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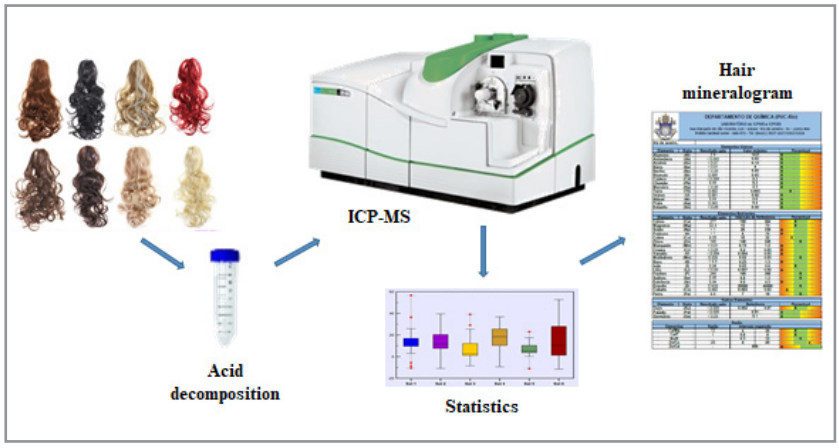
ARTICLE

Hair Mineralogram Analysis for Health Assessment: *Statistical Bias from Gender and Aesthetic Treatments*

Gabrielly Peregrino[1,2], Carlos G. Massone[2], Adriana Haddad Nudi[2], Tatiana Dillenburg Saint'Pierre*[2]

[1]*Instituto Federal do Rio de Janeiro (IFRJ), IFRJ Campus São Gonçalo, São Gonçalo, RJ, Brazil*

[2]*Pontifícia Universidade Católica do Rio de Janeiro (PUC-Rio), Rua Marquês de São Vicente, 225, Gávea, Rio de Janeiro, RJ, CEP 22451-900, Brazil*



The hair mineralogram is a complementary multielement analysis that provides information to aid in the diagnosis of a patient's health status; however, aesthetic treatments can affect the analysis results. This research aimed to identify standard patterns among mineralogram results and some variables, such as gender and the use of aesthetical treatments that can point out differences and causes of variation in elemental concentrations in hair. For this purpose, 151 hair samples were obtained from volunteers and analyzed by inductively coupled plasma mass spectrometry (ICP-MS). This work is pilot research, part of a project to encourage girls to the STEM area, called "Girls in Science", with financial support from the Brazilian Government. Mineralogram results were compared through statistical analysis. The results of natural hair indicate significant differences ($p<0.05$) between genders in the concentrations of Ca, Mg, Sr, and Mo, being higher in women. This behavior was related to the remodeling of minerals in bones, which is different between men and women. The metal concentration in natural hair from women was also compared among different skin colors and no significant differences were observed. Hair treatment, in contrast, has affected significantly the concentrations of many elements. Concentrations increased in hair submitted to dyeing only or with straightening, when compared to natural hair, especially for Ca, Mg, Sr, Ba, and Ni. These results confirm the recommendation of physicians to let the hair grow free of aesthetic treatments for at least 3 months before performing the mineralogram.

Keywords: Mineralogram, hair, ICP-MS, statistic, cosmetic treatment.

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INTRODUCTION

Nowadays, people are increasingly exposed to chemical substances, many of them being possible harmful to the health, although most people have little information about, or just ignore this fact. As well, the effects on the health of many products are not completely understood, mainly concerning the interactions among different substances. Besides, the lack of supervision by competent authorities and the use of low-quality raw materials have compounded this problem. This is especially true for cosmetic products, which can affect not only the consumers but also the professionals who apply them. Fortunately, it seems that most dyes employed in aesthetical treatment for hair are safe to the health [1].

Exposition to toxic elements can be identified by the analysis of hair, an exam called hair mineralogram, since these elements are excreted also by hair growing, besides other excretion routes. Better than urine analysis, which identifies only acute and recent exposure, hair mineralogram presents advantages also in comparison with blood analysis since elements are concentrated in hair and this is a non-invasive exam. In addition, hair samples are easier to collect and store, and can be employed for temporal monitoring, since hair has a growing rate of about 1 cm $month^{-1}$ [2–9].

The mineralogram is mainly employed by physicians to have an idea of the overall health condition of the patient, by the analysis of nutrient elements, as well as to identify health problems, such as those in thyroid and osteoporosis, that can be indicated by an imbalance in the concentration ratios of nutrient elements, mainly Ca, Mg, Na, K, and Fe. However, the main reason why the mineralogram is so few employed by physicians is that the interpretation of the results is still a challenge. Besides the high amount of data obtained with this exam, it is difficult to establish reference values, by the natural variations among different ethnic groups, genders, ages, food habits, etc [9–14].

Also, although hair has been employed for a long time as biopsy material for the determination of trace elements in the human body, there are also no consensus protocols for hair analysis. Before the 1980s, hair used to be analyzed by inductively coupled plasma optical emission spectrometry (ICP OES), then with the development of the much more sensitive inductively coupled plasma mass spectrometry (ICP-MS), new horizons for hair analysis have emerged. The problem is that, since it has allowed quantifying elements at trace levels, there was overvaluation of hair analysis, which triggered some misdiagnosis [15].

Then, as mentioned, besides the lack of a standardized reference method for hair analysis, the sample preparation procedure is also not a consensus. For example, some authors recommend a previous washing of the sample, while others alert for the possibility of removing endogenous substances by incorrect washing of the sample, which could interfere with the results [16–19]. As well, one requirement seems to be adopted: The analysis should be carried out in natural hair, it means, one should let the hair grow without permanent aesthetical treatments, such as dye or straightening, for at least 3 months before sample collection, since the products employed can incorporate or extract elements from the hair, hampering the interpretation of the results [20].

For these reasons, the objective of this pilot study was to evaluate a dataset of mineralogram results, employing statistical analysis, to identify distribution patterns and significant effects in the analysis results due to permanent aesthetical treatments, such as dyeing and/or straightening. This work was part of a project to encourage young women to sciences, in collaboration with a Rio de Janeiro state high school, and had financial support from the "Conselho Nacional de Desenvolvimento Científico e Tecnológico" (CNPq).

MATERIALS AND METHODS

Reagents

Water was ultra-purified by deionization (18 MΩ cm minimum resistivity) in a MilliQ system (Millipore, USA). Nitric acid (Vetec, São Paulo, Brazil) was purified by sub boiling bi-distillation in quartz still (Duo-PUR, Milestone, USA).

Analytical solutions were prepared with the multielemental standard solution Merck 23 (B, Ba, Bi, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Sr, and Zn at 1000 μg L^{-1} each one) and the monoelemental standard

solutions: As, Hg and Se 1000 µg L^{-1}, P 40 µg mL^{-1}, Be, I, Mo, S, Sb, and V 500 µg L^{-1}, U and Th 200 µg L^{-1}, all from Merck, Darmstadt, Germany. A 40 µg L^{-1} Rh solution (prepared from a 200 µg L^{-1} Rh solution VHG, Manchester, USA) was added online to all other solutions (blank, analytical solutions, and samples), as an internal standard. Nitric acid 10% v/v was added to all blank and analytical solutions to equalize the acid concentration of the sample solutions.

Samples

Samples were collected from 151 volunteers randomly selected, which answered a form with information about ethnic group, gender, age, food and sports habits, health treatments, and kind of hair aesthetical treatment when that was the case. No previous selection of variables was done. The volunteers were advised of the objective of the work before agreeing in giving the hair sample.

Samples were from 116 women and 35 men, from 4 to 88 years old. Table I summarizes the main characteristics of the volunteers.

Table I. Main characteristics of the volunteers

	Women	**Men**
Total	**116**	**35**
Ethnic group:		
White	86	29
Black	9	1
Brown	21	5
Hair treatment:		
Natural	31	34
Dye	45	1
Straightening	15	0
Dye + straightening	25	0

The hair samples were cut from the occipital region, close to the scalp, with a stainless steel scissor cleaned with ethanol. About 3 cm from the scalp were cut and the length was discharged. The samples were stored in identified plastic bags until analysis.

A certified reference sample of hair (NCS DC73347a) from China National Analysis Center for Iron and Steel (2015) was employed for checking the accuracy of the method.

Sample preparation

Sample preparation was adapted from the procedure developed by Miekeley et al. [21]. About 500 mg of the sample were weighted in 50 mL propylene flasks, cut into smaller pieces, and washed alternately with ultra-pure water and acetone, by standing for 10 min in an ultrasonic bath with each solvent. This washing procedure was repeated 2 more times and then, the samples were dried overnight in an oven at 60 ºC. Aliquots of 250 mg were weighted in an analytical balance (Adventurer, Ohaus, USA), added 2.5 mL HNO_3 conc. and stand overnight at room temperature. Then, the flasks were heated on a hot plate at 70 ºC for 1 h. After achieving room temperature, water was added to 25 mL final volume and the resulting solutions were analyzed by ICP-MS.

Analysis by ICP-MS

The ICP-MS spectrometer used for the determination of the elements was a quadrupole, model ELAN DRC II from PerkinElmer Sciex, USA. The operating conditions were optimized according to the

manufacturer's Daily Performance procedure and are presented in Table II. The monitored isotopes were chosen concerning the higher abundance and absence of spectral interferences.

Table II. Operational parameters used in the measurements by ICP-MS

Radio frequency power	1200 W
Plasma Ar flow rate	15.0 L min^{-1}
Auxiliary Ar flow rate	1.0 L min^{-1}
Nebulizer Ar flow rate	0.90 – 1.10 L min^{-1}
% of oxides (CeO^{+})	< 3%
% of bivalent ions (Ba^{2+})	< 3%
Background in *m/z*=220	1 cps
Dwell time	60 ms
Scan per reading	1
Number of replicates	3

Assessment of method accuracy

Figures of Merit

The analytical curves were constructed at different concentration ranges for different groups of analytes, according to the methodology developed in the Labspectro, by Carneiro et al. [22]. The correlation coefficient (R) values, given by the software of the spectrometer, above 0.99 have been accepted. The portion of the total variance explained by the regression was considered significant by ANOVA and F tests [23,24].

The limits of detection and quantification were calculated as 3 or 10 times, respectively, the standard deviation of 10 concentration measurements in the blank solution.

The reference material of hair was used to check for the accuracy of the method, by calculating the percentage of the obtained values in relation to the certified concentrations. For the purpose of this work, recovery values are considered acceptable [25].

Statistical analysis

The concentrations obtained from each element and the information filled out in the form by each volunteer were added to a spreadsheet in Microsoft Excel. The minimum, maximum, mean, median, and standard deviations of the elemental concentrations in hair of women and men measured in this work are presented in Table S1 (Supplementary Material).

The elements determined were divided into two groups, also following the work by Carneiro et al. [22], and the statistical analysis will be presented using the same division: Essential elements and others: B, Ca, Co, Cr, Cu, Fe, I, Mg, Mn, Mo, P, S, Se, Sr, V, and Zn; and toxic elements: As, Ba, Be, Bi, Cd, Hg, Ni, Pb, Sb, Th, and U.

The statistical treatment of the data was started by the Shapiro-Wilk normality test [26]. All statistical analyses were executed in R software and related packages [27–29]. Statistical significant assessments consider a 95% probability ($p < 0.05$). Differences among groups were determined based on the Man Whitney test for single comparisons (e.g. male versus female). In multiple comparisons, as in the different types of hair treatment, the Kruskal-Wallis test (KW) was performed. When Kruskal-Wallis test pointed significant differences among groups the Wilcoxon-Mann-Whitney test (WMW) was performed as *post hoc* method. The KW test indicates the presence or absence of difference among groups but, when the difference is identified, it does not identify in which groups the differences occur. Therefore, *post hoc*

testing (Wilcoxon-Mann-Whitney) is necessary to identify intra-group differences. In the *post hoc* method, the p (probability) values were adjusted according to Holm correction method to avoid type I error, common in multiple comparisons.

RESULTS AND DISCUSSIONS

Assessment of method accuracy

The method employed for the multielemental determination in hair samples was based on previous works of our research group [21,22] and is routinely employed at Labspectro, then figures of merit have already been determined. In this work, for checking the accuracy of the method, instrumental detection limits (LOD, μg L^{-1}) and quantification limits (LOQ, μg g^{-1}) of the method, for each element, were determined for comparison with values obtained previously, as well as the analysis of a certified reference material. The correlation coefficients of the analytical curves, not shown here, were better than 0.997 for all studied elements. The limits obtained in this work were in the same order or better than most of those obtained in previous works [21,22] and are shown in Table III, as well as the results for the certified sample, with the percentage recoveries related to the certified concentrations. The measured concentrations of all studied elements were from 80 to 114% of the certified values, confirming the accuracy of the method.

Table III. Instrumental detection limits (LOD) and quantification limits of the method (LOQ), and certified and measured concentrations obtained for the certified hair sample (average ± standard deviation, n=3). Recoveries are the percentage of obtained concentrations relative to the certified values (Rec).

Monitored isotope	LOD (μg L^{-1})	LOQ (μg g^{-1})	Certified (μg g^{-1})	Measured (μg g^{-1})	Rec (%)
^{75}As	3 x 10^{-2}	9.7 x 10^{-3}	0.28 ± 0.05	0.29 ± 0.01	105.2
^{11}B	2	5.2 x 10^{-1}	2.9 ± 0.5	2.4 ± 0.2	82.1
^{138}Ba	9 x 10^{-2}	3.0 x 10^{-2}	11.4 ± 0.6	10.3 ± 0.3	90.3
^{9}Be	8 x 10^{-2}	2.8 x 10^{-2}	0.11 ± 0.07	0.10 ± 0.01	94.1
^{209}Bi	2 x 10^{-3}	8.0 x 10^{-4}	0.021 ± 0.002	0.020 ± 0.001	96.4
^{44}Ca	4 x 10^{1}	1.5 x 10^{1}	1450 ± 20	1239 ± 19	85.5
^{114}Cd	5 x 10^{-3}	1.5 x 10^{-3}	0.07 ± 0.01	0.07 ± 0.01	97.3
^{59}Co	5 x 10^{-3}	1.6 x 10^{-3}	0.045 ± 0.009	0.041 ± 0.010	90.8
^{53}Cr	2 x 10^{-1}	6.2 x 10^{-2}	0.41 ± 0.12	0.47 ± 0.01	114.0
^{65}Cu	8 x 10^{-2}	2.7 x 10^{-2}	14.3 ± 1.6	13.5 ± 1.2	94.3
^{57}Fe	8	2.7	36 ± 5	31.0 ± 1.2	85.0
^{202}Hg	7 x 10^{-2}	2.3 x 10^{-2}	0.67 ± 0.1	0.64 ± 0.1	96.0
^{127}I	1 x 10^{-1}	3.6 x 10^{-2}	0.8 ± 0.2	0.7 ± 0.1	81.8
^{24}Mg	4 x 10^{-1}	1.3 x 10^{-1}	140*	126 ± 6	90.1
^{55}Mn	3 x 10^{-2}	9.9 x 10^{-3}	2.0 ± 0.3	1.8 ± 0.1	90.0
^{98}Mo	1 x 10^{-2}	3.7 x 10^{-3}	0.17 ± 0.03	0.16 ± 0.01	92.1
^{60}Ni	3 x 10^{-2}	1.0 x 10^{-2}	0.43 ± 0.12	0.40 ± 0.01	91.9

Table III. Instrumental detection limits (LOD) and quantification limits of the method (LOQ), and certified and measured concentrations obtained for the certified hair sample (average ± standard deviation, n=3). Recoveries are the percentage of obtained concentrations relative to the certified values (Rec). (Continuation)

Monitored isotope	LOD (μg L^{-1})	LOQ (μg g^{-1})	Certified (μg g^{-1})	Measured (μg g^{-1})	Rec (%)
^{31}P	2 x 10^{1}	7.4	140 ± 20	139 ± 3	99.6
^{208}Pb	3 x 10^{-2}	9.6 x 10^{-3}	5.7 ± 0.5	5.4 ± 0.3	94.8
^{34}S	1 x 10^{3}	4.1 x 10^{2}	41900 ± 1100	37652 ± 2125	89.9
^{121}Sb	7 x 10^{-3}	2.4 x 10^{-3}	0.065*	0.073 ± 0.002	112.3
^{82}Se	7 x 10^{-1}	2.3 x 10^{-1}	0.58 ± 0.12	0.64 ± 0.03	110.9
^{88}Sr	1 x 10^{-2}	3.6 x 10^{-3}	7.7 ± 0.4	6.2 ± 0.1	80.5
^{232}Th	4 x 10^{-2}	1.3 x 10^{-2}	0.064 ± 0.011	0.061 ± 0.001	95.8
^{238}U	3 x 10^{-3}	1.0 x 10^{-3}	0.099 ± 0.015	0.092 ± 0.001	92.7
^{51}V	2 x 10^{-2}	8.2 x 10^{-3}	0.50 ± 0.18	0.40 ± 0.01	86.3
^{66}Zn	8 x 10^{-1}	2.7 x 10^{-1}	137 ± 9	127 ± 5	92.7

*reference value, no uncertainty is given.

Statistical analysis

Verifying differences

Gender: Evaluating natural hair from men and women

In the first moment, all hair samples would be compared to verify differences promoted by different aesthetic treatments; however, only one male volunteer presented aesthetic hair treatment. Thus, the idea arose to evaluate whether variations due to gender affect the mineralogram results. For this, mineralogram of men and women, all with natural hair, were compared, and the sample from the only man with dyeing treatment was excluded.

As mentioned, one of the objectives of this work is to identify significant distribution patterns among the mineralogram results dataset, and then, initially, only natural hair samples were evaluated. Some differences were observed between women and men for nutrient and toxic elements. Gender differences are exposed in Figure 1.

As observed in Figure 1, the elements Ca, Mg, Sr, and Mo showed significant differences ($p<0.05$) between gender, and the concentrations of all of them were higher in women than in men, all with natural hair. It is expected that Ca, Mg, and Sr, which are in the same group IIA in the periodic table, have similar properties and, in this case, it is also expected that these elements have similar or correlated behavior in hair. Mg and Sr are related to the remodeling of Ca minerals in bones, which process has different rates between men and women, mainly during puberty and old age. The similar behavior of these elements, for both men and women, is confirmed by the strong correlations, presented in Figure 2. Then, the drop in the concentration of Ca and Mg happens concomitantly and the ratio between these elements remains practically steadied. Although, when the Ca/Mg ratio is evaluated, there were no significant differences between men (from 3.87 to 28.00) and women (from 1.69 to 30.54), according to the Man Whitney test ($p=0.39$). This indicates that the ratio should be also checked in addition to the absolute values as a parameter for evaluating the patient health.

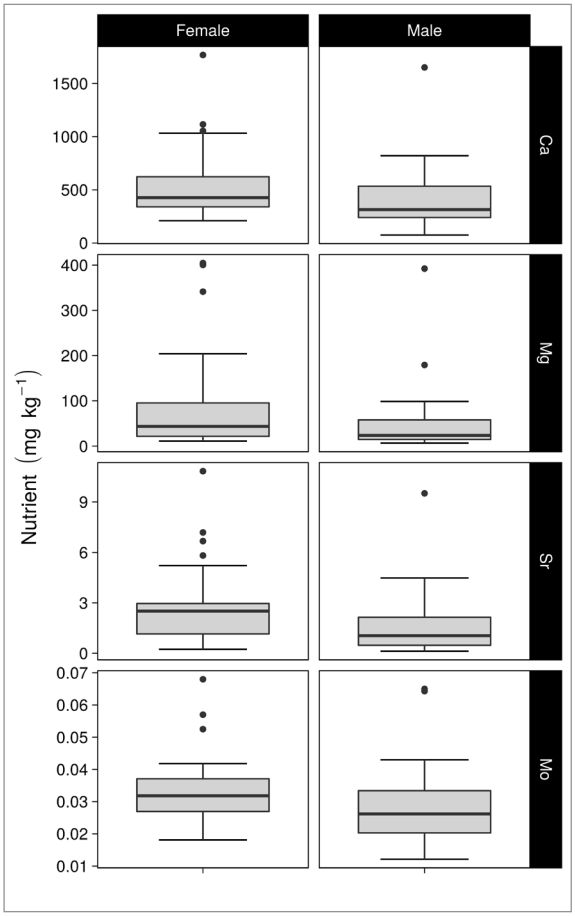


Figure 1. Statistical differences ($p<0.05$) observed between men (n=34) and women (n=31), all with natural hair.

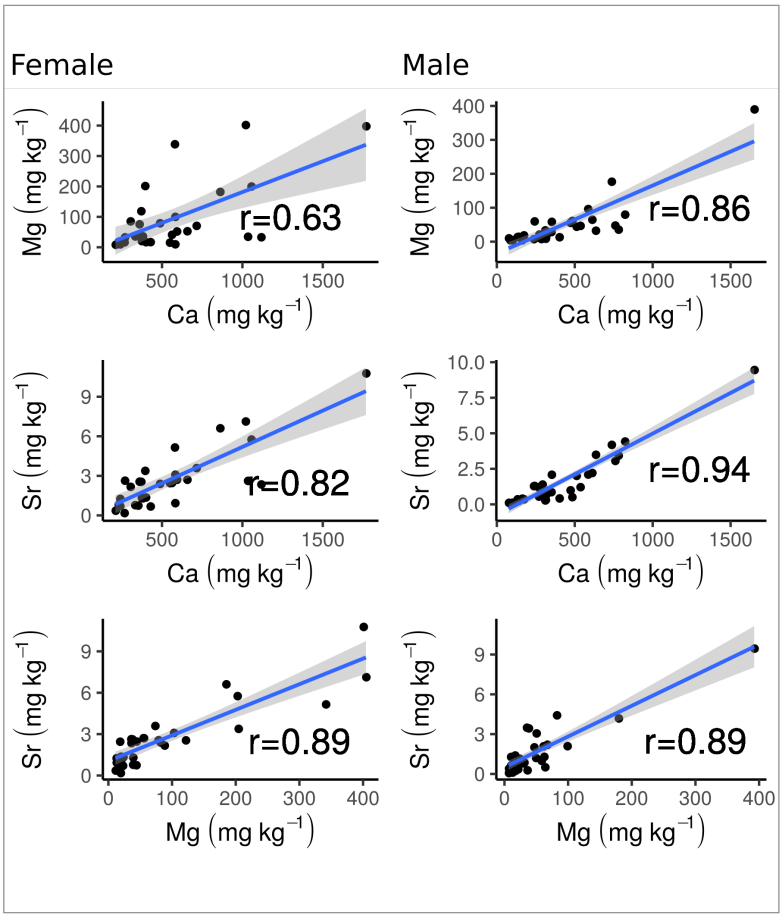


Figure 2. Correlations for Ca, Mg, and Sr in female (n=31) and male (n=34) samples, all with natural hair.

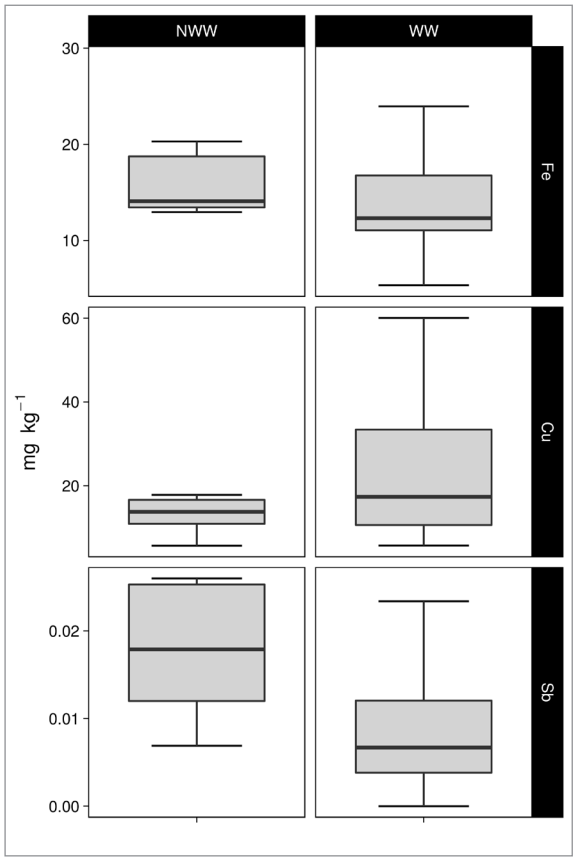
Our findings corroborate most works [6,10,16,30–34]. However, some authors also attribute the concentrations of these elements in hair to environmental, food, and socioeconomic factors. Szynkowska et al. [30] have attributed the differences in Sr concentrations as result of cosmetic treatment, but treated hair samples were excluded in our study, to minimize bias due to it. Also, few studies have found differences in other elements considering gender and each author attributes different causes to it. Luo et al. [35] reported great variability of mean concentrations of elements in hair from different countries and attributed it to environmental exposure, ethnic and geographic origin, and dietary habits, indicating that any country should determine its reference ranges. Higher concentrations of Au or Ni in women have been attributed to the use of jewels, while other metals higher in men were attributed to occupational exposure [31,36–38]

In this research, the reason for higher concentrations of Mo in women than in men could not be elucidated. It is important to mention that the differences were not due to outliers, since the differences are kept by excluding them.

Ethnic group: Evaluating natural hair from white and non-white women

At that moment, we asked ourselves whether differences among ethnic groups, which reflect differences in skin color and hair type, might also promote differences in the mineralogram results. Concerning skin color, we asked the volunteers to declare themselves as white, brown, or black; however, it can be said that, in the Brazilian people, there are no clear limits, but instead, a skin color gradient.

Then, we decided to check whether different ethnic groups present differences in the mineralogram results, by analyzing only samples from women with natural hair. Among these volunteers, most (21) declare themselves as white, 4 as brown, and 3 as black. Because of the skin color issues, already described, and few samples of each group, the differences in the mineralogram results were checked only for 2 groups, white (WW) and non-white women (NWW). Few elements (Cu, Fe, and Sb) presented statistical differences ($p<0.05$) for white and non-white women. However, when we apply the Grubbs test in 3 samples for Cu (285, 153, and 125 mg kg^{-1}) and 1 sample for Fe (182 mg kg^{-1}), all from white women, these values are considered outliers. By excluding them, the concentration ranges of these elements are, respectively, Cu: from 5.7 to 60.0 mg kg^{-1} for WW and from 5.7 to 34.3 mg kg^{-1} for NWW; Fe: from 5.3 to 23.9 mg kg^{-1} for WW and from 12.9 to 35.6 mg kg^{-1} for NWW. In this case, by excluding the outlier bias, there are no differences for Cu and Fe between women from different ethnic groups. The box plots for the concentration distribution of these elements are presented in Figure 3. The outliers were omitted for better range visualization.



Concerning Sb, two outlier values were identified for the group of non-white women (0.052 and 0.030 mg kg^{-1}). Differently from the results found for Cu and Fe, even excluding the outliers, the difference in Sb concentration between WW and NWW is still significant ($p<0.05$), although the concentration ranges are similar (NWW: from 0.007 to 0.026 mg kg^{-1} and WW: from below 0.0024 (LOD) to 0.023 mg kg^{-1}), as evidenced in Figure 3. Antimony is normally associated with pollution due to traffic, since it is a component of alloys employed in vehicle parts, such as breaks and batteries. It has some uses in medicine and other applications; however, we could not find a reason for NWW have higher concentrations of this element than WW in the hair mineralogram. It could be that social-economic differences are responsible for potentially higher exposure to pollution; however, unfortunately, social-economic data were not collected in this research.

Figure 3. Statistical differences ($p<0.05$) observed between white (WW, n=24) and non-white (NWW, n=7) women, all with natural hair. Outliers were excluded.

It is important to mention that, differently than observed in the study concerning differences between genders, in this case, we found outliers in only one of the groups compared and, by excluding them, there are no differences between groups. Maybe these values could be originated from a health problem not recognized in the questionnaires of the volunteers. These samples will be reanalyzed and the volunteers contacted after further analysis.

Aesthetic treatment: Evaluating natural and treated hair from all women

Natural and treated hair mineralogram results were compared only for women, to avoid gender bias observed herein. Also, all women samples were included, except for the 4 mentioned outliers found for Cu, Fe, and Sb. The elements that have presented differences in hair concentrations, when comparing natural

and hair with different aesthetical treatment (dyeing, straightening, or both), for all women, are presented in Figure 4. In this case, the outliers were included in the statistical analysis, since they can be caused by the aesthetical treatments, but omitted in the graphs for better range visualization. It is important to mention that there is a huge amount of different products for hair treatment available on the market. Even products not regulated by health surveillance can be found in Brazilian markets, which can pose risks to the health of consumers. Information about the brand or composition of the hair treatment was not requested for volunteers, since most of them do not know this information, especially when applied in beauty salons.

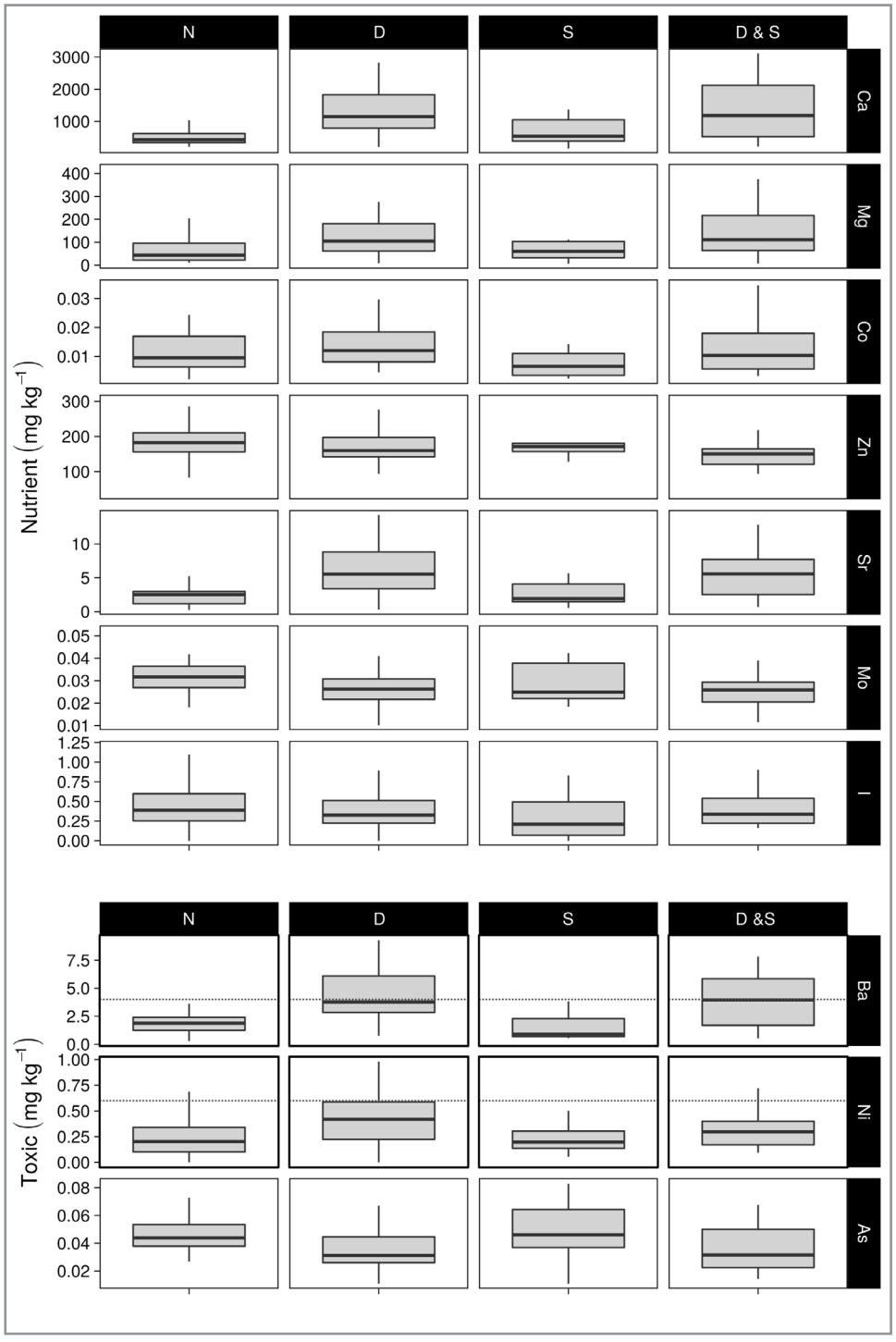


Figure 4. Statistical differences ($p<0.05$) observed among natural hair (N, n=27) and those with aesthetical treatment (D: dyeing n=45, S: straightening n=15, D & S: dyeing + straightening n=25) for all women (n=112) hair samples. Outliers were omitted for better range visualization. The dotted lines indicate the upper limits for toxic elements.

Significant differences ($p<0.05$) were observed for many elements, most of them presented increased concentrations in hair submitted to dyeing only (D) or with straightening (D+S) when compared to natural hair (N). This behavior was especially observed for the earth alkaline elements, Ca, Mg, Sr and Ba, and also for Ni. Surprisingly, the concentrations of Mo and As have decreased in hair submitted to treatments including dyeing, when compared to natural hair. For As, although not significant, a slight increase in concentration was observed in straightened hair. In fact, straightening only (S) seems not to affect significantly the mineralogram results, when compared to natural hair, only for I and Co, whose concentrations have decreased with this aesthetical treatment. Zn concentration has also decreased with straightening, but only when associated with dyeing. It can be that straightening treatments employ chemical substances to break the hair protein bounds, while dyeing treatments require extracting substances from the hair and adding colorful others, to allow changing the natural color, which can be more aggressive, concerning the hair composition, than straightening.

Still, concerning the essential elements, the lower and upper limits considered in this work were not indicated in the figure, since it would result in too much information, difficult to visualize. The common ranges of essential elements in the hair mineralogram of the Brazilian people were already established in the previous research of our group [22] and are present in Table IV. It is important to mention that, for the earth alkaline elements, about 22% for Ca, 39% for Mg, and 16% for Sr, of the samples with natural hair (only for women) presented concentrations above the upper limits, which are 684, 73 and 4.3 mg g^{-1}, respectively. The percentage of samples with concentrations above the upper limit increases to more than 71%, for Ca and Mg, and more than 64% for Sr, of the treated hair including dyeing (D or D+S). For Ca and Sr, the straightening also increased their concentration (not for Mg), but only about 10% more than those observed for natural hair. Among these elements, just a few samples presented concentrations below the lower limits (about 10%) and no tendency was observed for the different treatments. The other essential elements that presented statistical differences ($p<0.05$) among the hair treated or not, are Co, I, Zn, and Mo. For Co, about 6% of the natural hair samples presented values above the upper limit, which are increased to about 16% when D or D+S were applied. No difference was observed for S compared to N hair and less than 10% of the total female samples were below the lower limit. For I and Zn, the opposite effect was observed, about 42% and 32%, respectively, of the women with N hair, presented I and Zn concentration above the upper limits, and these numbers decrease to less than 30% and 25% respectively, above the limit for treated hair. Indeed, the Zn concentrations were below the lower limit for 10% of N and up to 40% for D+S hair. Decreasing in concentration was observed also for Mo, being about 15% of the N samples, 24% of D, 27% of S, and 32% of D+S samples, with concentrations below the lower limit. For Mo, less than 10% of the samples presented concentrations above the upper limits.

Table IV. Reference ranges for nutrient elements and upper limits for toxic elements employed in this work. The values were established in previous work by our research group [22].

Element	Lower limit (μg g^{-1})	Upper limit (μg g^{-1})	Element	Upper limit (μg g^{-1})
	Nutrient		**Toxic**	
B	NE	0.3	**As**	0.15
Ca	190	684	**Ba**	4.0
Co	0.003	0.03	**Be**	NE
Cu	10	32	**Bi**	0.03
Fe	7	18	**Cd**	0.3
I	0.05	0.6	**Cr**	0.3

Table IV. Reference ranges for nutrient elements and upper limits for toxic elements employed in this work. The values were established in previous work by our research group [22]. (Continuation)

Element	Lower limit (μg g^{-1})	Upper limit (μg g^{-1})	Element	Upper limit (μg g^{-1})
	Nutrient		**Toxic**	
Mg	13	73	**Hg**	2.3
Mn	0.15	1.2	**Ni**	0.06
Mo	0.02	0.05	**Pb**	9.3
P	161	257	**Sb**	0.03
S	39965	46000	**Th**	0.005
Se	0.8	1.5	**U**	0.02
Sr	0.6	4.3		
V	0.004	0.03		
Zn	140	239		

NE: not established

Still evaluating the limits for essential elements, it was interesting to observe that, among those that did not present statistical differences between natural and treated hair, some presented concentrations above the upper limits, such as Fe (from 27 to 50% of the samples) and S (from 24 to 40%), while others presented concentrations below the lower limits, such as B (from 56 to 73% of the samples), P (from 73 to 88%) and Se (from 28 to 72%). Men with natural hair presented more or less similar tendencies than women with natural hair, except for those elements with statistical differences between genders, as presented above.

Concerning toxic elements, from the 11 elements quantified, only As, Ba and Ni presented significant differences ($p<0.05$) in treated hair when compared to natural hair. A worrisome observation was the number of treated hair samples whose Ba and Ni concentration exceeded the upper limits. The dotted lines in Figure 4 indicate the upper limits for Ba (4 mg g^{-1}) and for Ni (0.6 mg g^{-1}). For As, only 2 samples presented values above the upper limit (0.15 mg g^{-1}) and were considered outliers. All the others had As concentrations below the reference value employed in this work. For this reason, the upper limit was not highlighted in the figure. However, when analyzing the values obtained for Ba and Ni, it was interesting to observe that about 45% and 30%, respectively, of the hair samples with dyeing, exceeded the upper limits. It corresponds to 20 samples for Ba and 14 for Ni among the 45 hair samples with dyeing. Also, among the hair with dyeing and straightening (n=25), 56% (n=14) of the samples presented Ba concentration above the upper limit, while only 12% (n=3) for Ni. Among the other toxic elements, which did not present statistical differences among treated and non-treated hair, only a few samples have surpassed the upper limits for some elements and were considered outliers.

Few works are reporting the mineral composition of hair treated with aesthetical products. Chojnacka et al. [39] also compared naturally colored hair with those that were artificially colored. They have found that colored hair contained more Sr, Ba, Ca, Mg, W, Mo, Ag, and Mn than in natural one. Also, the same authors reported lower V, Zr, Sb, Pb, As, Si, K, and Hg concentrations in colored hair. Moreover, they have also compared and found differences in the elemental composition of hair samples with different natural colors. However, they analyzed a total of 83 people, male and female, smokers and non-smokers, from children to elderly, with natural (5 different colors) and colored hair. Besides having too many variables, it is supposed that they analyzed few individuals of each hair color.

Massadeh et al. [40] also pointed out differences in the composition of natural and dyed hair, besides other variables, such as age and smoking habit. They have found higher concentrations for some elements, mainly heavy metals, such as Cd and Pb, and also Cu, Fe, and Zn, in dyed hair when compared to non-dyed ones. Differently from our results, they observed lower Ca concentrations in dyed than in non-dyed hair, but they also mention that the differences can be attributed to other variables, such as environment, food habits, and use of medicine. It reinforces the idea that the patient should let the hair grow up for, at least 3 months, before collecting the sample for the mineralogram exam, to exclude one more variable to the results, making more precise the diagnosis.

Verifying grouping

Cluster analyses based on Euclidean Distance and in Ward's method were applied to all women data, including the kind of aesthetic treatment, ethnic group, and age. This statistical evaluation allowed to segregate the samples (n=116) into 3 groups (Figure 5). It was interesting the relation of the 3 groups with age, shown in the density distribution graph in the upper left corner of Figure 5. Although the age is not normal and homogeneously distributed among the groups, by comparing the median ± sd of the age, G1, G2, and G3 could be related to younger (24 ± 13, n=28), middle-age (34 ± 16, n=9), and older (52 ± 16, n=79) women, respectively.

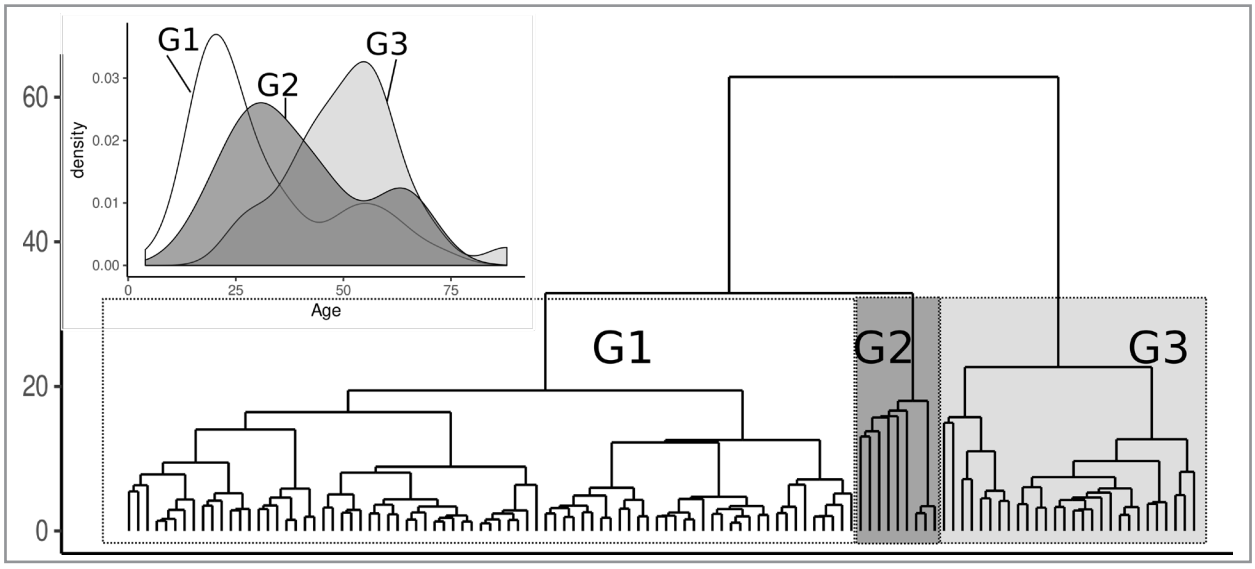


Figure 5. Cluster-based in Euclidean distance and Ward's Method applied to all women group. The density distribution graph is in detail.

Corroborating with the discussion in the "*Ethnic group"* item, the skin color was not relevant for clustering the data, since non-white women (NWW) were more or less equally distributed among groups, about 25% (n=20) in G1, 22% (n=2) in G2, and 28% (n=8) in G3. On the other hand, the dyeing hair treatment was not homogeneously distributed among groups, as discussed below.

The elemental concentrations determined in the 3 groups are presented in the box plot graphs in Figure 6. Only the elements that presented significant differences ($p<0.05$) in concentration among groups are presented, besides the distribution of hair treatment for each group in the lower-left corner of Figure 6.

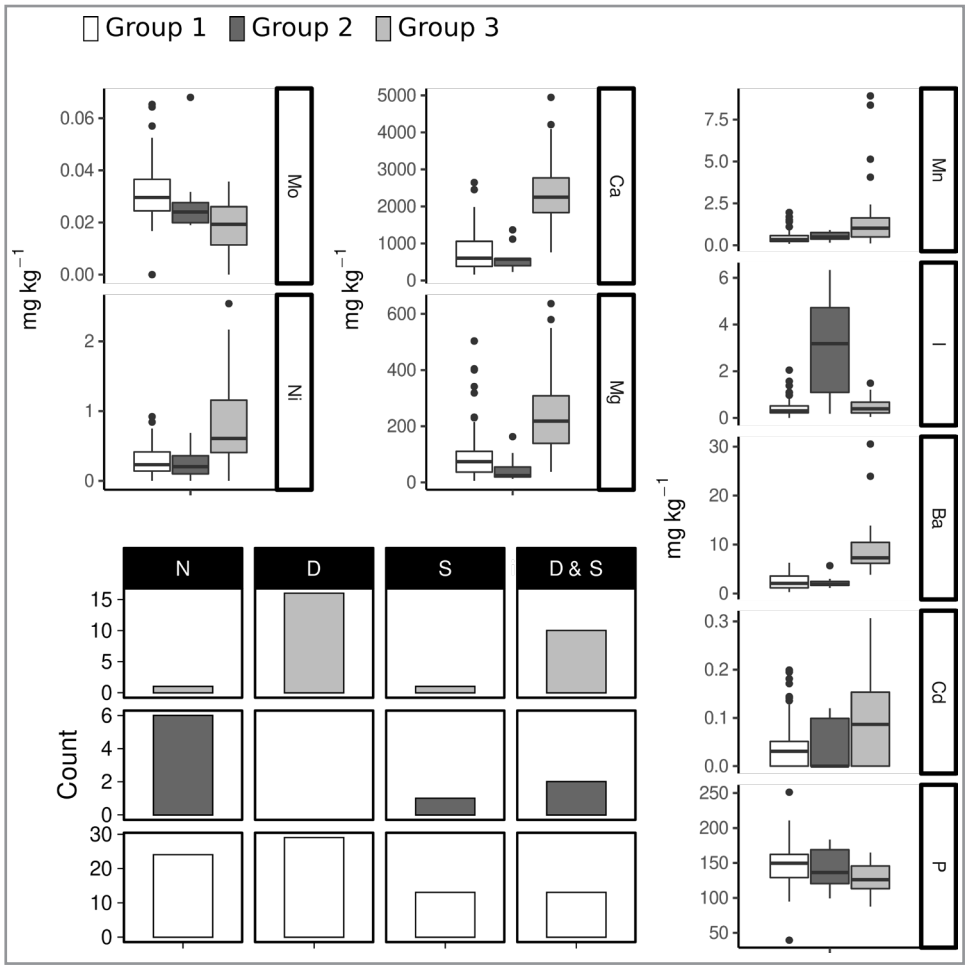


Figure 6. Boxplot of elemental concentrations determined in the 3 groups segregated in the dendrogram. The number of samples with each aesthetical treatment is in detail. D: dyeing, S: straightening, D & S: dyeing + straightening.

By analyzing Figures 5 and 6, it can be said that G3 is characterized by older age women with dyed hair (93%) and higher elemental concentrations, many of them essential. G1 and G2 are younger and middle-age women, with lower concentrations of most elements, when compared to G3 (except Mo and P). These two groups differ, one from each other, mainly by iodine concentration, being those for G2 much higher than for the other groups. It is important to mention that, although the clustering analysis indicates a relationship between the higher concentrations of elements in hair and older age, this correlation is not linear, at least for essential elements, since they are rather related to changes due to life cycles, mostly ruled by hormones. However, concerning toxic elements, it can be related to cumulative effects, since most of them are known for accumulating in body tissues over long periods of exposure. Also, as already mentioned, the hair treatments affect the mineralogram results, and then, a study concerning age should be repeated only for natural hair and with a larger number of samples.

Concerning the aesthetical treatment, almost all women in G3, those older and with the highest metal concentration, have their hair dyed, as expected, while in G2, whose median age is 34 years old, women with natural hair predominate. This group is more related to natural hair than to age, since G2 is distributed over a wide age range, combining the characteristics, in terms of metals concentration, of young and older women. In G1, the group of younger women and lower concentrations of most elements, many women have dyed hair, but many also have natural hair. Differently from G3, the high percentage of dyed hair in G1 (53%) is rather attributed to fashion than to white hair, but even that, present a lower concentration of metals, indicating that it is not possible to attribute a direct relationship between dyeing hair and any tendency in metals concentration.

CONCLUSION

This study fulfilled the main objective of involving high school students of a public school from the metropolitan region of Rio de Janeiro in scientific research conducted by a group of mostly female researchers/professors. The study evaluated the factors that affect the results of the capillary mineralogram, such as gender and use of aesthetic treatment. Although this study was carried out on a pilot scale, the variation between men and women, for Ca, Sr, and Mg, was observed, as well as that these elements must be evaluated by the concentration and by the relationship between them, concomitantly.

The main difference between natural hair and hair with aesthetical treatments was observed to the earth alkaline elements, Ca, Mg, Sr, and Ba, with increased concentrations in hair submitted to dyeing. The toxic elements As, Ba, and Ni also presented significant differences in treated hair when compared to natural hair. However, it was interesting to note that the treatments involving only straightening have significantly affected just a few elements (I and Co). It is important to reinforce that, even knowing these findings, it is difficult to preview the effects of any specific aesthetic treatment in any individual. Then, this work corroborates the doctors' recommendation, that the patient should let the hair grow up for, at least 3 months, before collecting the sample for the mineralogram exam.

It is also noteworthy that the sampling complexity must be better evaluated in future studies. Throughout this research, to eliminate possible interference in statistical evaluations, many samples were removed, such as those for women undergoing aesthetic treatment when comparing whites and non-whites. Despite this procedure, the presence of outliers remains and prejudiced statistical evaluation.

Cluster analysis performed herein was able to indicate a relationship between the higher concentrations of elements in hair and older age, not achieved from univariate analysis. This relationship is related to changes due to life cycles, mostly ruled by hormones, and is not linearly related to age values.

The limitations presented herein are intrinsic to pilot research but guide future studies and other researches to the ideal minimum number of samples or to limit a certain class during sampling. As the samples obtained were donated, the distribution of samples between the different classes is discovered after sampling and, perhaps, several sampling efforts by different classes is a more advantageous strategy than a generalist sampling.

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Compliance with Ethical Standards

The volunteers were advised of the objective of the work and the ethical procedures, before agreeing in giving the hair sample.

Conflicts of interest

The authors do not have any conflict of interest concerning the involved institutions or the Brazilian financial agencies.

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SUPPLEMENTARY MATERIAL

Table S1. The minimum, maximum, mean, median and standard deviations of the elemental concentrations (mg g^{-1}) measured in hair of women and men

	Women					Men				
	Nutrient									
Element	**Min**	**Max**	**Mean**	**Median**	**Std Dev**	**Min**	**Max**	**Mean**	**Median**	**Std Dev**
B	< LOQ	79.0	2.56	< LOQ	8.99	< LOQ	9.21	1.51	< LOQ	2.63
Be	< LOQ	0.03	0.00	< LOQ	0.01	< LOQ	0.03	0.01	< LOQ	0.01
Ca	158	4946	1174	882	969	75.6	1651	411	313	302
Co	0.00	0.34	0.02	0.01	0.05	< LOQ	0.02	0.01	0.01	0.01
Cr	0.33	1.14	0.60	0.56	0.18	0.31	1.61	0.59	0.57	0.22
Cu	5.68	5662	86.7	15.7	532	4.61	412	37.6	13.6	76.0
Fe	3.26	182	20.39	15.66	19.28	5.43	33.75	14.73	13.51	7.53
I	< LOQ	6.34	0.66	0.37	0.95	0.04	2.03	0.49	0.39	0.41
Mg	5.52	637	136	91.8	134	6.56	392	47.5	26.5	70.0
Mn	0.08	8.91	0.76	0.43	1.27	0.05	0.82	0.32	0.26	0.21
Mo	< LOQ	0.07	0.03	0.03	0.01	0.01	0.07	0.03	0.03	0.01
P	39.5	251	141	143	27.8	90.0	210	144	143	29.0
S	25459	82748	53047	54209	8151	41592	72756	52575	52337	6572
Se	0.25	262	3.18	0.82	24.56	0.56	1.85	0.99	0.93	0.29
Sr	0.24	22.8	5.37	4.08	4.58	0.13	9.51	1.61	1.01	1.83
V	0.01	0.18	0.08	0.07	0.03	0.03	0.20	0.08	0.08	0.04
Zn	34.6	2315	222	172	234	114	995	234	185	171

Table S1. The minimum, maximum, mean, median and standard deviations of the elemental concentrations (mg g^{-1}) measured in hair of women and men (Continuation)

	Women					Men				
	Toxic									
Element	**Min**	**Max**	**Mean**	**Median**	**Std Dev**	**Min**	**Max**	**Mean**	**Median**	**Std Dev**
As	0.01	0.40	0.04	0.04	0.04	0.02	0.14	0.06	0.06	0.02
Ba	0.30	30.5	4.00	2.84	4.23	< LOQ	5.43	1.61	1.54	1.22
Bi	< LOQ	0.34	0.03	< LOQ	0.07	< LOQ	0.15	0.02	< LOQ	0.04
Cd	< LOQ	0.31	0.05	0.03	0.07	< LOQ	0.41	0.04	< LOQ	0.08
Hg	< LOQ	5.77	0.62	0.38	0.73	0.03	8.56	0.96	0.38	1.67
Ni	< LOQ	2.54	0.43	0.32	0.45	< LOQ	0.69	0.20	0.17	0.17
Pb	0.05	11.5	0.97	0.52	1.34	0.09	19.5	2.02	0.90	3.46
Sb	< LOQ	0.05	0.011	0.01	0.009	< LOQ	0.19	0.02	0.01	0.03
Th	< LOQ	0.024	0.002	< LOQ	0.004	< LOQ	0.012	0.002	< LOQ	0.003
U	< LOQ	0.13	0.01	0.01	0.02	< LOQ	0.16	0.02	0.01	0.03