Peña-Fernández, A., González-Muñoz, M.. and Lobo-Bedmar, M.. (2014) '"Reference values" of trace elements in the hair of a sample group of Spanish children (aged 6–9 years) – Are urban topsoils a source of contamination?', Environmental toxicology and pharmacology, 38(1), pp. 141–152.

Available at https://doi.org/10.1016/j.etap.2014.05.011

Title: **"**Reference values" of trace elements in the hair of a sample group of Spanish children (aged 6-9 years) - are urban topsoils a source of contamination?

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ABSTRACT

Human hair is used as a biomonitor to evaluate the environmental exposure to contaminants in the individual. However, the use of human hair is controversial, mainly because reference levels for pollutants in hair have not yet been set. In the case of Spain, few biomonitoring studies have involved infants and children. A biomonitoring study was conducted to investigate the possible normal values of trace elements of toxicological concern in children aged 6-9 years from the city of Alcalá de Henares, Community of Madrid (Spain), following the methodology and strict inclusion criteria previously developed by our group. Levels of Al, As, Be, Cd, Cr, Cu, Hg, Mn, Ni, Pb, Sn, Ti, Tl, V and Zn were monitored in scalp-hair from 117 healthy children (47 boys and 70 girls) between April and May of 2001. The levels of trace elements here described could be considered as possible "reference values" for children aged 6-9 years resident in the Community of Madrid. These values might also be selected as a preliminary screening tool to evaluate if a Spanish child has been exposed to any of the contaminants studied here. This study also investigated whether local urban topsoils were a source of metals for this population.

Keywords:

Biomonitoring, metals and metalloids, human hair, children, reference values, Spain.

1. INTRODUCTION

Exponential urban and economic development has resulted in human populations settling in urban areas, and as a result being exposed to pollutants. Inorganic environmental pollutants such as metals and metalloids are progressively increasing in the urban environment due to their uses and inorganic characteristics. Trace elements can induce toxic, carcinogenic effects or oxidative stress even at long-term low-dose levels of exposure, and urban citizens can be long-term exposed to low levels without discernible symptoms (Peña-Fernández, 2011; Peña-Fernández et al., 2014a; Varrica et al., 2014).

In the metabolism of any living organism, removing toxic substances is a prerequisite for life. The elimination of a toxicant is defined as the net decrease of the amount in the body. In human beings, excretion is the passage of the toxic susbstance inside the body to the external environment via excreta: urine, faeces, sweat, etc. Many toxic xenobiotics such as metals and metalloids have a particular affinity for sulfhydryl groups. These groups are mainly in keratinized tissues such as nails or hair (Cespón-Romero and Yebra-Biurrun, 2007; Kordas et al., 2010; Rodrigues et al., 2008; Unkiewicz-Winiarczyk et al., 2009a, 2009b). Furthermore, it has been shown that human hair is one of the major excretion vehicles for trace elements that have been absorbed and metabolised (Cespón-Romero and Yebra-Biurrun, 2007; Sukumar, 2002). Therefore, these micro-pollutants are found in human hair at a concentration about 10 times higher than other biomarkers such as urine, blood or plasma (Olmedo et al., 2010).

Currently there is no scientific information on the kinetics of incorporation of trace elements in the hair (Pragst and Balikova, 2006; Rodrigues et al., 2008). This makes it difficult to establish reference values for these substances in human hair. The presence of metals and metalloids in hair may be: i) because metals are caught by hair directly from the bloodstream (known as endogenous elements); ii) because metals are incorporated by the hair once excreted by the sebaceous glands and/or sweat (semi-exogenous elements); or iii) due to external contamination with elements in the environment: air, water, soil, dust, cosmetics, etc. (exogenous elements) (Herber et al., 1983; Hopps, 1977).

Human hair can be used as a biomonitor to assess the environmental exposure to metals and metalloids in human beings (Bartell et al., 2004) and is considered a good matrix for estimating environmental exposures prior to more extensive and expensive studies involving the collection of blood and urine (Schumacher et al., 1996; Liu et al., 2008; Sanna et al., 2008; Lemos and de Carvalho, 2010; Sanna and Vallascas, 2011; Varrica et al., 2014). Hair offers several advantages in human biomonitoring: is stable and easily accesible for sampling analysis (Dongarra et al., 2012), reflects long-term exposure (Gil et al., 2011), and is better accepted by young people than other matrices such as blood. Hair is also considered a good biomonitor of environmental exposure for children, that can be more sensitive to environmental contamination (Callan et al., 2012; Demetriades et al., 2010) due to their less developed blood-brain barrier (Jarup, 2003), and the fact that they breathe more air and consume more food per unit weight than adults (Moya et al., 2004). Moreover, young people could be more likely to be affected by contaminated soils due to their higher sensitivity, higher absorption rates from the gastrointestinal tract and behavioural traits (Johnson and Bretsch, 2002; Ljung et al., 2007; Madrid et al., 2008; Yáñez et al., 2003).

Despite these advantages, human hair has several limitations that have been discussed comprehensively in Peña-Fernández (2011): a) several factors influencing metals and metalloids concentrations in hair such as age, gender, life-styles, etc.; b) the kinetic of incorporation of trace elements in hair is not well-known; and c) some trace elements have not shown correlation with other biological matrices. Therefore, the use of hair as a biomonitor can be controversial, especially due to the lack of information available to define a normal range for trace element levels in the general population (Barbosa et al., 2005; Kilic et al., 2004; Kordas et al., 2010). However, the use of the methodology and strict inclusion criteria developed by our group could facilitate the use of human hair as a screening tool in environmental bio-monitoring studies to help to identify individuals with higher exposures to metals and metalloids (Peña-Fernández et al., 2014a).

Due to the limited availability of information on levels of metals and metalloids in hair in Spain, a comprehensive study of Human Bio-monitoring (HBM) in hair of school children and teenagers was undertaken in one of the largest cities in the Community of Madrid, Spain: Alcalá de Henares (Peña-Fernández, 2011). This paper reports the results from the children aged 6 to 9 years.

The aims of the present study were: a) to determine the reference values of aluminium (Al), arsenic (As), beryllium (Be), cadmium (Cd), chromium (Cr), copper (Cu), mercury (Hg), manganese (Mn), nickel (Ni), lead (Pb), tin (Sn), titanium (Ti), thallium (Tl), vanadium (V) and zinc (Zn) in the hair of healthy school children (6-9 years) from Alcalá de Henares, Madrid, Spain; and b) to determine if urban metal-topsoils are a significant source for this particular group of children, since these soils are considered as a "target" for contaminants, especially for metals and metalloids. In urban environments, trace element concentrations in top soils can act as tracers of environmental pollution and as "health indicators" (Massas et al., 2009).

2. MATERIAL AND METHODS

2.1. Study design and recruitment

Hair samples were collected between April and May of 2001 from healthy children aged 6-9 years of Caucasian ethnicity who had been permanent residents of Alcalá de Henares, one of the most populated cities of the Community of Madrid, Spain. Alcalá de Henares is an UN World Heritage city (latitude: 40° 28' 49'' N - longitude: 3° 22' 9''), is 35 km from Madrid city and 15 km from the international airport of Madrid-Barajas. Samples were taken following the methodology and strict inclusion criteria developed by Peña-Fernández (2011), summarised in a pilot study in Peña-Fernández et al. (2014a). Written consent was obtained from the parents or legal guardians, as well as from the schools' directors. The study was performed following the guidelines of the Helsinki Declaration.

Hair samples 1-2 cm long were cut with stainless steel scissors from the nape of the neck, close to the occipital region of the scalp. The methodology and selection criteria were developed to ensure a robust study design and use of appropriate population sample, as there are several factors that may affect the presence of trace elements in human hair. The preliminary study presented in Peña-Fernández et al. (2014a) could be the basis for further environmental studies to identify environmental contamination that can affect the health of the inhabitants of cities.

2.2. Trace element levels in hair

The study included 117 children (6 to 9-years-old), 47 boys and 70 girls, who met the requirements for participation in this study, as described previously (Peña-Fernández et al., 2014a). The number of children was large enough to be representative of Alcalá's population, as required to establish valid reference values (Seifert et al., 2000).

The concentration of Al, As, Be, Cd, Cr, Cu, Hg, Mn, Ni, Pb, Sn, Ti, Tl, V and Zn were determined by Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES, Thermo Jarrel Ash ICAP 61), after acid digestion following the methodology described by Schumacher et al. (1991). Appropriate quality-assurance procedures and precautions were carried out to ensure reliability of the results. High purity de-ionised water (resistivity 18.2 MΩcm) obtained from a Milli-Q water purification system was used throughout the study. Teflon bombs were properly cleaned, and the reagents were of analytical grade. The quality control consisted of analyzing a reference standard every ten samples (Lobster hepatopancreas, NRC Canada, *TORT-2*). The mean recovery rates ranged between 78% and 110%. Limit of detection for each trace element are provided in Tables 4 to 6.

2.3. "Reference" levels of trace elements in hair

Reference values are intended to characterise the upper margin (usually the 90th or 95th percentile) of the current background exposure of the population to a given environmental pollutant at a given time. This population has to be large enough to cover a representative sample of the population, and should be well characterised under strict quality control (Ewers et al., 1999; Poulsen et al., 1997; Schulz et al., 2009). Reference values do not represent toxicologically derived biological exposure limits since they are statistically derived, and therefore they cannot be used to evaluate the health status of an individual. However, reference values can be used as guideline values to identify subjects who have had a high exposure to an environmental contaminant (Ewers et al., 1999; Seifert et al., 1999). These values can also be used to minimise errors in the interpretation of results; for example those derived from an exogenous contamination in hair biomarkers (Tsanaclis and Wicks, 2008).

The methodology and recommendations suggested by the International Union of Pure and Applied Chemistry (IUPAC) (Poulsen et al., 1997) were followed to establish possible reference values for Al, As, Be, Cd, Cr, Cu, Hg, Mn, Ni, Pb, Sn, Ti, Tl, V and Zn in the hair of healthy children aged 6-9 years living in Alcalá de Henares. Reference values were also suggested for male and female children separately. IUPAC recommend the non-parametric computation of the reference value within the 95% confidence interval of the 95th population percentile of the distribution of concentration of a specific compound in a human biomarker (Poulsen et al., 1997).

Although there are currently no official reference levels for trace elements in hair, levels of trace elements here monitored were compared with those proposed in the literature as "normal or reference" values: Seifert et al. (2000) and Beneš et al. (2003). These authors have suggested normal levels for some trace elements in comprehensive surveys carried out in their countries: Germany and Czech Republic, respectively. Values are shown in Tables 1 and 2, respectively.

2.4. Trace element levels in soils

To estimate the significance of topsoils from urban parks in Alcalá as a potential source of trace elements, total concentration of Al, As, Be, Cd, Cr, Cu, Hg, Mn, Ni, Pb, Sn, Ti, Tl, V and Zn were monitored in topsoils soil samples collected in July 2001. This study is comprehensively described in Peña-Fernández et al. (2014b) and below.

A total of 97 topsoil samples (0–3 cm depth) were randomly sampled from different parks in Alcalá de Henares. Soil samples were dried at room temperature for 2 weeks, ground and sieved with a 2 mm sieve to remove stones, coarse materials, and other debris. Soil samples of 0.7 g were treated with 5 ml of nitric acid (65% Suprapur, E. Merck, Darmstadt, Germany) in Teflon bombs for 8h at room temperature. Samples were then heated at 96º C for 12h. After cooling, the solutions were filtered and made up to 25 ml with Milli-Q demineralized water. Trace elements concentrations were analysed by inductively coupled plasma-mass spectrometry (ICP-MS, Perkin Elmer Elan 6000) following the methodology described in Peña-Fernández et al. (2014b). The results are summarized in Table 3.

2.5. Statistical analysis

Data for Al, Cu, Hg, Mn, Ni and Pb were logarithmically transformed to generate distributions that were close to a normal distribution. Differences were determined by using the Kruskal-Wallis non-parametric test and multiple linear regression analysis to evaluate significant differences between groups. Values of $p<0.05$ were considered to be significant. A Pearson correlation analysis between the different metals determined in the hair was conducted to investigate possible common contamination sources of trace elements by trying to identify significant correlations among them (Aelion et al., 2009; Gong et al., 2010). A correlation study was also conducted between the trace elements analysed in hair samples and those monitored in urban topsoils collected in Alcalá de Henares, to establish if urban topsoils were a significant source of these pollutants to the juvenile population studied. All calculations were performed using the statistical package SPSS 15.0.

3. RESULTS

Tables 4, 5 and 6 present the results obtained from the monitoring of Al, As, Be, Cd, Cr, Cu, Hg, Mn, Ni, Pb, Sn, Ti, Tl, V and Zn in hair of general, male and female child population living in Alcalá de Henares, respectively. Levels of Pb have been previously reported by our team in a preliminary survey carried out in the same area (Peña-Fernández et al., 2014a). "Reference values" suggested for metals and metalloids in hair were defined for general, male and female healthy children that live in Alcalá (Tables 4-6).

The possible influence of gender on the presence of metals and metalloids in the hair of the young Spanish population of Alcalá was also evaluated (Table 7). Of all trace elements monitored in children's hair, only **Al** ($p<0.001$) **and Cu** ($p<0.01$) have been shown to be gender-dependent. For both metals, their concentration in hair was nearly twice as great in the female child population as it was in the male child population. Specifically, for Al: 11.17 vs. 6.00 μg/g, and for Cu: 24.09 vs. 12.01 μg/g (Table 7). Contrarily, the levels of the other trace elements monitored were slightly higher in boys, except for Zn (Table 7).

A Pearson correlation study was conducted for Al, Cr, Cu, Hg, Mn, Ni, Pb, Sn, and Zn monitored in children's hair samples (Table 8). A logarithmic transformation was applied for all pollutants except Cr, Sn and Zn, which were considered directly, due to their normalised distribution in all hair samples. In general, no significant correlation for any of the trace elements considered was found except for Al and Sn. Al was correlated with most variables in this study, but with a very small correlation coefficient: positively with Cu ($r = 0.245$; $p<0.01$) and Pb ($r = 0.225$; $p<0.05$); and negatively with Mn ($r = -0.282$; $p<0.01$) and Sn ($r = -0.220$; $p<0.05$). By contrast, Sn was correlated just with Cu ($r = -0.203$; $p<0.05$).

To conclude, it was thought appropriate to conduct a statistical correlation study between the trace elements analysed in hair (Al, Cr, Cu, Hg, Mn, Ni, Pb, Sn, and Zn) and those found in topsoils from public parks in Alcalá, see Table 3 (Peña-Fernández et al., 2014b). All topsoils and hair samples were collected during the spring and summer of 2001. The results were: Al ($r = -0.144$; $p = 0.163$), Cr ($r = 0.042$; $p = 0.706$), Cu ($r = -0.039$; $p = 0.704$), Mn ($r = -0.076$; $p = 0.479$), Ni ($r = 0.114$; $p = 0.454$), Pb ($r = 0.047$; $p = 0.648$), Sn ($r = -0.059$; $p = 0.580$) and Zn ($r = 0.112$; $p = 0.296$). None of the levels of trace elements studied in hair were significantly correlated with levels measured in topsoils of public parks in Alcalá.

4. DISCUSSION

4.1. Concentrations and reference values of trace elements in children's hair in Alcalá

"Reference values" (CI-PP95) here defined (Tables 4-6) may be used in future studies of children living in the Community of (Alcalá's Community) due to their similar sources of metal exposure. It is recommended that these values be reviewed in the future if the exposure situation changes (Ewers et al., 1999).

Reference values are proposed for a general population's background exposure (Ewers et al., 1999), and therefore their use in different countries and regions will be limited. However, it was considered appropriate to compare the reference values for the Spanish population suggested here with those described in the literature for children living in two different European communities: Germany and Czech Republic (Beneš et al., 2003; Seifert et al., 2000), to avoid potential errors which could have arisen during the establishment of the reference values.

Hair levels of Al, Cu, Pb and Zn were similar to or lower than the reference values established for a German population of 6 to 14 year olds (Seifert et al., 2000) (Table 4 vs. 1), whereas the arithmetic means of **Cd** (0.52 vs. 0.096 μg/g) and **Cr** (0.66 vs. 0.106 μg/g), were higher in the population of Alcalá (Table 4).

Hair levels of Cu, Pb and Zn were similar to or lower than normal values (medians) described by Beneš et al. (2003) (Table 2) in a general Czech population of medium age 9.9 years, whereas levels of **Cd** (0.46 vs. 0.14 μg/g), **Cr** (0.64 vs. 0.22 μg/g) and **Hg** (0.75 vs. 0.19 μg/g) were higher in the hair of the children under study (Table 4). These results might indicate, *a priori*, that the general Spanish group monitored had suffered a slight environmental exposure to **Cd, Cr** and **Hg** that should be investigated. However, considering the limitations of the reference values proposed for hair to date for different human communities (Kilic et al., 2004), more studies would be needed to support this hypothesis.

Furthermore, **As, Be** and **Tl** were not detected in any of the hair samples monitored (n=117; Table 4). Because human hair allows retrospective investigation of past and chronic exposure to trace elements (Pragst and Balikova, 2006), and because the presence of trace elements in hair would be a reflection of environmental exposure (Chłopicka et al., 1998; Marques et al., 2007) as well as an indicator of mineral status of the individual (Štupar et al., 2007), it could be said that the child population monitored had not suffered chronic environmental exposure to As, Be and Tl. This hypothesis could also be supported because hair is one of the major excretion vehicles for trace elements that have been absorbed and metabolised (Cespón-Romero and Yebra-Biurrun, 2007; Sukumar, 2002).

It has been shown that human hair would be a suitable biomarker to assess environmental exposure to **As** (Hinwood et al, 2003). Thus, Díaz-Barriga et al. (1993) have linked high levels of As in children aged 3-6 years in Morales, San Luis Potosí, Mexico (geometric mean of 9,87 μg/g), with the proximity of an industrial smelting complex.

In relation to **Be**, Drolet-Vives et al. (2009) have pointed out that hair would be a good biological indicator for estimating mammal environmental exposure. There is little information in the literature regarding biomonitoring of this toxic metal in human hair.

Although there are very few studies that have measured **Tl** in human hair, Brockhaus et al. (1981) and Dolgner et al. (1983) have related Tl ultra trace levels in subjects under 20 years old in Lengerich, Germany, to the proximity of a large cement plant. Brockhaus et al. (1981) have proposed a normal range of Tl in human hair of 0.001-0.010 μg/g. In Spain, Nadal et al. (2005) did not detect levels of Tl in any of the hair samples collected from 134 children aged 12-14 years in Tarragona.

Furthermore, and unlike other trace elements monitored in the children's hair, **Cd** and **V** were detected in very few samples, specifically 22 and 24 (Table 4), respectively.

Cd was hardly detected at all in the studied population, possibly because the children did not smoke and had very little exposure to smoke. Tobacco fumes are described as one of the main sources of Cd (Rodushkin and Alexon, 2000). The arithmetic mean of Cd observed in the general child population of Alcalá (Table 4) was lower than that determined in the population aged 6-7 years (0.52 vs. 0.71 μg/g), and similar to that found in the population aged 8-9 years (0.52 vs. 0.59 μg/g), both in the province of Tarragona (Bosque et al., 1991).

The range of Cd found in children from Alcalá (0.16-0.99 μg/g; Table 4) was slightly higher than, but within the same order as, that described by Rodushkin and Alexon (2000) in the general population (1-76 year olds) of medium-sized cities in Switzerland (0.01-0.36 μg/g).

However, the presence of Cd was lower than that found in a population of 6-10 year olds from Hyderabad, Pakistan (Shah et al., 2011). The comparison for boys was 0.59 vs. 2.1 μg/g, and the comparison for girls was 0.48 vs. 1.5 μg/g.

V, which is rarely monitored in humans, was only detected in 24 samples. The mean concentration of V in hair Alcalá's children was higher than that quantified in 655 children aged 3-6 years living in small towns in Korea (0.44 vs. 0.08 μg/g) (Park et al., 2007).

The low number of samples in which Cd and V were detected indicates that the child population of Alcalá has not suffered chronic exposure to these metals; however, more studies are needed.

The results obtained for some of the rest of the trace elements, as well as a brief description of the possible sources of the exposure, are described below.

Al, along with Tl, is one of the trace elements which is least often examined in human biomonitoring studies, although it is a neurotoxic chemical (Unkiewicz-Winiarczyk et al., 2009b). Unkiewicz-Winiarczyk et al. (2009b) have suggested that hair could be a biological indicator of Al since the human body will excrete this metal through skin and its components. However, it has been found that the use of human hair as an indicator of environmental exposure to Al is limited if only considers hair as a route of excretion for this metal (Peña-Fernández, 2011). Although more studies are needed, the levels of metals in children's hair may be used to identify whether any child that lives in a given area has been exposed to any of these pollutants. Levels of Al in Alcalá's children were similar to the reference value described by Seifert et al. (2000) (9.05 vs. 9.20 μg/g; Table 4 vs. 1), and also similar to the levels observed by Park et al. (2007) in Korean children aged 3-6 years (9.05 vs. 8.78 μg/g).

Due to the lack of studies, the values found for Al were compared with different age groups. Thus, levels of Al in hair here monitored were similar to those described in 573 children aged 0-12 months from Marrakech, Morocco (9.05 vs. 9.50 μg/g) (Souad et al., 2006). Because the average concentration of Al observed in Morrocan soils (41,400.0 μg/g) was ten times higher than that monitored in Alcalá (5,797.7 μg/g; Table 3), the population studied in Alcalá must have been exposed to Al from other sources besides intake/ingestion of soil. It is reported that the dietary exposure pathway is one of the most significant for Al (Bakar et al., 2010). Another possible source of exposure is prophylactic vaccines containing Al adjuvants such as amorphous aluminium, aluminium phosphate or aluminium hydroxide, compounds which are employed in order to increase the immune response (Clapp et al., 2011).

Regarding **Cr**, it was observed that the mean concentration determined in hair of chidren from Alcalá was greater than that monitored in chidren aged 3-6 years in Korea (0.66 vs. 0.47 μg/g) (Park et al., 2007). However, it was lower than that monitored in 86 children aged 6-11 years in the region of Puglia, Italy for both sexes. For boys the comparison was 0.67 vs. 5.4 μg/g and for girls the comparison was 0.65 vs. 2.7 μg/g (Perrone et al., 1996). Also, it was lower than the concentration observed by Shah et al. (2011), both in girls (0.65 vs. 3.8 μg/g) and boys (0.67 vs. 3.8 μg/g) aged 6-10 years in Hyderabad, Pakistan.

Kempson et al. (2007) have suggested that hair would be a good matrix for monitoring of **Cu**, although there is controversy in this regard (Rodrigues et al., 2008). The arithmetic mean of Cu determined in the hair of the population of 1-10 year olds in Chakwal, Pakistan (Khalique et al., 2005), was similar to that found in the girls (22.12 vs. 24.09 μg/g) and lower than that found in the boys (8.06 vs. 12.01 μg/g) in Alcalá. Moreover, the levels of Cu were greater in the hair of children from Alcalá than in the hair of an Italian group aged 6-11 years, for boys (12.01 vs. 10.0 μg/g) and girls (24.09 vs. 9.7 μg/g) (Perrone et al., 1996).

Human hair is a good matrix to measure exposure to **Hg** (Budtz-Jørgensen et al., 2004; Peña-Fernández, 2011). The levels of total Hg in hair (0.16-4.86 μg/g; Table 4) are within the range found in the general population of Madrid, Spain (1.29-129.47 μg/g) (Gonzalez et al., 1985). They are also similar to the range (0.16-4.86 vs. 0.19-5.63 μg/g) and arithmetic mean (1.10 vs. 0.94 μg/g) found in the Spanish preschool population (Díez et al., 2009). The presence of total Hg in the hair here monitored was slightly lower than in other Spanish regions such as those found in the hair of children aged 4 years in the province of Granada (Freire et al., 2010).

The geometric mean of total Hg in the hair of Alcala's children was a little higher than that measured by Batista et al. (1996) in the hair of 233 children aged 6-16 years in the Provincia de Tarragona, Spain (0.82 vs. 0.77 μg/g). Moreover, the presence of Hg was higher in the hair of the Alcalá population than in other regions in Europe such as Germany (Wilhelm et al., 2002), Denmark (Budt-Jørgensen et al., 2004) or Czech Republic (Beneš et al., 2003). The higher presence of total Hg in the Spanish population could be attributed to the consumption of fish and seafood, since this is described as the largest source of exposure of Hg for humans (Martorell et al., 2011). It is considered that the Spanish population consumes more fish per capita than any other country in Europe (Welch et al., 2002).

With respect to **Mn**, the arithmetic mean in the hair of Alcalá's children was lower than that observed in the population aged 6-37 months in Montevideo, Uruguay (0.30 vs. 2.16 μg/g) (Kordas et al., 2010). Moreover, it was much lower than that monitored in 46 children aged 6 to 15 years in a small town in Canada (0.30 vs. 5.1 μg/g) (Bouchard et al., 2007). These last authors have linked the presence of Mn in hair with an elevated natural presence of Mn in drinking water. Bouchard et al. (2007) have proposed a maximum tolerable level of Mn in hair of 3 μg/g, above which would be observed damage to the nervous system in children; decreased verbal memory and IQ; as well as damage to the neuromuscular system. This tolerable level was not exceded in the children of Alcalá (0.11-1.00 μg/g; Table 3). These results could indicate that the school population here analysed had not suffered chronic exposure to Mn, since Eastman et al. (2013) have recently reported that hair may be an appropriate biomarker of environmental Mn exposure in children. However, more studies are needed because there is controversy in the literature in this respect. For example, Rodrigues et al. (2008) have suggested that hair would not be an appropriate biomarker of Mn in humans.

With respect to **Ni**, a lower concentration was observed in the population studied than in the hair of 134 boys (0.58 vs. 6.9 μg/g) and 132 girls (0.38 vs. 6.9 μg/g) aged 6-10 years from Hyderabad, Pakistan (Shah et al., 2011).

Sn is a trace element little monitored in children's hair, although it is neurotoxic (Moser et al., 2009). The geometric mean of Sn was higher in the population monitored (Table 4) than in the hair of children aged 0-15 years in the metropolitan area of New York, USA: 1.19 vs. 0.56 μg/g (Creason et al., 1975), although the age range was greater in the latter.

Ti is very rarely measured in human biomonitoring throughout the globe, despite the fact that it is widely used in many different applications (Bozkus et al., 2011). The range of Ti here observed (0.13-2.92 μg/g) (Table 4) was lower than that reported by Tomasseo-Ponzetta et al. (1998) in the general population (17-60 years old) of Indonesia (0.95-17.3 μg/g), although the age range considered was different.

With respect to **Zn**, a essential metal for humans (Qin et al., 2009), it was found that the average concentration (85.58 vs. 151.0 μg/g) and range (8.25-181.54 vs. 89.8-312.0 μg/g) in the hair studied (Table 4) were lower than those reported in 31 Polish children aged 6-10 years (Lech, 2002). Also, the levels of Zn were much lower in Alcalá's children's hair than those found in a similar study conducted on 266 children aged 6-10 years from Hyderabad, Pakistan, for both boys (82.84 vs. 225.1 μg/g) and girls (87.44 vs. 230.0 μg/g). This might indicate that the children in Alcalá have a deficiency of Zn that would be interesting to monitor further, since human hair is a good biomarker of Zn (Kempson et al., 2007), and there is evidence that low levels of Zn in children's hair may reflect a suboptimal status of this micronutrient (Gibson et al., 2008). The low levels of Zn found in hair from this Spanish group could be attributed to the intake of cereals, legumes and tubers, common food products in the Mediterranean diet, due to their high content in phytates, a substance that prevents the gastrointestinal absorption of Zn (Shah et al., 2011).

However, the levels of Zn in the hair of the studied population were similar to the reference range reported for the German population of 6-14 years of age (2.4-1,020 μg/g; Table 1) (Seifert et al., 2000) and the reference range suggested for Czech population of 9.9 years of age (8.0-642.0; Table 2) (Beneš et al., 2003). Moreover, the arithmetic mean of Zn in hair collected in Alcalá was higher than that observed in 655 Korean children of 3-6 years of age (85.58 vs. 69.99 μg/g) (Park et al., 2007).

4.2. Effect of gender on levels of trace elements in children's hair in Alcalá

The fact that all trace elements monitored in children's hair were slightly higher in boys except for Al and Cu could be attributed to the fact that boys are more active and spend more time playing outside in parks and playgrounds than girls, so will be more exposed to pollutants (Dongarrà et al., 2011; Kordas et al., 2010). However, this hypothesis has not currently been proved. In this study, there was no correlation between the trace elements studied in Alcalá's children's hair with those analysed in the public parks's soil samples although it may be due to the fact that these children espent their spare time playing in schoolgardens.

After monitoring the hair of 665 Korean children, aged 3-6 years, Park et al. (2007) reported that the levels of **Al** showed no sex dependence. However, significant differences in the content of Al in hair are found between sexes in older groups such as teenagers aged 11-13 years living in Palermo, Sicily, Italy (Dongarrà et al., 2011). This last study reported higher levels of Al in girls' hair. To date, there is no hypothesis that explains these significant differences. This is mainly because it is unknown which factors affect Al excretion through hair, especially at early ages. Al is a potential neurotoxin (González-Muñoz et al., 2008; Peña et al., 2005, 2007) that has been very little considered in human biomonitoring, despite the fact that it is commonly used to treat drinking water.

In relation to **Cu**, levels of this micronutrient have been found to be significantly higher in the hair and blood of the female population than the male population (Ghayour et al., 2005). Khalique et al. (2005) have attributed the significantly higher levels of Cu in the hair of Pakistani girls aged 1-10 years to metabolic and physiological differences related to gender. However, Perrone et al. (1996) have found no significant differences related to gender for Cu in the hair of 86 children aged 6-11 years from the region of Puglia, Italy.

In conclusion, some studies have reported significant differences between the sexes in the levels of some trace elements in hair in school aged populations (Bouchard et al., 2007; Khalique et al., 2005; Kordas et al., 2010), but others have found no significant differences (Ferré-Huguet et al., 2009; Nadal et al., 2005). The significant differences between sexes in the trace element content in hair for children and the general population could be mainly attributed to physiological, socioeconomical and cultural (e.g. nutritional habits) differences between countries and regions. However, food habits do not play an important role in the juvenile age-group, and the differences for that age-group may instead be caused by as yet unknown hormonal influences (Beneš et al., 2003). Therefore, the assessment of reference values for trace elements in hair for the human population in each country, and especially in each community, should be carried out for male, female and general populations separately.

4.3. Correlation analysis of trace elements in children's hair in Alcalá

The general absence of significant correlation between the trace elements monitored in children's hair may indicate that there is no common source of exposure to these pollutants in Alcalá's children. The correlation study of metals and metalloids in human hair can be used as a preliminary tool to identify possible pathologies which require further assessment (Afridi et al., 2011).

4.4. Correlation study of trace elements in soils and children's hair samples

No significant correlation was found between concentrations of the pollutants studied in soils and those monitored in children's hair. If hair is considered to be the main route of excretion for metals and metalloids, then this absence of significant correlation implies that Alcalá's topsoils are not a significant source of these pollutants for the age-group here considered. However, it would not be conclusive, since the apparent absence of correlation may reflect that (Creason et al., 1975): a) the exposure was too low to reveal such correlation, or, b) Alcalá's chidren were exposed to several sources. Further studies are therefore needed. For example, it is necessary to understand the bioaccesibility and bioavailability to humans of trace elements present in topsoils.

Although there are few studies that seek a correlation between the two media (soils and hair), there are some studies which have observed a significant correlation (Gebel et al., 1998; Nowak and Kozłowski, 1998). For example, Gebel et al. (1998) reported a positive and significant correlation between the levels of As found in soils and the levels of this metalloid in the hair of the general population in a northern region of Germany.

4. CONCLUSIONS

This study proposes for the first time potential "reference values" for a series of trace elements in the hair of a sample group of Spanish children aged 6-9 years. These values were based on a well-defined healthy group of non-exposed children living in Alcalá de Henares, a significant urbanised city in the Community of Madrid, Spain. These reference values were computed according to IUPAC criteria and may be used as a tool to detect and recognise children in the Community of Madrid who are at risk of exposure to Al, As, Be, Cd, Cr, Cu, Hg, Mn, Ni, Pb, Sn, Ti, Tl, V and Zn.

Our results also suggest that reference values for trace elements in human hair should be set for groups according to age, that male and female populations should be considered separately, and that the process should be carried out for each country or region individually, since age, gender and place of residence may affect levels of these pollutants in human hair. Although it has been described that the use of human hair as a biomonitor of environmental exposure for some metals, such as aluminium, may be limited, reference values of metals in children's hair may be used to identify whether any child that lives in the studied area has been exposed to these pollutants.

The methodology described in this study and the inclusion criteria developed for our group could be used in other regions to establish possible "reference values" for trace elements in children's hair in different communities, regions and countries to protect future generations.

ACKNOWLEDGEMENTS

In memoriam of Prof. Salvador Granero.

The above work is part of a doctoral thesis (Peña-Fernández, 2011) which has been funded through the program EIADES: "Technology Assessment and Remediation of Contaminated Sites" S0505/AMB-0296 and S2009/AMB-1478. Consejería de Educación, Comunidad de Madrid. Spain.

The authors would like to express their sincere appreciation to Karen Exley, for her writing assistance.

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Table 1. Summary results of concentration and reference values (95th percentile) in µg/g of trace elements in hair of children aged 6-14 years from Germany (Seifert et al., 2000).

Element	LoD	N	P95	Maximum	A.M	G.M
Al	1.0	638	23.1	125.0	9.2	6.57
Cd	0.006	704	0.30	3.66	0.096	0.048
Cr	0.02	641	0.25	0.89	0.106	0.088
Cu	0.4	711	71.0	793.0	24.8	14.9
Pb	0.1	711	5.4	26.9	1.61	1.02
Zn	2.4	711	230.0	1020.0	155.0	141.0

LoD = limit of detection; N= number of samples above LoD; P95 = percentile 95; A.M. = arithmetic mean; G.M. = geometric mean.

Table 2. Summary results of concentration and normal values (medians) in μg/g of trace elements in hair of children aged 9.9 years in the Czech Republic (Beneš et al., 2003).

Population group	Element	N	Range	Median	A.M.	G.M
Children 9.9 years	Cd	3,554	0.01-5.45	0.14	0.23	0.15
	Cr	3,427	0.01-25.0	0.22	0.40	0.25
	Cu	3,553	1.0-337.0	12.0	20.0	14.0
	Hg	3,427	0.01-28.0	0.19	0.27	0.2
	Pb	3,555	0.01-44.8	1.60	2.05	1.54
	Zn	3,556	8.0-642.0	124.0	128.0	117.0
Boys 10.1 years	Cd	1,741	0.01-3.17	0.12	0.2	0.13
	Cr	1,674	0.01-25.0	0.22	0.45	0.29
	Cu	1,740	1.0-337.0	10	16.0	12.0
	Hg	1,667	0.01-10.6	0.19	0.27	0.2
	Pb	1,741	0.01-25.7	1.56	2.03	1.49
	Zn	1,741	8.0-570.0	115	117.0	107.0
Girls 9.7 years	Cd	1,813	0.01-5.45	0.17	0.26	0.18
	Cr	1,753	0.01-9.73	0.22	0.35	0.31
	Cu	1,813	1.0-327.0	13	24.0	15.0
	Hg	1,760	0.01-28.0	0.2	0.28	0.24
	Pb	1,814	0.01-44.8	1.64	2.07	1.57
	Zn	1,815	10-642.0	135	140.0	105.0

N= number of samples above LoD; A.M. = arithmetic mean; G.M. = geometric mean

Table 3. Statistical summary of metals and metalloids in **urban soils** of Alcalá de Henares (μg/g) (Peña-Fernández et al. (2014b).

Element	A.M.	G.M.	Range
Al	5,797.7 ± 2,646.03	5,135.27	762.02-12,672.0
As	4.83 ± 2.10	4.45	1.87-11.68
Be	0.75 ± 0.50	0.62	0.17-2.57
Cd	0.11 ± 0.06	0.10	0.03-0.33
Cr	8.37 ± 3.67	7.41	1.32-16.45
Cu	10.78 ± 6.44	8.99	2.21-38.08
Hg	ND	ND	<0.002
Mn	99.27 ± 40.09	90.15	17.91-188.17
Ni	6.56 ± 0.49	6.54	4.49-7.15
Pb	41.32 ± 47.59	26.24	3.03-290.46
Sn	0.31 ± 0.08	0.30	0.16-0.58
Ti	77.91 ± 45.34	66.27	15.31-234.93
Tl	0.12 ± 0.05	0.11	0.03-0.25
V	9.05 ± 4.04	8.10	1.65-18.29
Zn	34.51 ± 16.50	29.94	5.81-78.67

A.M. = arithmetic mean (results are presented as mean values ± S.D.); G.M. = geometric mean; ND= Not detected.

Table 4. Concentrations of metals and metalloids (μg/g) in hair of **children aged 6 to 9 years** in Alcalá de Henares, Spain.

Element	LoD	N	% ≥ LoD	P5	P50	P95	P97.8	CI-PP95	A.M	G.M.	Range
Al	0.2	115	98.3	1.71	6.15	22.76	25.87	21.36-24.17	9.05 ± 7.68	6.35	0.88-38.63
As	0.02	0	0.0	/	/	/	/	/	ND	ND	<0.02
Be	0.05	0	0.0	/	/	/	/	/	ND	ND	<0.05
Cd	0.005	22	18.8	0.17	0.46	0.93	0.96	0.83-1.03	0.52 ± 0.24	0.46	0.16-0.99
Cr	0.01	114	97.4	0.44	0.64	0.92	0.97	0.90-0.95	0.66 ± 0.15	0.64	0.33-1.00
Cu	0.01	117	100.0	1.51	9.32	63.00	128.68	58.28-67.71	19.24 ± 26.02	11.27	1.14-138.84
Hg	0.01	114	97.4	0.25	0.75	3.35	3.57	3.18-3.52	1.10 ± 0.91	0.82	0.16-4.86
Mn	0.01	104	88.9	0.12	0.23	0.74	0.76	0.70-0.78	0.30 ± 0.20	0.25	0.11-1.00
Ni	0.02	58	49.6	0.11	0.23	1.04	1.49	0.96-1.12	0.42 ± 0.32	0.30	0.11-2.46
Pb	0.01	117	100.0	0.25	1.16	3.47	4.77	3.24-3.71	1.48 ± 1.29	1.03	0.12-7.85
Sn	0.01	110	94.0	0.59	1.28	2.33	2.58	2.23-2.43	1.29 ± 0.52	1.19	0.43-2.87
Ti	0.02	39	33.3	0.31	0.69	1.82	2.79	1.63-2.01	0.88 ± 0.60	0.73	0.13-2.92
Tl	0.01	0	0.0	/	/	/	/	/	ND	ND	<0.01
V	0.02	24	20.5	0.13	0.27	1.21	1.52	1.04-1.37	0.44 ± 0.41	0.32	0.12-1.77
Zn	0.02	116	99.1	11.95	87.16	147.88	154.16	139.32-156.44	85.58 ± 47.06	64.55	8.25-181.54

LoD = limit of detection; N= number of samples above LoD; % ≥ LoD = percentage above limit of detection; P5, P50, P95, P97.8 = percentiles; CI-PP95 = 95% confidence interval for 95PP (population percentile); A.M. = arithmetic mean (results are presented as mean values ± S.D.); G.M. = geometric mean; ND= Not detected.

Table 5. Concentrations of metals and metalloids (μg/g) in hair of **boys aged 6 to 9 years** in Alcalá de Henares, Spain.

Element	LoD	N	% ≥ LoD	P5	P50	P95	P97.8	CI-PP95	A.M	G.M.	Range
Al	0.2	47	100.0	1.68	3.70	18.70	21.40	17.13-20.26	6.00 ± 5.48	4.28	0.88-22.45
As	0.02	0	0.0	/	/	/	/	/	ND	ND	<0.02
Be	0.05	0	0.0	/	/	/	/	/	ND	ND	<0.05
Cd	0.005	7	14.9	0.26	0.62	0.87	0.90	0.70-1.04	0.59 ± 0.23	0.53	0.17-0.93
Cr	0.01	45	95.7	0.44	0.67	0.98	0.98	0.93-1.02	0.67 ± 0.16	0.65	0.33-1.00
Cu	0.01	47	100.0	1.27	7.96	38.31	58.99	34.33-42.29	12.01 ± 13.92	7.91	1.14-68.46
Hg	0.01	47	100.0	0.29	0.74	3.33	3.65	3.05-3.61	1.17 ± 0.99	0.84	0.16-4.15
Mn	0.01	42	89.4	0.14	0.22	0.75	0.93	0.68-0.82	0.33 ± 0.23	0.27	0.13-1.00
Ni	0.02	12	25.5	0.14	0.42	1.58	2.08	1.22-1.95	0.58 ± 0.64	0.40	0.11-2.46
Pb	0.01	47	100.0	0.18	1.24	4.36	6.55	3.90-4.81	1.71 ± 1.60	1.15	0.13-7.85
Sn	0.01	45	95.7	0.58	1.31	2.62	2.79	2.43-2.81	1.36 ± 0.65	1.22	0.43-2.87
Ti	0.02	13	27.7	0.39	0.85	1.69	1.70	1.42-1.95	0.95 ± 0.49	0.82	0.27-1.71
Tl	0.01	0	0.0	/	/	/	/	/	ND	ND	<0.01
V	0.02	13	27.7	0.14	0.27	1.47	1.64	1.20-1.73	0.48 ± 0.49	0.35	0.13-1.77
Zn	0.02	47	100.0	12.14	85.40	141.56	145.73	128.58-154.54	82.84 ± 45.41	62.83	8.82-147.96

LoD = limit of detection; N= number of samples above LoD; % ≥ LoD = percentage above limit of detection; P5, P50, P95, P97.8 = percentiles; CI-PP95 = 95% confidence interval for 95PP (population percentile); A.M. = arithmetic mean (results are presented as mean values ± S.D.); G.M. = geometric mean; ND= Not detected.

Table 6. Concentrations of metals and metalloids (μg/g) in hair of **girls aged 6 to 9 years** in Alcalá de Henares, Spain.

Element	LoD	N	% ≥ LoD	P5	P50	P95	P97.8	CI-PP95	A.M	G.M.	Range
Al	0.2	68	97.1	1.94	8.77	23.46	33.03	21.49-25.44	11.17 ± 8.29	8.34	1.15-38.63
As	0.02	0	0.0	/	/	/	/	/	ND	ND	<0.02
Be	0.05	0	0.0	/	/	/	/	/	ND	ND	<0.05
Cd	0.005	15	21.4	0.24	0.43	0.93	0.96	0.80-1.05	0.48 ± 0.24	0.43	0.16-0.99
Cr	0.01	69	98.6	0.44	0.64	0.88	0.91	0.85-0.92	0.65 ± 0.14	0.63	0.40-0.94
Cu	0.01	70	100.0	1.93	13.33	99.81	137.43	92.59-107.03	24.09 ± 30.81	14.31	1.15-138.84
Hg	0.01	67	95.7	0.25	0.76	3.03	3.40	2.82-3.23	1.05 ± 0.87	0.81	0.18-4.86
Mn	0.01	62	88.6	0.12	0.23	0.64	0.72	0.60-0.69	0.27 ± 0.17	0.23	0.11-0.76
Ni	0.02	46	65.7	0.11	0.22	0.98	1.26	0.89-1.08	0.38 ± 0.34	0.28	0.11-1.56
Pb	0.01	70	100.0	0.28	1.13	3.40	3.80	3.16-3.64	1.31 ± 1.02	0.96	0.12-4.82
Sn	0.01	65	92.9	0.60	1.28	1.91	2.16	1.81-2.01	1.24 ± 0.41	1.18	0.53-2.42
Ti	0.02	26	37.1	0.34	0.65	2.42	2.84	2.17-2.68	0.85 ± 0.66	0.69	0.13-2.92
Tl	0.01	0	0.0	/	/	/	/	/	ND	ND	<0.01
V	0.02	11	15.7	0.13	0.27	0.86	0.88	0.68-1.04	0.38 ± 0.30	0.29	0.12-0.89
Zn	0.02	69	98.6	11.57	87.39	153.10	161.16	141.68-164.52	87.44 ± 48.40	65.75	8.25-181.54

LoD = limit of detection; N= number of samples above LoD; % ≥ LoD = percentage above limit of detection; P5, P50, P95, P97.8 = percentiles; CI-PP95 = 95% confidence interval for 95PP (population percentile); A.M. = arithmetic mean (results are presented as mean values ± S.D.); G.M. = geometric mean; ND= Not detected.

Table 7. Concentrations of metals and metalloids (μg/g) in hair **of boys and girls aged 6 to 9 years** in Alcalá de Henares, Spain.

Element	Boys	Girls	*p*
Al	6.00 ± 5.48	11.17 ± 8.29	<0.001
As	ND	ND	/
Be	ND	ND	/
Cd	0.59 ± 0.23	0.48 ± 0.24	NS
Cr	0.67 ± 0.16	0.65 ± 0.14	NS
Cu	12.01 ± 13.92	24.09 ± 30.81	<0.01
Hg	1.17 ± 0.99	1.05 ± 0.87	NS
Mn	0.33 ± 0.23	0.27 ± 0.17	NS
Ni	0.58 ± 0.64	0.38 ± 0.34	NS
Pb	1.71 ± 1.60	1.31 ± 1.02	NS
Sn	1.36 ± 0.65	1.24 ± 0.41	NS
Ti	0.95 ± 0.49	0.85 ± 0.66	NS
Tl	ND	ND	/
V	0.48 ± 0.49	0.38 ± 0.30	NS
Zn	82.84 ± 45.41	87.44 ± 48.40	NS

Results are presented as mean values ± S.D. (in μg/g); NS= Differences are not statistically significant ($p > 0.05$); ND= Not detected.

Table 8. Correlation matrix of the trace elements monitored in children's hair in Alcalá

	Ln Al	**Cr**	**Ln Cu**	**Ln Hg**	**Ln Mn**	**Ln Ni**	**Ln Pb**	**Sn**	**Zn**
Ln Al									
Cr	0,099[a]								
	112[b]								
	0,299[c]								
Ln Cu	**0,245**	0,047							
	115	114							
	0,008	0,644							
Ln Hg	0,063	0,120	-0,001						
	113	111	114						
	0,510	0,209	0,996						
Ln Mn	**-0,282**	-0,135	-0,090	-0,033					
	103	101	104	102					
	0,004	0,180	0,365	0,740					
Ln Ni	0,119	-0,112	0,025	-0,050	0,007				
	57	57	58	55	55				
	0,377	0,407	0,851	0,715	0,962				
Ln Pb	**0,225**	0,174	-0,023	0,152	-0,044	0,060			
	115	114	117	114	104	58			
	0,016	0,065	0,808	0,106	0,655	0,656			
Sn	**-0,220**	0,070	**-0,203**	0,109	0,005	0,115	0,174		
	108	107	110	107	99	55	110		
	0,022	0,471	0,033	0,263	0,962	0,405	0,069		
Zn	-0,069	-0,052	0,157	0,061	0,093	-0,104	-0,122	-0,126	
	114	114	116	113	103	58	116	109	
	0,463	0,585	0,094	0,525	0,352	0,438	0,193	0,193	

a= Correlation coefficients (r); b= number of samples; c= significance value.