transcripts was apparent at ART termination (six-months postpartum) in women on Trizivir (ABC-based) when compared to those on Kaletra/Combivir (PI-based). Outliers greater than three standard deviations from the mean are indicated as white data points. ABC, abacavir; ART, antiretroviral therapy; PI, protease inhibitor.

number of inflammatory responses and could be associated with CVD via the concomitant induction of adhesion molecules and cytokines from endothelial and vascular smooth muscle cells [19].

This study found significantly lower levels of CCL5 transcription in women on an ABC-containing regimen. Low baseline CCL5 (RANTES) levels were an independent predictor of cardiac mortality in male patients followed up prospectively for 24-months for the occurrence of cardiac mortality and MI [20]. This followed an earlier study that associated increased serum levels of IL-8 and reduced levels

of CCL5 with the risk of CHD, after adjustment for known risk factors [21]. Although there are conflicting reports correlating elevated levels of CCL5 with adverse cardiac outcomes, it should be noted that measurement of circulating CCL5 can be error prone as it can be readily released by activated platelets during sample collection [22,23]. Nevertheless, when examining CCL5 transcription, single nucleotide polymorphisms that are known to downregulate CCL5 expression have been linked to cardiac events in a number of studies [24,25]. CCL5 can bind to both chemokine receptors CCR1 and CCR5, and the loss of either of these receptors is

Table 2a. Comparison of differentially expressed cytokine transcripts between treatment arms at six and twelve months

	ABC-based treatment arm		PI-based treatment arm		Fold change (SD)	p
	Mean	95% CI	Mean	95% CI		
Six months						
CCL2	0.003	0.001 to 0.004	0.002	0.001 to 0.003	1.46 (±0.53)	.366
CCL5	0.325	0.226 to 0.423	0.543	0.401 to 0.684	-1.67 (±0.33)	.035
CCR7	0.056	0.038 to 0.074	0.084	0.050 to 0.118	-1.50 (±0.38)	.304
CD40	0.459	0.302 to 0.616	0.349	0.234 to 0.464	1.32 (±0.31)	.342
CD40LG	0.060	0.043 to 0.077	0.033	0.024 to 0.042	1.82 (±0.35)	.027
CXCL1	0.009	0.006 to 0.012	0.007	0.004 to 0.009	1.36 (±0.31)	.263
CXCL5	0.049	0.033 to 0.064	0.051	0.034 to 0.068	-1.05 (±0.23)	.829
IFNG	10.68	5.16 to 16.20	8.81	4.52 to 13.09	1.21 (±0.42)	.639
IL8	0.018	0.010 to 0.026	0.006	0.004 to 0.008	3.16 (±0.87)	.020
LTA	0.335	0.222 to 0.448	0.119	0.080 to 0.158	2.82 (±0.65)	.008
NFKB1	0.003	0.002 to 0.004	0.004	0.003 to 0.005	-1.33 (±0.24)	.266
TOLLIP	0.067	0.029 to 0.104	0.028	0.014 to 0.043	2.34 (±0.86)	.140
Twelve months						
CCL5	24.32	13.72 to 34.92	12.33	7.80 to 16.87	1.97 (±0.54)	.198
CD40LG	0.150	0.105 to 0.196	0.171	0.113 to 0.230	-1.14 (±0.25)	.559
CXCL1	0.021	0.014 to 0.029	0.030	0.017 to 0.043	-1.40 (±0.38)	.610
IL8	0.030	0.017 to 0.042	0.039	0.020 to 0.058	-1.31 (±0.40)	.664
LTA	0.092	0.048 to 0.136	0.115	0.066 to 0.165	-1.25 (±0.39)	.601

ABC, abacavir; LTA, lymphotoxin alpha; PI, protease inhibitor.

associated with the positive and negative development of atherosclerosis, respectively, with the absence of CCR5 demonstrating a protective effect by reducing atherosclerotic plaque formation [26]. However, a well-defined consequence

of reduced CCL5 expression is the coupled upregulation of CCR5 [20,24]. This feedback mechanism may lead to adverse cardiovascular outcomes, as a protective effect for CVD, CAD and MI has been associated with lower levels of CCR5.

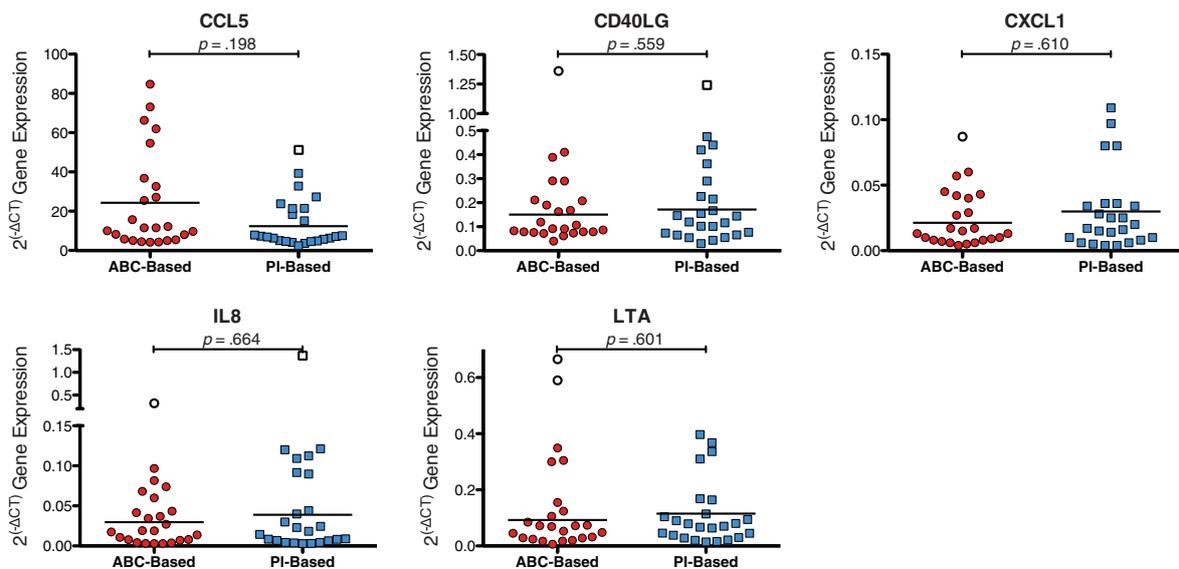


Figure 2. Differential expression of cytokine transcript after ART cessation. The Y-axis plots the $2^{(-\Delta C_q)}$ value, with a greater value indicative of higher gene expression. There was no difference cytokine gene transcription at 6-months after ART termination (12-months postpartum) in women on Trizivir (ABC-based) when compared to those on Kaletra/Combivir (PI-based). Outliers greater than three standard deviations from the mean are indicated as white data points. ABC, abacavir; ART, antiretroviral therapy; PI, protease inhibitor.

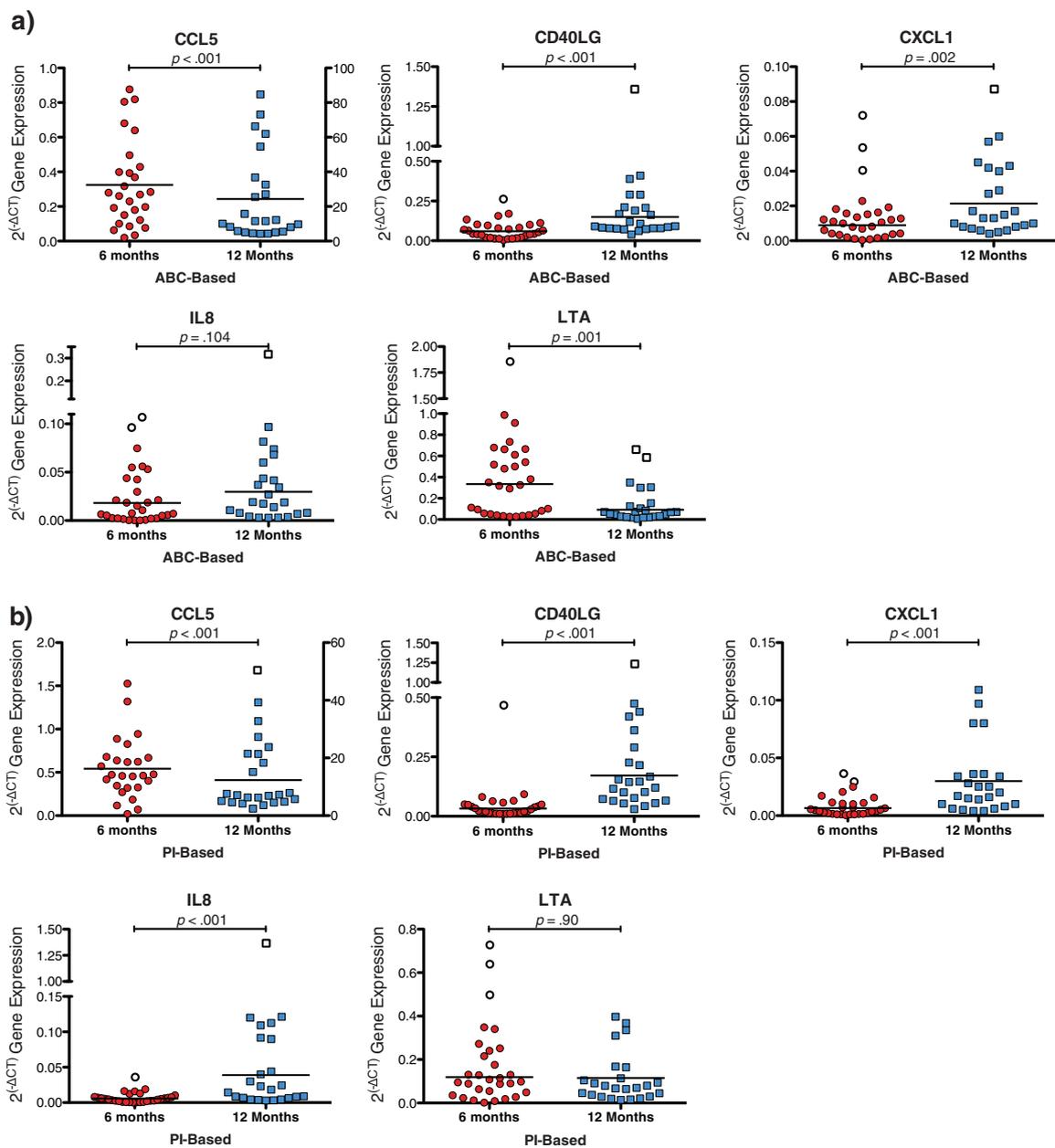


Figure 3. Differential expression of cytokine transcript between 6- and 12-months in individual treatment arms. The Y-axis plots the $2^{(-\Delta C_t)}$ value, with a greater value indicative of higher gene expression. Differential expression of cytokine transcripts was apparent at 6-months after ART termination (12-months postpartum) when compared to ART termination (6-months postpartum) in women in both treatment arms. Outliers greater than three standard deviations from the mean are indicated as white data points. ART, antiretroviral therapy.

IL-6 is a classical marker of systemic inflammation, yet our preliminary data showed that approximately 50% of samples in both arms had levels of IL-6 transcription that were below the limit of detection. This cytokine was subsequently excluded from the expanded analysis, as it would have resulted in data from only 15 samples from each arm. Furthermore, excluding those that had undetectable IL-6 would have introduced unwanted bias into the data analysis.

There are several limitations to the work outlined here. This sub-group analysis was matched by CD4 count and viral

load in an attempt to ensure the groups are alike but there may be unknown biases introduced that would be mitigated if we were able to use all randomized samples. All of the women in this study were breastfeeding at the six-month interval and previous data demonstrate that breastfeeding raises inflammatory cytokines, although it should increase cytokines equally in both [27]. However, it may underestimate the change between cytokine expression between the 6- and 12-month period. The period of virologic suppression was relatively short, and it is unknown if both

Table 2b. Comparison of differentially expressed cytokine transcripts between six and twelve months of individual treatment arms

	Twelve months		Six months		Fold change (SD)	p
	Mean	95% CI	Mean	95% CI		
ABC-based						
CCL5	24.32	13.72 to 34.92	0.325	0.226 to 0.423	74.83 (±19.29)	<.001
CD40LG	0.150	0.105 to 0.196	0.060	0.043 to 0.077	2.50 (±0.51)	<.001
CXCL1	0.021	0.014 to 0.029	0.009	0.006 to 0.012	2.33 (±0.52)	.002
IL8	0.030	0.017 to 0.042	0.018	0.010 to 0.026	1.67 (±0.48)	.104
LTA	0.092	0.048 to 0.136	0.335	0.222 to 0.448	-3.64 (±1.03)	.001
PI-based						
CCL5	12.33	7.80 to 16.87	0.543	0.401 to 0.684	22.71 (±4.95)	<.001
CD40LG	0.171	0.113 to 0.230	0.033	0.024 to 0.042	5.18 (±1.11)	<.001
CXCL1	0.030	0.017 to 0.043	0.007	0.004 to 0.009	4.29 (±1.25)	<.001
IL8	0.039	0.020 to 0.058	0.006	0.004 to 0.008	6.50 (±1.94)	<.001
LTA	0.115	0.066 to 0.165	0.119	0.080 to 0.158	-1.03 (±0.27)	.900

ABC, abacavir; LTA, lymphotoxin alpha; PI, protease inhibitor.

groups would have viral suppression in all reservoirs equally, and this may contribute to differences between the groups. While the lopinavir/ritonavir group may have side effects such as insulin resistance and lipid abnormalities contributing to increased inflammation, they would more likely bias towards the null and fail to demonstrate differences in inflammation rather than the results observed here.

Our data demonstrate the presence of an intracellular inflammatory response in those treated with ABC-based antiretrovirals. All of the differentially expressed genes identified in this study are under the transcriptional control of NF-κB, a well-established mediator of inflammation, and these data suggest its possible involvement in mediating inflammation during ABC-containing ART [28–32]. This correlates with our recently presented findings that Toll-like receptors may recognize ABC and stimulate an inflammatory response *in vitro* [33]. However, the interplay between multiple signalling pathways makes it difficult to isolate the transcriptional changes that are possibly attributable to ABC. It is possible that these differences in cytokine expression are a reflection of a greater reduction in systemic inflammation in those treated with a PI-based regiment, but we consider this to be unlikely as women in both arms attained full virologic suppression after nine months of ART. Our study was not designed to evaluate clinical cardiovascular events, and it is important to note that few cardiovascular events (and no MIs) occurred in either study arm in this group of young women with higher CD4 cell counts receiving PMTCT. In conclusion, our data suggest that ABC may directly stimulate the expression of proinflammatory cytokines or it may produce a secondary immunomodulatory effect of upregulated mediators such as CD40L. There is a need to expand the scope of research on ABC beyond the traditional biomarkers of systemic inflammation to understand cellular messaging and, ultimately, the mechanism of inflammation. While the pathways involved in mediating inflammation will need to be the subject of future investigation, it appears that

ABC-containing ART regimens have the capacity to upregulate intracellular inflammatory processes.

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Competing interests

None of the authors have any conflicts or potential conflicts of interest.

Authors' contributions

IJM, CFR, ME and RLS designed the study. ME, RLS, SL, MM and JM designed and conducted the parent study. AO collected the samples. SM processed the samples. IJM and CFR conducted RNA isolation and qRT-PCR. IJM, CFR, EvW, ME and RLS analyzed the data. All authors contributed to the drafting and revision of the manuscript.

Abbreviations

ABC, abacavir; ART, antiretroviral therapy; CVD, cardiovascular disease; GSK, GlaxoSmithKline; LTA, lymphotoxin alpha; MI, myocardial infarction; PI, protease inhibitor; PMTCT, prevention of mother-to-child transmission.

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