Urinary cobalt and ferritin in four-years-old children

Eva Junqué[a], Joan O. Grimalt[a*], Ana Fernández-Somoano[b,c,d] and Adonina Tardón[b,c,d]

[a]Institute of Environmental Assessment and Water Research (IDAEA-CSIC). Jordi Girona, 18. 08034-Barcelona. Catalonia. Spain

[b]IUOPA Medicine Department. University of Oviedo. Asturias. Spain.

[c]Spanish Consortium for Research on Epidemiology and Public Health (CIBERESP). Spain.

[d]Institute of Health Research of the Principality of Asturias-Foundation for Biosanitary Research of Asturias (ISPA-FINBA). Oviedo. Asturias. Spain.

Corresponding author: Joan O. Grimalt, joan.grimalt@idaea.csic.es

KEY WORDS: Cobalt, four-years-old children, urine analyses, ferritin, anemia

ABSTRACT

Cobalt (Co) is an essential trace element but may cause toxic effects upon occupational or environmental exposure. The present study is aimed to determine the urine concentrations of Co in four years-old children in the INMA-Asturias cohort (Spain) and to assess the factors determining the observed levels. This cohort is located in a heavily industrialized zone with strong potential for metal exposure.

Some diet components such as consumption of sweets were meaningfully associated with higher urine Co concentrations. Traffic pollution also showed a noteworthy positive association with Co levels. Family tobacco consumption did not show substantial association with the urine concentrations of this metal in the INMA-Asturias children.

A significant inverse association between urine Co and venous blood ferritin was found. Iron deficiency anemic children had significantly higher concentrations of Co than those with normal levels, e.g. median values 1.9 µg/g creatinine and 1.0 µg/g creatinine, respectively. This association could be explained by an increased expression of DMT1, a divalent metal transporter that captures higher levels of iron in deficiency states of this metal. This transporter is non-specific and not only captures iron but also other divalent metals such as Co. The presence of this metal in iron deficiency anemic children may represent an additional disturbing health factor that must be considered during treatment.

1. Introduction

Cobalt (Co) is a transition metal of widespread environmental occurrence. It is present in pigments, catalysts for oil and gas production, battery electrodes, orthopedic prostheses and others (NHANES, 2009). Human exposure to Co mainly depends on diet. Fish, green vegetables and fresh cereals are common sources (Unice et al., 2012).

Human metabolism uses this trace metal for cobalamin synthesis (vitamin B12) whose deficiency causes a wide range of hematological, gastrointestinal, psychiatric and neurological disorders (Briani et al., 2013). However, only a small fraction of Co intake is used for this purpose and the remaining ingested amounts are inorganic compounds with no essential function (Kim et al., 2006).

Gastrointestinal absorption of dietary Co can typically range from 10 to 35% (Unice et al., 2012). Intakes of 20% and 45% in males and females, respectively, are considered standard reference values in human biokinetic models (Unice et al., 2014). Cobalt deficiency has never been described in human metabolism (Simonsen et al., 2012). Occupational and accidental exposures to Co have been reported to originate asthma, allergic alveolitis, hypersensitivity pneumonitis, interstitial pneumonia (Nemery et al., 1992; Swennen et al., 1993; Leyssens et al., 2017), alterations of thyroid hormones (Prescott et al., 1992; Pausterbach et al., 2013), polycythemia (Pausterbach et al., 2013) and dermatitis (Leyssens et al., 2017).

An oral reference dose of 0.03 mg/kg-day has been recently proposed as maximum Co intake for non-cancer health effects in general population over lifetime exposure (Finley et al., 2012). However, these toxic effects are related to inorganic Co in free ionic state, not bound to albumin. Subjects with albumin alterations such as anephric, sickle cell or sepsis patients may undergo higher effects upon Co exposure (Pausterbach et al., 2013).

Animal studies have shown that iron depletion is associated with increases of the intestinal absorption of divalent metals such as Co (Flanagan et al., 1980). Hereditary hemochromatosis patients were found to accumulate both iron and Co (Nichols and Bacon, 1989). Correspondences between decreases of iron and increases of Co have been observed in blood of 15-17 years-old boys and blood and serum of 15-17 years-old girls (Barany et al., 2005). Statistically significant differences in concentrations of this metal in subjects with abnormal and normal iron status of this population were also observed (Barany et al., 2005).

Toxicokinetic modeling and Co intake studies have long demonstrated that urinary Co is a good measure for Co concentrations in the human body. $CoCl_2$ intake and absorption is reflected in the urine Co concentrations (Christensen et al. 1993). Furthermore, urinary Co excretion was found

to represent two thirds of daily intake in a group of women who self-measured their dietary inputs (Harp and Scoular, 1952).

Urine is therefore the preferred source of information for Co biomonitoring. Furthermore, it can be collected without invasive methods. This fluid has been widely used in large environmental studies on trace metals such as the German Environmental Survey for Children (GerES) and the National Health and Nutrition Examination (NHANES).

Interdependences between urinary Co concentrations and hemoglobin levels have been reported in pregnant women (Fort et al., 2015). However, the role of this metal in young children is still pending to be clarified which is important because the metabolism and organs of toddlers is still under formation (Angelova et al., 2014).

The present study is aimed to assess the influence of iron status and Co levels in four years-old children with iron deficiency anemia and to compare their Co concentrations with those of healthy individuals. The cohort selected for study corresponds to the sanitary area III of Asturias which is a strongly industrialized area of Spain (Fernandez-Somoano et al., 2011; Fernandez-Somoano and Tardon, 2014). The study is also devoted to investigate which are the most common exposure sources of this metal which, in general population, have been attributed to food and drinking water (Tchounwou et al., 2012) and, in some cases, cigarette smoking and traffic (ATSDR, 2008; Fort et al., 2016). Univariate and multivariate models of Co concentrations including diet, e.g. seafood, meat, vegetables, dairy products, etc, tobacco consumption and traffic exposure have therefore been implemented for elucidating potential body burden metal sources.

2. Materials and methods

2.1. *Population and study design*

Between 2009 and 2012, in the context of the INMA (childhood and environment) research network four years-old children were followed in the Hospital San Agustín (Avilés, Asturias) and 334 urine samples were collected. Parents were asked to complete a questionnaire that encompassed demographic information as well as lifestyle data or activities that may affect children's exposure to metals, e.g diet, family smoking habits, distances of family home to traffic areas, etc. Before the study, written informed consent was obtained from the parents of each child. This research was approved by the Asturias Regional Ethics Committee.

2.2. *Laboratory analytical methods*

Teflon vessels were cleaned after every use by rinsing with 7% HNO_3. Then, they were filled with 7% HNO_3, heated at 90ºC overnight, in an oven and finally rinsed with abundant MilliQ water. All polypropylene material was cleaned by soaking it into 7% HNO_3 for 48 hours, followed by rinsing with abundant MilliQ water. Digestion and dilution of the samples was performed to oxidize and remove organic matter and to keep the concentrations of inorganic solids to a minimum (Castillo et al., 2008; Krachler et al., 2009).

Two mL of each urine sample were introduced in clean Teflon vessels, together with 1 mL 60 % HNO_3 (Merck; Darmstadt, Germany) and 0.5 mL H_20_2 (Merck). They were then kept overnight in an oven at 90 ºC. After cooling, the vessels were opened and the samples were diluted with 16.5 mL of 1% HNO_3. These dissolutions were introduced into plastic tubes and stored in a refrigerator until instrumental analysis. Before analysis, an internal standard indium (10 ppb) was introduced and depending on sample density, they were diluted with MilliQ water to 30mL or 60mL to avoid spectral interferences. ICP-MS analysis was performed by a X-SERIES II device from Thermo Fisher Scientific.

One MilliQ water blank was processed in each batch of samples for possible contamination control. The analytical protocol was validated by processing a Bio-Rad Level 1 urine reference sample (Lyphochek Urine Metals Control 4770-03; Marnes-la-Coquette, France). Instrumental limit of detection (LOD) was 0.2 ng/mL. The Co urine concentrations were standardized to creatinine content.

Creatinine was determined by the Jaffé method (kinetic with target measurement, compensated method) with Beckman Coulter© reactive in AU5400 (IZASA®). Ferritin was measured in serum by immunoturbidimetry.

2.3. *Data analysis*

The Kolmogorov-Smirnov test was used to check for the normality of the distribution of the Co concentrations. Since it did not fulfil the normality requirements, the concentrations were log-transformed.

The associations of parental and children variables with metal concentrations were examined by univariate linear regression analysis. The variables showing a significant association at $p<0.20$ were retained for the multivariate regression model. A value equal to half limit of detection, 0.1 ng/mL, was used for non-detected concentrations. All statistics were performed by Stata 10.0.

3. Results and discussion

3.1.Population characteristics

The main socio-demographic characteristics of the children included in the study are presented in Table 1. More than half of the population were boys (52%). Concerning social class, 46% belonged to medium-low class, 22% to medium class and the remaining 32% to high and high-medium class. In thirty-five percent of the families at least one parent smoked regularly. Fifty-three percent of the families lived close to high traffic areas, either with continuous or episodic high traffic, 38% and 15%, respectively (Table 1). The means and standard deviations (SDs) of weight and height of these four years-old children were 18 (2.9) kg and 1.0 (4.5) m, respectively.

3.2.Cobalt concentrations

Cobalt was found above limit of detection in 84% of the samples analysed. The urine Co concentrations ranged between 0.15 and 7.5 ng/mL, with average concentrations of 0.66 ng/mL (SD: 0.66 ng/mL) and median of 0.48 ng/mL (Table 2). The average and median values normalized to creatinine were 1.3 µg/g creatinine (SD: 0.96 µg/g creatinine) and 1.0 µg/g creatinine, respectively, with a range of 0.10-5.8 µg/g creatinine (Table 2).

These median values were lower than those reported in 8-14 years-old children from Mexico (0.78 ng/mL; Lewis et al., 2018) or 5-11 years-old-children from Italy (0.96 ng/mL; Protano et al., 2016) and similar to those reported in 6-11 years-old children from Valencia (1.4 µg/g creatinine; Roca et al., 2016). In contrast, they were higher than those reported in 6-11 years-old children from the USA (NHANES, 2009).

In any case, the observed concentrations were not very different from those in these other studies. Thus, the present Asturias cohort did not reflect strong pollution inputs but the regular intake of Co due to diet and urban environmental exposure. In these conditions, the observed concentrations likely indicate the common internal exposure of the individuals to this metal as the Co burden excreted through urine is replaced by new Co contributions. The usefulness of urine concentrations as markers of internal exposure in cohorts not exposed to acute metal pollution events has also been shown in other cohort studies (Fort et al., 2014).

3.3.Regression analyses

Univariate modelling of the Co concentrations and diet covariates only showed significant relations with consumption of nuts ($\beta = 0.12$; $p < 0.05$) and sweets ($\beta = 0.16$; $p < 0.01$), involving higher excretion of this metal at higher consumption (Table 3). No significant association was observed with the other examined diet components.

Tobacco consumption in the family context did not involve any association (Table 3).

A statistically significant association between Co exposure and traffic intensity was identified, as home distances from streets with continuous high traffic were inversely correlated with Co urine concentrations ($\beta = -0.13$; $p < 0.05$; Table 3). Shorter distances to streets with continuous high traffic density involved higher levels of this metal. Conversely, study of the family home distances to the main industrial center in Asturias did not show significant associations with urine Co concentrations in children.

A strong inverse association between ferritin concentration in blood and Co urine excretion was also observed ($\beta = -0.41$; $p < 0.001$; Table 3).

Multivariate regression analysis with these variables was also performed, including adjustment for body mass index and sex. The multivariate model confirmed the strong association between Co urine concentration and blood ferritin ($\beta = -0.44$; $p < 0.001$), traffic sources ($\beta = -0.13$; $p < 0.05$) and sweets consumption ($\beta = 0.13$; $p < 0.05$; Table 4). However, the relationship with nuts consumption lost statistical significance and sea food was identified as a new diet source ($\beta = 0.18$; $p < 0.01$; Table 4).

3.4.Associations between ferritin concentration and Co excretion

The arithmetic mean (SD) concentration of ferritin was 29 (1.1) ng/mL. This intracellular protein can be used as a marker of iron deficiency anemia. One standard threshold to identify people with anemia is 12 µg/L (Wang et al., 2010). Grouping of the INMA-Asturias children according to this threshold shows 19 individuals with iron deficiency anemia (8%; Table 2). They show higher average Co urine concentrations, 1.25 ng/mL (SD: 1.2 ng/mL), than the non-anemic group, 0.60 ng/mL (SD: 0.55 ng/mL), and the difference is statistically significant ($p < 0.001$). The same difference is observed when comparing creatinine normalized data, 2.0 µg/g creatinine (SD 1.5 µg/g creatinine) in the anemic group and 1.2 µg/g creatinine (SD 0.80 µg/g creatinine) in the non-anemic group.

Another ferritin threshold used for differentiation between individuals with iron deficiency anemia is 16 µg/L (Hallberg et al., 1993). Grouping the INMA-Asturias cohort according to this threshold shows 49 individuals within the anemia group (22%; Table 2). Again, they show higher

average Co urine concentrations, 1.05 ng/mL (SD: 0.98 ng/mL) than the non-anemic group, 0.54 ng/mL (SD: 0.49 ng/mL) and the difference is statistically significant ($p < 0.001$). The same difference is observed when comparing creatinine normalized data, 1.85 µg/g creatinine (SD 1.2 µg/g creatinine) in the anemic group and 1.1 µg/g creatinine (SD 0.69 µg/g creatinine) in the non-anemic group.

As shown in the regression analyses (Tables 3 and 4), there was a strong inverse association between ferritin levels and Co excretion in the INMA-Asturias cohort. A statistically significant inverse relationship between iron status, using serum ferritin, and blood and serum concentrations of Co was found in a study conducted in adolescents from Sweden. The associations were significant at the age of 15 years old in both sexes and only in girls at 17 years old (Bárány et al., 2005). Another study in pregnant women from Catalonia, reported a statistically negatively association between Co urine concentrations and hemoglobin levels in the last pregnancy period but not during the first trimester (Fort et al., 2015). In a study performed in a population of menstruating women from Norway (Meltzer et al., 2010), inverse associations between blood levels of several divalent metals and ferritin were found. Among the metals included in this study, Mn, Co and Cd showed significantly relation but not Cu and Zn, being Co the one with the strongest association in the linear regression models (Meltzer et al., 2010).

It has been reported that intestinal absorption of Co in mice increased under iron deficiencies due to bleeding or diet (Flanagan et al., 1980). However, Co intestinal absorption can also increase under higher iron demand, such as during adolescence or late stages of pregnancy (Bárány et al., 2005; Fort et al., 2015). However, comparison of the results of the present study with four years-old children and those of the pregnant mother study shows higher Co intake in the former (increases of 63-90%) in comparison with the statistically significant differences of the latter (increases of 29%; Table 5). Furthermore, anemic four years-old children also exhibit higher absolute Co concentrations, 1.5-1.9 µg/g creatinine, than the mothers, 1.2 µg/g creatinine. Anemia may therefore involve higher exposure to Co in young children than in pregnant women which is consistent with the lower capacity of pollutant elimination of children due to the above mentioned organ and metabolism formation.

During the times of iron deficiency or increased iron demand, the divalent metal transporter 1 (DMT1) is found to have increased expression because this transporter is up-regulated by iron status. DMT1 transports inorganic iron in its oxidized form, but it has also the ability to transport other divalent ions (Gunshin et al., 1997) among which Co binds with more affinity than others, e.g. copper or zinc (Garrick et al., 2003). In the present study no significant associations between ferritin and copper on zinc urine levels were found ($\beta = -0.079$, $p = 0.23$ and $\beta = -0.082$, $p = 0.22$,

respectively). The present study also demonstrates a statistically significant negative correlation between iron status and Co levels in urine.

Hence, we propose that the higher iron demand occurring during childhood could promote the expression of DMT1 which could result into increased absorption of Co along with iron. This process has been considered to explain the association between iron status and metal concentrations in humans (Meltzer et al., 2010; Bárány et al., 2005). However further investigation is needed for a more comprehensive description of this association.

4. Conclusions

Cobalt concentrations were negatively associated to ferritin levels. The trend recorded important differences between children having iron deficiency anemia and children with normal iron levels, the former showing statistically significantly higher urine Co concentrations. The results are compliant with a common absorption mechanism for Co and iron that may be DMT1 mediated. Low iron supply may enhance absorption of Co in blood.

Acknowledgements

The authors would particularly like to thank all the participants for their generous collaboration and the staff from Hospital San Agustin in Aviles for their effort. This study was funded by grants from CIBERESP, FIS-FEDER (PI04/2018, PI09/02311, PI13/02429, PI18/00909), Obra Social Cajastur/Fundación Liberbank and Oviedo University. This paper was also supported by funding from the European Union project: EDC-MET (H2020-HEALTH/0490-825762).

References

Angelova, M.G., Petkova-Marinova, T.V., Pogorielov, M.V., Loboda, A.N., Nedkova-Kolarova, V.N., Bozhinova, A.N. 2014. Trace Element Status (Iron, Zinc, Copper, Chromium, Cobalt, and Nickel) in Iron-Deficiency Anaemia of Children under 3 Years. Anemia. 2014:718089.

ATSDR, 2008. Toxicological Profile for Cadmium (Draft). Agency for Toxic Substances and Disease Registry; U.S. Department of Health and Human Services, Atlanta, GA.

Bárány, E., Bergdahl, I.A., Bratteby, L.E., Lundh, T., Samuelson, G., Skerfving, S., et al., 2005. Iron status influences trace element levels in human blood and serum. Environ. Res. 98, 215-223.

Briani, C., Torre, C. D., Citton, V., Manara, R., Pompanin, S., Binotto, G., Adami, F. 2013. Cobalamin Deficiency: Clinical Picture and Radiological Findings. Nutrients. 5, 4521-4539.

Castillo, S., Moreno, T., Querol, X., Alastuey, A., Cuevas, E., Herrmann, L., Mounkaila., M., Gibbons, W. 2008. Trace element variation in size-fractionated African desert dusts. J. Arid Environ. 72,1034–1045.

Christensen, J.M., 1995. Human exposure to toxic metals: Factors influencing interpretation of biomonitoring results. Sci. Total Environ. 166, 89-135.

Fernández-Somoano A, Estarlich M, Ballester F, Fernández-Patier R, Aguirre-Alfaro A, Herce-Garraleta MD, Tardón A. 2011. Outdoor NO2 and benzene exposure in the INMA (Environment and Childhood) Asturias cohort (Spain). Atmos Environ 45(29): 5240-5246

Fernández-Somoano A, Hoek G, Tardon A. 2013. Relationship between area-level socioeconomic characteristics and outdoor NO2 concentrations in rural and urban areas of northern Spain. BMC Public Health 13(1): 71.

Fernandez-Somoano, A., Tardon, A., 2014. Socioeconomic status and exposure to outdoor NO2 and benzene in the Asturias INMA birth cohort, Spain. J. Epidemiol. Community Health 68 (1), 29-36.

Finley, B. L., Monnot, A. D., Paustenbach, D. J., Gaffney, S. H., 2012. Derivation of a chronic oral reference dose for cobalt. Regul. Toxicol. Pharm. 64, 491-503.

Flanagan P.R., Haist J., Valberg L.S., 1980. Comparative effects of iron deficiency induced by bleeding and a low-iron diet on the intestinal absorptive interactions of iron, cobalt, manganese, zinc, lead and cadmium. J. Nutr. 110, 1754-1763.

Fort, M. Cosín-Tomás, M., Grimalt, J.O., Querol, X., Casas, M., Sunyer, J., 2014. Assessment of exposure to trace metals in a cohort of pregnant women from an urban center by urine analysis in the first and third trimesters of pregnancy. Environ. Sci. Pollut. Res. 21, 9234-9241.

Fort, M. Grimalt, J.O., Casas, M., Sunyer, S. 2015. Interdependence between cobalt concentrations and haemoglobin levels in pregnant women. Environ Res. 136, 148–154.

Fort, M, Grimalt, J.O., Querol X., Casas, M., Sunyer, J. 2016. Evaluation of atmospheric inputs as possible sources of antimony in pregnant women from urban areas. Sci Total Environ. 544, 391-399.

Garrick, M.D., Dolan, K.G., Horbinski, C., Ghio, A.J., Higgins, D., Porubcin, M., et al., 2003. DMT1: A mammalian transporter for multiple metals. BioMetals 16, 41-54.

Gunshin, H., Mackenzie, B., Berger, UV., Gunshin, Y., Romero, M.F., Boron, W.F., et al., 1997. Cloning and characterization of a mammalian proton-coupled metal-ion transporter. Nature 388, 482-488.

Guxens, M., Ballester, F., Espada, M., Fernandez, M.F., Grimalt, J.O., Ibarluzea, J., Olea, N., Rebagliato, M., Tardon, A., Torrent, M., Vioque, J., Vrijheid, M., Sunyer J. on behalf of the INMA Project. 2012. Cohort Profile: The INMA—INfancia y Medio Ambiente—(Environment and Childhood) Project. Int. J. Epidemiol. 41, 930–940.

Hallberg, L., Bengtsson, C., Lapidus, L., Lindstedt, G., Lundberg, P.-A., Hultén, L., 1993. Screening for iron deficiency: an analysis based on bone-marrow examinations and serum ferritin determinations in a population sample of women. British J. Haematology 85, 787-798.

Harp, M. J., Scoular, F. I., 1952. Cobalt metabolism of young college women on self-selected diets. The Journal of nutrition. 47, 67-72.

Kim, J.H., Gibb, H.J., Howe, P.D., 2006. Cobalt and inorganic cobalt compounds. In: Kim, J.H., Gibb, H.J., Howe, P.D., editors. IPCS Concise International Chemical Assessment Documents, pp. 1-82.

Krachler, M., Heisel, C., Kretzer, J.P. 2009. Validation of ultratrace analysis of Co, Cr, Mo and Ni in whole blood, serum and urine using ICPSMS. J Anal At Spectrom. 24, 605–610.

Lewis R.C., Meeker J.D., Basu N., Gauthier A.M., Cantoral A., Mercado-García A., Peterson K.E., Téllez-Rojo M.M., Watkins D.J. 2018. Urinary metal concentrations among mothers and children in a Mexico City birth cohort study. Int J Hyg Environ Health. 221, 609-615.

Leyssens L., Vinck B., Van Der Straeten C., Wuyts F., Maes L. 2017. Cobalt toxicity in humans—A review of the potential sources and systemichealth effects. Toxicology. 387: 43-56.

Meltzer, H.M., Brantster, A.L., Borch-Iohnsen, B., Ellingsen, D.G., Alexander, J., Thomassen, Y., et al., 2010. Low iron stores are related to higher blood concentrations of manganese, cobalt and cadmium in non-smoking, Norwegian women in the HUNT 2 study. Environ. Res. 110(5), 497-504.

Nemery, B., Casier, P., Roosels, D., Lahaye, D., Demedts, M., 1992. Survey of cobalt exposure and respiratory health in diamond polishers. Am. Rev. Respir. Dis. 145: 610-616.

NHANES., 2009. Fourth National Report on Human Exposure to Environmental Chemicals. http://www.cdc.gov/exposurereport/.

Nichols, G. M., Bacon, B. R., 1989. Hereditary hemochromatosis: Pathogenesis and clinical features of a common disease. Am J Gastroenterol.. 84, 851-862.

Paustenbach, D. J., Tvermoes, B. E., Unice, K. M., Finley, B. L., Kerger, B. D., 2013. A review of the health hazards posed by cobalt. Crit. Rev. Toxicol. 43, 316-362.

Prescott, E., Netterstrom, B., Faber, J., Hegedus, L., Suadicani, P., Christensen, J.M., 1992. Effect of occupational exposure to cobalt blue dyes on the thyroid volume and function of female plate painters. Scand. J. Work Environ. Health 18, 101-104.

Protano, C., Astolfi, M. L., Canepari, S., Vitali, M. 2016. Urinary levels of trace elements among primary school-aged children from Italy: The contribution of smoking habits of family members. Sci Total Environ. 557–558, 378–385.

Roca, M., Sánchez, A., Pérez, R., Pardo, O., Yusà, V. 2016. Biomonitoring of 20 elements in urine of children. Levels and predictors of exposure. Chemosphere. 144:1698–705.

Simonsen, L. O., Harbak, H., Bennekou, P., 2012. Cobalt metabolism and toxicology-A brief update. Sci. Total Environ. 432, 210-215.

Swennen, B., Buchet, J.P., Stanescu, D., Lison, D., Lauwerys R., 1993. Epidemiological survey of workers exposed to cobalt oxides, cobalt salts, and cobalt metal. Br. J. Ind. Med. 50, 835-842.

Tchounwou, P.B., Yedjou, C.G., Patlolla, A.K., Sutton, D.J., 2012. Heavy metal toxicity and the environment. EXS 101, 133–164.

Unice, K.M., Monnot, A.D., Gaffney, S.H., Tvermoes, B.E., Thuett, K.A., 2012. Paustenbach DJ, et al. Inorganic cobalt supplementation: Prediction of cobalt levels in whole blood and urine using a biokinetic model. Food Chem. Toxicol. 50, 2456-2461.

Unice, K. M., Kerger, B. D., Paustenbach, D. J., Finley, B. L., Tvermoes, B. E., 2014. Refined biokinetic model for humans exposed to cobalt dietary supplements and other sources of systemic cobalt exposure. Chem. Biol. Interact. 216, 53-74.

Wang, W., Knovich, M. A., Coffman, L. G., Torti, F. M., Torti, S. V., 2010. Serum ferritin: Past, present and future. Biochim. Biophys. Acta 1800, 760-769.

TABLES

Table 1 Socio-demographic characteristics of the Asturias cohort.

Variables	N (%)	Total
Sex		
Boys	52	254
Girls	48	231
Social class		
I+II (most affluent)	32	156
III	22	107
IV+V (least affluent)	46	221
Physical exercise		
Sedentary	5	18
Moderately active	77	246
Very active	18	57
Tobacco use in the family		
No smokers	65	299
Mother or father smoking	35	157
Urban traffic intensity		
Continuous high traffic	38	120
Episodic high traffic	15	49
Low	47	148

Table 2. Concentrations of Co in the studied four-years-old children considering the whole population and grouping by several criteria related with iron deficiency anemia.

		Total (%)	Mean (SD)	P50	P90	*p*-value
Total	ng/mL	334	0.66 (0.66)	0.48		
	µg/g creatinine	334	1.3 (0.96)	1.0		
Ferritin < 12 µg/L	ng/mL	19 (8)	1.25 (1.2)	1.1	2.0	
	µg/g creatinine	19 (8)	2.0 (1.5)	1.9	2.9	
Ferritin ≥ 12 µg/L	ng/mL	208 (92)	0.60 (0.55)	0.45	1.3	p<0.001[a]
	µg/g creatinine	208 (92)	1.2 (0.80)	1.0	2.3	p<0.001[a]
Ferritin < 16 µg/L	ng/mL	49 (22)	1.05 (0.98)	0.80	2.0	
	µg/g creatinine	49 (22)	1.85 (1.2)	1.5	3.0	
Ferritin ≥ 16 µg/L	ng/mL	178 (78)	0.54 (0.49)	0.43	1.1	p<0.001[a]
	µg/g creatinine	178 (78)	1.1 (0.69)	0.92	2.05	p<0.001[a]

[a]Statistical significance of the mean differences after grouping by 12 µg/L or 16 µg/L ferritin

Table 3. Results of the univariate models of the Co concentrations in urine of four years-old children with different covariates.

	β[a]	*p*-value
Food items		
Dairy products	0.10	
Eggs	-0.02	
White meat	0.02	
Red meat	-0.02	
Lean fish	0.01	
Fatty fish	-0.03	
Shellfish	0.09	
Vegetables	0.08	
Fruit	-0.08	
Nuts	**0.12**	**0.05**
Legumes	0.04	
Pasta/cereal	0.08	
Potatoes	0.06	
Bread	-0.03	
Sweets	**0.16**	**0.01**
Tobacco consumption		
Smoking of some family member	-0.03	
Traffic		
Distance between home and a street with continuous high traffic intensity	**-0.13**	**0.05**
Others		
Ferritin	**-0.41**	**0.001**

[a]β Coefficients of the univariate regression models

Table 4. Results of the multivariate model of the Co concentrations in urine of four years-old children with the covariates of Table 3 and adjustment for infant body mass index and sex.

Predictor variable	**β**[a]	***p*-value**
Dairy products	0.03	
Seafood	**0.18**	**0.01**
Vegetables	0.07	
Fruit	-0.05	
Nuts	0.10	
Pasta/cereal	0.08	
Sweets	**0.13**	**0.05**
Traffic exposure[b]	**-0.13**	**0.05**
Ferritin	**-0.44**	**0.001**

[a]β Coefficients of the multivariate regression models.
[b]Family home distances to streets with continuous traffic.

Table 5. Comparison of the statistically significant median Co concentration differences between iron deficiency anemic and non-anemic four years-old children (this study) and mothers (Fort et al., 2015) (concentrations in µg/g creatinine).

	N(%)	Median	Ratio	% increase
Four years-old children				
anemic[a]	19 (8)	1.9	1.9	90%
non-anemic[b]	208 (92)	1.0		
anemic[c]	49 (22)	1.5	1.63	63%
non-anemic[d]	178 (78)	0.92		
Third trimester pregnant mothers (Fort et al., 2015)				
anemic[e]	109 (28)	1.2	1.29	29%
non-anemic[f]	282 (72)	0.93		

[a]ferritin < 12 µg/L. [b]ferritin ≥ 12 µg/L. [c]ferritin < 16 µg/L. [d]ferritin ≥ 16 µg/L. [e]hemoglobin < 11 g/dL. [f]hemoglobin > 11 g/dL.

Urinary cobalt and ferritin in four-years-old children

Eva Junqué[a], Joan O. Grimalt[a*], Ana Fernández-Somoano[b,c,d] and Adonina Tardón[b,c,d]

[a]Institute of Environmental Assessment and Water Research (IDAEA-CSIC). Jordi Girona, 18. 08034-Barcelona. Catalonia. Spain

[b]IUOPA Medicine Department. University of Oviedo. Asturias. Spain.

[c]Spanish Consortium for Research on Epidemiology and Public Health (CIBERESP). Spain.

[d]Institute of Health Research of the Principality of Asturias-Foundation for Biosanitary Research of Asturias (ISPA-FINBA). Oviedo. Asturias. Spain.

Corresponding author: Joan O. Grimalt, joan.grimalt@idaea.csic.es

KEY WORDS: Cobalt, four-years-old children, urine analyses, ferritin, anemia

ABSTRACT

Cobalt (Co) is an essential trace element but may cause toxic effects upon occupational or environmental exposure. The present study is aimed to determine the urine concentrations of Co in four years-old children in the INMA-Asturias cohort (Spain) and to assess the factors determining the observed levels. This cohort is located in a heavily industrialized zone with strong potential for metal exposure.

Some diet components such as consumption of sweets were meaningfully associated with higher urine Co concentrations. Traffic pollution also showed a noteworthy positive association with Co levels. Family tobacco consumption did not show substantial association with the urine concentrations of this metal in the INMA-Asturias children.

A significant inverse association between urine Co and venous blood ferritin was found. Iron deficiency anemic children had significantly higher concentrations of Co than those with normal levels, e.g. median values 1.9 µg/g creatinine and 1.0 µg/g creatinine, respectively. This association could be explained by an increased expression of DMT1, a divalent metal transporter that captures higher levels of iron in deficiency states of this metal. This transporter is non-specific and not only captures iron but also other divalent metals such as Co. The presence of this metal in iron deficiency anemic children may represent an additional disturbing health factor that must be considered during treatment.

1. Introduction

Cobalt (Co) is a transition metal of widespread environmental occurrence. It is present in pigments, catalysts for oil and gas production, battery electrodes, orthopedic prostheses and others (NHANES, 2009). Human exposure to Co mainly depends on diet. Fish, green vegetables and fresh cereals are common sources (Unice et al., 2012).

Human metabolism uses this trace metal for cobalamin synthesis (vitamin B12) whose deficiency causes a wide range of hematological, gastrointestinal, psychiatric and neurological disorders (Briani et al., 2013). However, only a small fraction of Co intake is used for this purpose and the remaining ingested amounts are inorganic compounds with no essential function (Kim et al., 2006).

Gastrointestinal absorption of dietary Co can typically range from 10 to 35% (Unice et al., 2012). Intakes of 20% and 45% in males and females, respectively, are considered standard reference values in human biokinetic models (Unice et al., 2014). Cobalt deficiency has never been described in human metabolism (Simonsen et al., 2012). Occupational and accidental exposures to Co have been reported to originate asthma, allergic alveolitis, hypersensitivity pneumonitis, interstitial pneumonia (Nemery et al., 1992; Swennen et al., 1993; Leyssens et al., 2017), alterations of thyroid hormones (Prescott et al., 1992; Pausterbach et al., 2013), polycythemia (Pausterbach et al., 2013) and dermatitis (Leyssens et al., 2017).

An oral reference dose of 0.03 mg/kg-day has been recently proposed as maximum Co intake for non-cancer health effects in general population over lifetime exposure (Finley et al., 2012). However, these toxic effects are related to inorganic Co in free ionic state, not bound to albumin. Subjects with albumin alterations such as anephric, sickle cell or sepsis patients may undergo higher effects upon Co exposure (Pausterbach et al., 2013).

Animal studies have shown that iron depletion is associated with increases of the intestinal absorption of divalent metals such as Co (Flanagan et al., 1980). Hereditary hemochromatosis patients were found to accumulate both iron and Co (Nichols and Bacon, 1989). Correspondences between decreases of iron and increases of Co have been observed in blood of 15-17 years-old boys and blood and serum of 15-17 years-old girls (Barany et al., 2005). Statistically significant differences in concentrations of this metal in subjects with abnormal and normal iron status of this population were also observed (Barany et al., 2005).

Toxicokinetic modeling and Co intake studies have long demonstrated that urinary Co is a good measure for Co concentrations in the human body. $CoCl_2$ intake and absorption is reflected in the urine Co concentrations (Christensen et al. 1993). Furthermore, urinary Co excretion was found

to represent two thirds of daily intake in a group of women who self-measured their dietary inputs (Harp and Scoular, 1952).

Urine is therefore the preferred source of information for Co biomonitoring. Furthermore, it can be collected without invasive methods. This fluid has been widely used in large environmental studies on trace metals such as the German Environmental Survey for Children (GerES) and the National Health and Nutrition Examination (NHANES).

Interdependences between urinary Co concentrations and hemoglobin levels have been reported in pregnant women (Fort et al., 2015). However, the role of this metal in young children is still pending to be clarified which is important because the metabolism and organs of toddlers is still under formation (Angelova et al., 2014).

The present study is aimed to assess the influence of iron status and Co levels in four years-old children with iron deficiency anemia and to compare their Co concentrations with those of healthy individuals. The cohort selected for study corresponds to the sanitary area III of Asturias which is a strongly industrialized area of Spain (Fernandez-Somoano et al., 2011; Fernandez-Somoano and Tardon, 2014). The study is also devoted to investigate which are the most common exposure sources of this metal which, in general population, have been attributed to food and drinking water (Tchounwou et al., 2012) and, in some cases, cigarette smoking and traffic (ATSDR, 2008; Fort et al., 2016). Univariate and multivariate models of Co concentrations including diet, e.g. seafood, meat, vegetables, dairy products, etc, tobacco consumption and traffic exposure have therefore been implemented for elucidating potential body burden metal sources.

2. Materials and methods

2.1. *Population and study design*

Between 2009 and 2012, in the context of the INMA (childhood and environment) research network four years-old children were followed in the Hospital San Agustín (Avilés, Asturias) and 334 urine samples were collected. Parents were asked to complete a questionnaire that encompassed demographic information as well as lifestyle data or activities that may affect children's exposure to metals, e.g diet, family smoking habits, distances of family home to traffic areas, etc. Before the study, written informed consent was obtained from the parents of each child. This research was approved by the Asturias Regional Ethics Committee.

2.2. *Laboratory analytical methods*

Teflon vessels were cleaned after every use by rinsing with 7% HNO_3. Then, they were filled with 7% HNO_3, heated at 90ºC overnight, in an oven and finally rinsed with abundant MilliQ water. All polypropylene material was cleaned by soaking it into 7% HNO_3 for 48 hours, followed by rinsing with abundant MilliQ water. Digestion and dilution of the samples was performed to oxidize and remove organic matter and to keep the concentrations of inorganic solids to a minimum (Castillo et al., 2008; Krachler et al., 2009).

Two mL of each urine sample were introduced in clean Teflon vessels, together with 1 mL 60 % HNO_3 (Merck; Darmstadt, Germany) and 0.5 mL H_20_2 (Merck). They were then kept overnight in an oven at 90 ºC. After cooling, the vessels were opened and the samples were diluted with 16.5 mL of 1% HNO_3. These dissolutions were introduced into plastic tubes and stored in a refrigerator until instrumental analysis. Before analysis, an internal standard indium (10 ppb) was introduced and depending on sample density, they were diluted with MilliQ water to 30mL or 60mL to avoid spectral interferences. ICP-MS analysis was performed by a X-SERIES II device from Thermo Fisher Scientific.

One MilliQ water blank was processed in each batch of samples for possible contamination control. The analytical protocol was validated by processing a Bio-Rad Level 1 urine reference sample (Lyphochek Urine Metals Control 4770-03; Marnes-la-Coquette, France). Instrumental limit of detection (LOD) was 0.2 ng/mL. The Co urine concentrations were standardized to creatinine content.

Creatinine was determined by the Jaffé method (kinetic with target measurement, compensated method) with Beckman Coulter© reactive in AU5400 (IZASA®). Ferritin was measured in serum by immunoturbidimetry.

2.3. *Data analysis*

The Kolmogorov-Smirnov test was used to check for the normality of the distribution of the Co concentrations. Since it did not fulfil the normality requirements, the concentrations were log-transformed.

The associations of parental and children variables with metal concentrations were examined by univariate linear regression analysis. The variables showing a significant association at $p<0.20$ were retained for the multivariate regression model. A value equal to half limit of detection, 0.1 ng/mL, was used for non-detected concentrations. All statistics were performed by Stata 10.0.

3. Results and discussion

3.1.Population characteristics

The main socio-demographic characteristics of the children included in the study are presented in Table 1. More than half of the population were boys (52%). Concerning social class, 46% belonged to medium-low class, 22% to medium class and the remaining 32% to high and high-medium class. In thirty-five percent of the families at least one parent smoked regularly. Fifty-three percent of the families lived close to high traffic areas, either with continuous or episodic high traffic, 38% and 15%, respectively (Table 1). The means and standard deviations (SDs) of weight and height of these four years-old children were 18 (2.9) kg and 1.0 (4.5) m, respectively.

3.2.Cobalt concentrations

Cobalt was found above limit of detection in 84% of the samples analysed. The urine Co concentrations ranged between 0.15 and 7.5 ng/mL, with average concentrations of 0.66 ng/mL (SD: 0.66 ng/mL) and median of 0.48 ng/mL (Table 2). The average and median values normalized to creatinine were 1.3 µg/g creatinine (SD: 0.96 µg/g creatinine) and 1.0 µg/g creatinine, respectively, with a range of 0.10-5.8 µg/g creatinine (Table 2).

These median values were lower than those reported in 8-14 years-old children from Mexico (0.78 ng/mL; Lewis et al., 2018) or 5-11 years-old-children from Italy (0.96 ng/mL; Protano et al., 2016) and similar to those reported in 6-11 years-old children from Valencia (1.4 µg/g creatinine; Roca et al., 2016). In contrast, they were higher than those reported in 6-11 years-old children from the USA (NHANES, 2009).

In any case, the observed concentrations were not very different from those in these other studies. Thus, the present Asturias cohort did not reflect strong pollution inputs but the regular intake of Co due to diet and urban environmental exposure. In these conditions, the observed concentrations likely indicate the common internal exposure of the individuals to this metal as the Co burden excreted through urine is replaced by new Co contributions. The usefulness of urine concentrations as markers of internal exposure in cohorts not exposed to acute metal pollution events has also been shown in other cohort studies (Fort et al., 2014).

3.3.Regression analyses

Univariate modelling of the Co concentrations and diet covariates only showed significant relations with consumption of nuts ($\beta = 0.12$; $p < 0.05$) and sweets ($\beta = 0.16$; $p < 0.01$), involving higher excretion of this metal at higher consumption (Table 3). No significant association was observed with the other examined diet components.

Tobacco consumption in the family context did not involve any association (Table 3).

A statistically significant association between Co exposure and traffic intensity was identified, as home distances from streets with continuous high traffic were inversely correlated with Co urine concentrations ($\beta = -0.13$; $p < 0.05$; Table 3). Shorter distances to streets with continuous high traffic density involved higher levels of this metal. Conversely, study of the family home distances to the main industrial center in Asturias did not show significant associations with urine Co concentrations in children.

A strong inverse association between ferritin concentration in blood and Co urine excretion was also observed ($\beta = -0.41$; $p < 0.001$; Table 3).

Multivariate regression analysis with these variables was also performed, including adjustment for body mass index and sex. The multivariate model confirmed the strong association between Co urine concentration and blood ferritin ($\beta = -0.44$; $p < 0.001$), traffic sources ($\beta = -0.13$; $p < 0.05$) and sweets consumption ($\beta = 0.13$; $p < 0.05$; Table 4). However, the relationship with nuts consumption lost statistical significance and sea food was identified as a new diet source ($\beta = 0.18$; $p < 0.01$; Table 4).

3.4.Associations between ferritin concentration and Co excretion

The arithmetic mean (SD) concentration of ferritin was 29 (1.1) ng/mL. This intracellular protein can be used as a marker of iron deficiency anemia. One standard threshold to identify people with anemia is 12 µg/L (Wang et al., 2010). Grouping of the INMA-Asturias children according to this threshold shows 19 individuals with iron deficiency anemia (8%; Table 2). They show higher average Co urine concentrations, 1.25 ng/mL (SD: 1.2 ng/mL), than the non-anemic group, 0.60 ng/mL (SD: 0.55 ng/mL), and the difference is statistically significant ($p < 0.001$). The same difference is observed when comparing creatinine normalized data, 2.0 µg/g creatinine (SD 1.5 µg/g creatinine) in the anemic group and 1.2 µg/g creatinine (SD 0.80 µg/g creatinine) in the non-anemic group.

Another ferritin threshold used for differentiation between individuals with iron deficiency anemia is 16 µg/L (Hallberg et al., 1993). Grouping the INMA-Asturias cohort according to this threshold shows 49 individuals within the anemia group (22%; Table 2). Again, they show higher

average Co urine concentrations, 1.05 ng/mL (SD: 0.98 ng/mL) than the non-anemic group, 0.54 ng/mL (SD: 0.49 ng/mL) and the difference is statistically significant ($p < 0.001$). The same difference is observed when comparing creatinine normalized data, 1.85 µg/g creatinine (SD 1.2 µg/g creatinine) in the anemic group and 1.1 µg/g creatinine (SD 0.69 µg/g creatinine) in the non-anemic group.

As shown in the regression analyses (Tables 3 and 4), there was a strong inverse association between ferritin levels and Co excretion in the INMA-Asturias cohort. A statistically significant inverse relationship between iron status, using serum ferritin, and blood and serum concentrations of Co was found in a study conducted in adolescents from Sweden. The associations were significant at the age of 15 years old in both sexes and only in girls at 17 years old (Bárány et al., 2005). Another study in pregnant women from Catalonia, reported a statistically negatively association between Co urine concentrations and hemoglobin levels in the last pregnancy period but not during the first trimester (Fort et al., 2015). In a study performed in a population of menstruating women from Norway (Meltzer et al., 2010), inverse associations between blood levels of several divalent metals and ferritin were found. Among the metals included in this study, Mn, Co and Cd showed significantly relation but not Cu and Zn, being Co the one with the strongest association in the linear regression models (Meltzer et al., 2010).

It has been reported that intestinal absorption of Co in mice increased under iron deficiencies due to bleeding or diet (Flanagan et al., 1980). However, Co intestinal absorption can also increase under higher iron demand, such as during adolescence or late stages of pregnancy (Bárány et al., 2005; Fort et al., 2015). However, comparison of the results of the present study with four years-old children and those of the pregnant mother study shows higher Co intake in the former (increases of 63-90%) in comparison with the statistically significant differences of the latter (increases of 29%; Table 5). Furthermore, anemic four years-old children also exhibit higher absolute Co concentrations, 1.5-1.9 µg/g creatinine, than the mothers, 1.2 µg/g creatinine. Anemia may therefore involve higher exposure to Co in young children than in pregnant women which is consistent with the lower capacity of pollutant elimination of children due to the above mentioned organ and metabolism formation.

During the times of iron deficiency or increased iron demand, the divalent metal transporter 1 (DMT1) is found to have increased expression because this transporter is up-regulated by iron status. DMT1 transports inorganic iron in its oxidized form, but it has also the ability to transport other divalent ions (Gunshin et al., 1997) among which Co binds with more affinity than others, e.g. copper or zinc (Garrick et al., 2003). In the present study no significant associations between ferritin and copper on zinc urine levels were found ($\beta = -0.079$, $p = 0.23$ and $\beta = -0.082$, $p = 0.22$,

respectively). The present study also demonstrates a statistically significant negative correlation between iron status and Co levels in urine.

Hence, we propose that the higher iron demand occurring during childhood could promote the expression of DMT1 which could result into increased absorption of Co along with iron. This process has been considered to explain the association between iron status and metal concentrations in humans (Meltzer et al., 2010; Bárány et al., 2005). However further investigation is needed for a more comprehensive description of this association.

4. Conclusions

Cobalt concentrations were negatively associated to ferritin levels. The trend recorded important differences between children having iron deficiency anemia and children with normal iron levels, the former showing statistically significantly higher urine Co concentrations. The results are compliant with a common absorption mechanism for Co and iron that may be DMT1 mediated. Low iron supply may enhance absorption of Co in blood.

Acknowledgements

The authors would particularly like to thank all the participants for their generous collaboration and the staff from Hospital San Agustin in Aviles for their effort. This study was funded by grants from CIBERESP, FIS-FEDER (PI04/2018, PI09/02311, PI13/02429, PI18/00909), Obra Social Cajastur/Fundación Liberbank and Oviedo University. This paper was also supported by funding from the European Union project: EDC-MET (H2020-HEALTH/0490-825762).

References

Angelova, M.G., Petkova-Marinova, T.V., Pogorielov, M.V., Loboda, A.N., Nedkova-Kolarova, V.N., Bozhinova, A.N. 2014. Trace Element Status (Iron, Zinc, Copper, Chromium, Cobalt, and Nickel) in Iron-Deficiency Anaemia of Children under 3 Years. Anemia. 2014:718089.

ATSDR, 2008. Toxicological Profile for Cadmium (Draft). Agency for Toxic Substances and Disease Registry; U.S. Department of Health and Human Services, Atlanta, GA.

Bárány, E., Bergdahl, I.A., Bratteby, L.E., Lundh, T., Samuelson, G., Skerfving, S., et al., 2005. Iron status influences trace element levels in human blood and serum. Environ. Res. 98, 215-223.

Briani, C., Torre, C. D., Citton, V., Manara, R., Pompanin, S., Binotto, G., Adami, F. 2013. Cobalamin Deficiency: Clinical Picture and Radiological Findings. Nutrients. 5, 4521-4539.

Castillo, S., Moreno, T., Querol, X., Alastuey, A., Cuevas, E., Herrmann, L., Mounkaila., M., Gibbons, W. 2008. Trace element variation in size-fractionated African desert dusts. J. Arid Environ. 72,1034–1045.

Christensen, J.M., 1995. Human exposure to toxic metals: Factors influencing interpretation of biomonitoring results. Sci. Total Environ. 166, 89-135.

Fernández-Somoano A, Estarlich M, Ballester F, Fernández-Patier R, Aguirre-Alfaro A, Herce-Garraleta MD, Tardón A. 2011. Outdoor NO2 and benzene exposure in the INMA (Environment and Childhood) Asturias cohort (Spain). Atmos Environ 45(29): 5240-5246

Fernández-Somoano A, Hoek G, Tardon A. 2013. Relationship between area-level socioeconomic characteristics and outdoor NO2 concentrations in rural and urban areas of northern Spain. BMC Public Health 13(1): 71.

Fernandez-Somoano, A., Tardon, A., 2014. Socioeconomic status and exposure to outdoor NO2 and benzene in the Asturias INMA birth cohort, Spain. J. Epidemiol. Community Health 68 (1), 29‑36.

Finley, B. L., Monnot, A. D., Paustenbach, D. J., Gaffney, S. H., 2012. Derivation of a chronic oral reference dose for cobalt. Regul. Toxicol. Pharm. 64, 491-503.

Flanagan P.R., Haist J., Valberg L.S., 1980. Comparative effects of iron deficiency induced by bleeding and a low-iron diet on the intestinal absorptive interactions of iron, cobalt, manganese, zinc, lead and cadmium. J. Nutr. 110, 1754-1763.

Fort, M. Cosín-Tomás, M., Grimalt, J.O., Querol, X., Casas, M., Sunyer, J., 2014. Assessment of exposure to trace metals in a cohort of pregnant women from an urban center by urine analysis in the first and third trimesters of pregnancy. Environ. Sci. Pollut. Res. 21, 9234-9241.

Fort, M. Grimalt, J.O., Casas, M., Sunyer, S. 2015. Interdependence between cobalt concentrations and haemoglobin levels in pregnant women. Environ Res. 136, 148–154.

Fort, M, Grimalt, J.O., Querol X., Casas, M., Sunyer, J. 2016. Evaluation of atmospheric inputs as possible sources of antimony in pregnant women from urban areas. Sci Total Environ. 544, 391-399.

Garrick, M.D., Dolan, K.G., Horbinski, C., Ghio, A.J., Higgins, D., Porubcin, M., et al., 2003. DMT1: A mammalian transporter for multiple metals. BioMetals 16, 41-54.

Gunshin, H., Mackenzie, B., Berger, UV., Gunshin, Y., Romero, M.F., Boron, W.F., et al., 1997. Cloning and characterization of a mammalian proton-coupled metal-ion transporter. Nature 388, 482-488.

Guxens, M., Ballester, F., Espada, M., Fernandez, M.F., Grimalt, J.O., Ibarluzea, J., Olea, N., Rebagliato, M., Tardon, A., Torrent, M., Vioque, J., Vrijheid, M., Sunyer J. on behalf of the INMA Project. 2012. Cohort Profile: The INMA—INfancia y Medio Ambiente—(Environment and Childhood) Project. Int. J. Epidemiol. 41, 930–940.

Hallberg, L., Bengtsson, C., Lapidus, L., Lindstedt, G., Lundberg, P.-A., Hultén, L., 1993. Screening for iron deficiency: an analysis based on bone-marrow examinations and serum ferritin determinations in a population sample of women. British J. Haematology 85, 787-798.

Harp, M. J., Scoular, F. I., 1952. Cobalt metabolism of young college women on self-selected diets. The Journal of nutrition. 47, 67-72.

Kim, J.H., Gibb, H.J., Howe, P.D., 2006. Cobalt and inorganic cobalt compounds. In: Kim, J.H., Gibb, H.J., Howe, P.D., editors. IPCS Concise International Chemical Assessment Documents, pp. 1-82.

Krachler, M., Heisel, C., Kretzer, J.P. 2009. Validation of ultratrace analysis of Co, Cr, Mo and Ni in whole blood, serum and urine using ICPSMS. J Anal At Spectrom. 24, 605–610.

Lewis R.C., Meeker J.D., Basu N., Gauthier A.M., Cantoral A., Mercado-García A., Peterson K.E., Téllez-Rojo M.M., Watkins D.J. 2018. Urinary metal concentrations among mothers and children in a Mexico City birth cohort study. Int J Hyg Environ Health. 221, 609-615.

Leyssens L., Vinck B., Van Der Straeten C., Wuyts F., Maes L. 2017. Cobalt toxicity in humans—A review of the potential sources and systemichealth effects. Toxicology. 387: 43-56.

Meltzer, H.M., Brantster, A.L., Borch-Iohnsen, B., Ellingsen, D.G., Alexander, J., Thomassen, Y., et al., 2010. Low iron stores are related to higher blood concentrations of manganese, cobalt and cadmium in non-smoking, Norwegian women in the HUNT 2 study. Environ. Res. 110(5), 497-504.

Nemery, B., Casier, P., Roosels, D., Lahaye, D., Demedts, M., 1992. Survey of cobalt exposure and respiratory health in diamond polishers. Am. Rev. Respir. Dis. 145: 610-616.

NHANES., 2009. Fourth National Report on Human Exposure to Environmental Chemicals. http://www.cdc.gov/exposurereport/.

Nichols, G. M., Bacon, B. R., 1989. Hereditary hemochromatosis: Pathogenesis and clinical features of a common disease. Am J Gastroenterol.. 84, 851-862.

Paustenbach, D. J., Tvermoes, B. E., Unice, K. M., Finley, B. L., Kerger, B. D., 2013. A review of the health hazards posed by cobalt. Crit. Rev. Toxicol. 43, 316-362.

Prescott, E., Netterstrom, B., Faber, J., Hegedus, L., Suadicani, P., Christensen, J.M., 1992. Effect of occupational exposure to cobalt blue dyes on the thyroid volume and function of female plate painters. Scand. J. Work Environ. Health 18, 101-104.

Protano, C., Astolfi, M. L., Canepari, S., Vitali, M. 2016. Urinary levels of trace elements among primary school-aged children from Italy: The contribution of smoking habits of family members. Sci Total Environ. 557–558, 378–385.

Roca, M., Sánchez, A., Pérez, R., Pardo, O., Yusà, V. 2016. Biomonitoring of 20 elements in urine of children. Levels and predictors of exposure. Chemosphere. 144:1698–705.

Simonsen, L. O., Harbak, H., Bennekou, P., 2012. Cobalt metabolism and toxicology-A brief update. Sci. Total Environ. 432, 210-215.

Swennen, B., Buchet, J.P., Stanescu, D., Lison, D., Lauwerys R., 1993. Epidemiological survey of workers exposed to cobalt oxides, cobalt salts, and cobalt metal. Br. J. Ind. Med. 50, 835-842.

Tchounwou, P.B., Yedjou, C.G., Patlolla, A.K., Sutton, D.J., 2012. Heavy metal toxicity and the environment. EXS 101, 133–164.

Unice, K.M., Monnot, A.D., Gaffney, S.H., Tvermoes, B.E., Thuett, K.A., 2012. Paustenbach DJ, et al. Inorganic cobalt supplementation: Prediction of cobalt levels in whole blood and urine using a biokinetic model. Food Chem. Toxicol. 50, 2456-2461.

Unice, K. M., Kerger, B. D., Paustenbach, D. J., Finley, B. L., Tvermoes, B. E., 2014. Refined biokinetic model for humans exposed to cobalt dietary supplements and other sources of systemic cobalt exposure. Chem. Biol. Interact. 216, 53-74.

Wang, W., Knovich, M. A., Coffman, L. G., Torti, F. M., Torti, S. V., 2010. Serum ferritin: Past, present and future. Biochim. Biophys. Acta 1800, 760-769.

TABLES

Table 1 Socio-demographic characteristics of the Asturias cohort.

Variables	N (%)	Total
Sex		
Boys	52	254
Girls	48	231
Social class		
I+II (most affluent)	32	156
III	22	107
IV+V (least affluent)	46	221
Physical exercise		
Sedentary	5	18
Moderately active	77	246
Very active	18	57
Tobacco use in the family		
No smokers	65	299
Mother or father smoking	35	157
Urban traffic intensity		
Continuous high traffic	38	120
Episodic high traffic	15	49
Low	47	148

Table 2. Concentrations of Co in the studied four-years-old children considering the whole population and grouping by several criteria related with iron deficiency anemia.

		Total (%)	Mean (SD)	P50	P90	*p*-value
Total	ng/mL	334	0.66 (0.66)	0.48		
	µg/g creatinine	334	1.3 (0.96)	1.0		
Ferritin < 12 µg/L	ng/mL	19 (8)	1.25 (1.2)	1.1	2.0	
	µg/g creatinine	19 (8)	2.0 (1.5)	1.9	2.9	
Ferritin ≥ 12 µg/L	ng/mL	208 (92)	0.60 (0.55)	0.45	1.3	p<0.001[a]
	µg/g creatinine	208 (92)	1.2 (0.80)	1.0	2.3	p<0.001[a]
Ferritin < 16 µg/L	ng/mL	49 (22)	1.05 (0.98)	0.80	2.0	
	µg/g creatinine	49 (22)	1.85 (1.2)	1.5	3.0	
Ferritin ≥ 16 µg/L	ng/mL	178 (78)	0.54 (0.49)	0.43	1.1	p<0.001[a]
	µg/g creatinine	178 (78)	1.1 (0.69)	0.92	2.05	p<0.001[a]

[a]Statistical significance of the mean differences after grouping by 12 µg/L or 16 µg/L ferritin

Table 3. Results of the univariate models of the Co concentrations in urine of four years-old children with different covariates.

	β[a]	*p*-value
Food items		
Dairy products	0.10	
Eggs	-0.02	
White meat	0.02	
Red meat	-0.02	
Lean fish	0.01	
Fatty fish	-0.03	
Shellfish	0.09	
Vegetables	0.08	
Fruit	-0.08	
Nuts	**0.12**	**0.05**
Legumes	0.04	
Pasta/cereal	0.08	
Potatoes	0.06	
Bread	-0.03	
Sweets	**0.16**	**0.01**
Tobacco consumption		
Smoking of some family member	-0.03	
Traffic		
Distance between home and a street with continuous high traffic intensity	**-0.13**	**0.05**
Others		
Ferritin	**-0.41**	**0.001**

[a]β Coefficients of the univariate regression models

Table 4. Results of the multivariate model of the Co concentrations in urine of four years-old children with the covariates of Table 3 and adjustment for infant body mass index and sex.

Predictor variable	**β[a]**	***p*-value**
Dairy products	0.03	
Seafood	**0.18**	**0.01**
Vegetables	0.07	
Fruit	-0.05	
Nuts	0.10	
Pasta/cereal	0.08	
Sweets	**0.13**	**0.05**
Traffic exposure[b]	**-0.13**	**0.05**
Ferritin	**-0.44**	**0.001**

[a]β Coefficients of the multivariate regression models.
[b]Family home distances to streets with continuous traffic.

Table 5. Comparison of the statistically significant median Co concentration differences between iron deficiency anemic and non-anemic four years-old children (this study) and mothers (Fort et al., 2015) (concentrations in µg/g creatinine).

	N(%)	Median	Ratio	% increase
Four years-old children				
anemic[a]	19 (8)	1.9	1.9	90%
non-anemic[b]	208 (92)	1.0		
anemic[c]	49 (22)	1.5	1.63	63%
non-anemic[d]	178 (78)	0.92		
Third trimester pregnant mothers (Fort et al., 2015)				
anemic[e]	109 (28)	1.2	1.29	29%
non-anemic[f]	282 (72)	0.93		

[a]ferritin < 12 µg/L. [b]ferritin ≥ 12 µg/L. [c]ferritin < 16 µg/L. [d]ferritin ≥ 16 µg/L. [e]hemoglobin < 11 g/dL. [f]hemoglobin > 11 g/dL.