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Metal(loid)s role in the pathogenesis of amyotrophic lateral sclerosis: Environmental, epidemiological, and genetic data

Marina Tesauro [a,**], Maurizio Bruschi [b,1], Tommaso Filippini [c], Sandra D'Alfonso [d], Letizia Mazzini [e], Lucia Corrado [d], Michela Consonni [a], Marco Vinceti[c,g], Paola Fusi [f,2], Chiara Urani [b,2,*]

[a] Department of Biomedical, Surgical and Dental Sciences, University of Milan, Via C. Pascal, 36, 20133, Milan, Italy
[b] Department of Earth and Environmental Sciences, University of Milano – Bicocca, Piazza della Scienza, 1, 20133, Milan, Italy
[c] CREAGEN-Environmental, Genetic and Nutritional Epidemiology Research Center, Department of Biomedical, Metabolic and Neural Sciences, University of Modena and Reggio Emilia, Via Campi, 287, 41125, Modena, Italy
[d] Department of Health Sciences, CAAD, UPO University, Via Solaroli, 17, 28100, Novara, Italy
[e] ALS Centre Department of Neurology, Maggiore della Carità University Hospital, Corso Mazzini, 18, 28100, Novara, Italy
[f] Department of Biotechnology and Biosciences, University of Milano-Bicocca, Piazza della Scienza, 2, 20126, Milan, Italy
[g] Department of Epidemiology, Boston University School of Public Health, Boston, 715 Albany Street, MA 02118, USA

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ABSTRACT

Amyotrophic Lateral Sclerosis (ALS) is a progressive neurodegenerative disorder of the motor system. The etiology is still unknown and the pathogenesis remains unclear. ALS is familial in the 10% of cases with a Mendelian pattern of inheritance. In the remaining sporadic cases, a multifactorial origin is supposed in which several predisposing genes interact with environmental factors. The etiological role of environmental factors, such as pesticides, exposure to electromagnetic fields, and metals has been frequently investigated, with controversial findings. Studies in the past two decades have highlighted possible roles of metals, and ionic homeostasis dysregulation has been proposed as the main trigger to motor-neuron degeneration. This study aims at evaluating the possible role of environmental factors in etiopathogenesis of ALS, with a particular attention on metal contamination, focusing on the industrial Briga area in the province of Novara (Piedmont region, North Italy), characterized by: *i)* a higher incidence of sporadic ALS (sALS) in comparison with the entire province, and *ii)* the reported environmental pollution. Environmental data from surface, ground and discharge waters, and from soils were collected and specifically analyzed for metal content. Considering the significance of genetic mechanisms in ALS, a characterization for the main ALS genes has been performed to evaluate the genetic contribution for the sALS patients living in the area of study. The main findings of this study are the demonstration that in the Briga area the most common metal contaminants are Cu, Zn, Cr, Ni (widely used in tip-plating processes), that are above law limits in surface waters, discharge waters, and soil. In addition, other metals and metalloids, such as Cd, Pb, Mn, and As show a severe contamination in the same area. Results of genetic analyses show that sALS patients in the Briga area do not carry recurrent mutations or an excess of mutations in the four main ALS causative genes (*SOD1*, *TARDBP, FUS, C9ORF72)* and for *ATXN2* CAG repeat *locus.* This study supports the hypothesis that the higher incidence of sALS in Briga area may be related to environmental metal(loid)s contamination, along with other environmental factors. Further studies, implementing analysis of genetic polymorphisms, as well as investigation with long term follow-up, may yield to key aspects into the etiology of ALS. The interplay between different approaches (environmental, chemical, epidemiological, genetic) of our work provides new insights and methodology to the comprehension of the disease etiology.

* Corresponding author. Department of Earth and Environmental Sciences, University of Milano – Bicocca, Piazza della Scienza, 1, 20133, Milan, Italy.
** Corresponding author. Department of Biomedical, Surgical and Dental Sciences, University of Milan, Via C. Pascal, 36, 20133, Milan, Italy.
E-mail addresses: marina.tesauro@unimi.it (M. Tesauro); chiara.urani@unimib.it (C. Urani)

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[2] Integrated Models for Prevention and Protection in Environmental and Occupational Health, (MISTRAL) Interuniversity Research Center.

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1. Introduction

Amyotrophic Lateral Sclerosis (ALS) is a progressive neurodegenerative disorder of the motor system. It is the third most common neurodegenerative disease after Alzheimer's Disease and Parkinson's Disease and the most common form of adult-onset Motor Neuron Disease (Shang et al., 2015). ALS has a rapid progression and is uniformly fatal: death usually occurs following respiratory failure within 2–4 years from symptoms onset (Al-Chalabi and Hardiman, 2013).

ALS is a rare condition with an increased incidence in the last decades: currently the mean incidence rate is 2.8/100,000 in Europe and 1.8/100,000 in North America, while the mean prevalence rate is 5.4/100,000 in Europe and 3.4/100,000 in North America. ALS incidence increases with age, with a peak in the seventh decade and it is likely that the overall incidence of ALS will increase as the world population ages (Chiò et al., 2013; Goutman and Feldman, 2020). The disease is slightly more common in men than in women, with an incidence of 1.5:1, and the incidence in men and women is about the same in familial ALS (Zarei et al., 2015). In North Italy during the 1995 to 2014 period, the mean annual incidence rate, adjusted for age and sex, in the 2001 Italian population was 2.78 per 100,000 population (95% CI 2.57–2.96) (men: 3.06 per 100,000; women: 2.51 per 100,000; rate ratio for men to women, 1.22:1) (Chiò et al., 2017).

The different increase of ALS incidence in men and women points to an effect of exogenous factors with a differential effect on the two sexes, acting on a genetic background. ALS is familial in only about 10% cases, with a family history, and sporadic (sALS) in the remaining 90%, without apparent genetic link (Renton et al., 2014).

Advances in genetics have greatly contributed to the knowledge of ALS, with the discovery of Mendelian variants in at least 25 known genes that account for up to 70% of all cases of familial ALS and 10% of sporadic form. More specifically, mutations in four major genes namely, *C9ORF72, SOD1*, *TARDBP, FUS* account for 60% of familial cases and 10% of sporadic cases, while mutations in each of the remaining ALS genes have been identified in a small fraction of ALS patients (Brown and Al-Chalabi, 2017).

The observation of high frequencies of ALS in Guam, combined with a strong association with dietary habits of the indigenous population, supported the assumption of an environmental basis for ALS (Spencer et al., 2009; Al-Chalabi and Hardiman, 2013). In particular, in Guamanian patients Gellein et al. (2003) highlight that the plausible hypothesis regards imbalances in essential and toxic metals (e.g., Cd, Co, Cu, Fe, Zn), with significantly elevated Cd concentrations in brain tissues in ALS patients. The incidence decline of ALS occurring with rapid changes in the epidemiology of the population, strongly suggests that environmental factors associated with former lifestyle of the population were involved. An interesting study (Román, 1996) reports a correlation between the presence of specific metals (e.g., Fe, Co, Zn) in environmental matrices and ALS in Guam island.

Indeed, current theories posit that the disease is a culmination of multiple genetic and environmental factors, with inevitable gene-environment interaction forming different steps along the pathway to the disease (Gellein et al., 2003; Al-Chalabi and Hardiman, 2013; Ingre et al., 2015; Wang et al., 2017; Chiò et al., 2018).

The role of environmental factors has been frequently reported in systematic reviews and meta-analysis of observational studies (see for example Yu et al., 2014; Oskarsson et al., 2015; Bozzoni et al., 2016; Wang et al., 2017; Riancho et al., 2018). Toxins from cyanobacteria, infectious agents, and mushrooms (Cox et al., 2016; Castanedo-Vazquez et al., 2019; French et al., 2019; Spencer et al., 2019; Fiore et al., 2020), hydrazine from fungal or industrial origin (Spencer, 2019; Spencer et al., 2019), cigarette smoking (Calvo et al., 2016), alcohol consumption (de Jong et al., 2012), physical work (Huisman et al., 2013), agonistic sport activities (Chiò et al., 2005; Gotkine et al., 2014; Visser et al., 2019), military service (Binazzi et al., 2009; Drouet et al., 2010; Beard and Kamel, 2015), head trauma (Filippini et al., 2020a; McKee et al., 2010; Seelen et al., 2014), exposure to electromagnetic fields (Hakansson et al., 2003; Gunnarsson and Bodin, 2018), pesticides (Chiò et al., 2009; Malek et al., 2012; Kamel et al., 2012; Vinceti et al., 2012; Vinceti et al., 2017), and Pb (Filippini et al., 2020b; Oh et al., 2007) are the environmental culprits most commonly cited for ALS (Zufiria et al., 2016), sometimes in controversial ways.

As far as in the early '80s, case-control studies (Roelofs-Iverson et al., 1984) suggested an association between heavy metals, such as Hg, Pb, or other toxic metals and ALS.

Studies in the past two decades have highlighted possible roles of metals in the pathogenesis of ALS, and ionic homeostasis dysregulation has been proposed as the main trigger to motor-neuron degeneration (Kamel et al., 2002; Johnson and Atchinson, 2009; Sutedja et al., 2009; Sirabella et al., 2018). Higher concentrations of different essential and non-essential metals (Cu, Zn, Mn, Al, Cd, Co, Pb, V and U) were found in cerebrospinal fluid and blood plasma from ALS patients, supporting the hypothesis of their involvement in the pathogenesis of ALS (Vinceti et al., 1997; Vinceti et al., 2017; Roos et al., 2013).

A cause-effect relationship between heavy cadmium exposure and ALS has also been suggested in nickel-cadmium battery workers. Noteworthy, Cd increases the permeability of the blood-brain barrier, and affects the activity of Cu-Zn superoxide dismutase and other enzymes involved in reactive oxygen species scavenging (Bar-Sela et al., 2001). Finally, as recently highlighted (Garza-Lombò et al., 2018), exposure to toxic metals (e.g., Al, As, Cd, Pb, and Hg) is linked to several neurological disorders, including neurodegeneration.

In this work, we have collected environmental data from surface, ground and discharge water, and from soils of the province of Novara (Piedmont region, North Italy) and, particularly, of an industrial area, Briga area, located in the same province where metal contamination was mainly due to specific metallurgic activities. In this latter area of study, a previous epidemiologic work has shown evidence of a clear increase of mortality for ALS in the population (Uccelli et al., 2007). A recent study by our group (Tesauro et al., 2017) found incidence rates in the period 2002–2012 of 3.98, 5.14, and 2.97 cases/100,000 persons in Novara province, and 4.65, 4.27, and 4.98 in Briga area, for the total population, men and women, respectively. Stratifying for sex and age, incidence rates were higher in men in the 45–54 years age group and in women aged 65–74 years. Male to female ratio is 0.75 and 1.54, respectively in Briga area and in the rest of the province of Novara. A population-based case control study in the same area showed a positive association with ALS risk for occupational history, especially for exposure to solvents (OR 4.11 95% CI 1.29–13.05), electromagnetic fields (OR 1.2 95% CI 0.25–5.67), pesticides in particular fungicides (OR 2.97 95% CI 0.24–36.75), and heavy metals such as Pb and Hg (OR 4.43 95% CI 0.88–22.29 and OR 2.92 95% CI 0.24–35.30, respectively) (Gagliardi, 2017). In particular, about metals, in the same area and in other provinces increased ALS risk for Pb (OR 3.66, 95% CI 1.63–8.20), and for Cd (OR 1.79, 95% CI 0.23–13.73) were found (Filippini et al., 2020a; Filippini et al., 2020b).

Due to the suggested relation between metals and ALS, this study aims at evaluating the possible role of environmental factors in the etiopathogenesis of ALS, with a particular focus on metal contamination. The specific features that prompted this study are the higher incidence of sALS in Briga area in comparison with the entire province, and the reported high levels of environmental contamination in the same area. Considering the significance of genetic mechanisms in ALS, a characterization for the main ALS genes has been performed to evaluate the genetic contribution for the ALS patients living in the area of study.

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In addition, the interference of toxic metals on the homeostasis of essential metals, and on the function of enzymes (e.g., SOD1) with relevant roles in neurodegeneration are herewith discussed.

2. Material and methods

2.1. The area of study

The study area (Fig. 1) is the province of Novara, located in Piedmont region (North Italy) with an extension of 1,340 km^2. In particular, the Briga area here described includes 28 municipalities, defined with a radius of 10 Km from the centroid Briga Novarese, and considered as an ALS cluster (Uccelli et al., 2007).

The population was almost of 370,000 people in 2019 with a density of 276 inhabitants/km^2. Generally, corn and rice represent the major cultivations in the South area, where manufacturing industries are also present along with one of the most important Italian oil refinery. In the Northern part of the province, and especially in the Briga area, there is a large number of metallurgic industries devoted to the production of valves and taps, which increased from 69 in 1961 to 300 in 1991, encompassing 110 and 3083 workers respectively. Around 22% of workers in that area were involved in tap production units, and in addition some family members of these workers were involved in 'home-work' (Rabellotti, 2003), such as the preparation of souls for mergers, mostly performed by women (Rogatti, 2013; Tesauro et al., 2017). In most recent years, in the three main municipalities of Briga area, Gozzano, Briga Novarese and Borgomanero, there were still 56 (4.66/km^2), 20 (4.2/km^2) and 29 (0.9/km^2) production units respectively in the metalworking sector (ARPA, 2010).



Fig. 1. Area of study: Briga area in the red circle (modified from d-maps.com). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.2. Environmental data and processing

The environmental data of the entire province of Novara were collected from the Regional Agency for the Protection of the Environment (Agenzia Regionale per la Protezione Ambientale - ARPA) of Piedmont and other sources (Cremonesi, 1996, study commissioned by Province of Novara). Environmental data collection covered the same years of the epidemiological analysis (2002–2012), followings years until 2018, and a previous period (1989–1995) in which an extensive monitoring campaign has been carried out in the Briga area.

Old chemical database is very fragmented, because of difficulties in obtaining paper-based data in old archives, not used for a long time, and inhomogeneities in chemical parameters analyzed. On the other hand, more recent digital data is easily accessible and homogeneous.

As for the analysis of soil in the Briga area, a campaign was carried out in the period May–April 2009 and analysis was carried out for the following metals and metalloids: As, total Cr, Cu, Ni, Pb, Sn, and Zn (ARPA, 2010).

Data for surface and deep water, drinking water, and soil, were collected, processed, and compared with past (Legge 319/76) and current standards (D.Lgs 152/06). In addition, air quality data from the same ARPA database of Piedmont on areas not necessarily corresponding to the area of study were collected (data not shown).

2.3. Genetic analyses

The ALS patients of the previous epidemiological study (Tesauro et al., 2017), followed at the ALS tertiary clinical center at Novara Hospital (Italy), were enrolled for genetic analysis. According to medical history, all considered cases were classified as sALS. DNA was collected based on their availability at the time of the study.

The ALS cases have been screened for mutations in the 4 main ALS causative genes (*SOD1*, *TARDBP, FUS, C9ORF72)* and for *ATXN2* CAG repeat *locus* whose intermediate alleles (>30 CAG repeats) represent the main ALS susceptibility genetic factor in sALS. The genetic analysis was performed on two different groups: the group belonging to Briga area composed of 11 patients (7 men and 4 women with a mean age at onset of 62 years old, higher in women, 67 years old, and lower in men, 59 years old), and a second group used as a reference composed of 42 patients (28 men and 14 women, where the mean age at onset of the disease is of 65 years old, same for men and 63 for women), living in the territory of Novara province but outside the investigated area.

The study was approved by the local hospital ethics committee. A blood sample of the patients was drawn after informed consent to genetic analysis for research purposes.

Genomic DNA was isolated from peripheral blood lymphocytes using a standard protocol. The coding exons and 30bp of the flanking intron-exon boundaries of *SOD1*, *TARDBP* (exon 6), and *FUS* (exons 13, 14 and 15) have been PCR amplified, sequenced using the BigDye Terminator v3.1 sequencing kit (Applied Biosystems Inc.), and run on an ABI Prism 3100XL genetic analyzer. These exons were selected as the vast majority of known pathogenic variants are known to lie within these mutational hotspots. A repeat-primed PCR assay was used to screen for the presence of the GGGGCC hexanucleotide expansion in the first intron of *C9ORF72* (DeJesus-Hernandez et al., 2011). The *ATXN2* CAG repeat in exon 1 (Ref Seq NM_002973.3) was amplified using a fluorescent primer and sized by capillary electrophoresis on an ABI 3100XL genetic analyzer (Applied Biosystems, Foster City, CA).

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3. Results

3.1. Chemical data reveal a heavy environmental contamination by metal(loid)s on different matrices

The following paragraphs show all data on metal contamination in the considered matrices. All concentrations reported in the Tables are expressed as mean, median and maximum levels. The most relevant data to be considered is the maximum level, which better stresses the amount of environmental contamination close to the industrial discharge and in point-sources. In addition, the number of samples exceeding the law limits has to be taken into account.

Soil contamination. Metal concentrations in soil were compared to those measured for the Novara province (see Table 1). We found that the Briga area was characterized by a metal contamination, in particular for Zn, As, Sn, Pb and Cu, related to anthropic activities and discharges in soil. For Zn and As this was due to several sampling points with concentration values far above the law limits, whereas for Sn, to a spreader diffusion of the contamination with a large number of points with concentration higher than the background value determined for the Novara province. The concentrations of Zn and As were well correlated suggesting a common anthropic origin. Contamination of these two elements was on average higher in the eastern part of the Briga area with respect to the western border. Cu and Pb featured localized contamination with few points characterized by concentrations higher than the background value. Other metals (Cr, Ni, Co, V) did not show differences with respect to the value determined for the Novara province.

Surface water contamination. A high metal contamination in surface waters has been revealed by the monitoring campaign carried out in the period 1989–1995. This has been shown in particular for the Tancognino, La Grua and Agogna rivers.

Tancognino river in the period considered featured a dramatic metal contamination. Concentration values as high as 378 mg/L, 34 mg/L, 68 mg/L, 125 mg/L and 247 mg/L have been measured for Cr(III), Cr(VI), Cu, Ni and Zn, respectively, associated with pH values as low as 2.3 (see Table 2). Even if several samples showed small metal contents with values lower than law limits, most of the samples highlighted very high concentrations for several metals, clearly suggesting frequent discharges of industrial wastes from metallurgic plants in the Tancognino river. It is important to note that the law limits considered for the comparison are those referred to the discharge of waste in surface waters, which are higher than the law limits for the metal concentration of the water body itself. The La Grua river was also highly contaminated by metals, even if only Cu was determined to be systematically higher than the limit law. Agogna river is the receptor of both Tancognino and La Grua rivers. The metals content was high also for this river, with sporadic exceeding of the law limit for Cu, Zn, Al and Cr. The water quality of these rivers was considerably improved in the subsequent period, due to more strictly monitoring and control of industrial water discharges. Table 2 reports metal concentrations in the period 2012–2018, showing their decreased levels to values systematically lower than the limit law for all the metals considered.

Discharge in surface water, wastewater and soil. Results of the monitoring campaign carried out in the period 1989–1995 by collecting and analyzing samples at several point sources and discharging in the fresh surface waters of the Briga area also showed a severe metal contamination. Concentration values as high as 24.7, 68, 125 and 378 mg/L have been measured for Zn, Cu, Ni and Cr(III), respectively, with several exceeding the law limit for all of the metal considered, with the exception of Mn (see Table 3). These emissions clearly explain the high level of metal contamination found in fresh surface waters due to point discharges from metallurgic activities. Discharges in wastewaters collected at several point sources in the same period give a picture similar to the one presented for discharge in surface water; very high content of Zn, Cu, Ni and Cr(III) were found, in many cases exceeding the standards. The monitoring campaign of water discharges in soil carried out in the period 1989–1995 also showed severe metal contamination, in particular for Ni and Cu, as well as for Cd (see Table 3).

Groundwater contamination. In the period considered 2003–2008, the sampling at the piezometers, as well as at deep wells of the water supply systems, did not show exceeding in the law limits for metal concentrations (see Table 4). In particular, values measured in the period 2009–2018 in the Briga area do not reveal differences with respect to those reported for the Oleggio and Novara control areas. On the other hand, groundwater analysis from contaminated sites showed evidence of local metal contamination. In one contaminated site frequent exceeding the law limits for Al, Mn and Ni were observed, with

Table 1
Descriptive statistics of concentrations of metal(loid)s (mg/kg) in soil in Briga area, province of Novara and comparison with law limit and background value (BV).

	mg/kg	As	Cr	Cu	Ni	Zn	Pb	Sn
	Law limits [a]	20	150	120	120	150	100	1
Briga area (3*3km)	BV	14	150	51	130	105	55	4.3
	n. samples	25	25	25	25	25	25	25
	Mean	16.7	75.1	51.8	34.0	134.9	65.9	6.0
	Median	9.8	64.0	40.0	31.0	100.0	51.0	5.4
	Max	41.0	280.0	210.0	88.0	360.0	210.0	11.0
	n > law limit	8	1	1	0	8	3	25
	n > BV	9	1	7	0	11	10	20
Novara province (9*9km)	n. samples	16	16	16	16	16	16	16
	Mean	6.8	64.8	23.1	27.3	70.3	48.6	3.8
	Median	7.5	56.5	24.5	27.0	58.0	34.5	3.7
	Max	12.0	180.0	53.0	38.0	187.0	120.0	5.8
	n > law limit	0	1	0	0	1	2	16
	n > BV	0	1	1	0	1	5	3

[a] Law limits according to Table 1 annex 5 D.Lgs 152/2006.

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Table 2
Metal(loid)s concentrations (in μg/L) in the surface waters (Tancognino, La Grua and Agogna rivers) determined in the various monitoring campaigns of the Briga area.

Law limits [a]	As	Cd	Cr (III + VI)	Ni	Cu	Zn	Mn	pH
	≤500	≤20	≤2000	≤2000	≤100	≤500	≤2000	5.5–9.5
Tancognino river								
1989–1995								
n. samples	3	7	35	43	48	48	39	48
Mean	4	13	14900	17700	3100	1420	96	6.2
Median	5	5	860	5210	325	315	40	6.7
Max μg/L (Min)	5	4	378000	125000	68000	24700	1600	9.0 (2.3)
n > law limit (n < law limit)	0	0	16	27	29	18	0	0 (9)
2000–2008								
n. samples	1	20	40	20	20	20	20	–
Mean	<3	<0.5	3.9	8.3	7.0	13.3	5.2	–
Median	<3	<0.5	2.2	5.2	6.8	10	5	–
Max	<3	<0.5	24	52	13	38	7.8	–
n > law limit	0	0	0	0	0	0	0	
La Grua river								
1989–1995								
n. samples	–	–	20	20	22	23	12	25
Mean	–	–	91	280	180	120	24	7.1
Median	–	–	45	180	85	90	20	6.9
Max	–	–	670	800	1190	430	60	8.6
n > law limit	–	–	0	0	10	0	0	0
2012–2018								
n. samples	12	31	39	31	31	31	31	61
Mean	<3	0.34	3.5	6.8	8.1	43.0	16.1	6.9
Median	<3	<0.5	2.2	4.5	7	<10	5	7
Max	<3	<0.5	24	52	23.2	920	300	7
n > law limit	0	0	0	0	0	0	0	0
Agogna river								
1989–1995								
n. samples	6	0	19	22	19	27	15	28
Mean	2.8	–	156	186	564	198	87	6.77
Median	2.5	–	40	140	40	0.07	40	6.93
Max	5	–	1240	680	4650	1480	360	8.68
n > law limit	0	–	3	0	4	3	0	3
2012–2018								
n. samples	12	48	48	48	48	48	48	53
Mean	<3	0.40	2.1	1.9	5.0	34.9	7.7	7.03
Median	<3	<0.5	<2	<2	<5	37	<5	7.05
Max	<3	<0.5	3.9	3.6	7	67	44.7	7.7
n > law limit	0	0	0	0	0	0	0	0
2012–2018 Armeno – control								
n. samples	37	169	120	169	121	122	121	119
Mean	3	<0.5	<2	2.2	<5	81.2	7.9	6,9
Median	<3	<0.5	<2	<2	<5	89	<5	7
Max	3.2	0.7	<2	10	8.9	137	116.8	7
n > law limit	0	0	0	0	0	0	0	1

[a] Law limits refer to the limit for the discharge in surface water according to Table 3 annex 5 D.Lgs. 152/2006.

values as high as 1260, 780 and 270 μg/L, respectively. In a second contaminated site frequent exceeding in the law limits were determined for Cr and Ni, whereas in a third contaminated site exceeding in the law limits were determined for Ni and Mn. "Cascina Beatrice" was an extended wetland area of about 350,000 m^2 in which flowed the Tancognino River. This site was classified as contaminated area in the Regional Registry of Contaminated Sites (see reference) due to a large metal contamination of soil and groundwater. Indeed, the monitoring campaign conducted in the period 2004–2008 using a network of 11 piezometers showed a severe metal contamination for Al, Ni, and Mn, with values as high as 3190, 203, 1260 μg/L, respectively, as well as a contamination above law limits for Cu. The study conducted in the period 2003–2008 highlighted the presence of site-specific metal contamination (Al, Mn, Ni, Cu) even if it was not demonstrated diffuse metal contamination of deep groundwater in the Briga area.

3.2. Genetic analyses

To test whether genetic mutations are clustered among the ALS patients belonging to the area of high ALS incidence (Briga area), we performed a mutational screening for the main ALS genes in the ALS patients belonging to the subset of ALS patients of Briga area and the rest of Novara province and referred to the tertiary ALS Centre in Novara. DNA samples were available for 11 out of 35 ALS patients belonging to

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Table 3
Metal(loid)s concentrations (in μg/L) of discharge in surface water, groundwater and soil determined in the various monitoring campaigns of the Briga area.

Discharges in fresh surface waters in Briga area 1990–1995									
	As	**Cd**	**Cr(III)**	**Cr(VI)**	**Ni**	**Cu**	**Zn**	**Mn**	**pH**
Law limits [a]	≤500	≤20	≤2000	≤200	≤2000	≤100	≤500	≤2000	5.5–9.5
n. samples	3	7	35	36	43	48	48	39	15
Mean	4	10	14920	4450	17740	3130	1420	1000	6.22
Median	5	5	860	3950	5210	325	315	40	6.70
Max (Min)	5	40	378000	34200	125000	68000	24700	1600	9.01 (2.25)
n > law limit (n < law limit)	0	1	16	20	27	29	18	0	(9)
Discharges in wastewaters in Briga area 1990–1995									
	As	**Cd**	**Cr (III)**	**Cr(VI)**	**Ni**	**Cu**	**Zn**	**Mn**	**pH**
Law limits [a]	≤500	≤20	≤4000	≤200	≤4000	≤400	≤1000	≤4000	5.5–9.5
n. samples	–	–	9	1	3	10	7	7	15
Mean	–	–	1110	20	820	910	3230	130	7.10
Median	–	–	70	6	110	120	150	90	6.8
Max (Min)	–	–	5470	730	5220	18100	60000	760	12.5 (5.01)
n > law limit	–	–	1	2	1	4	2	0	3
Discharges in soils in Briga area 1990–1995									
	As	**Cd**	**Cr (III)**		**Cr(VI)**	**Ni**	**Cu**	**Zn**	**Mn**
Law limits [b]	≤50	n.p. [c]	≤1000 Cr(tot)		≤200	≤100	≤500	≤200	6.0–8.0
n. samples	1	1	6	3	4	10	12	8	13
Mean	1	1	120	180	410	96860	10030	420	8.47
Median	20	70	70	10	120	170	120	220	8.79
Max (Min)	20	70	300	510	1390	961000	112500	1900	12.00 (5.81)
n > law limit	0	1	0	0	1	5	3	4	9

[a] Law limits refer to the limit for the discharge in surface water and wastewater according to Tab 3 annex 5 D.Lgs. 152/2006.
[b] Law limits refer to the limit for the discharge in soil according to Tab 4 annex 5 D.Lgs. 152/2006.
[c] n. p. means not permitted.

Briga area, and for 42 out of 71 patients from the rest of Novara province, enrolled from the previous incidence study (Tesauro et al., 2017).

Regarding the Briga area, we found only one mutation in 1 patient, namely the GGGGCC pathological expansion in *C9ORF72* gene, representing the most common mutation in sporadic and familial ALS cases in the Piedmont region (Chiò et al., 2012, 2020) as well as worldwide (Brown and Al-Chalabi, 2017). In the larger reference group of patients living outside the Briga area, 6 patients carried the GGGGCC pathological expansion in *C9ORF72* gene, 1 patient carried a known missense mutation (p.L144F) in *SOD1* and 3 patients carried intermediate alleles (>30 CAG repeats) at *ATXN2* CAG repeat *locus*.

Altogether, these data do not identify any genetic defect in the main ALS genes clustered in ALS patients belonging to the Briga area.

4. Discussion

Interactions between genes, lifestyle and environmental factors are proposed to contribute to ALS etiology (Ingre et al., 2015; Cicero et al., 2017; Wang et al., 2017; Castanedo-Vazquez et al., 2019; Swash and Eisen, 2020). In the present study, we took advantage of an area (Briga area) with a higher ALS incidence compared to Novara province (Italy), and the rest of Europe, to analyze the role of genetic factors and of metal(loid)s in ALS.

Case-control studies and meta-analyses report associations (positive or inverse) between increased risk of ALS and different metals (Roos et al., 2013; Bocca et al., 2015; Peters et al., 2016; Sheykhansari et al., 2018; Filippini et al., 2020b).

Based on recent and previous data on metal exposure and the possible risk to develop ALS, we have performed an analysis of chemical contamination of environmental matrices in a heavily polluted area of the Novara province (Briga area).

The main findings of this study are the demonstration that in the Briga area the most common metal contaminants are Cu, Zn, Cr, Ni (widely used in tip-plating processes), that are highly present and above law limits in surface, discharge waters, and soil. In addition, other metals and metalloids, such as Cd, Pb, Mn, and As, although not frequently assessed in monitoring campaigns, show a severe contamination in the same area.

Results of genetic analyses on available DNA samples show that ALS patients in the Briga area did not carry recurrent mutations or an excess of mutations in the major ALS related genes. Indeed, 1 out of 11 ALS patients carried the pathogenic hexanucleotide GGGGCC expansion on *C9ORF72*, the most common mutation in familial ALS (34.2%), but also found in sporadic cases (5.9%) worldwide (van Blitterswijk et al., 2012). Moreover, these data are consistent with frequency reported in a mutational analysis on a population-based ALS registry in the same region (Chiò et al., 2012).

We cannot exclude that mutations in other minor causative ALS genes are clustered in these patients and thus associated with the high ALS incidence in the Briga area. Nevertheless, the results showing no recurrent mutations on 10 out of 11 sALS are consistent with the hypothesis according to which environmental exposure may be implicated in the higher incidence of the disease in this area.

Different environmental matrices are connected to each other through powders, particulate matters (PM), dry deposition, evaporation processes, rain, snow, wind and other factors. In this way, pollutants can transfer in unpredictable concentrations and reach the humans through ingestion, inhalation or contact. This aspect has to be taken into account as possible synergic factor, particularly where heavy and diffused pollution occurs, as in the observed Briga area. The same transfer mechanism can apply to the metals highlighted as heavy contaminants in this study.

We collected data on air quality and PM but not strictly referred to the Briga area, and for this reason the data are not shown. Po Valley, where Briga area is located, is one of the most developed and industrialized area in Italy, characterized by a heavy air pollution. Negative health outcomes from outdoor pollution and particularly from PM exposure are well documented (Lucchini et al., 2012; Santurtun et

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Table 4
Metal(loid)s concentrations (in μg/L) of deep groundwater (drinking water) determined in the various monitoring campaigns of the Briga area.

Deep Groundwater in Briga area 2003–2008							
	As	**Cd**	**Cr(III + VI)**	**Ni**	**Cu**	**Zn**	**Mn**
Law limits[a]	10	5	50	20	1000	3000	50
n. samples	19	19	19	19	19	1	19
Mean	<0.8	<0.12	1.2	1.6	11.9	<50	1.9
Median	<0.8	<0.12	<1.2	1	<5	<50	<1.2
Max	<0.8	<0.12	2	4	68	<50	7
n>law limit	0	0	0	0	0	0	0
Deep Groundwater in Briga area 2009–2018							
	As	**Cd**	**Cr(III + VI)**	**Ni**	**Cu**	**Zn**	**Mn**
Law limits[a]	10	5	50	20	1000	3000	50
n. samples	16	68	80	79	79	79	80
Mean	<3	<0.5	2.7	2.9	11.0	57.7	5.5
Median	<3	<0.5	<2	<2	<5	<50	<5
Max	<3	<0.5	<5	14.3	73	163	27
n > law limit	0	0	0	0	0	0	0
Deep Groundwater in Novara and Oleggio (control) areas 2009–2018							
	As	**Cd**	**Cr(III + VI)**	**Ni**	**Cu**	**Zn**	**Mn**
Law limits[a]	10	5	50	20	1000	3000	50
n. samples	6	28	34	34	28	34	34
Mean	<3	<0.5	3.9	2.82	<5	<50	9.54
Median	<3	<0.5	4.9	<2	<5	<50	<5
Max	<3	<0.5	7.1	5	<5	<50	73
n>law limit	0	0	0	0	0	0	2

[a] Law limits refer to the limit for the groundwater according to Tab 2 annex 5 D.Lgs. 152/2006.

al., 2016), while studies on indoor PM are emerging. Only in recent years, it has been recognized that indoor concentrations of many pollutants, including particles, are the sum of outdoor contaminants and of those produced directly indoor (Smielowska et al., 2017).

An occupational exposure to metals is expected in tap production units, representing the main economic sources of the study area. Many different works describe the occupational risk factors, including metal exposure, and ALS (see among others, Bar-Sela et al., 2001; Capozzella et al., 2014; Gunnarsson and Bodin, 2018, 2019; Filippini et al., 2020b). The observed increased disease risk for women, and particularly for elderly women, is peculiar for the Briga area when compared to other studies. Women in past decades traditionally supported at their homes familial metallurgic activities in the process of tap manufacture (Rabellotti, 2003), thus being exposed to metals both through outdoor and indoor activities (i.e., supporting working activity and usual house works). Referred to indoor exposure, a recent study demonstrates that the concentrations of nanoparticles in indoor environments are greatly underestimated and related to domestic activities (Manigrasso et al., 2017). These particles can be translocated to the brain through olfactory bulb and play an important role in the etiopathogenesis of neurodegenerative diseases (i.e. Alzheimer's disease) (Manigrasso et al., 2019). In addition, translocation through circumventricular organs that lack a blood-brain barrier is possible (Kakaroubas et al., 2019).

As firstly described in Guam population for brain tissues (Gellein et al., 2003), toxic metals, such as Cd, are found in *locus ceruleus* (LC) neurons from human autopsies (Pamphlett et al., 2018), and in the olfactory bulbs of experimental animals exposed to Cd through diet or inhalation (Sunderman, 2001). The primary olfactory neurons represent a likely way to transport Cd to their terminations in the olfactory bulb and into the brain (Arvidson, 1994). In the nervous system, Cd tends to accumulate in the choroids plexus at concentrations much greater than those found in the cerebrospinal fluid (CSF) and in other brain areas (Pamphlett and Kum Jew, 2013; Wang and Du, 2013, and references therein).

Due to their physiological functions (Sirabella et al., 2018), metal homeostasis is strictly regulated as their accumulation or deficiency leads to human diseases. In particular, ion dyshomeostasis and metal excess resulting in oxidative stress have been proposed as the main triggers of the cascade of events leading to motor neurons degeneration and loss. Defects in cellular Ca^{2+} signaling, and Zn^{2+} and Cu^{2+} homeostatic regulation are reported in ALS cases (Sirabella et al., 2018). Intracellular Zn concentration is strictly regulated by Zn importers, exporters, and binding proteins such as metallothioneins. Neuronal injury as a consequence of *in vivo* Zn mobilization and release occurs in several neurological disorders, including ALS (Bitanihirwe and Cunningham, 2009). In addition, Zn and Cu are metals with structural the first and catalytic functions the second in Cu/Zn-superoxide dismutase 1 (SOD1). This enzyme is mutated in around 20% of familial ALS cases, leading to a strong reactive oxygen species (ROS) generation. The increased ROS production, among which the superoxide anion, and the interaction with cell signaling pathways are possibly responsible of DNA damage and apoptosis of motor neurons. Thus, it is suggested that mutations do not cause ALS *via* loss of function, rather than through the alternate toxic gain of function (Hilton et al., 2015; Sirangelo and Iannuzzi, 2017).

Interestingly, Cd, among the metals present with a severe contamination in the study area of this work, is an inactive-redox heavy metal acting with mechanisms described as 'Trojan-horse' (Martelli et al., 2006). Cd is able to activate neuronal-specific and non specific mechanisms possibly involved in neurodegeneration in a neuronal human model (Forcella et al., 2020), and chronic exposure even to low level concentrations of Cd are able to induce dysregulation of essential metals such as Cu and Zn (Urani et al., 2015; Satarug et al., 2018).

Metal ion homeostasis is crucial for correct brain functions, and excess metal levels leading to metal dyshomeostasis have been proven to be a possible risk factor for neurodegenerative diseases including ALS, as demonstrated by chemical distribution of trace elements in post-mortem brain tissues (Gellein et al., 2003; Grochowski et al., 2019). In addition, to the causative role of metal dyshomeostasis in the process of oxidative damage, it is known the role of SOD1 mutation on Cu dysregulation in ALS, and the role of demetalled SOD1 in aggregates formation, typical hallmarks of ALS (Sirabella et al., 2018). These processes suggest a mutation-like effect of metal dysregulation in the formation of a non-functional SOD1 that could be one leading cause in oxidative stress generation of motor neurons.

Some limitations of this study should be noted, including the relatively small number of patients analyzed for genetic variants, and the absence of a thorough history of all possible environmental and other factors to which patients might be exposed. Only limited information was available, such as the exposure to solvents, some hobbies (photography, painting, hunting), occupation (welders, farmers, metallurgic workers), and some dietary habits. However, this information suggests important clues in relation to ALS etiology, such as photography and metallurgic occupation to be further taken into account.

5. Conclusions

This study supports the hypothesis that the previously described ALS cluster in Briga area may be related to environmental metal contamination, even supported by genetic analyses, although other environmental factors could have contributed to such disease excess.

Briga area is in fact one of the most important world tap manufacture district involving most people in that kind of activity, thus, being

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occupationally exposed to metals. This population during life span was exposed to indoor and outdoor environment, characterized by an elevated metal contamination.

An aspect to be implemented or included in future analyses regards the information about food exposition. The study design of the present work refers to previous epidemiological data collection not comprising specific information on metal dietary intake. Additionally, as observed in our chemical data collection in a very long period on the different matrices, the study area shows a substantial improvement of the environmental quality. As a consequence, it will be interesting to note whether a decreased ALS incidence rate will be observed, especially in the long term follow-up, also supported by further analyses able to identify genetic polymorphism. The outcomes of the new research may yield to key aspects into the etiology of ALS.

Despite the numerous studies carried out so far, the main risk factor(s) of this lethal neurodegenerative disease are still unknown. However, the interplay between different approaches and scientific visions (environmental, chemical, epidemiological, genetic, mechanistic) provides not only new insights to the comprehension of the disease, but also new hypothesis-driven approaches for stimulating future studies.

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Credit authors statement

Marina Tesauro: Conceptualization, Formal analysis, Investigation, Data collection and elaboration, Writing-original draft, writing-review & editing; Maurizio Bruschi: Conceptualization, Formal analysis, Investigation, Data elaboration, Writing-original draft; Tommaso Filippini: Data elaboration and review; Sandra D'Alfonso: Data collection and elaboration, Writing-original draft, writing-review, Funding acquisition; Letizia Mazzini: Data collection, writing-review, Funding acquisition; Lucia Corrado: Data collection and elaboration, Funding acquisition; Michela Consonni: Data collection, writing-review; Marco Vinceti: Writing-review; Paola Fusi: Conceptualization, review; Urani Chiara: Conceptualization, Formal analysis, Investigation, Writing-original draft, writing-review & editing, Funding acquisition.

Uncited references

; ; ; Decreto Legislativo 3 aprile, 2006; Gunnarsson et al., 1992; ; Swash and Eisen, 2020; Vinceti et al., 1997.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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ARPA Piemonte Rete di Monitoraggio per le Acque Sotterranee della Regione Piemontehttp://www.arpa.piemonte.it/approfondimenti/temi-ambientali/acqua/rete-di-monitoraggio/rete-di-monitoraggio-delle-acque-sotterranee

ARPA Piemonte registry of contaminated sites of Piemonte regionhttps://www.regione.piemonte.it/web/temi/ambiente-territorio/ambiente/amianto-bonifiche-terre-rocce-scavo/datipiemonteit-anagrafe-dei-siti-contaminati

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