Persistence of HIV-drug-resistance among South African Children given Nevirapine to Prevent Mother-to-Child-Transmission

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Abstract

Objectives: We set out to examine the prevalence and persistence of mutations conferring high- level non-nucleoside-reverse-transcriptase (NNRTI)-resistance in a cohort of HIV-infected children who had failed prophylaxis to prevent mother-to-child-transmission (PMTCT).

Design: A prospective observational cohort study at the Pediatric HIV Clinic at Kalafong Provincial Tertiary Hospital in Pretoria, South Africa.

Methods: Children referred for initiation of ART were enrolled from July 2010 through February 2013. HIV-drug-resistance testing was performed using the oligonucleotide ligation assay (OLA) on dried blood spots (DBS) collected at enrollment and monthly follow-up visits for two years.

*Result*s: South African children who failed HIV-prophylaxis had a high prevalence of NNRTI- resistant HIV (46/88; 52%). Among children with NNRTI-resistance, the frequency of the predominant resistant variant in each child's HIV-quasispecies was high (median 96%) at study entry (median age 7.5 months), and in 26/27 followed a median of 13 months persisted at a high frequency (median 89%).

Conclusions: Our finding that infants who fail HIV-prophylaxis frequently have long-lived NNRTI-resistant HIV suggests that resistance will likely persists through 36 months of age, when children qualify for NNRTI-based antiretroviral therapy. These children may

benefit from HIV-drug-resistance testing to guide selection of their treatment.

Keywords: HIV, Nevirapine, Non-nucleoside-reverse-transcriptase-inhibitors, Infants, Children, HIV Drug Resistance, Oligonucleotide Ligation Assay, transmitted resistance

Introduction

Antiretrovirals (ARVs) are used to prevent mother-to-child-transmission (PMTCT) of human immunodeficiency virus type-1 (HIV). In 2000, the World Health Organization (WHO) recommended a single dose (sd) of nevirapine (NVP) be administered to HIV-infected women in labor and their newborns within 72-hours of birth [1], as a simple inexpensive strategy for PMTCT. Sd-NVP has been associated with transmission and selection of non-nucleoside- reverse-transcriptase-inhibitor (NNRTI)-resistant-HIV [2-4], which can lead to treatment failure when young infants are given NVP-based antiretroviral therapy (ART) [5, 6]. While decay of NVP-resistant-HIV from infants' plasma following sd-NVP has been described [2], other studies have found that NNRTI-resi stance can persist within infected infants [3, 7]. The current 2015 WHO PMTCT Guidelines recommend life-long maternal ART throughout gestation and nursing, as well as infant NVP-prophylaxis for the first 6-weeks of breastfeeding [8]. HIV-infected infants who have taken 6-weeks of NVP-prophylaxis appear to have delayed decay of NNRTI- resistant-HIV compared to sd-NVP; however, this has been evaluated in only small cohorts (median n=10 subjects, range: 10-13) followed until 6-12 months of age [9-11].

Despite scale-up of ART in resource-limited settings, children have relatively few ART options. Based on clinical trials [6], protease inhibitor (PI)-based-ART is recommended for HIV- infected children <3 years of age and NNRTI-based-ART for older children. PI-ART is more costly, interacts with tuberculosis treatment, has long-term toxicities and suspensions are unpalatable. Virologic failure during PI-ART often selects lamivudine (3TC) resistance, which is part of nearly all NNRTI-ART regimens, and this resistance may contribute to the lower efficacy of NNRTI-ART following PI-ART. A better understanding is needed of: (1) the decay versus persistence of NNRTI-resistance; and (2) if persistent NVP-resistance is cross-resistant to efavirenz (EFV)-based-ART and diminishes suppression of HIV-replication in children >3 years of age. To address the former knowledge-gap, we examined the prevalence of mutations conferring high-level NNRTI-resistance in a cohort of HIV-infected children who had failed MTCT-prophylaxis, and the persistence of these mutations over time. We hypothesized that the persistence of NNRTI-resistant-HIV would correlate with a greater duration of NVP- prophylaxis.

Methods

A prospective observational cohort study at the Pediatric HIV Clinic at Kalafong Provincial Tertiary Hospital in Pretoria, South Africa enrolled children aged 0-5 years of age, referred for initiation of ART from July 2010 through February 2013. Children who (1) had not received PMTCT prophylaxis, (2) were severely ill or at high risk of mortality,

and (3) would not be available for prospective monthly follow-up were not eligible to participate in the study. The duration of NVP administration to infants varied due to changes in South Africa's clinical guidelines for PMTCT in 2010. These guidelines recommended 6 weeks of daily NVP be given to infants born to HIV-infected mothers on lifelong ART, or if mothers were not taking ART, for the duration of breastfeeding. The study was approved by the Human Subjects Boards at Seattle Children's Hospital and the University of Pretoria, with the consent of a guardian required for enrollment.

Whole blood was collected at enrollment and monthly for two years. Five-hundred pL of the specimen was divided across four sample-areas on Whatman FTA™ cards (GE Healthcare Life Sciences) that contain a proprietary chemical-coated matrix that lyses cells and denatures proteins on contact, allowing DNA to adhere to the card. The remaining blood was separated into plasma and peripheral blood mononuclear cells (PBMCs). Plasma HIV RNA was quantified (Nuclisens EasyQ HIV-1 VL Assay v2.0; bioMeRIEUX, SA) according to the manufacturer's instructions. Amplifiable HIV DNA templates within one 3mm-punch of a dried blood spot (DBS) were quantified by real-time polymerase chain reaction (PCR) of HIV *gag* [3], and then >150 viral templates were amplified from one or more punches by nested HIV*pol* PCR [3], with amplicons from each specimen combined and tested for HIV-drug-resistance using an oligonucleotide ligation assay (OLA) for codons K103N, V106M, Y181C, and G190A, optimized for HIV subtype C, the predominant subtype in South Africa [3]. A standard curve of 0, 2, 5, 10, 25, 50, 75, 100% mutant in each OLA plate allowed quantification of

HIV-resistant sub-populations >2%. Clinical data, including infant feeding (breast or formula), were collected from each mother's and child's medical record.

Children with and without mutations at enrollment were compared by exposure groups (sd-NVP versus extended NVP-prophylaxis) using the Fisher's exact test, and by age using the Mann-Whitney test. A paired-sample t-test was used to compare the frequency of mutants at enrollment and last visit in the sub-population analysis of the cohort.

Results

A total of 103 (87%) of 118 children referred for initiation of ART were enrolled into the study based on inclusion/exclusion criteria, study nurse availability, and refusal of three families due to collection of extra blood specimens. Eighty-eight HIV-infected children with a documented history of NVP-prophylaxis and with a DBS collected at >1 time-point were included in this analysis. Their median age was 7.5 months (IQR: 4-15). Their HIV-prophylaxis included sd-NVP at birth in 40 (45%) and extended NVP-prophylaxis (median 6 weeks; IQR: 4- 9) in 48 (55%) infants. During the study, 73/88 (83%) infants initiated ART with lopinavir/ritonavir (LPV/r) plus abacavir (ABC) and 3TC; 5/88 (6%) initiated NVP- or EFV- ART with 3TC and ABC or zidovudine (ZDV), and 10/88 (11%) did not initiate ART.

At enrollment, NNRTI-associated mutations were detected by OLA in 46/88 (52%; 95% CI, 42-64%), with Y181C detected in 33%, K103N in 23%, G190A in 13%,

and V106M in 10%. A single mutant codon was detected in 27/88 (31%) and >2 mutants in 19/88 (22%). Children with mutant viruses were younger compared to those with exclusively wild-type virus (median 4.5 months of age, IQR: 2-8.5 vs. 13.5 months, IQR: 7-24; P<0.0001). NNRTI-resistance was detected more frequently among those who took NVP-prophylaxis for a longer duration (17/40; 43% receiving sd-NVP vs. 29/48; 60% receiving extended NVP-prophylaxis), although this difference did not reach statistical significance (P=0.1334).

Among children with NNRTI-resistance at enrollment, the codon with the highest proportion of resistance represented a median of 96% (IQR: 82-100%) of the child's HIV- quasispecies. Detection of low frequencies of NNRTI-resistance (2-25%) within a child's HIV- quasispecies was uncommon (8/88; 9%).

During the study, nearly half of children were lost-to-follow-up (LFTU) prior to study- month-9, this included 17/88 (20%) who participated only in the enrollment visit and 22/88 (25%) who exited within 8 months of enrollment. A total of 49/88 (56%) children participated in the study for >9 months. Follow-up in the latter group extended for a median of 13 months (IQR: 12-16) until children reached a median of 21 months of age (IQR: 17-32). This longitudinally followed sub-population of 49 children was mostly breastfed (12/18 (66%) in the sd-NVP group and 29/31 (94%) in the extended-NVP group), and all were treated with LPV/r-ART, except one child (Subject 49) was given EFV-based-ART. At enrollment, the age of the longitudinally followed sub-population

(median 8 months; IQR: 3-16), prevalence of NNRTI-resistant-HIV (27/49; 55%) and mutant frequency did not differ from the greater study population, including those children LFTU. Among the 27 children with resistance in this sub-population, NNRTI-resistant-HIV persisted over the entire period in 26/27 (96%). The single child with loss of resistance over time (Subject 14) had received sd-NVP; he was 26 months of age at enrollment when V106M was detected at a frequency of 3%, when LPV/r-ART was initiated. The frequency of the predominate NNRTI-resistant variant in each child's quasispecies did not decay significantly during follow-up (medians nd IQR: 98%; 84-100% vs. 89%; 76-100%, P=0.2585, at enrollment and last visit, respectively).

Of note, during the study NNRTI-resistance was selected in only one child (Subject 49). At enrollment only wild-type virus was found, but 6 weeks after initiating EFV-ART both V106M and G190A were detected (Figure 1).

	ID	Duration of NVP	Age at Enr (mo)	Age LPV/r ART initiated (mo)_	Mo of followup	K103N			V106M			Y181C			G190A			Feeding (breast or formula)	Plasma HIV RNA <400 c/mL at last study visit	Maternal ART at Enr.
						Enr	6mo	12mo	Enr	6mo	12mo	Enr	6mo	12mo	Enr	6 mo	12mo			
a. sdNVP	1	sdNVP	1	1	12	wt	wt	wt	98%	98%	98%	24%	28%	44%	wt	wt	wt	breast	No	3TC,TDF,EFV
	2	sdNVP	2	4	14	7%	wt	12%	20%	15%	11%	wt	wt	wt	wt	wt	wt	breast	Yes	Not enrolled
	3	sdNVP	2	2	15	100%	100%	o o	6%	wt	wt	84%	84%	100%*	wt	wt	wt	formula	Yes	3TC,TDF,EFV
	4	sdNVP	5	5	13	2%	wt	6%*	wt	7%	2%*	86%	82%	75%*	3%	5%	13%*	formula	No	Not on ART
	5	sdNVP	7	7	14	wt	wt	wt	wt	wt	4%	wt	wt	wt	wt	wt	wt	breast	No	Not enrolled
	6	sdNVP	7	9	13	wt	wt	8%	wt	wt	wt	wt	wt	wt	wt	wt	wt	breast	Yes	3TC,TDF,EFV
	7	sdNVP	8	8	12	7%	8%	15%	wt	wt	wt	86%	73%	80%	wt	wt	wt	formula	Yes	Not on ART
	8	sdNVP	9	9	16	wt	wt	wt	wt	wt	wt	98%	88%	88%*	wt	wt	wt	breast	No	Not enrolled
	9	sdNVP	9	9	9	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	breast	No	Not enrolled
	10	sdNVP	12	13	9	wt	wt	wt	wt	wt	wt	14%	30%	72%	wt	wt	wt	breast	Yes	3TC,TDF,EFV
	11	sdNVP	20	20	26	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	breast	Yes	Not on ART
	12	sdNVP	21	22	11	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	formula	Yes	Not on ART
	13	sdNVP	23	23	20	wt	wt	wt	wt	wt	wt	90%	100%	98%*	wt	wt	wt	formula	Yes	Not on ART
	14	sdNVP	26	26	15	wt	wt	wt	3%	wt	wt	wt	wt	wt	wt	wt	wt	breast	Yes	3TC,TDF,EFV
	15	sdNVP	26	28	13	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	formula	Yes	3TC,TDF,EFV
	16	sdNVP	27	27	11	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	breast	No	Not on ART
	17	sdNVP	27	27	14	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	breast	Yes	Not on ART
	18	sdNVP	44	44	17	wt	wt	wt	wt	wt	wt	wt ^	wt	wt	wt	wt	wt	breast	Yes	3TC,TDF,NVP
I	19	60 days	2	2	14	wt	wt	wt	wt	wt	wt	91%	75%	100%*	wt	wt	wt	breast	No	Not on ART
	20	42 days	2	2	11	wt	wt	wt	wt	wt	wt	wt wt	wt		ind#	ind#	ind#	breast	Yes	3TC,TDF,EFV
	21	30 days	2	3	12	100%	100%	100%	wt	wt	wt	wt wt	wt		wt	wt	wt	breast	Yes	Not on ART
	22	30 days	2	3	14	wt	wt	wt	wt	wt	wt	wt wt	wt		wt	wt	wt	breast	No	3TC,D4T, EFV
	23	90 days	3	6	14	36%	38%	22%*	wt	wt	wt	56%	44%	69%^	wt	wt	wt	breast	No	Not on ART
	24	30 days	3	7	17	6%	12%	8%*	wt	wt	wt	82%	42%	77%*	wt	2%	wt	formula	Yes	Not on ART
	25	60 days	3	5	16	100%	100%	o o	wt	wt	wt	57%	59%	50%^	wt	wt	wt	breast	Yes	Not on ART
	26	70 days 30	3	4	17	wt	wt	wt	wt	wt	wt	80%	50%	82%*	wt	6%	6%	breast	No	3TC,TDF,EFV
	27	days 42	3	3	13	wt	wt	wt	wt	wt	wt	7%	4%	3%	20%	15%	18%*	breast	Yes	3TC,TDF,EFV
	28	days 42	3	3	16	wt	wt	wt	100%	100%	92%*	wt	wt	wt	100%	100%	92%*	breast	No	3TC,TDF,LPV/r
	29	days 21	4	7	16	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	breast	Yes	3TC,AZT,NVP
	30	days 7	4	4	13 9	11%	14%	20%*	wt	wt	wt	92%	90%	88%*	wt	wt	wt	breast	Yes	Not on ART
	31	days 60	4	4	12	wt	wt	wt	wt	wt	wt	100%	100%	80%	wt	wt	wt	breast	Yes	3TC,TDF,EFV
	32	days 180	7 7	7 7	9	100%	100%	100%	45%	6%	wt	wt	wt	wt	wt	wt	wt	breast	Yes	Not enrolled
	33	days 180	7	7	9	100%	nt	100%	wt	nt	wt	wt	nt	wt	wt	nt	wt	breast	Yes	3TC,TDF,EFV
	34	days 180	7	7	15	wt	wt	wt	wt	wt	wt	99%	98%	91%	8%	3%	5%	breast	No	Not enrolled
	35	days 14	9	9	12	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	breast	No	Not on ART
	36	days 42	9	9	18	wt	wt	wt	wt	wt	wt	73%	83%	44%	100%	100%	100%	breast	No	Not on ART
	37	days 28	10	10	12	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	breast	Yes	3TC,TDF,NVP
	38	days				wt	wt	wt	wt	wt	wt	wt	16%	wt	wt	wt	wt	breast	Yes	Not enrolled
b. Extended NVP prophylaxis	39	24 days	12	13	10	100%	100%	27%	wt	wt	wt	wt	wt	wt	wt	wt	wt	formula	Yes	Mother died
	40	42 days	12	13	17	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	breast	Yes	Not on ART
	41	14 days	12	13	12	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	breast	Yes	Not on ART
	42	14 days	13	13	12	100%	100%	100%	wt	wt	wt	wt	wt	wt	wt	wt	wt	breast	Yes	3TC,TDF,NVP
	43	180 days	13	13	9	100%	100%	100%	wt	wt	wt	86%	98%	98%	wt	wt	wt	breast	No	Not on ART
	44	180 days	15	16	16	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	breast	Yes	3TC,TDF,EFV
	45	42 days	16	16	16	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	breast	Yes	3TC,D4T, EFV
	46	30 days	18	18	15	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt	breast	No	3TC,AZT,NVP
	47	30 days	20	20	13	14%	40%	48 %*	wt	wt	wt	wt	wt	wt	wt	wt	wt	breast	No	3TC,TDF,NVP
	48	49 days		38		wt	wt	wt				wt	wt	wt	wt	wt	wt			
	49	7 days	45	45 (EFV)	12	wt	wt	wt	wt	90%	68%	wt	wt	wt	wt	16%	14%	breast	No	3TC,TDF,EFV

wt., wild-type; ind., indeterminant; mo., month; nt, not tested; 3TC, lamivudine; TDF, tenofovir; EFV, efavirenz; LPV/r, lopinavir boosted ritonavir

*-resistance mutation detected at last visit when last visit >12mo; #-G190S detected by consensus sequencing 25-49% | 15-24% | 5-14% | 2-4%

Key: Q5-100% I 50-74% ^ | 0%(wt)~

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Figure 1. HIV-nevirapine (NVP)-resistance genotypes and clinical parameters of 49 infants followed longitudinally. Cohort (n=88) was composed of Pretorian infants and young children who failed NVP-prophylaxis for prevention of mother-to-children-transmission of HIV (PMTCT). NVP-resistance (K103N, V106M, Y181C, and G190A) was evaluated by an oligonucleotide ligation assay (OLA) at enrollment (Enr), and ~6 (+/-3) months and ~12 (+/-3) months after enrollment. Study participants (n=49) followed for >9 months are shown grouped by duration of NVP exposure: (a) single dose (sd-) NVP (n=18) and (b) extended NVP prophylaxis (n=31). Within each group participants are listed by increasing age at enrollment. These participants were followed from enrollment when their median age was 8 months (IQR: 316) for 13 (IQR: 12-16) months to a median age of 21 (IQR: 17-32) months. The median frequency of the predominate mutant variant within each individual's HIV quasispecies at enrollment and last visit of follow-up were 98% (IQR: 84-100%) vs. 89% (IQR: 76-100%), respectively (P=0.2585). Data on infants' feeding, infants' antiretroviral therapy (ART) regimen, and infant's plasma HIV RNA (<400 copies/ml) (Nuclisens EasyQ HIV-1 VL Assay v2.0; bioMeRIEUX, SA) at last visit, as well as maternal ART are shown. Note all children received lopinavir/rt-based ART, except for Child-49, who received an efavirenz-based regimen; he was the only child who at enrollment had wild-type HIV and during ART selected HIV-drug-resistant variants.

Discussion

This study of ART-naive HIV-infected children who failed NVP-prophylaxis has three novel observations: (1) The prevalence of NNRTI-mutations was relatively high (>50%) among older infants/young children; (2) NNRTI-mutations predominated in these children's HIV quasispecies; and (3) NNRTI-mutations persisted without significant decay across the cohort or within individual's quasispecies, including those >3 years of age. Taken together, these findings suggest infants who fail WHO-recommended MTCT prophylaxis have a high risk of NNRTI- resistant variants establishing long-lived viral reservoirs.

Our observation that NNRTI-resistant HIV prior to ART was more prevalent in younger children could be interpreted as suggesting that resistance decays as children age, as observed in previous studies [2, 3, 9-11]. However, we rarely detected decay within a child's quasispecies in this cohort. We suspect that the higher rate in younger children and the persistence of NNRTI- resistant HIV is due to the convergence of the following recent trends: (1) A longer duration of NVP-prophylaxis in more recently born infants; (2) Scale-up of PMTCT programs, resulting in a greater proportion of infected infants receiving early diagnosis; and (3) Increasing rates of transmitted-drug-resistance to women of childbearing age [12].

The prevalence of NVP-associated mutations in our cohort is within the range of previous studies involving children who had been exposed to sd- or extended-NVP prophylaxis, 27% [13] to 60% [3, 14, 15]. However, previous studies of sd-NVP involved younger children and demonstrated that resistance decayed to clinically insignificant

levels in most infants by 12 months [2, 3, 9-11]. In sharp contrast to the younger cohorts [9-11], decay of resistance was exceedingly rare in our cohort, suggesting a difference in HIV dynamics.

The persistence of NNRTI-resistant-HIV DNA in the absence of selective drug pressure suggests NNRTI-resistant-HIV is part of the long-lived viral reservoir thought to be established during acute infection, either through selection of randomly generated mutants by NVP [3] or as a result of maternal transmission of NNRTI-resistant-HIV, which occurs more frequently during breastfeeding [4]. Among the 26 children with persistence of NNRTI-resistance over a median of 13 months of study follow-up, ongoing selection of NNRTI-resistant variants may have occurred in only five infants (19.2%; Subjects 1, 8, 26, 34, and 47) due to virus replication while breastfeeding from a mother receiving NNRTI-based-ART (n=3; 11.5%) or from a mother with unknown ARV-status (n=2; 7.7%). In the remaining 21 (80.7%) children, selection of NNRTI-resistance is not a likely mechanism of persistence, as their plasma HIV RNA was <400 copies/mL (n=12), or their mothers were receiving LPV/r-based ART (n=1) or untreated (n=8)
(Figure 1).

Persistence of NNRTI-resistant-HIV has been described previously in smaller study populations [2, 3, 7, 9-11]. Of concern is that high concentrations of archived NNRTI-resistance could result in treatment failure of the NNRTI-based-ART, even at

three or more years of age, when the WHO guidelines assume NNRTI-resistant-HIV has decayed. We contend that the stable frequency of NNRTI-resistant variants we observed in young children suggests viral decay dynamics differ from past observations in infants who took sd-NVP; and that NNRTI-resistance in children with longer periods of selection by NVP or with transmitted-drug-resistance may last for years or indefinitely, as observed in adults [16]

Limitations of our study include (1) maternal history was not available for all participants, limiting our assessment of ongoing selection for NNRTI resistance during breastfeeding of a few infants; (2) because mothers and infants were not enrolled and followed prospectively from prior to birth, we could not determine the onset of HIV drug resistance and whether it appeared to have been transmitted to the infant or selected by NVP prophylaxis; and (3) analyses of DBS by the OLA may have affected our estimates of the frequency of NNRTI- resistant DNA in a child's quasispecies. Specifically, the occasional variation in mutant frequency in our longitudinal assessment of DBS may have been due to changes in the number of viral templates amplified from the DBS. Given validation of OLA quantification of mutant by "next-generation-sequencing" [17], the variation in NNRTI-resistant DNA may alternatively been due to the turnover of HIV-infected and uninfected PBMC populations. Furthermore, we may have underestimated resistance due to primer and/or probe sequence biases affecting our assays on DBS.

In summary, NNRTI-resistant-HIV was prevalent among older infants and young children who failed MTCT-prophylaxis and this resistance persisted at stable high levels

without evidence of decay in nearly all children. If these data are confirmed in studies of children >3 years of age, the high prevalence of NNRTI-resistance would warrant testing for HIV-drug- resistance and evaluation of cross-resistance prior to prescription of NNRTI-ART regimens, or introduction of ARV regimens for infant prophylaxis and/or therapy that has a higher genetic barrier to resistance, as observed with the integrase inhibitor dolutegravir [18].

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U.D.F. provided patient samples and clinical data. R.K., S.O. performed experiments. R.K. analyzed data and wrote first draft. All authors contributed to critical revision of the manuscript.

Potential conflicts of interest: None

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