Original Article

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Compare antiretroviral drug concentrations in hair and plasma across EFV-based regimens in China

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SUMMARY: Effective antiretroviral therapy (ART) depends on adequate drug exposure. Plasma ART concentrations provide a short-term assessment of drug exposure, and hair promises to be an alternative matrix for measuring long-term exposure. We aimed to determine the association between plasma and hair ART concentrations and explore the therapeutic concentrations in hair. A cohort study in which HIV-infected adults receiving tenofovir disoproxil fumarate (TDF) + lamivudine (3TC) + efavirenz (EFV) regimen for at 6 months were recruited and paired hair and plasma samples collected at about 6±1 months of ART. Previously validated liquid chromatography and tandem mass spectrometry methods were used to measure ART concentrations in plasma and hair. Among 74 participants enrolled, 47 used a 400 mg dose of EFV daily and 27 used 600 mg EFV daily. Hair and plasma EFV concentrations were strongly correlated, with particularly strong association observed in the 600 mg EFV group. The hair EFV concentration of female participants was significantly higher than in male participants, which might be the inter-individual variations in the drug metabolism and dissolution and life habits. The concentrations of TDF and 3TC in hair are too low to determine effective threshold and relationship with plasma drugs concentrations. The accumulation and correlation of hair and plasma EFV concentrations promise to determine a therapeutic range in hair. The therapeutic range for EFV in hair needs to be calculated in order to give quantitative results more value within the field of drug exposure assessment.

Keywords: Antiretroviral therapy, Hair analysis, Plasma, Drug concentrations, LC-MS/MS

1. Introduction

Antiretroviral therapy (ART) is a combination of antiretroviral drugs, which block viral spreading and reduces HIV-related mortality (*1-2*). Adequate exposure to ART drugs is key to remaining virologic suppression, preserving immune function and preventing viral resistance (*3-5*). Therefore, measuring drug exposure would benefit forecasting treatment outcomes (*6-9*). Conventionally, drug exposure is measured by determining concentrations of parent drugs or metabolites in plasma, however, plasma concentrations typically offer only a short-term assessment of drug exposure and is easily affected by external conditions (*10*).

More recently, hair has been considered as an alternative matrix for measuring drug exposure, showing some advantages. Drug concentrations in hair provide a window of detection up to weeks or months (*11-12*). Hair collection is easier than blood sampling since it is non-invasive, and the samples can be stored at room temperature (*13*). Therefore, hair analysis provides a valuable advantage by enabling assessment of long-term medication adherence and estimating average drug exposure over extended periods (*14-16*). Analysis of drug concentration in hair is now routinely used in following scenarios including doping control, diagnosis of anti-psychotic drug abuse and chronic intoxication, criminal assaults, and detection of excessive alcohol abuse (*17-18*). In recent years, liquid chromatography and tandem mass spectrometry (LC-MS/MS) method has been developed for quantitative determination of some conventional ART drugs in plasma or hair among people living with HIV (*19-24*). Some studies suggested the application of LC/MS/MS have shown the relationship between ART levels in hair and virologic outcomes, which can predict virologic failure, medication compliance and drug

resistance (*20,23-25*).

The global scale-up of ART necessitates the development of multifactorial evaluation paradigms to adequately measure therapeutic effectiveness. The predominantly recommended regimens in China include two nucleotide reverse transcriptase inhibitors (NRTIs) and one non-nucleoside reverse transcriptase inhibitor (NNRTI), encompassing tenofovir disoproxil fumarate (TDF) + lamivudine (3TC) + efavirenz (EFV). An EFV-based regimen (with a 600 mg dose of EFV, known as EFV600) was preferred as first-line treatment for HIV-1 infection by World Health Organization until June 2018, after which clinical treatment gradually transitioned to 400 mg of EFV (*26*). In plasma, a number of studies have determined pharmacokinetic pattern and therapeutic range of some antiretroviral drugs, but little research exists to assess ART concentration level in hair (*21-22,24*). Hence, in the present study we aimed to determine the association between plasma and hair ART concentrations across different EFV-based regimens and explore the ART concentration level in hair.

2. Materials and Methods

2.1. Study design and population

We conducted a cohort survey to recruit HIV-positive people from March 2019 and February 2020 in a designated ART hospital located in Wenshan prefecture, Yunnan province, China. Eligible participants were HIV adults (aged ≥ 18 years old), infected through heterosexual transmission, and had received ART for 6 months with viral loads < 50 copies/mL. The participants used a 300 mg dose of TDF daily, a 300 mg dose of 3TC daily and EFV (with a 400 or 600 mg dose of EFV daily) as a regimen throughout the study.

The baseline demographic characteristics, laboratory results and clinical characteristics were extracted from their clinical records, including age, gender, ART regimens, CD4 cell counts and so on. Hair and blood samples were collected from participants at about 6±1 months of ART. Then, hair samples were assayed for drug concentration among patients whose plasma HIV-1 RNA levels were lower than 50 copies/mL.

2.2. Sample collection and testing

We drew 5 mL of whole blood for HIV-1 viral load using COBAS Amp liPrep/COBAS Taq Man HBV test, v2.0 (Roche Diagnostics, Germany), a fully-automated system that employs real-time PCR technology with a limit of detection of 50 copies/mL. An additional 5 mL whole blood sample was drawn for probing the plasma ART concentrations (in the time window between 12±1 hour after self-reported medication intake). For the determination of plasma ART concentrations, blood samples were centrifuged at 3500 rpm for 10 minutes. Plasma was transferred into labelled cryovials that were frozen at -80°C until analysis.

All participants provided at least 30 strands of 1cm hair close to the scalp as possible in the posterior vertex region, since hair grows at an average of 1 cm/month. The hair samples were placed in small plastic bags and stored at room temperature in the dark to avoid excessive exposure to moisture until analysis, using methods previously published (*19,27*). We washed and dried the hair segments with acetone, and than transferred the hair samples into a frozen grinder for full grinding (-30°C, 4 minutes) to obtain hair powder. Accurately weighed 10 mg of hair powder, added the extraction solution containing internal standard (TDF-D6, 3TC-$^{15}N_2$, EFV-^{13}C) for vortex extraction (37°C, 1h). Followed by centrifugation, we transferred the supernatant into a 96-hole plate, dried the extract with nitrogen, and mixed with deionized water. The mixture was whirled and used for analysis. The validation was done under a solution of standards spiked with blank hair matrices that were the hair strands 1 cm away from the scalp from a healthy female adult.

2.3. Liquid chromatography and tandem mass spectrometry

LC-MS/MS is utilized as the primary method for detecting the concentrations of the ART drugs because of high sensitivity, specificity and less analysis time. Hair and Plasma concentrations are measured by using LC-MS/MS; hair concentrations with an assay range of TDF (0.04-8 ng/mg), 3TC (0.15-30 ng/mg), and EFV (0.4-80 ng/mg); plasma concentrations with an assay range of TDF (4-800 ng/mL), 3TC (15-3000 ng/mL), and EFV (4–8000 ng/mL).

The LC-MS/MS system consists of Applied Biosystems Sciex Triplequad 4500MD triple quadrupole tandem mass spectrometer, SciexExionLC controller, auto-diagnostics (AD) liquid phase pump, AD column heater and AD autosampler. The high performance liquid chromatography (HPLC) conditions are as follows: the column is a Phenomenex Luna Omega; the mobile phase A is composed of methanoic acid and HPLC-grade water; the mobile phase B is composed of methanoic acid and methanol. The column oven temperature is maintained at 40°C and test is 6 min. Data processing was performed using Analyst 1.6.3 software. All drug concentration tests were conducted by Calibra Lab at DIAN Diagnostics (Hangzhou, Zhejiang, China).

2.4. Statistical analysis

Descriptive data were summarized using median and interquartile range (IQR) for continuous data and percentages for categorical data. The X^2 test was used

to compare proportions, and the two-tailed *t*-test (for normal variables) or the Mann-Whitney test (for skewed variables) was used to compare continuous variables. Statistical Analysis System (SAS 9.4, SAS Institute Inc., Cary, NC, USA) and SPSS Statistics 26 (SPSS Inc., USA) were used for statistical analysis, including Spearman's correlation. Multivariate regression modeling was performed in a forward stepwise manner with predictor variables being added to the model if they demonstrated *P* values > 0.05.

2.5. Ethics statement

The present study obtained ethical approvals from the institutional review board (IRB) at the National Center for AIDS/STD Control and Prevention of the China Center for Disease Control and Prevention (NCAIDS, China CDC) and the approval number was X190111540. Each participant provided written informed consent.

3. Results

3.1. Demographics of participants

A total of 74 participants receiving the 3TC+TDF+EFV regimen were enrolled in the survey. Among them, 47 participants used a 400 mg dose of EFV daily and the rest used a 600 mg dose of EFV daily. The average and standard deviation (SD) age in years of the participants was 44.8±11.1 years. Most participants (68.9%) were between the ages of 18 and 50 years, and fifty-four percent were male. Thirty-five percent of the participants had baseline CD4 counts below 200 cells/μL. There was no statistically significant difference in baseline characteristics between the two groups of participants. The demographic and clinical characteristics of all participants are listed in Table 1.

3.2. Stratified analysis of drug concentrations in plasma and hair

Distribution of plasma drug median concentrations are shown in Table 2. In the 400 mg EFV group, plasma TDF, 3TC and EFV median and interquartile range (IQR) concentrations were 73.75 (53.31-95.47) ng/mL, 220.73 (146.95-361.95) ng/mL and 1457.68 (990.17-2000.84) ng/mL, respectively; plasma TDF, 3TC and EFV median and IQR concentrations were 74.22 (60.32-114.71) ng/mL, 272.03 (131.62-334.22) ng/mL and 2349.73 (1702.76-2832.02) ng/mL in the 600 mg EFV group.

Table 3 summarizes the distribution of hair drug median concentrations. In the 400 mg EFV group, hair TDF, 3TC and EFV median and IQR concentrations were 0.05 (0.01-0.08) ng/mg, 0.87 (0.55-1.23) ng/mg and 2.36 (1.84-3.46) ng/mg, respectively; hair TDF, 3TC and EFV median and IQR concentrations were 0.01 (0.01-0.06) ng/mg, 0.61 (0.35-0.89) ng/mg and 3.39 (2.14-5.43) ng/mg in the 600 mg EFV group. In the 600 mg EFV group, hair EFV median (IQR) concentrations of males was 2.18 (1.95-2.41) ng/mg, and the concentrations of females was 5.06 (4.11-8.10) ng/mg. Hair EFV median concentration was significantly higher in females than males in the 600 mg EFV group ($p < 0.05$).

Neither age nor baseline CD4 count showed a significant difference between the median ART concentrations in hair and plasma in both groups (all $p > 0.05$). Plasma EFV median concentration was significantly higher in the 600 mg EFV group than in the 400 mg EFV group ($p < 0.05$), as was hair concentration ($p < 0.05$).

3.3. Scatterplots of the correlation between hair and plasma drug concentrations

Scatterplots of the correlation between hair and plasma drug concentrations are presented in Figure 1. Spearman's correlation coefficients were used to assess the relationship between the concentrations of ART drugs in the two matrices. The results indicated that hair and plasma EFV concentrations were correlated

Table 1. Demographics of participants (*n* = 74)

Characteristics	Overall, *n* (%)	400 mg EFV Regimen, *n* (%)	600 mg EFV Regimen, *n* (%)	*P* value
Overall	74 (100.0)	47 (63.5)	27 (36.5)	
Sex				
Male	40 (54.1)	27 (57.5)	13 (48.2)	0.44
Female	34 (45.9)	20 (42.5)	14 (51.8)	
Age, years				
average (SD; Range)	44.8 (11.1; 22-74)	45.4 (11.3; 22-74)	44.3 (10.5; 26-74)	0.54
18-50	52 (70.3)	30 (63.8)	21 (77.8)	0.21
> 50	22 (29.7)	17 (36.2)	6 (22.2)	
Baseline CD4 Count (cells/μL)				
Median count; IQR	302 (169, 387)	312 (172, 405)	252 (163, 356)	0.57
≤ 200	25 (33.8)	16 (34.1)	10 (37.0)	0.79
> 200	49 (66.2)	31 (65.9)	17 (63.0)	

Abbreviation: SD, Standard Deviation; IQR, Interquartile Range; EFV, efavirenz.

Table 2. The stratified analysis of drug concentrations in plasma

Variable	400 mg EFV Regimen (n = 47)			600 mg EFV Regimen (n = 27)		
	TDF	3TC	EFV	TDF	3TC	EFV
Total	73.75 (53.31-95.47)	220.73 (146.95-361.95)	1457.68[a] (990.17-2000.84)	74.22 (60.32-114.71)	272.03 (131.62-334.22)	2349.73[a] (1702.76-2832.02)
Sex						
Male	72.18 (53.31-93.55)	231.21 (161.24-360.17)	1434.26 (1106.14-2138.85)	69.66 (61.04-113.32)	248.55 (155.68-339.21)	2289.75 (1640.17-2988.86)
Female	76.21 (55.19-113.17)	214.24 (104.88-457.88)	1583.98 (924.84-1888.72)	84.69 (48.98-115.42)	279.84 (83.41-356.54)	2395.53 (1904.86-3406.07)
P value	0.52	0.70	0.48	0.85	0.96	0.62
Age, years						
18-50	71.88 (57.55-95.17)	211.86 (120.20-324.02)	1424.78 (982.22-2021.98)	69.66 (57.89-113.16)	272.03 (137.97-322.11)	2377.73 (1810.24-2988.85)
> 50	85.51 (53.20-98.12)	231.21 (163.98-440.90)	1504.97 (1000.01-2085.06)	97.08 (51.46-132.53)	283.31 (98.71-422.22)	2265.41 (1267.88-3605.53)
P value	0.58	0.35	0.86	0.36	0.79	0.52
Baseline CD4 Count (cells/uL)						
≤ 200	79.27 (54.74-101.60)	198.86 (161.26-327.93)	1481.33 (966.23-1881.94)	89.85 (71.21-103.43)	201.83 (83.41-356.54)	2182.40 (1904.86-2456.92)
> 200	72.17 (53.09-93.75)	252.77 (111.47-378.19)	1434.26 (1010.23-2085.37)	66.49 (52.04-114.87)	276.24 (175.69-334.22)	2672.59 (1640.17-3431.22)
P value	0.50	0.62	0.91	0.23	0.53	0.29

Abbreviation: DC, drug-concentration; SD, standard deviation; TDF, tenofovir disoproxil fumarate; 3TC, lamivudine; EFV, efavirenz; [a]Mann-Whitney *U* test: Plasma EFV median concentrations were significantly higher in the 600mg EFV group than those in the 400mg EFV group ($p < 0.05$).

Table 3. The stratified analysis of drug concentrations in hair

Variable	400 mg EFV Regimen (n = 47)			600 mg EFV Regimen (n = 27)		
	TDF	3TC	EFV	TDF	3TC	EFV
Total	0.05 (0.01-0.08)	0.87 (0.55-1.23)	2.36[b] (1.84-3.46)	0.01 (0.01-0.06)	0.61 (0.35-0.89)	3.39[b] (2.14-5.43)
Sex						
Male	0.01 (0.01-0.08)	0.89 (0.54-1.41)	2.37 (1.72-3.15)	0.01 (0.01-0.07)	0.76 (0.44-1.23)	2.18 (1.95-2.41)
Female	0.01 (0.06-0.09)	0.85 (0.62-1.21)	2.36 (2.01-4.36)	0.01 (0.01-0.06)	0.53 (0.31-0.83)	5.06 (4.11-8.10)
P value	0.45	0.59	0.25	0.82	0.16	< 0.01
Age, years						
18-50	0.01 (0.01-0.07)	0.87 (0.54-1.19)	2.34 (1.73-3.28)	0.01 (0.01-0.07)	0.61 (0.35-0.89)	2.41 (2.05-5.72)
> 50	0.07 (0.01-0.09)	0.87 (0.56-1.51)	2.36 (1.91-4.05)	0.01 (0.01-0.02)	0.65 (0.44-1.07)	3.87 (3.09-7.74)
P value	0.05	0.74	0.56	0.25	0.68	0.29
Baseline CD4 Count (cells/uL)						
≤ 200	0.07 (0.01-0.09)	0.88 (0.76-1.61)	2.27 (1.86-3.37)	0.01 (0.01-0.06)	0.53 (0.32-0.67)	3.76 (2.22-4.58)
> 200	0.01 (0.01-0.07)	0.84 (0.51-1.16)	2.36 (1.71-3.86)	0.01 (0.01-0.07)	0.76 (0.37-1.15)	2.41 (2.12-7.70)
P value	0.14	0.17	0.59	0.64	0.13	0.96

Abbreviation: DC, drug-concentration; SD, standard deviation; TDF, tenofovir disoproxil fumarate; 3TC, lamivudine; EFV, efavirenz; [b]Mann-Whitney *U* test: Hair EFV median concentrations were significantly higher in the 600mg EFV group than those in the 400mg EFV group ($p < 0.05$).

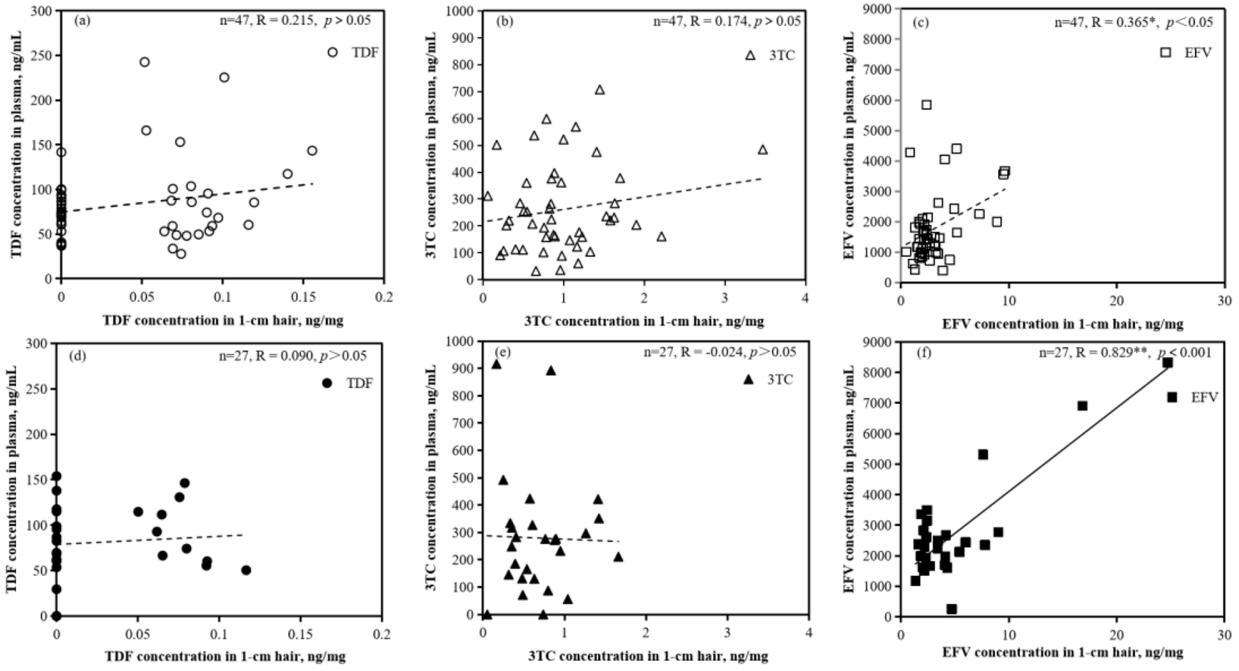

Figure 1. Scatterplots showing the correlation between plasma and hair TDF(a), 3TC(b), EFV(c) concentrations in 400mg EFV group; TDF(d), 3TC(e), EFV(f) in 600mg EFV group. Spearman's correlation coefficients used to assess the relationship between drug concentrations in hair and plasma are shown.

in 400 and 600 mg groups (all $p < 0.05$). Hair and plasma EFV concentrations were strongly correlated in the 600 mg group (correlation coefficients, 0.829; $p < 0.001$). In contrast, TDF and 3TC showed no significant correlation between plasma concentrations and hair concentrations in both groups (all $p > 0.05$).

4. Discussion

Our study first revealed the distribution and association of TDF, 3TC and EFV in plasma and hair among HIV patients of virologic suppression across different EFV-based regimens. Strong correlation was observed between plasma and hair EFV concentrations. The concentrations of 3TC and TDF in hair are too low to be reliably detected, making them difficult to apply in clinical practice. Hair concentrations could be a substitute for plasma testing, provided that corresponding concentration relationships are established through further research.

In the study cohort, hair and plasma EFV concentrations showed correlation, with particularly strong association observed in the 600 mg dose group. Nevertheless, no statistically significant correlation was observed between plasma and hair concentrations for TDF, nor was 3TC. So this phenomenon was mainly caused by differences in the physiochemical properties of ART drugs. Lipophilic molecules can easily penetrate membranes and diffuse into hair (*28*). EFV has the highest lipophilicity and the lowest solubility in the blood, which is easier to incorporate into hair than the other drugs, resulting in a higher correlation (*29*). Therefore, it is expected to find a therapeutic concentration of EFV in hair through accurate predictive models. In contrast, 3TC and TDF contains multiple polar groups which accelerate its dissolution, absorption and transport in the blood, but are not conducive to absorption into hair (*30*). Data from the study showed 3TC and TDF hair concentrations of some participants fell below the detection limits of LC-MS/MS. In this case, we were unable to determine effective hair concentrations threshold for the two ART and relationship between hair ART concentrations and a measure of treatment response. Therefore, we believe that not all ART drugs are suitable for measuring concentrations in hair as an alternative to plasma testing. Under conditions of high adherence, one appropriate drug can be selected as a substitute for the entire regimen.

EFV can be used as a representative drug to observe its distribution differences in hair. Our findings reveal a notable disparity in the distribution of EFV concentrations within hair samples when stratified by gender. The concentration of EFV in the hair of female participants was significantly higher than that observed in male participants. The might be the inter-individual variations in the drug metabolism of HIV patients resulting from their physiological characteristics (*i.e.*, weight) and in the drug incorporation into the hair shaft and the drug dissolution out of hair resulting from the

irradiation of sunlight and life habits (*e.g.*, hair washing and cutting frequency) (*31-32*). Further, previous study has demonstrated that CYP2B6 enzyme polymorphisms significantly influence efavirenz pharmacokinetics, which may partially account for the observed interindividual variability even within the same gender (*33*). Therefore, the life habits of participants need to be controlled before they provide hair samples, including less frequency shampooing, especially when detecting low concentrations of ART drugs. Further analysis could include performing the comparisons separately by gender, or considering gender as one of the confounders in multivariate analysis. Genotyping of clinically relevant CYP2B6 polymorphisms may be performed when necessary to evaluate efavirenz metabolic status.

ART concentrations in hair as a substitute for plasma ART concentrations in HIV patients has consistently presented several challenges. Previous studies have used hair drug concentration as a tool for measuring ART exposure. However, the choice of the multivariate statistical models involving hair concentration as the outcome variable in these studies have not rigorously determined ART drugs concentration thresholds in hair and lacked a large enough sample size to validate hair drug concentration in relation to amount in plasma (*16,17,21,24*). On the other hand, current studies lack a standardized protocol for sampling, collection, and processing hair. The hair sampling sites and length have a great impact on measured concentration. The way of collecting hair samples was mostly cutting or grinding 1cm hair segment closest to the scalp into a fine powder (*18,22,23,34*). Though the drug contents in the 1cm hair segment closest to the scalp can probably reflect drug usage during the past month (*35-36*), hair growth rate actually depends on scalp region, age, gender, ethnicity and inter-individual variability. Hair concentrations testing as a substitute for ART exposure measuring have a long way to go before addressing these challenges.

The limitations of our study were: first, the sample size was limited and the types of data collected were relatively simple. This limited the extent to which data could be analyzed and did not allow for certain associations to be investigated. Second, the observational study mainly relied on extraction of routine clinical records. High quality exposure data are needed to further study exposure-response relations and establish joint pharmacokinetic modelling of plasma and hair drug concentrations. Moreover, we did not strictly control physiological characteristics and life habits (*e.g.*, hair washing and cutting frequency) of participants before collecting their hair samples. Finally, since there is no standard protocol for measuring drug levels in hair, our results may not represent the actual drug concentration.

5. Conclusion

In conclusion, we have shown the distribution and correlation of three antiretroviral drugs exhibited pronounced variability in hair. EFV has the high accumulation resulting in a strong correlation between EFV concentrations in hair and plasma. It is crucial to perform follow-up work in which the therapeutic range for EFV in hair needs to be calculated through accurate predictive models and a standard protocol for the sampling, collection and processing of hair should be established.

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