HIV-1 genotypes and phenotypes associated with mother to child transmission

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Chapter 4

Chloroquine treatment increases HIV-1 plasma viral loads in breastfeeding mothers without influencing the gp120 envelope genotype

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ABSTRACT

Chloroquine (CQ) treatment has previously been shown to decrease human immunodeficiency virus type 1 (HIV-1) viral load in infected individuals. It is also shown that CQ accumulates in breast milk cells with the predicted potential to reduce viral load in breast milk (BM) and thereby restrict HIV-1 transmission via breastfeeding. Here a Phase I/II, randomized controlled study was conducted to evaluate the effect of CQ on modulating HIV-1 activity in breastfeeding mothers: the CHARGE study. Thirty mothers from Rwanda (CQ treated n=20; placebo n=10) were enrolled in a 16 week study. CQ levels in plasma correlated to those in BM, and CQ levels were 2.5 fold higher in BM. We found a positive correlation between CQ concentrations and $CD4^+$ T-cell counts in plasma of the mothers. We found HIV-1 plasma loads increased significantly in mothers treated with CQ ($p=0.01$) and with no change observed in the placebo group. Three children were infected during birth or the breast feeding period; all of them in the group of CQ treated mothers. No major differences in amino acid sequences relating to charge, length or number of potential N-linked glycosylation sites were observed in the variable regions of the HIV-1 gp120 envelope gene. Additionally, no differences in neutralization potential of gp120 Env viruses were found following CQ treatment. These data caution against the use of CQ in reducing HIV-1 MTCT via breastfeeding.

INTRODUCTION

The anti-malarial drugs Chloroquine (CQ) and Hydroxychloroquine (HCQ) are cheap and widely available. These are both weak bases that affect acid vesicles leading to dysfunction of several enzymes such as those involved in protein post translational modification. In HIV-1 infected individuals CQ treatment may therefore lead to impaired glycosylation of the HIV-1 envelope protein [1-5]. This could decrease the capacity by which HIV-1 can undergo viral capture and transfer to $CD4^+$ T lymphocytes via cells expressing DC-SIGN [6], modulate neutralizing antibody (NAb) epitopes (as seen for 2G12 [1] as well as reduce the glycan shield protecting against Abs binding gp120. We amongst others have shown that in cell culture CQ can interfere with virus replication in $CD4^+$ T lymphocytes and that this inhibition is likely conferred at the cellular level [6]. The altered endosomal pH also reduces IL-6 production [7], followed by down-regulation of HIV-1 production in chronically infected T-cells and monocyte cell lines [2,8]. CQ also inhibits Tat mediated transactivation of HIV-1 transcription [9]. For these reasons it has been postulated that the treatment of HIV-1 infected individuals with CQ or HCQ may result in lower viral loads as well as a reduction in their capacity to transmit HIV-1.

Several clinical trials have studied the effect of CQ and HCQ on HIV-1 replication *in vivo* [3,4,10]. In two of these trials treatment with HCQ resulted in a decrease in viral loads with no measurable effect on the CD4 counts [3,4]. A decrease in plasma IL-6 expression levels was observed together with a decrease of total serum IgG [4]. These results make HCQ and CQ promising candidates in HIV-1 treatment strategies. CQ has been shown to accumulate in human milk [11-13] and has been shown not to be toxic in breastfed infants [14]. According to the 2011 UNAIDS Progress report 390,000 children were newly infected with HIV-1 in 2010 with the majority resulting from mother-to-child transmission (MTCT). Known maternal risk factors associated with MTCT are high plasma viral loads, low CD4 T-cell numbers coinciding with advanced maternal immune deficiency and prolonged labor [15]. In populations where replacement feeding is

not feasible it has been estimated that 41% of MTCT occur *in utero* (IU), 20% *peri-partum* (PP) and the remaining 39% during prolonged breastfeeding (BF) [16]. We performed a study "CHARGE" to assess the potential role of CQ in modulating HIV-1 activity in breastfeeding mothers.

MATERIALS AND METHODS

Study participants

The study was approved by an Independent Ethics Committees (IEC) in the Netherlands, the STEG-METC (ref no R01-089). In the absence of an operational IEC in Rwanda at the time, the Ministry of Health's Treatment and Research AIDS Center (TRAC), the Rwandan National Malaria program (PNLP) and the "Cellule de recherché" at the CHK acknowledged the approval of the Dutch IEC. All women provided written informed consent for both themselves and their children. Thirty Rwandese HIV-1 infected pregnant women were randomized (2:1) to receive either placebo (n=10) or CQ 200 mg once daily from the day of birth for a duration of 16 weeks (n=20) (Table 1). All women received a single-dose of 200 mg Nevirapine at the start of labor to reduce *intra-partum* transmission of HIV-1. Regular counseling on exclusive breastfeeding was provided. At inclusion the patients had to complete the following conditions: at least 18 years of age, between 32 and 35 weeks of gestation, antiretroviral therapy-naive, intending to breastfeed their child(ren), able to provide written informed consent for both themselves and their expected infant(s) and likely to complete the 18 weeks. Women were excluded if they had received any CQ treatment within 6 weeks prior to study drug administration, if there was evidence of severe fetal anomalies, serious disease or laboratory abnormalities incompatible with study participation or any CQ related ocular toxicity or other adverse effects that occurred due to previous CQ administration. The study was approved by an Independent Ethics Committee (IEC) in the Netherlands. In the absence of an operational IEC in Rwanda at that time, the Ministry of Health's Treatment and Research AIDS Center (TRAC) the Rwandan National Malaria program (PNLP) and the "Cellule de recherché" in CHK acknowledged the approval of the Dutch IEC.

Viral load and $CD4^+$ T-cell count

At intake blood from pregnant women was screened for HIV-1 infection using a rapid assay. At the day of delivery and 8 and 16 weeks blood, plasma and breast milk samples were taken and stored. HIV-1 viral loads were measured using the RNA PCR assay from Roche Diagnostics (Mannheim, Germany) with a lower limit of detection of 25 copies/ml (Table 2). Immunologic and virologic testing of mothers was performed in two laboratories (National HIV/AIDS Reference Laboratory, Kigali, Rwanda and the Joint Clinical Research Centre, JCRC, Kampala, Uganda).

Measuring CQ concentration

To a 1.0 ml plasma sample (patient samples were supplemented with blank plasma to this volume if required) in a glass conical tube, 25 µl of internal standard solution (50 µg/ml diaminonaphthalene in methanol), 1 ml of 1M sodium hydroxide and 6 ml diethylether were added. After shaking for 10 min, the mixture was centrifuged for 5 min at ca. $2x10^3$ *g*. After storage of the tube

for 1 h at -30°C, the organic layer was poured into a second tube and evaporated at 40°C under a gentle stream of nitrogen. Finally, the residue was reconstituted in 200 µl eluent using vortex-mixing and 50 µl was injected onto the HPLC column using partial loop filling.

To a 1 ml milk sample (patient samples were supplemented with drug-free cow milk to this volume if required) in a glass conical tube, 6 ml *n*-hexane was added. The tube was shaken for 10 min and centrifuged for 5 min at ca. $2x10^3$ *g*. After storage of the tube for 1 h at -30°C, the organic layer was discarded. After thawing the aqueous layer, 1 ml of 1M sodium hydroxide and 6 ml diethylether were added and the procedure was continued according to the pre-treatment of plasma samples. Chromatographic conditions: Fifty µl injections were made on a Symmetry C_{18} column (100×4.6 mm, d_p = 3.5 µm, average pore diameter = 10 nm, Waters), protected by a Symmetry C_{18} pre-column (20×3.8 mm, d_p = 5 µm, Waters). The column temperature was maintained at ambient temperature (23-29°C). The eluent comprised 9% (v/v) acetonitrile and 91 % (v/v) phosphate buffer (45mM, pH 3.0) and the eluent flow rate was 1.0 mL/min. The UV detection wavelength was 340 nm.

HIV-1 infection status of the children

Presence of HIV-1 viral RNA in the plasma at the day of birth was tested by RT-PCR for the HIV-1 V3 region using primer 3'V3Not (GCG CGG CCG CCC CCT CTA CAA TTA AAA CTG TG) in the RT reaction, followed by the first PCR using 3'V3Not and 5'V3Not (GCG CGG CCG CAC AGT ACA ATG TAC ACA TGG). In the second PCR primers 5'Ksi (ATA AGC TTG CAG TCT AGC AGA AGA AGA) and 3'Ksi (ATG AAT TCT GGG TCC CCT CCT GAG GA) were used. At week 16 the infection of the infants was established by viral load measurements as described above.

Sequencing of the HIV-1 gp120 envelope region and analysis

Viral RNA was isolated from plasma using a silica-based method [17]. The C1-C4 envelope region was amplified by RT-PCR as previously described [18]. Viral RNA was converted to cDNA and then subjected to a nested PCR amplifying a fragment covering the V1 to C4 region of the gp120 gene. The primers that were used for the first PCR were A1053 (5'-GAAAGAGCAGAAGACAGTGGCAATGA-3') and A1262 (5'-CTGACGGTACAGGCCAGACAATTATTGTC-3'). For the Nested PCR A1322 (5'-TCTTGGGAGCAGCAGGAAGCAC-3') and A0385 (5'-GAGGATATAATCAGTTTATGGGA were used as primers. PCR products were cloned into the TOPO II vector (Invitrogen, Carlsbad, CA, USA) and sequenced bi-directionally using the BigDye Terminator Cycle Sequencing kit (ABI, Foster City CA, USA) and analyzed using an ABI 377 automated sequencer (ABI) or the Thermo Sequenase fluorescence-labeled primer cycle-sequencing kit (Amersham International, Little Chalfont, UK) according to the manufacturer's instructions. The primers used for the sequencing were A1360 (5'-GAGCCAATTCCYATACAT TATTG-3'), SP6 (5'-ATTTAGGTGACACTATAG-3') and T7 (5'-TAATACGACTCACTATAGGG-3'). Sequences were assembled using CodonCode Aligner (CodonCode Corporation, Dedham, MA) and aligned with ClustalW and manually edited using Textpad (Helios Software Solutions). Phylogenetic analysis of the aligned sequences was performed using the neighbor-joining method of MEGA36 (Tamura, Dudley, Nei, and Kumar 2007). The distance matrix was generated by Kimura's two-parameter estimation and the tree topology was

confirmed by the maximum-likelihood option. Bootstrap values greater than 70% were considered significant based on 100 replications. Sequences obtained from the Los Alamos database were included as references. The presence of potential N-linked glycosylation sites (PNGS) was determined using the program available from the HIV sequence database (http://hiv-web.lanl.gov/content/hiv-db/GLYCOSITE/glycosite.html).

Subtype analyses

HIV-1 subtype was determined through analyzing the gp120 V3 region sequences. The Subtype Reference Alignments of the Los Alamos Data Base (www.hiv.lanl.gov/content/sequence/NEWALIGN/align.htm) year 2005 were used as reference sequences.

Determination of IgG concentrations

IgG concentrations in plasma were measured using the Cobas C502 (Roche, Roche Diagnostics, Darmstadt, Germany) according to the instructions of the manufacturer**.**

Statistics

All statistical comparisons were performed with the Mann-Whitney test or the paired t-test using GraphPad Prism version 5.00. P-values < 0.05 were considered statistically significant.

RESULTS

Clinical parameters of study individuals

Thirty HIV-1 positive mothers were included in this study (Table 1). Each mother received a single dose of Nevirapine at time of delivery with the 20 CQ treated mothers receiving daily doses of 200 mg CQ for a period of 16 weeks. Base-line, week 0, week 8 and week 16 plasma and BM samples were taken from each mother, with a week 0 and week 16 plasma sample taken from each infant. CD4 counts of the mothers were measured at baseline, week 0, week 8 and week 16. Patients 100, 150, 160, 400, 220, 260 and 280 stopped treatment between week 8 and 16. Three children died of unknown causes within the 16 week period (101, 161 and 401), 2 children born from mothers in the control group and 1 from a CQ treated mother. Seven mothers were lost for follow up during the 16 week period, 2 CQ treated and 5 placebos, indicating that dropout is not CQ treatment related. The HIV-1 subtype was determined by analyzing the V3 region of gp120. One CQ treated mother (250) carried an A/C recombinant, (Table 1) and the subtype of 2 mothers (020 and 240) could not be determined. The remaining 27 mothers were infected with either subtype A (n=20, 12 CQ treated and 8 placebo) or subtype C (n=7, 6 CQ treated and 1 placebo) reflecting the viral subtypes circulating within the geographical region. Four children were found to be positive at the day of birth indicating HIV-1 transmission *in utero*. Three children were infected either during delivery or during the breastfeeding period, notably all from CQ treated mothers.

Table 1. Characterization of study participants

Mothers							Children			
			Plasma IgG g/L		Plasma	Sequenc	RNA		Viral Load	Sequence
Mother	CQ	Subtype	Week 0	Week 16	neutralization	analysis	Week 0	Week 16	Week 16	analysis
20	+	ND	ND	ND	-	-	-	-	-	-
30	+	A	ND	ND	-	-	-	-	-	-
40	+	A	ND	ND	-	-	-	-	-	-
50	+	A	ND	ND	-	+	-	-	-	+
60	-	A	ND	ND	-	+	-	-	-	+
90	-	A	17.400	26.500	-	+	+	+	750000	+
100	-	A	29.400	38.200	+	-	+	+	ND	-
110	-	A	ND	ND	-	+	-	-	-	+
130	+	C	19.500	25.900	+	+	-	+	7590	+
150	-	A	ND	ND	-	-	-	-	-	-
160	+	A	23.000	37.000	+	+	-	-	-	+
180	+	C	ND	ND	-	-	-	-	-	-
190	+	C	ND	ND	-	+	-	-	-	+
200	-	A	20.400	31.100	+	+	-	-	-	+
210	+	C	ND	ND	-	-	-	-	-	-
220	+	A	ND	ND	-	-	-	-	-	-
240	-	nd	ND	ND	-	-	-	-	-	-
250	+	A/C	36.100	79.300	+	+	+	+	269000	+
260	-	A	ND	ND	-	-	-	-	-	-
270	+	C	16.500	31.300	+	-	-	-	-	-
280	-	C	ND	ND	-	-	-	-	-	-
290	+	A	20.600	29.500	+	+	+	+	531000	+
300	+	A	27.900	40.100	+	+	-	+	747000	+
340	+	A	ND	ND	-	+	-	-	-	+
350	+	A	ND	ND	-	-	-	-	-	-
360	+	A	ND	ND	-	-	-	-	-	-
370	+	A	20.200	40.300	+	+	-	+	144000	+
380	+	A	16.600	28.500	+	+	-	-	-	+
390	+	C	ND	ND	-	-	-	-	-	-
400	-	A	ND	ND	-	-	-	-	-	-

CQ concentrations higher in BM than in plasma

In previous studies patients received a daily dose of 800 mg HCQ, which corresponds to 500 mg CQ [3,4]. The mean plasma HCQ concentrations found in these trials were 316.3 and 347.1 ng HCQ /ml respectively at week 8, corresponding with 197.7 CQ and 216.9 ng CQ /ml and 435 ng HCQ /ml at week 16 corresponding with 271.9 ng CQ /ml. In our trial the mothers received a daily dose of 200 mg CQ, with mean plasma CQ concentrations of 759.7 ng CQ /ml (95%CI 467.8-1052) at week 8 and 478.2 ng CQ /ml (95%CI 351.7-604.6) at week 16 (Fig 1A and 1B). The CQ levels were comparable with those in the previous trials at week 16 and higher at week 8, thereby indicating differences in efficiency of uptake between HCQ and CQ.

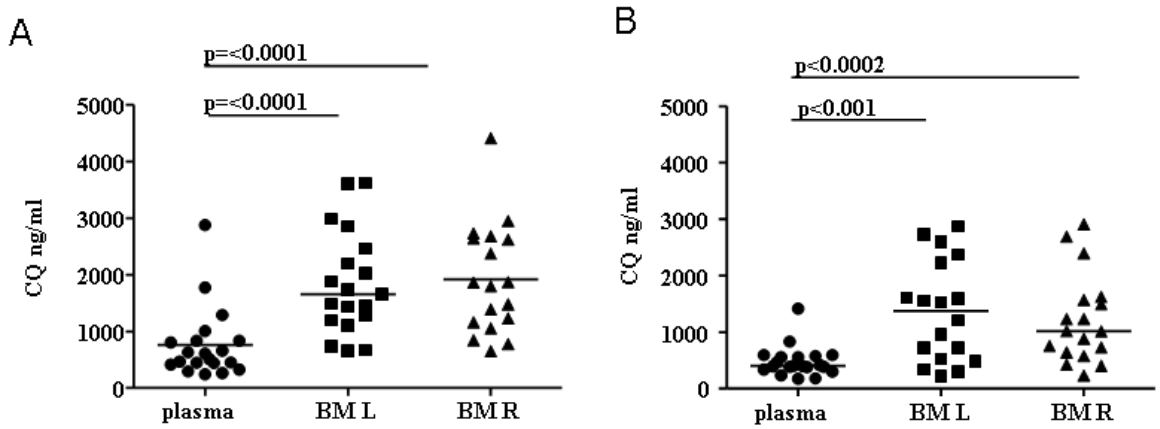

Figure 1. CQ concentration in ng/ml in plasma and breast milk after 8 weeks (**A**) and 16 weeks (**B**) of treatment.

Earlier reports demonstrated accumulation of CQ in BM cells [19]. We measured the CQ levels of milk of both right and left breast at 8 and 16 weeks after initiation of treatment and compared these with the CQ levels in blood plasma at the same time point. The CQ concentrations in plasma correlated with those in BM at week 8 (r^2 0.50, $p<0.0001$) and at week 16 (r^2 0.49, $p<0.0001$) (data not shown). The CQ levels in the right and left breast were found to be similar at both time points ($p=0.94$ and 0.79 respectively). The mean CQ concentrations were 1831 ng/ml (95%CI 1134-2648) and 1658 ng/ml (95%CI 1198-2457), respectively at week 8 and 1017 ng/ml (95%CI 232.0-1581) and 1369 ng/ml (95%CI 512.3-2265) at week 16 (Fig. 1A and Fig. 1B), making the CQ level in BM higher than observed in plasma ($p<0.0001$ at week 8 and $p<0.001$ at week 16). These results indicate that the CQ concentration in BM was approximately 2.5 fold higher in BM than in blood plasma supporting earlier findings that CQ accumulates in BM.

CQ treatment did not decrease BM HIV-1 load

It has been hypothesized that the accumulation of CQ in BM would lower VL and therefore lower the risk of HIV-1 transmission through breast feeding [19]. We measured HIV-1 VL in BM from both the right and left breast at baseline and after 8 and 16 weeks of CQ treatment. The VL load of the left and right breast correlated at all time points (r^2 0.80 $p<0.0001$, r^2 0.81 $p<0.0001$ and r^2 0.56 $p<0.0005$ respectively). We found no significant difference in BM VL between baseline and week 8 and 16 samples in the CQ treated mothers (Fig. 2A and 2B). No correlation was found between the CQ concentration and the VL in BM (data not shown). The three mothers who transmitted HIV-1 to their child either during delivery or via breast feeding (130, 300 and 370) were all from the CQ treated group. They had a mean BM-VL of 214 RNA copies/ml (95%CI 25-367) and a mean BM-CQ concentration of 1542 ng/ml (95%CI 845-2620) which was not different from the remainder within group.

CQ treatment increased plasma HIV-1 load

We determined the effect CQ treatment had on influencing plasma HIV-1 VL (Table 2). We performed paired t-tests of baseline versus week 8 and week 16 VL from treated and non-treated mothers. We found no significant increase or decrease in both the groups. There was no correlation between CQ levels and VL (data not shown), Three mothers (210, 290, 360) with high CD4 T-cell counts at

Table 2. Synonymous and nonsynonymous analysis of gp120 sequence

	ΔS*			ΔN*			ΔS/ΔN		
	Placebo (#)	CQ (#)	p value	Placebo (#)	CQ (#)	p value	Placebo (#)	CQ (#)	p value
V1V2	0.940 (± 0.72)	0.936 (± 0.83)	0.99	0.862 (± 0.61)	0.919 (± 0.95)	0.92	0.857 (± 0.68)	3.032 (± 4.21)	0.34
V3	0.406 (± 0.48)	2.023 (± 3.99)	0.44	1.602 (± 2.13)	1.526 (± 2.77)	0.96	0.603 (± 0.86)	1.960 (± 3.91)	0.51
V4	1.176 (± 1.21)	0.336 (± 0.59)	0.11	0.617 (± 0.55)	1.713 (± 2.00)	0.38	1.355 (± 1.53)	0.453 (± 0.73)	0.17

* ΔS and ΔN were determined by dividing the average distance of week 0 by week 16
\# standard deviations

baseline (1355, 1168 and 846 cells/µl respectively) showed CQ levels >1200ng/ml in their plasma at week 8. Two of these mothers showed a more than tenfold increase in plasma VL between baseline and week 8. We found no significant difference in VL between baseline and the week 16 samples in the placebo group but we did find a significant increase (P=0.010 and 0.012, respectively) of viral load within the CQ treated group at 8 and 16 weeks after treatment (Fig. 3). CQ treatment in our cohort associated with increased HIV-1 VL in plasma. To investigate if CQ treatment has a different effect on the two viral subtypes in our group, we compared the VL of subtype A and C infected, treated mothers and found no difference (data not shown) indicating that the effect of CQ is not subtype specific.

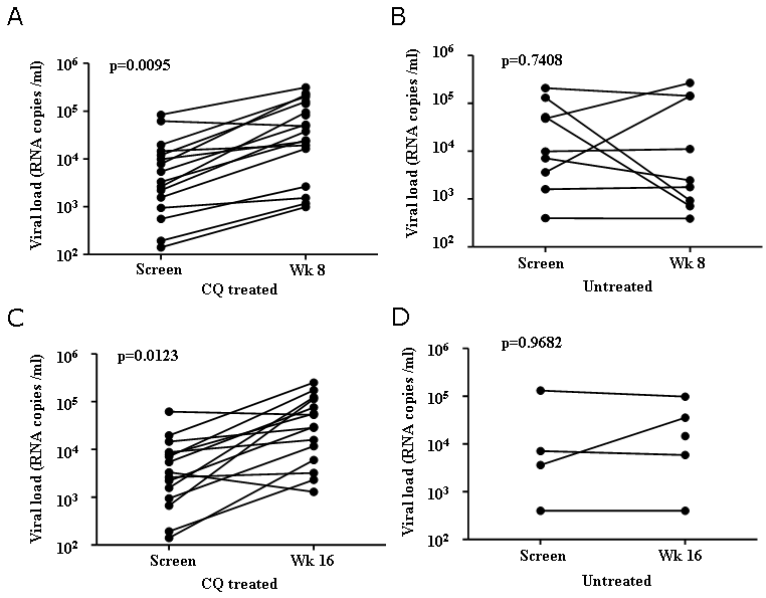

Figure 3. Viral loads in plasma after 8 weeks CQ (**A**) or placebo treatment (B) or 16 weeks CQ (**C**) or placebo treatment (**D**).

No effect of CQ treatment on CD4 T-cell counts

The increase in VL in our cohort could be the result of an increase in number of target cells for HIV-1 replication. In two earlier studies with HCQ treatment no measurable effects were seen regarding changes to CD4 counts [3,4]. The mean $CD4^+$ T-cell count at week 8 of both the treated and the placebo mothers were shown to increase, but not significantly (p= 0.145 and p= 0.076 respectively), confirming the earlier data (Fig. 4).

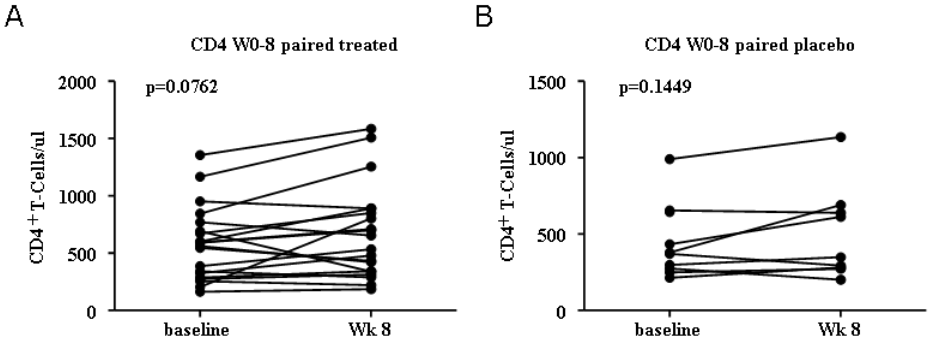

Figure 4. CD4+ T-cell counts in the CQ (**A**) and the placebo group (**B**) after 8 weeks of treatment.

The effect of CQ treatment on the gp120 envelope gene

The observed increase in HIV-1 VL could be the result of genotypic, hence phenotypic, changes in the viral population within the CQ treated group. Changes have been previously reported for HIV-1 cultured in the presence of CQ [1,6]. We have shown that HIV-1 cultured in the presence of CQ can increase the V1V2 charge, decrease the overall length of the V4 region, and induce loss of a PNGS in the V3 region [6]. To determine the effect of CQ treatment *in vivo*, we amplified gp120 envelope protein genes from plasma from mothers receiving CQ (n=10) and from placebo mothers (n=4) at baseline and 16 weeks after therapy initiation. In addition, we amplified the gp120 *env* genes from BM sample of 2 CQ treated mothers (130 and 160,) and 1 placebo mother (090) for both time-points. Ten clones from each sample were sequenced. We analyzed the V1V2, V3 and V4 regions for alterations in charge, length and PNGS patterns. The data acquired from the BM samples were similar to those from the corresponding plasma samples in all analyses.

i) Charge of variable regions

In the V1V2 region, the V3 region as well as in the V4 region there was variation in charge with decreases as well as increases in both the CQ treated and placebo mothers. No statistical differences were found between the baseline and the week 16 sample in overall charge in either of the variable regions analyzed.

ii) Length of variable regions

We found no statistical differences in the length of the V1V2 regions between baseline and week 16 samples in the CQ treated as well as placebo mothers The V3 length did not alter in any of the mothers in either the treated or the placebo group. Three of the CQ treated mothers acquired an insertion within the V4 region between baseline and week 16. Mother 190 had an insertion of 7 Amino Acids (aa), mother 300 of 8aa and mother 380 of 5aa. No insertions were found in the plasma of the mothers and in BM. The VL in the 3 mothers showing an insert in the V4 region increased with 1.4 log (14.3 (95% CI 11.0 – 17.7) fold) at week 8 and 1.15 log at week 18. The VL in the CQ treated mothers with no insert in V4 increased 0.27 log (12.6 (95% CI -0.06 – 25.3) fold) (p=0.05) at week 8 and 0.28 at week 16. When tested we found no overall correlation between the length of V4 and viral load in all mothers (data not shown).

iii) Alterations in variable region PNGS patterns

We determined loss or gain of PNGS in all three variable regions studied. We found no major changes in number of PNGS between baseline and week 16 in the V1V2 region in either the CQ treated or the placebo mothers. All baseline clones of mother 160 had a V3 PNGS at position 301 (HXB2 numbering) and which was absent at week 16. All clones from mother 200 also demonstrated the absence of PNGS on position 301 indicating that the loss of this PNGS is not associated with CQ treatment. No difference was observed in the number of PNGS in the V4 region, but in the CQ treated mothers the mean number of PNGS increased significantly between baseline and week 16 from 4.2 (95%CI 3.6 – 4.9) to 4.9 (90%CI 4.1 – 5.6) (p=0.014). No correlation was found between the number of PNGS and VL.

iv) No difference in the synonymous versus non-synonymous mutation rates of gp120 after CQ treatment

Overall our results indicate that CQ does not alter charge, length or PNGS patterns of the gp120 envelope protein *in vivo*. The ratio between non-

synonymous and synonymous mutations in a gene over time is a measure for positive or negative pressure on these mutations. We therefore calculated the synonymous (ΔS) as well as the non-synonymous (ΔN) mutation rates and determined the ΔS/ΔN between baseline and week 16 samples for both the CQ and the placebo group. We found no statistical differences between the two groups in ΔS, ΔN or the ΔS/ΔN ratio, indicating that there was no evolutionary pressure on the gp120 protein by CQ (Table 2).

Plasma IgG levels are not different between CQ and placebo treated mothers

The lower endosomal pH caused by CQ treatment is postulated to reduce IL-6 production [7] along with a decrease of total serum IgG [4]. We measured the plasma levels of total IgG in a random selection of mothers in our cohort. In both the CQ mothers and the placebo treated mothers the IgG levels were enhanced at 16 weeks after delivery compared with the baseline levels ($p < 0.005$ and $p < 0.004$ respectively). This increase of IgG levels was not different between the CQ treated and placebo mothers ($p = 0.19$), indicating CQ does not modulate IgG production (Fig. 5).

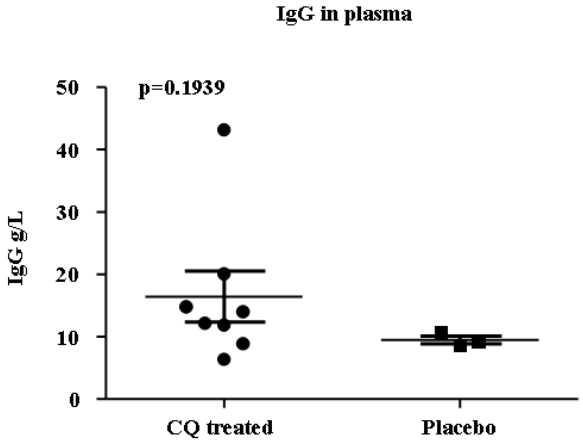

Figure 5. Increase of plasma IgG concentrations from the day of delivery to 16 weeks later in the CQ and the placebo treated group

CQ did not alter the neutralizing antibody responses

To identify whether the NAb responses were altered by CQ treatment we analyzed data from chapter 5. When comparing neutralization of the JRFL gp120 Env pseudo-typed virus with mother plasma (baseline and week 16 - 18 weeks after delivery) no differences were found between the early and late samples (data not shown). We also analyzed the autologous virus neutralization capacity and found no differences between early and late plasma samples for their ability to neutralize virus, again indicating that CQ treatment did not modify HIV-1 neutralization potential.

DISCUSSION

Here we present the results of the CHARGE study which was performed to study the influence of CQ treatment on modulating HIV-1 activity during the breast feeding period. Earlier reports had demonstrated the accumulation of CQ in cells present in BM [19]. Our findings show that the CQ concentration is approximately 2.5 fold enhanced in BM compared to blood plasma and confirm that CQ accumulates to higher levels in BM. In spite of higher CQ levels, no change was observed in BM viral loads between baseline and either week 8 or week 16 after treatment initiation. In contrast to previous reports describing a decrease in HIV-1 viral loads after HCQ treatment [3,4] our study demonstrated a significant increase in VL in the CQ treated mothers whilst no change was observed in the placebo group. The discrepancy in VL changes between our study and the earlier reports cannot be readily explained by the fact that our mothers received a lower daily dose of CQ. On the contrary, the concentration of CQ in the plasma of the mothers after 8 weeks of treatment was higher than in the earlier studies, possibly relating to differences in efficiency of uptake between HCQ and CQ. In concordance with earlier reports we fount no effect of CQ on $CD4^+$ T-cell counts.

On comparing the gp120 envelope sequences of CQ treated and placebo mothers we observed no overall differences in the amino acid composition of the variable regions. However, we did find numerous insertions within the V4 region in 3 of our CQ treated mothers after 16 weeks of treatment. It is not clear if these insertions are induced by CQ, but these inserts in V4 were not observed in the non-treated mothers nor were they observed in BM clones. Interestingly, the mothers demonstrating insertions within V4 showed an increase in VL of 1.15 logs after 16 weeks of treatment, higher than the increase of 0.28 log seen for the remaining CQ treated mothers. Inserts in the V4 region have previously been associated with immune evasion [20] and we suggest that the higher increase in VL in the mothers with these inserts may be caused by immune evasion and partly explain the results. There was also no significant change in the ΔS/ΔN ratio between the different groups of individuals, indicating that the increase in viral load does not stem from CQ exerting pressure on the gp120 envelope resulting in altered genotypes, hence phenotypes. We identified that the alterations observed *in vitro* culture under CQ pressure did not translate to differences *in vivo.*

It has previously been reported that CQ treatment can decrease the glycosylation pattern of the HIV-1 envelope [1]. We have also shown that the *in vitro* passaging of HIV-1 in the presence of CQ can provide alterations to HIV-1 that can be linked to alteration within the glycosylation patterning of the gp120 envelope [6]. We did not find such alterations *in vivo*, however, it should be noted that we only looked at alterations to envelope amino acid sequences and may have missed post-translational modification differences. The lack of observed genotypic differences could be due to the short course of CQ treatment (16 weeks in comparison to the *in vitro* culturing of 30 weeks). Longer treatment may see the emergence of viruses with selected mutations that are preferentially fixed. Alternatively, the alterations *in vivo* could be directed by host genetic or immune factors and therefore the alterations will be specific to the host environment in which they are selected. Since we have shown that the modifications observed with *in vitro* culturing can modify regions associated with escape from neutralizing antibody responses, namely alterations in the length of variable regions and PNGS patterns, each individual may select different viruses based on pre-existing immunity etc. Decrease of the number of PNGS for

example could lead to better Ab recognition of the virus resulting in enhanced neutralization of HIV-1, possible explaining why these variants are not found in patient plasma. Apart from an insertion in the V4 region we did not find differences in the viral population between the CQ treated and placebo groups that could explain for the increase in viral load. Another possibility is that some of the patients developed resistance to CQ, which results from mutations outside the gp120 region, however, this is highly unlikely given timings and the number of mothers demonstrating an increased in viral loads. We also show that NAb responses of the plasma's have not changed and it will be interesting to further analyze the viruses from the patients in this study to identify whether the viruses isolated have altered capacities to be neutralized with either monoclonal Abs or the longitudinal sera from their respective host.

It has been shown for $CD8^+$ T-lymphocytes that CQ can induce their activation state through the increased presentation of antigen [21]. It could be argued that if the same scenario prevails for $CD4^+$ T-lymphocytes then the increase in viral load observed could reflect an enhanced presentation of antigen via the MHC-class II molecule by APC, resulting in heightened cellular activation and increased viral replication. However, this would not explain the lack of increase of VL in earlier CQ or HCQ studies [3,4]. Our results support a study where mothers receiving CQ did not show a decrease in plasma VL or BM VL [10]. Since the previous studies, where reduction in plasma HIV-1 viral loads were shown, were composed predominantly of male participants we may be looking at a male versus female phenomenon and more so one which is determined by hormonal differences brought about by pregnancy or child delivery and lactation. Our results would suggest for the analysis of larger cohorts, however, ethical issues naturally will prevent such studies from being performed and we will have to rely on the information available from the limited individuals studied to-date.

The adverse effect of CQ on HIV-1 viral load observed in plasma could have implications for the potential use of CQ as a prophylaxis for HIV-1, especially in breastfeeding women. It will be interesting to decipher the specific cause and/or mechanism leading to the increased viral loads observed. CQ is now considered to be a safe and cheap alternative to conventional HIV-1 treatments, and is being considered for large-scale use in resource-limited settings. Our results indicate caution when treating HIV-1 patients with CQ and especially pregnant women and those breastfeeding where the likelihood of transmitting HIV-1 to their infants may be increased. Although all mothers received Nevirapine, which reduces transmission during delivery by approximately 50% [22], we can not identify which transmissions took place via breast feeding. Remarkably all three *post partum* transmissions were in the group of CQ treated mothers.

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