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Blood metal levels and amyotrophic lateral sclerosis risk: a prospective cohort

Running head: Blood metal levels and ALS risk

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ABSTRACT

Objective: Metals have been suggested as risk factor for amyotrophic lateral sclerosis (ALS), but only retrospective studies are available to date. We compared metal levels in prospectively collected blood samples from ALS patients and controls, to explore whether metals are associated with ALS mortality.

Methods: A nested ALS case-control study was conducted within the prospective EPIC cohort. Cases were identified through death certificates. We analyzed metal levels in erythrocyte samples obtained at recruitment, as biomarker for metal exposure from any source. Arsenic, cadmium, copper, lead, manganese, mercury, selenium and zinc concentrations were measured by inductively coupled plasma-mass spectrometry. To estimate ALS risk, we applied conditional logistic regression models.

Results: The study population comprised 107 cases (65% female) and 319 controls matched for age, sex and study center. Median time between blood collection and ALS death was 8 years (range 1-15). Comparing the highest with the lowest tertile, cadmium (odds ratio (OR) 2.04, 95% confidence interval (CI) 1.08-3.87) and lead (OR 1.89, 95%CI 0.97-3.67) concentrations suggest associations with increased ALS risk. Zinc was associated with a decreased risk (OR 0.50, 95%CI 0.27-0.94). Associations for cadmium and lead remained when limiting analyses to non-current smokers.

Interpretation: This is the first study to compare metal levels before disease onset, minimizing reverse causation. The observed associations suggest that cadmium, lead and zinc may play a role in ALS etiology. Cadmium and lead possibly act as intermediates on the pathway from smoking to ALS.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a relentlessly progressive and fatal neurodegenerative disease involving the central and peripheral nervous system of, as yet, unknown etiology. The disease is characterized by loss of motor neurons, resulting in progressive muscle weakness, swallowing difficulties, and finally death due to respiratory failure. Most patients die within three to four years after the first symptoms, although survival is variable.[1] ALS is the most common adult onset motor neuron disease with an estimated incidence of 2.6 to 3.0 per 100.000 person years.[1]

Exposure to metals has been suggested as a possible risk factor for ALS for many years.[2, 3] Exposures may occur in a wide range of occupational settings, for example welding, plumbing, manufacturing, metal smelting and refining, but also occur in the general population through contaminated food or drinking water, air pollution, cigarette smoking, medication or dietary supplements.[4, 5]

Previous studies on the association between metal exposure and ALS risk were limited by cross-sectional or case-control designs, where metal concentrations were measured only after disease onset.[e.g. 6-8] As such, these studies did not allow inference on temporality and were unable to rule out reverse causality. Observed differences in blood metal levels in ALS patients could be the consequence of disease progression, rather than representing pre-disease exposure levels. For example, reduced physical activity resulting from widespread muscle weakness (a key clinical feature of ALS), may give rise to an increase in bone turnover leading to a raised release of lead blood levels. Metals have also been found in the locus coeruleus and motor neurons of ALS patients, but the significance of this finding in disease etiology remains unclear.[9]

To determine whether increased burden of metals contributes to the etiopathogenic mechanisms leading to ALS, and not result from the disease, prospective data are needed. A prospective design, however, is challenging for relatively rare diseases such as ALS. The EPIC cohort, with more than 500,000 subjects and a follow-up time of up to 15 years, offered a unique opportunity to compare pre-diagnostic blood metal levels of ALS cases and controls. For the first time, results from an ALS case-control study nested in a prospective cohort are presented here. We hypothesize that ALS patients

have increased blood metal levels before disease onset, including the neurotoxicants lead, manganese and mercury, and possibly also arsenic, cadmium, copper, selenium and zinc.

METHODS

Study population

A nested case-control study was conducted within the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort.[10, 11] This cohort is a large prospective study initiated in 1992 which was designed to investigate the relationship between diet, lifestyle and environmental factors and the incidence of cancer and other chronic diseases. Recruitment took place between 1993 and 1999. In total, over half a million (520,000) people, with the vast majority aged between 35 and 70 years, were recruited in ten European countries: Denmark, France, Germany, Greece, Italy, the Netherlands, Norway, Spain, Sweden and the United Kingdom. Data on diet and lifestyle was obtained through structured questionnaires at baseline. At the same time, anthropometric measurements and blood samples were taken.

The EPIC study was approved by the ethical committee of the International Agency for Research on Cancer (IARC) and by the ethical review boards of each participating center. All participants signed an informed consent.

ALS cases were defined as those subjects for whom "motor neuron disease" (G12.2 according to the 10th revision of the International Statistical Classification of Diseases, Injuries and Causes of Death) was reported as an immediate, antecedent or underlying cause of death. In this and other ALS studies, mortality has been shown to be a good proxy for disease incidence, given the almost invariable 100% fatality rate and the relatively short duration of the disease.[12] A total of 168 people who died from ALS had been identified within the EPIC cohort after a median follow-up time of 8 years (range 1 to 15). Blood samples from the Nordic countries were not available in the central biobank, resulting in 107 cases included for analyses. Three controls per case were selected by incidence density sampling matched by age at recruitment, sex and study center (to account for center specific differences in questionnaire design, blood collection procedures, etc.).

Biological samples

A 30 ml sample of blood was obtained from each participant at recruitment into the study. Plasma, serum, erythrocytes, and buffy coat were separated by centrifugation, aliquoted into 0.5 ml plastic

straws and stored in liquid nitrogen at -196°C until they were picked for analyses. Metals are to a large extent bound to erythrocytes in blood. Given the relatively long lifetime of erythrocytes (around 3 months), erythrocyte concentrations of these metals have been shown to be a relevant biomarker of ongoing exposure.[4]

The erythrocyte samples were prepared for inductively coupled plasma-mass spectrometry (ICP-MS) analysis by a direct alkali dilution method.[13] Briefly, samples were diluted 1:25-50 with an alkali solution consisting of 2% butanol (Honeywell Research Chemicals, Seelze, Germany), 0.05% EDTA (Sigma-Aldrich, St. Louis MO, USA), 0.05% Triton X-100 (Sigma-Aldrich), 1% NH_4OH (Romil, Cambridge, UK) and 20 µg/g internal standards. Before analysis, the diluted samples were centrifuged at 2000 rpm for 5 min.

The Agilent 7700x ICP-MS (Agilent Technologies, Tokyo, Japan) equipped with an octopole reaction system (ORS) collision/reaction cell technology was used for measuring concentrations of eight elements (isotope): arsenic (^{75}As), cadmium (^{111}Cd), copper (^{63}Cu), lead (^{208}Pb), manganese (^{55}Mn), mercury (^{202}Hg), selenium (^{78}Se) and zinc (^{66}Zn). The elements germanium (^{72}Ge), rhodium (^{103}Rh), lutethium (^{175}Lu), and iridium (^{193}Ir) were included as internal standards. The majority of the elements were measured in helium mode (^{55}Mn, ^{63}Cu, ^{66}Zn, ^{75}As, ^{111}Cd; gas flow 5ml/min), whereas ^{78}Se was measured in hydrogen mode (gas flow: 5-6 ml/min) and ^{202}Hg and ^{208}Pb in standard mode (no gas). One ppm of a gold standard (Sigma-Aldrich) was added to all solutions to stabilize mercury.

The limit of detection (LOD) for each element was determined as 3 x SD of analyzed blanks (alkali solution) and as signal/noise=3. The limit of quantification (LOQ) was determined as 10 x SD of analyzed blanks. As quality control, two commercially available whole blood reference materials: Seronorm level-1 WB, lot 1702821 and Seronorm level-2 WB, lot 1406264 (SERO, Billingstad, Norway) were analyzed (Supplemental Table 1). Blanks and reference materials were treated together with the collected erythrocyte samples and analyzed in the beginning, in the middle and at the end of each analysis.

Statistical analyses

Values of metal concentrations below the LOD were replaced by LOD/√2 [n=1 (0.2%) for cadmium and n=9 (2.1%) for mercury]. No values between LOD and LOQ were obtained. Metal levels were log-transformed to normalize their distribution.

Tertiles of metal concentrations were calculated based on the distribution among controls. Conditional logistic regression models for the matched case-control sets were applied to estimate the odds ratio (OR) and 95% confidence intervals (95% CI) of ALS in relation to each of the blood metal levels before disease onset. P values for linear and flexible spline trends were based on the continuous levels. We examined cigarette smoking, body-mass index, physical activity and alcohol consumption as possible confounding factors, but these variables did not modify the risk estimates (P>0.1) and were therefore not included in the models. We also examined educational level as possible confounder, since lower education has been suggested to be associated with higher risk of ALS, although this association was largely explained by smoking.[14] In our data, educational level was highly unbalanced across study centers and we observed no consistent association. As such, we decided not to adjust for education in our models.

We performed several sensitivity analyses to test the robustness of our findings, as follows: i) exclude cases who died within 3 years (median survival time from symptom onset) of recruitment to further minimize possible reverse causation; ii) exclude current smokers at baseline, since cigarette smoking is a recognized source of metals and a risk factor for ALS[12, 15]; and iii) exclude ever smokers. For the analyses on non-current and never smokers, unconditional logistic regression models were applied to preserve statistical power, adjusted for the matching variables sex, age at recruitment and study center.

Exposure to metals may result, among other sources, from cigarette smoking or dietary intake (*e.g.* methylmercury and selenium from fish and selenium and cadmium from cereals). We, therefore, looked at the correlations between metal levels to identify patterns in our data (with smoking status or intakes of fish/shellfish and cereal).

All statistical analyses were carried out in SAS v.9.4 (SAS Institute, Cary, North Carolina, USA). Ethical approval for the project has been provided by the International Agency for Research on Cancer (IARC) Ethics Committee.

RESULTS

The study population comprised 107 cases (65% female) and 319 controls (Table 1). The median age at recruitment was 60 and the median time between blood collection at recruitment and ALS death was 8 years (ranging from 1 to 15 years). Among the controls, 13% were smokers at baseline, compared with 16% among cases.

Table 2 shows the erythrocyte metal concentrations (ng/g) for both cases and controls. Correlations between metal concentrations as well as with possible determinants of exposure are presented in Table 3. Exploration of possible sources of metals indicated correlations between particularly cadmium and to a lesser extent lead levels with cigarettes per day, as well as arsenic, mercury and selenium levels with fish/shellfish consumption. No correlation was observed between metal concentrations and cereal consumption. Among cases, no association between the metal concentrations and time to ALS death was observed (data not shown).

Logistic regression models suggested that cadmium and lead levels were associated with an increased risk of ALS (Table 4). The ORs for tertiles of cadmium levels were 2.16 (95% CI 1.18-3.97) and 2.04 (95% CI 1.08-3.87) for the second and third tertile compared to the first, respectively. The corresponding ORs for lead were 1.83 (95% CI 0.99-3.35) and 1.89 (95% CI 0.97-3.67), respectively. No strong trends on the continuous scale were observed. In the opposite, zinc levels were associated with decreased risk for ALS (highest vs lowest tertile: OR 0.50, 95% CI 0.27-0.94).

Sensitivity analyses excluding cases who died within three years of recruitment (n=13) revealed virtually the same results (Table 4). An interaction of metal levels with smoking status and the risk for ALS was observed for cadmium (p=0.009), but not for lead (p=0.626). The observed associations for both cadmium and lead remained when excluding current smokers at baseline, showing a clear linear

trend for cadmium (p=0.023) (Table 5). These associations weakened when further limiting the models to never smokers, but the numbers became small. No interactions between zinc and cadmium or zinc and lead were observed.

DISCUSSION

Our study is the first prospective study investigating blood metal levels and the risk of ALS to date. The metal concentrations in blood from ALS patients, before disease onset, and controls indicated that lead and cadmium may be associated with an increased risk of ALS, with evidence of an inverse association for zinc.

Previously observed positive associations between lead level and risk of ALS were based on the comparison of (post-disease onset) blood levels,[6, 7, 16] as well as on assessments of occupational exposure to lead, using registry data.[17] Our data support these observations. The relatively small magnitude of increase in risk observed for lead in our study may reflect the multifactorial nature of ALS, in which lead is one of the potential steps in the multistage etiologic process leading to the disease.[18] Among relevant etiologic factors, genetic variants have been discussed in the literature, such as the highly penetrant *C9orf72* repeat expansion. The *C9orf72* repeat expansion is present in about 8% of the sporadic ALS cases in European populations,[19] but unfortunately, we had no information on genetic variants.

Further associations between other metals and the risk of ALS have also been reported,[3] but much less studied. Our new findings, particularly for cadmium and zinc, warrant further research.

Cigarette smoking is one of the few established risk factors for ALS, which also appeared to be a risk factor in our study population.[12] The mechanisms or the causing components of cigarette smoke for the development of ALS, however, are still unclear. Cigarette smoking is a recognized source of metals: the tobacco plant takes up cadmium[20] which is inhaled by the smokers and smokers were shown to have higher cadmium blood levels than non-smokers.[21] Smoking is also associated with higher blood lead levels and with lower selenium and zinc levels.[22] This pattern was also reflected in our data. We

nevertheless observed the same associations with blood metal levels among participants who gave up smoking or never have smoked cigarettes, suggesting a possible role of cadmium and lead in ALS etiology, independent of cigarette smoking. Future studies should explore mechanisms of actions by conducting formal mediation analysis.

Our study is the first ever to investigate metal levels in blood samples from ALS patients collected prior to disease and to compare these samples to those from matched controls from the same source population. The prospective design of the EPIC study allowed an insight on temporality, arguing against reverse causality which may be suspected based on results of classical case-control studies. Similar to the blood samples, the data on lifestyle factors such as smoking have also been collected before the onset of disease, ruling out the distortion of risk estimates by recall bias. The lack of association between metal levels and time to ALS death confirms this assumption.

Survival of ALS is highly variable, ranging from a few months to over ten years.[1] In our study population we had no clinical data to confirm the time of onset. As such we cannot be sure that all included samples were truly pre-symptomatic. Sensitivity analyses limiting to those who died more than three years after recruitment resulted in similar results, but also a three-year cut-off remains arbitrary. Some excluded participants may have been without symptoms at time of blood collection, while among those who remained in the analyses there may have been long survivors for whom the disease process started already before recruitment. Furthermore, although EPIC is a large cohort, our overall number of ALS cases was not high due to the rarity of the disease. Our study would have benefitted from a larger number of cases, which could be achieved by extended follow-ups of mortality in the future. Larger numbers would also have allowed for further restricting analyses by time till death.

The lifespan of erythrocytes is typically 120 days in healthy adults.[23] The observed metal concentrations therefore reflect only exposure close to the time point when the blood samples were collected. The observed concentrations, however, will be correlated to the overall retention of the metals in the body, unless people drastically changed their exposure conditions. The latter is unlikely, since we analyzed blood samples from subjects before disease onset. As such, our data will have well reflected the relative ranking of exposure levels of both cases and controls. Possibly, we have

underestimated occupational exposures to metals, which may have been higher in the more distant past. Given the typically low prevalence of high metal exposed workers in the general population,[24] however, our results are unlikely to be affected by missing possible occupational exposures.

The coefficient of variation for cadmium was relatively high for the low-level reference sample with 34% (Supplemental Table 1), whereas the coefficients for the other elements were all below 15%. Cadmium showed somewhat unstable signals close to LOD. We therefore categorized the metal concentrations into tertiles, to avoid giving samples with low concentrations and less confident measurements too much weight.

In conclusion, the metal concentrations in blood from ALS patients (obtained before disease onset) and controls indicate that cadmium and lead may be associated with an increased risk of ALS and zinc with a decreased risk. This is the first study to evaluate pre-disease metal levels in blood, thus minimizing reverse causation. Even though our results are inconclusive, we saw a positive association for lead, for which there was the strongest *a priori* evidence. Our observations suggest that these metals may play a role in ALS etiology and warrant further studies in other populations, although few cohorts have stored pre-symptomatic erythrocytes.

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Author Contributions

SP, VG, PV, LM, RCT, MMB, DP, SG, RT, AE, TV, FM, TK, VK, AA, FG, JHG, MRB, SM, AB, AT, MJ, EW, and RV contributed to the conception and design of the study; SP, KB, VG, ML, MK, PV, JV, LvdB, LM, RCT, MMB, DP, SG, RT, AE, TV, FM, TK, VK, AA, FG, JHG, MRB, SM, AB, AT, MJ, EW, and RV contributed to the acquisition and analysis of data; SP and RV contributed to drafting the text and preparing the figures.

Potential Conflicts of Interest

Nothing to report.

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Chapter 6 - Metals and neurodegeneration

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Content

1. Metals and the risk of neurodegeneration

1.1. Neurotoxicants

Exposure to a toxic agent can result in neurotoxicity, which is characterized by adverse changes in the structure or function of the nervous system. Several metals and metalloids have been identified as such neurotoxicants, including aluminium (Al), manganese (Mn), arsenic (As), mercury (Hg) and lead (Pb) (Klaassen 2008). Also cadmium (Cd) and selenium (Se) have shown toxic effects on nerve cells (Klaassen 2008). While excessive exposure to manganese and selenium are known to cause neurotoxicity, these metals are also nutritionally essential in the human body (Goyer et al. 2004). No beneficial effects are known for aluminium, arsenic, cadmium, lead and mercury (Goyer et al. 2004).

Roughly, neurotoxicity can be grouped into acute and chronic effects. Acute effects occur shortly after exposure, whereas chronic effects may become apparent many months, years or even decades later and generally result from repeated exposures at lower levels. Neurodegenerative disorders are an example of such chronic effects. It has been suggested that neurodegenerative diseases are partly caused by occupational and environmental exposures (Pearce and Kromhout 2014), which may include exposures to metals. Early life environmental exposures have also been implicated in neurodegeneration (Landrigan et al. 2005; Logroscino 2005). Evidence of possible associations between exposure to metals and the onset of neurodegenerative disorders is very limited. Here we summarize what is known to date.

1.2. Alzheimer's disease and other dementias

The largest epidemiological literature relates to aluminium and Alzheimer's disease, which is suggestive of an association, but not at all conclusive (Miu and Benga 2006; Tomljenovic 2011). The emphasis has been on aluminium exposure through drinking water, which is not likely to be the primary source of exposure (see 2.1). Moreover, most of these studies derived exposure estimates in an ecological design, *i.e.* assigning exposures at a group level rather than individually. Killin et al. (2016)

reviewed the literature regarding environmental risk factors for dementia, not limited to Alzheimer's disease, and concluded that there is moderate evidence for aluminium exposure to increase dementia risk. This positive association was mainly observed in the larger and better quality studies. Large, prospective longitudinal studies with a robust measure of exposure at baseline and a clinical diagnosis of dementia were considered of highest quality.

Cadmium has also been associated with Alzheimer's disease, but results are as yet not conclusive (Peng et al. 2017). Likewise, mercury has been suggested to have a role in the aetiology of Alzheimer's disease (Mutter et al. 2010). Occupational studies showed that chronic exposure to mercury affected memory and attention, which are relevant to Alzheimer's disease. No studies reporting on an association between mercury exposure and Alzheimer's disease specifically have been published. Results from autopsies have been inconclusive with regards to accumulation of mercury in the brain of Alzheimer patients (Mutter et al. 2010). Six epidemiological studies have explored the association between lead exposure and Alzheimer's disease (Chandra et al. 1987; French et al. 1985; Gun et al. 1997; Heyman et al. 1984; O'Flynn et al. 1987; Shalat et al. 1988), without finding an association. However, these studies all suffered from important limitations in exposure assessment.

Consistent positive associations between occupational metal exposure and non-vascular dementia have been found in a large prospective cohort study in the Netherlands (Koeman et al. 2015). No distinction between specific metals could be made, however, hindering to identify which metal could be accountable for the observed association.

1.3. Amyotrophic lateral sclerosis (ALS) and motor neuron diseases

The association between lead exposure and ALS risk has been studied in various case-control studies and most report a positive relationship, up to over a four-fold increase (Kamel et al. 2005; Wang et al. 2014). However, these studies were predominantly reliant on self-reported exposure. One of those

studies additionally presented risk estimates based on exposure assessment by (blinded) industrial hygienists, showing no association (McGuire et al. 1997). The latter exposure assessment method was considered more reliable, minimizing possible recall bias, *i.e.* bias that occurs when the diagnosis affects reporting of exposure by cases. Therefore, the observed increased risk of ALS due to lead exposure may have been influenced by this bias, whereas possibly no real association exists.

Roos et al. (2013) analysed metal concentrations in cerebrospinal fluid and blood plasma of ALS patients and compared these with controls. Concentrations of aluminium, cadmium, manganese and lead in cerebrospinal fluid of ALS patients appeared to be significantly higher. Such increases were not observed for blood plasma levels, suggesting accumulation of metals in cerebrospinal fluid (Roos et al. 2013). Other studies have reported elevated levels of lead in both blood and bone among ALS cases (Fang et al. 2010; Garzillo et al. 2014; Kamel et al. 2002), but a causal relation has not yet been established.

Similar to lead, McGuire et al. (1997) revealed no significant associations with ALS for exposure to aluminium, cadmium, manganese or mercury in their US case-control study when based on expert assessment of occupational exposures. With regards to selenium, an increased risk of ALS with increasing levels of selenium intake through drinking water was reported in an Italian case-control study (Vinceti et al. 2010).

(Sutedja et al. 2009) applied strict quality criteria to the methodology when reviewing epidemiological studies that looked into the association between metal exposure and ALS risk. Only studies based on objective exposure assessments (*i.e.* not self-reports) for full job histories or monitoring results collected repeatedly were considered sufficiently reliable. When these criteria were applied, no significant results were revealed for unspecified metals (Sutedja et al. 2009). Overall, evidence about associations between metal exposure and the risk of ALS is yet inconclusive.

1.4. Parkinson's disease and parkinsonism

Chronic exposure to manganese is known to cause 'manganism', which is a form of parkinsonism (Cannon and Greenamyre 2011). Exposure to welding fumes has been associated with parkinsonism and suggested as risk factor for Parkinson's Disease, possibly due to manganese in welding fumes (Antonini 2003; Racette et al. 2001; Racette et al. 2012). However, welding fume can contain many other metals (IARC 1990), and these other metals may also have added to the observed risks among welders (Racette et al. 2012).

Positive associations between bone lead levels and Parkinson's disease were observed in US case-control studies (Coon et al. 2006; Weisskopf et al. 2010). The relevant exposure window was possibly many years or decades before the onset of disease. This conclusion was drawn because the association with Parkinson's disease was found for lead levels in the more compact cortical bone, representing cumulative exposure to lead over many years, whereas no association was observed for lead levels in the less dense cancellous bone tissue (Weisskopf et al. 2010). Among female nurses an association has been reported between airborne mercury exposure and risk of Parkinson's disease, whereas no evidence was found for any association with other airborne metals (Palacios et al. 2014).

1.5. Other neurodegenerative disorders

For multiple sclerosis no evidence has been reported with regards to metals specifically (Cannon and Greenamyre 2011).

2. Occurrence of metal exposure

We will here describe the occurrence of metal exposure, focusing on the metals and metalloids that have been identified as neurotoxicants, as described in 1.1.

2.1 Aluminium

Occupational exposure to aluminium and its compounds predominantly occurs among workers in industries producing or using aluminium (Krewski et al. 2007). Another major intake source of aluminium is chronic use of aluminium-containing antacids, buffered aspirin and other medical preparations. Food and drinking water can also contain aluminium and air pollution is another possible source of exposure (Krewski et al. 2007).

2.2 Arsenic

Arsenic is ubiquitously distributed in the environment, *e.g.* in geothermal waters, weathering of rocks and forest fires. In addition to natural sources, humans encounter arsenic from manmade sources through soil, water, air and food.

Occupational exposure to arsenic may occur in pesticide manufacturing and application, wood preservation, smelting and refining metallic ores, semiconductor manufacturing, and glassmaking (Burgess 1995; Tchounwou et al. 2012). Non-occupational exposure to arsenic mainly occurs via food, with intake from air, water and soil being less substantial. However, exposure from water and soil may become significant in areas of arsenic contamination as a result of natural mineral deposits or mining sites, pesticide application or industrial waste disposal (Tchounwou et al. 2012).

2.3 Cadmium

Cadmium is used in several applications, including nickel-cadmium batteries; pigments used in plastics, ceramics and glass; stabilizers for polyvinyl chloride (PVC); engineering coatings on steel and non-ferrous metals; and as a component of various specialized alloys. The largest consumers of cadmium are Japan, the United States, Belgium, the United Kingdom, France and Germany.

Occupational exposure to cadmium may occur during production processes related to the aforementioned products (IARC 1993).

After occupational settings, cigarette smoke and contaminated food are the main sources of exposure (Tchounwou et al. 2012). About 10-15% of cadmium in the atmosphere originates from natural sources, predominantly from volcanic activity (IARC 1993), but most result from emissions from human sources. Cadmium levels in the air are higher in the proximity of cadmium-related industries.

2.4 Lead

Multiple sources can cause human exposure to lead. Its abundance and widespread usage makes lead a well-recognized environmental and occupational toxicant, particularly in the urban environment. Lead has been used in car batteries, gasoline, paints, ceramics, ammunition, pipes, solders, cosmetics, shielding for X-rays, and in corrosion and acid resistant materials (Sanders et al. 2009). Inhalation of contaminated dust particles or aerosols, and ingestion of contaminated food or water are the major routes of human exposure to lead (Tchounwou et al. 2012).

Elimination of lead in gasoline and the reduction of lead levels in paints, pipes, and food and drink cans significantly decreased lead exposure since the 1970s (Tchounwou et al. 2012). However, human exposure continues from deteriorating household paint in older homes, contaminated soil and drinking water, and from lead used in industrial processes or hobbies (Sanders et al. 2009; Tchounwou et al. 2012). Occupational exposures to lead may occur in a wide range of workplaces, such as in construction, printing, ceramic, glass making, primary and secondary metal manufacturing, electric and electronic industries, as well as through welding, firing ranges and waste incineration (Koh et al. 2014).

2.5 Manganese

Manganese is a nutritionally essential metal and diet is the major route of exposure for most people. The highest concentrations can be found in grains, rice and nuts, but manganese can also be a component of dietary supplements (Farina et al. 2013). Common sources of occupational exposure to manganese include welding processes, power plant and coke oven combustion and steel production (Farina et al. 2013; Klaassen 2008; Racette et al. 2012). Manganese exposure may also result from the fuel additive methylcyclopentadienyl manganese tricarbonyl (MMT), which has been used to enhance the octane in gasoline in many countries, including the US and Canada (Davis 1998).

2.6 Mercury

Occupational exposure may occur in chlorine-alkaline factories, thermometer factories and mercury extraction plants, as well as among dental care workers (Mutter et al. 2010). The latter is due to the application of dental amalgam fillings, containing mercury. People carrying such fillings, consisting of 50% of mercury, are also exposed. Mercury from fillings evaporates at a slow rate, but its release is faster when the fillings are put in place or removed (Mutter et al. 2010). Environmental exposure to mercury may results from volcanic activity, coal combustion and other industrial processes (Chen et al. 2016).

Organic mercury, methylmercury, accumulates in the aquatic food chain through waste water due to human activities (Hong et al. 2012). Fish is the major contributor to mercury exposure through people's diet, so populations relying on fish diets can be exposed to high levels of organic mercury (Mutter et al. 2010).

2.7 Selenium

Environmental selenium can occur both in organic and inorganic form. Selenium in food is essential in the organic form, whereas inorganic selenium can be found in drinking water and occupational settings (Vinceti et al. 2010). Occupational exposure to selenium has been described sparsely and can,

together with drinking water, be considered only a minor source of exposure. Large regional differences exist in the selenium concentrations in food systems, as the concentration highly depends on the soil selenium. Soil selenium is influenced by weathering of selenium containing rocks, volcanic activity and vicinity of coal burning (Combs 2001). Cereal grains, meats and fish are the largest contributors to the selenium content in most diets and food is also being enriched in selenium in some places (Combs 2001).

3. Mechanisms of chronic neurotoxicity

Neurotoxicity is influenced by age and genetics: certain gene variants affect an individual's ability to respond to metals (Caito and Ascher 2015). Major mechanisms are shared between virtually all neurodegenerative diseases and involve initiation or potentiation by neurotoxicants: blood-brain-barrier (BBB) disruption, protein aggregation, oxidative stress and mitochondrial dysfunction leading to neuroinflammation. Lipid soluble neurotoxicants can easily move across the biological membranes of the BBB by simple diffusion. Neurotoxicants that share chemical structures with endogenous molecules, but are not lipid soluble, can enter through carrier-mediated transport. If not lipophilic nor mimicking a biological entity, access to the central nervous system is not gained. However, in the majority of neurodegenerative diseases, the BBB is disrupted. Whether BBB disruption contributes to the pathogenic mechanism or is part of the pathology remains to be determined. In addition to BBB disruption, protein aggregation is a major unifying mechanistic theme in neurodegenerative diseases, each disease producing a characteristic aggregation pattern. Mostly cytoplasmic protein aggregation is a pathological feature observed in many neurodegenerative diseases. Oxidative stress (*i.e.* having too many reactive species for available antioxidants) results in biomolecular damage caused by an attack of these reactive species upon the constituents in living organisms. The last major feature of neurodegenerative diseases is mitochondrial dysfunction which typically occurs early in the disease process. Environmental toxicants that affect mitochondrial function have been linked to various

degrees epidemiologically to neurodegenerative diseases and have shown in animals to elicit neurodegeneration (Cannon and Greenamyre 2011).

3.1 Aluminium

Aluminium sensitive areas are the frontal cortex and the hippocampus (Maya et al. 2016). Aluminium has no physiological role, but toxic consequences are thought to be related to dysregulation of other essential metals or ions (Maya et al. 2016): increase in iron accumulation, the production of oxygen reactive species and induction of the formation of neurofibrillary tangles and a pro-inflammatory response (Chin-Chan et al. 2015; Maya et al. 2016; Yegambaram et al. 2015). Aluminium has mostly been studied in relation to Alzheimer's disease and mainly *in vitro*, where cultured rat cortical neurons show conformational changes in amyloid ß and enhanced aggregation with the formation of fibrillary deposits on the cultured neurons (Chin-Chan et al. 2015).

3.2 Arsenic

Arsenic is thought to influence cognitive function by alternating the amyloid pathway (Chin-Chan et al. 2015). Exposure to arsenic has been associated with brain inflammatory responses and oxidative stress through impaired cellular protein degradation and autophagy leading to intracellular protein aggregation and neurofibrillary tangle formation, extracellular accumulation of amyloid ß, reticulum endoplasmic stress and mitochondrial dysfunction (Chin-Chan et al. 2015; Escudero-Lourdes 2016)

3.3 Cadmium

Also for cadmium there is evidence linking exposure with amyloid ß overproduction and increased size and number of senile plaques in the cerebral cortex and hippocampus. It was suggested that both the non-amyloidogenic pathway as well as degradation of amyloid ß are targets of cadmium exposure. *In vitro,* cadmium causes self-aggregation of a Tau peptide leading to astrocyte and neural cell toxicity (Chin-Chan et al. 2015; Yegambaram et al. 2015).

3.4 Lead

Many mechanisms have been described by which lead could result in neurotoxicity. The absorption of lead depends on the specific compound to which an individual is exposed. Lead exposure targets various voltage- and ligand-gated ionic channels, such as the N-methyl-D-aspartic acid (NMDA) receptor, subtypes of the voltage- and calcium-gated potassium channels, cholinergic receptors and voltage-gated calcium channels, altering the cellular excitability and mainly resulting in an increase of the cytoplasmic calcium concentration (Garza et al. 2006; Wennberg 1994). Mechanisms described for nervous-system damage more specifically include lipid peroxidation, excitotoxicity, alterations in neurotransmitter synthesis, storage and release, interference with second-messenger systems, and damage to the astroglia and oligodendroglia (Garza et al. 2006). Histologically, myelin loss and axonal degeneration have been described (Wennberg 1994). Furthermore, lead is thought to alter the ability of astrocytes to regulate the excitotoxic glutamate levels (Struzynska 2009).

3.5 Manganese

Chronic manganese intoxication gives symptoms similar to Parkinson's disease. Manganese increasing the dopamine autoxidation seems one of the mechanisms behind the clinical picture of parkinsonism. Manganese toxicity causes a reduction in striatal concentrations of dopamine due to accumulation in the mitochondria (Cannon and Greenamyre 2011; Farina et al. 2013; Wennberg 1994). Elevated intra-mitochondrial manganese interferes with oxidative respiration, leading to excessive production of redox oxidative species and consequently mitochondrial dysfunction. Lastly, astrocytes function as major homeostatic regulators and as storage site for manganese. Increased accumulation of manganese in astrocytes has been shown to alter glutamate homeostasis and elicit neurotoxicity: it decreases astrocytic glutamate uptake and reduces the expression of the astrocytic glutamate:aspartate transporter leading to increased extracellular glutamate levels and neuronal excitability (Farina et al. 2013).

3.6 Mercury

In contrast to elementary mercury, of which only a certain amount passes the BBB where it is oxidated to mercury ions, organic mercury, mostly targets the central nervous system. Brain regions that are most severely affected by organic mercury toxicity are the cerebral and cerebellar cortices. Crucial events are the interaction of mercury with sulfhydryl-containing proteins (*i.e.* neurotransmitter receptors, transporters, antioxidant enzymes, etc.), as well as with non-protein thiols (*i.e.* glutathione, cysteine). These events alter protein function and lead to disturbed proper homeostasis of neuronal and glial cells. Mercury toxicity results in glutamate dyshomeostasis, namely extracellular glutamate levels are increased. Consequently, there is calcium influx leading to oxidative stress and mitochondrial collapse (Farina et al. 2013; Tasleem Jan et al. 2015).

3.7 Selenium

The differential toxicity and metabolism of various organic and inorganic selenium forms makes it improper to generalize the term 'selenium neurotoxicity', whereas each neurotoxic effect should be referred to as a specific poisoning by a specific selenium compound. However, the neurotoxicity of inorganic selenium is shown to exceed that of the organic form. In general, the neurotoxic effects inducible by selenium compounds include among others an increase of central nervous system dopamine levels and metabolites, alteration of cholinergic signalling and degeneration of cholinergic neurons, inhibition of glutamate uptake and prostaglandin D synthase, decrease of total antioxidant status, gangliosides and sulphydryl groups, activity of adenosine deaminase, succinic dehydrogenase and acetylcholine esterase, and finally increase of thiobarbituric acid reactive substances and lipid peroxidation (Vinceti et al. 2014).

3.8 Interaction between heavy metals

Metal mixtures are believed to play a role in the development of neurodegenerative diseases, potentially acting synergistically rather than displaying simple, additive effects (Yegambaram et al. 2015). Various metals share transporters or are controlled by overlapping signalling pathways. Neurotoxicity of perinatal and childhood mixed exposure to lead and other metals is well studied: lead and arsenic appear to have a synergistic effect on cognition and behaviour, whereas lead with cadmium, mercury or manganese seems to work in an antagonistic way (Chen et al. 2016). As stated previously, the toxic consequences of aluminium exposure are generally caused by the disruption of homeostasis of essential metals such as magnesium, calcium and iron. Aluminium can mimic the biological functions of these metals triggering biochemical alterations (Maya et al. 2016). Cadmium interacts with iron leading to decreased haemoglobin and haematocrit concentrations (Jaishankar et al. 2014) and selenium is thought to counteract neurotoxicity of other elements, *e.g.* mercury, cadmium and aluminium. The ability to form complexes might also induce a longer persistence of the elements, which possibly leads to long-term release of these heavy metals in the brain (Vinceti et al. 2014).

Table 1. Overview of metals that have been identified as neurotoxicants

Metal	**Evidence of association with neurodegenerative disorders**		**Major sources of human exposure**
	Suggested	Established	
Aluminium (Al)	AD, dementia (ns)		Contaminated drinking water or food, medication or supplements, industrial processes (production and use of aluminium), air pollution
Arsenic (As)	AD, dementia (ns)	-	Contaminated drinking water or food, industrial processes (e.g. pesticide manufacturing and application, smelting and refining, semiconductor manufacturing, glassmaking)
Cadmium (Cd)	AD	-	Tobacco smoking, plastic stabilizers, pigments, batteries, industrial processes (e.g. welding, plating)
Lead (Pb)	AD, dementia (ns), ALS, PD	-	Contaminated drinking water or food, tobacco smoking, paint, gasoline, industrial processes (e.g. ceramics, soldering, plumbing, firing ranges)
Manganese (Mn)	AD, dementia (ns), PD	Parkinsonism	Food, gasoline, pesticides, industrial processes (e.g. steel factories, welding, mining plants)
Mercury (Hg)	AD, PD	-	Dental amalgams, contaminated fish, industrial processes (e.g. chlorine-alkaline and thermometer factories, mercury extraction plants), volcanic activity and coal combustion
Selenium (Se)	ALS	-	Food

AD=Alzheimer's disease; ALS=amyotrophic lateral sclerosis; PD=Parkinson's disease; ns=not specified

4. Challenges in metal exposure and neurodegeneration epidemiology

Epidemiological evidence of associations between metal exposure and neurodegenerative disorders is scarce. There are several challenges impeding the investigation of these associations, which are addressed below.

4.1 Case ascertainment

Registration of neurodegenerative disorders at a national level is not yet common practice, although registrations are more and more being established (Pearce and Kromhout 2014). Also, neurodegenerative disorders are largely under-reported on death certificates, although registration is generally better for ALS, due to its poor prognosis, than for Parkinson's and Alzheimer's diseases. Lack of registration may result in case-ascertainment bias. For example, if only the more severe cases are being registered or only cases in a specific area, the selection of cases in the analyses is not a random sample. The results from an epidemiological study based on such data may therefore not be translatable to all cases of that particular disease.

Diagnosis of neurodegenerative disorders is largely symptom-based, which complicates accurate diagnosis. Moreover, overlap between the various disorders exists (Chin-Chan et al. 2015; Pearce and Kromhout 2014). Studies often report on different disorders combined, for example Parkinson's disease with parkinsonisms together, Alzheimer's disease with other dementias, or motor neuron diseases as one group. However, such grouping could obscure existing associations for one of the specific diseases, since the disease aetiologies may be different. Grouping may also hinder comparison between studies.

4.2 Disease mechanisms

Although the disease mechanism of neurodegenerative disorders is still poorly understood (Al-Chalabi et al. 2014; Pearce and Kromhout 2014), they are considered to be multifactorial and to follow a

multistep process (Al-Chalabi et al. 2014; Lai et al. 2002). Interactions between genetic factors and environmental risk factors, such as metals, may play a role and strictly dividing cases into genetic and sporadic therefore seems too simplistic (Cannon and Greenamyre 2011). Besides, studying one factor at the time may be insufficient to identify possible risk factors. In a multistep model, the same step might result from different agents, suggesting different possible pathways and therefore reducing the statistical power to identify a specific risk factor (Al-Chalabi et al. 2014). Moreover, there is experimental evidence that Parkinson's disease and other neurologic diseases related to aging may be determined by exposures occurring early in the development (*i.e.* prenatal or in early life) (Logroscino 2005), further complicating identifying causal risk factor(s).

4.3 Retrospective assessment of exposure

Neurodegenerative disorders typically have very long latency periods and the exact onset is usually unknown. Many studies use onset of symptoms as onset of disease, but the disease process may well have started much earlier. It is nearly impossible to know at which point to assess exposure. Furthermore, the desired time resolution may not be available.

In addition to long latency, neurodegenerative disorders are relatively rare, making it harder to include sufficient cases in a population. For rare diseases and diseases with long latency, the case-control design is therefore generally the only way to investigate potential risk factors. Where cohort studies suffer from insufficient numbers of cases or long durations before the disease develops, the statistical power of case-control studies is optimised by focussing on including cases and matching controls. Limiting analyses to a certain health outcome offers the opportunity to study a wide range of risk factors. Because subjects are identified at time of disease diagnosis for the cases, information about exposures, lifestyle factors and other determinants can be collected for their full lifetime up to the time of the disease onset. The collection of information on medical and lifestyle factors is important

because these factors may confound or modify an exposure–disease association and should be controlled for (Teschke et al. 2002).

Such lifetime information is crucial, but the inevitable retrospective character makes exposure assessment the most difficult part in case-control studies. Particularly gaining accurate information on the early life environment can be problematic. Many studies rely on self-reported exposures, as these are fairly easy to collect via interviews or questionnaires. However, metals are amongst the most difficult exposures for cases and controls to recognise and report in questionnaires (Lai et al. 2002). Additionally, self-reports are prone to recall bias, because exposures are generally reported after cases have been diagnosed (Rothman et al. 2008). Knowledge of disease diagnosis may improve memory or provoke false memories and as such affect the reporting of exposure. The disease itself may also affect memory of patients, which may particularly be true for Alzheimer and other dementias, but also for Parkinson or ALS patients with co-existing dementias. McGuire et al. (1997) showed associations between occupational exposure and ALS, both based on self-reported exposure and exposure assessment by an expert panel that was unaware of the subjects' disease status. Very different conclusions were drawn on the two approaches for lead, warranting caution for the interpretation of studies fully relying on self-reported exposures.

Expert assessments are often considered the best exposure assessment method in occupational settings (Teschke et al. 2002). However, experts may also be hindered by insufficient information to reliably assess exposure. Exposure to metals is highly task-specific and therefore very detailed information is needed to distinguish levels of metal exposure between workers with the same job title. Job-specific modules (asking questions about tasks, work conditions and other exposure determinants) could offer such detailed information rather than simply job titles (Stewart et al. 1998).

On the other hand, occupational exposure to metals may be uncommon among study participants, as was the case in a recent ALS case-control in the US (Yu et al. 2014), making it inevitable to treat metals as one group. Another complication is disentangling the risks following exposure to the different metals, because exposures to metals are often correlated (Palacios et al. 2014; Sadetzki et al. 2016) and synergistic effects between metals may exist. Since humans may be exposed to a variety of metals it would be interesting to explore the combined effect of specific metal exposures, which would require large cohorts and detailed exposure assessment.

4.4 Biomarkers of exposure

Biomarkers of exposure provide the option to assess the internal concentration of a specific metal. Internal concentrations best reflect the actual exposure, capturing all sources of exposure, *i.e.* occupationally, environmentally and diet (Goyer et al. 2004). Ideally, a biomarker of exposure can be measured in samples that are easily collected, analyses are sensitive and reliable, and the marker is specific for a particular metal, providing information about level of exposure (Goyer et al. 2004). Concentrations highly depend on elimination half-lives and where in the body the metal accumulates. For example, recent exposure to lead is reflected in blood, while bone lead levels are considered more useful for cumulative lifetime exposure assessment (Kamel et al. 2005). Biomarkers may not be appropriate if metals do not accumulate in tissue, and suitable biomarkers have not been identified for all metals. To assess exposure to inorganic selenium, for example, available biomarkers are not able to reliable reflect exposure (Vinceti et al. 2010).

When comparing biomarkers for metal exposure between cases and controls, one has to be aware of possible reverse causality. The disease may have changed the way the body metabolizes or reacts to metals. If samples are measured after disease onset, levels may have been affected by the disease process, resulting in significant bias.

5. Future directions

In comparison with cancer and respiratory diseases, associations with environmental or occupational exposures have not been investigated as extensively for neurodegenerative disorders (Pearce and Kromhout 2014). With regards to metals, research has mainly been focused on neurodevelopmental disorders, rather than neurodegenerative outcomes. Armon (2003) described criteria for studies to classify the evidence of risk factors for ALS, which are also applicable to other neurodegenerative disorders. The best evidence comes from either prospective or retrospective cohort studies with parallel controls, or population-based case-control studies (Armon 2003). To be informative, such studies should include large numbers and should be well-performed. Quality studies include the assessment of exposure before disease onset, preferably quantifying exposure, and sources of biases and confounding are identified and accounted for (Armon 2003). To date, not many studies on metal exposure and neurodegenerative disorders meet these criteria.

Future research should also be focussed on understanding the role of metals in the pathology of neurodegenerative disorders. Is there actually a causal relationship between metal exposure and the disease? Are metals accumulated as a result of the disease? What are the mechanisms? What are the critical time windows in the life span? Levels of metals in stored tissue of patients collected before their disease onset may provide more insights when compared with tissue from control subjects. Another way to limit reverse causality is the approach of Mendelian randomisation studies. Such studies randomize groups based on genetic variants that influence the risk factor of interest, as a marker for lifelong exposure (Brennan 2004). Large cohort studies, with a comprehensive description of the lifelong exposure history (the 'exposome'), sufficient time of follow-up and a biobank are needed to study such questions. Such large cohorts provide the framework to study environment, lifestyle, genetic variations, and chronic disease (Wild 2005).

A multidisciplinary approach could help improving future studies. Gorell et al. (1999) suggested collaboration between occupational toxicologists, industrial hygienists, neurologists, (environmental) epidemiologists and statisticians. Combining strengths could ensure the best assessments of the disease under study, case and control ascertainment and enrolment from suitable population bases, and measurement of occupational and/or environmental metal exposure (Gorell et al. 1999).

In conclusion, future epidemiological studies should be large and high-quality studies, utilizing better quality exposure assessment to provide the crucial evidence on metal exposures as possible risk factors for neurodegenerative disorders.

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