Traces of heavy metals in children toenails as a bio-indicator of environmental exposure in Forlì (Northern Italy): an observational study

Tracce di metalli pesanti nelle unghie dei bambini come bioindicatore di esposizione ambientale in Forlì: uno studio osservazionale

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ABSTRACT

OBJECTIVES: to assess the concentration of heavy metals in the nails of children aged 6-9 years residing in Forlì (Emilia-Romagna Region, Northern Italy).
DESIGN: biomonitoring survey.
SETTING AND PARTICIPANTS: in March 2017, a total of 236 toenail samples were collected, 221 of them were eligible; the concentration of 23 metals were measured in these eligible samples.
MAIN OUTCOME MEASURES: a spatial analysis was conducted, considering home addresses as grouped in the four macroareas in which the local territory is administratively divided.
RESULTS: In the two North-Center and East areas – which include various industrial operations, two waste incinerators and a motorway – the total concentration of all metals resulted 60% higher than in the West and South areas. Given the lack of Italian reference values, comparison tests between areas were performed for aluminum (Al), cadmium (Cd), iron (Fe), manganese (Mn), copper (Cu), and zinc (Zn), which concentrations were detectable in over 50% of the subjects.
Higher concentrations were observed in the East area compared with the other areas, with statistical significance for Al (vs North-Center), Cu and Zn (vs West), and Al and Mn (vs South). Further comparisons showed significantly higher concentration of Cu in Nord-Center vs West, which in turn had higher concentrations of Zn compared to the Southern area. By applying a Tobit regression to evaluate possible confounding factors, a marginally significant correlation resulted for the nail concentration of Mn among children practicing outdoor sports and eating locally grown vegetables. The consumption of local vegetables was at the limits of significance also for Cd.
CONCLUSIONS: the data obtained came from a voluntary and crowdfunded study and suggest a possible relationship between the exposure to air pollutants and subsequent accumulation of metals in the nails. Further and more detailed epidemiological studies are warranted to identify the exposure sources and to yield preventive intervention.

Keywords: biomonitoring, toenails, metal exposure, primary prevention

RIASSUNTO

OBIETTIVI: osservare la concentrazione di metalli pesanti nelle unghie di bambini di 6-9 anni di età residenti nel territorio del Comune di Forlì.
DISEGNO: indagine di biomonitoraggio.
SETTING E PARTECIPANTI: nel mese di marzo 2017 sono stati raccolti 236 campioni, 221 dei quali sono stati considerati idonei. In questi, sono stati misurati i valori della concentrazione di 23 metalli.
PRINCIPALI MISURE DI OUTCOME: è stata condotta un'analisi spaziale, nella quale i soggetti sono stati ripartiti e valutati aggregando secondo il domicilio dei donatori nelle 4 macroaree in cui è suddiviso amministrativamente il territorio della città.
RISULTATI: nelle 2 aree Est e Centro-Nord, comprendenti zona industriale, due inceneritori di rifiuti e autostrada, è stata trovata una concentrazione media di tutti i metalli del 60% superiore a quella delle aree Ovest e Sud. Test di confronto fra aree, in assenza di valori italiani di riferimento, sono stati eseguiti solo per alluminio (Al), cadmio (Cd), Ferro (Fe), manganese (Mn), rame (Cu) e zinco (Zn), risultati presenti con concentrazioni rilevabili in oltre il 50% dei soggetti. A Est sono stati osservati valori di concentrazione più elevati nel confronto con le altre aree, con differenze significative per Al (vs Nord+Centro), per Cu e Zn (vs Ovest) e per Al e Mn (vs Sud). Negli ulteriori confronti, il Nord+Centro mostra una concentrazione significativamente più elevata di Cu nei confronti dell'Ovest, che a sua volta ha concentrazione di Zn più elevata del Sud. Applicando una regressione di Tobit per valutare eventuali fattori di confondimento, si è os-

WHAT IS ALREADY KNOWN

- **The concentration of heavy metals measured in nails can correlate with the presence of these metals in the environment and for a prolonged exposure.**
- **The presence of some of these metals, considered as toxic at high concentrations (like Al, Fe, Mn) or carcinogenic (like Cd), could provide indications for the risk of developing chronic diseases.**

WHAT THIS PAPER ADDS

- **No reference values are available for the concentration of the metals in toenails of healthy children in Italy.**
- **The presence of 23 heavy metals in toenails of 236 children aged 6-9 years residing in the municipality of Forlì.**
- **The data were aggregated in four areas and showed 60% higher concentration in the East and North areas, which include industrial operations, two waste incinerators, and a motorway section, compared to the West and South areas.**

servata una globale correlazione al limite della significatività per la concentrazione di Mn nei ragazzi che fanno sport all'aria aperta e che consumano frutta e verdura coltivate in loco. Il consumo di frutta e verdura locali è ai limiti di significatività anche per il Cd.

CONCLUSIONI: i dati ottenuti da questo studio osservazionale, spontaneo e autofinanziato, suggeriscono una possibile relazione tra l'esposizione a inquinanti ambientali e il conseguente accumulo di metalli nella unghie. Ulteriori e più approfonditi studi epidemiologici dovranno essere promossi per individuare le possibili fonti di inquinamento e per stimolare e programmare interventi preventivi.

Parole chiave: biomonitoraggio, matrice biologica unghie, esposizione a metalli pesanti, prevenzione primaria

INTRODUCTION

Epidemiologists are discussing the effective ability to highlight the environmental risks in a timely manner that should yield preventive intervention. Some authors argue that inconclusive trials, or trials with highly uncertain results, may delay the environmental remediation and increase the insecurity in the population. In addition, a long latency is usually needed to recognize the effects of environmental exposures.[1,2] In this context, biomonitoring initiatives are increasing within communities located in polluted areas. Linking environmental monitoring and health screening allows a faster assessment of the health status of exposed populations.[3] Many environmental contaminants can be reliably detected in biological matrices (blood, plasma, urine, cerebrospinal fluid, breast milk, and even hair and nails), allowing an accurate in vivo estimate of population exposure.[4] In particular, the search for heavy metals in the nails is non-invasive and relatively inexpensive, and has been used in many parts of the world to monitor the intake of these environmental pollutants.[5]

Metals are naturally widespread in the environment in variable concentrations. Some of them are essential, within physiological concentrations, while others are toxic components of air pollution and pose hazards to human health.[6,7] Several studies indicated a correlation between the concentration of metals in the nails and the environmental levels if the exposures took place in the long term: from 2 to 12 months, before cutting.[5,8,9] This biomarker can be predictive of chronic diseases including cardiovascular, respiratory, and neurological diseases, and cancer.[10-16] Data reported in the international literature generally concern populations exposed to specific polluting sources. In Italy, the average concentration of metals in the nails are reported in the ISTISAN 10/22 Reports of the Italian National Institute of Health (*Istituto superiore di sanità*, ISS) and are based on case reports of adults suffering from specific diseases: a basic reference on values regarding healthy childhood does not exist.[17]

OBJECTIVES

The ISDE (International Society of Doctors for the Environment) section of Forlì-Cesena (Emilia-Romagna Region, Northern Italy), in collaboration with the Forlì District, promoted a biomonitoring study among children aged 6-9 years residing in the Forlì area. This observational study was designed to assess the environmental exposure to metals in the four areas in which the 41 districts of the Municipality of Forlì are grouped, by determining the concentration of heavy metals in toenails. The comparison of aggregate data among the four macroareas was aimed to provide indications for further assessment of environmental media in the areas with higher concentrations of metals in toenails and, in the absence of reference data, to promote awareness on potential risks from environmental exposure.

MATERIALS AND METHODS

Public meetings and events were organized between December 2016 and March 2017 with district managers and parents of the Forlì area to provide information and promote this voluntary and crowdfunded study. The managers of the four public macroareas (North-Centre, West, South, East) of the Municipality of Forlì provided access to 8 facilities and volunteers for the sample collection. Parents who voluntarily joined the initiative took their children, born in the years 2008-2011 and survey subjects, to the sampling sites. Parents were invited to sign an informed consent, after receiving information on the study objectives, methods, and risks/benefits. They completed questionnaires designed to check for possible environmental conditions or personal behaviours able to influence the concentration of metals in toenails of their children (see table S1, on-line supplementary materials). Parents collected the samples by cutting their children's toenails with ceramic scissors, previously washed in a weakly acidic solution (10% acetic acid) to avoid contamination. The nail fragments were immediately placed in a test tube sealed with a screw cap. Personal information of each subject was eliminated and replaced by ID codes, used for test tube, questionnaire, and informed consent.

LABORATORY ANALYSIS

The test tubes were sent to the EUROLAB Srl laboratory in Turin, Italy (*http://www.eurolabtorino.com* accreditation number 0571 for quality assurance according to UNI EN ISO 17025). The nail samples, placed in 10 ml PP test tubes, were immersed in a 70% ethanol solution, without stirring or sonication, for a period of 10 minutes; 1 ml of ethanol was sufficient to completely immerse the sample. Three consecutive washings of the samples were performed

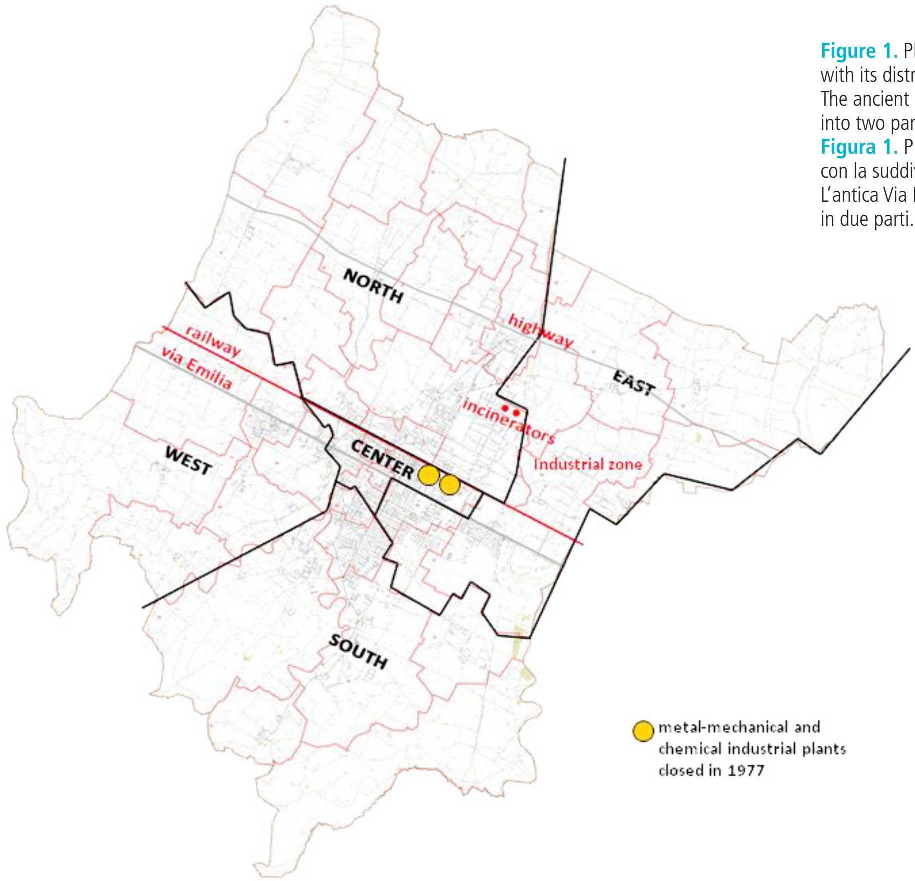

Figure 1. Plan of the Municipality of Forlì, with its districts and macroareas. The ancient Emilia road divides the territory into two parts.
Figura 1. Pianta del Comune di Forlì, con la suddivisione in quartieri e macroaree. L'antica Via Emilia divide il territorio in due parti.

with acetone (about 1 ml) and sonicated for 1 minute. The parts of acetone were excluded and the samples were rinsed twice with ultra-pure water. After extracting the water, the samples were allowed to dry at room temperature. The dry samples were weighed on an analytical scale with precision ±0.0001 g. Lastly, they were mineralized through acid digestion assisted in PTFE containers by means of microwaves, at controlled temperature and pressure, in the presence of an acid mixture composed of nitric acid, hydrochloric acid, and hydrogen peroxide. For each batch of samples which was processed, appropriate controls were analysed along the entire analytical process, in order to verify any environmental or system contamination. Multi-elemental measurement was carried out for 23 metals: Aluminium (Al), Antimony (Sb), Arsenic (As), Barium (Ba,) Beryllium (Be), Boron (B), Cadmium (Cd), Cobalt (Co), Chromium (Cr), Iron (Fe), Manganese (Mn), Mercury (Hg), Molybdenum (Mo), Nickel (Ni), Lead (Pb), Copper (Cu), Selenium (Se), Thallium (Tl), Thorium (Th), Tungsten (W), Uranium (U), Vanadium (V,) Zinc (Zn). Analyses were conducted with Agilent Technologies 7500cx ICP-MS and Agilent Technologies 7900 ICP-MS equipped with a Helium and Hydrogen fluxed collision cell. By comparing the intensities between the signal obtained from a standard of known concentration and the sample, a concentration in µg/L was obtained to relate to the weight of the sample, in order to obtain the concentration of each analyte.[18-20]

STATISTICAL ANALYSIS

No reference values are currently available for metal concentration in nails.[17] For this reason, aggregate data from participants living in the four macroareas of the Forlì territory were compared. For each metal, descriptive statistics – including median, range, 1st and 3rd quartiles – were reported for the overall population and by area of residence. Since many of the metals showed concentrations below the limit of detection (LOD), the analysis was conducted on values showing at least 50% of detectable metal concentration. A preliminary analysis was performed through a Wilcoxon test to identify differences in the distribution for each element according to the study area. A more in depth analysis was conducted adjusting by covariates including:

- passive smoke;
- practice of outdoor sports (Never/Occasionally vs Often/Everyday);
- hobbies with use of chemicals (N/O vs O/E);
- consumption of locally grown vegetables (N/O vs O/E).

Tobit models were used to estimate the association between the study area and individual metal concentration, adjusting for the covariates and considering the left censored data of the distributions. Metal concentrations were log-transformed to meet the normal assumption levels.[21] Two-sided alpha with a threshold of 5% for statistically significance was considered. Analysis were performed in R (version 3.5.1).

ETHICAL RESPECT OF PERSONAL DATA

All information derived from the questionnaires and the overall evaluation of the data were filed electronically in a special computer programme developed by Global Sistemi - Forlì (Prot. No. VAN / OFF / 201609DC09V40) used exclusively for collection purposes, in compliance with the current EU and Italian data protection legislation. Access to personal data is allowed only to the study PI and to authorized IT collaborators. The results of the analyses were aggregated for the 4 macroareas and not for individual participants. The Romagna Ethical Committee (CEROM) approved this observational study. The study, whose cost amounted to 12,079.07 euros, was entirely funded by voluntary donations from the impacted communities. The cash flow statement was publicly disclosed.

RESULTS

Collection of nail samples was carried out between March 4th and April 8th, 2017 and provided a total of 236 specimens. no material was found in 3 test tubes and the examined material considered insufficient for an evaluation in 12 cases. The number of suitable samples was 221 (93 females and 128 males) and represents the 5% of the entire population of the same age residing in the municipal area. More in detail, at December 31st, 2017, the total number of children aged 6-9 years in the municipality of Forlì was 4,354, distributed among the 4 macroareas:

- Centre-North: 1,860 (tested children: 2.8%);
- West: 510 (tested children: 9.4%);
- South 1,040 (tested children: 6.3%);
- East: 944 (tested children: 5.8%).

The chicking of the donors' address led to the following breakdown of cases for macroareas:

- Centre-North: 52 (23.5%);
- West: 48 (21.7%);
- South: 66 (29.8%);
- East: 55 (24.8%).

Molybdenum (Mo), thallium (Tl), and tungsten (W) were not found in any sample, because the concentration was always lower than the LOD. From the descriptive statistics (table 1), only Al, Cd, Fe, Mn, Cu, and Zn were detected in at least 50% of the samples.

In table 2, the significant difference between area of residence considered in pairs at each time was tested. Most of the significant differences were identified in the values found in the Eastern area, significantly higher than those found in the other 3 areas of the municipality. In particular, differences were found in Al, Cu, Zn, and Mn between the Eastern and Centre-North areas, and between the West and South, respectively. A Tobit regression was performed to test for the association between the area of residence and each nail metal concentration adjusting for the covariates (outdoor sports, hobbies with chemicals usage, passive smoke, and local vegetables consumption) to avoid bias (table 3) from other exposure sources. This analysis showed that subjects residing in the Eastern area show higher values of Al concentration compared to the rest of the subjects. All the other metals were still higher on average in the Eastern area, but only Mn in the South area and Cu in the Western area had statistically significantly lower concentration compared to the East area. Only Zn had a significantly higher concentration in the Western area compared to the Eastern one. For all other variables, in the questionnaire no reliable or valid answers were obtained to be considered in the statistical analysis, as well as no significant differences were observed for either sex or the four different age groups (6, 7, 8, and 9 years old).

To allow spatial analyses, we aggregated the data of the Centre-North and East areas, comparing them to the West and South areas (table 4). The rough assessment of the total load of metals in the Centre-North and East areas was about 60% higher than the one of the other two areas (West+South). The averages of concentrations of Ba and Cr (3.5 times) and Ni (almost 3 times) appeared clearly higher in the Centre-North+East; those of Al (+50%), Be (+10%), Fe (+90%), Mn (+30%), Hg (+50%), Pb (+80%), and Cu (+40%) were also higher compared to the West+South. Sb (+30%), Cd (+42%), and Se (+33%) prevailed in West+South macroareas.

DISCUSSION

Forlì is a town with 117,000 inhabitants located in Emilia-Romagna Region (Northeastern Italy), its territory has historically surrounded by land devoted to agriculture with cereal crops, vineyards, and orchards, managed intensively for industrial production. Drinking water, coming largely from the reservoir of Ridracoli, situated in a National Park, is almost free of pesticide residues, but this is not true for surface waters. In the last century, a metal-mechanical and a chemical industrial plant, both large and closed some tens of years ago, were located in neighbourhoods between the Centre and North areas. Today, the Forlì industrial zone is developed near the city in the East area, with many small-to-medium sized metalworking. On the border between the North and East areas, there is an incinerator for urban waste (120-180,000 tons/year) and one for 32,000 tons/year hospital waste.

A previous study on mortality of the population living near the incinerators, carried out in the early 2000s and published in 2011, was based on a mathematical model of dispersion of heavy metals according to the main winds.[22] It drew a small area of greatest fallout in the North and an equally intense and much more extensive area in the East. Finally, the North and East areas are crossed by the A14 motorway and by the new ring road connections.

METAL (mg/kg)	OVERALL (No. 221)			EAST (No. 55)			CENTRE-NORTH (No. 51)			WEST (No. 48)			SOUTH (No. 67)		
	<LOD%	Median (Q1, Q3)	Range	<LOD%	Median (Q1, Q3)	Range	<LOD%	Median (Q1, Q3)	Range	<LOD%	Median (Q1, Q3)	Range	<LOD%	Median (Q1, Q3)	Range
Aluminum	36.7	63 (0, 160)	(0, 2500)	20.0	120 (42.5, 190)	(0, 2500)	49.0	15.5 (0, 107.5)	(0, 530)	41.7	58 (0, 180)	(0, 770)	37.3	60 (0, 100)	(0, 690)
Antimony	57.5	0 (0, 0.085)	(0, 2.6)	45.5	0.033 (0, 0.095)	(0, 0.43)	52.9	0 (0, 0.115)	(0, 0.64)	60.4	0 (0, 0.198)	(0, 2.6)	68.7	0 (0, 0.034)	(0, 0.44)
Arsenic	98.2	0 (0, 0)	(0, 0.46)	94.5	0 (0, 0)	(0, 0.46)	98.0	0 (0, 0)	(0, 0)	100.0	0 (0, 0)	(0, 0)	100.0	0 (0, 0)	(0, 0)
Barium	71.5	0 (0, 2)	(0, 560)	58.2	0 (0, 4.1)	(0, 20)	60.8	0 (0, 4.2)	(0, 560)	91.7	0 (0, 0)	(0, 14)	76.1	0 (0, 0)	(0, 130)
Beryllium	71.5	0 (0, 0.025)	(0, 3)	60.0	0 (0, 0.036)	(0, 0.24)	74.5	0 (0, 0)	(0, 0.52)	83.3	0 (0, 0)	(0, 0.35)	70.1	0 (0, 0.017)	(0, 0.41)
Boron	99.1	0 (0, 0)	(0, 24)	100.0	0 (0, 0)	(0, 0)	98.0	0 (0, 0)	(0, 0)	100.0	0 (0, 0)	(0, 0)	98.5	0 (0, 0)	(0, 24)
Cadmium	25.8	0.022 (0, 0.061)	(0, 3.9)	18.2	0.027 (0.011, 0.064)	(0, 0.55)	21.6	0.0185 (0.006, 0.045)	(0, 0.11)	39.6	0.0295 (0, 0.06)	(0, 1.7)	25.4	0.024 (0.002, 0.058)	(0, 0.28)
Cobalt	99.1	0 (0, 0)	(0, 2.4)	98.2	0 (0, 0)	(0, 2.4)	98.0	0 (0, 0)	(0, 0)	100.0	0 (0, 0)	(0, 0)	100.0	0 (0, 0)	(0, 0)
Chromium	87.8	0 (0, 0)	(0, 240)	83.6	0 (0, 0)	(0, 46)	82.4	0 (0, 0)	(0, 240)	87.5	0 (0, 0)	(0, 42)	95.5	0 (0, 0)	(0, 13)
Iron	22.2	69 (18, 170)	(0, 38000)	12.7	78 (46, 145)	(0, 3800)	21.6	73 (37, 180)	(0, 5100)	29.2	125 (0, 220)	(0, 940)	25.4	58 (8, 100)	(0, 1600)
Manganese	43.0	0.92 (0, 3.5)	(0, 250)	27.3	1.6 (0, 4)	(0, 61)	41.2	1.3 (0, 3.4)	(0, 36)	47.9	1.115 (0, 4.275)	(0, 20)	53.7	0 (0, 2.2)	(0, 27)
Mercury	85.1	0 (0, 0)	(0, 3)	80.0	0 (0, 0)	(0, 0.52)	78.4	0 (0, 0)	(0, 0.8)	95.8	0 (0, 0)	(0, 0.24)	86.6	0 (0, 0)	(0, 3)
Nickel	78.3	0 (0, 0)	(0, 91)	76.4	0 (0, 0)	(0, 24)	72.5	0 (0, 0.143)	(0, 91)	79.2	0 (0, 0)	(0, 27)	83.6	0 (0, 0)	(0, 3.1)
Lead	90.5	0 (0, 0)	(0, 68)	92.7	0 (0, 0)	(0, 68)	88.2	0 (0, 0)	(0, 6.2)	83.3	0 (0, 0)	(0, 16)	95.5	0 (0, 0)	(0, 3.5)
Copper	28.5	4.7 (0, 7.4)	(0, 39)	16.4	5.5 (4.15, 7.55)	(0, 20)	17.6	5.2 (3.9, 7.35)	(0, 35)	52.1	0 (0, 8.2)	(0, 26)	29.9	4.5 (0, 7.35)	(0, 14)
Selenium	92.3	0 (0, 0)	(0, 0.23)	90.9	0 (0, 0)	(0, 0.1)	92.2	0 (0, 0)	(0, 0.23)	97.9	0 (0, 0)	(0, 0.23)	89.6	0 (0, 0)	(0, 0.22)
Thorium	98.6	0 (0, 0)	(0, 0.52)	98.2	0 (0, 0)	(0, 0.34)	96.1	0 (0, 0)	(0, 0.037)	100.0	0 (0, 0)	(0, 0)	100.0	0 (0, 0)	(0, 0)
Uranium	97.7	0 (0, 0)	(0, 0.12)	96.4	0 (0, 0)	(0, 0.064)	98.0	0 (0, 0)	(0, 0)	97.9	0 (0, 0)	(0, 0.12)	98.5	0 (0, 0)	(0, 0.004)
Vanadium	96.4	0 (0, 0)	(0, 6.1)	90.9	0 (0, 0)	(0, 6.1)	96.1	0 (0, 0)	(0, 0.29)	100.0	0 (0, 0)	(0, 0)	98.5	0 (0, 0)	(0, 1.1)
Zinc	0.5	87 (77, 100)	(0, 840)	1.8	83 (77, 90)	(0, 150)	0.0	84 (77.25, 100)	(61, 660)	0.0	93 (87.75, 110)	(60, 270)	0.0	85 (76.5, 98.5)	(60, 250)

LOD: limit of detection / *limite di rivelabilità*

Table 1. Overall nail metal concentrations summary statistics: median value, range, 1st and 3rd quartiles. Overall population and by area of residence
Tabella 1. Statistica generale della concentrazione dei metalli rinvenuti nelle unghie: valori mediani, range, 1° e 3° quartile. Popolazione totale e per le 4 macroaree.

METAL (mg/kg)	EAST *VS* NORTH-CENTRE	EAST *VS* WEST	EAST *VS* SOUTH	CENTRE-NORTH *VS* WEST	CENTRE-NORTH *VS* SOUTH	WEST *VS* SOUTH
Aluminum	0.001	0.077	0.004	0.196	0.339	0.545
Cadmium	0.085	0.339	0.607	0.801	0.283	0.610
Iron	0.789	0.607	0.057	0.813	0.180	0.086
Manganese	0.396	0.515	0.008	0.952	0.127	0.199
Copper	0.946	0.039	0.065	0.048	0.117	0.337
Zinc	0.224	<0.001	0.257	0.056	0.722	0.013

Table 2. Analysis conducted on 6 metals detected in at least 50% of the samples with a detectable metal concentration comparing the 4 macroareas. All p-values were computed by Wilcoxon test.
Tabella 2. Analisi condotta sui 6 metalli rinvenuti con una concentrazione rilevabile in almeno il 50% dei campioni nel confronto fra le 4 macroaree. Tutti i valori (p-value) sono stati valutati con test di Wilcoxon.

	ß COEFFICIENTS (95% CONFIDENCE INTERVALS)					
	Aluminum	**Cadmium**	**Iron**	**Manganese**	**Copper**	**Zinc**
Centre-North vs East	-1.3# (-1.9; -0.7)	-0.5 (-1.2; 0.2)	0.002 (-0.6; 0.6)	-0.4 (-1.2; 0.3)	-0.02 (-0.3; 0.3)	0.1 (-0.01-0.1)
West vs East	-0.8^ (-1.4; -0.2)	-0.4 (-1.1; 0.3)	-0.1 (-0.7; 0.5)	-0.4 (-1.1; 0.4)	-0.4* (-0.7; -0.1)	0.1# (0.1; 0.2)
South vs East	-0.8§ (-1.4; -0.2)	-0.1 (-0.8; 0.5)	-0.5^ (-1.1; 0.1)	-0.9^ (-1.6; -0.2)	-0.2 (-0.5; 0.04)	0.03 (-0.03; 0.1)
Outdoor sports	0.5^ (-0.01; 0.9)	-0.1 (-0.6; 0.4)	0.1 (-0.4; 0.5)	0.6* (0.05; 1.2)	0.04 (-0.2; 0.3)	-0.01 (-0.1; 0.05)
Hobbies involving chemicals	0.2 (-0.3; 0.6)	-0.1 (-0.6; 0.3)	0.4^ (-0.01; 0.9)	0.2 (-0.3; 0.8)	-0.2^ (-0.4; 0.02)	0.1§ (0.02; 0.1)
Passive smoke	0.5^ (-0.06; 0.9)	0.05 (-0.5; 0.6)	0.01 (-0.5; 0.5)	0.05 (-0.6; 0.7)	0.01 (-0.2; 0.2)	-0.05 (-0.1; 0.01)
Vegetables local consumption	0.04 (-0.5; 0.6)	0.6* (0.06; 1.2)	0.3 (-0.2; 0.8)	0.7* (0.05; 1.3)	-0.04 (-0.3; 0.2)	0.01 (-0.04; 0.1)
Constant	4.2# (3.7; 4.7)	-3.9# (-4.4; -3.3)	4.1# (3.6; 4.6)	-0.2 (-0.8; 0.4)	1.7# (1.5; 1.9)	4.4# (4.4; 4.5)
Observations (No.)	218	218	218	218	218	214

^ $p<0.1$, * $p<0.05$, § $p<0.01$, # $p<0.001$

Table 3. Tobit models results, allowing to estimate the association between area of residence and each metal concentration, adjusted for covariates, and considering the left censored data present in metals distribution: ß coefficients (95% confidence intervals).
Tabella 3. Risultati del modello di Tobit che consentono di stimare l'associazione tra area di residenza e concentrazione di ciascun metallo, aggiustato per le covariate e considerando censurati i dati di sinistra presenti nella distribuzione dei metalli: coefficienti ß (intervalli di confidenza al 95%).

The Forlì area is divided into two parts by the line of the ancient Emilia road, which crosses the city separating the Northeast from the Southwest. This division, although administratively determined by the Municipality of Forlì, also allows separating important environmental aspects of the territory as in the North-East areas are located industrial production sites, two waste incinerators, and the prevalence of road traffic. The South-West areas have a predominantly agricultural characterization and include some small areas of craftsmanship. In this observational study, carried out by evaluating the concentration of heavy metals in the nails of children aged 6-9 years, an initial rough estimate shows that the average of metals found in the nails of children living in the North/Central and Eastern areas is about 60% higher than that found in children who live in the South-West and is characterized by typical elements of "industrial production" such as Al, Cr, Fe, Ni. Other metals considered toxic as Cd, Mn, Sb, Hg, and Pb seem to be more widely present in all 4 macroareas, probably because being predominantly "ubiquitous" (except Mn) and, therefore, dispersed in larger territories; perhaps, also because they can be correlated with eating habits or lifestyles to be considered transversal and, finally, because some (especially the Cd) are related to the use of chemicals in agriculture.[23] The albeit weak correlation that emerges for the concentrations of Mn and Cd with the habit to play outdoor and the consumption of local vegetables seems to confirm these last observations.[24]

More detailed statistical evaluations performed on the 6 metals found in over 50% of subjects confirm their prevalence in the East area, with statistically significant peaks for Al, Cu, Zn, and Mn in comparison with the rest of the city. From this simple observational study, it is certainly not possible to track the point sources of metals emission which

METALS	EAST	NORTH-CENTRE	NORTH-CENTRE +EAST	WEST	SOUTH	WEST+SOUTH	RATIO (E+NC)/W+S
	Median mg/kg	Median mg/kg	mg/kg	Median mg/kg	Median mg/kg	mg/kg	
Alluminum	218.6182	72.0980	147.4121	107.9792	85.8209	95.1507	1.50
Antimony	0.0616	0.0884	0.0746	0.2039	0.0349	0.1060	0.70
Arsenic	0.0189	0.0000	0.0097	0.0000	0.0000	0.0000	
Barium	2.7127	13.3647	7.8894	0.6438	3.2776	2.1686	3.60
Beryllium	0.0265	0.0278	0.0271	0.0171	0.0290	0.0240	1.10
Boron	0.0000	0.0000	0.0000	0.0000	0.3582	0.2074	
Cadmium	0.0572	0.0287	0.0433	0.1078	0.0497	0.0742	0.58
Cobalt	0.0436	0.0000	0.0224	0.0000	0.0000	0.0000	
Chromium	1.6600	5.8490	3.6958	1.8917	0.4746	1.0713	3.40
Iron	216.5455	364.1765	288.2914	189.0208	130.7313	155.2743	1.90
Manganese	3.3876	3.5108	3.4475	2.9444	2.3334	2.5907	1.30
Mercury	0.0809	0.0812	0.0810	0.0213	0.0790	0.0547	1.50
Molybdenum	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	
Nickel	0.6355	2.2447	1.4175	0.8608	0.2154	0.4871	2.90
Lead	1.6691	0.3098	1.0085	1.1500	0.1055	0.5453	1.80
Copper	5.8400	6.1902	6.0102	4.1333	4.5925	4.3992	1.40
Selenium	0.0061	0.0081	0.0070	0.0048	0.0146	0.0105	0.67
Thallium	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	
Thorium	0.0062	0.0007	0.0035	0.0000	0.0000	0.0000	
Tungsten	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	
Uranium	0.0013	0.0000	0.0007	0.0025	0.0001	0.0011	
Vanadium	0.2618	0.0057	0.1373	0.0000	0.0164	0.0095	
Zinc	86.2000	108.7647	97.1660	103.7708	91.4478	96.6364	1.01
All metals	538	577	557	413	320	359	1.60

Table 4. Aggregated metal levels data of the North-Centere+East areas compared to the West+South areas and their ratio.
Tabella 4. Dati delle concentrazioni dei metalli aggregati dell'area Nord-Centro+Est nel confronto con Ovest+Sud e loro rapporto.

should not be found in children's tissues, nor it is possible to assume a real risk due to the detection of these metals in children's nail tested in the absence of reference values. The presence of Cu and Zn may not be relevant to hypothesize health risks, while Al and Mn could be the subjects of further research. In particular, Mn and Fe, widely used in the steel industry, are essential trace elements and become toxic when they reach high concentrations in early life exposure windows.[13,25,26] In a report of 2015, the Environment Protection Agency of the Emilia-Romagna Region stated: "As for the background values [...] iron, sulfates, and manganese are considered endemic. As regards the Forlì territory, the old former industrial site Orsi-Mangelli present (from 1926 to 1977: note of the author) in the city centre is mainly mentioned".[27]

Therefore, the assessment of the environmental load should be considered in the areas of the Centre and in the outskirts of the North-East of Forlì, both for Al and – especially – for Mn, which we found in concentrations similar to those of highly polluted sites described in other reports.[28,29] Concern is also raised by the high content of Al in the East and North areas, for which less direct effects on health were known, but which were also found in significant concentrations.[30] Finally, the content of Cd, present in 75% of the examined subjects – transversally in all areas and probably also linked to the agricultural use of chemicals – should not be overlooked for its harmful competition with the Zn and its possible endocrine and carcinogenic effects.[31-34] Further and more detailed studies, inclusive of studies considering other types of investigations possibly on different biological matrices including environmental concentrations in soil and water, should be promoted to identify the potential sources of environmental pollution and their health impact for the inhabitants of the Forlì district. This observational study, based on a voluntary participation and subject to possible selection bias, carried out a simple comparison of the data observed among the 4 macroareas of the city. In the absence of comparative data in the literature and prebuilt toxicological limits for healthy children, it may serve as a starting hypothesis and stimulus for further planning.

Raising awareness from public health authorities is a desirable outcome of this study, which has certainly achieved one of the initial aims, i.e., raising awareness among a large part of the population, given the high participation of parents and young people to the initiative and the associated public fundraising. Finally, as explicitly reported in the ISTISAN 10/22 Reports of the Italian National Institute of Health «the Reference Values are limited to five biological matrices, including blood, serum, urine, cerebrospinal fluid (CSF) and breast milk, while for no metal VRs have been defined to date in matrices such as hair and nails»,[17] the data reported in this paper concerning 221 healthy children aged 6-9 years collected in an Italian provincial town may serve as a useful reference for other biomonitoring studies.

CONCLUSIONS

The assessment of metals concentration in toenails proved to be a simple, non-invasive, and relatively inexpensive method to verify the accumulation of several elements present in the environment.[4,5] The East and North areas of the Forlì territory, characterized by the presence of industrial plants, two waste incinerators, and major road traffic arteries, showed an average of 60% higher content of heavy metals in toenails of examined children than that found in their peers residing in the Western and Southern areas. In particular, in the East area, statistically significant peaks were recorded for Al and Mn in comparison with the other 3 areas. This observational study, which compared the values of concentration of heavy metals in children of 4 different areas, does not allow to track the emission of potentially toxic metals nor can indicate risk values, and further and more detailed studies are warranted to identify any sources of environmental pollution.[35] In the absence of Italian Reference Values, this study can provide a useful basis of comparison for other biomonitoring determinations, and the wide voluntary participation in the initiative encourages the promotion of further forms of environmental biomonitoring.[36]

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